

Vaccination of Balb/c mice against experimental visceral leishmaniasis with the GP36 glycoprotein antigen of *Leishmania donovani*

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Abstract

Leishmania donovani GP36 glycoprotein is the main antigen of the FML Fucose Mannose Ligand (FML) complex specifically recognized by sera of kala-azar human patients. The GP36 was isolated by chemical elution + sonication and used for Balb/c mouse vaccination in combination with saponin, by the s.c. route, inducing a strong and specific protective effect against experimental visceral leishmaniasis shown by the increase of: specific IgG antibodies (82.6%), mainly IgG2a, the delayed type of hypersensitivity to promastigote lysate (37.8%, $P < 0.001$), the in vitro cellular proliferative response to GP36 of ganglia lymphocytes (53.5%, $P < 0.005$) and the decrease of liver parasite burden (68.1%, $P < 0.025$). Saponin treated controls reacted significantly differently from GP36 vaccinated animals at all the assayed variables ($P < 0.05$). GP36 induced significant protection against murine visceral leishmaniasis at concentrations commonly used for vaccination with recombinant antigens. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Human visceral leishmaniasis or kala-azar is a severe disease, lethal if not treated soon after the onset of symptoms, caused by parasites of the *Leishmania donovani* complex. Clinical signs in humans include: malaise, anemia, hepato-splenomegaly, hypergammaglobulinemia, fever, cachexia and progressive suppression of the cellular immune response. About 500000 human cases of kala-azar are registered annually. The disease develops with endemic characteristics in Asia, Europe and America with important localized epidemic bursts. 90% of all human cases occur in Bangladesh, Brazil, India and Sudan. Between 1977–1987, 185000 new cases

were registered in Bihar (India). 40000 obits due to the disease took place in Sudan and 400000 annual new cases with 5–7% fatality were reported in India [1]. In America, kala-azar is a canid zoonosis transmitted by sandflies. The control of canine kala-azar should therefore reduce the availability of parasites to sandflies and thus reduce the human disease incidence [2]. The current strategy for control of kala-azar, as recommended by the World Health Organization (WHO), is based on detection and destruction or treatment of infected dogs, treatment of human cases and vector control [2]. Since the efficacy of this control has been shown to be inconsistent, the development of vaccines has been given priority and is considered to be urgent by the World Health Organization.

First and second generation vaccines against leishmaniasis were primarily based on either of two technologies from which antigenic material was derived. These

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were: (1) live attenuated or killed forms of whole organisms: mixed *Leishmania* strains — *L. amazonensis* for humans, in Brazil [3], *L. braziliensis* or *mexicana* with or without BCG in Venezuela [4] and *L. major* with BCG in Iran [5] and (2) defined native or recombinant protein components of the organism obtained by biochemical purification or by genetic engineering. Most second-generation vaccines can be further subdivided into three categories according to their composition: live vaccines, defined subunits and crude fractions. Live vaccines include: attenuated but live parasites [6–8], and recombinant bacteria and viruses carrying *Leishmania* antigens [9,10]. This is the case of the gp63 surface protease gene of *L. major* expressed in *Salmonella typhimurium* [9], and of vaccinia virus expressing the gp46/M-2 gene of *L. amazonensis* [10]. Among the defined subunit vaccines, the gp63 antigen has been studied most extensively [11–13]. The LeIF recombinant analogue of eukaryotic ribosomal protein [14] and the LACK protein or cDNA formulations proved to be protective immunogens for mice [15] while chemically defined antigens of *Leishmania* such as: Lipophosphoglycan (LPG) [16] and Glycoinositolphospholipids (GIPLS) [17] were considered candidates for vaccine formulation. All these antigens, however, demonstrated their protective effect only at Phase I–II levels (safety and immunogenicity, with or without experimental parasitic challenge). Among the crude fractions, the LPGAP, a group of peptides that co-elute with the lipophosphoglycan of *L. donovani* (LPG), were responsible for its previously described immunogenic properties [18]. The DP72, a native glycoprotein of *L. donovani* protected Balb/c mice from experimental visceral and cutaneous leishmaniasis [19]. Finally, the third-generation vaccines are composed of cDNA encoding leishmanial antigens cloned into a eukaryotic expression vector. Preliminary trials with a third-generation vaccine, composed of plasmid DNA containing gp63-cDNA showed that Balb/c mice immunized with this formulation developed significant resistance against cutaneous leishmaniasis [20].

In previous reports, the protective potential of a *L. donovani* promastigote glycoproteic complex was analyzed [21]. This glycoprotein-enriched fraction was named FML ligand, since it contains the neutral sugars fucose, mannose, glucose and galactose and behaved as a ligand that strongly inhibits the in vitro infection of murine macrophages by promastigotes and amastigotes of *L. donovani* [21,22]. This inhibition was species-specific for the genus *Leishmania* [23]. The FML antigen is present on the surface of the parasite throughout the life cycle [22] being a potent immunogen in rabbits and mice [22,24,25] and a sensitive, predictive and specific antigen in serodiagnosis of human [26] and canine kala-azar [27].

Saponins were considered to be the best adjuvants in several different experimental models [28–30] with bacterial adjuvants as a second choice, mainly when cell-mediated immunity is required. They are conjugates of triterpenes, or glycoalkaloids and glycidic moieties, they have been extensively used in vaccination of mice, canine and non-human primate experimental models. Their adjuvant activities were outstanding in vaccines against *Trypanosoma cruzi* [28], cytomegalovirus [29], syncytial respiratory virus [31] and HIV [32]. They are being tested, with WHO support, in large clinical assays against human malaria, melanoma and HIV infection [33].

With the FML antigen of *L. donovani* in combination with Riedel De Haën saponin administered via the intraperitoneal (i.p.) route, an average protection of 87.7% ($P < 0.01$) and 84% ($P < 0.001$) was achieved against visceral leishmaniasis, in the isogenic CB hamster [34] and Balb/c mouse models, respectively [24]. Animals treated with saponin or saline only, showed significantly lower reactivities ($P < 0.01$ and $P < 0.001$, respectively) with no protective effect. No toxic effects were detected at the dosage used [34,24]. No significant differences in antibody response or liver infection were observed among control animals treated only with saline, saponin or FML [34,24]. Protection criteria included maintenance of the delayed type of hypersensitivity, the reduction of splenomegaly [34] and parasitic load, the increase of the in vitro splenocyte proliferation against leishmanial antigen, and the increase in anti-FML specific antibody response [34,24]. The FML was further used in combination with saponin, aluminum hydroxide ($Al(OH)_3$) and Freund's incomplete adjuvant (FIA) in vaccines tested in an outbred murine model of visceral leishmaniasis, either through the i.p. or subcutaneous (s.c.) route [25]. In that work saponin was also the best adjuvant for the FML-vaccine against *L. donovani*, followed by $Al(OH)_3$. The humoral response was significantly higher in the groups treated with FML + saponin or FML + $Al(OH)_3$ than in controls, both before and after infection [25]. Animals immunized by the ip route developed higher antibody titers. A significant and specific reduction of parasitic load in relation to saline (85%, $P < 0.01$) and saponin ($P < 0.025$) controls, was seen in animals treated with FML + saponin by the i.p. route. Coincidental with this reduction, an increase in antibodies of the IgG2a subtype was detected only in animals treated with FML + saponin i.p. A reduction of 88% in parasitic load was achieved by the combination of FML + $Al(OH)_3$ subcutaneously, but the $Al(OH)_3$ treatment itself accounted for 68% of this protection. In our conditions, vaccination with FML + saponin i.p. was superior to other treatments and had no toxic effect due to saponin [25].

FML electrophoretic (SDS-PAGE) analysis disclosed the presence of several proteic bands. Two of them: 36 and 55 kDa, were also stained for carbohydrates [21]. In Western blots, rabbit anti-FML hyperimmune serum reacted with the 36 kDa band [22]. 23 hybridomas secreting IgG and seven hybridomas secreting IgM were cloned. Twenty-two IgG clones recognized the 36 kDa band of FML, and a single clone recognized the 55 kDa band. All the IgM clones recognized only the 55 kDa band. No clone recognized both bands, or other subfractions of the FML. The integrity of the GP36 glycidic moiety was necessary for its antigenic function [22]. No cross reactivity among these two FML fractions was detected. No antigenic homology could be detected among the 36 and 55 kDa bands of FML and the gp63 major surface leishmanial antigen [22]. The 36kDa glycoprotein was identified as the major FML antigenic fraction, a surface glycoprotein antigen of *L. donovani*, and designated 'GP36' [22].

The FML antigen was 100% sensitive and 96% specific in the diagnosis of human kala-azar [26]. In FML–Western blot analysis, the GP36 glycoprotein was the specific marker of human visceral leishmaniasis, labeled only by kala-azar patients' sera. No labeling was detected with sera of patients with tegumentar leishmaniasis, Chagas Disease, or normal controls [35]. Conversely, the 55 kDa component of FML was recognized by normal sera from kala-azar endemic and non-endemic areas, and sera from patients with tegumentar leishmaniasis, kala-azar or Chagas' disease. This antigen is thus non-specific [35].

In the present investigation we pursued the isolation of the GP36 glycoprotein antigen and the analysis of its protective potential in vaccination of Balb/c mice against experimental visceral leishmaniasis.

2. Material and methods

2.1. Isolation of GP36 glycoprotein by chemical elution + sonication

Isolation and chemical characterization of the FML obtained from stationary-growth phase promastigotes of *L. donovani* Sudan (LD 1S/MHOM/SD/00-strain 1S) was performed as previously described [21]. Briefly, promastigotes were submitted to an aqueous extraction followed by heat inactivation and centrifugation. The aqueous supernatant was lyophilized and fractionated by gel filtration on a Bio-Gel P-10 column yielding the FML glycoproteic complex in void volume [21]. For GP36 glycoprotein isolation, the FML antigen was submitted to chemical elution + sonication according to Estalote et al. [36]. The FML was fractionated through SDS-PAGE, under denaturing conditions, in 10% slab baby gels as described [21]. After they were run, gels

were stained with Coomassie Brilliant Blue R stain. Sections of the gels corresponding to GP36 glycoprotein were excised and further disrupted into 2–3 mm slices, incubated with 1% SDS, 1% NaHCO₃, for 2 h at 22°C with agitation (150 rpm), followed by 16 h at 4°C. The eluted GP36 was submitted to sonication in a Sonifier Cell Disruptor B15 (Branson) (6 cycles × 5 s) and centrifuged at 1180 g, 4°C × 30 min. Pellets were discarded and supernatants were dialyzed overnight, against 400 volumes of distilled water. The protein content of GP36 was monitored using a calibration curve relating the SDS-PAGE densitometric profile of known concentrations of BSA to the GP36 sample [36]. Images were acquired using Adobe Photoshop and the NIH Image 1.58 program for densitometric analysis. An anti-GP36 serum was obtained. Briefly, the FML antigen was fractionated by single comb SDS PAGE and the band corresponding to GP36 antigen was excised and homogenized in 0.5 ml PBS (pH 7.2). A three months old rabbit was immunized twice, with a week interval, by footpad injection of 840 µg of GP36 antigen emulsified in an equal volume of Complete Freund's adjuvant (Difco, Detroit, MI) and a third time using the Incomplete adjuvant. Fifteen days after immunization, three intravenous injections of 10 µg of GP36, obtained through chemical elution + sonication, were given at three day intervals. Serum was collected two weeks after the last antigen injection.

2.2. Isolation of GP36 glycoprotein by chemical 'T cell blot'

This was performed according to Kaye et al. [37]. Briefly, FML proteins were applied to a single comb of a 10% SDS-PAGE gel and electrophoretically transferred to a nitrocellulose membrane (0.45 µm; Sigma Co.) in a Western blot apparatus (250 mA × 2 h at 4°C). The region corresponding to GP36 was identified using Rosso Ponceau S (Carlo Erba, Italia) in acetic acid and excised. The nitrocellulose strips (NCP) (5 mm) were dried at room temperature for 12 h, fractionated in small pieces, solubilized with 2 ml dimethylsulfoxide (DMSO) (Sigma Co.) for 2 h at room temperature and precipitated with 5 ml carbonate buffer (pH 9.6). The precipitated NCP were then washed five times in sterile PBS and stored frozen at –20°C [37]. The protein content of GP36 was monitored using a calibration curve that correlates known concentrations of BSA to their SDS-PAGE densitometric profile [36]. Images were acquired using Adobe Photoshop and the NIH Image 1.58 programs for densitometric analysis. Strips containing FML antigen were eluted by the same protocol and used for control of in vitro proliferation experiments.

2.3. Mice

Female outbred Balb/c mice (3-month-old) were obtained from the central animal care facilities, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, RJ, Brazil.

2.4. Immunization and infection of mice

Animals were immunized through the s.c. route, with three weekly doses of GP36 glycoprotein (16 µg) obtained through chemical elution + sonication, and 100 µg of Riedel De Haën saponin (R) in 0.2 ml saline solution. The inoculation was performed in the hind footpads. Saline and adjuvant-treated animals were included as controls. Seven days after immunization, animals were challenged by intravenous injection of 2×10^7 amastigotes (*Leishmania (L.) donovani* LD-1S/MHOM/SD/00-strain 1S), obtained from infected hamster spleens, as previously described [38]. Intradermal reaction against promastigote lysate of *L. donovani* was determined and sera of animals collected, 7 days after last vaccine injection and 15 days after infection. Anti-FML IgM, IgG and IgG subtypes of immunoglobulins were monitored by the FML-ELISA assay. Fifteen days after infection, animals were sacrificed, their *in vitro* ganglia cell proliferation against *L. donovani* antigens was determined and their liver and spleen parasite loads assessed as Leishman–Donovan Units of Stauber on Giemsa-stained imprints (LDU = number of amastigotes/1000 cell nuclei \times mg organ weight).

2.5. FML-ELISA assay

The anti-FML antibody levels were assayed in pools of sera collected before and after infection from all the vaccinated groups using the FML-ELISA as previously described [25,26], with 2 µg antigen per well and goat anti-mouse IgG peroxidase conjugate (Sigma) in a 1:1000 dilution in blocking buffer. For the detection of specific antibody types and isotypes against FML (2 µg/well), serial dilutions of immune mouse sera (15 days after infection) were incubated with the antigen, washed and further treated with goat anti-mouse IgM peroxidase conjugate (Sigma) at 1 in 4000 dilution, or with goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 horseradish peroxidase conjugated antibodies (Southern Biotechnology Associates, Birmingham, AL, USA) at 1 in 1000 dilution for 1 h in blocking buffer. The reaction was developed with O-phenyldiamine (Sigma), interrupted with 1 N sulphuric acid, and monitored at 492 nm. Sera were analysed by double-blind tests, in triplicate. Positive and negative control sera were included in each test. All results of serology were expressed as log₂ end-point titres. According to conventional serology,

titrations differing by two or more dilutions are significant.

2.6. Delayed type hypersensitivity (intradermal reaction to promastigote lysate)

This was determined by injecting mice intradermally, in the right front footpad, with 10^7 freeze-thawed stationary phase promastigotes of *L. donovani* in 0.1 ml sterile saline solution, measuring the footpad thickness with a Mitutoyo apparatus, both before and 0, 24, 48 and 72 h after injection. Controls were performed by injecting each animal with 0.1 ml saline in the left hind footpad. At each time, the values of the saline control were subtracted from the reaction due to *Leishmania* antigen. Previous experiments carried out in CB hamsters demonstrated that 24 h after inoculation saline treated footpads returned to base levels [34].

2.7. In vitro ganglia cell proliferation against *L. donovani* antigens

To assess the specific proliferative *in vitro* response, popliteal ganglia were aseptically removed and disrupted in Hank's saline solution (Sigma Co.) using a Dounce homogenizer. The mononuclear cells were separated by centrifugation at 400 g for 5 min at 4°C and resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 10 mM HEPES, 0.2g/ml L-glutamine, 0.04 mM 2-mercaptoethanol and antibiotics (200 U/ml of penicillin and 200 µg/ml of streptomycin). The cell suspension was distributed in flat-bottomed microtiter plates (Nunc, Roskilde, Denmark), each well containing 10^6 cells in a final volume of 100 µl. RPMI supplemented medium was added as negative control. 0.4 µg of Concanavalin A, 1.31 µg FML, 0.15 or 0.6 µg of GP36 or 1.25 µg *L. donovani* protein (10^6 freeze and thawed promastigotes) were added to triplicate wells as a stimulus for lymphocyte proliferative responses. Cells were further incubated for periods of 1–5 days at 37°C under a 5% CO₂ atmosphere. Cell proliferation was monitored as described by Mossman [39]. Briefly, 10 µl of a 5 mg/ml solution of [3-(4,5-dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide] (MTT) (Sigma Co.) were added to each well and the plates further incubated for 4 h at 37°C under a CO₂ atmosphere. Reaction was interrupted by the addition of 100 µl 10% SDS in 0.04N HCl. Plates were further incubated at 37°C for 17 h in the dark and absorbancy reading was performed in an Elisa BioRad Microplate Reader Model 550 at 570 nm.

2.8. Statistical analysis

This was performed by a standard *t* test [40].

3. Results

3.1. Isolation of GP36

Fig. 1. shows the GP36 antigen isolated from FML through chemical elution and 'T cell blot'. Isolation by both methods yielded a large and diffuse band in the 36 kDa region (Fig. 1C, 1D). This result is expected for a glycoproteic band. In Fig. 1E, GP36 obtained through chemical elution was treated with an anti-GP36 rabbit monospecific hyperimmune sera. The sharper appearance of the blot band is probably related to the lower concentration of antigen in this nitrocellulose strip (Fig. 1E) and the high antibody titer of the anti-GP36 rabbit serum. A more defined appearance of the band could also correspond to an enhanced reactivity of the serum against proteic epitopes of the molecule. However, this serum showed reactivity against both the carbohydrate and proteic epitopes of GP36 antigen. Indeed, when nitrocellulose strips containing the GP36 antigen were submitted to a mild hydrolysis with 0.1–10 mM sodium *m* periodate in sodium acetate buffer (pH 4.5) and further incubation with 50 mM sodium borohydride solution according to Woodward et al. [41], the carbohydrate epitopes were destroyed and still the anti-GP36 serum labeled the proteic moiety of the antigen. These results were confirmed by an ELISA assay (unpublished results).

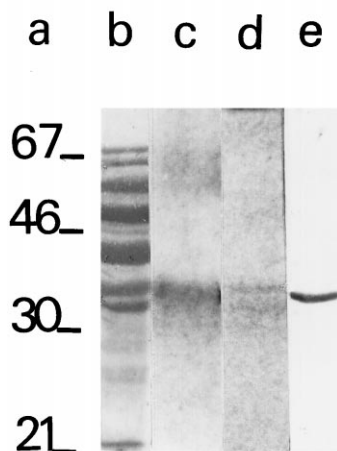


Fig. 1. Polyacrylamide gel electrophoresis of *L. donovani* antigens: lane A, low molecular weight size standards: Bovine Serum Albumin (67 kDa), Ovalbumin (46 kDa), Carbonic Anhydrase (30 kDa) and Trypsin inhibitor (21 kDa) (Amersham, Pharmacia Biotech); lane B, 90 μ g of FML antigen; lane C, 0.5 μ g GP36 antigen obtained by chemical elution + sonication; lane D, 0.6 μ g GP36 glycoprotein obtained through 'T cell blot'; lane E, 0.04 μ g GP36 glycoprotein obtained by chemical elution + sonication reacted with rabbit anti-GP36 hyperimmune serum (1:1000) and goat-anti rabbit peroxidase conjugate (1:2000). Lane A–D, Coomassie blue stained antigens; lane E, Immunoblot.

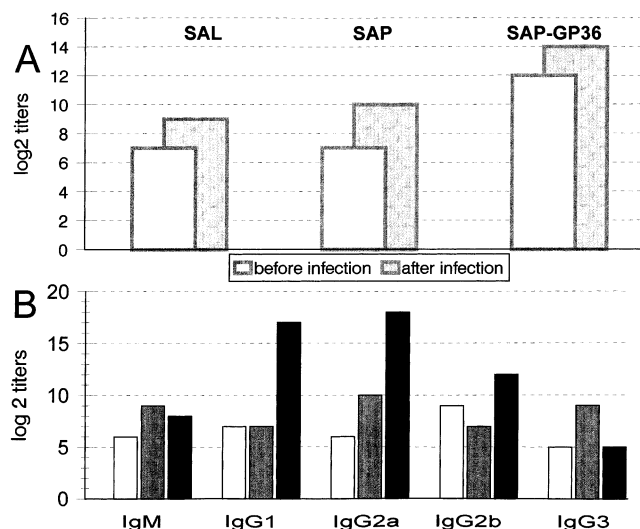


Fig. 2. Anti-FML IgG antibodies in animals vaccinated with the GP36 glycoprotein antigen of *L. donovani* and Riedel de Haën saponin. Balb/c mice were immunized with three doses of 16 μ g of GP36 glycoprotein obtained through chemical elution + sonication and 100 μ g of saponin (SAP-GP36) by the subcutaneous route (two independent experiments). Control animals received only saline (SAL) or saponin (SAP). The y axis represents the log₂ titers of the FML-ELISA absorbance values in the pool of sera obtained from 12 animals for each treatment. (A) Anti-FML IgG immunoglobulins, before and after the challenge. (B) Anti-FML IgM and IgG subtypes of immunoglobulins obtained in sera 15 days after infection. For each set of antibody titers, the vertical bars represent, from left to right, the results of: saline control, saponin and saponin-GP36 treated animals.

3.2. Antibody response in GP36 vaccinated and control mice

Balb/c mice were immunized through the s.c. route, with either three doses of GP36 glycoprotein (16 μ g) and 100 μ g saponin or saponin or saline as controls. Animals were challenged by intravenous injection of 2×10^7 *L. donovani* amastigotes and their sera was collected, 7 days after last vaccine injection and 15 days after infection. Anti-FML IgM, IgG and IgG subtypes of immunoglobulins were monitored by the FML-ELISA assay. The results represent the mean average of log₂ end-point titers for total anti-FML immunoglobulins in pools of sera from each group of animals (two independent experiments, $n = 12$). Fig. 2A shows the anti-FML total antibodies achieved in the immunized animals, either before or after the challenge. The humoral response was specific, e.g. significantly higher in the groups treated with GP36 + saponin than in saponin or saline controls, both before and after infection. Finally, the highest antibody levels were achieved by animals that received the complete vaccine, after the challenge (Fig. 2A).

The IgM and IgG isotypes of specific anti-FML antibodies were measured in all groups of animals,

fifteen days after infection. As seen in Fig. 2B, a significant, although non-specific, increase in IgM antibody titres was observed in GP36 + saponin immunized animals. According to conventional serology, titration differing in two or more dilutions is significant. One desirable feature of adjuvant activity is the capacity to selectively induce the production of protective IgG subclass antibodies that are directed specifically to the antigen. An increase in antibodies of the IgG2a and IgG2b subtypes was detected only in animals treated with GP36 + saponin. Also, a significant and specific enhancement of the IgG1 subclass was observed. This effect was expected as a consequence of the saponin treatment. The IgG3 antibody titers were similar in both the vaccinated and control groups. This immunoglobulin subtype profile composition characterizes a protective response (Fig. 2B).

3.3. Cell mediated immune response in GP36 immunized mice

Cellular immunity against leishmanial parasites was assessed in vaccinated animals by skin-testing with freeze-thawed stationary phase promastigotes of *L. donovani* and by measuring the capacity of ganglion cells from immunized and infected mice to respond to the antigen preparation by in vitro lymphocyte proliferation.

The increase in footpad thickness (IDR) was measured 0, 24 and 48 h after antigen injection. Fig. 3A summarizes the IDR results in vaccinated animals before infection. The swelling was significantly greater in vaccinated animals than in controls, both at 24 and 48 h after antigen injection ($P < 0.005$). Saponin controls were also significantly different from GP36-saponin treated animals, either ($P < 0.005$). These results indicate that the intradermal response corresponds to a delayed type of hypersensitivity considered as a signal of protection or resistance against infection by *L. donovani*. The IDR responses of two independent experiments of vaccination with GP36 and saponin were then evaluated, 48 h after antigen injection. After infection, the thickness increase was significantly greater in animals treated with GP36 and saponin than in those treated with adjuvant ($P < 0.025$) or saline ($P < 0.001$), characterizing a protective response against the infection with *L. donovani*.

Fig. 4 shows the proliferative response of ganglia lymphocytes against GP36 and FML antigens isolated through 'T cell blot'. This evaluation was performed 15 days after challenge. As expected in heavily infected animals a low proliferation is seen against the promastigote protein of *L. donovani*. However, ganglion cells responded to GP36 antigen. Lymphocyte proliferation was significantly greater than control ($P < 0.005$) in cells of GP36 vaccinated animals incubated for 24 h either with 0.15 and for 24 and 48 h with 0.6 µg of GP36. The

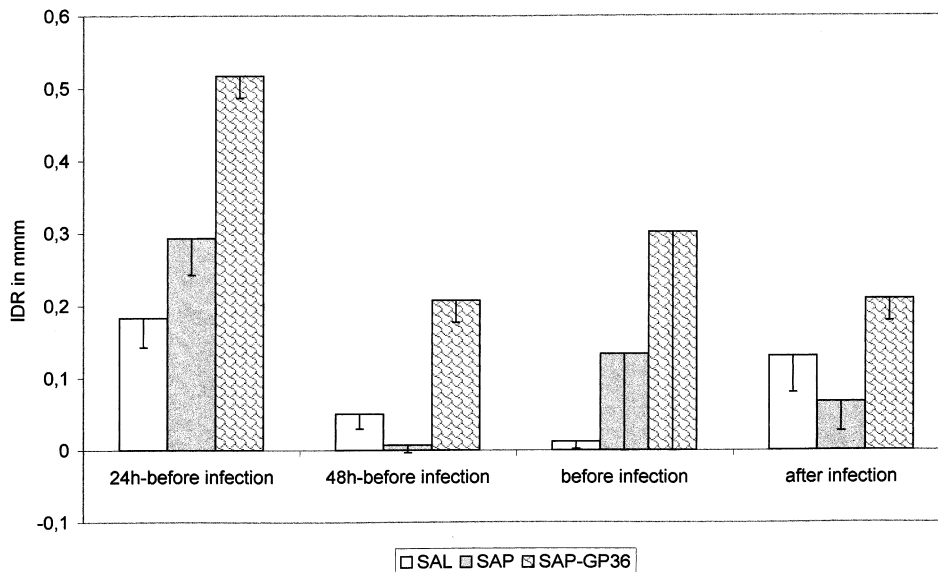


Fig. 3. Delayed type hypersensitivity in Balb/c mice immunized with saponin-GP36. Balb/c mice were immunized with three doses of 16 µg of GP36 glycoprotein obtained through chemical elution + sonication and 100 µg of saponin (SAP-GP36) by the subcutaneous route. Control animals received only saline (SAL) or saponin (SAP). From right to left: the intradermal reaction, before infection, 24 and 48 h after injection with 10^7 freeze-thawed stationary phase promastigotes of *L. donovani*, and the IDR responses of two independent experiments of vaccination with GP36 and saponin ($n = 12$) evaluated at 48 h after antigen injection. At each time, the values of the saline control were subtracted from the reaction due to *Leishmania* antigen. The y axis represents the thickness of skin test in mm. Horizontal bars represent the standard deviation.

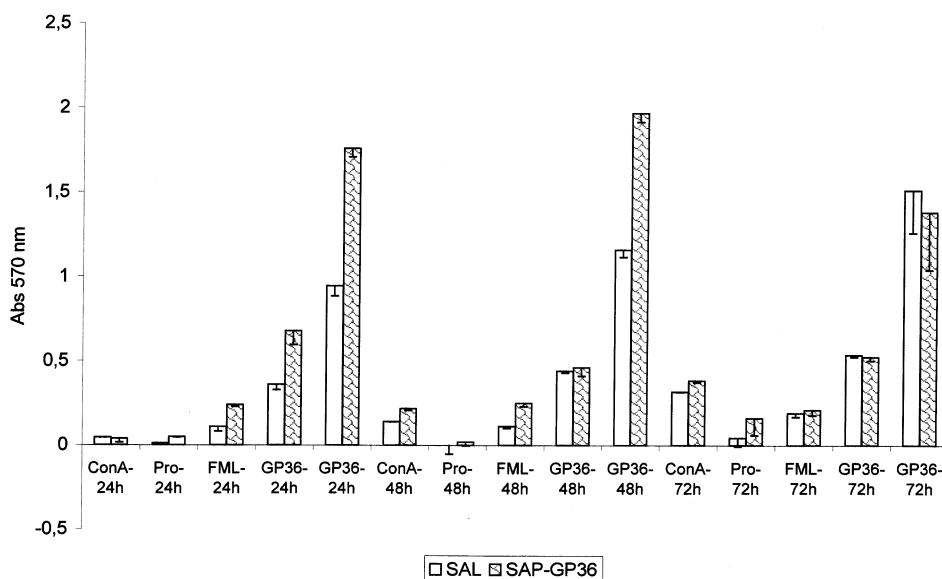


Fig. 4. Proliferative response of ganglia lymphocytes against GP36 antigen isolated through 'T cell blot'. This evaluation was performed in saponin-GP36 vaccinated animals (SAP-GP36) and saline controls (SAL), 15 days after challenge. Triplicates of 10^6 cells, in RPMI supplemented medium, were treated with $0.4 \mu\text{g}$ of Con A, $1.25 \mu\text{g}$ *L. donovani* protein (10^6 freeze and thawed promastigotes), $1.31 \mu\text{g}$ of FML, $0.15 \mu\text{g}$ and $0.6 \mu\text{g}$ of GP36 antigen and incubated for 24–72 h at 37°C under a 5% CO_2 atmosphere. Cell proliferation was monitored as described by the MMT method. Horizontal bars represent the standard deviation. The y axis represents the absorbency readings at 570 nm.

response was more pronounced against $0.6 \mu\text{g}$ of the antigen.

3.4. Protection against experimental kala-azar

Previous experiments on the mouse model demonstrated that parasites are evident in liver touch biopsies, 15 days after inoculation with 2×10^7 amastigotes of *L. donovani* [24,25]. The reduction of liver parasitic load in response to GP36 + saponin vaccine is shown in Fig. 5. Animals treated either with saline or saponin developed LDU values close to 1000, while a specific and significant protective effect was achieved in animals vaccinated with GP36 + saponin that showed 68.1% reduction of liver parasitic load. Differences from the saline and saponin control groups were significant ($P < 0.025$ and $P < 0.05$, respectively). Although the Balb/c model is considered susceptible to visceral leishmaniasis, usually this strain does not develop fatal disease [38]. In our investigation, however, 6/20 animals treated with saline and 3/20 treated with saponin died of kala-azar while all animals vaccinated with GP36 + saponin survived until the end of the experiment.

Finally, the survival of vaccinated mice, the reduction of parasite load in the liver, the enhancement of: the anti-*L. donovani* IgG2a and IgG2b specific immunoglobulins, the intradermal reaction to promastigote lysate and the in vitro lymphocyte proliferation against GP36 confirm the protective potential of the GP36 + saponin vaccine formulation against experimental visceral leishmaniasis in the Balb/c mice model.

4. Discussion

4.1. GP36 isolation

The present investigation aimed at the isolation and analysis of the protective potential of GP36 glycoprotein in vaccination against experimental murine visceral leishmaniasis. Previous studies showed that GP36 was a serologically specific marker of human kala-azar [35]. The chemical elution + sonication method, that had proved to be efficient in purification of the Band-3 major antigen from erythrocyte lysate [36], allowed the isolation of the GP36 antigen from FML preparations, showing an apparent molecular weight of 36 kDa (SDS-PAGE) and its use in vaccination of Balb/c mice. On the other hand, and although with a lower yield, the use of the 'T cell blot' method was preferred, in cases of cell proliferation in vitro assays. Different from chemically eluted antigen, no SDS or NaOH contamination was present in these antigen preparations, avoiding any cell toxicity.

The chemical elution + sonication method was also worthy for the GP36 aminoacid and sugar analysis. As previously described in FML antigen [21] and in glycoproteic fractions formerly isolated from *L. donovani* by Olafson et al., [42], a majority of acid and non-polar residues were detected among GP36 components: 13.1% aspartic acid, 11.0% glutamic acid, 7.6% glycine, 10.2% alanine, 7.4% valine and 10.1% leucine. On the other hand, fucose and mannose that were previously characterized in the FML glycidic moiety [21] were disclosed

in GP36 glycidic moiety. The analysis of acetylated residues performed by gas-chromatography disclosed the presence of two different types of fucose residues (2,3-Me₂-Fucose, 2,4-Me₂-Fucose) and a majority of 2,3,6,-Me₃-Mannose units and tri-Me₃-Galactose residues corresponding to short linear chains of 4-*O*-substituted mannopyranose alternating with 3-*O* and 4-*O* substituted fucopyranose residues (unpublished results). This is the first description of fucose in the genus *Leishmania*. Fucose and mannose were shown to be the more active sugars in competition with promastigotes for the *L. donovani* interiorization receptor on the macrophage surface [21]. The mannose–fucose receptor (MFR) constitutes one of the major routes of entry for promastigotes into murine macrophages [43] and human monocytes [44]. Co-incubation with FML inhibited the promastigote interiorization at very low concentrations (2–6 µg/ml), achieving a ‘plateau’ of 70–80% inhibition. This kind of curve is typical of specific ligands acting on a saturable number of receptors of the macrophage surface [21]. GP36, then, is an *L. donovani* specific antigen that contains the sugar ligands necessary for macrophage-parasite recognition and penetration.

Cumulating evidence in the literature describes *Leishmania* antigens with m.w. ranging from 32 to 35 kDa recognized by sera raised against membrane preparations of *L. donovani* and *L. chagasi* (reviewed in [22]). Therefore, investigations focused on crude or purified antigens, obtained under denaturing conditions or not, confirmed the existence of a glycoproteic compo-

nent with apparent m.w. 30–36 kDa in the *L. donovani* complex (reviewed in [22]).

4.2. GP36 immunoprotective potential against experimental murine visceral leishmaniasis

The efficacy of a vaccine formulation relies not only in the specificity of its antigen but also on the ability of the adjuvant to trigger an efficient protective response. For an immunosuppressor intracellular parasite like *L. donovani*, a good adjuvant should guarantee the maintenance of a strong humoral and cellular immune response against the pathogen. In our models with FML-vaccine, the Riedel de Haën saponin gave the best adjuvant performance: the reduction in liver parasitic load of vaccinated animals was 87% ($P < 0.01$) for CB hamsters [34], 84.4% ($P < 0.001$) for Balb/c [24] and 85% ($P < 0.01$) for Swiss Albino mice [25]. Although considered haemolytic in vitro [53], the Riedel de Haën saponin used in combination with FML was not harmful either for hamsters or mice [24,25,34]. Aluminium hydroxide, on the other hand, showed to be haemolytic even when tested at concentrations allowed for use in humans [25]. For all these reasons we choose the Riedel de Haën saponin for the vaccine formulation with the GP36 antigen of *L. donovani*.

In GP36-saponin vaccination experiments the protective effect was evident by the significant and specific increase of: total anti-FML IgG antibodies, intradermal reaction to promastigote lysate, in vitro ganglion cell proliferation and decrease of liver parasitic load

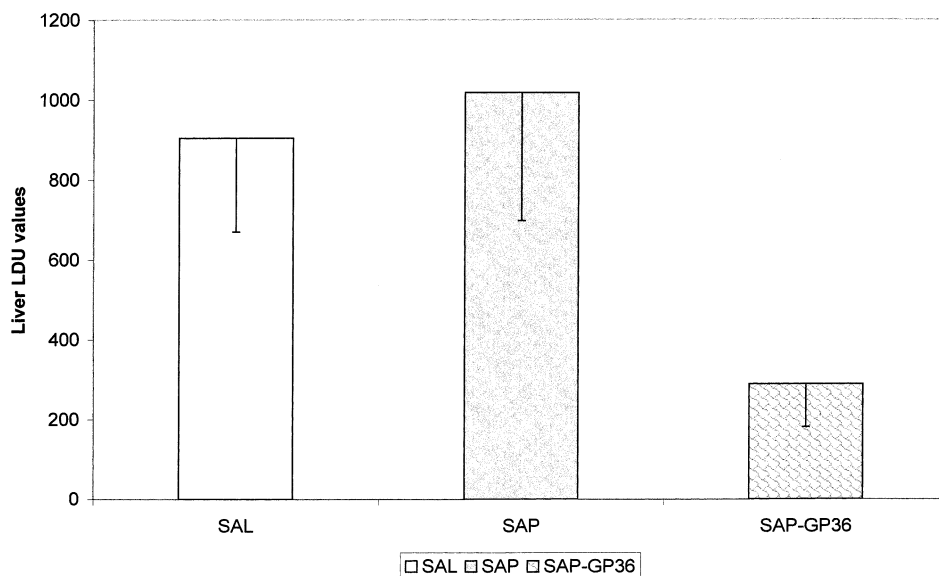


Fig. 5. Liver parasitic burden in saponin-GP36 vaccinated Balb/c mice after infection with *L. donovani*. Animals vaccinated with three doses of 16 µg of GP36 and 100 µg of saponin by the subcutaneous route were challenged with an intravenous injection of 2×10^7 amastigotes. Control animals received only saline (SAL) or saponin (SAP), as indicated. Fifteen days after infection mice were sacrificed and parasites were counted in Giemsa stained liver touch biopsies. Vertical bars represent the mean values of LDU based on parasite counts in 1000 cells for 12 mice in each group. Horizontal bars represent the standard deviation.

(68.1%) when compared to the saline control. Also, a specific increase of anti-FML IgG1, IgG2a and IgG2b antibody subtypes was observed. All these effects were specifically related to the antigen and not to the saponin adjuvant.

If humoral response is related to protection against disease, two aspects should be considered in the interpretation of the success of a vaccination protocol: (1) the quantitative aspect of reaction measured as the total IgG end-point titers and (2) the qualitative protective response, indicated by the increase in IgG2a and/or IgG2b subtypes of immunoglobulins against the candidate antigen. These subtypes are specifically related to a protective response. Regarding the quantitative IgG response: the increase in anti-FML IgG titer after amastigote challenge was of two titer units in GP36 vaccinated animals (12–14), and of three units (7–10) in saponin treated animals. Although a stronger response could be expected of GP36 vaccinated animals, one must consider that, before infection, the IgG antibodies in GP36 treated group, were already significantly higher than controls (12:7:7), and this is one of the desired effects of a vaccination protocol. Furthermore, this anti-GP36 serum specifically recognizes the GP36 band in Western blot analysis against the purified GP36 (Fig. 1E) and the total FML complex (not shown).

On the other hand, the relatively low IgG global response after amastigote infection, could be masking, the protective effect, evident by the IgG2a and IgG2b increase, found only in GP36 vaccinated animals and not in controls. Regarding the analysis of the qualitative antibody response, measured in IgG immunoglobulin subtypes, and considering a difference of two or more titer units as significant, we showed that GP36 vaccinated animals display significantly higher titers than saline controls in IgM (8:6), IgG1 (17:7), IgG2a (18:6) and IgG2b (12:9), while saponin treated animals show increases in IgM (9:6), IgG2a (10:6) and IgG3 (9:5), only. Therefore, although an unspecific increase in the protective IgG2a subtype is seen in saponin treated animals, the difference between this and the titer achieved by GP36 vaccinated animals is highly significant (10:18). These results demonstrate that although diluted in the total IgG global response, the antibody protective response achieved by the GP36 vaccinated animals is strongly significant, specific and correlated to other protection results (IDR increase, and liver LDU decrease).

After challenge, high titers of IgG1 were found in GP36 vaccinated animals (IgG1:IgG2a:IgG2b: 17:18:12). In previous investigation, using the FML antigen and saponin for Swiss Albino vaccination against visceral leishmaniasis, Santos et al. [25] also described high levels of IgG1. Indeed, the ratio of IgG1, IgG2a and IgG2b titers was: 14:12:14, for FML

vaccinated group, and 8:8:9 for saponin treated animals. Using the same protocol, the magnitude of the increase in all subtypes was then higher in GP36 vaccinated animals, than in FML vaccinated animals, despite the homologous nature and higher concentration of antigen dosis (150 µg of FML) used in Santos' work [25]. An increase in IgG1 or IgE titers suggests a TH2 response. Our results indicate then a mixed TH1/TH2 response. This kind of profile is the expected for vaccine formulations using saponin. Cumulative evidence in literature have proved that [31,45,46] and although the response is mixed in all systems, saponins induced a highly protective response reducing, parasitemia [25,28,47], viremia [31,45] or bacteremia [46]. Furthermore, saponins are considered promising adjuvants and are now being tested even in human large clinical trials against malaria, HIV and melanoma [33]. The mixed response is also evident by the combined cytokine response (IFN and IL4 or IL10) achieved by mice immunized with antigen and saponin [48,50]. Previous experiments in Balb/c mice vaccinated with GP36 and saponin disclosed a mixed response: supernatants of Concanavalin A in vitro stimulated ganglia lymphocytes of GP36-saponin vaccinated animals secreted 5.624 ng/ml of γ IFN and 4.020 ng/ml IL10. No detectable levels of these cytokines were found in supernatants of saponin controls (unpublished results). Recent experiments with FML-QuilA saponin vaccinated Balb/c mice, also showed increased levels of both γ IFN and IL10 in animal sera. 93.9 pg/ml of γ IFN and 169.4 pg/ml of IL10 were detected in sera of vaccinated animals while 61.3 pg/ml of γ IFN and 84.7 of pg/ml IL10 were detected in saline controls ($P < 0.05$). Concomitantly, these animals showed the increase in anti-FML IgG1, IgG2a and IgG2b antibody titers (19:18:18) when compared to saline controls (10:12:12) and the 33% reduction in liver LDU load (Santos et al., unpublished results).

Regarding the cell mediated immune response in GP36 immunized mice, we showed that the in vitro proliferative response was significantly enhanced in cells treated with GP36. The absorbance ratio at 570 nm between SAP-GP36 and SAL-stimulated cultures was 1.88 (0.15 µg–24 h), 1.86 (0.6 µg–24 h) and 1.7 (0.6 µg–48 h). In previous work, with FML saponin vaccinated Balb/c mice, similar ratios (3.0) were achieved [24]. These values, although lower than those generally achieved by thymidine incorporation assays, are the expected from MTT assays [39]. They correspond to the proliferative response of GP36-vaccinees that was significantly higher than that of controls and this is correlated to the protective effect showed by the reduction on liver parasite load and animal survival. No differences between results of colorimetric MTT assays, radioisotope assays or visual inspection of wells for the same system were described [39]. Furthermore, different

from tegumentar leishmaniasis, the suppression of the cellular immune response is expected in animals and humans suffering from visceral leishmaniasis. This is seen both, in the in vitro leishmania T-cell proliferation assays [49,50] as well as in the intradermal reaction [50]. Therefore, the achievement of a significant proliferative response and of a positive intradermal reaction after infection with *L. donovani* in GP36-vaccinated animals is worthy.

In this investigation, the IDR reaction reached its maximum at 24 h and was still present 48 h after injection. Even though at 48 h, when the response is expected to be lower, we found significant differences between the vaccinated and unvaccinated heavily infected groups, indicating the protective effect. This behaviour discloses a delayed type of hypersensitivity to the leishmanial antigen and not merely a Jones–Mote response. We previously detected a maximal reactivity 24 h after antigen injection in CB hamsters vaccinated with FML + saponin [34]. In that case, a histological analysis was performed and the microscopic observation of hematoxylin-eosin stained samples showed typical mononuclear cell infiltration.

In this investigation we used 16 µg of GP36 for each vaccination dose. This is a small amount of antigen equivalent to that used in protocols using recombinant antigens [12,51]. Recently, a cystein proteinase, the GP63 glycoprotein and the acid phosphatase of *L. mexicana* were used in combination with IL12, Detox, 4'-monophosphoryl lipid A, QS-21 saponin, BCG and *Corynebacterium parvum*, in mouse vaccination against tegumentar leishmaniasis, achieving different degrees of protection against challenge with promastigotes [12]. All vaccine formulations contained a mixture of the three recombinant antigens, each at a concentration of 2.5 µg/dose [12]. Also, protection against *L. major* infection was obtained with the recombinant LACK protein and rIL-12 [15]. Mice received two doses of 50 µg of recombinant protein and 100 µg of cytokine cDNA. As previously described for the FML antigen vaccine, in this investigation we used Balb/c mice and 100 µg of Riedel de Haën saponin as adjuvant. Immunization with 16 µg GP36 determined a 68.1% ($P < 0.025$) reduction in liver LDU while the use of 150 µg of FML (75 µg of protein) induced a 85% average reduction of parasitic load [24,25]. In vaccination with FML then, a 20% enhancement of protective response was achieved using a five-fold increased concentration of antigen [24,25]. GP36 antigen represents therefore, not only a specific antigenic marker but a very active immunogenic component of the FML complex that might be considered a good candidate for development of vaccine formulations against visceral leishmaniasis.

Using the purified dp 72 native protein an 81–82% of reduction in liver parasitic load was obtained in two different investigations [19,52] using the Balb/c model.

In the first study, the vaccine protocol comprised 10 µg of dp 72 in combination with 100 µg of *C. parvum* and 2×10^7 *L. donovani* promastigotes [19]. Similar to the present study, challenge was performed with 7.5×10^6 amastigotes and animals were sacrificed 18 days after infection. An average of 60 LDU was found in livers of saline treated controls [19], while infection levels ranging from 2000 to 3000 LDU were expected [24] for this strain considered to be susceptible [53]. The low degree of infection achieved by controls complicates the interpretation of the dp 72 protective power in this model. In the second investigation, 700 LDU were detected in liver of adjuvant controls and 300 in vaccinated animals [52]. Specificity is however difficult to evaluate since no data about the adjuvant control is presented [54].

Balb/c mice are considered susceptible to experimental visceral leishmaniasis, however they do not develop the fatal disease. To our knowledge, the only report of fatal kala-azar in mice described the disease in Swiss albino animals inoculated with 2×10^7 amastigotes [54]. In our study, 6/20 saline treated and 3/20 saponin controls died of visceral leishmaniasis while no death was observed in the GP36 + saponin vaccinated group. This would be the first description of fatal kala-azar in the Balb/c model.

In the present work we showed the strong immunoprotective potential of the *L. donovani* GP36 glycoprotein which might be considered as a potent antigen candidate for a second generation vaccine. As described for recombinant antigens, it might be used at low dosages, and due to the denaturation process included in its isolation protocol it is very stable and can be easily used in large scale field assays.

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