THE ADJUVANT EFFECT OF SAPONIN (Q*UILLAJA BARK*) IN THE VACCINE AGAINST MURINE CUTANEOUS LEISHMANIASIS

EFEITO ADJUVANTE DA SAPONINA (QUILLAJA BARK) NA VACINA CONTRA LEISHMANIOSE CUTÂNEA MURINA

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ABSTRACT: Experimental murine infection with *Leishmania major* constitutes an important model to study host immune responses to this parasite, enabling the development of new strategies for vaccination. The purpose of this study was to evaluate the adjuvant effect of saponin on the experimental infection with *L. major* in a murine model. Groups of female BALB/c mice were immunized with either soluble *Leishmania* antigen (SLA), SLA + saponin, saponin alone or control vehicle. Three days after the last shot mice were infected in the left paw with promastigotes of *L. major* and accompanied for 10 weeks. Serum antibody levels were analyzed by ELISA and western blot and the progression of the paw lesions was measured and compared to the uninfected side. Our results demonstrated low levels of serum antibodies, that recognized mostly antigens of 80-90 kDa in mice immunized with SLA + saponin. In this same group we observed a slower progression of the paw lesions, which suggests that saponin plus SLA contributed to a better protection against leishmaniasis and may be used for the development of immunization schedules to this disease.

UNITERMS: Leishmania major; Saponin; Antibodies; Adjuvant

INTRODUCTION

Leishmania parasites are dimorphic *Trypanosomatidae* being the amastigote the form that parasites mammals. In this stage, the parasites replicate in macrophages, within structures called parasitophorous vacuoles (ALEXANDER ; RUSSELL, 1992). Vertebrate hosts present a broad spectrum of the disease, from self-limited skin ulcers to a progressive visceral disease that may be lethal to the host. Human leishmaniasis can have distinct clinical manifestations depending upon the species of the parasite*: Leishmania major, L. amazonensis* and *L. donovani* will cause the cutaneous (CL), and visceral (VL) leishmaniasis, respectively (BARRAL et al., 1991; SILVEIRA et al., 1991).

The utilization of experimental models to reproduce human leishmaniasis allowed the establishment of immunological parameters associated to the disease. For example, in resistant animals, infection by *L. major* manifests as a self-limited skin disease, whereas susceptibility is characterized by metastatic lesions and death (CHATELAIN et al*.*,1992; SCOTT et al., 1989; SCOTT, 1991). Resistance was associated with a T_H1 response, with secretion of IL-2 and IFN-g, whereas in susceptible animals a T_H2 response is predominant, favoring the humoral immune response (HEINZEL et al*.,* 1991; SCOTT et al*.,*1988).

BALB/c mice, which are highly susceptible to infection by *L. major*, develop a progressive and fatal disease. In the progressive stage of the disease, lymphoid cell populations in these animals contain a high T_H2/T_H1 ratio (BOGDAN et al*.,* 1990; LIEW; DHALIWAL, 1987). Adoptive transfer of T_H1 clones result in protection, while transfer of T_H2 clones lead to disease exacerbation (SCOTT et al*.,* 1988). Therefore, it is important to consider factors that will influence the differentiation from T_H0 to either T_H1 or T_H2 in vaccine design.

DNA vaccines have resulted in protection in experimental cutaneous leishmaniasis, apparently by the inducement of a T_H1 immune response (SJOLANDER et al., 1998). Follador et al. (2002) demonstrated that *L. (L.) amazonensis* (IFLA/BR/67/PH8) antigens were combined with recombinant human GM-CSF as an adjuvant, enhanced IFN-gð production and the number of IFN-gð responders, indicating an improvement in immune response to *Leishmania* antigens.

New approaches for adjuvant production have given promising results, such as the expression of IL-12 subunits

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delivered by adenoviral vectors. Using this approach, BALB/c mice simultaneously infected with *L. major* and the IL-12 vector developed a specific cellular response with local production of IFN-gð (GABAGLIA et al., 1999).

The use of adjuvants such as saponin have demonstrated satisfactory results for intracellular parasites such as *Trypanosoma cruzi* (SCOTT et al., 1984), *Toxoplasma gondii* (KHAN et al., 1991), cytomegalovirus (BRITT et al., 1995) and *Leishmania donovani* (PALATNIK-de-SOUZA et al., 1994a; PALATNIK-de-SOUZA et al.; 1994b; SANTOS et al., 1999; SANTOS et al., 2002). Saponin has several fractions of which A and C are strong candidates for human use, since they have demonstrated low toxicity. Fraction C stimulates IgG2a production whereas fraction A induces the secretion of IFN-g and IL-12 without a concomitant increase in IgG2a (JOHANSON; LOVGREN-BENGTSSON, 1999). Saponin has also been shown to induce class I MHC antigen-restricted cytotoxic T lymphocytes (WU et al., 1994), which favors a T_H1 immune response.

In the present study we aimed to evaluate the protective effect of saponin associated with soluble antigen of *L. major* in a BALB/c model, by analyzing the development and progression of cutaneous lesions as well as the presence of parasites in popliteal lymphnodes after inoculation of parasites in the footpad.

MATERIAL AND METHODS

Mice: Female BALB/c mice between 6 and 8 weeks of age were obtained from the animal breeding facility of this university and housed in 12-hour dark/light intervals.

Parasites: *L. major*, strain MRHO/SU/59/P was obtained from the Immunology Laboratory, Ribeirão Preto Faculty of Medicine. The parasites were inoculated in the footpads of BALB/c mice and the lesion size measured with a dial caliper (Mitutoyo Corporation, Tokyo, Japan). When the diameter of the footpads reached 8mm or more the animals were sacrificed and parasites harvested under sterile conditions by maceration of subcutaneous tissue from the sites of lesion. Parasites were then cultured at 28°C in Schneider Medium (Sigma Chemical Co., St. Louis, MO), containing 2 mM L-glutamine (Sigma), 20% fetal bovine serum (FBS, Life Technologies, Gaithesburg, MD), 100 U/ml penicillin G, 100 µg/ml streptomycin (Sigma) and 2 mM HEPES (Sigma).

Preparation of soluble *Leishmania* antigen: SLA was prepared according to Scott et al. (1987) with some modifications. Briefly, *L. major* promastigotes from cultures in stationary phase were harvested and washed four times by centrifugation at 2200 x *g*, 4°C, 15 minutes each, in sterile phosphate buffered saline (PBS). The concentration of parasites was adjusted to 1 x 10^6 parasites/ml in PBS containing 50 µg/ml leupeptin (Sigma) and 1.6 mM phenylmethylsulfonyl fluoride (PMSF, Sigma) and incubated on ice bath for 10 minutes, followed by six successive cycles of freezing in liquid nitrogen and thawing at 37°C. The lysate was centrifuged at 2200 x *g*, 4°C, 15 minutes and the supernatant collected and centrifuged at 10000 x *g* at 4°C for 30 minutes. The supernatant was then filtered through a 0.22 µm membrane and stored at -70°C until use. SLA protein concentration was determined by the Lowry method using bovine serum albumin (BSA) as standard.

Electrophoretic analysis of *Leishmania* antigens: *L. major* antigenic profile was evaluated in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). The proteins were visualized by silver staining and the molecular weight was compared to the following protein markers run on the same gel: Phosphatase B, 112 kDa; bovine albumin, 84 kDa; ovoalbumin, 84 kDa; carbonic anhydrase, 34.9 kDa; trypsin inhibitor, 28.7 kDa; and lysozyme, 20 kDa (Life Technologies, Gaithesburg, MD, USA).

Immunization: Female BALB/c mice were distributed in four groups, five animals per group, as follows: Group I, control (PBS); Group II, SLA (25 µg) + saponin (7.5 µg/animal; saponin from *Quillaja bark*, Sigma Chemical CO, USA); Group III, saponin (7.5 µg/animal); Group IV, SLA (25 µg/animal). Animals in each group were immunized with three doses. The first immunization was done subcutaneously in the left footpad. After 15 days, the animals were immunized intradermically in the left flank and the last boost was done in the same site after 10 days. Three days after the last immunization, the animals were infected in the right footpad with 1x10^6 metacyclic promastigotes of *L. major*. The lesion size was measured weekly during 10 weeks with a dial caliper (Mitutoyo) and expressed as the mean difference in thickness between the infected footpad and the contralateral uninfected footpad. At the end of 10 weeks, mice were sacrificed and popliteal lymphnodes were collected, measured and processed for immunohistochemistry.

Serum samples: Animals immunized and infected with *L. major* were individually identified and bled through the orbital plexus with capillary glass tubes. The bleedings were done on days 15, 40 and 70 post-infection and the sera collected were stored at -20°C until use.

Immunoblotting: SLA was applied into 12% acrylamide gels, subjected to electrophoresis and then transferred to nitrocellulose membranes (0.45 µm pore size), according to the methods of Towbin et al. (1979) ; Burnett (1981). After transfer, nitrocellulose membranes were cut into strips and blocked with 0.05% Tween 20 and 5% non-fat milk in PBS (PBS-TM) for 2h at room temperature on a slow shaker. The strips were then incubated overnight at 4°C with murine serum samples (control and/or infected with *L. major*) at 1:100 dilution in PBS-TM, followed by washes and the addition of peroxidase conjugate (rabbit IgG anti-mice IgG, Sigma Chemical Co.) at 1:2000 dilution

for 2 hours at room temperature on a slow shaker. The blots were developed with a solution of 10 mg/ml of diaminobenzidine (DAB) and 0.03% of H_2O_2 in PBS, and the reaction stopped by extensive washes in distilled water.

Detection of anti-*Leishmania* antibodies by ELISA: Specific antibodies against *Leishmania* were quantified in individual serum samples by an indirect ELISA technique. Polystyrene microplates (Corning) were coated with 50 µl of a 10 µg/ml solution of SLA in carbonate-bicarbonate buffer (0.06M, pH 9.6) for 18 hours at 4°C. Microplates were then washed in 0.05% Tween 20 in PBS (PBS-T), followed by the addition of serum samples diluted 1:40 in PBS-T. After a 45 minute incubation at 37°C, microplates were washed and the conjugate (peroxidase-labeled goat IgG anti-mouse IgG, Sigma Chemical Co.) was added at a dilution of 1:1000 in PBS-T and incubated at 37°C for 45 minutes. The microplates were washed and the substrate (0.5 mg/ml ortho-phenylenediamine and 0.03% H_2O_2 in citrate-phosphate buffer, pH 5.5) added for 15 minutes. Color development was stopped with the addition of 2N H_2SO_4, after which the reactivity was determined by reading at 492 nm. Results were expressed as an ELISA index (EI), calculated by the formula EI = (S - B)/(N - B), where S is the mean absorbance value of the sample, B is the blank value and N is the mean absorbance value of the negative control.

Immunohistochemistry: To look for the presence of *L. major* in popliteal lymphnodes we performed immunohistochemistry. The lymphnodes were measured with a dial caliper (Mitutoyo) and fixed in 10% formalin buffered with PBS followed by paraffin embedding. Sections of 3 mðm thickness were first deparaffinized by immersion in xylene and then permeabilized in 1% Triton-X-100 for 30 minutes, followed by a blocking step with 2% normal goat serum for another 30 minutes. Sections were then incubated with rabbit serum against *L. major* diluted 1:100 in Tris Buffer Saline (TBS) overnight at 4°C, washed in TBS and incubated with biotinylated goat anti-rabbit IgG. After washes, the tissues were incubated with HRP-streptavidin reagent (Dako Corporation, USA) for 30 minutes, followed by rinsing and development with diaminobenzidine (Sigma Chemical Co., USA) and hydrogen peroxide.

Statistical analysis: Data were analyzed using the Instat™ software and represented as the mean ± standard deviation for each group. The Kruskal-Wallis test was used to determine the significance of antibody indices between groups, and values were considered significant when $p < 0.05$. To analyze statistical differences between groups in relation to lymphnode size we performed the Student-Newman-Keuls multiple comparisons test.

RESULTS

Adjuvant effect of saponin on the experimental infection by *L. major*

BALB/c mice inoculated with SLA (25 mg/animal) and/or saponin (7.5 mg/animal), as well as the control group (PBS) were monitored for 10 weeks. We observed that in the group injected with both SLA and saponin there was an 80% protection against disease progression when compared to the controls (p£ 0.01), whereas in the groups treated with either SLA or saponin alone there was only 40% protection (Figure 1). Also in the group treated with SLA plus saponin the lesion size was smaller as compared to controls (2.55 e 5.88 mm, respectively, p £ 0,05) (Figure 1).

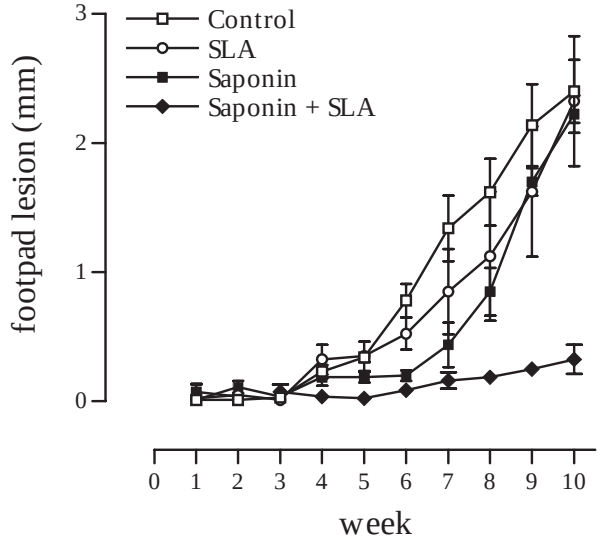

Figure 1. BALB/c mice immunized with saponin + SLA are more resistant to *L. major* infection. Non-immunized mice (control) and mice immunized with either 25 mðg SLA and/or 7.5 mðg saponin/animal were infected with $1x10^6$ metacyclic promastigotes of *L. major* in the footpad. The course of infection was monitored weekly, for ten weeks, by measuring the footpad lesions. Each point represents the difference between the mean of infected and non-infected footpads plus standard deviation (n=5). Data are representative of three independent experiments.

Determination of antibody level by ELISA

To evaluate specific antibody level present in serum samples of immunized BALB/c mice we performed an ELISA assay. In sera from the control group IgG antibody level were higher than in the other groups, with a peak at 70 days post-infection (EI=37.8, Figure 2). In the saponin + SLA group the antibody level were significantly lower (EI=10.3, Figure 2).

SLA antigenic profile recognized by immunized mice

To determine the specificity of the antibodies raised after immunization serum samples were analyzed by western blotting. The most abundant antigenic fraction was of approximately 79 kDa and was recognized by 100% of the sera. As for the other antigenic fractions, there was variation according to the experimental group (Table 1).

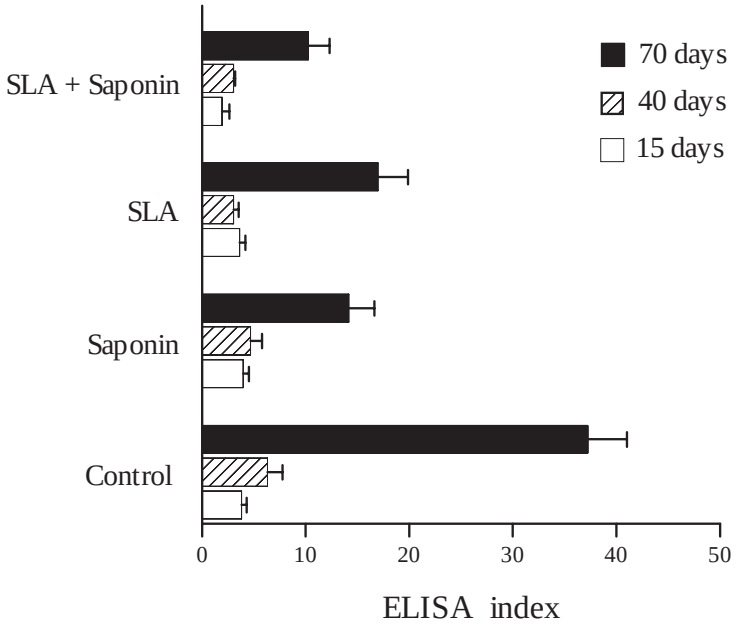

Figure 2. Levels of specific IgG against *L. major*. Serum samples from immunized mice were collected at 15, 40 and 70 days after challenge and analyzed by indirect ELISA. Open, hatched and closed bars represent sera from 15, 40 and 70 days, respectively. Data from three independent experiments are shown as ELISA index plus standard deviation.

Table 1. Percentage recognition of antigenic fractions of SLA, as determined by western blotting, in serum samples of mice submitted to different immunization schedules and challenged with *L. major*.

	Recognition of antigenic fractions per group[a]			
Antigenic fraction (kDa)[b]	Control(n=4)	SLA(n=4)	Saponin(n=4)	SLA+Saponin(n=5)
99	100	33	100	100
91		100	100	80
79	100	100	100	100
68	100	100	50	
57	100		75	
51		100	50	20
42	100	33		
39		66	75	
36	50	66		
27		33		
23	50		25	
18		100		

[a]BALB/c mice were pre-immunized as described in material and methods, after which they were infected with 10^6 *L. major* promastigotes. Serum samples from immunized mice were diluted 1:50 and added to nitrocellulose strips containing immobilized SLA. Results are expressed as the percentage of antibody recognition per group and per antigenic fraction.
[b]SLA presented a protein profile ranging from 18-99 kDa.

The group immunized with SLA + saponin recognized antigenic bands at 80-90 kDa, which may be related to a protective profile.

Immunohistochemistry and measurement of popliteal lymphnode size

To determine the presence of the parasite in regional lymphnodes we performed immunohistochemistry. Popliteal nodes from animals immunized with SLA + saponin were significantly smaller (9.42 ± 4.71 mm^2) than SLA (29,83 ± 10.30 mm^2, p £ 0.05), saponin (22.50 ± 3.27 mm^2, p £ 0.05) or control (29.31 ± 5.01 mm^2, p £ 0.05). Moreover, lymphnodes from the SLA + saponin group had almost no detectable parasites (Figure 3D). Inoculation of either saponin or SLA alone reduced the parasite load on the lymphnodes (Figure 3B and C) when compared to the control group (Figure 3A), without a significant decrease in organ size.

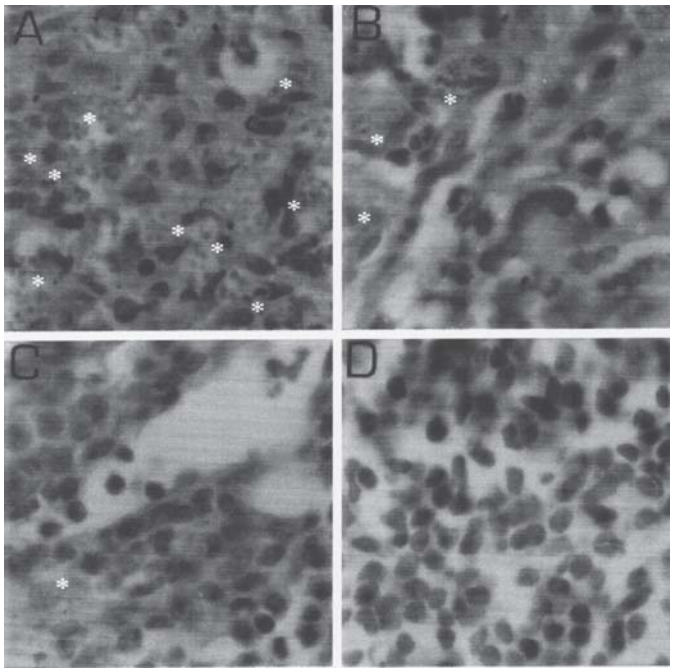

Figure 3. *Leishmania major* amastigotes in popliteal lymphnodes. Lymphnodes from control mice (A) and mice immunized with saponin alone (B), SLA alone (C) or SLA + saponin (D) were embedded in paraffin and sections were stained with antibodies against *L.major*. Note that control mice (A) had several macrophages (*) infected with amastigotes, whereas mice injected with either saponin (B) or SLA (C) alone had fewer infected cells. *L. major* amastigotes were practically undetectable in mice immunized with saponin + SLA (D). Magnification, x 40.

DISCUSSION

The use of adjuvant is often necessary in vaccines for proper stimulation of the immune system toward raising a protective response. Various substances have been tested as adjuvant, nonetheless only aluminum salts are approved by the United Stated Food and Drug Administration (FDA) for human use. However, alum is inconsistent to generate a cellular immune response (COX; COULTER, 1997), which is of great importance for intracellular parasites.

Saponin has been presented as a good adjuvant because of its low toxicity. Scanlen et al. (2002) demonstrated that a crude saponin (saponin Q) induced a less severe local inflammatory reaction than did purified saponin fractions Quil-A and QS-21, being suitable for use as adjuvants. In view of that, in the present work we evaluated the effect of a crude saponin preparation as an adjuvant with soluble antigen of *L. major* in an experimental vaccination schedule in BALB/c mice. Saponin, together with a fucose-manose surface ligand (FML) from *L. donovani* promastigote glycoprotein complex gave 84.4 and 87% protection in BALB/c and hamster models, respectively, against *L. donovani* (PALATNIK-de-SOUZA et al., 1994a ; PALATNIK-de-SOUZA et al., 1994b). An other study, using saponin-*Trypanosoma cruzi* antigen and saponin-ovalbumin in immunization of the mice, was observed high interleukin-10 production predominates, in lymphnode cells, indicating preferential priming towards a TH2 type response (TADOKORO et al., 1996). In the present work we observed an 80% protection in BALB/c mice, which are highly susceptible to *Leishmania*, after immunization with soluble *Leishmania* antigen plus saponin. Palatnik-de-Souza et al. (1994b) demonstrated high titers of specific antibodies against a 68 kDa antigen, while we

have here described an overall low titer of antibodies in sera from immunized animals challenged with *L. major*. This discrepancy may be due to differences in the species of *Leishmania* tested (*L. donovani/ L. major*) as well as diverse route and immunization schedules.

A study by Santos et al. (1999) showed that the FML antigen of *L. donovani* in combination with saponin induced primarily an antibody response in Swiss mice, correlating decrease in parasite load with high titers of IgG2a. In the present work, we observed low antibody level in immunized animals challenged with *L. major,* suggesting effects in another arm of immune response, T cell mediated immunity. Borja-Cabrera et al. (2002) observed that FML-QuilA vaccine induced a significant, long lasting and strong protective effect against canine kala-azar in the field.

Results obtained in this work suggest that saponin may function as an immunomodulator since when it is administered together with SLA there is an apparent protection against disease progression in immunized but not in control animals. This may be due to the preferential stimulation of cellular immunity by saponin. Furthermore, we also detected low levels of specific antibodies, which suggest that protection may not be directly associated with humoral response. It is interesting to note that the regional lymphnodes of mice immunized with the combination of SLA + saponin were of small size and had characteristics of a fibrotic tissue, suggesting a process of wound healing of the areas affected by *Leishmania.*

Overall, our results demonstrated the generation of a protective immune response against *Leishmania major* in this model, probably T cell mediated immunity, and that saponin might be useful as adjuvant in vaccination schedules agat *Leishmania.*

RESUMO: A infecção experimental em camundongos com *Leishmania major* constitui um importante modelo para o estudo da resposta imune do hospedeiro. O presente trabalho avaliou efeito da saponina sobre a leishmaniose, em modelo murino. Foram utilizados camundongos BALB/c, fêmeas, distribuídas em quatro grupos, os quais foram imunizados com antígeno solúvel de *Leishmania* (SLA), SLA + saponina, saponina apenas, ou veículo (PBS). Três dias após a última dose, os camundongos foram infectados, no coxim plantar, com formas promastigotas de *L. major* e, a evolução da doença monitorada por dez semanas. A presença de anticorpos específicos foi analisada nos soros por ELISA e Western Blot e o tamanho da lesão, nas patas, mensurada com especímetro de Mitutoyo. Nos animais imunizados com SLA + saponina, observou-se progressão mais lenta da lesão baixos níveis de anticorpos no soro e reconhecimento de antígenos entre 80 e 90 kDa. Estes resultados sugerem que a adição de saponina ao antígeno potencializou a resposta imune do hospedeiro, conferindo proteção parcial contra a leishmaniose.

UNITERMOS: *Leishmania major*, Saponina, Anticorpos, Adjuvante

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