A rabies vaccine adjuvanted with saponins from leaves of the soap tree (*Quillaja brasiliensis*) induces specific immune responses and protects against lethal challenge

Anna Carolina A. Yendo a, b, Fernanda de Costa a, b, Samuel P. Cibulski c, d, Thais F. Teixeira c, d, Luana C. Colling a, Mauricio Mastrogiovanni e, Silvia Soulé e, Paulo M. Rohe c, d, Grace Gosmann b, Fernando A. Ferreira e, Arthur G. Fett-Neto a, *

a Plant Physiology Laboratory, Center for Biotechnology and Department of Botany, Federal University of Rio Grande do Sul (UFRGS), Av. Bento Gonçalves, 9500, Porto Alegre, RS 91501-970, Brazil
b Faculty of Pharmacy, UFRGS, Av. Ipiranga 2752, Porto Alegre, RS 90610-000, Brazil
c Fepagro Animal Health – Institute of Veterinary Research “Desidério Finanmor” (IPPDF), Estrada do Conde 6000, Eldorado do Sul, RS 92990-000, Brazil
d Virology Laboratory, Microbiology Department, FCS/UFGRS, Av. Sarmento Leite, 500, Porto Alegre, RS 90070-150, Brazil
e Laboratory of Carbohydrates and Glycoconjugates, Hygiene Institute, Universidad de la Republica (Udelar), Montevideo, Av. Dr. Alfredo Navarro, 3051 Montevideo, Uruguay

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ABSTRACT

*Quillaja brasiliensis* (Quillajaceae) is a saponin producing species native from southern Brazil and Uruguay. Its saponins are remarkably similar to those of *Q. saponaria*, which provides most of the saponins used as immunoadjuvants in vaccines. The immunostimulating capacities of aqueous extract (AE) and purified saponin fraction (QB-90) obtained from leaves of *Q. brasiliensis* were favorably comparable to those of a commercial saponin-based adjuvant preparation (Quil-A®) in experimental vaccines against bovine herpesvirus type 1 and 5, poliovirus and bovine viral diarrhea virus in mice model. Herein, the immunogenicity and protection efficacy of rabies vaccines adjuvanted with *Q. brasiliensis* AE and its saponin fractions were compared with vaccines adjuvanted with either commercial Quil-A or Alum. Mice were vaccinated with one or two doses (on days 0 and 14) of one of the different vaccines and serum levels of total IgG, IgG1 and IgG2a were quantified over time. A challenge experiment with a lethal dose of rabies virus was carried out with the formulations. Viral RNA detection in the brain of mice was performed by qPCR, and RNA copy-numbers were quantified using a standard curve of *in vitro* transcribed RNA. All *Q. brasiliensis* saponin-adjuvanted vaccines significantly enhanced levels of specific IgG isotypes when compared with the no adjuvant group (*P* ≤ 0.05). Overall, one or two doses of saponin-based vaccine were efficient to protect against the lethal rabies exposure. Both AE and saponin fractions from *Q. brasiliensis* leaves proved potent immunological adjuvants in vaccines against a lethal challenge with a major livestock pathogen, hence confirming their value as competitive or complementary sustainable alternatives to saponins of *Q. saponaria*.

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1. Introduction

Rabies is a widespread zoonosis caused by a neurotropic virus, classified in the genus *Lyssavirus* of the family *Rhabdoviridae*, which affects wild and domestic animals, as well as humans. The domestic dog acts as reservoir and transmitter of rabies virus in 95% of human cases [1], and infection induces acute encephalitis lethal in almost 100% of cases. More than 70,000 human deaths per year caused by the canine variant have been reported [1]. The only existing way to prevent the disease is prompt treatment, including vaccination as an obligatory component and anti-rabies immunoglobulin administration as a supplement [2]. More than 15 million patients receive post-exposure therapy annually [3], but the costs associated with post-exposure prophylaxis are high. For instance, in Asia, the direct and indirect economic burdens associated to post-exposure rabies prophylaxis are estimated at around US $1.5 billion [1].

In fact, vaccination of reservoirs such as dogs and cats are most efficient in preventing transmission of rabies to humans [4,5].
However, rabies vaccines must still meet safety and immunogenicity requirements. Modified live rabies vaccines have been largely superseded by inactivated vaccines in view of the risk of reversion to virulence; inactivated rabies vaccines, on their turn [6], are less potent than modified live vaccines. In addition, some rabies vaccines are still prepared in nervous tissues of animals; such vaccines may induce considerable adverse effects such as allergic reactions of varying degrees of severity and occasionally culminating with serious neuropathological conditions [7]. A significant advance to maximizing the potency of currently available vaccines is the incorporation of adjuvants into the formulation. Apart from its numerous immunostimulating effects, adjuvants can also decrease the required amount of antigen to be incorporated into the vaccine, and thus reduce costs of production. However, the addition of adjuvants to vaccines must be carefully evaluated in order to ensure that not only the immunogenic potential of the vaccine is stimulated, but also to ensure that no undesirable side effects are caused by the preparation [8]. In addition, the cost of the vaccine must always be taken into account, since the majority of countries afflicted by the rabies burden have low incomes and, for those, production costs are critical. Thus, besides the immunobiological issues commented above, there is a continuous demand for vaccine prepa-
rations with low cost, ease of use, and with no side effects [2], for both veterinary and human formulations.

*Quillaja brasiliensis* (Quillajaceae) is native from southern Brazil and Uruguay, and its saponins are remarkably similar to those of *Q. saponaria* barks, major source of saponins with immunoadjuvant activity in vaccines [9,10]. Activity comparable to that of commercial saponin-adjuvant Quil-A® was reported for the aqueous extract (AE) and a purified saponin fraction (QB-90) obtained from leaves of *Q. brasiliensis* in experimental vaccines against bovine herpesvirus type (BoHV) 1 and 5, human poliovirus and bovine viral diarrhea virus (BVDV) in mice [11–14]. Leaves of *Q. brasiliensis* are a readily renewable source of saponins compared to the tree destructive source of *Q. saponaria* barks [15]. Herein the immunogenicity and protection to challenge provided by an anti-rabies vaccines adjuvanized with aqueous extract (AE) and several saponin fractions from *Q. brasiliensis* was compared to commercially available adjuvants: one saponin-based adjuvant (Quil-A) and a classical adjuvant, aluminum hydroxide (Alum), mainly aiming toward new veterinary vaccines.

2. Material and methods

2.1. Plant material and preparation of aqueous extract and saponin fractions QB-80, QB-90, Fraction B and Fraction 3

*Q. brasiliensis* leaves were from adult plants growing in Canguçu, RS, Brazil (31°23′42″ S–52°40′32″ W) (voucher ICN 142953) and Parque Batle, Montevideo, Uruguay (voucher MVFQ 4321). Air-dried powdered leaves were extracted in distilled water (1:10, w/v) for 8 h, filtered, partitioned with ethyl acetate and lyophilized, yielding AE. Then AE was submitted to further purification through reverse-phase chromatography and gradient of water and methanol yielding fraction QB-90 [9]. QB-80 was obtained using the same protocol. Fraction B was obtained by solid-phase extraction on reversed phase C-18 eluted with a stepwise gradient of MeOH and water of AE obtained from air-dried leaves harvested in Montevideo [16]. Fraction 3 was purified from leaf AE of Brazilian trees using ultrafiltration and ion chromatography (manuscript in preparation). QB-90, Fraction B and Fraction 3 were also analyzed by TLC. Quil-A was purchased from Bremntag Biosector (8047-15-2, Denmark) and Alum from Omega Produtos Químicos Ltda. (Itaberaba, SP, Brazil).

2.2. Rabies virus preparation

Inactivated rabies virus Pasteur strain suspension for vaccine preparation was provided by IPVDF (Instituto de Pesquisas Veterinárias Desidério Finamor, Brazil) (Labovet®, batch 003/11 B). For in vivo rabies challenge assay, Challenge Virus Standard (CVS) strain was propagated in mice brain [17]; DL50 was measured according to Reed and Muench [18] by intramuscular inoculation of CVS dilutions.

2.3. Mice and immunizations

Female Swiss mice of the CF-1 breed (60-days old, Fundação Estadual de Produção e Pesquisa em Saúde – FEPPS, Porto Alegre, RS, Brazil) were acclimatized for 72 h prior to use. Mice were maintained at 22 ± 2 °C, a cycle of 12/12 h day/night, and fed with standard pellet diet and tap water *ad libitum*.

Animals were divided into 9 groups, with six mice each. Rabies vaccine formulations were diluted in MEM (Modified Eagle’s Medium) and contained 150 μL of rabies antigen (Pasteur strain, titer 7.5 IU/ml) and 50 μL of one of the following adjuvants: QB-90 (50 μg), QB-90 (100 μg), QB-80 (50 μg), QB-80 (100 μg), AE (400 μg), Quil-A (50 μg) or Alum (200 μg). QB adjuvants were tested in two concentrations to check for possible dose response. AE was used at higher amount considering the lower concentration of bioactive saponins in the complex extract. Negative control had no adjuvant (antigen only), using MEM as vehicle. Formulations were filter sterilized and kept at 4 °C until use. Animals were immunized subcutaneously on the hind neck with vaccines; a booster injection was given 2 weeks later. Blood was collected on days 0, 28, 42, 84 and 112 post-inoculation of the first dose of vaccine via tail vein and sera were kept frozen until processed for antibody titers.

2.4. Immunoassays

Anti-rabies IgG (total), IgG1 and IgG2a were quantitated in each serum sample by an indirect ELISA. ELISA plates (Nunc-Immuno MicroWell®) were coated with 50 μL of the same rabies antigen used for mice immunization, diluted (1:100, v/v in carbonate-bicarbonate buffer, pH 9.6) and incubated for 16 h at 4 °C. After antigen adsorption, plates were washed once with 100 μL of phosphate buffered saline containing 0.05% Tween-20 (PBS-T), blocked with 80 μL of PBS-T, containing 2% casein, and incubated for 30 min at 37 °C. Next, plates were washed twice with PBS-T. Sera were diluted in PBS-T (1:100 to IgG total and IgG1 and 1:50 to IgG2a) and added to wells in duplicate. After 1 h at 37 °C, plates were washed three times with wells-T and incubated with adequate dilutions of peroxidase conjugated anti-mouse IgG, anti-mouse IgG1 or anti-mouse IgG2a for 1 h at 37 °C. After washing, 100 μL of OPD (ortho-phenylenediamine) Sigma® (one tablet diluted in 50 mL of distilled water) with 0.03% of H2O2 were added to each well. After 15 min of incubation in darkness at room temperature, reaction was stopped by addition of 1 M HCl (25 μL/well). Optical density was obtained in a microplate reader at 492 nm. Data were expressed as the mean OD value of samples minus the mean OD of control wells (without sera addition).

2.5. Delayed type hypersensitivity (DTH) assay

DTH responses were evaluated in three animals of each group on day 112. Ten μL of rabies virus suspension used for immunization were injected intradermally in the right hind footpad of mice. Footpad thickness was measured with a caliper rule, both before and 24 h after injection. As controls, animals were injected with 10 μL saline in the left hind footpad. Rabies-specific response of
each animal was calculated based on values of its injected footpad minus the average of the basal (control) swelling.

2.6. Challenge of mice against rabies

Groups of six Swiss mice of the CF-1 breed (45–60 days old, Universidade Federal de Pelotas, Pelotas, RS, Brazil) were used. Half of the groups was subcutaneously immunized once (day 0) and half was immunized twice (day 0 and day 14) with the rabies vaccine. The formulations of rabies vaccines had 150 μL of rabies antigen (Pasteur strain, titer 7.5 IU/mL) and 50 μL of one of the following adjuvants: QB-90 (50 μg), QB-90 (100 μg), QB-80 (50 μg), QB-80 (100 μg), AE (400 μg), Fraction 3 (50 μg) (immunized only twice), Fraction B (50 μg) (immunized only twice), Quil-A (50 μg) and Alum (200 μg). Negative control had no adjuvant (antigen only), using MEM. Formulations were filter sterilized and kept at 4°C until use. Fraction 3 and B groups had blood collected on day 28 to quantify total IgG, IgG1 and IgG2a in sera. All mice were challenged via intramuscular injection with 100 μL volumes containing 25xDL50 of Rabies virus strain CVS three weeks after booster (day 35) [17]. This dose led to 100% mortality of non-vaccinated mice. Intramuscular inoculation was chosen based on the main route of infection. All surviving mice were euthanized at day 21 post challenge (day 56). Animals were kept in isolated cages after challenge, under 12 h day–night cycle and with water and food ad libitum.

2.7. Virus quantification

Brains were collected from all animals at the end of the experiment (21 days after challenge) by euthanizing the animals or when animals died along the experiment. RNAs were stabilized with RNalater® (Ambion®). RNA was extracted by Pure Link® RNA Mini kit (Ambion®) and cDNA synthesized by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems®) according to manufacturer’s guidelines and stored at −80°C until use. qPCR for the detection of viral RNA copies was done with SYBR Green and primers that amplify rabies viral sequences. Reactions were performed in 15 μL volumes, with mixtures containing 5 μL of synthetized cDNA, 7.5 μL of 2X Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen®) and 250 nM each of forward (CTAGGGTGAATAAAGGT) and reverse (CTCAATGGAATCCCGAC) primers. A StepOne Real-Time PCR System (Applied Biosystems®) was used for amplification and detection under the following conditions: PCR activation at 95°C for 2 min followed by 40 cycles of amplification (15 s at 95°C and 60 s at 60°C). RNA copy-numbers were quantified using a standard curve of in vitro transcribed RNA of known quantities. For standard curve, CVS RNA was extracted with Trizol®, cDNA synthesized with random primers and amplimers cloned in pCR2.1 vector (Invitrogen®). Colonies containing the insert were selected in kanamycin plus X-Gal/ IPTG medium (Thermo Fischer Scientific®). Plasmidial DNA was extracted according to Sambrook and Russell [19]. RNA was synthesized in vitro by T7 RNA polymerase and quantified in a Qubit® fluorometer (Invitrogen®).

2.8. Statistical analyses

Results were analyzed by ANOVA followed by Tukey test (P ≤ 0.05) whenever appropriate, using the statistics package SPSS 20.0 for Windows. Data were expressed as mean ± standard deviation (SD). Final data on percent survival of rabies infected mice after challenge by CVS strain were analyzed by confidence intervals overlap (P ≤ 0.05).

2.9. Ethical agreement

All experiments complied with the International Legislation on the Use and Care of Laboratory Animals with approval of the Committee for Animal Experiments from Desidério Finanor Veterinary Research Institute (processes 05/2011 and 24/2014). An in vivo model was chosen to provide clear evidence of efficiency and safety of Q. brasiliensis saponin-adjuvanted vaccines. The choice of mice as experimental animals considered suitability based on published studies, costs, and adequacy of facilities for maintenance. An effort was made to use the minimum adequate number of animals, so the suitable number of replicates was estimated as 6 animals per treatment. Although there are humane end points outlined for in vivo rabies studies, we had to refrain from using these in order to be able to measure viral titers in brains.

3. Results and discussion

3.1. Immunogenicity of saponins from Q. brasiliensis

Levels of anti-rabies total IgG were significantly enhanced by all adjuvanted vaccines compared with the control group (without adjuvant) (Fig. 1A) on day 42 of the experiment. QB-80 and QB-90 fractions and AE induced antibody response characterized by higher titers of total IgG, although not different from that of Quil-A. Surprisingly, even not differing statistically from almost all saponin groups, IgG1 titers with QB-90 50 μg were not significantly different from those of the control group (Fig. 1B). However, its IgG2a titers were as high as the other Q. brasiliensis saponin adjuvanted vaccines (Fig. 1C). Serum IgG2a titers did not increase in comparison to no adjuvant group in mice immunized with Alum or Quil-A, but were significantly higher in QB-80 and QB-90, independently of the dose. AE induced a response comparable to Quil-A. QB-80 100 μg immunization led to high IgG levels in all sampling days (Fig. S1).

Total IgG, IgG1 and IgG2a results are consistent with those previously obtained when the individual viral antigens BoHV-1, BoHV-5, human poliovirus and BVDV were co-administered with QB-90 [11–14]. Results emphasize differences in isotype profile of mice immunized with alum or saponin preparations: IgG2a response was significantly higher in Q. brasiliensis groups than Alum group. Except for AE, equivalent to Quil-A in IgG2a yield, all Q. brasiliensis fractions induced higher concentrations of this immunoglobulin (Fig. 1C).

Fractions QB-80 and QB-90 are a complex mixture of saponins, containing some structures similar to those of Quil-A. Chromatographic analysis reveals peculiar compounds in the Brazilian fractions (Fig. S2). Structure elucidation is still underway, but the in vivo assays suggest that these peculiarities in composition may explain immunoadjuvant differences between fractions.

In mouse, Th1 responses are usually associated with stimulation of IgG2a, IgG2b and IgG3 isotypes, and production of cytokines IL-2, TNF-β and IFN-γ. Th2 responses stimulate the production of IgG1 and cytokines IL-4, IL-5, IL-10 and IL-13 [20–22]. The isotype pattern elicited by Q. brasiliensis saponins appears to include both antibody responses. Th1-type is evidenced by the high levels of IgG2a, and Th2, by the elevated titers of IgG1.

After 112 days, the same immunoglobulin profile that had been seen at 42 days was observed, (Fig. S1). The induction of humoral (Th2) response and the suggestion of a cellular (Th1) activation by Q. brasiliensis saponins further provide evidence that such saponin extracts may be used as adjuvants in vaccines against a wide range of viral pathogens.
Supplementary Figs. S1 and S2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.03.070.

3.2. Immunogenicity of Fraction B and Fraction 3

As alternatives for purifying bioactive saponins, two distinct procedures were carried out. The first led to purification of the fraction named Fraction B from AE of Uruguayan trees. The second led to the fraction named Fraction 3, isolated from AE of Brazilian trees. In order to compare their immunoadjuvant activity, an additional immunogenicity analysis was performed with these two groups, with sera collected from mice on day 28 after the first immunization of the challenge experiment.

The results showed induction profiles of the total immunoglobulin and isotypes IgG1 and IgG2a similar to the previous experiment (Fig. 2). The two treatments were statistically equivalent in all cases, indicating that formulations containing different sources of
Q. brasiliensis saponins obtained by several extraction protocols are able to promote similar immunological responses.

3.3. Delayed type hypersensitivity assay

Delayed type hypersensitivity (DTH) assays are indirect methods for evaluating cellular responses, often used to compare the ability of adjuvants to stimulate such kind of immune responses [23]. This is due to immunocompetent cells, including CD4+ T lymphocytes, CD8+ cytotoxic T lymphocytes (CTLs), macrophages and other cells secreting inflammatory cytokines, such as interleukin (IL-1), IL-2, colony stimulating factors, chemokines, and other mediators, migrating to the inoculation site. In this site, cellular recruitment, edema and fibroblastic proliferation take place [24,25].

DTH assay was carried out to evaluate a possible cellular response in animals previously immunized. Local DTH was significantly higher in animals immunized with any one of the Q. brasiliensis saponins adjuvanted vaccines compared to that of control animals or those treated with Alum or Quil-A adjuvanted vaccines (Fig. 3). DTH reactions of QB-90 and Quil-A were shown when tested as adjuvants in vaccines prepared with bovine herpesvirus antigen or human poliovirus antigen [12,13], whose cytokines quantification testified this cellular response profile. Herein, reactions are expanded to other Q. brasiliensis saponin fractions. Since positive DTH reaction may be a consequence of memory Th1 CD4+ T cells [26,27], results suggest that Q. brasiliensis vaccine formulations possibly promote CD4+ T-cells and yield a Th1 profile pattern of stimuli of enhanced immune responses.

3.4. Protective efficacy of candidate vaccines

All control animals of the challenge assay died of rabies infection 7 to 9 days post challenge (Fig. 4). Before dying mice showed typical signs of rabies including hunched-back posture, lethargy and hind-leg paralysis. Protection of immunized mice after this lethal challenge correlated well with induced antibody titers (Fig. 1). Overall, apparent protection against lethal challenge by adjuvanted
rabies antigen was better than that provided by antigen alone. This protection depended on the dose, but even after a single immunization with saponins Quil-A, QB-90 100 µg, or QB-80 in both doses, all mice were protected against virus exposure. Statistical analyses of the last time point in the challenge experiment indicated important differences. Further discrimination among treatments was probably difficult because of the inherent low power of non-parametric tests and the need to minimize the number of animals used.

The dead mouse that had been immunized twice with QB-90 50 µg had lower viral RNA copies in its brain compared to other dead animals (Fig. 5). The RNA viral counting was also analyzed by relative expression using β-actin gene as normalizer, and the results corroborated this profile (data not shown). The challenged survivor animals had no viral RNA detectable by qPCR (Fig. 5), unlike reported by Koraka et al. [28]. This might be due to the different viral administration pathways (intracranial versus intramuscular) used. In the present study, only a non-protective vaccine would allow virus passage into the brain, inevitably leading to death.

The results from QB-90, QB-80 and Fraction B indicated that the two populations of Q. brasiliensis trees studied, despite their distant geographical distribution, yielded similar saponins with strong adjuvant activity, as revealed by comparison of TLC profiles (Fig. S2) and adjuvant assays. One animal died when immunized once with AE vaccine whereas all mice immunized twice with AE vaccine survived after rabies infection. This is a noteworthy activity of the AE adjuvant, since bioactive saponins are present in much lower concentrations in extract.

Although Alum is the major adjuvant in veterinary vaccine formulations, it is unable to elicit cell-mediated Th1 or CTL responses required to control most intracellular pathogens. Commercial fraction Quil-A has been successfully used for veterinary applications, but it is known that severe local reactions and granulomas, besides hemolysis associated with saponin affinity for cholesterol [29,30], can be attributed to its components, making it difficult to increase the virus range for human vaccines, for example. Q. brasiliensis saponins have some advantages. They can induce immune responses similarly to Quil-A, showing significantly less in vivo and in vitro toxicity [11–14]. Accumulation of bioactive saponins in leaves and methods for propagation of Q. brasiliensis facilitate renewal of catalytically active biomass [31].

Q. brasiliensis fractions are a complex mixture of saponins, somewhat similar to Q. saponaria Quil-A, but with some distinct structures. Although these components have not been fully elucidated, the results suggest that composition differences can lead to distinct intensities of response against rabies infection with Q. brasiliensis fractions. Q. brasiliensis fractions also share similarities, but differences can lead to immunoadjuvant activity alterations. Associating saponin fractions or combining them with AE may prove useful to improve adjuvant yields with same efficiency. Reducing the number of immunization doses with effective protection using these adjuvants is an interesting path to be explored for lowering production costs.

4. Conclusion

Saponin adjuvanted vaccines against rabies, a relevant urban and rural infectious virus, were evaluated in mice. Q. brasiliensis saponin formulations appear to have stimulated both Th1 and Th2 immune responses producing high antibodies titers in sera, even after 112 days after immunization. DTH assay indirectly indicates possible cellular response stimulation. One dose of vaccine formulations conferred moderate protection against lethal rabies virus challenge, whereas animals immunized twice were all protected. These results, particularly those of the challenge experiment, further support the use of Q. brasiliensis saponins as a viable alternative and/or complement to Quil-A adjuvant in veterinary rabies vaccines. As data on the use of Q. brasiliensis adjuvants against a range of virus types with favorable activity compared to commercial adjuvants accumulate, the perspective of possible use in a future adjuvanted human vaccine is promising. In fact, adjuvants have been regarded as a topic of interest in experimental rabies vaccines for humans [32]. For this purpose, however, more toxicity and security studies must be performed.
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