Comparative Immunology, Microbiology and Infectious Diseases 45 (2016) 1–8

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Quillaja brasiliensis saponins induce robust humoral and cellular responses in a bovine viral diarrhea virus vaccine in mice

Samuel Paulo Cibulski[a,b,1], Fernando Silveira[c,*,1], Gustavo Mourglia-Ettlin[d], Thais Fumaco Teixeira[a,b], Helton Fernandes dos Santos[a,b], Anna Carolina Yendo[e], Fernanda de Costa[e], Arthur Germano Fett-Neto[e], Grace Gosmann[f], Paulo Michel Roehe[a,b]

[a] *FEPAGRO Saúde Animal, Instituto de Pesquisas Veterinárias Desidério Finamor, Laboratório de Virologia, Eldorado do Sul, RS, Brazil*
[b] *Departamento de Microbiologia Imunologia e Parasitologia, Laboratório de Virologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*
[c] *Laboratorio de Carbohidratos y Glicoconjugados, Departamento de Desarrollo Biotecnológico – Facultad de Medicina, Universidad de la República (UdelaR), Av. Alfredo Navarro 3051, CP 11600, Montevideo, Uruguay*
[d] *Cátedra de Inmunología, Departamento de Biociencias – Facultad de Ciencias/Química, Universidad de la República (UdelaR), Av. Alfredo Navarro 3051, CP 11600, Montevideo, Uruguay*
[e] *Laboratório de Fisiologia Vegetal, Centro de Biotecnologia e Departamento de Botânica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*
[f] *Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga 2752, Porto Alegre 90610-000, RS, Brazil*

ARTICLE INFO

Article history:
Received 17 September 2015
Received in revised form
16 December 2015
Accepted 19 January 2016

Keywords:
Vaccine
Adjuvant
Quillaja brasiliensis
QB-90
BVDV
Immune response
Cellular immunity

ABSTRACT

A saponin fraction extracted from *Quillaja brasiliensis* leaves (QB-90) and a semi-purified aqueous extract (AE) were evaluated as adjuvants in a bovine viral diarrhea virus (BVDV) vaccine in mice. Animals were immunized on days 0 and 14 with antigen plus either QB-90 or AE or an oil-adjuvanted vaccine. Two-weeks after boosting, antibodies were measured by ELISA; cellular immunity was evaluated by DTH, lymphoproliferation, cytokine release and single cell IFN-γ production. Serum anti-BVDV IgG, IgG1 and IgG2b were significantly increased in QB-90- and AE-adjuvanted vaccines. A robust DTH response, increased splenocyte proliferation, Th1-type cytokines and enhanced production of IFN-γ by $CD4^+$ and $CD8^+$ T lymphocytes were detected in mice that received QB-90-adjuvanted vaccine. The AE-adjuvanted preparation stimulated humoral responses but not cellular immune responses. These findings reveal that QB-90 is capable of stimulating both cellular and humoral immune responses when used as adjuvant.

1. Introduction

Bovine viral diarrhea (BVD) is a viral disease with major significance in cattle, where it may be responsible for huge economic losses [1]. Acute bovine viral diarrhea virus (BVDV) infections are common, usually resulting in mild disease characterized by fever, increased respiratory rate, diarrhea and leucopenia. Although animals generally recover, the effect of BVDV on the immune system may impair the host's resistance to disease [2]. In addition, BVDV infections in pregnant cows can give rise to abortions, malformations, reduced size at birth, decreased growth curve and birth of persistently infected calves; the latter is a major source for virus perpetuation and dissemination of the infection in herds [2].

Vaccination aims to induce the development of acquired immunity by inoculation of immunogenic components of a particular pathogen or closely related microorganisms [3]. Nonliving vaccine antigens, especially purified or recombinant subunit vaccines, are often poorly immunogenic and require additional components to help stimulate protective immunity based on antibodies and effector T cell functions [4,5]. Thus, adjuvants capable of enhancing both humoral and cellular immune responses are a subject of high interest to vaccinology as well as to the vaccine industry [6].

Triterpenoid saponins extracted from *Quillaja saponaria* Molina have a long usage record as adjuvants in veterinary vaccines [6,7]. Saponins extracted from an alternative three, *Quillaja brasiliensis*, native to South America, have been evaluated as immune enhancer [8–10]. In particular, a saponin fraction extracted from *Q. brasiliensis* named QB-90 and aqueous leaves extracts (AE) of this species

* Corresponding author.
E-mail address: fernandosilveiragonzalez@gmail.com (F. Silveira).
[1] Contributed equally to this work.

http://dx.doi.org/10.1016/j.cimid.2016.01.004
0147-9571/

were demonstrated to stimulate both humoral and cellular immune responses to viral antigens at levels comparable to those induced by *Q. saponaria* saponins [8–10].

The aim of this study was to evaluate the potential adjuvant capacity of QB-90 and AE when added to experimental vaccine preparations formulated with a model viral antigen (bovine viral diarrhea virus; BVDV) and inoculated into mice. These were compared to classical adjuvants as Alum and IFA.

2. Material and methods

2.1. Vaccine adjuvants

Q. brasiliensis leaves were collected from adult plants naturally growing in the municipality of Canguçu, RS, Brazil. Extracts from leaves of *Q. brasiliensis* were prepared from air-dried powdered leaves resuspended in distilled water (1:10, w/v), filtered, partitioned with ethyl acetate and lyophilized. The product was then submitted to further purification through reverse-phase chromatography to obtain fraction QB-90, as described in detail previously [10]. Quil A® was purchased from Brenntag Biosector (Denmark). Alum was obtained from Omega Produtos Quimicos Ltda. (Brazil). Incomplete Freund's Adjuvant (IFA) was purchased from Sigma (USA).

2.2. Cytotoxicity

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 Quil A, QB-90 and AE, the three preparations were tested for induction of haemolysis over a range of concentrations (10–2000 μg/mL) on 0.5% rabbit red blood cells (RBCs), as previously described [9]. 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-bottomed microtiter plates. The haemolytic activity was expressed as the end point dilution capable of inducing haemolysis in 50% of the RBCs (HD_{50}).

2.3. Antigen production

MDBK (Madin-Darby bovine kidney cells) were obtained from ATCC (originally CCL-22™) and cultured at 37 °C in a 5% CO_2 incubator in Eagle's minimal essential medium (E-MEM; Gibco) supplemented with 10% fetal bovine serum and antibiotics (penicillin 100 IU/mL; streptomycin 100 μg/mL). Cells were subcultured every 3–4 days following standard procedures [11]. For virus multiplication, an autochthonous cytopathogenic BVDV1 isolate (EVI001/94) was inoculated onto nearly confluent monolayers of MDBK cells at a multiplicity of infection of 0.01. When cytopathic effect was evident in about 90% of monolayers, cells and supernatants were harvested and frozen at −80 °C. Subsequently, the supernatant was thawed, clarified by low speed centrifugation (1500 × *g* for 10 min) aliquoted and used as virus' stocks. Infectious titers obtained were around $10^{8.3}$ 50% tissue culture infectious doses per mL ($TCID_{50}$) before inactivation with binary ethylenimine (BEI) [12]. The inactivated virus suspension was used as antigen in the vaccine formulations.

2.4. Vaccination of mice

Female Swiss mice (6–7 weeks old) were purchased from the Fundação Estadual de Produção e Pesquisa em Saúde (FEPPS, Porto Alegre, Brazil). Prior to the beginning of the experiments, the animals were acclimatized for 1 week under controlled temperature (22 ± 2 °C) and humidity, with a cycle of 12/12 h day/night and fed with standard pelleted food and tap water *ad libitum*.

Mice were then divided in ten groups of seven; each group was immunized subcutaneously on the hind neck with one of the following: 150 μL of BVDV antigen in the presence of 50 μL of QB-90 (containing 100, 50 or 10 μg per dose); AE (containing 400, 200 or 100 μg per dose); Quil A (50 μg per dose); alum (100 μg per dose); incomplete Freund's adjuvant (IFA, 150 μL of antigen emulsified with 150 μL of oil) or without adjuvant (antigen only). The formulations of BVDV vaccines were filtered through 0.22 μm (Millipore) and kept at 4 °C until use. A booster injection with the same volume of vaccine preparations (200 μL) was applied 2 weeks later (day 14). Blood were collected *via* tail vein on days 0, 14 and 28 post-inoculation of the first dose of vaccine and kept frozen at −20 °C until processed further.

All experiments were performed in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Treaty Series–No. 170 revised 2005) and the procedures of the Brazilian College of Animal Experimentation (COBEA). The project was approved by the Ethics Commission on Animal Experimentation (CEUA) of FEPAGRO Animal Health, Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF).

2.5. Immunoassays for antibodies

Anti-BVDV IgGt (total), IgG1 and IgG2a were determined in each serum sample in an indirect ELISA. ELISA plates (Nunc) were coated with the same BVDV antigen used for preparation of the vaccine antigens above and diluted (1:100, v/v) in PBS (pH 7.2) for 18 h at 4 °C. After adsorption, plates were washed two times with 200 μL of PBS containing 0.05% Tween-20 (PBS-T). Wells then received 180 μL of a blocking solution (PBS-T with 5% non-fat dry milk) and incubated for 60 min at 37 °C. Subsequently, the plates were washed twice with PBS-T. Sera were appropriately diluted in PBS-T (1:300 to IgGt and IgG1 and 1:100 to IgG2a) and added to duplicate wells. After 1 h at 37 °C, plates were washed three times with PBS-T and incubated with adequate dilutions of peroxidase conjugated anti-mouse IgG, anti-mouse IgG1 or anti-mouse IgG2a (Sigma) for 1 h at 37 °C. After washing, 100 μL of OPD (ortho-phenylenediamine, Sigma) with 0.03% of H_2O_2 were added to each well. After 30 min of incubation in the dark at room temperature, the reaction was stopped with the addition of 1 M HCl (25 μL/well). Optical density was determined in a microplate reader set to 492 nm. Data were expressed as the mean OD value of the samples minus the mean OD recorded in control wells.

2.6. Delayed type hypersensitivity (DTH) assay

DTH responses were evaluated in three animals of each group on day 28 post inoculation of the first dose. The assay was performed by intradermal (ID) injections of 10 μL of the same BVDV antigen used for production of the vaccines, in the right hind footpad. The thickness of the footpad was measured with a caliper 24 h before and 24 h after ID injections. The BVDV-specific response of each animal was calculated based on values of its injected footpad minus the average of the basal swelling [13].

2.7. Splenocyte proliferation assay

Twenty eight days after the priming, spleens were collected under aseptic conditions, immersed in RPMI 1640 medium (Invitrogen) and mechanically dissociated to obtain a homogeneous cell suspension. Erythrocytes were lysed with ACK (Ammonium-Chloride-Potassium) lysis buffer. Subsequently, cells were pelleted by low speed centrifugation (380 × *g* at 4 °C for 10 min), washed