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Vaccination of Swiss Albino mice against experimental visceral leishmaniasis with the FML antigen of *Leishmania donovani*

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Abstract

The FML antigen of *Leishmania donovani* in combination with saponin, aluminum hydroxide (Al(OH)₃) and Freund's incomplete adjuvant (FIA) was used in vaccines tested in an outbred murine model of visceral leishmaniasis, either through intraperitoneal or subcutaneous routes. 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 the infection. Animals immunized by the i.p. route developed higher antibody titres. 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. Coincidentally 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 (s.c.), 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. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Within the broad range of clinical manifestations of human leishmaniasis, visceral leishmaniasis, also known as Kala-azar, elicits the most severe symptoms. Kala-azar is a chronic and frequently lethal disease caused by parasites of the *Leishmania donovani* complex. The disease is fatal if untreated after the onset of symptoms. Clinical signs include: malaise, anaemia,

hepato-splenomegaly, hypergammaglobulinemia, fever, cachexia and progressive suppression of the cellular immune response [1]. Chemotherapy directed against Kala-azar is not always successful and frequently has undesirable toxic effects. The disease is spreading in Asia, Africa and America, probably due to the resistance of sandfly vectors to insecticides and the resistance of parasites to the specific chemotherapy. Undernutrition and acquired immunosuppressive syndromes proved to be risk factors for Kala-azar [1,2].

For all these reasons, the prophylactic vaccines against Kala-azar are considered to be the most efficient tool for disease control. Indeed, the development of vaccines has been given priority and is considered to be urgent by the World Health Organization. A

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first generation of vaccines against leishmaniasis was composed of formulations including killed parasites (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]). All these formulations were developed against cutaneous but not visceral leishmaniasis and were used in large clinical trials on human populations of endemic areas. Most second-generation vaccines can be divided into three categories according to their composition: live vaccines, defined subunits and crude fractions. Live vaccines include genetically modified *Leishmania* that would cause abortive infection in man, e.g. *L. major* lacking dihydrofolate reductase/thymidylate synthetase [6]. Also included in this category are recombinant bacteria and viruses carrying *Leishmania* antigens, which have been tested in animal models. This is the case of the gp63 surface protease gene of *L. major* expressed in *Salmonella typhimurium* and of vaccinia virus expressing the gp46/M-2 gene of *L. amazonensis* [7,8]. Among the defined subunit vaccines, the gp63 antigen has been studied most extensively [9], while the LeIF recombinant analogue of eukaryotic ribosomal protein [10] and the LACK protein or cDNA formulations proved to be protective immunogens for mice [11,12]. 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 [13]. Finally, the DP72, a native glycoprotein of *L. donovani* protected Balb/c mice from experimental visceral and tegumentar leishmaniasis [14]. 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. This immunization protocol seemed to preferentially induce a Th1 response [15].

Although a great number of antigens have been tested for protection against the cutaneous disease with in vitro cell or mouse models, no vaccine against human visceral leishmaniasis is yet available. It has been claimed that the presentation of the antigen to the immune system, rather than the selection of it, might be crucial in the success of a vaccine formulation. In this sense, the empirical approach used for the first-generation vaccines (killed parasites) has been considered the most rational and it has provided a large amount of information about the reactivity of the human target population to the vaccine [16]. Indeed, only these kinds of vaccines have so far attained the level of analysis of detailed safety studies, field efficacy and clinical trials in humans [16].

One of the most critical problems in large-scale vaccination trials is the stability of a vaccine formulation. In the specific case of a leishmanial vaccine, pre-treat-

ment of the antigen should guarantee at least the inactivation of the very potent proteases expressed by the parasite [17]. This might have been one of the factors responsible for the lower effectiveness detected in the first-generation vaccines [3,4]. In a previous report based on an experimental Kala-azar model, the protective potential of a fraction containing a glycoprotein complex purified from promastigotes of *L. donovani* was analysed. The protocol for isolating this antigen involves aqueous extraction followed by denaturing conditions that determine the further stability of the vaccine. This glycoprotein-enriched fraction was named FML ligand, since it contains the neutral sugars fucose, mannose, glucose and galactose [18]. The FML antigen is present on the surface of the parasite throughout the life cycle [19]. It strongly inhibits the in vitro infection of murine macrophages by promastigotes and amastigotes of *L. donovani* [18,19]. This inhibition was species-specific for the genus *Leishmania* [20]. The ligand is a potent immunogen in rabbits and mice. No cross reactivity was observed between FML and the gp63 antigen of *Leishmania* by monoclonal antibodies in ELISA and Western blots [19]. Furthermore, in a serological assay (FML-ELISA), FML was 100% sensitive and 96% specific in the diagnosis and prognosis of human Kala-azar [21] and useful as a tool for evaluating the prevalence of anti-*L. donovani* antibodies in the sera of blood donors from a Kala-azar endemic area [22].

With the FML antigen of *L. donovani* in combination with saponin administered via the intraperitoneal route, an average protection of 87.7% ($p < 0.01$) and 84% ($p < 0.001$) against Kala-azar in the isogenic CB hamster and Balb/c mouse models, respectively, was achieved [23,24]. Animals treated with saponin alone reacted significantly differently ($p < 0.01$ and $p < 0.001$, respectively) and no toxic effects were detected at the dosage used [23,24]. No significant differences in antibody response or liver infection were observed among control animals treated only with saline, saponin or FML [23,24]. Protection criteria included maintenance of the delayed type of hypersensitivity, the reduction of splenomegaly [23] and parasitic load, the increase of the in vitro splenocyte proliferation against leishmanial antigen and the increase in anti-FML specific antibody response [23,24].

Vaccination with glycoconjugates of *Leishmania* proved to have opposite effects when different immunization routes were used [25–27]. Immunization with the lipophosphoglycan (LPG) or LPG + gp63 of *L. mexicana* conferred protection against the disease when administered by the intraperitoneal (i.p.) route while no effect was detected on cutaneous lesions in mice vaccinated by the subcutaneous (s.c.) route [25]. Liew [26] obtained protection against leishmanial infection by the i.p. or intravenous route, while the s.c. im-

munization intensified the disease. In murine cutaneous leishmaniasis, both a higher protective response [28] and disease exacerbation [29] were already observed after i.p. immunization. In the case of visceral leishmaniasis this possibility needs to be carefully analyzed since the disease is frequently lethal and the parasite induces an immunosuppressive response. Although the i.p. route is not expected to be used in a final human vaccine, it is therefore, very important to exclude any possibility of a deleterious effect of a new vaccine administered by any route.

Furthermore, different degrees of protection were achieved by vaccine formulations in which the same antigen was used in different adjuvants. In other infectious disease models (human cytomegalovirus, *Trypanosoma cruzi*, *Toxoplasma gondii*) saponins offered the best adjuvant performance when compared to aluminum hydroxide or Freund's adjuvant [30–32]. While the Freund's water–oil systems are particulate adjuvants that enhance the antibody response against the antigen, saponins are non-particulate adjuvants that trigger simultaneously both the humoral and cellular immune specific response [33]. This effect is particularly interesting in the case of an intracellular parasite such as *L. donovani* whose development in the host would not be limited by an exclusive antibody response.

In this investigation, the analysis of the protective potential of the FML antigen of *L. donovani* on experimental visceral leishmaniasis, was followed comparing the adjuvant potential of saponin, Al(OH)₃ and Freund's adjuvant in vaccine formulations administered either by the i.p. or s.c. routes. An outbred model, Swiss Albino mice, was used in order to analyse if the highly protective effect already shown in inbred models [23,24] could also be achieved in this genetically heterogeneous strain, as a first step of pre-clinical development of the FML-vaccine against Kala-azar.

2. Material and methods

2.1. Mice

Female outbred Swiss Albino 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.

2.2. Adjuvants

Saponin was purchased from Riedel De Haën, Saponin pure (R) (8047-15-2) EINECE (WG); alumi-

num hydroxide (Al(OH)₃) from Reagen (Brazil) and Freund's incomplete adjuvant (FIA) from Sigma.

2.3. Immunization and infection of mice

Animals were immunized with three weekly doses of the FML antigen of *L. donovani* [23,24] (150 µg) and 100 µg of Riedel De Haën saponin (R), 500 µg of aluminum hydroxide (Al(OH)₃) or 0.1 ml of FIA, either by the i.p. or s.c. routes. The s.c. inoculation was performed in the back. Saline and adjuvant-treated animals were included as controls. 7 days after immunization, animals were challenged by intravenous injection of 2×10^7 amastigotes (*L. donovani* LD-1S/MHOM/SD/00-strain 1S), obtained from infected hamster spleens, as previously described [34]. Animals were sacrificed 15 days after infection and their liver and spleen parasite loads were monitored in Leishman–Donovan units of Stauber on Giemsa-stained imprints (LDU = number of amastigotes/1000 cell nuclei \times mg organ weight). Sera of animals before and after infection were analysed by FML-ELISA.

2.4. FML-ELISA assay

The anti-FML antibody levels were assayed in all the vaccinated groups using the FML-ELISA as previously described [21], with 2 µg antigen per well and goat anti-mouse IgG peroxidase conjugate (Sigma) in a 1:1000 dilution in blocking buffer. Results are represented as the absorbance readings at 492 nm of a pool of sera at a dilution of 1:80.

For the detection of specific antibody types and isotypes against FML (2 µg/well), serial dilutions of immune mouse sera (7 days after the third vaccine injection) were incubated with the antigen, washed and further treated with goat anti-mouse IgG and IgM peroxidase conjugate (Sigma) at 1 in 5000 and 1 in 4000 dilution, respectively, in blocking buffer, or with goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 horseradish peroxidase conjugated antibodies (Southern, Biotechnology Associates, Birmingham, AL) 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. Results were expressed as log₂ titres. According to conventional serology, titration differing in two or more dilutions is significant.

2.5. Statistical analysis

This was performed by a standard *t*-test [35].

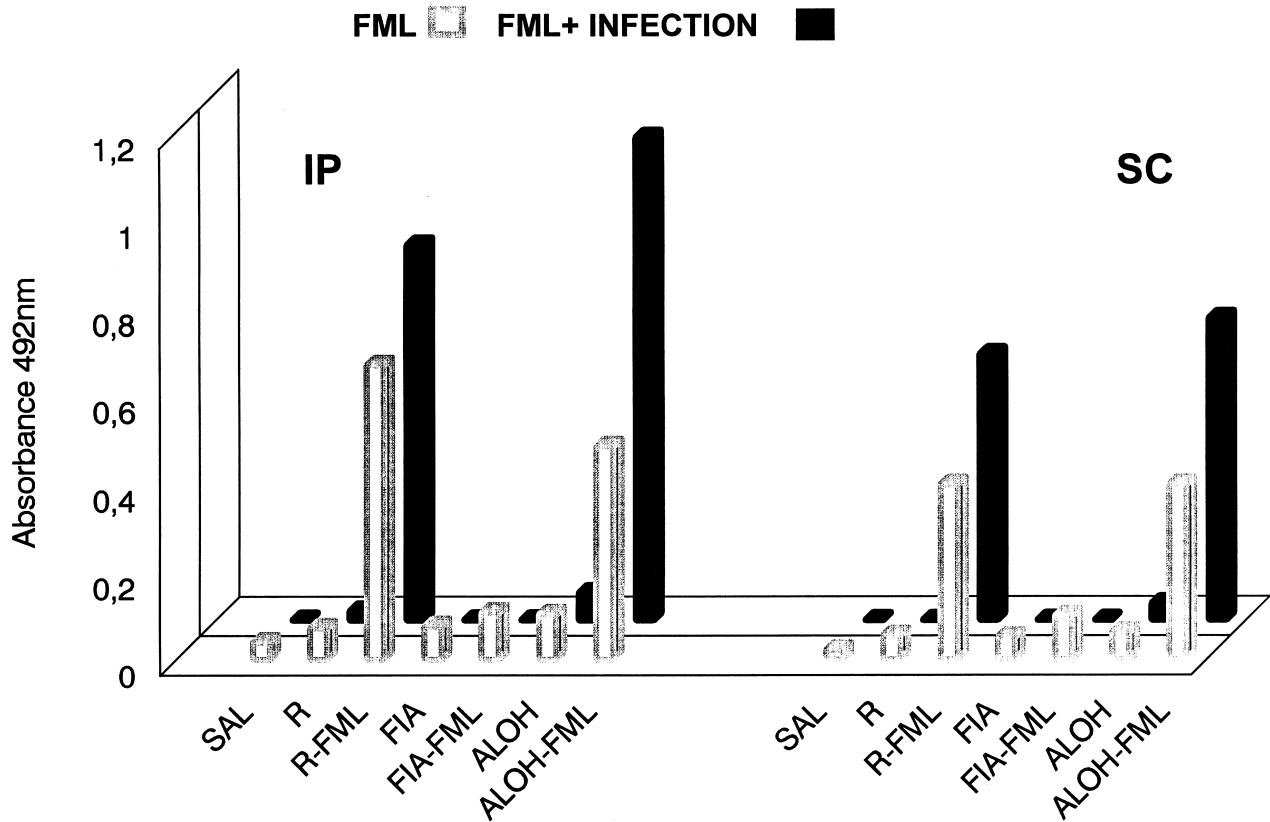


Fig. 1. Anti-FML IgG antibodies in animals vaccinated with the FML antigen of *Leishmania (L.) donovani* and Riedel De Haën saponin (R), Freund's incomplete adjuvant (FIA) or aluminum hydroxide (AlOH). Swiss Albino mice were immunized with three doses of 100 μ g of saponin, 100 μ l of FIA or 500 μ g of aluminum hydroxide in FML (150 μ g) by the intraperitoneal (i.p.) or subcutaneous (s.c.) route. Control animals received only saline (SAL) or adjuvant, as indicated. The y-axis represents the FML-ELISA absorbance values (492 nm) of sera at a dilution of 1:80 in the pool of sera obtained from 6 to 15 animals for each treatment.

3. Results

3.1. Relative efficacy of saponin, aluminum hydroxide and FIA in the antibody response

Swiss Albino mice were immunized with FML in combination with Riedel De Haën's saponin, Al(OH)₃ or FIA, either by i.p. or s.c. routes. Fig. 1 shows the anti-FML total antibody scores achieved in the immunised animals, either before or 15 days after the challenge with 2×10^7 amastigotes of *L. donovani*, i.v. The results represent absorbance readings at 492 nm for total anti-FML immunoglobulins in pools of sera from each group of animals ($n = 6-15$). The humoral response was specific, e.g. significantly higher in the groups treated with FML + saponin and FML + Al(OH)₃, than in controls treated with only the adjuvant or saline, both before and after the infection. Animals immunized by the i.p. route gave higher antibody titres than the ones treated by the s.c. route. Finally, the antibody levels were significantly greater for the groups treated with FML + saponin or FML + Al(OH)₃, after the parasite challenge. Animals that

received FIA, either with FML or not, showed a very low response, irrespectively of the immunization route.

3.2. Liver parasitic load

The reduction of parasitic load in liver in response to each FML-vaccine formulation, is shown in Fig. 2. A significant protective effect was observed in the groups treated with FML + saponin either by the i.p. ($p < 0.01$) or the s.c. route ($p < 0.005$), representing a protection of 85 and 89%, respectively, in relation to saline control. On the other hand, 67% protection ($p < 0.025$) was achieved in the animals treated with FML + FIA i.p. and 88% in the FML + Al(OH)₃ s.c. group. However, comparison of these values with those achieved in the control group for each adjuvant revealed that the FML + saponin i.p. group was the only one that developed a specific protection. Indeed, the animals treated with saponin alone showed values similar to those treated with saline ($p > 0.05$), although they were different ($p < 0.025$) from those vaccinated with FML + saponin.

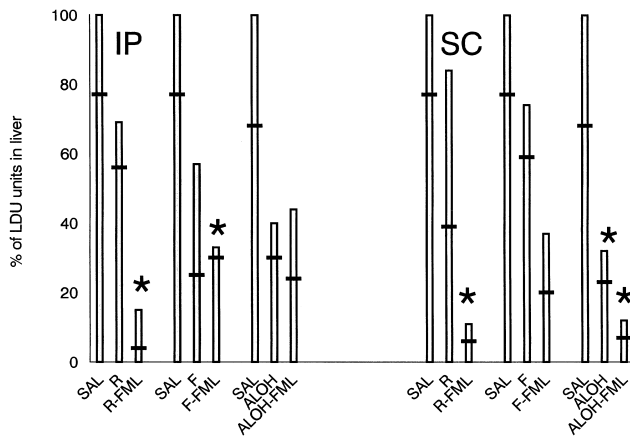


Fig. 2. Liver parasitic burden in FML-vaccinated Swiss Albino mice after infection with *Leishmania donovani*. Animals vaccinated with three doses of 100 µg of saponin, 100 µl of FIA or 500 µg of aluminum hydroxide (AlOH) in FML (150 µg) by the intraperitoneal (i.p.) or subcutaneous (s.c.) route were challenged with an intravenous injection of 2 × 10⁷ amastigotes. Control animals received only saline (SAL) or adjuvant, as indicated. 15 days after infection mice were sacrificed and parasites were counted in liver imprints. Vertical bars represent the percentage mean values of LDU based on parasite counts in 1000 cells for 5–16 mice in each group, relative to controls treated with saline. Horizontal bars represent the standard deviation. (*) Mean values significantly different (*p* < 0.05) from saline control.

3.3. IgM and IgG isotypes of specific anti-FML antibodies

Since saponin, then Al(OH)₃ showed the best performance as adjuvant and further analysis of the IgM and IgG subtype specific anti-FML antibody titres of sera in both groups of vaccinated animals was performed. As seen in Fig. 3, a specific and significant increase in IgM antibody titres was observed only in i.p. immunized animals, both for the saponin + FML and Al(OH)₃ + FML groups. According to conventional serology, titration differing in two or more dilutions is significant [33]. An increase in IgG antibodies was evident for both adjuvants and both immunization routes but, like IgM, higher values were achieved for the i.p. route. A significant and specific increase in antibodies of the IgG1 subclass was achieved with both adjuvants in animals treated by the i.p. route and only with Al(OH)₃ in the s.c. group.

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. Coincidentally with the reduction of parasite load in liver (85%, *p* < 0.01), as seen in Fig. 3, an increase in antibodies of the IgG2a subtype was detected only in animals treated with FML + saponin i.p. IgG2b increased in both adjuvant groups treated by the i.p. route and developed lower levels in the s.c.-treated animals. The IgG3 antibodies were similar in both the FML-vaccinated and control groups.

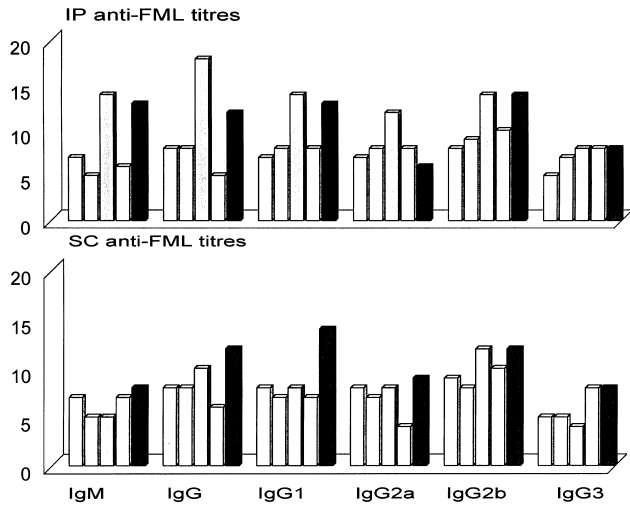


Fig. 3. IgM and IgG isotypes of specific anti-FML antibodies in sera of animals immunized with FML and saponin or Al(OH)₃ either by the i.p. or the s.c. route. The ordinate corresponds to log₂ titres of the sera obtained 7 days after immunization with FML and one of the adjuvants. The IgM and IgG-subclass titres (IgG1, IgG2a, IgG2b and IgG3) were determined by the FML-ELISA assay on pooled sera of 3–5 mice per group. Titration was performed in triplicate. For each set of antibody titres, the vertical bars represent, from left to right the results of: saline control, saponin, saponin + FML, Al(OH)₃ and Al(OH)₃ + FML treated animals.

4. Discussion

In previous work, saponin (Riedel De Haën) conferred in conjunction with FML-vaccine, 84.4 and 87% average levels of protection in the Balb/c and CB hamster isogenic models, respectively [23,24]. In the present work, a significant reduction in parasitic load (85%, *p* < 0.01) was obtained using the FML-saponin vaccine against experimental visceral leishmaniasis in mice. In the mice immunized with FML + saponin i.p. the reduction in liver parasitic burden was correlated with an increase in specific total IgG as well as IgG2a and IgG2b anti-FML antibodies. This level of protection (85%) is higher than that reported by Jaffe et al. [14], who vaccinated Balb/c mice with the DP72 glycoprotein (81.1%), and difficult to achieve, especially since outbred animals were used.

This investigation indicates that the i.p. immunization route was more effective than the s.c. route. Previously, significant protection was obtained in the CB hamster and Balb/c mouse models with the same

vaccine administered by the i.p. route [23,24]. In response to three doses of *Periandra mediterranea* saponin injected with the FML antigen of *L. donovani*, anti-FML increased levels of IgG2a were detected only in the i.p. group [36]. With different vaccination protocols, Russell [25], Liew [26] and Rodrigues et al. [27], also obtained better results when immunizing through this route. However, it was recently shown that the s.c. route was more successful in Balb/c mice vaccination with the GP36 glycoprotein + saponin [37] and in mongrel dogs vaccination with FML + QuilA saponin [38]. Indeed, the locus of injection, and not merely the immunization route, seems to be determinant. Whereas in the present work the s.c. injections were performed on the back of the animals, the GP36 immunized Balb/c mice [37] received the s.c. dose in their footpads and the dogs were vaccinated in the flank [38]. It seems that in our conditions also the presentation of the antigen rather than its composition constitutes the crucial factor in vaccine success [16].

Saponins are considered to be the best adjuvants for different experimental models [30,31,33] with bacterial adjuvants as a second choice, mainly when cell-mediated immunity is required. Britt et al. [30], showed that QS-21 saponin in combination with gB antigen of human cytomegalovirus, induced higher levels of virus binding and virus neutralizing antibodies than gB combined with either Freund's adjuvant or aluminum hydroxide. A wide range of adjuvants including alhydrogel, saponin, *Corynebacterium parvum*, oil adjuvants and several MDP analogues have been compared for the adjuvants activities in protection of mice against *Trypanosoma cruzi* infection. Only saponin showed to be effective [31]. Regarding their adjuvant activities and immunostimulating complex (ISCOM) formation, saponins were also more potent than glycoalkaloids in murine vaccination against different antigens [33]. In this work saponin was also the best adjuvant for the FML-vaccine against *L. donovani*, an intracellular parasite of human macrophages and monocytes. Although the adjuvant activity of saponins has been extensively demonstrated, an undesirable haemolytic effect has restricted their use with human vaccines. In previous work, the haemolytic activity of plant saponins was related to the chemical nature of their nucleus [36]. The haemolytic activity of steroid saponins from *Agave sisalana* and *Smilax officinalis* as well as a commercial saponin from Riedel De Haën, was higher than that of saponins with a triterpenoid nucleus (*Bredemeyera floribunda*; *Periandra mediterranea*) [36]. In the same investigation it was demonstrated that Al(OH)₃ and Freund's complete adjuvant in the concentration used for human and animal vaccination, respectively, are haemolytic [36]. Furthermore, even the low haemolytic effect detected in triterpenoid saponins could be abolished by removal of their

glycidic moiety [36]. The use of Riedel De Haën's saponin, although haemolytic in vitro, did not lead to any detectable toxic effect, lethality or anaemia in CB hamsters or Balb/c mice [23,24]. Knowing the chemical structure of any saponin chosen as adjuvant should make it possible to avoid undesirable effects without any loss in adjuvant potential.

The life cycle of the *L. donovani* complex is described as mediated by the phlebotomine sand-flies, which inoculate promastigotes into the human skin. Parasites are engulfed by local macrophages, where they divide by binary fission. Macrophages eventually die and release amastigotes, which spread the infection among cells of the mononuclear macrophagic system in the body. In early studies, infected monocytes, as well as neutrophils and eosinophils were described in the blood, as potential carriers of parasites from skin to the target organs. Therefore *Leishmania* are intracellular parasites not expected to be free in peripheral blood circulation. In this work, a slight non-specific protective effect was detected in animals vaccinated with FML + Al(OH)₃ through the s.c. route. Aluminum hydroxide is one of the few adjuvants licensed for use in humans. It is useful in bacterial vaccines, eliciting a mainly humoral response against the antigen (IgG1 and IgE). Therefore, it was not expected to contribute any protection against an intracellular parasite such as *L. donovani*. In the Kala-azar experimental model used in this and other investigations [14,23,24,34], animals are infected by an intravenous injection of a large number of parasites (1–2 × 10⁷). This inoculum might temporarily simulate a high bacteremia, common in bacterial diseases, that could be partially removed by a strong antibody response. One must keep in mind that this kind of infection, though useful as a strong experimental challenge, differs considerably from the biological cycle of *Leishmania* in nature, which starts with the interiorization of several parasites into skin macrophages and does not normally develop into high parasitaemia. This difference between the experimental model and the natural biological cycle of *Leishmania* can explain the slight protective effect developed by vaccination with FML + Al(OH)₃. On the other hand, control animals that received only Al(OH)₃ showed 68% reduction of liver parasitic load. However, as expected for a control group, no specific IgG or IgM anti-FML antibody response was detected. This fact suggests that the Al(OH)₃ treatment itself also induced a non-humoral, non-specific protective response against infection by *L. donovani*.

Finally, the achievement of 85% specific protection in an outbred animal model such as the Swiss Albino mouse encourage us to pursue analysis of the prophylactic effect of the FML-vaccine toward Kala-azar in more complex, heterogeneous models.

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References

- [1] Modabber F. Leishmaniasis. In: Tropical disease research progress, 1991–1992. Geneva: World Health Organization, 1993. p. 77–87.
- [2] Badaró R, Jones TC, Carvalho EM, Sampaio D, Reed SG, Barral A, Teixeira R, Johnson WDJ. New perspectives on a subclinical form of visceral leishmaniasis. *J Infect Dis* 1986;156:1003–11.
- [3] Antunes CM, Mayrink W, Magalhães PA, Costa A, Melo MN, Dias M, Michalick MSM, Williams P, Lima AO, Vieira JBF, Schetini APM. Controlled field trials of a vaccine against New World cutaneous leishmaniasis. *Int J Epidemiol* 1986;15:572–80.
- [4] Castes M, Blackwell J, Trujillo D, Formica S, Cabrera M, Zorrilla G, Rodas A, Castellanos PL, Convit J. Immune response in healthy volunteers vaccinated with killed leishmanial promastigotes plus BCG. I: skin-test reactivity T-cell proliferation and interferon- γ production. *Vaccine* 1994;12:1041–51.
- [5] Bahar K, Shidani B, Dowlati Y, Deihimi I, Fesharki R, Ale-Agha S, Sepehrzad C, Shafiyi A, Ehsassi S. Overview of human vaccine studies using killed *L. major*. In: 13th International Congress for Tropical Medicine and Malaria. Faculty of Tropical Medicine, Mahidol University, Bangkok, 1993. p. 183 Abstracts.
- [6] Cruz A, Coburn CM, Beverley SM. Double targeted gene replacement for creating null mutants. *Proc Natl Acad Sci USA* 1991;88:7170–4.
- [7] Yang DM, Fairweather N, Button LL, McMaster WR, Kahl LP, Liew FY. Oral *Salmonella typhimurium* (AroA-) vaccine expressing a major leishmanial surface protein (gp63) preferentially induces T helper 1 cells and protective immunity against leishmaniasis. *J Immunol* 1990;145:2281–5.
- [8] McMahon-Pratt D, Rodriguez D, Rodriguez JR, Zhang Y, Manson K, Bergman C, Rivas L, Rodrigues JF, Lohman KL, Ruddle NH, Esteban M. Recombinant vaccinia viruses expressing GP-46/M-2 protect against *Leishmania* infection. *Infect Immun* 1993;61:3351–9.
- [9] Russo DM, Burns Jr. JM, Carvalho EM, Armitage RJ, Grabstein KH, Button LL, McMaster WR, Reed SGJ. Human T cell responses to gp63, a surface antigen of *Leishmania*. *J Immunol* 1991;147:3575–80.
- [10] Sheiky YAW, Benson D.R. DR, Elwasila M, Badaró R, Burns JM, Reed SG. Shared antigens between *Leishmania* and *Trypanosoma cruzi*: characterization of the *Leishmania chagasi* acidic ribosomal protein PO. *Infect Immun* 1994;62:1643–51.
- [11] Mougneau E, Altare F, Wakil AE, Zheng S, Coppola T, Wang ZE, Waldmann R, Locksley RM, Glaichenhaus N. Expression and cloning of a protective *Leishmania* antigen. *Science* 1995;268:563–6.
- [12] Gurunathan S, Sacks DL, Brown DR, Reiner SL, Charest H, Glaichenhaus N, Seder RA. Vaccination with DNA encoding the immunodominant LACK parasite antigen confers protective immunity to mice infected with *Leishmania donovani*. *J Exp Med* 1997;186:1137–47.
- [13] Jardim A, Tolson DL, Turco SJ, Pearson TW, Olafson RW. The *Leishmania donovani* lipophosphoglycan T lymphocyte reactive component is a tightly associated protein complex. *J Immunol* 1991;147:3538–44.
- [14] Jaffe CL, Rachamim N, Sarfstein R. Characterization of two proteins from *Leishmania donovani* and their use for vaccination against visceral leishmaniasis. *J Immunol* 1990;144:699–706.
- [15] Xu D, Liew FY. Genetic vaccination against leishmaniasis. *Vaccine* 1994;12:1534–6.
- [16] Modabber F. Vaccines against leishmaniasis. *Ann Trop Med Parasitol* 1995;89:83–8.
- [17] Bouvier J, Etges R, Bordier C. Identification of the promastigotes surface protease in seven species of *Leishmania*. *Mol Biochem Parasitol* 1987;24:73–9.
- [18] Palatnik CB, Borojevic R, Previato JO, Mendonça Previato L. Inhibition of *Leishmania donovani* promastigote internalization into murine macrophages by chemically defined parasite glycoconjugate. *Infect Immun* 1989;57:754–63.
- [19] Palatnik de Sousa CB, Dutra HS, Borojevic R. *Leishmania donovani* surface glycoconjugate GP36 is the major immunogen component of the fucose-mannose ligand (FML). *Acta Trop* 1993;53:59–72.
- [20] Palatnik CB, Previato JO, Mendonça-Previato L, Borojevic R. A new approach to phylogeny of *Leishmania*: species-specificity of glycoconjugate ligands for promastigote internalization into murine macrophages. *Parasitol Res* 1990;76:289–93.
- [21] Palatnik de Sousa CB, Gomes EM, Paraguai de Souza E, Luz K, Palatnik M, Borojevic R. *Leishmania donovani*: titration of antibodies to the fucose mannose ligand as an aid in diagnosis and prognosis of visceral leishmaniasis. *Trans R Soc Trop Med Hyg* 1995;89:390–3.
- [22] Luz KG, Gomes EM, da Silva VO, Machado FCS, Araújo MAF, Fonseca HEM, Freire T, d'Almeida JB, Palatnik M, Palatnik-de-Souza CB. Prevalence of anti-*Leishmania donovani* antibody among Brazilian blood donors and polytransfused hemodialyzed patients. *Am J Trop Med Hyg* 1997;57:168–71.
- [23] Palatnik de Sousa CB, Moreno MB, Paraguai de Souza E, Borojevic R. The FML vaccine (fucose-mannose ligand) protects hamsters from experimental Kala-Azar. *Cienc Cult (J Braz Assoc Adv Sci)* 1994;46:290–6.
- [24] Palatnik de Sousa CB, Paraguai de Souza E, Gomes EM, Borojevic R. Experimental murine *Leishmania donovani* infection immunoprotection by the fucose mannose ligand (FML). *Braz J Med Biol Res* 1994;27:547–51.
- [25] Russell DG. Immunity to leishmaniasis: what properties delineate a protective antigen? In: 20th Forum in Immunology, 1987. p. 774–81.
- [26] Liew FY. Cell mediated immunity in experimental cutaneous leishmaniasis. *Parasitol Today* 1986;2:264–70.
- [27] Rodrigues MM, Mendonça-Previato L, Charlab R, Barcinski

- MA. The cellular immune response to a purified antigen from *Leishmania mexicana* subsp. *amazonensis* enhances the size of the leishmanial lesion on susceptible mice. *Infect Immun* 1987;55:3142–8.
- [28] Russell DG, Alexander J. Effective immunization against cutaneous Leishmaniasis with defined membrane antigens reconstituted into liposomes. *J Immunol* 1988;140:1274–9.
- [29] Frankenburg S, Axelrod O, Kutner S. Effective immunization of mice against cutaneous leishmaniasis using an intrinsically adjuvanted synthetic lipopeptide vaccine. *Vaccine* 1996;14:923–9.
- [30] Britt W, Fay J, Kensil C. Formulation of an immunogenic human cytomegalovirus vaccine: response in mice. *J Infect Dis* 1995;171:18–25.
- [31] Scott MT, Bahr G, Modabber F, Chedid L. Adjuvant requirement for protective immunization of mice using *Trypanosoma cruzi* 90K cell surface glycoprotein. *Int Arch Aller Appl Immunol* 1984;74:373–7.
- [32] Khan IA, Ely KH, Kasper LH. A purified parasite antigen (p30) mediates CD8+ T cell immunity against fatal *Toxoplasma gondii* infection in mice. *J Immunol* 1991;147:3501–6.
- [33] Bomford R, Stapleton M, Winsor S, Beesley JE, Jessup EA, Price KR, Fenwick GR. Adjuvanticity and ISCOMs formation by structurally diverse saponins. *Vaccine* 1992;10:572–7.
- [34] Bradley DJ, Kirkley K. Regulation of *Leishmania* population within the host. The variable course of *Leishmania donovani* infections in the mice. *Clin Exp Immunol* 1977;30:119–29.
- [35] Fisher RA, Yates F. Student's *t* test of significance. In: *Statistical tables for agricultural, biological and other research workers*. Edinburgh: Oliver and Boyd, 1957. p. 157.
- [36] Santos WR, Bernardo RR, Peçanha LT, Palatnik M, Parente JP, Palatnik de Sousa CB. Haemolytic activities of plant saponins and adjuvants. Effect of *Periandra mediterranea* saponin on the humoral response to the FML antigen of *Leishmania donovani*. *Vaccine* 1997;15:1024–9.
- [37] Paraguai de Souza E, Santana DM, Passeri de Aguiar R, Palatnik de Sousa CB. Vaccination of *Balb/c* mice with GP36 antigen of *L. donovani* and saponin. *Acta Parasitol Turcica* 1997;21:38.
- [38] Borja-Cabrera GP, Bernardo RR, Paraguai de Souza E, Palatnik M, Palatnik de Sousa CB. Vaccination against experimental canine Kala-azar with the FML antigen of *Leishmania donovani* and the Quil-A saponin. *Mem Inst Oswaldo Cruz* 1997;92:209.