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Identification of suitable adjuvant for vaccine formulation with the *Neospora caninum* antigen NcSRS2

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ABSTRACT

The parasite *Neospora caninum* is the main cause of abortion in cattle in many countries around the world, so a vaccine is a rational approach method for the control of the disease. An effective vaccine should be able to prevent both, the horizontal and vertical transmission of *N. caninum*. In this study, the immune vaccinal response of the recombinant protein rNcSRS2 of *N. caninum* expressed in *Pichia pastoris* and formulated with water-in-oil emulsion, xanthan gum, and alum hydroxide was assessed in an experimental murine model. Groups of 10 Balb/c mice were subcutaneously inoculated with two doses of prNcSRS2 twenty-one days apart. After the second immunization, four mice from each group were euthanized, and splenocytes were stimulated *ex vivo* with recombinant protein. The IgG dynamics were evaluated by indirect ELISA, and the splenocytes cytokines transcription by qPCR. All groups elicited specific antibodies against prNcSRS2, with the water-in-oil group showing significantly ($p \leq .05$) elevated titers compared to the other groups. The prNcSRS2 protein alone did not induce a significant *ex vivo* splenic transcription level of IFN-γ, TNF-α, IL-4, IL-10, and IL-12 cytokines, except for IL-17A, and the adjuvant associations with the prNcSRS2 protein induced different cytokine transcription profiles. The water-in-oil emulsion modulated the expression of TNF-α; the xanthan gum modulated IL-4, IL-10, and IL-12; and alum hydroxide modulated IFN-γ, TNF-α, IL-4, IL-10, and IL-12. In conclusion, it was found that the association of the recombinant prNcSRS2 protein with different adjuvants induced different levels of specific antibody, and a distinct splenic cytokine profile in an adjuvant-dependent manner. The mechanisms of adjuvancity activity is complex, so adjuvant formulation may help in the design of efficient vaccine to control Neosporosis.

1. Introduction

Neospora caninum infects a very wide range of livestock and causes important economic losses to the cattle industry [1]. The predominant route of transmission is the endogenous transplacental infection from a pregnant dam [2], and cows of any reproductive age may abort, with most abortions occurring at five to six months of gestation [3]. The development of a vaccine has been proposed as the most suitable control strategy [4]. It has been proposed that the incorporation of tachyzoite host cell adhesion/invasion protein would be ideal for a *N. caninum* vaccine as effective immune responses could block parasite dissemination and fetal transmission [5]. The surface protein NcSRS2 is involved in the *N. caninum* adhesion process and in host cell invasion [6] and has been regarded as a promising antigen candidate for use in vaccines.

However, recombinant subunit vaccines are often poorly immunogenic and require additional components to stimulate protective immunity [7]. Adjuvants are required to enhance the immunogenicity of antigens, ideally eliciting both humoral and cellular immune responses. A vaccine formulation (antigen/adjuvant) should be developed to activate balance immune responses that are likely to address pathogen evasion strategies. Thus, this study was undertaken to investigate the immune modulatory effect of adjuvants on the immune responses to the *N. caninum* recombinant antigen NcSRS2.

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2. Materials and methods

2.1. Parasite culture and recombinant prNcSRS2 preparation

The *N. caninum* isolate NC-1 was propagated in Vero cells using Dulbecco's modified essential medium supplemented (Cultilab, Campinas, Brazil) with 10% fetal calf serum (FCS, Cultilab, Campinas, Brazil), at 37 °C in a humidified atmosphere of 5% CO_2. The *Pichia pastoris* strain X33 (Invitrogen Tech, Carlsbad, CA, USA) was grown in yeast extract peptone dextrose (YPD) medium (1% yeast extract, 2% peptone and 2% D-glucose) or in 1.5% YPD agar at 30 °C supplemented with 100 μg/ml of zeocin. Briefly, the recombinant clone (positive by dot blotting and by colony PCR) was selected and inoculated into a 3-L baffled fermenter containing 1 L of BMMY broth (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 0.00004% biotin, 0.5% methanol, 100 mM potassium phosphate, pH 6.0), and the culture was incubated at 28 °C for approximately 48 h until an OD600 of 2–6 was reached. Expression was induced by the addition of methanol (Sigma-Aldrich) at 1% of the final concentration as previously described [8].

2.2. Vaccination of the mice

Female Balb/c, 4–6 weeks of age, were used in all experiments. The mice were housed in eight groups of 10 animals each, and food and water were provided *ad libitum*. Each mouse was subcutaneously inoculated with a 200-μL volume as follows: group 1 with PBS alone, group 2 with 20 μg recombinant prNcSRS2, group 3 with 20 μg recombinant NcSRS2 with water-in-oil emulsion (Montanide ISA 61VG, SEPPIC, São Paulo, Brazil), group 4 with 20 μg recombinant NcSRS2 adjuvanted with xanthan gum, and group 5 with 20 μg recombinant NcSRS2 adjuvanted with 2% Alhydrogel® (Brenntag Nordic, Haslev, Denmark). Group 6 was given PBS with water-in-oil emulsion; group 7 was given PBS with xanthan gum; and group 8 was given PBS with 2% Alhydrogel®. After 21 days, a boost inoculation was repeated in all groups with the same dose of vaccine. Blood samples were collected through the retro-orbital plexus before immunization and on days 7, 14, 21, 28 and 35 post-vaccination. The sera were collected and stored at −20 °C until use. The chemical characteristics of xanthan have been previously described [9], and LPS-exposure tests yielded low amounts of endotoxins (<0.2 EU/ml) (data not show).

All protocols were reviewed and approved by the Ethics Committee on Animal Experimentation (CEEA No. 9339) of the Universidade Federal de Pelotas (UFPel). The CEEA of UFPel is accredited by the Brazilian National Council for the Control of Animal Experimentation (CONCEA). The mice used in the study were provided by the animal unit at UFPel.

2.3. Dynamics of serum IgG antibodies against prNcSRS2

Antibody responses were monitored by an indirect ELISA using prNcSRS2 as the antigen. ELISA plates (Polysorp Surface, Nunc, Sigma-Aldrich, St. Louis, MO, USA) were coated overnight at 4 °C with 50 ng of recombinant protein per well per well in pH 9.6 carbonate-bicarbonate buffer. The plates were then washed three times using PBS-T (10 mM PBS with 0.05% Tween 20) and blocked using 10 mM PBS with 5% non-fat milk at 37 °C for 1 h. To determine the best serum dilution, a checkerboard titration was performed using different antigen concentrations and sera dilutions (pool of the sera from the 35th day). After three washes with PBS-T, the positive and negative control sera and serum samples, all in duplicate, were diluted to 1:100 in 10 mM PBS – T and incubated at 37 °C for 1 h. After three additional washes, anti-mouse IgG conjugated to peroxidase (Sigma-Aldrich, St. Louis, MO, USA) and diluted at 1:4000 in 10 mM PBS-T was added at 100 μL/well, which was followed by incubation at 37 °C for 1 h. After five washes, 100 μL of the substrate (o-phenylenediamine dihydrochloride; OPD tablets, Sigma-Aldrich) in phosphate–citrate buffer (0.4 mg/mL) containing 0.04% of 30% (v/v) hydrogen peroxide, pH 5.0, was added to each well, and the plates were incubated in the dark at room temperature for 15 min followed by the addition of 50 μL of stop buffer (1N H_2SO_4). Mean optical density (OD) at 492 nm was determined for all test wells using a microtiter plate reader (Multiskan MCC/340 MKII), and an intra-plate ELISA was performed. The IgG1 and IgG2a isotype levels were evaluated by ELISA using pooled sera. Briefly, the plates were coated as described for ELISA above and then 50 μL/well of pooled sera diluted to 1:200 in PBS-T was added to the wells, and the plates were incubated at 37 °C for 90 min. After this period, the plates were washed three times with PBST, and after adding 50 μL/well of anti-mouse IgG1 isotype antibody (Sigma-Aldrich) diluted 1:10,000 in PBS, they were incubated at 37 °C for 120 min. The same protocol was performed using anti-mouse IgG2 isotype antibody.

2.4. Ex vivo spleen stimulation and cytokine transcripts

Thirty-five days after the first inoculation, four mice per group were euthanized, and their spleens were removed. The splenocytes were suspended in Hank's solution, centrifuged and suspended in cell lysis solution (chloride ammonia 0.8%). Another wash was performed with Hank's solution, and the cells were suspended in RPMI 1640 (Cultilab, Campinas, Brazil) with 10% fetal bovine serum (Cultilab, Campinas, Brazil). The cells were incubated for 24 h at 37 °C in an atmosphere of 5% CO_2 and then stimulated with either prNcSRS2 (2.5 μg mL^{-1}) or concanavalin A (Con A, 10 μg mL^{-1}). The cells were incubated for 24 h under the same conditions and then collected in TRIzol (Invitrogen Tech, Carlsbad, CA, USA) and stored at −70 °C. Total RNA was extracted from the cells, and cDNA synthesis was performed from ~300 ng/μL of RNA according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Quantitative real-time polymerase chain reaction (qPCR) (MxPro-Mx3005P) was used to quantify the cytokines. The reaction was performed in a final volume of 12.5 μL containing 1 μL of cDNA, 6.25 μL of SYBR Green (Invitrogen Tech, Carlsbad, CA, USA), 0.5 μM of each primer and 4.25 μL of RNase-free water (Gibco-BRL, San Francisco, CA, USA). The samples underwent (1) denaturation at 95 °C for 5 min; (2) 40 cycles of amplification under the following conditions: 95 °C for 30 s, 60 °C for 60 s and 72 °C for 60 s; (3) final extension at 72 °C for 5 min. All analyses were performed in duplicate, and a control without cDNA was included to eliminate contamination or unspecific reactions. The value of the threshold cycle (cycle threshold – CT) was defined by the number of PCR cycles required for the fluorescence signal to exceed the threshold detection value. Beta-actin and GAPDH genes were used as endogenous reference controls, but subsequently, β-actin was selected as the internal reference standard based on its efficiency (M-value of 1.8 and 1.98 for GAPDH and β-actin, respectively). The primer sequence used was described elsewhere [10].

2.5. Statistical analysis

The differences in serum ELISA antibody titers between groups were analyzed by two-way ANOVA, and pair-wise comparisons were made using Tukey's multiple comparisons test, considering p-values of ≤ 0.05 to be significant. The cytokine results were expressed as the means ± S.E.M., and statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparisons test to investigate statistical differences obtained from