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Vaccine 24 (2006) 7129–7134
Vaccine
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Adjuvant activity of *Quillaja brasiliensis* saponins on the immune responses to bovine herpesvirus type 1 in mice

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Received 17 June 2006; accepted 26 June 2006
Available online 12 July 2006

Abstract

The chemical characterization of aqueous extracts (AE) of barks, leaves and branches and the saponin fraction denominated QB-90 obtained from *Quillaja brasiliensis*, a native species from Southern Brazil, show remarkable similarities to *Quillaja saponaria* saponins which are known as adjuvants in vaccine formulations. *In vivo* toxicity assays of AE and QB-90 showed not to be lethal for mice in doses ranging from 50 to 1600 μg and 50–400 μg, respectively. Experimental vaccines prepared with bovine herpesvirus type 1 (BHV-1) antigen and either AE (barks 100 μg, leaves 400 μg, branches 400 μg) or QB-90 (100 μg) were able to enhance the immune responses of mice in a comparable manner to saponins from *Q. saponaria* (QuilA, 100 μg). BHV-1 specific IgG, IgG1 and IgG2a antibody levels in serum were also significantly enhanced by AE, QB-90 and QuilA compared to control group ($p < 0.05$). These results showed that AE and QB-90 from *Q. brasiliensis* are potential candidates as adjuvants in vaccines.

Keywords: *Quillaja brasiliensis*; Saponin; QB-90; BHV-1; Adjuvant

1. Introduction

Quillaja brasiliensis (A. St.-Hil. et Tul.) Mart. is native to Southern Brazil, commonly known as soap tree due to the capacity of its leaves and barks to produce abundant foam in water [1]. We had previously presented the first chemical analysis carried out on this species [2]. From *Q. brasiliensis* leaves, a new diterpene, named 19-*O*-β-D-glucopyranoside of 16-hydroxy-lambertic acid was isolated and identified together with quercetin and rutin. After acid hydrolysis of the aqueous leaves extract, one prosapogenin was isolated and identified as 3-*O*-β-D-glucuronopyranosyl-quillaic acid (Fig. 1). Since these first studies, we proceed to the phytochemical studies and determination of the immunoadjuvant properties of this Brazilian *Quillaja* species.

A number of studies have focused on the use of saponins as immunological adjuvants. Particular attention has been drawn to the economically important Chilean tree *Quillaja saponaria* Molina whose bark extract furnishes saponins, such as, QuilA [3]. QuilA has been incorporated into immunostimulating complexes (ISCOMs) and used in many immunogens such as equine influenza virus, feline leukemia virus and bovine mastitis vaccines [4,5]. *Quillaja* saponins were further purified to allow adjuvant formulations for human vaccine use, such as, melanoma, HIV-1 and malaria vaccines [6–12]. Chemical structural comparisons suggest that the known adjuvant saponins have the same triterpene backbone including the aldehyde at carbon 4 (quillaic acid) and glucuronic acid, two oligosaccharide chains, one of which is acylated by two fatty acid residues in tandem [13–16].

Considering that the overexploitation of the *Q. saponaria* bark has caused important ecological damage and a consi-

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doi:10.1016/j.vaccine.2006.06.059

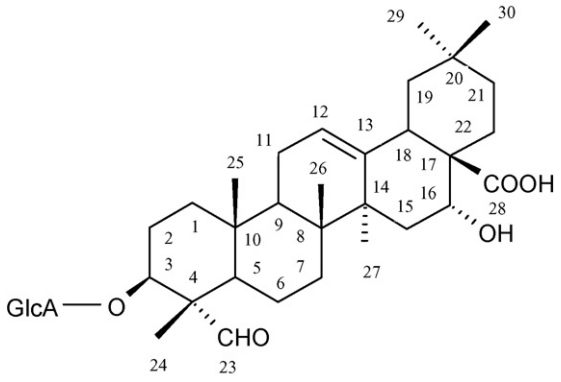

Fig. 1. Compound 3-*O*-β-D-glucuronopyranosyl-quillaic acid.

derable shortage of the available supplies, the possible use of *Q. brasiliensis* saponins could provide another possible source of such compounds, decreasing the pressure on *Q. saponaria* exploitation and adding to the possibilities of sustainable exploitation [17].

Herein we describe the preparation of aqueous extracts from the leaves, barks and branches of *Q. brasiliensis*, the isolation from its leaves of one saponin fraction named QB-90 and their structural characterization by hydrolysis and NMR. The immunological properties of these extracts and QB-90 were compared to those of *Q. saponaria* saponins on the induction of specific immune responses to bovine herpesvirus type 1 (BHV-1) antigen following immunization of mice.

2. Materials and methods

2.1. Materials

QuilA® was from Superfos®, 3-*O*-β-D-glucuronopyranosyl-quillaic acid was isolated previously from *Q. brasiliensis* leaves [2]. ^{1}H NMR spectra were measured in methanol-d_4/D_2O (9:1) with an INOVA VARIAN 300 MHz spectrometer.

2.2. Plant material

Q. brasiliensis (A. St.-Hil. et Tul.) Mart. were collected in Caçapava do Sul, State of Rio Grande do Sul, Brazil. A herbarium specimen is deposited at the Herbarium of the Botany Department of the Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil (ICN 124818).

2.3. Extraction and purification of saponins

Air-dried powdered leaves, barks and branches from *Q. brasiliensis* were extracted, separately, in water (1:10, w/v) under constant stirring at room temperature for 8 h. These extracts were filtered and lyophilized to obtain the leaves extract, the barks extract and the branches extract.

The leaves extract was submitted to further purification. It was applied to silica Lichroprep® (Merck, 40–63 μm particle size) using as eluate a stepwise gradient of aqueous 0–100% MeOH. Elution of saponins was monitored by thin-layer chromatography (TLC). TLC was performed on silica gel aluminum plates (Aldrich) using $CHCl_3$:MeOH:H_2O:AcOH (30:20:3:0.2, v/v) or *n*-BuOH:AcOH:H_2O (5:1:4, v/v) as solvent and anisaldehyde-sulphuric acid followed by heating as spray reagent. Fractions containing similar saponins were pooled together and evaporated to dryness. Fraction QB-90 was obtained and its immunological properties were determined.

2.4. Acid hydrolysis and NMR of AE, and QB-90

Aqueous extracts of leaves, barks and branches, QB-90 and QuilA were submitted, separately, to acid hydrolysis (reflux for 2 h in 1 M H_2SO_4 in EtOH 70%) as previous work [2]. Their residues were chromatographed using Si gel and $CHCl_3$:MeOH (10:1, v/v) as eluant and anisaldehyde/sulphuric acid for detection. These same samples were submitted to ^{1}H NMR spectroscopy in order to visualize the fingerprint of the metabolites in these extracts.

2.5. Animals

Female Swiss mice (7–8 weeks old) of the CF-1 breed were purchased from the Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS), Porto Alegre, RS, Brazil, and acclimatized for 72 h prior to use. Rodent laboratory chow and tap water were provided *ad libitum*. Mice were maintained under controlled temperature (22 ± 2 °C) and humidity under a 12/12 h light/dark cycle. All the procedures were carried out in strict accordance with the International Legislation on the Use and Care of Laboratory Animals and were approved by the University Committee for Animal Experiments/UFRGS (project number 2003112).

2.6. Toxicity assays of AE and QB-90

Toxicity of *Q. brasiliensis* extracts prepared from leaves, barks and branches were tested by subcutaneous administration on the back of mice ($n = 4$) using 200 μl of appropriate dilutions (50, 100, 200, 400, 800 and 1600 μg) of each extract dissolved in phosphate buffered sterile saline (PBS). The toxicity of the fraction QB-90 was tested in mice ($n = 6$) by subcutaneous administration of appropriate dilutions (50, 100, 200, 400, and 800 μg) of QB-90 dissolved in 200 μl of PBS. Each dilution was administered in two weekly doses. The mice were monitored daily for 14 days. Sterile saline-treated animals were included as control group. Toxicity was assessed by lethality, local swelling, loss of hair and development of skin lesions.

2.7. Virus and cells

A bovine herpesvirus type 1 (BHV-1) recombinant vaccine strain was propagated on Madin Darby bovine kidney cells (MDBK, ATCC CCL-24) following standard procedures [18]. Cells were routinely maintained in Eagle's minimal essential medium (E-MEM, Gibco) supplemented with 6% foetal calf serum (FCS, Nutricell) and enrofloxacin (Baytril; Bayer). When cytopathic effect was evident in 90–100% of the monolayers, supernatant medium and cells were harvested and frozen at −70 °C. This was then clarified by low speed centrifugation (1500 × *g*) and the supernatant use as antigen. Virus titrations were performed on microtitre plates following usual methods [19,20]. Titres were expressed as $\log_{10}$ $TCID_{50}$ per 50 μl. The infectious virus titre of antigen before inactivation was 10^6 $TCID_{50}$/50 μl.

2.8. Immunization protocols

First, the evaluation of the adjuvant activity of AE from different parts of *Q. brasiliensis* was performed in groups of six mice as follows: Group 1: 400 μg of leaves extract; Group 2: 100 μg of barks extract; Group 3: 400 μg of branches extract; Group 4: 100 μg of QB-90.

The second experiment was the determination of the dose–response curve of QB-90 through its serial dilutions (50–200 μg) in PBS and it was performed in groups of eight animals.

In both experiments PBS was used as the vehicle. All samples were filtered through 0.22 μm Micropore® filters and kept at 4 °C until use. Animals were inoculated subcutaneously twice, on days 0 and 28 with 150 μl of BHV-1 antigen adjuvanted with 50 μl of different concentrations of saponin in a total vaccine volume of 200 μl. A control preparation was formulated with QuilA (100 μg) as adjuvant. Another control without adjuvant (antigen only) was also included. Sera from inoculated mice were collected on days 0, 28, 42, 56, 84 and 112 post-inoculation (p.v.) of the first dose of vaccine, and frozen for subsequent determination of specific antibody titres in immunoassays.

2.9. Immunoassays

The titres for IgG, IgG1 and IgG2 a specific anti-BHV-1 were determined in pooled sera by an indirect ELISA as previously described [18]. ELISA plates were coated with the same BHV-1 antigen preparation used for preparation of the samples. Coating was performed in a previously determined dilution (1:6400, v/v) in bicarbonate buffer (pH 9.6) overnight at 4 °C. Wells were then washed three times with PBS containing 0.05% Tween 20 (PBS-T). One hundred microliters of the sera collected from mice (diluted 1:50, v/v in PBS-T) were added to duplicate wells and incubated for 1 h at 37 °C. Subsequently, plates were washed three times in PBS-T. Next, an appropriate dilution (1:1500 in PBS-T) of anti-mouse IgG peroxidase conjugate (DAKO, Denmark) or anti-mouse IgG1 or IgG2 a peroxidase conjugate (VMRD) was added to wells in 100 μl volumes. Plates were then incubated for another hour at 37 °C. After washing, 100 μl of substrate (*ortho*-phenylenediamine Sigma® 10 mg; 0.003% H_2O_2) were added to each well. Plates were then incubated for 5 min at 37 °C, when the reaction was terminated by adding 50 μl/well of 2N H_2SO_4. The optical density (OD) was measured in an ELISA plate reader (Multiskan, Titertek) at 492 nm. Data were expressed as the mean OD value of the samples minus the mean OD value of control wells.

2.10. Statistical analysis

The data were expressed as mean ± standard errors and examined for their statistical significances by ANOVA and Tukey test performed on SPSS for Windows. Differences in *p*-value of ≤0.05 were considered significant.

3. Results

3.1. Characterization of AE and QB-90

From the dry weight of *Q. brasiliensis* extracts, approximately 6% (leaves), 10% (barks) and 4% (branches) were extractable in water. One gram of the aqueous extract from leaves gives 15 mg of QB-90. Aqueous extracts of leaves, barks and branches, QB-90 and, QuilA were submitted, separately, to TLC after acid hydrolysis. The presence of 3-*O*-β-D-glucuronopyranosyl-quillaic acid (Rf = 0.4) was detected by co-TLC in all acid hidrolisate samples. This compound was previously isolated by us [2] and it is a prosapogenin of the saponins found in *Q. saponaria* demonstrating that the different parts of *Q. brasiliensis* should have saponins similar to the first one [3].

^{1}H NMR was carried out on aqueous extracts of leaves, barks and branches, QB-90 and QuilA, separately, in deuterated methanol:H_2O in order to visualize the chemical profile of these extracts. The aldehyde proton resonance (H-23, $\delta = 9.45$) characteristic of the quillaic acid was presented in all samples together with the signals of methyls ($\delta = 0.7$–1.5). The characteristic signals of *Quillaja* saponins were also presented as those of aliphatic acid portion ($\delta = 2.4$–2.7), the signals of sugars hydrogens ($\delta = 3.0$–5.5) together with the anomeric ones ($\delta = 4.3$–5.5). It was also possible to verify in the ^{1}H NMR spectrum of QuilA the presence of one singlet ($\delta = 1.98$) attributed to the acetyl in the carbon 3 of the fucosyl attached to C-28 [15,16].

It was possible to demonstrate by detailed comparison that ^{1}H NMR spectra of QB-90 and the aqueous extracts (AE) from *Q. brasiliensis* are very similar to the ^{1}H NMR spectra of QuilA (saponin mixture from *Q. saponaria* bark).

3.2. Toxicity assays of AE and QB-90

When applied by the s.c. route to mice, no lethality was detected within the concentration range of the *Q. brasiliensis*

Table 1
Toxicity *in vivo* of aqueous extracts from the barks, leaves and branches of *Quillaja brasiliensis*[a]

	Extract/dose (μg)					
	50	100	200	400	800	1600
Barks	0/4	0/4	0/4	3/4	4/4	4/4
Leaves	0/4	0/4	0/4	0/4	1/4	1/4
Branches	0/4	0/4	0/4	0/4	1/4	2/4

[a] Results are expressed as number of animals that showed local swelling after the second subcutaneous injection of saponins.

extracts evaluated. Local swelling or loss of hair was not detected in mice inoculated with two doses of 50–200 μg of all extracts. However *Q. brasiliensis* bark extract, after the second administration, caused a local swelling in three out of four mice inoculated with 400 μg of such extract, as well as in all four animals inoculated with 800 and 1600 μg of bark extract (Table 1).

In mice inoculated with QB-90, after the first immunization, local swelling at the injection site was detected in one out of six mice inoculated with 400 μg and in three out of six animals that received 800 μg. Three out of six mice died after the second immunization with 800 μg of QB-90.

Taking into account the results, the doses of extracts and QB-90 which did not induce toxic reactions were selected to be tested as adjuvants.

3.3. Immunological studies of AE and QB-90

To estimate the adjuvant effect of the vaccine preparations with different amounts of saponins, the specific anti-BHV-1 IgG, IgG1 and IgG2 a responses of inoculated mice were evaluated.

In relation to experiment 1, where aqueous extracts (AE) from the leaves, barks and branches of *Q. brasiliensis* were used as adjuvants, 28 days after the first immunization (Fig. 2(a)) a significant rise ($p < 0.03$ to <0.01) was detected in total specific anti-BHV-1 IgG levels in all saponin formulations in comparison to the control. The vaccine prepared with QuilA induced significantly higher rises in IgG levels than AE and QB-90.

Total specific anti-BHV-1 IgG, IgG1 and IgG2a profiles on days 42 (data not shown) and 56 (that is 28 days after the second immunization) (Fig. 2(b)) are similar to all samples. On day 56 there are not significant differences in IgG and IgG1 levels among any of the saponin-adjuvanted formulations with the exception of the branch extract that presented a significant rise ($p < 0.05$). Relating to IgG2a, there are not significant differences in any of the saponin formulations tested. To all saponin samples, total specific IgG levels are significantly higher than IgG1 and this latter one is higher than IgG2a titres. Antibody levels in the sera of all samples are higher than the control ($p < 0.05$). It was observed similar specific anti-BHV-1 IgG, IgG1 and IgG2a profiles on days 56 and 84 (data not shown) to all samples.

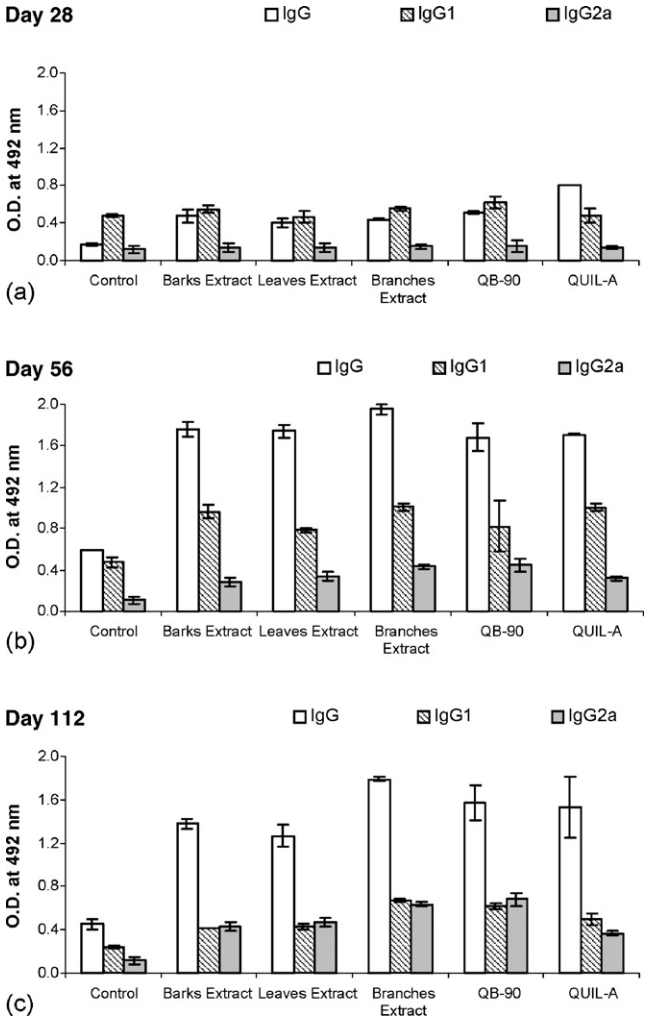

Fig. 2. BHV-1 specific serum IgG, IgG1 and IgG2a antibodies in mice immunized s.c. on days 0 and 28 with vaccines prepared with: (i) inactivated BHV-1 antigen without adjuvant (control); antigen adjuvanted with *Quillaja brasiliensis* extracts from: (ii) barks (100 μg); (iii) leaves (400 μg); (iv) branches (400 μg); (v) QB-90 (100 μg) and (vi) QuilA® (100 μg). Sera were collected on days 28, 42, 56, 84 and 112 after the initial dose of vaccine (p.v.) and antibodies measured by ELISA as described in the text. (a) Sera collected on day 28 p.v.; (b) sera collected on day 56 p.v.; (c) sera collected on day 112 p.v. The values are presented as mean ± S.E. ($n = 6$).

On day 112 (Fig. 2(c)), all saponin-adjuvanted vaccines significantly continue to enhance the total specific IgG and IgG1 levels in mice, as compared with control ($p < 0.05$), although these antibody levels were significantly lower on day 112 when compared to sera collected in previous days. Relating the total specific IgG levels in mice, there were not significant differences among QB-90, QuilA and bark extract ($p > 0.05$).

In relation to the determination of the dose–response curve of QB-90, no significant differences were detected on the profiles of antibody responses examined in those groups of mice that received vaccine formulations with different amounts of QB-90 (50–200 μg) (Fig. 3). QB-90 was also found to stimulate IgG, IgG1 and IgG2a antibody responses to BHV-1

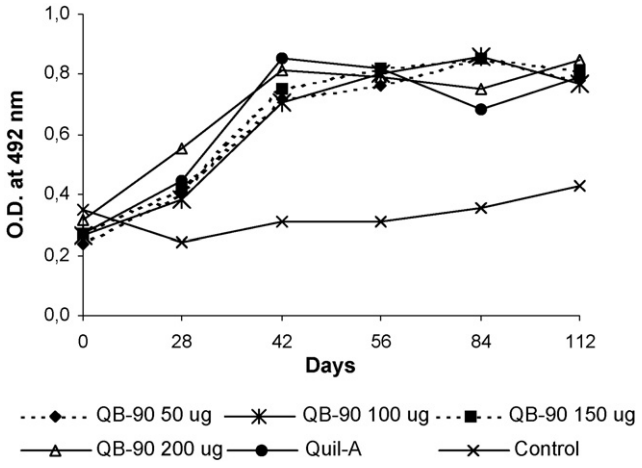

Fig. 3. BHV-1 specific serum of total IgG antibody in mice immunized s.c. on days 0 and 28 with vaccines prepared with inactivated BHV-1 antigen without adjuvant (control) or either in combination with QB-90 (50, 100, 150 and 200 μg/dose) or QuilA® (100 μg). Sera were collected on day 28, 42, 56, 84 and 112 and antibodies measured by ELISA as described in the text. The values are presented as mean ± S.E. ($n = 8$).

to levels equivalent to those obtained with QuilA (100 μg, $p > 0.05$). The significant higher antibody levels detected in formulations containing QB-90 and QuilA in comparison to the control was observed until day 112.

4. Discussion

Adjuvants play an important role in increasing the efficacy of a number of different vaccines. Such compounds may also play a role in determining the type of immune response generated. *Q. saponaria* saponins have for decades been studied for its important immune adjuvant activity. Presently, several pre-clinical and clinical experiments are in course in order to evaluate vaccines with *Quillaja* saponins as adjuvant, including vaccines to HIV, malaria and even tumors such as melanomas [6–12].

In this paper it was possible to demonstrate the potential adjuvant activity of aqueous extracts (AE) of leaves, barks and branches from *Q. brasiliensis*, a native plant from Southern Brazil, together with a purified saponin fraction named QB-90 obtained from leaves of *Q. brasiliensis*.

The chemical characterization of AE and QB-90 described in this paper indicates that they have the prosapogenin 3-*O*-β-D-glucuronopyranosyl-quillaic acid as the main component of their saponins. Through the use of acid hydrolysis and ^{1}H NMR spectroscopy, these extracts and QB-90 show remarkable structural similarities to *Q. saponaria* saponins which are known as outstanding adjuvants in vaccines.

BHV-1 specific IgG, IgG1 and IgG2a antibody levels in serum were also significantly enhanced by the different aqueous extracts (AE) and QB-90 and, this latter one presented adjuvant activity at doses from 50 to 200 μg.

Considering that the overexploitation of *Q. saponaria* barks has caused important ecological damage and a shortage of its resources, the possible use of the leaves of *Q. brasiliensis* to obtain adjuvant saponins should contribute to a stable supply of saponins through this new raw material and their sustainable exploitation [17].

In this study, bovine herpesvirus type 1 (BHV-1) was used as an indicator antigen in order to demonstrate the immune activity of saponins of *Q. brasiliensis*. Our results demonstrated the low s.c. toxicity and the immune potentiating responses of formulations using aqueous extracts (AE) from the leaves, barks and branches of *Q. brasiliensis* altogether with different concentrations of the saponin fraction QB-90 isolated from the leaves of *Q. brasiliensis*.

In conclusion, *Q. brasiliensis* saponins could significantly aid the induction of antibodies to BHV-1 in immunized mice. These results showed that AE and QB-90 from *Q. brasiliensis* are potent immunological adjuvants that must be further studied to determine their potential as adjuvants in vaccines.

Acknowledgements

We would like to thank Prof. Gilson R.P. Moreira (Departamento de Zoologia, UFRGS, Brazil) and Marcos Sobral (Programa de Pós-Graduação em Ciências Farmacêuticas/UFRGS) for locating, collecting and identifying the plant material; Prof. Clarisa B.P. de Sousa (Centro de Ciências da Saúde/UFRJ, Brazil) who kindly supplied us QuilA®; Prof. Teresa Dalla Costa Laboratory for the kind supply of Eppendorf Centrifuge (5417R). This work was supported by CAPES and CNPq (Brazil).

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