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Leaf saponins of *Quillaja brasiliensis* enhance long-term specific immune responses and promote dose-sparing effect in BVDV experimental vaccines

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ABSTRACT

Saponin-based adjuvants are promising adjuvants that enhance both humoral and T-cell-mediated immunity. One of the most used natural products as vaccine adjuvants are *Quillaja saponaria* bark saponins and its fraction named Quil A®. Despite that, its use has been restricted for human use due to safety issues. As an alternative, our group has been studying the congener species *Quillaja brasiliensis* saponins and its performance as vaccine adjuvants, which have shown to trigger humoral and cellular immune responses comparable to Quil A® but with milder side effects. Here, we studied a semi purified aqueous extract (AE) and a previously little characterized saponin-enriched fraction (QB-80) from *Q. brasiliensis* as vaccine adjuvants and an inactivated virus (bovine viral diarrhea virus, BVDV) antigen co-formulated in experimental vaccines in mice model. For the first time, we show the spectra pattern of the *Q. brasiliensis* saponins by MALDI-TOF, a novel and cost-effective method that could be used to characterize different batches during saponins production. Both AE and QB-80 exhibited noteworthy chemical similarities to Quil A®. In addition, the haemolytic activity and toxicity were assessed, showing that both AE and QB-80 were less toxic than Quil A®. When subcutaneously inoculated in mice, both fractions promoted long-term strong antibody responses encompassing specific IgG1 and IgG2a, enhanced the avidity of IgG antibodies, induced a robust DTH reaction and significantly increased IFN-ɣ production in T $CD4^+$ and T $CD8^+$ cells. Furthermore, we have proven herein that AE has the potential to promote dose-sparing, substantially reducing the dose of antigen required for the BVDV vaccines and still eliciting a mixed Th1/Th2 strong immune response. Based on these results, and considering that AE is a raw extract, easier and cheaper to produce than commercially available saponins, this product can be considered as candidate to be escalated from experimental to industrial uses.

1. Introduction

Bovine viral diarrhea virus (BVDV) is a major pathogen responsible for diseases in ruminants worldwide – especially in dairy and beef cattle populations – that leads to significant economic losses. Although the name of the virus refers to a specific disease, BVDV is a pathogen that affects multiple organs in many ruminant species. The heaviest burden of BVDV infections is displayed through reproductive losses, as infections of pregnant cows may give rise to abortions, foetal malformations, and persistently infected (PI) calves, being the latter a major strategy for virus perpetuation and dissemination of the infection in herds [1]. PI calves are often

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weak at birth but may be phenotypically normal and are important to the epidemiologic aspects of viral propagation as they constitute a biological reservoir [2].

BVDV is an enveloped, single-stranded RNA virus, and is the prototypic member of the *Pestivirus* genus within the family *Flaviviridae* [3]. There are two species of BVDV [4,5]. BVDV1 includes the classical BVDV isolates, which are commonly used as laboratory reference, and is subdivided into types 1a and 1b [6]. BVDV2 comprises the BVDV strains associated with high mortality, acute and peracute infections [7]. Vaccination is widely used to control manifestations of BVDV infections. Yet, such control still may be improved by rational vaccine design [8]. Modified live virus (MLV) and inactivated or killed virus (KV) BVDV vaccines for cattle have been available for more than 50 years. In attempting to overcome BVDV's antigenic diversity, MLV and KV vaccines nowadays are often designed to include both BVDV1 and BVDV2 antigens [7]. In addition, different adjuvants such as alum, saponins, or combination of these have been added to vaccine preparations in order to enhance immune responses [9]. Vaccine adjuvants have been used in order to induce a faster and stronger immune response that correlates with increased protection or allow dose-sparing, very useful in the case of expensive or difficult-to-produce antigens, affording a decrease in the manufacturing cost of vaccines [10,11]. Among these, the most widely used adjuvants approved for human and veterinary vaccines (mainly parenteral injections) are alum compounds [12], which primarily stimulate the production of antibodies. However, in some infections, particularly those caused by intracellular pathogens such as BVDV, antibodies are not sufficient to induce protection [13,14]. In such cases, stimulation of antigen-specific $CD4^+$ and $CD8^+$ T-cells is not only required but essential for eliciting protective responses [15].

In attempting to increase stimulation of cell mediated responses, many natural products have been targeted as potential adjuvants and are currently under investigation [16,17]. Among these, triterpenoid saponins extracted from *Quillaja saponaria* Molina, and in particular, a partially purified mixture of saponins, named Quil A® [18,19], is one of the most widely used saponin-based adjuvant for veterinary vaccines. Although it has shown to stimulate both humoral and cellular immune responses with the generation of T helper 1 (Th1) and cytotoxic cell (CTLs) responses [20,21], its use for human vaccines has been restricted due to safety issues such as local reactions, haemolytic activity and occasional events of systemic toxicity [18,22]. As an alternative, our group has been studying *Quillaja brasiliensis* saponins and their performance as vaccine adjuvants. *Q. brasiliensis* is the second of the two species recognized in the *Quillaja* genus, a native tree from southern Brazil and Uruguay. Saponin fractions obtained from *Q. brasiliensis* leaf extracts have proven to be highly immunogenic, yet inducing milder side effects than Quil A® [23]. Aqueous extracts (AE) and some purified fractions (named QB-90 and QB-80), have demonstrated to possess adjuvant potential similar to Quil A®. Both *Q. brasiliensis* saponins fractions and Quil A® showed similar patterns of antibody induction (IgG and its subclasses) and stimulation of cellular immunity biased towards Th1-associated responses [23–27]. Recently, the immune response elicited against BVDV antigen by formulations adjuvanted either with QB-90 or AE was reported [27]. In this previous study, we found that, the more purified fraction QB-90 was better in stimulating both cellular and humoral immune responses than AE, although the latter was also able to elicit a strong immune response. Nevertheless, AE is a saponin raw extract, and as such, is easier and cheaper to produce than the more purified saponin fractions [28].

Considering these encouraging results, in this study we analyze in more detail the long term adjuvant effect of AE in experimental BVDV vaccines. The purified *Q. brasiliensis* saponins fraction QB-80, which has been shown to elicit protective immunity when combined with a rabies antigen [26], was evaluated in terms of its ability to induce an immune response in mice when included in experimental vaccines for BVDV. Prior to conducting these experiments, AE and QB-80 were partially characterized by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) for the first time. We also examined the haemolytic activity of AE, and the *in vivo* toxicity of QB-80. Formulations containing BVDV antigen and *Q. brasiliensis* AE, or QB-80 as adjuvants, were compared to alum adjuvanted or unadjuvanted formulations, in terms of their ability to induce specific humoral and cellular immune responses. Finally, the dose-sparing effect of AE formulations was assessed.

2. Materials and methods

2.1. AE and QB-80 characterization by MALDI-TOF

AE and QB-80 were characterized by MALDI-TOF and compared to the commercially available saponins Quil A®. MALDI-TOF measurements were conducted on a Microflex LR MALDI-TOF (Bruker Daltonics, Billerica, MS, USA) with a 337 nm nitrogen laser operated in positive ion lineal mode with delayed extraction and optimized in the m/z range of 0–20 kDa. Calibrations were performed with a peptide calibration standard mix (Bruker Daltonics). The laser was fired 100 times at each of ten locations for each sample well on a 96 well plate for a cumulative 1000 shots per sample well taken at 30% intensity. 1 μL of 1 mg/mL AE, QB-80 or Quil A® solutions were mixed with 1 μL of matrix solution (2,5-dihydroxybenzoic acid, 10 mg/mL in sterile H_2O with 1% TFA) at a 1:1 ratio. The sample and matrix mixes were spotted onto a 96 well stainless steel plate and allowed to air dry for 15 min at room temperature.

2.2. In vitro and in vivo toxicity assays

The haemolytic activity of saponins is one of the main indicators of cytotoxicity. In order to make a comparative evaluation of the haemolytic activities of AE, crude saponin fraction obtained from roots and rhizomes of *Gypsophila paniculata* (White Saponin, Merck®), *Q. saponaria* saponins (Acros Organics™), purified saponins fractions from *Q. brasiliensis* leaves extract (QB-90 and QB-80) and Quil A® (a purified fraction of saponins from barks of *Q. saponaria*, Brenntag) were tested over a range of concentrations (10–2000 μg/mL) for induction of haemolysis with 0.5% rabbit red blood cells (RBCs) as previously described [23]. Physiological saline solution and *Q. saponaria* saponins at 250 μg/mL were used as indicators of 0% and 100% haemolysis, respectively. Samples were tested in quadruplicate in V-bottom microtitre plates. The haemolytic activity was expressed as the end point dilution capable of inducing haemolysis in 50% of the RBCs (HD_{50}).

The acute toxicity of QB-80 was assessed in 8-weeks old CD1 male mice as previously described [29]. Briefly, mice (n = 5) were subcutaneously injected in the neck scruff with 100 μL of QB-80 or Quil A® solutions containing 150, 75, 37.5 or 18.75 μg of saponins per dose. The control group was injected with saline solution only. Mice were monitored for 72 h and signs of toxicity (lethality, local swelling, loss of hair, and piloerection) were assessed.

2.3. Vaccine adjuvants and antigen production

Q. brasiliensis (A. St.-Hil. et Tul.) Mart. leaves were collected from native adult plants naturally growing in the municipality of Canguçu, RS, Brazil (31°23′42″S-52°40′32″W). A voucher specimen is deposited at the UFRGS Herbarium (ICN 142953). AE were prepared from air-dried powdered leaves resuspended in distilled

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