Veterinary Immunology and Immunopathology 154 (2013) 36–41

Contents lists available at SciVerse ScienceDirect

Veterinary Immunology and Immunopathology

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

Research paper

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

Alejandra G. Díaz[a,e], María Clausse[a,e], Fernando A. Paolicchi[b], María A. Fiorentino[b], Giselle Ghersi[d], Vanesa Zylberman[d], Fernando A. Goldbaum[c,d], Silvia M. Estein[a,e,*]

[a] *Laboratorio de Inmunología, Depto. SAMP, Centro de Investigación Veterinaria de Tandil (CIVETAN)- CONICET, Facultad de Ciencias Veterinarias, Universidad Nacional del Centro de la Provincia de Buenos Aires (U.N.C.P.B.A.), Tandil, Buenos Aires, Argentina*
[b] *Laboratorio de Bacteriología, Departamento de Producción Animal, Instituto Nacional de Tecnología Agropecuaria (INTA), Balcarce, Argentina*
[c] *Fundacion Instituto Leloir e Instituto de Investigaciones Biológicas Buenos Aires-CONICET, Argentina*
[d] *Inmunova S.A., Argentina*
[e] *Comisión Nacional de Investigaciones Científicas (CONICET), Argentina*

ARTICLE INFO

Article history:
Received 18 December 2012
Received in revised form 4 April 2013
Accepted 5 April 2013

Keywords:
Chimera BLSOmp31
Ram
Immune response
Short immunization schedule

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.

© 2013 Elsevier B.V. All rights reserved.

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

* Corresponding author at: Laboratorio de Inmunología, CIVETAN, CONICET, Facultad de Ciencias Veterinarias, U.N.C.P.B.A., Tandil, Pinto 399, Buenos Aires, Argentina. Tel.: +54 11 22494439850; fax: +54 11 22494439850.
E-mail address: silmares@vet.unicen.edu.ar (S.M. Estein).

0165-2427/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.vetimm.2013.04.003

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)

were sensitized with rBLSOmp31 and were incubated with diluted sera. After the plates were washed, a donkey anti-sheep immunoglobulin–horseradish peroxidase conjugate (Sigma, St. Louis, MO) was added and the reaction developed with 2,2′-azino-di (3-ethylbenzothiazolinesulphonic acid) (ABTS)–H_2O_2. Absorbance was taken at 405 nm in automatic ELISA reader (Titertek, Multiskan EX, Labsystems). Antibody levels (IgG) were expressed as the arithmetic mean ± SD of the O.D. obtained for rams included in each group.

Isotypes of IgG were determined by using monoclonal antibodies specific for ovine IgG1 and IgG2 kindly provided by Dr Tony Pernthaner (AgResearch, New Zealand). Bound antibodies were detected by a goat anti-mouse IgG (whole-molecule) conjugated to horseradish peroxidase (Sigma) (1/3000). Enzyme activity was revealed as indicated to determine IgG levels. Isotypes of IgG were expressed as the arithmetic mean ± SD of the O.D. obtained for rams included in each group.

2.3.2. Serological diagnosis tests

Serum samples were assayed in I ELISA against HS from *B. canis* and in RSAT to evaluate the serological response against *B. ovis* as previously described (López et al., 2005; Estein et al., 2009). Buffered plate agglutination test (BPAT) was performed to evaluate serological interference induced against smooth *Brucellae* (OIE, 2009).

2.3.3. Complement-mediated bacteriolysis assay

Sera collected at weeks 0, 22 and 56 were tested for their ability to promote *in vitro* complement-mediated killing of *B. ovis*. This assay was performed in 96-well, flat bottomed polystyrene micro-titer plates (Linbro, Italy) as previously described (Estein et al., 2004). Briefly, 1.0×10^4 UFC of *B. ovis* PA/mL (50 μL) were suspended in PBS containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$ and mixed with ram heat inactivated serum (50 μL). The mixtures were shaken at 100 rpm for 90 min at 37 °C. As source of complement, 40 μL of fresh sheep serum was dispensed in appropriate wells. Mixtures were shaken at 100 rpm for 120 min at 37 °C. Controls, in duplicate, included antibody–*B. ovis* mixtures with (i) heat inactivated complement sources and (ii) without complement. Next, 50 μL of each well was plated in duplicate on required medium. The mean percentage of bacterial killing (%*K*) was calculated as: $\%K = 100 \times 1 -$ (mean number of CFU/mL after incubation/mean number CFU/mL before incubation).

2.4. Cellular assays

Cell-mediated immune response was investigated both *in vivo* by intradermal reaction to BLSOmp31 and *in vitro* by antigenic stimulation of whole blood cell cultures followed by an ELISA detection of IFN-γ.

2.4.1. Intradermal reaction to BLSOmp31

Twenty two and fifty six weeks after the first immunization, all rams were injected intradermally into wool-free region of the inside thigh with BLSOmp31 (10 μg in 0.1 mL). The skin thickness was measured with a Vernier caliper just before injection (initial skin thickness) and at 72 h later (Estein et al., 2004).

2.4.2. Interferon gamma assay

Blood samples were collected in heparinised tubes (BD Vacutainer®) at weeks 0, 22 and 56 after first immunization. Whole-blood cultures were incubated with chimera BLSOmp31 (20 μg/well) in 5% CO_2 atmosphere as previously described (Estein et al., 2009). Control cultures were incubated with Pokeweed mitogen (Sigma) (10 μg/well) and PBS. Supernatants were harvested after 48 h of culture and stored at −80 °C until assayed for IFN-γ content.

Interferon-γ was assayed by using an ELISA kit (Mabtech, Sweden). Concentrations of ovine IFN-γ were calculated using a standard curve with serial dilution of recombinant ovine IFN-γ. Data was expressed in pg/mL.

2.5. Statistical analysis

Data from ELISA and IFN-γ assay were analyzed by ANOVA followed by Tukey *post hoc* tests. The analysis was performed using INFOSTAT, 2008. Graphs were performed using Graph Pad software, version 5.0, San Diego, CA.

3. Results

3.1. A short immunization schedule with chimeric BLSOmp31 induces a vigorous humoral immune response that decreases significantly after one year

Rams immunized with BLSOmp31 formulated in IFA presented significantly higher specific IgG antibody levels ($p < 0.001$) than control group at 4 weeks after the first vaccination. These antibody levels remained stable until week 22 without significant differences within the group. Afterwards, antibody titers fell slowly and gradually to the end of the experiment (Fig. 1).

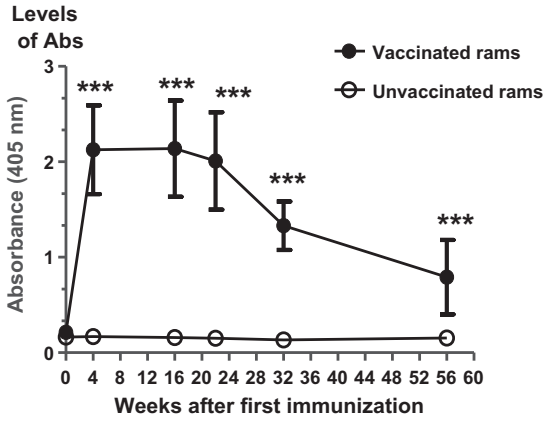

Fig. 1. Antibody response to BLSOmp31 was determined in individual ram sera from each immunization group at 0, 4, 16, 22, 32 and 56 weeks after the first immunization. Sera were diluted 1/200 and assayed against purified BLSOmp31. Data are expressed as arithmetic mean ± S.D. absorbances units. Values significantly different from non-immunized group are indicated by ***$p < 0.001$.

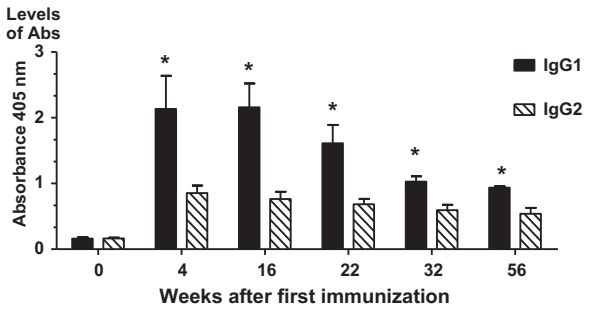

Fig. 2. Mean anti-BLSOmp31 IgG1 and IgG2 titers from vaccinated rams with BLSOmp31 in IFA. Sheep were injected i.m. with 500 μg at time 0 and 4 weeks. Data are expressed as arithmetic mean ± S.D. absorbances units. Values significantly different from non-immunized group are indicated by $^{*}p < 0.05$.

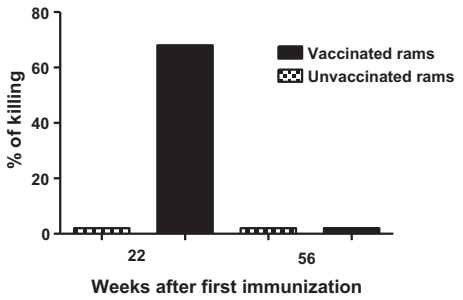

Fig. 3. Bactericidal activity of immunized ram sera against *B. ovis* in the presence of ovine serum as a source of complement after 2 h of incubation. Ram sera from vaccinated and unvaccinated groups were taken at weeks 22 and 56 post first immunization. % Killing (*i.e.* the percentage of bacteria killed) = 100 × 1 − (the mean number of CFU/mL after incubation/mean number CFU/mL before incubation). Data are representative of two separated experiments.

3.2. Chimeric BLSOmp31 elicits a mixed humoral Th1–Th2 immune response in immunized rams during the whole immunization schedule

As IgG1 and IgG2 are considered to be markers of Th2 and Th1 responses, respectively, anti-BLSOmp31 specific antibodies of both isotypes were also measured. As shown in Fig. 2, rBLSOmp31-immunization elicited high levels of anti-BLSOmp31 IgG1 as well as IgG2 antibodies.

Rams injected with PBS did not show specific anti-BLSOmp31 during the whole immunization schedule.

3.3. Specific antibodies against BLSOmp31 do not interfere with conventional serological brucellosis diagnosis tests

Sera taken from any of the animals immunized with BLSOmp31 were seronegative in both tests performed to evaluate immune response to *B. ovis* (I ELISA *B. canis* and RSAT). As expected, the seroagglutination (BPAT) was always negative with all the sera from immunized rams throughout the study (Table 1).

3.4. Specific antibodies elicited by the chimera BLSOmp31 were able to mediate complement bacteriolysis of B. ovis

Sera from rams immunized with chimera rBLSOmp31 formulated in IFA showed bactericidal activity at week 22 after first immunization (73.94%) (Fig. 3). This activity disappeared at week 56 after the first immunization showing a high correlation with lower levels of IgG specific antibodies. Unvaccinated rams (PBS) did not exhibit bactericidal activity at any time. A pool of sera taken before the immunization did not show any bactericidal activity indicating that BLSOmp31 specific antibodies induced by immunization were essential for bacterial killing (data not shown). Heat inactivation of the complement sources completely abolished bactericidal activity, confirming that complement was essential (data not shown).

3.5. Chimeric BLSOmp31 stimulates high levels of specific IFN-γ in whole-blood of immunized rams and stimulates in vivo cellular immune responses that decrease significantly after one year

To test *in vivo* correlates of cellular mediated immunity (CMI), rams were intradermally tested with rBLSOmp31.

Table 1
Serological tests to detect anti-*Brucella ovis* and anti-smooth *Brucella* antibodies in rams vaccinated with BLSOmp31 formulated in AFI.

Ram	Weeks post immunization														
	Week 4			Week 16			Week 22			Week 32			Week 56		
	%P	RSAT	BPAT	%P	RSAT	BPAT	%P	RSAT	BPAT	%P	RSAT	BPAT	%P	RSAT	BPAT
1	30.4	Neg	Neg	23	Neg	Neg	24.5	Neg	Neg	21.4	Neg	Neg	21.4	Neg	Neg
2	6.1	Neg	Neg	30.4	Neg	Neg	30.5	Neg	Neg	27.4	Neg	Neg	16.1	Neg	Neg
3	16.4	Neg	Neg	23	Neg	Neg	24.5	Neg	Neg	21.4	Neg	Neg	21.4	Neg	Neg
4	19.3	Neg	Neg	30.4	Neg	Neg	30.5	Neg	Neg	27.4	Neg	Neg	16.1	Neg	Neg
5	21.5	Neg	Neg	6.1	Neg	Neg	8.1	Neg	Neg	6.4	Neg	Neg	16.4	Neg	Neg
6	18.4	Neg	Neg	16.4	Neg	Neg	14.7	Neg	Neg	9.2	Neg	Neg	19.3	Neg	Neg
7	11.8	Neg	Neg	19.3	Neg	Neg	12.3	Neg	Neg	10.6	Neg	Neg	21.5	Neg	Neg
8	20	Neg	Neg	22.5	Neg	Neg	25.4	Neg	Neg	19.1	Neg	Neg	18.4	Neg	Neg
9	12.8	Neg	Neg	19.3	Neg	Neg	12.3	Neg	Neg	10.6	Neg	Neg	11.5	Neg	Neg
10	23	Neg	Neg	21.5	Neg	Neg	25.6	Neg	Neg	19.2	Neg	Neg	18.4	Neg	Neg

IELISA-*B. canis* %P, cut-off value: >39; BPAT: buffered plate antigen test; RSAT: rapid slide agglutination test; Neg: negative.

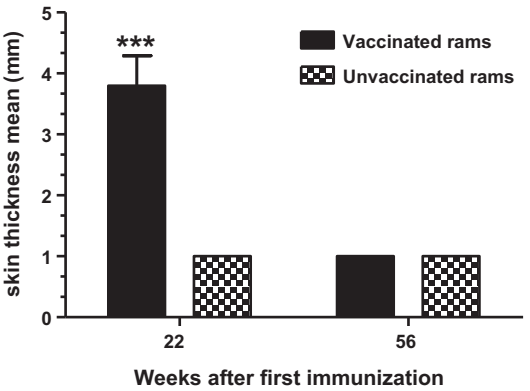

Fig. 4. Increase in the skin thickness following an intradermal injection with purified BLSOmp31 at week 22 and 56 after the first immunization. Measure was taken at 90 h after the injection. Values represent the mean differences between skin thickness after and before injection in the same animal. Values significantly different from non-immunized group are indicated: ***$p < 0.001$.

As illustrated in Fig. 4, at week 22 after the first immunization, immunized animals exhibited a marked local increase in skin thickness when compared with unvaccinated rams. However, when intradermic reaction was performed at 56 weeks after the first immunization, all vaccinated rams were negative.

3.6. IFN gamma determination

Immunization with BLSOmp31 stimulates high levels of IFN-γ in whole-blood cultures at week 22 after the first immunization with respect to negative control ($p < 0.001$). At week 56, IFN-γ levels were not detected in culture supernatants of cells from immunized rams. This cytokine was not observed in culture supernatants of whole blood cells from unvaccinated rams (Fig. 5).

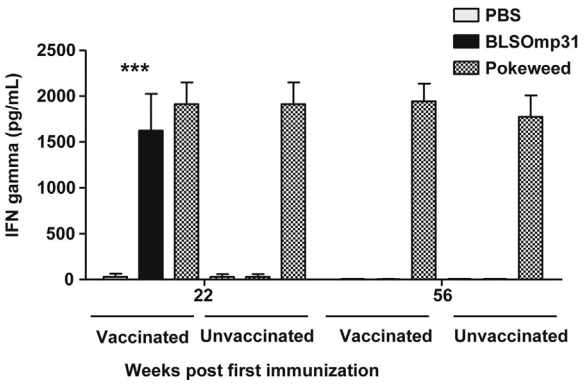

Fig. 5. Determination of antigen-specific IFN-γ response of whole-blood cells from immunized rams with BLSOmp31 in IFA. IFN-γ in cell supernatants was quantified (pg/mL) by mAb-capture ELISA. Each value represents the mean of duplicates ± S.D. of the response cells from individual rams. ***Significantly different from the same stimulus in unvaccinated rams ($p < 0.001$).

4. Discussion

For practical and economic reasons, veterinarian vaccines need to be applied in simple and short schedules. Thus, in the present work we examined along 56 weeks the evolution of the immune response elicited by immunization with only two doses of the candidate subunit vaccine BLSOmp31 against ovine brucellosis. We examined the immunogenicity and protective mechanisms against *B. ovis* of this vaccine in Polled Dorset rams. Rams immunized twice with adjuvanted BLSOmp31 developed high levels of serum IgG antibodies, similar to that found in previous studies using three doses of the same vaccine. In addition, different age or breed had no impact in the immunogenicity of the chimera BLSOmp31 since the antibodies titers elicited by the short immunization scheme were similar to those already reported (Estein et al., 2009). However, the strong humoral immune response induced with two doses of this vaccine declined slowly and gradually to the end of the experiment. The structural characteristics of BLS could explain the strong B cell response elicited when rams are immunized with BLSOmp31 (Zylberman et al., 2004). The polymeric nature of BLS, *i.e.* a repetitive and spatially ordered presence of a B- and Th1-epitopes derived from an exposed loop of the protein Omp31 would produce a strong signal transduction mediated by B cell receptors in rams, the susceptible host of *B. ovis* infection (Estein et al., 2009). Our results suggest that an annual boost of this subunit vaccine may be required to sustain a protective immune response.

BLSOmp31 induced high titers of IgG1 and IgG2, which is in agreement with its natural capacity of induce a mixed Th2/Th1 response (Velikovsky et al., 2003). The proportion of isotypes in an immune response is regulated by the balance of Th1/Th2 interleukins (Th1 cytokines promote IgG2 whereas Th2 cytokines favors IgG1), by the quantity and quality of the antigen and by the adjuvant used (Estes and Brown, 2002). In addition, IFA is a potent adjuvant known to induce primarily humoral response prone to produce more IgG1 antibodies than IgG2 (Th2 dominated response) (Yip et al., 1999). This further supports the notion that chimera BLSOmp31 induce a mixed T-helper response when we used IFA, adjuvant widely used in commercial veterinary vaccines.

The elicited antibodies after BLSOmp31 immunization did not interfere with the serological tests used for diagnosis of ovine brucellosis caused by *B. ovis* or *B. melitensis.* Combined eradication programs based on test, slaughter and vaccination, are the best method of brucellosis control in domestic animals under moderate to high prevalence conditions (Moriyón et al., 2004). Therefore, the assessment that the antibodies elicited by the BLSOmp31 vaccine do not interfere with the conventional serological tests used for diagnosis is considered a gold standard in combined eradication programs. This fact would be one of the main benefits of BLSOmp31 over the reference living attenuated *B. melitensis* Rev 1 vaccine.

Since bactericidal antibodies are believed to be important for protective immunity against *B. ovis*, we tested the sera of the individual rams for their ability to promote *in vitro* complement-mediated killing of this bacteria.

As previously reported (Estein et al., 2009), ram specific antibodies elicited by the chimera BLSOmp31 were able to mediate complement bacteriolysis of *B. ovis*. Also, cells from immunized rams stimulated *in vitro* with BLSOmp31 produced IFN-γ indicating that immunization with chimeric protein induces a Th1 cellular response mediated by activation of macrophages, which are the major effectors mediating the killing of the bacterium (Estein et al., 2009). In addition, BLSOmp31 elicited an *in vivo* hypersensitivity response after BLSOmp31 intradermal injection.

In conclusion, we have shown that two doses of chimera BLSOmp31 in IFA induced both lytic antibody and Th1-cell-mediated immune responses. Future studies with this vaccination schedule will be performed to evaluate the effect of annual booster in the immune response and protection against *B. ovis* in lambs and rams.

Conflict of interests

The authors have declared that no competing interests exist.

Authors' contributions

Conceived and designed the experiments: SME. Performed the experiments: SME, AGD, MAF, FAP, VZ, GG, MC. Analyzed the data: SME. Contributed reagents/materials/analysis tools: SME, FAP. Wrote the paper: SME. AGD, FAG, MAF, FAP, VZ critically revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by grant from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET): PIP 614 (to SME) and AESA 202811 (INTA). SME, FAG and VZ are members of the Research Career of CONICET. AGD, MC and GG are recipients of a fellowship from CONICET (Argentina). We thank Sergio Dinino for animal care and Rosana Malena for your technical assistance (INTA).

References

Alton, G.G., Jones, L.M., Angus, R.D., Verger, J.M., 1988. Techniques for the Brucellosis Laboratory. Institute National de la Recherche Agronomique, INRA, Paris, France.

Blasco, J.M., Marín, C.M., Barberán, M., Moriyón, I., Díaz, R., 1987. Immunization with *Brucella melitensis* Rev 1 against *B. ovis* infection of rams. Vet. Microbiol. 14, 381–392.

Blasco, J.M., 1990. *Brucella ovis*. In: Nielsen, K., Duncan, J.R. (Eds.), Animal Brucellosis. CRC Press, Boca Ratón, FL, pp. 352–378.

Blasco, J.M., Diaz, R., 1993a. *Brucella melitensis* Rev 1 vaccine as a cause of human Brucellosis. Lancet 342, 805.

Blasco, J.M., Gamazo, C., Winter, A.J., Jiménez de Bagués, M.P., Marín, C., Barberán, M., Moriyón, I., Alonso-Urmeneta, B., D'ıaz, R., 1993b. Evaluation of whole cell and subcellular vaccines against *Brucella ovis* in rams. Vet. Immunol. Immunopathol. 37, 257–270.

Bowden, R.A., Estein, S.M., Zygmunt, M.S., Dubray, G., Cloeckaert, A., 2000. Identification of protective outer membrane antigens of *Brucella ovis* by passive immunization of mice with monoclonal antibodies. Microbes Infect. 2, 481–488.

Cassataro, J., Estein, S.M., Pasquevich, K.A., Velikovsky, C.A., de la Barrera, S., Bowden, R., Fossati, C.A., Giambartolomei, G.H., 2007a. Vaccination with the recombinant *Brucella* outer membrane protein 31 or a derived 27-amino-acid synthetic peptide elicits a CD4+ T helper 1 response that protects against *Brucella melitensis* infection. Infect. Immun. 73, 8079–8088.

Cassataro, J., Pasquevich, K.A., Estein, S.M., Laplagne, D.A., Zwerdling, A., de la Barrera, S., Bowden, R.A., Fossati, C.A., Giambartolomei, G.H., Goldbaum, F.A., 2007b. DNA vaccine coding for the chimera BLSOmp31 induced a better degree of protection against *Brucella ovis* and a similar degree of protection against *Brucella melitensis* than Rev 1 vaccination. Vaccine 25, 4437–4446.

Estein, S.M., Cassataro, J., Vizcaíno, N., Zygmunt, M., Cloeckaert, A., Bowden, R.A., 2003. The recombinant Omp31 from *Brucella melitensis* alone or associated with rough lipopolysaccharide induces protection against *Brucella ovis* infection in BALB/c mice. Microbes Infect. 5, 85–93.

Estein, S.M., Cheves, P.C., Fiorentino, M.A., Cassataro, J., Paolicchi, F.A., Bowden, R.A., 2004. Immunogenicity of recombinant Omp31 from *Brucella melitensis* in rams and serum bactericidal activity against *B. ovis*. Vet. Microbiol. 102, 203–213.

Estein, S.M., Fiorentino, M.A., Paolicchi, F.A., Clausse, M., Manazza, J., Cassataro, J., Giambartolomei, G.H., Coria, L.M., Zylberman, V., Fossati, C.A., Kjeken, R., Goldbaum, F.A., 2009. The polymeric antigen BLSOmp31 confers protection against *Brucella ovis* infection in rams. Vaccine 27, 6704–6711.

Estes, D.M., Brown, W.C., 2002. Type 1 and type 2 responses in regulation of Ig isotype expression in cattle. Vet. Immunol. Immunopathol. 90, 1–10.

Kittelberger, R., Hansen, F., Hilbink, M.F., De Lisle, G.W., Cloeckaert, A., 1995. Selective extraction of bacterial macromolecules by temperature-induced phase separation in Triton X-114 solution. J. Microbiol. Methods 24, 81–92.

Kittelberger, R., Diack, D.S., Vizcaíno, N., Zygmunt, M.S., Cloeckaert, A., 1998. Characterization of an immuno-dominant antigen in *Brucella ovis* and evaluation of its use in an enzyme-linked immunosorbent assay. Vet. Microbiol. 59, 213–227.

Laplagne, D.A., Zylberman, V., Ainciart, N., Steward, M.W., Sciutto, E., Fossati, C.A., Goldbaum, F.A., 2004. Engineering of a polymeric bacterial protein as a scaffold for the multiple display of peptides. Proteins 57, 820–828.

López, G., Ayala, S.M., Escobar, G.I., Lucero, N.E., 2005. Use of *Brucella canis* antigen for detection of ovine serum antibodies against *Brucella ovis*. Vet. Microbiol. 105, 181–187.

Marín, C.M., Barberán, M., Jiménez de Bagüés, M.P., Blasco, J.M., 1990. Comparison of subcutaneous and conjunctival routes of Rev 1 vaccination with prophylaxis of *Brucella ovis* infection in rams. Res. Vet. Sci. 48, 209–215.

Menzies, P.I., 2012. Vaccination programs for reproductive disorders of small ruminants. Anim. Reprod. Sci. 130, 162–172.

Moriyón, I., Grillo, M.J., Monreal, D., Gonzalez, D., Marin, C., Lopez-Goñi, I., Mainar- Jaime, R.C., Moreno, E., Blasco, J.M., 2004. Rough vaccines in animal brucellosis: structural and genetic basis and present status. Vet. Res. 35, 1–38.

Muñoz, P.M., Estevan, M., Marín, C.M., De Miguel, M.J., Grilló, M.J., Barberán, M., 2006. Brucella outer membrane complex-loaded microparticles as a vaccine against *Brucella ovis* in rams. Vaccine 24, 1897–1905.

Office Internationale des Epizootie (OIE), 2009. Caprine and ovine Brucellosis (excluding *Brucella ovis*). In: Manual of Standards for Diagnostic Tests and Vaccines. OIE, Paris (chapter 2.4.2).

Rosas, G., Fragoso, G., Ainciart, N., Esquivel-Guadarrama, F., Santana, A., Bobes, R.J., Ramírez-Pliego, O., Toledo, A., Cruz-Revilla, C., Meneses, G., Berguer, P., Goldbaum, F.A., Sciutto, E., 2006. *Brucella* spp. lumazine synthase: a novel adjuvant and antigen delivery system to effectively induce oral immunity. Microbes Infect. 8, 1277–1286.

Velikovsky, C.A., Goldbaum, F.A., Cassataro, J., Estein, S.M., Bowden, R.A., Bruno, L., Fossati, C.A., Giambartolomei, G.H., 2003. Brucella lumazine synthase elicits a mixed Th1–Th2 immune response and reduces infection in mice challenged with *Brucella abortus* 544 independently of the adjuvant formulation used. Infect. Immun. 71, 5750–5755.

Yip, H.C., Karulin, A.Y., Tary-Lehmann, M., Hesse, M.D., Radeke, H., Heeger, P.S., Trezza, R.P., Heinzel, F.P., Forsthuber, T., Lehmann, P.V., 1999. Adjuvant-guided type-1 and type-2 immunity: infectious/noninfectious dichotomy defines the class of response. J. Immunol. 162, 3942–3949.

Zylberman, V., Craig, P.O., Klinke, S., Braden, B.C., Cauerhff, A., Goldbaum, F.A., 2004. High order quaternary arrangement confers increased structural stability to *Brucella* spp. lumazine synthase. J. Biol. Chem. 279, 8093–8101.