Vaccine 27 (2009) 6704–6711

Contents lists available at ScienceDirect

Vaccine

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

The polymeric antigen BLSOmp31 confers protection against *Brucella ovis* infection in rams

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ARTICLE INFO

Article history:
Received 15 July 2009
Received in revised form 24 August 2009
Accepted 25 August 2009
Available online 11 September 2009

Keywords:
Brucella ovis
BLSOmp31
Immunogenicity
Protection
Rams

ABSTRACT

We have engineered the polymeric vaccine BLSOmp31 by decorating the highly immunogenic and decameric *Brucella* lumazine synthase with an exposed loop of the *Brucella* outer membrane protein Omp31. In the present study, we have immunized different groups of rams with the recombinant chimera rBLSOmp31 in two different adjuvants (Incomplete Freund Adjuvant—IFA and QUIL A) and with the plasmid pCIBLSOmp31 administered either by i.m. injection alone or by using electroporation. In addition, we have used a heterologous prime-boost strategy consisting of repeated pCIBLSOmp31 electroporation priming followed by a single protein boost. Both, chimera rBLSOmp31 in IFA and the prime-boost strategy induced the highest IgG specific antibodies with bacteriolytic activity. While electroporation-enhanced humoral immune responses as compared to pCIBLSOmp31 injection alone, the highest levels of specific IFN-γ and protection against bacterial challenge were achieved with prime-boost (76%) and chimera rBLSOmp31 in IFA (63%). Taken together these results strongly support the usefulness of the chimera BLSOmp31 as a vaccine against *Brucella ovis* in ovine brucellosis.

1. Introduction

Brucella ovis and *B. melitensis* are the etiologic agents of ovine brucellosis. *B. ovis* causes a clinical or subclinical chronic disease in sheeps that is characterized by epididymitis and decreased ram fertility, abortions in ewes and increased lamb mortality, with severe economic losses [1,2]. 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 [1,2].

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, an attenuated smooth strain used to control *B. melitensis* infection in small ruminants, gives heterologous protection against *B. ovis* and is currently considered the best vaccine for the prophylaxis of ovine brucellosis [3,4]. However, there are significant limitations associated with its use, namely the development of antibodies that can interfere with serological diagnosis, virulence for humans and the prohibition of its use in countries considered free of *B. melitensis* [5]. Consequently, there is a need for new brucellosis vaccines to be developed. Different researchers have previously demonstrated that experimental subcellular vaccines based on outer membrane complex preparations of *B. ovis* (hot saline [HS] antigenic extracts) incorporated in selected adjuvants confer protection against *B. ovis* in mice and rams as effective as *B. melitensis* Rev. 1 vaccine [6,7].

Innovative approaches including recombinant protein and plasmid DNA vaccines are still under development. In large animals, DNA vaccines have demonstrated safety but only modest immune responses were achieved [8,9]. Recently, electroporation-enhanced delivery of plasmid DNA have been used in large animals and have resulted in increased DNA uptake in muscle, leading to robust

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doi:10.1016/j.vaccine.2009.08.097

trans-gene expression levels and enhanced humoral and cellular immune responses [10]. In addition, prime-boost strategies, the sequential delivery of a vaccine antigen by different modalities, are being developed to generate potent humoral and cellular immune responses which have led to superior levels of protection against a number of veterinary and human diseases [11–13]. While the exact underlying mechanism remains unsolved, multiple studies have demonstrated that plasmid DNA encoded antigens "primes" the immune system to recognize the same antigens much more effectively when they are delivered later as recombinant protein leading to a substantial increase in immune responses [14].

We have previously reported that recombinant *B. melitensis* Omp31 extract conferred protection against *B. ovis* in mouse model [15]. This immunogen induced potent antibody and cellular immune responses in sheep and reduced histopathological lesions in the reproductive tract of immunized rams after challenge with *B. ovis* [16]. Moreover, serum antibodies efficiently killed *B. ovis in vitro* in the presence of ovine serum [16]. On the other hand, recombinant Omp31 (rOmp31) or DNA vaccine (pCIOmp31) also conferred protection in mice against *B. ovis* associated with the stimulation of different immune mechanisms [17]. Also, pCIOmp31 priming followed by rOmp31 boosting led to moderately improved protection against *B. ovis* challenge [18].

The enzyme lumazine synthase from *Brucella* spp. (BLS) is highly immunogenic [16] and it has shown to confer partial protection against *B. abortus* as a DNA vaccine (pCIBLSOmp31) or as recombinant protein (rBLSOmp31) [19,20]. BLS is a remarkably stable decameric protein [21,22] and has adjuvant properties when a foreign antigen is covalently attached to it [23]. 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 rBLSOmp31 based on the addition to the N-termini of BLS of a 27-mer peptide containing the exposed loop epitope of Omp31 [24]. Immunization with rBLSOmp31 in Incomplete Freund Adjuvant (IFA) or pCIBLSOmp31 conferred similar protection to *B. melitensis* Rev. 1 against *B. ovis* infection in BALB/c mice [24].

In the present work, we have studied the immunogenicity and protective capacity of the BLSOmp31 antigen in sheep following different modalities of delivery. In addition, we have evaluated the bactericidal activity of specific antibodies in presence of complement against *B. ovis*. The results presented herein show that BLSOmp31 is an excellent candidate for the development of a subunit vaccine against ovine brucellosis.

2. Materials and methods

2.1. Bacterial strains

B. ovis PA-76250 (PA, for short) and *B. ovis* REO198 were obtained from the INRA *Brucella* culture collection (Laboratoire de Pathologie Infectieuse et Immunologie, INRA-Nouzilly, France). *B. ovis* PA was used as challenge strain after two serial passages in BALB/c mice and re-isolation from spleens. Bacterial suspensions were prepared as previously described [15]. 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 72 h at 37 °C in a 5% CO_2 atmosphere. For infection, cells were harvested, spectrophotometrically adjusted in PBS to a OD_{600} = 0.165 (approximately 10^9 CFU mL^{-1}). Exact numbers of cells were assessed retrospectively by dilution and spreading on the required medium [15]. *B. ovis* REO 198 was used for preparation of HS extract (see below).

2.2. Antigens for immunization and immunological assays

2.2.1. Recombinant protein

For immunizations, antibody determinations by indirect enzyme-linked immunosorbent assay (ELISA) and interferon-gamma (IFN-γ) assay, purified rBLSOmp31 was used. Chimera rBLSOmp31 was expressed in *E. coli* and purified as previously reported [24].

2.2.2. Plasmid pCIBLSOmp31

For immunizations, DNA vaccine coding for BLSOmp31 (pCIBLSOmp31) was constructed and characterized as previously [21].

2.2.3. Hot saline extract

For immunizations, HS from *B. ovis* REO 198 was prepared as recommended [25]. Protein was quantified by the bicinchoninic acid method (BCA Protein Assay, Pierce, Rockford, IL).

2.3. Experimental design

2.3.1. Animals

Seventy Romney Marsh and Corriedale male lambs, 4–5 months old, were obtained from Instituto Nacional de Tecnología Agropecuaria (INTA), Buenos Aires Province, Argentina. Animals were distributed at random in seven groups (10 rams per group) and were carefully identified. Animals were fed on natural pasture experiment and maize–corn concentrate. All the animals had free access to water. Animal procedures and management protocols were approved by the Ethics Committee according to the Animal Welfare Policy (act 087/02) of the Faculty of Veterinary Medicine (U.N.C.P.B.A, Tandil, Argentina; http://www.vet.unicen.edu.ar).

2.4. Vaccination schedules

2.4.1. Immunization with recombinant protein

Two groups were immunized with 2 mL of chimera rBLSOmp31 (500 μg) emulsified in IFA (Sigma, St. Louis, MO) or in QUIL A (Accurate Chemical & Scientific Corporation) adjuvants. Both groups were immunized three times with an interval of 4 weeks between immunizations by intramuscular or subcutaneous injection, respectively (Table 1).

2.4.2. Immunization with plasmid pCIBLSOmp31

Two groups were immunized with pCIBLSOmp31, either by intramuscular injection alone or by using electroporation-enhanced delivery. Both groups were vaccinated three times with an interval of 4 weeks between immunizations (Table 1). For this purpose, lambs were sedated by intramuscular injection of

Table 1
Vaccination schedule of rams with chimera BLSOmp31 using different strategies of immunization.

Vaccine or control (n = 10)	Weeks of immunization			
	−4	0	4	8
PBS		□	□	□
Prime-boost	●	●	●	▲
Electroporated pCIBLSOmp31		●	●	●
Nonelectroporated pCIBLSOmp31		○	○	○
rBLSOmp31 + IFA		▲	▲	▲
rBLSOmp31 + QUIL A		△	△	△
HS extract + IFA		■	■	■

(□) PBS, intramuscular (i.m.); (●) pCIBLSOmp31, 500 μg, i.m. with electroporation (Elgen 1000, Inovio, San Diego); (○) pCIBLSOmp31, 500 μg, i.m.; (▲) chimera rBLSOmp31 + IFA, 500 μg, i.m.; (△) chimera rBLSOmp31 + Quil A, 500 μg, subcutaneous (s.c); (■) HS extract from *B. ovis* + IFA, 800 μg, i.m.

100 μL of xylacine 2% (Richmond, Argentina). About 15 min later the animals were electroporated with pCIBLSOmp31. This plasmid (500 μg) was dissolved in phosphate-buffered saline (PBS) and delivered to the hamstring muscle in a total volume of 600 μL using the Elgen 1000 electroporation system (Inovio BC, San Diego). The Elgen 1000 injector uses two standard 1 mL syringes and was equipped with 21G × 2′ hypodermical needles which also serve as electrodes. Plasmid pCIBLSOmp31 was injected into muscles during insertion of two needles to ensure optimal distribution of the injectate [26] and was immediately followed by delivery of 5× 20 ms pulses at 400 mA at 4 Hz. The remaining group was vaccinated with pCIBLSOmp31 by standard intramuscular injection without electroporation (Table 1).

2.4.3. Immunization with prime-boost strategy

Lambs of prime-boost group were immunized with pCIBLSOmp31 by using the intramuscular electroporation-enhanced delivery as described above, followed by a final intramuscular booster (fourth injection) performed with chimera rBLSOmp31 in IFA (Table 1).

2.4.4. Control groups

A positive control group was vaccinated with 2 mL of HS extract (800 μg/ram) emulsified in IFA. Rams were vaccinated three times with an interval of 4 weeks between immunizations by intramuscular injection. In addition, an unvaccinated negative control group injected with PBS was included (Table 1).

2.5. Challenge and slaughter

At the time of experimental challenge, all rams were transferred to other facilities until the end of the experiment. Challenge inoculation was performed 34 weeks after the last immunization, with *B. ovis* PA (virulent strain). Each ram received 7.35×10^9 colony forming unit (CFU) (as shown by viable counts assessed retrospectively) in a total volume of 120 μL, both conjunctivally (60 μL) and preputially (60 μL). All rams were allocated in the same pasture. Rams were slaughtered 32 weeks after challenge and subjected to bacteriological examinations. The efficacy of the challenge infection was determined by detecting antibodies in R-LPS (rough lipopolysaccharide) ELISA and agglutination *B. canis* test (see below).

2.6. Clinical inspections

All rams were clinically examined on inoculation day and at different intervals after challenge. Genital organs were carefully observed and palpated to detect the presence of eventual lesions in testicles and epididymides.

2.7. Analysis of humoral immune response

2.7.1. Specific antibody response

Blood samples were collected using 10 mL vacutainer tubes (BD Vacutainer®) prior to immunization and then at different intervals (every 2 weeks until week 10). Additional samples were obtained at weeks −4, 0, 4, 6, 8, 10, 13, 21, 24, 34 (before challenge), 42 (time of challenge) and weeks 57, 59, 62, 64, 68, 70 and 74 (post-challenge).

Serum reactivities against chimera rBLSOmp31 were determined by ELISA. Briefly, plates (NUNC, Maxisorp, Denmark) were sensitized with 100 μL per well with rBLSOmp31 (0.3 μg/well) in PBS pH 7.2, at 4 °C overnight. Blocking was done with PBS plus 0.05% Tween 20 and 3% skim milk (San Regim). Ram sera were diluted 1/200 in the blocking solution and were incubated for 1 h at 37 °C. Bound antibodies were detected by a donkey anti-sheep IgG (whole-molecule) conjugated to horseradish peroxidase (Sigma, St. Louis, MO) (1/3000; 1 h at 37 °C). Enzyme activity was revealed by incubation with 1 mM 2,2′-azino-di (3-ethylbenzothiazoline-sulphonic acid) (ABTS) and 4 mM hydrogen peroxide (H_2O_2) in citrate buffer pH 4.5. After 20 min with shaking at room temperature, absorbance was read at 405 nm in automatic ELISA reader (Titertek, Multiskan EX, Labsystems). Antibody levels (IgG) were expressed as the arithmetic mean ± SEM of the O.D. obtained for rams included in each group.

2.7.2. R-LPS ELISA and B. canis agglutination test

Serum samples were assayed in ELISA against R-LPS from *B. ovis* as previously described [27]. Given that *B. ovis* is a rough strain that cross-reacts with *B. canis*, *B. canis* agglutination test was performed mixing 10 μL of serum with 10 μL of antigen on a 25 mm × 75 mm glass slide for 1–2 min. The antigen was prepared at Laboratorio de Inmunología, Facultad de Ciencias Veterinarias (U.N.C.P.B.A., Tandil, Argentina) using the strain (M−) variant of *B. canis*, kindly provided by Prof. L. Carmichael.

2.7.3. Complement-mediated bacteriolysis assay

Pooled sera collected at weeks 11 (3 weeks after the last immunization), 42 (at the time of challenge) and 57 (15 weeks after challenge) were tested for the ability to promote *in vitro* complement-mediated killing of *B. ovis*. This assay was performed in 96-well, flat bottomed polystyrene micro-titre plates (Linbro, Italy) as previously described with modifications [16]. 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 were mixed with ram pooled heat inactivated serum (50 μL). The mixtures were shaken at 100 rpm for 90 min at 37 °C to allow antibody–*Brucella* interaction to occur. 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–*Brucella* mixtures with (i) both 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 × 1 − (mean number of CFU mL^{-1} after incubation/mean number CFU mL^{-1} before incubation).

2.8. Interferon-gamma (IFN-γ) assay

Blood samples were collected in heparinised tubes (BD Vacutainer®) at weeks −4, 13, 21, 24 (before challenge), 42 (time of challenge), 57, 62 and 74 (post-challenge). Whole-blood cultures were incubated with chimera rBLSOmp31 (20 μg/well) in 5% CO_2 atmosphere. Control cultures were incubated with Pokeweed mitogen (SIGMA) (10 μg/well) and PBS. Supernatants were harvested after 48 h of culture and were assayed for bovine IFN-γ by using a sandwich ELISA (Bovigam TM, Biocor Animal Health, U.S.A.).

2.9. Bacteriological examination

Animals were slaughtered at 32 weeks after challenge for bacteriological studies. Each ram was necropsied and samples of spleen, liver, ampullae, testicles, epididymides, vesicular glands, cranial (retropharyngeal) and inguinal lymph nodes were taken. Portions of the organs were homogenized by using a Stomacher (Stomacher 80 Biomaster Seward, Norfolk, United Kingdom) after adding saline. One milliliter of each homogenate was seeded in Skirrow medium and incubated at 37 °C in a 5% CO_2 atmosphere [28]. Colonies were counted after 10 days of incubation. *Brucella* colonies were identified by morphology, Gram staining, catalase, urease, oxidase tests and agglutination with anti-R serum [25]. A ram or necropsy organ was considered as infected when at least one *B. ovis* PA was isolated from any samples tested. An organ was considered as severely

infected when more than 150 *B. ovis* CFUs were isolated. An infected ram was considered as severely infected when at least one of its necropsy organs were found severely infected.

2.10. Statistical analysis

Data from ELISA and IFN-γ assay were analyzed by ANOVA followed by Tukey *post hoc* tests. Irwin Fisher tests were used to analyze bacteriological data (significance was set at $p < 0.05$). The analysis was performed using INFOSTAT, 2008. Graphs were performed using Graph Pad software, version 4.0, San Diego, CA.

3. Results

3.1. Prime-boost strategy and rBLSOmp31 in IFA stimulated the best levels of specific antibodies

Seventy lambs divided in seven groups were immunized as indicated in Section 2 (Table 1). No clinical abnormalities were associated after vaccination at the sites of injection or electroporation. Five rams of different groups died during the course of the experiment for reasons not related to vaccination.

To evaluate the anti-rBLSOmp31 immune response (IgG) elicited by different schedules of immunization, sera from all rams were collected at selected times during the experiment. Rams immunized with chimera rBLSOmp31 in IFA or in QUIL A presented higher antibody levels than animals immunized with pCIBLSOmp31 ($p < 0.001$) in all samples. Immunization with chimera rBLSOmp31 in IFA also elicited strong specific IgG response after the first immunization but it did not increase after booster, and it remained at similar levels following bacterial challenge. In contrast, immunizations with the chimera rBLSOmp31 in QUIL A reached specific antibodies levels similar to chimera rBLSOmp31 in IFA after the last booster, but the levels of antibodies declined faster and were lower at the time of challenge (Fig. 1).

In rams vaccinated using the prime-boost regimen, antibody production was enhanced when pCIBLSOmp31 electroporated priming was followed with chimera rBLSOmp31 in IFA immunization. In this group, antibody levels after the last boost were similar to that of rams immunized with chimera rBLSOmp31 in IFA ($p > 0.05$) and remained at high levels until challenge (Fig. 1). In particular, electroporation-enhanced immunization gave significantly higher levels of antibodies as compared to pCIBLSOmp31 injection alone ($p < 0.01$). In addition, electroporation also gave a considerable boosting effect with increasing antibody levels after each subsequent immunization. In contrast, pCIBLSOmp31 injection alone induced only weak humoral immune responses against chimera rBLSOmp31 similar to immunization with HS extract in IFA ($p > 0.05$) (data not shown).

Sera from unvaccinated rams exhibited low background absorbance until *B. ovis* inoculation. Challenge was able to boost this response in all groups. Prime-boost strategy and immunization with chimera rBLSOmp31 in IFA showed the best antibody levels that remained stable until rams were slaughtered.

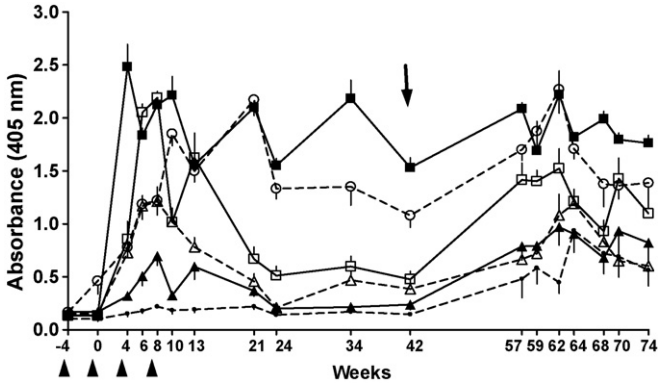

Fig. 1. Kinetics of the humoral immune response (IgG) elicited after immunization with prime-boost strategy (pCIBLSOmp31/rBLSOmp31) (○), electroporated pCIBLSOmp31 (△), nonelectroporated pCIBLSOmp31 (▲), chimera rBLSOmp31+IFA (■), chimera rBLSOmp31+ QUIL A (□), unvaccinated rams (—-). The top arrow indicates the time of *B. ovis* infection. Rams were immunized as indicated in Section 2 and bled at the indicated weeks. Specific antibodies against rBLSOmp31 were evaluated by indirect ELISA. Each value represents the mean ± SEM of animals per group.

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

Sera from rams immunized with the prime-boost strategy, chimera rBLSOmp31 formulated in IFA and HS formulated in the same adjuvant showed similar bactericidal activity after the last booster (around 50%) while sera from animal immunized with chimera rBLSOmp31 in QUIL A or electroporated pCIBLSOmp31 showed low percentages of bacteriolysis (20% or 16.3%, respectively) (Fig. 2). At the time of challenge, the percentage of killing remained similar in these groups. In contrast, sera from nonelectroporated pCIBLSOmp31 or unvaccinated rams (PBS) did not exhibit bactericidal activity. After the inoculation, bactericidal activity only increased in sera from rams immunized with PBS (Fig. 2). A pool of sera taken before the immunization did not show any bactericidal activity indicating that BLSOmp31 specific antibodies induced by some of the strategies employed 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).

3.3. Inmunization with chimera BLSOmp31 did not induce antibodies against R-LPS ELISA

Sera from rams vaccinated with BLSOmp31 employed in different methods of immunization were negative in R-LPS ELISA. In contrast, and as expected, sera from rams vaccinated with chimera as a protein or plasmid, or HS extract were positive in *B. canis* agglutination test because it detects antibodies directed against outer membrane antigens including immunodominant Omp31. Also, after challenge, all rams were seropositive for R-LPS antibodies using either technique (data not shown).

3.4. Prime-boost strategy stimulated the best levels of IFN-γ in whole-blood of immunized rams

Immunization with pCIBLSOmp31/rBLSOmp31 stimulated the highest levels of IFN-γ in whole-blood cultures at weeks 21 and 24 (13 and 16 weeks after last booster) with respect to negative control ($p < 0.01$). Immunization with chimera rBLSOmp31 formulated with either of adjuvants or electroporated pCIBLSOmp31 induced levels of IFN-γ with similar kinetics. At week 42 (time of challenge), IFN-γ levels were low but at weeks 57 and 62 this cytokine increased in all groups, mainly in groups immunized with chimera rBLSOmp31 in IFA or QUIL A and in rams vaccinated with prime-boost strategy. However, in samples collected at week 74 (before the slaughter), prime-boost strategy stimulated increased levels of IFN-γ with significant differences ($p < 0.001$) respect to other groups (Fig. 3).

3.5. Chimera BLSOmp31 protected against B. ovis infection

To evaluate protective activity conferred by different vaccines rams were challenged with *B. ovis* PA. Rams were clinically examined at different intervals after challenge and genital organs were

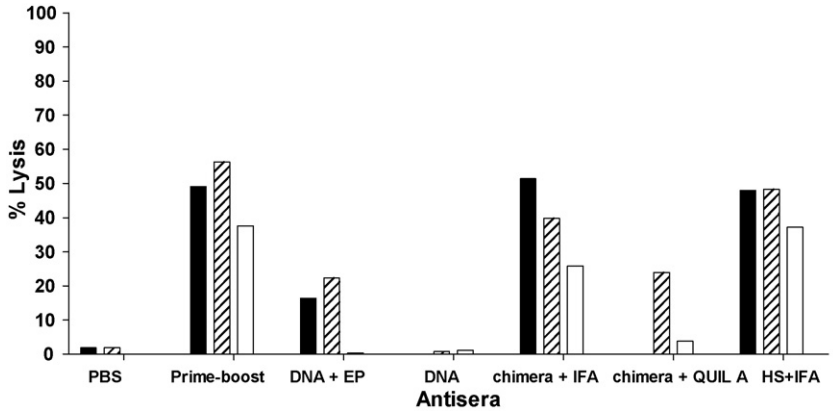

Fig. 2. Bactericidal activity of pooled immunized ram sera against *B. ovis* in presence of ovine sera as a source of complement after 2 h of incubation. Serum samples from groups were taken after the last boost ■ (week 11), before ▨ and after de inoculation □ (weeks 42 and 57, respectively). % Killing (i.e. the percentage of bacteria killed) = 100 × 1 − (the mean number of CFU mL^{-1} after incubation/mean number CFU mL^{-1} before incubation). Data are representative of two separated experiments.

carefully palpated to detect the presence of lesions in epididymides and testicles. Thirty-two weeks after the challenge, rams were slaughtered as described in Section 2.5 for bacteriological studies.

At genital inspection, no palpable testicular alterations were noticed before challenge in any of vaccinated or control rams. After challenge, only one ram from prime-boost group showed unilateral epididymal lesion. Genital alterations were detected in 50% of rams immunized with chimera rBLSOmp31 in IFA or HS extract also in IFA. In other groups, most of the animals showed testicular alterations at palpation and/or increase in epididymal tone. All unvaccinated rams manifested unilateral or bilateral epididymitis.

The bacteriological results obtained after the necropsy of *B. ovis* are summarized in Table 2. The best protection achieved were prime-boost (only 2 out of 9 rams were infected, i.e. 77.8% of protection) and chimera rBLSOmp31 in IFA (3 out of 8 rams infected, 62.5% of protection); with significant differences with respect to unvaccinated control group ($p = 0.0001$ and $p = 0.023$, respectively). In addition, prime-boost regimen was the most efficient strategy conferring 88.9% protection against severe *B. ovis* infection versus 44.5% obtained with electroporated plasmidic DNA and 75% with chimeric protein in IFA. In contrast, other groups of immunization, including control HS group, showed low percentages of protection (0–20%) although even in this group statistical differences from unvaccinated control were significant ($p = 0.016$).

Prime-boost strategy, chimera rBLSOmp31 and HS extract in IFA showed low percentages of samples containing *B. ovis* ranging from 7% to 28%. In addition, the numbers of *B. ovis* isolated from a majority of organs in these groups were lower. In contrast, nonelectroporated and unvaccinated groups showed high percentages of infection and *B. ovis* were isolated with uncountable CFU in most of the organs (data not shown).

The distribution of *B. ovis* in the different organs is shown in Table 3. This table shows the same tendency as Table 2. Although prime-boost strategy and chimera in IFA immunization conferred significant protection, some organs became infected with only a few colonies (data not shown). In contrast, rams from nonprotected groups had more organs infected with *B. ovis*, and their colonies could not be counted (data not shown). As expected, most

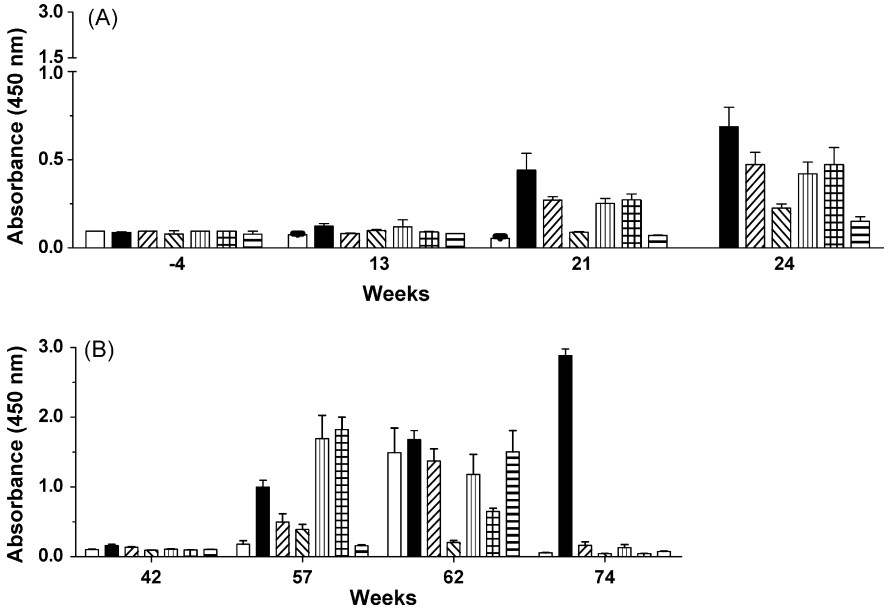

Fig. 3. Antigen-specific IFN-γ response of whole-blood cells from immunized rams. Samples from groups: prime-boost strategy (pCIBLSOmp31/rBLSOmp31) ■, electroporated pCIBLSOmp31 ▨, nonelectroporated pCIBLSOmp31 ▨, chimera rBLSOmp31 + IFA ▥, chimera rBLSOmp31 + QUIL A ▦, HS extract + IFA ▤, PBS + IFA □ were taken at weeks panel (A) −4, 13, 21 and 24 (before the challenge); panel (B) 42 (time of challenge), 57, 62 and 74 (post-challenge). Results are expressed as OD values and error bars indicate standard error from the mean.

Table 2
Protective efficacy of chimera BLSOmp31 against experimental *B. ovis* infection in rams after bacteriological culture.

Groups of immunization	Number/total of rams		Difference with unvaccinated control[c]	Number/total samples		Difference with unvaccinated control[d]
	Infected/total (%)[a]	Severely infected/total (%)[b]		Infected/total (%)[a]	Severely infected/total (%)[b]	
PBS (unvaccinated)	10/10(100.0)	10/10(100.0)		93/120(77.5)	64/116(55.2)	
Prime-boost	2/9(22.2)	1/9(11.1)	0.0001	8/108(7.4)	1/108(0.9)	<0.0001
Electroporated pCIBLSOmp31	8/9(88.8)	5/9(55.5)	0.474	34/108(31.5)	16/108(14.8)	0.0002
Nonelectroporated pCIBLSOmp31	10/10(100.0)	10/10/(100.0)	0.50	87/120(72.5)	71/120(59.2)	0.77
rBLSOmp31+IFA	3/8(37.5)	2/8(25.0)	0.023	14/96(14.6)	7/96(7.3)	<0.0001
rBLSOmp31+QUIL A	7/9(77.7)	5/9(55.5)	0.21	41/108(37.9)	18/108(16.7)	0.019
HS extract+IFA	8/10(80.0)	5/10(50.0)	0.0163	34/120(28.3)	19/120(15.8)	<0.0001

Groups of rams were immunized three times with 4 weeks between injections. Prime-boost group received three injections with electroporated pCIBLSOmp31 and last immunization with chimera rBLSOmp31 in IFA (Table 1). Thirty-four weeks after last immunization, rams were inoculated with *B. ovis* PA (conjunctivally and preputially) and were necropsied thirty-two weeks later. Genital and extragenital organs were cultured in the search of *B. ovis* colonization.

[a] A given ram or necropsy organ was considered as infected when at least one *B. ovis* PA CFU was isolated from any of the samples seeded.

[b] A given ram or necropsy organ was considered as severely infected when more than 150 *B. ovis* PA CFUs were isolated from any of the samples seeded.

[c] Irwin Fisher statistical differences of the % of infected rams/total rams.

[d] Irwin Fisher statistical differences of the % of infected samples/total samples.

positive isolates of *B. ovis* were obtained from epididimydes, ampullaes and vesicular glands although extragenital infection was also detected.

4. Discussion

In the present work we have evaluated the immune response and protective efficacy elicited for a novel acellular vaccine against ovine brucellosis. The chimeric antigen BLSOmp31 was tested using different delivery systems: the recombinant protein emulsified in two different adjuvants, a DNA vaccine with or without electroporation and a mixed (prime-boost) regimen of immunization.

BLSOmp31 protected against contagious rams epididymitis when the vaccine was applied using a prime-boost strategy or formulated in IFA. In both cases the applied vaccine was highly immunogenic and able to induce long-lasting lytic antibodies and interferon specific cellular responses. In agreement, both regimes showed high levels of protection against challenge with *B. ovis*. To our knowledge this is the first report demonstrating protection of large animals with an electroporation-enhanced plasmidic DNA vaccine in a prime-boost regimen. As such, these results provide an important "proof of principle" demonstrating the efficacy of electroporation in the veterinary field. Furthermore, the vaccination with BLSOmp31 does not interfere with current serological tests for diagnosis of ovine brucellosis as R-LPS ELISA. These advantages make BLSOmp31 an attractive vaccine candidate against *B. ovis* and warrants further evaluation in larger field studies to establish the full prophylactic activity of the antigen against *B. ovis* infection.

Which are the reasons that should explain the efficacy of this chimeric engineered vaccine? We have previously reported that a chimera constructed with the scaffold protein BLS decorated with 10 copies of a B- and Th1-epitope derived from an exposed loop of the protein Omp31 was immunogenic [29,30] and induced similar protection against *B. ovis* than the Rev.1 vaccine in BALB/c mice [29]. It is hypothesized that the stability conferred to the inserted peptide, the degree of repetitiveness and spatially ordered display on the protein surface [22] and the ability of BLS to activate dendritic cells increases the immune response against the Omp31 peptide [31]. As evidenced here, the immunogenic properties of this polymeric antigen are conserved in larger animals like rams, the natural host of *B. ovis* infection.

In this experiment we used chimera rBLSOmp31 formulated in two different adjuvants widely used in commercial veterinary vaccines: IFA, a potent adjuvant known to induce primarily humoral response, and QUIL A, which may selectively stimulate Th1 activity since it directs antigens into endogenous processing pathways and enhance costimulatory activity [32]. Recombinant BLSOmp31 in oil adjuvant induced higher and longer lasting antibody levels as compared with chimera in QUIL A. In addition, sera from rams immunized with chimera rBLSOmp31 in IFA were far more potent in promoting killing of *B. ovis* in presence of ovine complement. In previous studies, we reported that detergent-extracted recombinant Omp31 in oleic adjuvant induced lytic antibodies in rams [16]. In this experiment, the 27-mer Omp31 inserted peptide was able to induce antibodies with similar bactericidal activity to HS extract, the reference vaccine in our study. Also the chimera in IFA stimulated production of IFN-γ indicating that both mechanisms could be involved in protection against *B. ovis* in different stages of infection.

Plasmid DNA vaccines have been used in a number of human clinical trials. However only modest immune responses were obtained. While progress has been made in areas such as codon optimization, improved promoter sequences and inclusion of genetic adjuvants, it has become increasingly clear that enhanced

Table 3
Distribution of *B. ovis* among the different organs of vaccinated and unvaccinated rams.

Groups of immunization	Epididymides		Testicles		Ampullaes		V.[a] glands		Spleen	Liver	Lymph nodes	
	L[b]	R[c]	L	R	L	R	L	R			Cranial	Inguinal
PBS ($n=10$)	10	10	6	6	10	10	10	10	2	6	8	7
Prime-boost ($n=9$)	2	1	1	1	0	1	0	1	0	0	1	1
Electroporated pCIBLSOmp31 ($n=9$)	5	4	1	1	1	5	1	5	2	3	3	4
Nonelectroporated pCIBLSOmp31 ($n=10$)	10	10	6	6	9	9	9	10	2	3	5	8
Chimera rBLSOmp31 + IFA ($n=8$)	2	2	2	2	1	1	1	1	0	0	0	1
Chimera rBLSOmp31 + QUIL A ($n=9$)	5	5	3	3	4	4	5	5	0	1	2	3
HS extract + IFA ($n=10$)	7	4	3	4	2	1	2	6	0	1	1	3

Total of genital or extragenital organs per group from which *Brucella ovis* was isolated. Homogenates of portions of genital and extragenital organs were seeded in Skirrow medium as described in Section 2.

[a] Vesicular glands.

[b] Left.

[c] Right.

delivery is a critical factor for pDNA vaccine potency in larger species. Electroporation-enhanced immune responses have previously been demonstrated in sheep as well as cattle, goats, pigs, non-human primates [33–35], and more recently in man [36]. It is clear that this method may typically increase overall antigen expression by more than a 100-fold as electroporation effects increased intracellular plasmid levels by allowing more pDNA to enter the cells thereby making more template available for transcription. However, the electroporation procedure may also have a direct adjuvant effect by creating local muscle damage at the site of treatment leading to release of "danger signals" stimulating innate immune responses [37]. In corroboration with previous reports, we also found that electroporation-enhanced delivery significantly increased the immunogenicity of the pDNA encoded pCIBLSOmp31 and resulted in both higher levels of antibodies and antigen induced IFN-γ production as compared intramuscular delivery alone. Although electroporation-enhanced immunization did not significantly reduce the percentage of rams infected (88.8%) as compared with pDNA injection without electroporation (100%), it nevertheless reduced the percentage of rams severely infected more efficiently than it was achieved by simple intramuscular vaccine delivery (55.5% vs. 100%).

Several studies have shown that DNA vaccines are effective for priming immune response. However, immunogenicity of these vaccines in mice and domestic animals could be enhanced by DNA priming followed by protein boosting [11,12,38]. In effect, our previous results with Omp31 have demonstrated that the combination of a plasmid DNA priming step followed by a final boost with the homologous protein resulted in improved cellular as well as antigen-specific humoral responses and led to superior levels of protection against infection in mice [18]. In the present study, BLSOmp31 was used to evaluate whether priming rams with electroporation-enhanced pCIBLSOmp31 immunization followed by subsequently boosting with rBLSOmp31 in IFA would further improve immunogenicity and protective activity over immunization with plasmid or protein-adjuvant alone. Both the prime-boost vaccination regimen as well as rBLSOmp31 in IFA induced potent antigen-specific humoral and cellular immune responses. In particular, the prime-boost strategy increased the generation of long-lasting immune memory responses as evident by the higher antigen induced IFN-γ levels 28 weeks after challenge. These results could be in part related with spontaneous re-infections in rams because all animals were allocated in a common sheep pen.

Overall the prime-boost regimen was the most efficient strategy conferring highest protection against *B. ovis* infection. In addition, bacterial burden in the total of organs sampled was much lower in rams vaccinated with the prime-boost regimen as compared with other groups. The higher degree of protection against *B. ovis* would correlate with the magnitude of the specific humoral response and with the levels of bacteriolytic antibodies induced. On the other hand, the prime-boost regimen induced effector and memory Th1 cells that produced higher levels of IFN-γ that led to improved protection against a challenge with *B. ovis*.

An important consideration in the assessment of candidate vaccine efficacy is the infective dose used in experimental vaccination-challenge. In our study, each ram received a total volume of 120 μL of 7.35×10^9 CFU, both conjunctivally (60 μL) and intrapreputially (60 μL). The intensity of the inoculum was adequate but rather high in comparison with other experiments in rams [8,9]. In this context all unvaccinated rams were infected and the percentage of infection in rams vaccinated with the reference acellular vaccine (HS extract in IFA) was 80.0% and the percentages of infected organs were 28.3%. However, other authors obtained 33.3% or 40.0% of infection in rams when they used HS encapsulated in polymer microparticles or emulsified in other oleic adjuvants [8,9]. Hence, the discrepancy in protection efficacy obtained with HS in our experiment may be attributed to the higher dose of inoculum and the use of IFA. This adjuvant was chosen because it conferred effective protection in brucellosis mouse model [15].

In summary, the present work shows clear evidence to postulate the veterinarian field application of the BLSOmp31 vaccine in the prevention of ram contagious epididymitis.

Acknowledgements

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT-Argentina) (to S.M.E., J.C. and to F.A.G.). S.M.E., J.C., C.A.F., G.H.G. and F.A.G. are members of the Research Career of CONICET. M.C. is recipient of a fellowship from CIC (Argentina). C.A.F. is also a member of the Facultad de Ciencias Exactas, Universidad Nacional de La Plata. We thank María Rosa Ortíz and Rosana Malena for technical assistance and J. Llamas and S. Dinino for animal care (INTA). They are grateful to Pedro Soto and Hilda Echevarría for assistance in the bacteriological culture and to Edgardo Rodríguez for technical assistance in statistic analysis (F.C.V., U.N.C.P.B.A).

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