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Research paper

Immune response and serum bactericidal activity against *Brucella ovis* elicited using a short immunization schedule with the polymeric antigen BLSOmp31 in rams

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ABSTRACT

Brucella ovis is the etiologic agent of ovine brucellosis. The control measures for this disease are periodical diagnosis by serological tests and/or bacteriological culture and culling of positive animals. Vaccination with *Brucella melitensis* Rev 1 is recommended when prevalence is high. This attenuated strain vaccine gives protection against *B. ovis* but it has important disadvantages associated with the development of antibodies interfering with serodiagnosis, virulence for humans and the prohibition of its use in countries considered free of *B. melitensis*. Consequently, there is a need for new safe and effective brucellosis vaccines to be developed. We have previously reported that the polymeric subcellular vaccine BLSOmp31 confers protection against experimental challenge with *B. ovis* when rams are immunized three times. In the present work we evaluated and characterized, along 56 weeks after the first immunization of adult rams, the evolution of the immune response elicited by BLSOmp31 using a short immunization schedule.

1. Introduction

Brucella ovis causes a clinical or subclinical chronic disease in sheep that is characterized by epididymitis and decreased ram fertility, abortions in ewes and increased lamb mortality, with severe economic losses (Blasco, 1990; Alton et al., 1988). Control measures in flocks include culling of animals positive to serological tests and/or bacteriological culture. Vaccination is the only practical means of controlling the disease in countries with moderate to high incidence (Blasco, 1990).

A vaccine against *B. ovis* should be able to prevent infection and clinical lesions or, at least diminish the degree of infection in vaccinated animals. *B. melitensis* Rev 1, a smooth strain used to control *Brucella melitensis* infection in small ruminants, gives heterologous protection against *B. ovis* and is currently considered the best vaccine for the prophylaxis of ovine brucellosis (Blasco et al., 1987; Marín et al., 1990). However, due to its live

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attenuated nature, Rev 1 displays many drawbacks, including residual virulence and interference with serodiagnosis (Blasco and Diaz, 1993a). Consequently, there is a need for new brucellosis vaccines to be developed (Menzies, 2012). Different researchers have previously demonstrated that experimental subcellular vaccines based on outer membrane complex preparations of *B. ovis* (hot saline antigenic extracts (HS)), incorporated in selected adjuvants, are as effective as *B. melitensis* Rev 1 vaccine against *B. ovis* in mice and rams (Blasco et al., 1993b; Muñoz et al., 2006).

The outer membrane protein Omp31 is a major protein in the HS extracts and appears as immunodominant antigen in the course of *B. ovis* infection (Kittelberger et al., 1995, 1998). In addition, passive protection experiments in mice have shown that an anti-Omp31 monoclonal antibody conferred passive protection against *B. ovis* infection (Bowden et al., 2000). We have previously reported that detergent-extracted recombinant Omp31 (rOmp31 extract) from *B. melitensis* produced in *Escherichia coli* was immunogenic and conferred protection in mouse model, and reduced histopathological lesions in the reproductive tract of immunized rams after challenge with *B. ovis* (Estein et al., 2003, 2004). Moreover, serum antibodies efficiently killed *B. ovis in vitro* in the presence of ovine serum. On the other hand, recombinant Omp31 (rOmp31) also conferred protection associated with CD4+ mediated Th1 T cells stimulation in mice infected with *B. ovis* (Cassataro et al., 2007a).

The enzyme lumazine synthase from *Brucella* spp. (BLS) is highly immunogenic (Cassataro et al., 2007b), is a remarkably stable decameric protein (Velikovsky et al., 2003; Laplagne et al., 2004) and has adjuvant properties when a foreign antigen is covalently attached to it (Zylberman et al., 2004). Given the fact that Omp31 and the carrier BLS have been implicated in the generation of protective cellular and humoral immune responses, we have generated a recombinant chimera BLSOmp31, based on the addition at the N-termini of BLS of a 27-mer peptide containing the exposed loop epitope of Omp31 (Rosas et al., 2006). Immunization with BLSOmp31 in Incomplete Freund Adjuvant (IFA) conferred similar protection against *B. melitensis* Rev 1 and *B. ovis* infections in BALB/c mice (Cassataro et al., 2007b).

Recently, our group has evaluated the immunogenicity and protective efficacy elicited for BLSOmp31 against ovine brucellosis using different strategies of immunization. Chimera BLSOmp31, emulsified in IFA and administered three times, induced the highest levels of IgG specific antibodies with bacteriolytic activity, high levels of specific IFN gamma and protected 63% of vaccinated rams against *B. ovis* challenge (Estein et al., 2009).

In the present work, we have evaluated the humoral and cellular immune responses and the bactericidal activity of specific antibodies against *B. ovis* induced by immunizing adult sheep with BLSOmp31 chimera in IFA, following a short immunization schedule. The results presented herein suggest that subunit BLSOmp31 vaccine should require an annual booster to sustain a stable and protective immune response.

2. Materials and methods

2.1. Bacterial strains and antigen production

B. ovis PA-76250 (PA, for short) was obtained from our own laboratory collection and was used for bactericidal assay. Briefly, this strain was grown on Tryptone Soya Agar (Britania, Argentina) supplemented with 0.5% yeast extract (Britania, Argentina) and 5.0% sterile equine serum for 24 h at 37 °C in a 5% CO_2 atmosphere. For bactericidal assay, cells were harvested, spectrophotometrically adjusted in Phosphate Buffer Solution (PBS) to O.D. 600 = 0.165 (approximately 10^9 colony-forming units (CFU)/mL) (Ultrospec III, Pharmacia). Exact numbers of cells were assessed retrospectively by dilution and spreading on the required medium (Estein et al., 2003).

Strain *E. coli* BL21 (DE3) (Stratagene, La Jolla, CA) was used for expression of recombinant chimera BLSOmp31 (Cassataro et al., 2007a). This antigen was used for immunizations, antibody determinations by indirect enzyme-linked immunosorbent assay (ELISA) and interferon-gamma (IFN-gamma) assay.

2.2. Experimental design

2.2.1. Animals

Ten 2-year-old Polled Dorset rams belonging to the brucellosis free flock from the Instituto Nacional de Tecnología Agropecuaria (INTA, Argentina) were used. All animals were clinically normal and bacteriologically negative to *B. ovis*. In addition, sera were negative by Rapid Slide Agglutination test (RSAT) (Estein et al., 2009) and indirect ELISA against *B. canis* (IELISA-*Brucella canis*) (López et al., 2005).

Animals were randomized into two groups and were carefully identified. Rams were fed on natural pasture and maize–corn concentrate. Animal procedures and management protocols were approved by the Ethics Committee according to the Animal Welfare Policy (act 087/02) of the Facultad de Veterinaria, (Universidad Nacional del Centro de la Provincia de Buenos Aires, Tandil, Argentina; http://www.vet.unicen.edu.ar).

2.2.2. Vaccination schedule

Rams ($n = 6$) were vaccinated subcutaneously (two injections, four weeks apart) with 2 mL of chimera rBLSOmp31 (500 μg/ram) emulsified in IFA (Estein et al., 2009). Unvaccinated control group ($n = 4$) injected with phosphate buffered saline (PBS) was included. Reactions at injection sites were evaluated visually and by palpation following each immunization.

2.3. Analysis of humoral immune response

2.3.1. Specific antibody titer and isotyping

Blood samples to obtain serum were collected by jugular venipuncture using 10 mL vacutainer tubes (BD Vacutainer®) before immunization and at 4, 16, 22, 32 and 56 weeks after first immunization.

Serum reactivity against BLSOmp31 was determined by ELISA as described previously (Estein et al., 2009). Briefly, polystyrene plates (Maxisorp; Nunc, Roskilde, Denmark)