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Quillaja brasiliensis saponins are less toxic than Quil A and have similar properties when used as an adjuvant for a viral antigen preparation

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ABSTRACT

In this study, a preparation of saponins (QB-90U) extracted from leaves of *Quillaja brasiliensis* collected in Uruguay was evaluated as a vaccine adjuvant by comparison with alum and the well known saponin-based adjuvant, Quil A. The haemolytic activity and cellular toxicity of the saponin preparations were also evaluated. QB-90U was only slightly haemolytic and showed a low cytotoxicity when compared to Quil A. The adjuvant properties of QB-90U were assayed by sub-cutaneous immunization of mice with a preparation of inactivated bovine herpesvirus 5 (BoHV-5) either with no adjuvant or adjuvanted with QB-90U, Quil A or alum. Serum levels of anti-BoHV-5 IgG, IgG1, IgG2a, IgG2b and also IgG3 were significantly increased by QB-90U and were of the same order as those elicited by Quil A. Furthermore, high titres of neutralizing antibodies were found to be present in the serum of immunized animals from both groups. The cellular response induced by QB-90U did also reproduce the one elicited by Quil A. In fact, a robust DTH response was observed in mice immunized with both saponin preparations; as well as increased splenocytes levels of Th1-type cytokines, namely IFN- γ and IL-2. Taken together, the above results confirm and extend our previous observation regarding the similarity of the responses elicited by Quil A and the saponin preparation from *Q. brasiliensis* (Fleck et al., 2006) and indicate that QB-90U is worth of further studies as a safe and potent vaccine adjuvant.

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1. Introduction

Alum is the most widely employed adjuvant in human vaccine formulations [1]. It appears to induce a local pro-inflammatory reaction leading to a T helper 2 (Th2) type response [2] with enhanced production of antibodies to co-administered antigens [3]. The small number of other currently approved vaccine adjuvants for human use does not usually elicit the desired protective, sustained immune responses. In addition, alum is a poor inducer of cell-mediated immunity [4], which contributes to the elimination of virus and other intracellular pathogens as well as cancer cells. Thus, there is a broadly recognized need for the development of new adjuvants [5,6].

In this context, the adjuvant potential of natural products and of saponins in particular, has been largely explored. Saponins are natural steroidal or triterpenic glycosides with many biological and pharmacological activities, including potential adjuvant properties [7,8]. Actually, triterpenoid saponins extracted from *Quillaja saponaria* Molina have a long usage record as adjuvants in veterinary vaccines [9]. In some cases, saponins may show an alum-type adjuvant effect [10], but they have been mostly studied for their capacity to stimulate cell-mediated immunity. A partially purified mixture of saponins from *Q. saponaria*, called Quil A [11], is the most widely used and studied saponin-based vaccine adjuvant. It is known to stimulate both humoral and cellular responses against co-administered antigens, with the generation of T helper 1 (Th1) and cytotoxic cells (CTLs) responses. The ability to elicit this type of immune response makes them ideal for use in vaccines directed against intracellular pathogens, virus, as well as in therapeutic cancer vaccines [7,12]. However, in spite of its recognized adjuvant potential, the use of Quil A in human vaccines

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has been restricted due to undesirable side effects, including local reactions, haemolytic activity and even systemic toxicity [7,11]. The haemolytic activity of saponins has been shown to be closely related to their structure, both the aglycone type and the oligosaccharide residues [13,14] and, for this reason, considerable efforts have been undertaken over the last decades for the discovery of new plant saponins with improved adjuvant activity and reduced toxicity [7,9,15].

Quillaja brasiliensis (A. St.-Hil. et Tul.) Mart. is a tree native to Southern Brazil and Uruguay. It is commonly known as “soap tree” in view of the capacity of its leaves and bark to produce abundant foam in water due to their high saponin content. Some of us have been involved in the chemical characterization of the saponins present in the leaves of *Q. brasiliensis* [16] and, in particular, in one saponin fraction, named QB-90, which was found to have similarities with Quil A [17]. Furthermore, we have shown that QB-90 presents low toxicity when subcutaneously administered to mice and that it strongly potentiates the immune response to a viral antigen (bovine herpesvirus type 1, BoHV-1) [17].

In this work, we contribute to improve the knowledge of the adjuvant activity of the saponins fraction named QB-90U prepared from leaves of *Q. brasiliensis* collected in Uruguay, in comparison to two of the most commonly used adjuvants (alum and Quil A). We analyze the haemolytic activity and cytotoxicity of QB-90U and evaluate its potential as vaccine adjuvant using another viral antigen as model, by comparing its performance with those of Quil A and alum. For the latter purpose, we assess the antibody (IgG and its subclasses) and cellular (DTH assay) responses of mice immunized with a preparation of inactivated BoHV-5. In addition, we specifically evaluate whether QB-90U is capable of inducing the generation of Th1 CD4⁺ T cells by assessing the expression levels of Th1 cytokines in splenocytes from immunized mice.

2. Materials and methods

2.1. Plant material and preparation of QB-90

Q. brasiliensis (A. St.-Hil. et Tul.) Mart. leaves were collected in Parque Battle, Montevideo, Uruguay. The samples were identified by Eduardo Alonso of the Botany Department, Facultad de Química, UdelaR, and a voucher sample was kept at the Herbarium of the Faculty (MVFQ 4321).

Air-dried powdered leaves were extracted in distilled water (1:10, w/v) under constant stirring at room temperature for 8 h. The extract was then filtered and lyophilized to obtain the aqueous extract from which fraction QB-90U was purified following the procedure described by Fleck et al. [17]. Briefly, the aqueous saponin extract was applied to a silica Lichroprep column and eluted with a stepwise gradient of aqueous methanol 0–100% methanol. The fractions were analyzed by TLC, and those with a similar saponin composition were pooled together to give the QB-90U fraction.

2.2. Assays of saponin toxicity

2.2.1. Haemolytic activity assay

The haemolytic activity of QB-90U and Quil A (BRENNTAG, Denmark) was assessed as described before [10], except that guinea pig red blood cells at a 1% concentration were used for the assays. Concentration ranges from 500 µg/mL to 50 µg/mL (500, 250, 230, 200, 180, 160, 150, 130, 110, 100, 70 and 50 µg/mL) and from 110 µg/mL to 10 µg/mL (110, 100, 80, 60, 50, 30, 20, 15 and 10 µg/mL) were used for QB-90U and Quil A, respectively, each sample was tested in triplicate. Saline and *Q. saponaria* saponins (250 µg/mL) were used as references for 0% and 100% haemolysis, respectively. The mixture of *Q. saponaria* saponins was prepared by

dialysis against distilled water from a commercial sample [10]. The haemolytic activity was expressed as the concentration producing 50% of the maximum haemolysis (HD₅₀).

2.2.2. Cell cytotoxicity assay

Cytotoxicity was determined using the MTT assay, in general following the original procedure [18]. Briefly, VERO cells (African Green Monkey Kidney, ATCC CCL-81) were cultured in Eagle's minimal essential medium (E-MEM) supplemented with 10% foetal bovine serum (FBS, GIBCO) and antibiotics (penicillin 100 UI/mL; streptomycin 100 µg/mL) (E-MEM/FBS). Cells were seeded at a concentration of 4.0×10^4 per well on 96-well microplates and maintained at 37 °C under a humid atmosphere with 5% CO₂. After 18 h, the medium was removed and 100 µL of E-MEM/FBS containing different concentrations (100, 150, 200 and 300 µg/mL) of either QB-90U or Quil A were added to each well in triplicate. The plates were incubated as above; after 48 h, 50 µL of 2 mg/mL MTT (Sigma Chemical Co., Saint Louis, MO, USA) were added to each well and the cells were incubated for a further 4 h. The plates were centrifuged (1400 × g for 5 min) and the supernatant containing the untransformed MTT was carefully removed. Ethanol (100 µL/well) was added to solubilize the formazan crystals, and the optical density (OD) was measured in an ELISA reader (Anthos 2020) at 550 nm with a 620 nm reference filter. The amount of formazan produced was directly proportional to the number of living cells in culture. Results were expressed as the percent OD of each culture in comparison with the OD of untreated control cells.

2.3. BoHV-5 antigen preparation

Madin Darby Bovine Kidney cells (MDBK; originally ATCC CCL-22) were routinely multiplied in E-MEM/FBS [19]. For virus production, monolayers of MDBK were grown overnight in 150 cm² flasks and infected with BoHV-5 strain A663 [20,21] at a multiplicity of infection of 0.1. When cytopathic effect was evident in 90–100% of the monolayers, the flasks were frozen at –70 °C, thawed, and the medium was clarified by low speed centrifugation. The viral suspension was inactivated with binary ethylenimine (BEI) as described previously [22]. The median tissue culture infectious doses (TCID₅₀) before inactivation was 10^{7.8}/mL. The suspension of inactivated virus (to which we refer as BoHV-5) was used as antigen for adjuvant testing and for all assays except for the serum neutralization test.

2.4. Mouse immunization protocol

Female Rockefeller mice (5–6-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. Mice were maintained under controlled temperature (22 ± 2 °C) and humidity with a 12/12 h light/dark cycle chow and tap water were provided *ad libitum*. 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.

Mice were divided into six groups, each consisting of six animals. The formulations of BoHV-5 were prepared under aseptic conditions, filtered through 0.22 µm and kept at 4 °C until use. Animals were inoculated subcutaneously (in the hind neck) twice, on days 1 and 14, with 150 µL of BoHV-5 antigen plus 50 µL saline (no adjuvant group), or with either alum (Omega Produtos Químicos Ltda., 200 µg), Quil A (50 µg) or QB-90U (100 µg) suspended or dissolved in 50 µL saline (alum, Quil A and QB-90U, groups, respectively). Mice were bled prior to inoculations (on days 0 and 14) and

Table 1
Primer sequences used for RT-qPCR.

Gene name	Forward primer 5'–3'	Reverse primer 5'–3'	Prod. size
β-Actin	GCTTCTTTGCAGCTCCTTCGT	CGTCATCCATGCGCAACTG	68
IL-2	CCTGAGCAGGATGGAGAATTACA	CTTTC AATTCTGTGGCCTGCTGGG	92
INF-γ	TCAGCAACAGCAAGCGGAAA	CCGCTTCTGAGGCTGGAT	143

2 weeks after the second immunization (day 28); sera were kept frozen until processed.

2.5. Immunoassays for antibodies

Anti-BoHV-5 IgG (total), IgG1, IgG2a, IgG2b, and IgG3 were determined for each serum sample by ELISA, carried out essentially as previously described [10]. ELISA plates (Greiner Bio-One) were coated with the BoHV-5 suspension used for mouse immunization diluted (1:100, v/v) in carbonate-bicarbonate buffer pH 9.6 at 37 °C for 1 h. Plates were then washed three times with PBS containing 0.05% Tween 20 (PBS-T) and blocked with BSA (1% in PBS) at 37 °C for 1 h. Sera (100 μL of appropriate dilutions in PBS-T) were added in duplicates and incubated for 1 h at 37 °C. Subsequently, plates were washed three times with PBS-T. Next, 100 μL of appropriate dilutions in PBS-T of anti-mouse IgG (Sigma Chemical Co.), IgG1 (Caltag Laboratories), IgG2a, IgG2b, or IgG3 (Zimed Laboratories) were added to the wells and plates were incubated for another hour at 37 °C. After washing, 100 μL of OPD (orthophenylenediamine, Sigma Chemical Co.) with H₂O₂ were added to each well, plates were incubated for 15 min at 37 °C and the reactions were stopped by adding 50 μL/well of 1 N HCl. The OD was measured in an ELISA plate reader (Anthos 2020) at 492 nm. Antibody titres were expressed in arbitrary units (AU) referred to a standard calibration curve prepared with a pool of positive sera. IgG3 titres were expressed in OD because they were much lower than those for the other isotypes. All the samples were diluted 1/100 for the determination of IgG3 titres.

2.6. Serum neutralization test

The presence of neutralizing antibodies to BoHV-5 in mouse sera was analyzed in a virus neutralization test with the constant virus, varying serum method, in 96-well cell culture plates, as previously described [23]. The test was performed against 100 TCID₅₀/50 μL of BoHV-5 strain A663.

2.7. Delayed type hypersensitivity (DTH) assay

Delayed type hypersensitivity responses were evaluated in three mice from each group on day 28 as previously described [10]. Briefly, mice were subcutaneously injected in one footpad of the hind limb with 10 μL of the BoHV-5 suspension used for immunization. The thickness of the injected footpads was measured 24 h later with a calliper. The swelling of mice from the control group injected with saline was considered to be derived from the puncture procedure (basal swelling). The BoHV-5-specific DTH response of each animal was calculated based on the thickness of its injected footpad minus the average of the basal swelling.

2.8. Evaluation of cytokine gene expression

Spleens were collected in RPMI 1640 (Gibco) under aseptic conditions 120 days after the second immunization, minced and mechanically dissociated to obtain a homogeneous cell suspension. Erythrocytes were lysed with ammonium chloride (0.8%, w/v). After centrifugation (380 × g at 4 °C for 10 min), the cell pellets were washed three times in RPMI and suspended in

complete medium: RPMI 1640 supplemented with 0.05 mM 2-mercaptoethanol, 100 IU/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 10% FBS. Splenocytes (>95% viability as assessed by trypan blue exclusion) were seeded onto microtubes (100 μL of 5 × 10⁶ cells/mL), concanavalin A (Invitrogen, 5 μg/mL final concentration) or RPMI 1640 plus BoHV-5 suspension were added to a final volume of 200 μL in triplicate. The tubes were incubated at 37 °C in a humid atmosphere containing 5% CO₂ for 16 h, after which 0.5 mL of Trizol (Invitrogen) were added; the tubes were stored at –80 °C until use. RNA extraction was performed according to the manufacturer's instructions. RNA quality and quantity were assessed by spectrophotometric measurements at 260/280 nm (Nanodrop); 1 μg of total RNA was treated with DNase-I (Invitrogen) and immediately subjected to cDNA synthesis with random primers (Invitrogen) and M-MLV reverse transcriptase (Invitrogen).

Real-time PCR was performed using the QuantiTect[®] SYBR[®] Green PCR Kit (Qiagen) in a Rotor-Gene 6000 (Corbett), as follows. Primers (see Table 1) were used at a final concentration of 0.9 μM. The cycling conditions were 15 min at 95 °C, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min during which the fluorescence data were collected. The expression level of the genes of interest

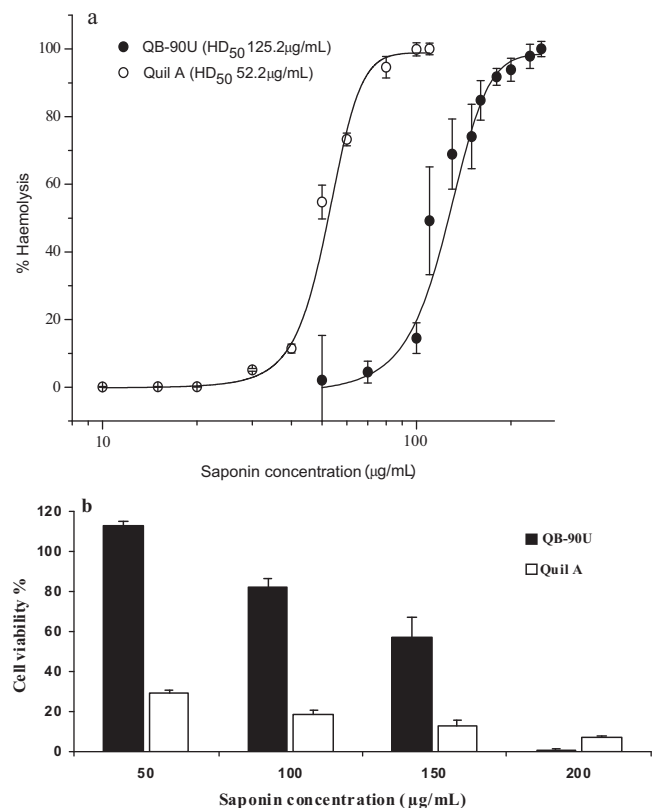


Fig. 1. *In vitro* toxicity of QB-90U and Quil A. (a) Haemolytic activity expressed as percent haemolysis (%haemolysis) referred to saline and *Q. saponaria* saponins (250 μg/mL), which were used as 0% and 100%, respectively. (b) Cytotoxicity assayed on VERO cells; cell viability was measured 48 h after treatment with the indicated saponin concentration. In (a) and (b), results are presented as the mean value ± S.D (n = 3).

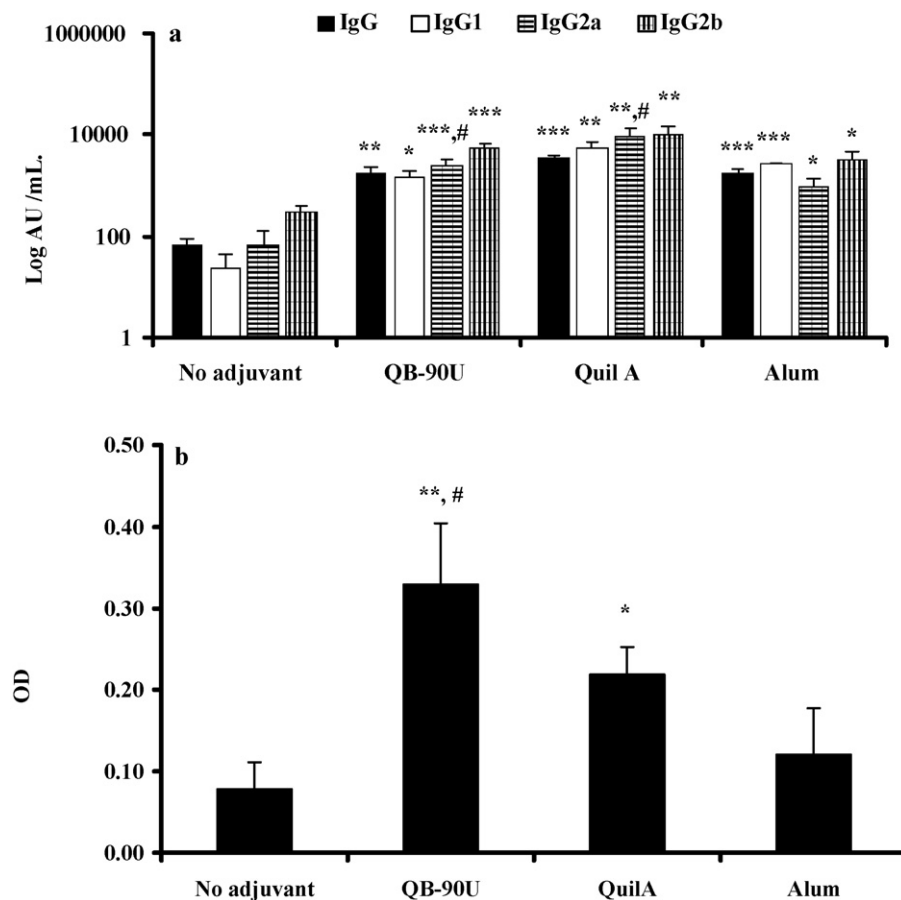


Fig. 2. Antibody response of immunized mice. Serum titres of anti-BoHV-5 total IgG and of IgG1, IgG2a, IgG2b (a) and IgG3 (b) isotypes, 2 weeks after the second antigen dose, administered either with no adjuvant or formulated with QB-90U, Quil A or alum. The values are expressed as the mean value \pm S.E. ($n=6$) in arbitrary units (a) or OD (b). Significant differences are indicated: * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$), with the group immunized with no adjuvant; and #, with the alum group ($P < 0.05$).

was normalized using β -actin as housekeeping gene. The relative mRNA amount in each sample was calculated using the $2^{-\Delta\Delta Ct}$ method [24] where $\Delta Ct = Ct_{\text{gene of interest}} - Ct_{\text{Actb}\beta\text{Act}}$, and expressed as relative mRNA level in the test group compared to the non-stimulate control group.

2.9. Statistical analysis

The data were expressed as mean \pm standard error (S.E.) or standard deviation (S.D.) and examined for statistical significance with the Student's *t*-test. *P*-values of less than 0.05 were considered to be statistically significant.

3. Results and discussion

3.1. QB-90U is significantly less toxic than Quil A

Fig. 1a shows the haemolytic activities of QB-90U and Quil A. Their respective HD_{50} values were $125 \pm 5 \mu\text{g/mL}$ and $52 \pm 2 \mu\text{g/mL}$, and their haemolytic activities at the concentrations used for vaccination (100 and $50 \mu\text{g/mL}$) were about 15% and 55%, respectively. Thus, compared with Quil A, QB-90U was only slightly haemolytic at the concentration used for immunization. Its low haemolytic activity allowed including QB-90U in the inoculated preparation at a higher concentration than is possible for Quil A.

A similar result was obtained in the cytotoxicity assay, which is shown in Fig. 1b. Indeed, the toxicity of Quil A against VERO cells was much higher than that of QB-90U. At a concentration of $100 \mu\text{g/mL}$, more than 80% of cells were viable after incubating

for 48 h at 37°C with QB-90U, while at the same concentration of Quil A just about 20% were viable. At $50 \mu\text{g/mL}$, the concentration used for immunization with Quil A, a viability of approximately 30% was observed with this saponin fraction, whereas no toxicity was detected with QB-90U.

These results on the *in vitro* toxicity of QB-90U and Quil A agree with previous reports on their *in vivo* toxicity in mice [11,15,17]. Taken together, they indicate that QB-90U is significantly less toxic than Quil A, despite being obtained from a species closely related to *Q. saponaria*, and of experimental evidence of structural similarity to Quil A [17].

3.2. QB-90U induces the production of high titres of anti BoHV-5 IgG containing neutralizing antibodies

The levels of anti-BoHV-5 IgG as well as IgG1, IgG2a and IgG2b were significantly enhanced by QB-90U, alum and Quil A, compared with the control group (Fig. 2a). Interestingly, mice immunized with either QB-90U or Quil A presented a similar increase of serum IgG2a titres which were significantly higher than those obtained for the alum group ($P < 0.05$). The titres of anti-BoHV-5 IgG3 are separately represented for clarity purposes (Fig. 2b). A significant increase was detected in mice immunized using QB-90U and Quil A compared with either the control or the alum groups.

The results in Fig. 2 show that QB-90U induces a strong antibody response characterized by high titres of total IgG with enhanced production of IgG1, IgG2a, IgG2b and IgG3 isotypes, with no statistical differences with the one elicited by Quil A. In terms of the production of total IgG, IgG1 and IgG2a, these results are consistent

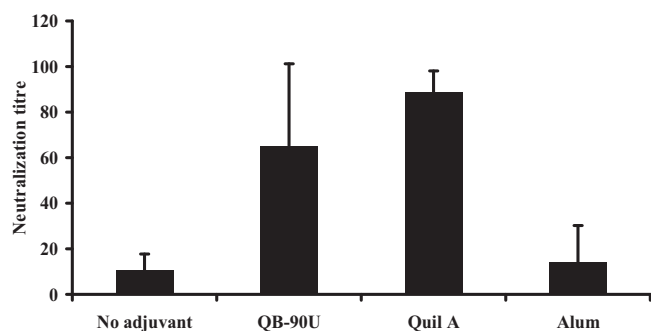


Fig. 3. Neutralizing antibodies in the serum of immunized mice. Serum titres of BoHV-5-neutralizing antibodies, 2 weeks after the second antigen dose, administered using no adjuvant or with either QB-90U, Quil A or alum as adjuvants.

with those previously obtained when the viral antigen BoHV-1 was co-administered with QB-90 [17]. Furthermore, they highlight differences in the isotype profile of mice immunized with alum or the saponin preparations: the IgG2a response was significantly higher in the QB-90U and Quil A groups than in the alum group; and only the saponin preparations led to a significant increase in the titres of IgG3. In the mouse, Th1 responses are usually associated with enhanced isotype switching to IgG2a and IgG3 (which are promoted by IFN- γ), whereas Th1 responses stimulate the production of IgG1 (which is promoted by IL-4) [25,26]. In this context, and although not conclusive, the isotype pattern elicited by QB-90U – rather similar to the one obtained with Quil A – indicate that it is capable of inducing an antibody response with a Th1-type bias, as evidenced by the high levels of IgG2a and the production of IgG3. In addition, the elevated titres of IgG1 suggest that Th2 CD4⁺ T cells are also involved in the response against the administered antigen.

Fig. 3 shows the titres of neutralizing antibodies against BoHV-5 in sera from the different groups. The titres from the QB-90U or Quil A groups were more than four times higher than those from the alum and control groups. These results point to the secretion of elevated titres of high affinity antibodies against the administered antigen in mice immunized with the saponin preparations, an effect that is crucial to generate protective immunity against a viral infection.

3.3. Similar to Quil A, QB-90U induced the generation of CD4⁺ T cells with a Th1 phenotype

Fig. 4 summarizes the results of the DTH assay for the different groups of mice. A significant response was observed in the QB-90U,

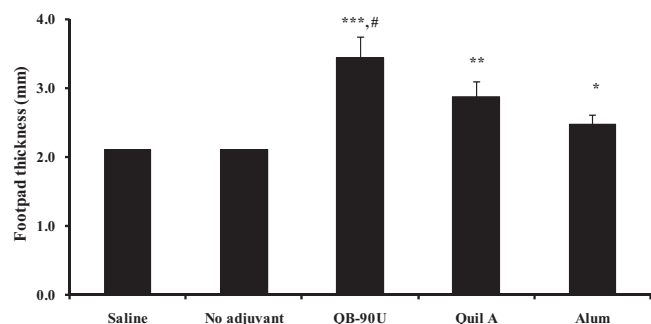


Fig. 4. Analysis of delayed type hypersensitivity (DTH) reaction of immunized mice. The assay was carried out 2 weeks after the second immunization of mice with the BoHV-5 preparation either with no adjuvant or formulated with QB-90U, Quil A or alum. Mice injected only with saline solution (no antigen) were also used as a control group (Saline). The DTH response is expressed as the mean value \pm S.D. ($n = 3$); significant differences are indicated: * ($P < 0.01$), ** ($P < 0.001$) and *** ($P < 0.0001$), with the group immunized with no adjuvant; # ($P < 0.001$) with the alum group.

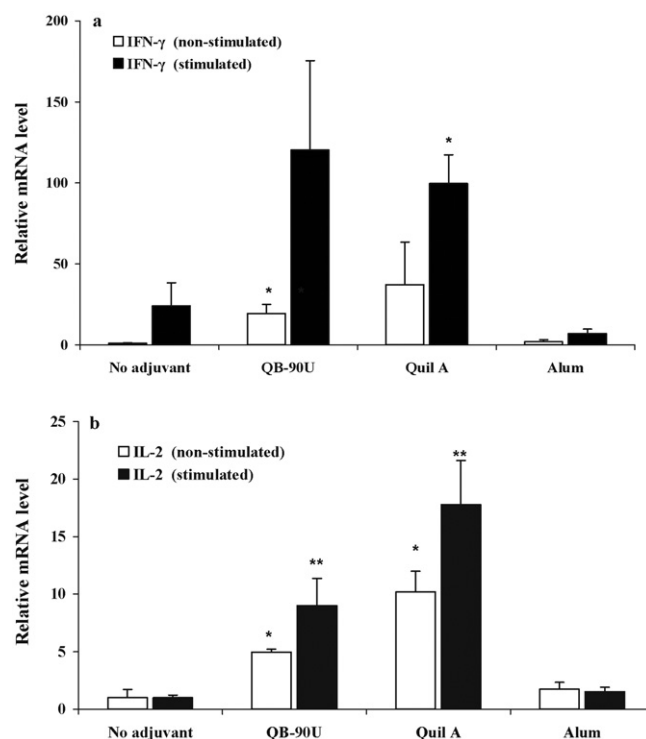


Fig. 5. Analysis of Th1 cytokine expression of immunized mice. mRNA expression levels of (a) IFN- γ and (b) IL-2 in BoHV-5-stimulated and non-stimulated splenocytes from mice immunized with the preparation of BoHV-5 using no adjuvant or formulated with QB-90U, Quil A or alum, 120 days after the second antigen dose. The mRNA levels relative to the one of non-stimulated splenocytes from the group immunized with no adjuvant are presented as mean values \pm S.E. ($n = 3$). Significant differences with the group immunized with no adjuvant are indicated * ($P < 0.05$) and ** ($P < 0.01$).

Quil A and alum groups ($P < 0.0001$, $P < 0.001$ and $P < 0.01$, respectively) albeit in the latter case the response was milder. Actually, the DTH response of mice immunized with QB-90U was also significantly higher than the one of the alum group ($P < 0.01$). Taking into account that a positive DTH reaction is attributed to memory Th1 CD4⁺ T cells [27], these results indicate that, similar to Quil A [28], QB-90U is capable of stimulating the generation of Th1 cells against the administered antigen.

The response elicited by QB-90U, specifically the profile of IgG subclasses and the positive DTH reaction, led us to analyze the expression of Th1 cytokines to confirm the capacity of this saponin preparation to induce the differentiation of T cells with a Th1 phenotype. Fig. 5 shows the relative expression levels of IFN- γ and IL-2, in antigen-stimulated and non-stimulated splenocytes, 120 days after the second immunization. Higher levels of IFN- γ and IL-2 mRNA relative to the control group were observed in mice from the QB-90U and Quil A groups. In the case of IFN- γ , the differences were statistically significant in non-stimulated splenocytes from mice of the QB-90U group ($P < 0.05$) and in antigen stimulated splenocytes from animals immunized with Quil A ($P < 0.05$). In the case of IL-2, significant differences were observed in all assayed samples, that is, in antigen stimulated and non-stimulated splenocytes from mice of the QB-90U ($P < 0.01$ and $P < 0.05$, respectively) and Quil A ($P < 0.01$ and $P < 0.05$, respectively) groups. As somehow expected, no significant differences were detected in the expression of IFN- γ or IL-2 in mice from the alum group.

The expression pattern of Th1 cytokines in mice from the QB-90U group – very similar to the one of the Quil A group and markedly different from the alum group – showed that this saponin

fraction from *Q. brasiliensis* did promote the generation of CD4⁺ T cells with a Th1 phenotype.

4. Conclusion

Considered globally, our results show that the saponin fraction from *Q. brasiliensis* that we named QB-90U is a safe preparation whose adjuvant effect resembles the one of Quil A, when used for immunization with a viral antigen (BoHV-5). Indeed, both saponin fractions stimulated the production of high antibody titres, containing neutralizing antibodies, and a strong DTH response. Similar patterns of IgG subclasses were observed in immunized mice, which suggested the involvement of Th2 (high IgG1 levels) as well as Th1 (high IgG2a and IgG3 levels) CD4⁺ cells in the antibody response; the participation of the latter was specifically confirmed through the detection of increased expression of IL-2 and INF- γ . The low *in vitro* (this work) and *in vivo* (our previous study [17]) toxicity of QB-90U and its high effectiveness to generate strong humoral and cellular responses towards a co-administered viral antigen allow us to propose that this saponin fraction can be considered as an interesting alternative to Quil A adjuvants.

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