

## Protective vaccination against murine visceral leishmaniasis using aldehyde-containing *Quillaja saponaria* saponins

C.B. Palatnik de Sousa<sup>a,\*</sup>, W.R. Santos<sup>a</sup>, C.P. Casas<sup>a</sup>, E. Paraguai de Souza<sup>a</sup>, L.W. Tinoco<sup>d</sup>, B.P. da Silva<sup>b</sup>, M. Palatnik<sup>c</sup>, J.P. Parente<sup>b</sup>

<sup>a</sup> Instituto de Microbiologia, “Prof. Paulo de Góes”, Universidade Federal do Rio de Janeiro (UFRJ), CCS, Cidade Universitária, Ilha do Fundão, CP 68040, CEP 21941-590, Rio de Janeiro, RJ, Brazil

<sup>b</sup> Núcleo de Pesquisas em Produtos Naturais, Rio de Janeiro, Brazil

<sup>c</sup> Hospital Universitário Clementino Fraga Filho-Faculdade de Medicina UFRJ, Rio de Janeiro, Brazil

<sup>d</sup> Departamento de Bioquímica, Universidad Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Received 30 July 2003; accepted 6 January 2004

Available online 10 April 2004

### Abstract

The presence of aldehyde groups at C-23 and C-24 of the triterpen aglycon moiety was disclosed in <sup>1</sup>H NMR spectra of both the Riedel de Haen saponin (R) ( $\delta$  9.336) and *Quillaja saponaria* QuilA saponin ( $\delta$  9.348). The sign of the C-28 acylated linked moiety ( $\delta$  176) was present in both saponins, while the  $\delta$  171 at C-28 (carboxy group) corresponding to the deacylated saponin, was only detected in the QuilA preparation, indicating 50% of hydrolysis of the ester moiety, probably due to the storage in aqueous solution. The normoterpen moiety was present in both saponins (signals at  $\delta$  14–18). The chemical removal of saponin glycidic moieties gave rise to their saponin fractions. Their <sup>1</sup>H NMR spectra showed the presence of two signals ( $\delta$  9.226 and 9.236) for saponin R and two signals ( $\delta$  9.338 and 9.352) for the QuilA saponin. The intensity of the signals suggested two conformational isomers of saponin R in the ratio 53% of equatorial aldehyde group to 47% of axial aldehyde group, and two conformational isomers of QuilA saponin in the ratio 76% of equatorial aldehyde group to 24% of axial aldehyde group. The chemical treatment abolished the saponin slight *in vivo* toxicity, reduced their hemolytic potential, did not affect their aldehyde contents, but gave rise to an enriched axial aldehyde-containing saponin R with enhanced potential on antibody humoral response (anti-IgM, IgG, IgG1, IgG2a, IgG2b and IgG3) and to an enriched equatorial aldehyde-containing QuilA-saponin that induced a mainly cellular specific immune response (increased intradermal response to leishmanial antigen and IFN $\gamma$  sera levels) and effective protection against murine infection by *L. donovani* (77% reduction in liver parasitic load). Our results suggest that the Riedel de Haen saponin is probably a *Quillaja saponaria* saponin.

© 2004 Elsevier Ltd. All rights reserved.

**Keywords:** *Leishmania donovani*; Kala-azar; Visceral leishmaniasis; Vaccine; *Quillaja saponaria* Molina; Saponins; Saponin

### 1. Introduction

Pathogen-associated microbial patterns (PAMPs) are evolutionary conserved structural elements common to microbial pathogens (lipopolysaccharide, bacterial lipoproteins, peptidoglycans and CpG motifs) that can induce a pro-inflammatory innate immune response. They are recognized by receptors on the innate immune antigen presenting cells (APCs). This recognition leads to activation of pro-inflammatory cytokines and chemokines and final up-regulation of membrane-bound co-stimulatory molecules

resulting in subsequent activation of innate immune response (reviewed in [1]). Effective adjuvants may provide a non-self, microbial signal or a host-derived danger signal both essential for induction of co-stimulatory molecules.

Adjuvants can be classified depending or not whether they have direct immunostimulatory effect on APC or function as antigen delivery system [2]. LPS derived Monophosphoryl Lipid A, unmethylated CpG motifs and saponins are the three main groups of direct immunostimulatory adjuvants [2]. *Quillaja saponaria* saponins are the adjuvant of veterinary vaccines against canine babesiosis [3], HIV [4] and foot and mouth disease [5], among others. Cumulative references in literature point out their outstanding and specific adjuvant potential [3–5]. The QS21 purified saponin, isolated from QuilA mixture was already used in clinical trials

\* Corresponding author. Tel.: +55-21-256-26742;

fax: +55-21-560-8344/8028.

E-mail address: [clarisaps@infolink.com.br](mailto:clarisaps@infolink.com.br) (C.B. Palatnik de Sousa).

with malaria peptide vaccine [6] and to low dose HIV-1 gp120 [7]. QS-21 is currently under clinical evaluation for various vaccines and has been tested in more than 3000 patients in 60 clinical trials (reviewed in [8]). Some structural characteristics of *Quillaja saponaria* saponins include the presence of an aldehyde group at C-4 and oligosaccharide chains attached to position C-3 and C-28 of their triterpen aglycone [9]. A unique feature of the quillaja saponins appears to be the presence of two normoterpene ester moieties linked linearly to a fucosyl residue attached at the C-28 position. The aldehyde group is crucial for the saponins adjuvanticity, mainly for stimulation of TH1 response [9]. On the other hand, the acylated C-28 linked hydrophobic moiety is responsible for stimulating the production of cytotoxic T cell response (CTL) against exogenous proteins [9,10].

*Leishmania donovani*, the agent of human and canine visceral leishmaniasis is an obligatory intracellular parasite that provokes a severe and frequently lethal disease whose control requires a well-established cellular immune response. For the development of a vaccine against canine visceral leishmaniasis, we analyzed the effect of several adjuvants in formulation with the fucose–mannose ligand of *Leishmania donovani* (FML) antigen [11] in Phase 1-2a trials on the murine model: alumin, Freund incomplete adjuvant (FIA) [12], BCG (*Bacille Calmette Guérin*), Interleukin 12 (IL-12) and Riedel de Haen, QuilA and QS21 saponins [11]. The best performances were obtained with formulations containing QuilA, Riedel de Haen and QS21 saponin (73–93% protection) [11]. We also recently reported that prophylactic canine vaccination with the FML-QuilA vaccine lead to 95% of protection against canine visceral leishmaniasis in exposed vaccinated dogs (80% of vaccine efficacy) of an endemic area for human and canine visceral leishmaniasis [13]. After vaccination with FML-QuilA, a pronounced decrease in human kala-azar cases was also detected [13]. The effect of the FML-QuilA vaccine was noted as well in the immunotherapy treatment of *L. donovani* and *L. chagasi* infected dogs [14]. In this case, the adjuvant effect was demonstrated in the enhanced or sustained higher levels of the protective antibody subtype (IgG2) and delayed type of hypersensitivity response to the leishmanial antigen, and in the reduced number and extent of clinical signs. Also, CD4 lymphocyte percent values in peripheral blood mononuclear cells (PBMC), that are normally decreased in canine kala-azar, correlating with dog infectivity to phlebotomines [15–17], remained within the range of normality, as compared with normal uninfected dogs from the endemic area. Noteworthy, the CD8 percents were significantly increased in all immunotherapy treated dogs over the normal values, as expected for the enhanced CTL response, after a Quillaja saponin vaccine treatment [9].

It has been recently demonstrated that the lipophilic acyl side chain of Quillaja saponins which is responsible for the remarkable stimuli for CTL production against exogenous proteins also appears to be responsible for the Quillaja saponin toxicity and instability under physiological

conditions [9]. The deacylated saponins are significantly less toxic and capable of eliciting a TH2 response while fail to stimulate either a lymphoproliferative response or the formation of CTL (CD8) [9]. Spontaneous deacylation would occur in aqueous solution or mild alkaline conditions (reviewed in [18]). This deacylation process is disadvantageous for vaccine formulation since storage would lead to a loss of vaccine capacity [9,18]. In order to stabilize vaccine formulations semi-synthetic analogs of quillaja saponins were designed by replacing the unstable lipophilic ester moiety with other hydrophobic chain linked by a stable amid-bond to the carboxyl group of glucuronic acid in C-3 position of triterpen (GPI-0100) [9,8]. This was performed either on total Quillaja saponin mixture (GPI-0100) [9] or in QS21 purified saponin (RDS-1)[8]. While GPI-0100 can stimulate a Th1 antibody isotype profile (IgG2a) as well as CTL production against exogenous antigens [9] RDS-1 showed moderate IgG2a response but did not sustained the CTL response at any dose evaluated [8]. Even in the case of recovered activity the reacylated adjuvant however, was 20-fold less potent than the native one [8,9].

On the other hand, the aldehyde of the Quillaja saponins may form an imine group (Schiff base) with amino groups on the surface of certain T cell-receptors to provide a co-stimulatory signal that is transduced by the receptor [9,19]. The aldehyde group may replace the B-7 ligand as a co-stimulatory signal and thus bias the immune system toward a Th1 response [9]. This signal would not be down regulated by CTLA-4 receptor [9,19]. Chemical modification of the Quillaja saponin aldehyde-group but not of the carboxyl group of glucuronic acid [20] results in the complete loss of their adjuvanticity. Effective immunopotentiatory, aldehyde-containing drugs were developed base on the discovery that small exogenous Schiff-base forming molecules could substitute for the natural donor of carbonyl group and provide a co-stimulatory signal to CD4<sup>+</sup> T helper cells [19].

Previous work demonstrated the hemolytic activities of saponins are related to their chemical structure [21–23]. The hemolytic effect is stronger in steroid than in triterpen saponins [21,22]. Also, in triterpen saponins, the size of the attached glycidic chains modulates the HD<sub>50</sub> [21]. The saponin of *Periandra mediterranea*, for instance, has a single sugar chain attached to carbon C-3 via oxygen; this chain is composed of two residues of glucuronic acid. The low hemolytic effect of this triterpen saponin (HD<sub>50</sub> = 250 µg ml<sup>-1</sup>) was abolished by removal of the glycosidic moiety (HD<sub>50</sub> > 500 µg ml<sup>-1</sup>) [21]. This might be one of the cases in which the membranolytic activity may be attributed to the oligosaccharide moiety of the saponin [23]. The analysis of saponin and saponin fractions isolated from *Bredemeyera floribunda* showed that also in this case the removal of the glycidic moiety abolished the undesirable hemolytic activity maintaining however the adjuvant potential [24].

In the present investigation we aimed to: (1) evaluate the integrity of the QuilA saponin preparation that was stored

in frozen aqueous solution for 0–5 years before use in the prophylactic and immunotherapeutic vaccination of dogs against canine visceral leishmaniasis [13,14]; (2) compare its chemical structure to the undisclosed and also highly potent Riedel De Haen saponin and (3) analyze the potential effect of the total chemical removal of the glycidic moieties on the possible reduction of in vivo and in vitro toxicity and on the adjuvant potential of both triterpen saponins in the murine model of visceral leishmaniasis.

## 2. Material and methods

### 2.1. Isolation and purification of saponins

One gram of Riedel De Haën Saponin pure (R) (8047-15-2, EINECE, West Germany) and of 1 g of *Quilaja saponaria* Molina, QuilA purified saponin (Superfos Biosector a/s, and, Kvistgaard, Denmark) were treated with 100 ml 2N H<sub>2</sub>SO<sub>4</sub> at 100 °C for 1 h. The reaction mixture was cooled at room temperature and slowly deposited white amorphous powder which was collected by filtration, washed with water, and dried. The residue was subjected to column chromatography on silica gel (10 g, 0.6 × 10 cm), eluted with chloroform-methanol (97.5:2.5, (v/v)) (5 ml each eluent). The fractions 40–45 ml showed similar thin layer chromatography profiles in the above described solvent system (*R<sub>f</sub>* 0.40). The product (96 mg) was identified as quillaic acid by spectroscopic methods.

### 2.2. NMR spectroscopy

100 mg of commercial saponin R and 100 mg of QuilA saponin mixture (Superfos Biosector, Denmark) and 100 mg of each respective saponin fraction were solubilized in purified aqueous solution. All the samples were further exchanged with D<sub>2</sub>O, with intermediate lyophilization and then dissolved in 0.6 ml DMSO-*d*<sub>6</sub>. The <sup>13</sup>C and <sup>1</sup>H NMR spectra were acquired on a Bruker DRX-400 NMR spectrometer with tetramethylsilane ( $\delta = 0.00$ ) used as internal standard on a 100.613 MHz frequency using a 5 mm Broad Band inverse probe at 25 °C.

### 2.3. Hemolytic assay

Normal human red blood cell suspension (0.6 ml of 0.5%) was mixed with 0.6 ml diluent containing 5, 10, 20, 30, 40, 50, 100, 250 and 500  $\mu\text{g ml}^{-1}$  concentrations of the Riedel De Haën and the QuilA total saponins and saponin fractions in saline solution. Mixtures were incubated for 30 min at 37 °C and centrifuged at  $70 \times g$  for 10 min. Free hemoglobin in the supernates was measured by absorbance at 412 nm [21]. Saline and distilled water was included as minimal and maximal hemolytic controls. The hemolytic percent developed by the saline control was subtracted from

all groups. The adjuvant concentration inducing 50% of the maximum hemolysis was considered the HD<sub>50</sub> (graphical interpolation). Each experiment included triplicates at each concentration. A series of 2–5 independent experiments was performed for the analysis of each HD<sub>50</sub>. Human red blood cells for the hemolytic assay, were obtained from healthy adult blood bank donors (Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, RJ, Brazil) [21]. The red blood cell suspension was prepared by finally diluting the pellet to 0.5% in saline solution.

### 2.4. In vivo saponin toxicity

Toxicity (assessed by lethality, local pain, local swelling, loss of hair) was tested in Swiss Albino female mice. 200  $\mu\text{g}$  of either Riedel De Haën or QuilA saponins dissolved in sterile saline were injected subcutaneously (s.c.) on the back in mice, as three weekly doses. The mice were monitored during 7 days. As positive controls, the toxic effect of total Riedel De Haën and QuilA saponin mixtures were monitored.

### 2.5. Immunization and infection of mice

Animals were immunized with three weekly doses of the FML antigen of *L. donovani* (150  $\mu\text{g}$ ) [11] and 100  $\mu\text{g}$  of Riedel De Haën saponin (two experiments, each one with  $n = 10$  each), QuilA saponin (one experiment with  $n = 10$ ) in 200  $\mu\text{l}$  sterile saline solution through the subcutaneous route in the back of 2 month old Swiss Albino females. Saline and adjuvant-treated animals were included as controls ( $n = 10$  for each group). Isolation and chemical characterization of the fucose mannose ligand (FML) obtained from stationary-growth phase promastigotes of *L. donovani* Sudan (LD 1S/MHOM/SD/00-strain 1S) was performed as previously described [25]. 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 [25]. The FML-vaccine is registered as a Patent: INPI number: PI1100173-9 (18.3.97). Federal University of Rio de Janeiro, Brazil. Ten days after immunization, animals were challenged by intravenous injection of  $2 \times 10^8$  amastigotes (*Leishmania (L.) donovani* LD-1S/MHOM/SD/00-strain 1S), obtained from infected hamster spleens. Animals were sacrificed 30 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 per 1000 cell nuclei × mg organ weight). Sera of animals before and after infection were analyzed by FML-ELISA and delayed type of hypersensitivity assay against leishmanial antigen was measured 7 days after complete immunization, before infection, and 30 days after infection.

## 2.6. Immunological assays

The anti-FML antibody levels were assayed in all the vaccinated groups using the FML-ELISA as previously described [11], with 2 µg antigen per well and: goat anti-mouse IgG or anti-IgM peroxidase conjugate (Sigma) or goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 horseradish peroxidase conjugated antibodies (Southern, Biotechnology Associates, Birmingham, AL, USA) in a 1:4000 dilution in blocking buffer. The reaction was developed with *O*-phenyldiamine (Sigma), interrupted with 1N sulfuric acid, and monitored at 492 nm. Sera were analyzed by double-blind tests, in triplicate. Positive and negative control sera were included in each test. Results were expressed as log<sub>2</sub> titers. According to conventional serology, titrations differing by two or more dilutions are significant.

The delayed type hypersensitivity (intradermal reaction to promastigote lysate) was determined by injecting mice intradermally, 10 days after the complete immunization, and 27 days after infection, in the right hind footpad, with 10<sup>7</sup> 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 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 [11].

The Gamma interferon (IFN-γ) level in serum was measured by enzyme-linked immunosorbent assay (ELISA) [11]. Capture and biotinylated monoclonal antibodies for gamma interferon (IFN-γ) (R4-6A2, XMG1.2) and the recombinant cytokine was purchased from Pharmingen (San Diego, Calif., USA). Levels of IFN-γ were determined following manufacturer's instructions (Pharmingen). Briefly, anti-cytokine capture antibody was diluted (1 µg ml<sup>-1</sup>) in binding solution, incubated overnight at 4 °C on Nunc maxisorb immunoplates, washed, blocked and further incubated with sera samples diluted in glycerol (1:1) for 2 h, at room temperature. The biotinylated anti-cytokine detection antibody (1 µg ml<sup>-1</sup>) diluted in blocking buffer, was added and incubated for 1 h at room temperature. Development of the reaction was performed with streptavidin-horseradish peroxidase (Dako) and *O*-phenyldiamine (Sigma) and stopped with sulfuric acid 1N. Reading of the optical density was done in a microplate reader set to 492 nm. A standard curve using preparations with known concentration of mouse recombinant r-IFN-γ was performed for each assay. The detection limit was 15 pg ml<sup>-1</sup> [11].

Means were compared by a standard *t*-test, ANOVA analysis, simple factorial test and by one way ANOVA, Student–Newman–Keuls method (SPSS for windows). Correlation coefficient analysis was determined on a Pearson bivariate, two-tailed test of significance (SPSS for windows).

## 3. Results

### 3.1. NMR analysis of saponin

Samples of the Riedel de Haen saponin (R) and of *Quillaja saponaria* Molina QuilA saponin mixture were both solubilized in purified aqueous solution (100 mg ml<sup>-1</sup>) showing pH = 5.5. After lyophilization, the <sup>13</sup>C NMR spectra of both saponins in DMSO-*d*<sub>6</sub> were recorded. Results are shown in Fig. 1. Two signals at δ -210.8 suggest the presence of aldehyde groups at C-23 and C-24 (Fig. 1). Also, the characteristic sign of the C-28 acylated linked moiety (δ 176) is present in both saponins, while the δ 171 at C-28 (carboxy group) corresponding to the deacylated saponin, is only detected in the QuilA preparation, indicating 50% of hydrolysis of the ester moiety, probably due to the storage in aqueous solution. The saponin R, freshly analyzed from the lyophilized preparation showed no signal of deacylation. The presence of the normoterpen moiety was confirmed in both saponins (signals at δ 14–18).

The <sup>1</sup>H NMR spectra of both saponins is shown on Fig. 2. The presence of aldehyde groups at C-23 and C-24 is disclosed by the signals δ 9.336 and 9.348 in saponin R and saponin QuilA, respectively. This was confirmed by the <sup>1</sup>H NMR of the sapogenins which showed the presence of two signals at δ 9.226 and 9.336 for sapogenin R and two signals at δ 9.338 and 9.352 for the QuilA sapogenin (Fig. 3). The intensity of the signals suggested two conformational isomers of sapogenin R in the ratio 53% of equatorial aldehyde group and 47% of axial aldehyde group, and two conformational isomers of QuilA sapogenin in the ratio 76% of equatorial aldehyde group and 24% of axial aldehyde group. Our results suggest that the Riedel de Haen saponin is probably a *Quillaja saponaria* saponin.

### 3.2. In vivo toxicity assay and in vitro hemolysis

The hemolytic activities of the sapogenin fractions were compared to their respective total saponin preparations. The HD<sub>50</sub> are represented in Table 1. The QuilA saponin was the most hemolytic, followed by saponin R. The hemolytic effect was drastically decreased in sapogenins. No lethality was detected after treatment with three doses of 200 µg of each saponin, nor local pain or swelling (Table 1). Only loss

Table 1  
Toxicity in vivo and haemolytic effect of saponins

| Adjuvant        | Local pain | Swelling | Loss of hair | Lethality | HD50 (µg ml <sup>-1</sup> ) |
|-----------------|------------|----------|--------------|-----------|-----------------------------|
| R saponin       | 0/9        | 0/9      | 2/9          | 0/9       | 25                          |
| R sapogenin     | 0/9        | 0/9      | 0/9          | 0/9       | 238                         |
| QuilA saponin   | 0/9        | 0/9      | 4/9          | 0/9       | 3                           |
| QuilA sapogenin | 0/9        | 0/9      | 0/9          | 0/9       | 298                         |

Note: Results are expressed as number of mice per group that showed any reactivity within 7 days after each one of the three subcutaneous injections of 200 µg saponin in the back of each animal.

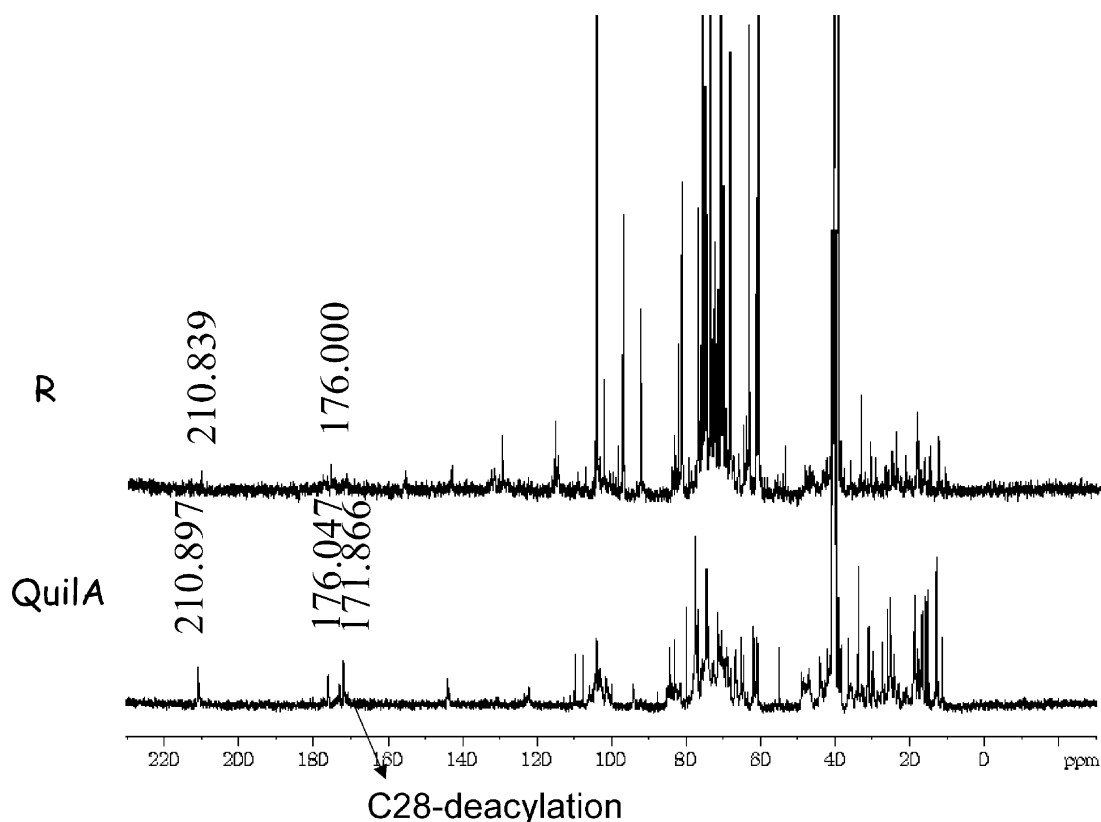


Fig. 1. Characteristic carbon signals in NMR spectra of 100 mg of the QuilA saponin (A) and 100 mg of the saponin R (B) in 0.6 ml DMSO- $d_6$  at 25 °C. Some assignments are indicated.

of hair at the local of injection was detected in 4 animals treated with QuilA and 2 animals treated with saponin R. These slight deleterious effects were abolished after chemical treatment of the saponins. Indeed no *in vivo* toxicity was found in the saponin treated mice.

### 3.3. Vaccination assays with saponin adjuvants

Animals were immunized with three weekly doses of the FML antigen of *L. donovani* and Riedel De Haën (R) or QuilA (Q) saponins. Seven days after immunization, and 30 days after infection with *Leishmania donovani* amastigotes, sera of animals were collected and analyzed by FML-ELISA (Fig. 4). ANOVA analysis disclosed significant variation among treatments only for IgG ( $P = 0.003$ ) and for IgG1 ( $P = 0.012$ ). According to conventional serology, titration differing in two or more dilutions is significant. Before infection, and although all FML-treated groups showed increase in antibody titers over their saline controls, only the saponin R-FML treatment led to a specific increase in anti-IgM, IgG, IgG1, IgG2a, IgG2b and IgG3 antibodies over their respective adjuvant control, showing also the highest absorbency values. The QuilA saponin-FML vaccine, on the other hand, showed mainly non-specific reactions. Comparing to the antibody titers before infection, no significant increases in any type or subtype of antibodies

were disclosed after infection (ANOVA analysis;  $P > 0.05$ ). On the other hand, after infection, significant increases over saline and adjuvant controls were only found in saponin R-FML treated animals, for IgG, IgG1 and IgG2a and in saponin QuilA-FML treated animals for IgG1 and IgG2b. The chemical removal of R saponin glycidic moieties released then a potent humoral aldehyde-containing triterpen adjuvant that still enhances the synthesis of the protective subtypes of immunoglobulins. The same treatment on QuilA saponin, yielded also an aldehyde-triterpen saponin that induced however, mostly lower and unspecific antibody responses against FML antigen.

The increases in anti-FML immunoglobulin titers were highly significantly correlated between all classes and subtypes, but IgM and IgG3 ( $P = 0.000$ – $0.044$ ).

The intradermal response to the *L. donovani* f/t lysate antigen is represented in Fig. 5 (top). The footpad swelling was evaluated at 0, 24, 48 and 72 h after antigen injection. The IDR values were already subtracted from their respective saline control. The values obtained before infection are represented on the left and those obtained after infection on the right. ANOVA analysis disclosed highly significant differences in IDR responses between all different treatments ( $P < 0.0005$ ;  $F = 6.181$ ) only at 24 h. Also, IDR values were significantly higher, after infection than before, at all tested times ( $P < 0.0005$ – $0.025$ ).

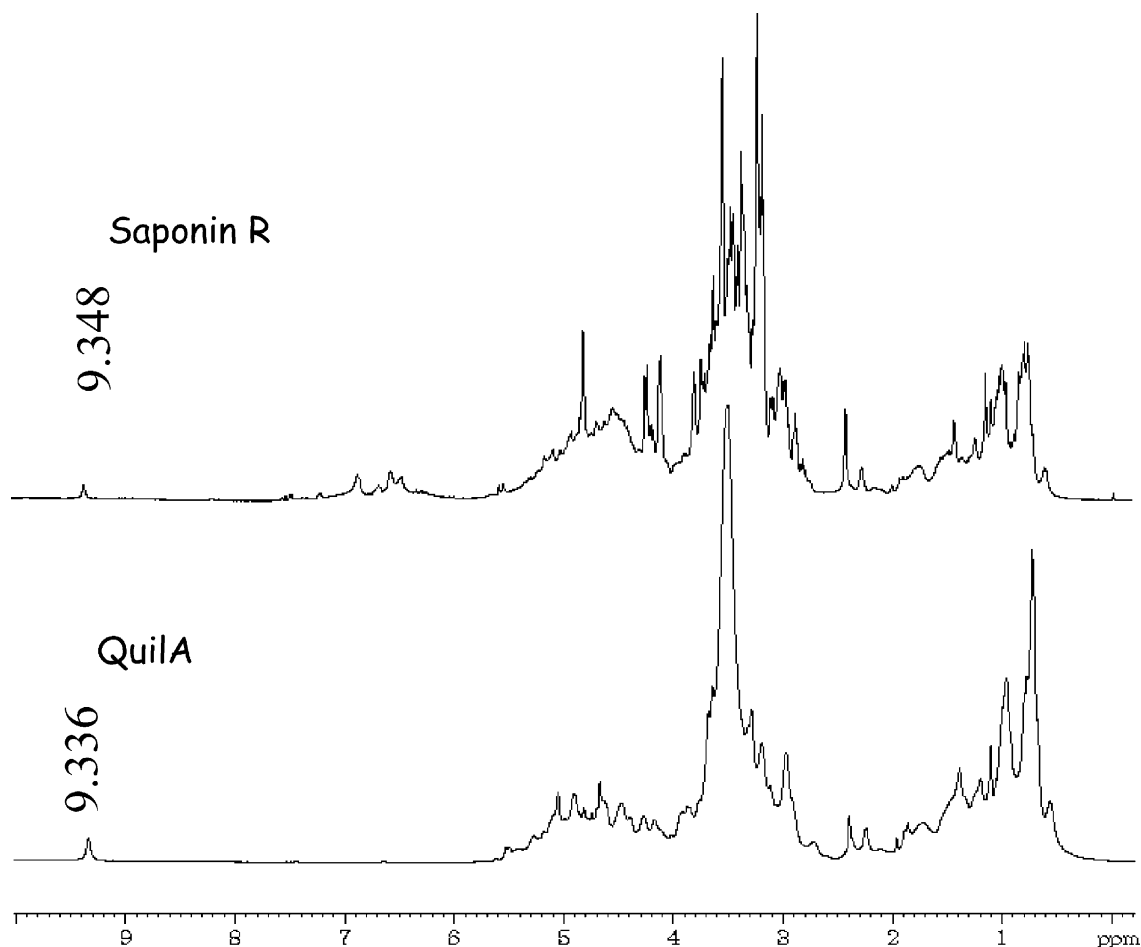


Fig. 2. Characteristic hydrogen signals in NMR spectra of 100 mg of the QuilA saponin (A) and 100 mg of the saponin R (B) in 0.6 ml  $\text{DMSO-}d_6$  at 25 °C. Some assignments are indicated.

As shown in Fig. 5, significant increases in intradermal reaction to *L. donovani* antigen were only detected 24 h after injection ( $P = 0.0001$ ). At this time, only the QuilA sapogenin and the QuilA sapogenin-FML groups were different from their saline control (Student–Newman–Keuls test, level 0.50). Therefore, animals treated with QuilA sapogenin-FML, but not with R sapogenin-FML induced a significant IDR reaction over their saline controls. These enhancements however, were the result of the unspecific effect of the QuilA sapogenin treatment which itself enhances IDR. Indeed, while the chemical removal of the saponin glycidic fractions completely abolished the IDR induction potential of the R saponin it only reduced the potency and specificity of the QuilA saponin treatment.

The analysis of interferon levels was carried out in sera of vaccinated and control animals, 30 days after infection. The results, summarized in Fig. 5 (center). The most pronounced significant and specific  $\text{IFN-}\gamma$  increase (53.4%) was found in animals treated with QuilA sapogenin-FML. This increase was significantly different from that of the saline control ( $P < 0.005$ ) and the sapogenin control ( $P < 0.05$ ) groups.

These results pointed out that the chemical removal of the triterpen glycidic fractions abolished the induction of  $\text{IFN-}\gamma$  secretion of the Riedel de Haen saponin while maintained that of the QuilA saponin.

The reduction of liver parasitic load in response to each FML-vaccine formulation, is shown in Fig. 5 (bottom). No significant differences were detected between the saline, adjuvant control and the R sapogenin-FML vaccine treatment. The only specific reduction of liver parasitic load was achieved by the QuilA-FML sapogenin treated animals which showed 77% of protection, significantly different from saline ( $P < 0.005$ ) and from sapogenin controls ( $P < 0.025$ ). This means that protection was related to the antigen-adjuvant formulation and not merely to a non-specific adjuvant effect.

Taken together, the chemical removal of triterpen glycidic moieties from Riedel De Haen and QuilA saponins abolished their slight toxicity, did not affect their aldehyde contents, but gave rise to an enriched axial aldehyde-containing R-sapogenin with enhanced potential on antibody humoral response and to an equatorial enriched aldehyde-containing QuilA-sapogenin that induced a mainly cellular specific

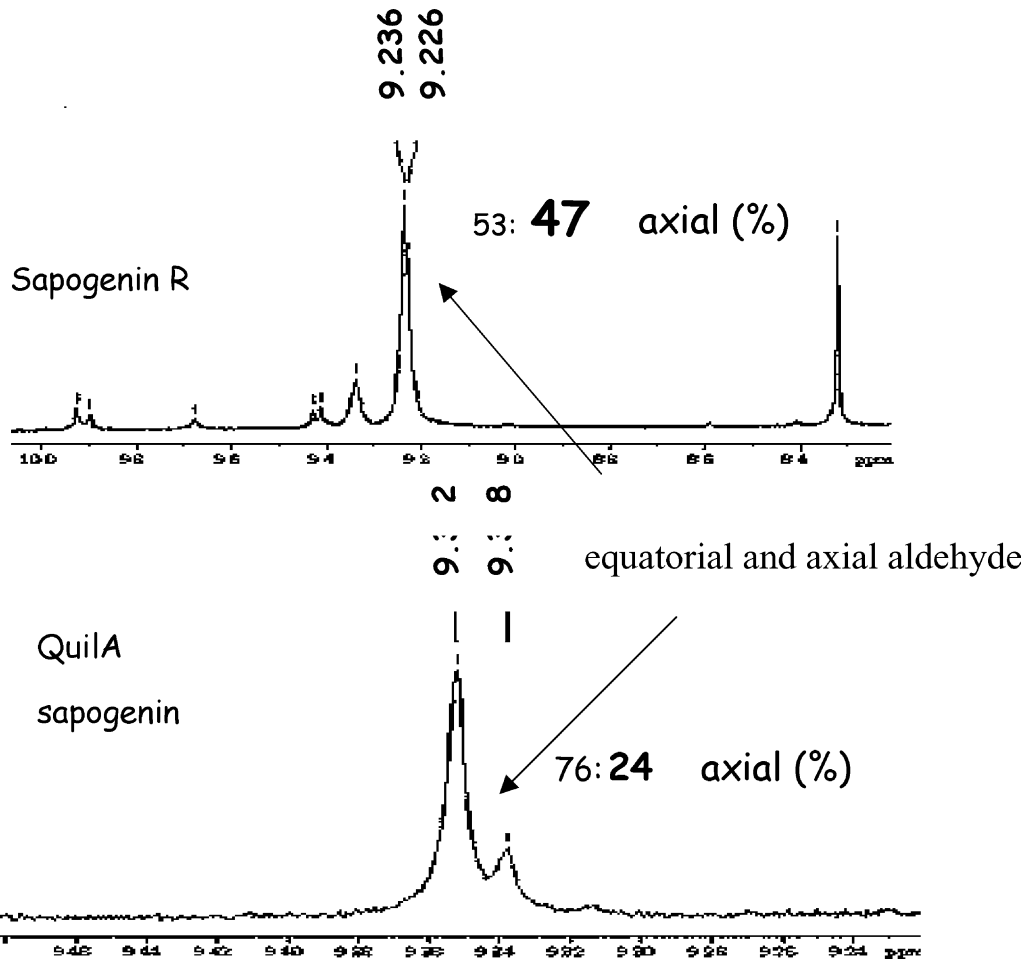


Fig. 3. Characteristic hydrogen signals in NMR spectra of 100 mg of the sapogenin R (A) and 100 mg of the sapogenin QuilA (B) in 0.6 ml DMSO-*d*<sub>6</sub> at 25 °C. Some assignments are indicated.

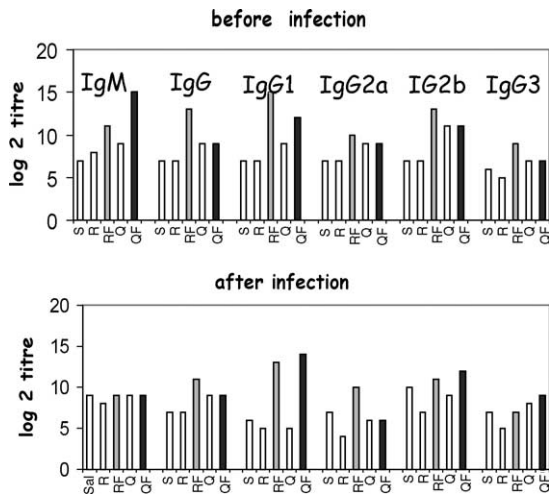


Fig. 4. Anti-FML IgG, IgM, IgG1, IgG2a, IgG2b and IgG3 antibodies titers before and after infection. Swiss Albino mice were vaccinated with the FML antigen of *Leishmania donovani* (F) and sapogenin R (R) or sapogenin QuilA (Q). Control animals received only saline (S) or adjuvant, as indicated. The y axis represents the FML-ELISA log<sub>2</sub> titers of the pool of sera obtained from each treatment (two experiments, each with *n* = 10), before and after infection.

immune response and effective protection against murine infection by *L. donovani*.

#### 4. Discussion

Although *Quillaja saponaria* saponins have been widely used for effective vaccination against experimental cancer, viral, bacterial and protozoan infections (reviewed in [26]) and commercial veterinary vaccines. Some slight toxic undesirable effects however, still limit their wide use for human vaccination [26]. Several clinical trials in humans are being performed using QS21 saponin at lower dosages than recommended for veterinary vaccines. The saponin fatty acid moiety is also the responsible for the saponin toxicity evident by hemolytic activity [11,27], mice lethality [18,27] and loss of hair [11]. This moiety is also responsible for saponin instability and is spontaneously lost at mild alkaline conditions or aqueous solutions by deacylation what leads to reduction of toxicity and of their capacity to stimulate an effective cytotoxic T cell response which is crucial for vaccines against obligatory intracellular parasites [18]. Aiming to reduce tox-

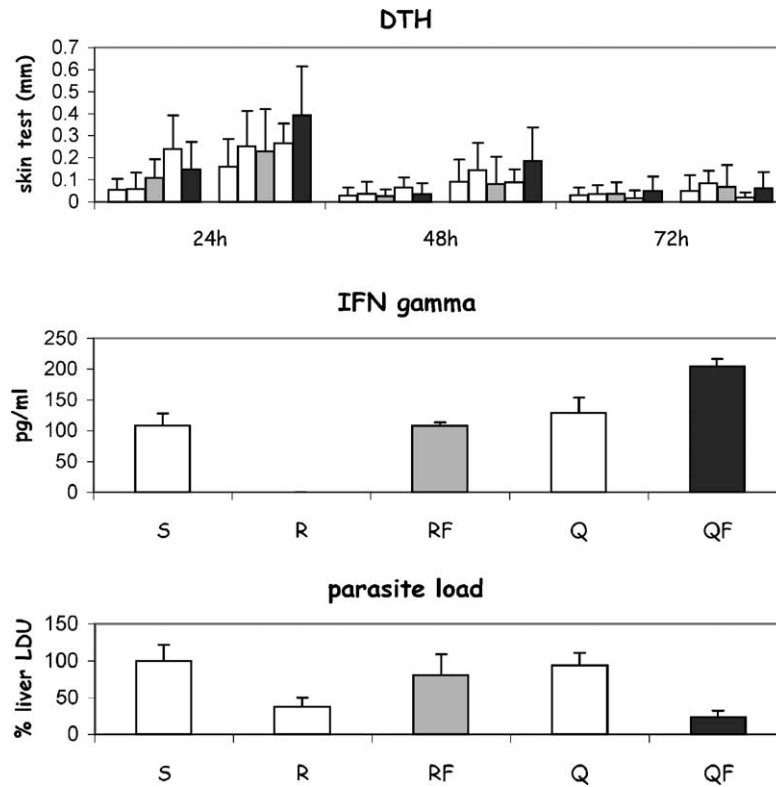


Fig. 5. (A) Delayed type hypersensitivity in Swiss Albino mice immunized with the FML antigen of *Leishmania donovani* (F) and and saponin R (R) or saponin QuilA (Q). Control animals received only saline (S) or adjuvant, as indicated. The y axis represents the thickness of skin test in mm. From right to left: the intradermal reaction, both before and after infection, at 24, 48 and 72 h after injection with  $10^7$  freeze-thawed stationary phase promastigotes of *L. donovani*. At each time, the values of the saline control were subtracted from the reaction due to *Leishmania* antigen. (B) Analysis of interferon- $\gamma$  levels in sera of vaccinated and control animals, after infection with *L. donovani*. The y axis represents the levels of interferon- $\gamma$ , detected by a specific ELISA assay, expressed as the ratio achieved by each treatment group over its saline control. (C) Liver parasitic burden in FML-vaccinated Swiss Albino mice after infection with *Leishmania donovani*. The y axis represents the liver parasitic load in percent of Leishman Donovan units (LDU = number of amastigotes per 1000 cell nuclei  $\times$  milligram organ weight), 30 days after infection with  $10^8$  amastigotes of *L. donovani*, by the endovenous route. Horizontal lines represent the standard deviation. (two experiments, each with  $n = 10$ ).

icity without loss of adjuvant potential, several strategies were designed. Combinations of QS21 and of QS7, another predominant *Quillaja saponaria* saponin which lacks the long hydrophobic C-28 attached moiety, reduced the previous described QS21 toxicity [28] maintaining its adjuvant potential. Another approach was the development of semi-synthetic saponins by chemical deacylation of the C-28 linked moiety and replacement by another lipophylic chain, linked by a stable amid bond to the saponin glucuronic acid attached to C-3. This has been performed on the entire saponin mixture (GPI-0100) [9] or to the purified QS21 saponin [8]. Both treatment reduced toxicity but also adjuvant potential in a 20 fold. In this investigation, we described an alternative method, which completely removes toxicity of *Quillaja saponaria* saponins and sustains adjuvant potential.

In previous investigation, working with the same infection model, we described a typical Th1/Th2 antibody response against the FML antigen using the Riedel de Haen saponin or the QuilA or the QS21 saponin with strong specific enhancements of IgG1, IgG2a and IgG2b [11]. The QS21FML and QuilAFML groups achieved the highest IgG2a response. QuilAFML developed the strongest DTH

and QS21FML animals showed the highest serum IFN- $\gamma$  concentrations. The reduction of parasitic load in liver in response to each FML-vaccine formulation was: 73% ( $P < 0.005$ ) for saponin R-FML, 93% ( $P < 0.005$ ) for saponin QuilAFML and 79.2% ( $P < 0.025$ ) for QS21FML treated animals, respectively. Protection was specific for saponin RFML and QS21FML while the QuilA saponin treatment itself induced 69% of LDU reduction [11]. In the present investigation, although a typical Th1 humoral response was lost for the QuilA saponin, a Th1 cellular immune response remains still active. Indeed, active and specific IDR, secretion of IFN- $\gamma$  and a pronounced reduction of LDU in liver were detected after FMLQuilA-saponin vaccination. The increase of serum IFN- $\gamma$  levels was not correlated to the increase in IgG2a neither for QuilA saponin-FML [11] nor for QuilA saponin-FML treatment (this investigation). A slight reduction in potency at these variables was found: footpad thickness average was 0.7 mm for saponin QuilA-FML [25] and 0.4 mm for saponin QuilA-FML (this investigation). This difference however was not significant. Protection in liver parasitic load reduction was 93% for QuilA saponin-FML [11] and

77% for QuilA saponin-FML, the difference being not significant.

Our results indicate that the sole presence of the intact triterpen-aldehyde at C-23 might be enough to maintain a strong Th1 response. In agreement with that, Soltysik demonstrated the abolishment of adjuvant potential of QS-21 by the chemical treatment of this specific aldehyde [20]. Furthermore, our results suggest that the proportion of conformational isomers of the triterpen-aldehyde is crucial for the integrity of the Th1 adjuvant response. It seems that axial aldehyde are more important in humoral immune response while equatorial aldehyde are more relevant to the cellular protective immune response.

Regarding the integrity of QuilA saponin in aqueous solution, as described by Marciani [9], our QuilA preparation showed indeed 50% of deacylation. The pH of the saponin solution was maintained in acid conditions even after 5 years of storage. Confirming Marciani's results [18], the slight toxic effects: hemolysis and loss of hair in local of injection were indeed stronger in QuilA saponin than in Riedel de Haen saponin that did not show deacylation at all. Previous immunotherapeutic vaccination of dogs with 1mg QuilA saponin each dose also led to mild apathy, local pain and edema that lasted for 24 h after vaccine injection [14]. No such effects were detected in the experimentally infected dogs submitted to immunotherapy with Riedel de Haen saponin. The cellular immune response induced in dogs by the FML/QuilA showed that despite deacylation, protection against visceral leishmaniasis was effective with normal CD4 and enhanced CD8 lymphocytes.

In this investigation, we confirmed the importance of the triterpen-aldehyde of Quillaja saponins in inducing a co-stimulatory signal and pointed out the biological relevance of the spatial position of this aldehyde relative to the triterpen aglycone. These results might help in the further development of semi-synthetic saponins and immunopotentiatory drugs. Our results also indicate that the Riedel de Haen saponin might be a *Quillaja saponaria* derived fraction.

## Acknowledgements

This study received financial support from: National Council for Scientific and Technological Development (CNPQ); MCT/PRONEX (Brazilian Ministry of Science and Technology); "José Bonifácio" University Foundation of the Federal University of Rio de Janeiro (FUJB-UFRJ), Rio de Janeiro State Research Foundation (FAPERJ), Council for Graduate Studies-UFRJ (CEPG-UFRJ), Brazil, and Fort Dodge Animal Health, Brazil and USA. Dr Carmen Nogueira from the Hemotherapy Service of the Hospital Universitário Clementino Fraga Filho-Faculdade de Medicina UFRJ, Rio de Janeiro, is gratefully acknowledged for the human red blood cell suspensions.

## References

- [1] Schijns VEJC. Mechanisms of adjuvant activity: initiation and regulation of immune responses by vaccine adjuvants. *Vaccine* 2003;21:829–31.
- [2] Singh M, O'Hagan DT. Recent advances in veterinary vaccine adjuvants. *Int J Parasitol* 2003;33:469–78.
- [3] Schetters TPM, Kleuskens J, Scholtes N, Bos HJ. Vaccination of dogs against *Babesia canis* infection using parasite antigens from in vitro culture. *Parasite Immunol* 1992;14:295–305.
- [4] Bomford R, Stapleton M, Winsor S, Mc Night A, Andronova T. The control of the antibody isotype response to recombinant immunodeficiency virus gp120 antigen by adjuvants. *AIDS Res Hum Retrovir* 1992;8:1765–71.
- [5] Cox SJ, Barnett PV, Dani P, Salt JS. Emergency vaccination of sheep against foot-and-mouth-disease: protection against disease and reduction in contact transmission. *Vaccine* 1999;17:1858–68.
- [6] Nardin EH, Oliveira GA, Calvo-Calle JM, Castro ZR, Nussenzweig RS, Schmeckpeper B, et al. Synthetic malaria peptide vaccine elicits high levels of antibodies in vaccines of defined HLA genotypes. *J Infect Dis* 2000;182:1486–96.
- [7] Evans TG, McElrath MJ, Matthews T, Montefiori D, Weinhold K, Wolff M, et al. NIAID AIDS Vaccine Evaluation Group. QS-21 promotes an adjuvant effect allowing for reduced antigen dose during HIV-1 envelope subunit immunization in humans. *Vaccine* 2001;19:2080–91.
- [8] Liu G, Anderson C, Scaltreto H, Barbon J, Kensil CR. QS-21 structure/function studies: effect of acylation on adjuvant activity. *Vaccine* 2002;20:2808–15.
- [9] Marciani DJ, Press JB, Reynolds RC, Pathak AK, Pathak V, Gundy LE, et al. Development of semi-synthetic triterpenoid saponin derivatives with immune stimulating activity. *Vaccine* 2000;18:3141–51.
- [10] Cox JC, Coulter AL. Adjuvants: a classification and review of their modes of action. *Vaccine* 1997;15:248–56.
- [11] Santos WR, de Lima VMF, Paraguai de Souza E, Bernardo RR, Palatnik M, Palatnik de Sousa CB. Saponins, IL12 and BCG adjuvant in the FML-vaccine formulation against murine visceral leishmaniasis. *Vaccine* 2002;21:30–43.
- [12] Santos WR, Paraguai de Souza E, Palatnik M, Palatnik de Sousa CB. Vaccination with the FML antigen (Fucose Mannose Ligand) of *Leishmania donovani* in the Swiss Albino model. *Vaccine* 1999;17:2554–61.
- [13] Borja-Cabrera GP, Correia Pontes NN, da Silva VO, Paraguai de Souza E, Santos WR, Gomes EM, et al. Long lasting protection against canine kala-azar using the FML-QuilA saponin vaccine in an endemic area of Brazil (São Gonçalo do Amarante). *Vaccine* 2002;20:3277–84.
- [14] Borja-Cabrera GP, Cruz Mendes A, Paraguai de Souza W, Okada LYH, Trivellato FAA, Kawasaki JKA, et al. Effective immunotherapy against canine visceral leishmaniasis with the FML-vaccine. *Vaccine* 2004;22:2234–43.
- [15] Guarga JL, Moreno J, Lucientes J, Gracia MJ, Peribanez MA, Alvar J, et al. Canine leishmaniasis transmission: higher infectivity among naturally infected dogs to sandflies is associated with lower proportions of T helper cells. *Res Vet Sci* 2000;69:249–53.
- [16] Bourdoiseau G, Bonnefont C, Hoareau E, Boehringer C, Stolle T, Chabanne L. Specific IgG1 and IgG2 antibody and lymphocyte subset levels in naturally *Leishmania infantum*-infected treated and untreated dogs. *Vet Immunopathol* 1997;59:21–30.
- [17] Moreno J, Nieto J, Chamizo C, Gonzalez F, Blanco F, Barker F, et al. The immune response and PBMC subsets in canine visceral leishmaniasis before and after chemotherapy. *Vet Immunopathol* 1999;30:181–95.
- [18] Marciani DJ, Pathak AK, Reynolds RC, Seitz L, May RD. Altered immunomodulating and toxicological properties of degraded *Quillaja saponaria* Molina saponins. *Int Immunopharmacol* 2001;1:813–8.

- [19] Rhodes J. Covalent chemical events in immune induction: fundamental and therapeutics aspects. *Immunol Today* 1996;17:436–41.
- [20] Soltysik S, Wu JY, Recchia J, Wheeler DA, Newman MJ, Coughlin RT, et al. Structure/function studies of QS21 adjuvant: assessment of triterpen-aldehyde and glucuronic acid roles in adjuvant function. *Vaccine* 1995;13:1403–10.
- [21] 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.
- [22] Takechi M, Tanaka Y. Haemolytic time course differences between steroid and triterpenoid saponins. *Planta Med* 1995;61:76–7.
- [23] Osbourn A. Saponins and plant defense: a soap story. *Trends Plant Sci* 1996;1:4–9.
- [24] Santos WR. Effect of the FML-vaccine on experimental kala-azar. Comparative analysis of commercial adjuvants and purified saponins. MSc Thesis. Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, RJ, Brazil, 1998 p. 69.
- [25] Palatnik CB, Borojevic R, Previato JO, Mendonça-Previato L. Inhibition of *Leishmania donovani* promastigote internalization into murine macrophages by chemically defined parasite glycoconjugate. *Infect Immunol* 1989;57:754–63.
- [26] Waite DC, Jacobson EW, Ennis FA, Edelman R, White B, Kammer R. Three double-blind, randomized trials evaluating the safety and tolerance of different formulations of the saponin adjuvant QS21. *Vaccine* 2001;19:3957–67.
- [27] Kensil CR, Patel U, Lennick M, Marciani DJ. Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria* Molina cortex. *J Immunol* 1991;146:431–7.
- [28] Kensil CR. QS21: an update. Abstracts of the modern vaccine adjuvants and delivery systems meeting. Dublin, Ireland: MVADS2003, 4–6 June 2003, p. 29.