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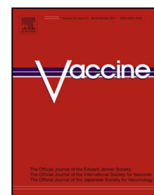

The vaccine candidate BLSOmp31 protects mice against *Brucella canis* infection

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A B S T R A C T

Canine brucellosis represents a major reproductive problem worldwide and it is considered a zoonotic disease. New approaches are therefore urgently needed to develop an effective and safe immunization strategy against *Brucella canis*. In the present study, BALB/c mice were subcutaneously immunized with the recombinant chimera rBLSOmp31 formulated in different adjuvants. The different strategies induced a vigorous immunoglobulin G (IgG) response, with high titers of IgG1 as well as IgG2. Besides, spleen cells from rBLSOmp31-immunized mice produced gamma interferon and IL-4, suggesting the induction of a mixed Th1–Th2. Vaccination with rBLSOmp31-IFA formulation provided the best protection levels comparable with that given by control vaccines. None of the immunization strategies induced serological interference in diagnosis. Hitherto, this is the first report that a recombinant vaccine confers protection against *B. canis* in mice.

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1. Introduction

Canine infection by *Brucella canis*, a rough specie of *Brucella*, constitutes a serious problem for dog breeders and pet owners [1]. Canine brucellosis is often devastating for kennels, it leads to negative effects on the quality of life of infected animals, and it is also of importance to public health because can be eventually transmitted to humans, who develop a mild to moderate disease [1,2]. Once established, the disease is very difficult to eradicate since serologic diagnosis is not always carried out routinely. Currently, disease surveillance is based solely on the early detection of infected animals by serological and clinical examinations, and culturing of the bacteria to confirm infection. Unfortunately, definitive diagnosis is not always possible and a negative result does not confirm the absence of the infection [3]. An infected animal must be castrated, treated with antibiotics and periodically monitored by serology. However, treatment with antibiotics is not entirely reliable, as it is impossible to guarantee complete elimination of the bacteria. Moreover, it is expensive, it has to be carried out for long periods of time, and it does not prevent the neutering and consequent loss of valuable breeding dogs [1,4].

Repeated experience in brucellosis control has shown that the stealthy spread of the disease in animals can only be prevented or reduced by the use of vaccines [5]. Unfortunately, efforts to develop an effective vaccine against canine brucellosis have been unsuccessful to date. Notwithstanding, few investigations have considered the possibility of developing an effective vaccine to protect healthy populations. Since Carmichael's pioneering work in the 1980s [6], there have been no further research studies in this matter. In that work, a less mucoid strain (M-) of *B. canis* was used to infect dogs. The results demonstrated that the M- variant met some of the criteria for an immunizing agent. Nevertheless, the studies failed to provide unequivocal assurance of acceptable attenuation

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and later communications demonstrated the zoonotic nature of the strain [7].

Consequently, it would be desirable the development of a safe and effective vaccine against *B. canis* which would induce immunity, yet not provoke serological responses that interfere with the diagnosis of canine brucellosis. Innovative approaches including recombinant subunit vaccines have numerous advantages: they are completely inert, their compositions are predetermined, their production can be better controlled, and they yield homogeneous products [8].

Cell surface and intracellular components of *Brucella* spp. have been assessed as protective antigens against *Brucella ovis*, the other rough specie of *Brucella* genus and aetiological agent of ovine brucellosis. Among these immunogens, outer membrane protein 31 (Omp31) as a recombinant protein, DNA vaccine or by prime-boost protocols elicited different immune responses and conferred protection against *B. ovis* in BALB/c mice and rams [9–12]. In addition, the enzyme lumazine synthase from *Brucella* spp. (BLS) is a highly immunogenic and stable decameric protein [13,14] with adjuvant properties when a foreign antigen is covalently attached to it [15,16]. Given the fact that Omp31 and the carrier BLS have been implicated in the generation of protective cellular and humoral immune responses, our group has generated a chimera BLSOmp31 as a recombinant protein or DNA vaccine (rBLSOmp31 and pCIBLSOmp31, respectively). For that purpose, we genetically fused at the N-termini of BLS a 27-mer peptide containing the exposed loop epitope of Omp31 [17]. The immunization with rBLSOmp31 in Incomplete Freund Adjuvant (IFA) and prime-boost strategy conferred protection against *B. ovis* in BALB/c mice and in rams [17,18].

Based on our previous results that indicated that chimera BLSOmp31 conferred protection against *B. ovis* infection [17,18], in the present study we have evaluated the immunogenicity, serological interference, safety and protective efficacy elicited by this acellular vaccine against *B. canis* in BALB/c mice using various formulations.

2. Materials and methods

2.1. Animals

Six to 8-weeks-old female BALB/c mice (obtained from Universidad de Buenos Aires, Argentina) were acclimated and randomly distributed into experimental groups. Mice were housed in appropriate conventional animal care facilities with filtered air and handled following international guidelines required for animal experiments under our Faculty Animal Welfare Comission (Acta 087/02, F.C.V., U.N.C.P.B.A, Tandil, Argentina; http://www.vet.unicen.edu.ar).

2.2. Bacterial strains

B. canis RM6/66, *B. canis* less mucoid strain (M-, for short) and *B. ovis* PA76250 were obtained from our *Brucella* culture collection. Bacterial suspensions were prepared as previously described [9].

2.3. Production of BLSOmp31 immunogens

Recombinant rBLSOmp31 was expressed in *Escherichia coli* (*E. coli*) BL21(DE3) (Stratagene, La Jolla, CA), and was purified by fast-protein liquid chromatography in a Mono-Q and a Superdex-200 column as previously described [17]. DNA vaccine coding for BLSOmp31 (pCIBLSOmp31) was amplified in *E. coli* JM109 (Stratagene, La Jolla, CA) and isolated using "megaprep" plasmid isolation columns (Genelute, Sigma) as previously described [19].

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

Vaccine ($n = 5$)	Adjuvant	Days of immunization			
		0	15	30	45
pCIBLSOmp31	None		●	●	●
Prime-boost	IFA	●	●	●	■
rBLSOmp31	Quil A			■	■
rBLSOmp31	Montanide			■	■
rBLSOmp31	AH gel			■	■
rBLSOmp31	IFA			■	■
B. canis M- bacterin	IFA			▲	▲
Live *B. ovis* PA76250	None			★	
PBS	–			▭	▭

●, pCIBLSOmp31: 100 μg in 100 μL of PBS (i.m.); ■, rBLSOmp31: 30 μg in 200 μl (s.c.); ▲, *B. canis* M- bacterin: 6.3×10^8 CFU in 200 μL (s.c.); ★, live *B. ovis* PA76250: 1×10^9 CFU in 200 μL (i.p.); ▭, PBS: 200 μL s.c.

2.4. Adjuvants

Aluminum hydroxide (AH) gel was prepared as described previously [20]. To adsorb the antigen, AH gel was incubated with rBLSOmp31, washed, and the final pellet was suspended in phosphate-buffered saline (PBS). Incomplete Freund Adjuvant was prepared mixing Marcol 52 (Biogenesis, Argentina) with 10% of Arlacel (Sigma, St. Louis, MO, USA). Montanide IMS3012 VGPR (Seppic, France) and Quil A (Brenntag Biosector, Denmark) were used according to the manufacturer's instructions.

2.5. Control vaccines

Heat killed *B. canis* M- suspension (6.3×10^8 CFU/mice) emulsified in IFA and a suspension of live *B. ovis* PA76250 (1×10^9 CFU/mice) were used as control vaccines as previously described [21].

2.6. Immunizations and experimental design

Mice were randomly separated in groups ($n = 10$). Different groups received the vaccines according with the immunization schedule (Table 1). Mice immunized with pCIBLSOmp31 were injected three times (days 0, 15 and 30) by intramuscular (i.m.) route (100 μg in 100 μL of PBS). Prime-boost group was immunized with the same plasmid schedule followed by a final subcutaneous (s.c.) booster (fourth injection) performed with rBLSOmp31-IFA formulation. Recombinant BLSOmp31 formulated in different adjuvants were administered two times (days 30 and 45) by s.c. route (30 μg in 200 μL).

B. canis M- bacterin was injected twice (days 30 and 45) and live *B. ovis* PA76250 only one dose, by s.c. and by i.p. routes, respectively [19].

2.7. Specific BLSOmp31 enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected every 2 weeks until sacrifice. Serum reactivity to rBLSOmp31 was determined by indirect ELISA as described previously [17]. Data were expressed as mean ± SD absorbance units [9].

Antibody isotyping of immune sera of immunized mice was also done to determine the Th1/Th2 bias of immune response. The conditions were the same for isotype determination of the antibodies, except that sheep anti-mouse IgG1 and IgG2a-specific antibodies (Sigma) were used and were detected by incubation with anti-sheep IgG–horseradish peroxidase conjugate. The cutoff value for the assay was calculated as the mean specific optical density plus

3 standard deviations (S.D.) for 20 sera from nonimmunized mice assayed at dilutions of 1/100. The titer of each serum was calculated as the last serum dilution yielding a specific optical density higher than the cutoff value.

2.8. *B. canis* agglutination test

Serum samples were assayed by *B. canis* agglutination test mixing 10 μL of serum with 10 μL of antigen on a glass slide for 1–2 min. The antigen was a 7% *B. canis* M- suspension prepared at Laboratorio de Inmunología, Facultad de Ciencias Veterinarias (U.N.C.P.B.A., Tandil, Argentina) [22].

2.9. Cytokine determination

In order to characterize the cellular immune response induced by the protein based strategies that yielded significant protection, we further analyzed the elicited cellular immune responses in mice immunized with the more promising formulations. Thirty mice were randomly separated in three groups ($n = 10$) and vaccinated as described above with rBLSOmp31 formulated in IFA, AH or Quil A, respectively. Thirty days after last immunization, the mice were sacrificed, and the spleens were removed and homogenized in complete medium. Cells were cultured at 4×10^6 mL^{-1} in duplicate wells with rBLSOmp31 (5 μg/mL), concanavalin A (ConA; 2.5 μg/mL) (Sigma) or with culture medium alone. Cultures were incubated for 48 h at 37 °C in 5% CO_2. Gamma interferon (IFN-γ) and interleukin-4 (IL-4) in culture supernatants were measured by sandwich ELISA using commercially available reagents according to the manufacturer's instructions (Pharmingen, San Diego, CA).

2.10. Mice protection test

Thirty days after the last immunization mice were challenged by i.p. inoculation with 5.5×10^5 CFU *B. canis* RM6/66 in 200 μL of PBS [21]. Mice were killed 30 days after being challenged, and their spleens were removed aseptically and weighed. Each experiment was conducted at least twice. To calculate the numbers of *B. canis*, each spleen was homogenized, serially diluted in sterile saline, plated on Brucella Agar as described previously [9]. Number of CFU per spleen was counted after incubation for 4 days at 37 °C [9] and the results were represented as the mean log CFU ± S.D. per group [9]. When no colonies grew, the spleen was considered infected by a maximum of five bacteria [23].

To differentiate challenge strain from live *B. ovis* PA76250 (a CO_2 dependent strain), the number of CFU/spleen from the virulent challenging strain was calculated by subtraction of numbers obtained in non-selective (with CO_2) and selective conditions (without CO_2) and then logarithmically transformed [24].

2.11. Statistical analysis

The CFU data were normalized by log transformation and evaluated by ANOVA followed by Dunnett's *post hoc* test. The Kruskal–Wallis test and ANOVA were used to compare antibody and cellular responses, respectively (InStat; GraphPadv4).

3. Results

3.1. Recombinant BLSOmp31 based vaccines develop higher specific IgG responses than DNA or prime-boost strategies

To evaluate the humoral immune response elicited by the different regimens of immunization, anti-BLSOmp31 immunoglobulin G (IgG) antibodies were measured by specific indirect ELISA in sera from immunized and control mice. Mice injected with PBS and whole cell vaccines served as controls. Recombinant BLSOmp31-IFA and rBLSOmp31-AH gel formulations elicited strong specific IgG antibodies after the second boost. The chimera in Montanide induced similar antibody levels than rBLSOmp31-Quil A formulations but significantly lower than rBLSOmp31-IFA and rBLSOmp31-AH gel ($P < 0.05$). In contrast, immunization with pCIBLSOmp31 or prime-boost strategies elicited lower antibody responses than the other rBLSOmp31 immunization groups. *B. canis* M- bacterin and *B. ovis* PA76250 induced only weak humoral immune responses against the chimera rBLSOmp31 ($P > 0.05$). After the i.p. challenge with virulent *B. canis*, antibody levels significantly increased also in the PBS group ($P > 0.05$) but the rise was not detected until the second bleeding (30 days) (Fig. 1).

As IgG1 and IgG2a are considered to be markers of Th2 and Th1 responses, respectively, anti-BLSOmp31 specific antibodies of both isotypes were also measured (Fig. 2). All the protein-based strategies elicited a vigorous humoral response, as seen by the higher specific IgG1 and similar IgG2a antibodies titers induced ($P < 0.05$). Immunization with pCIBLSOmp31, *B. canis* M- bacterin or live *B. ovis* PA76250 induced low titers, with IgG2a values higher than IgG1 ($P > 0.05$).

Table 2
Serologic results for *B. canis* Rapid Slide Agglutination Test (RSAT) of mice vaccinated by different strategies.

Vaccine	Adjuvant	After last immunization	After challenge with *B. canis*
PBS	–	Neg	++
pCIBLSOmp31	None	Neg	++
Prime-boost	IFA	Neg	+++
rBLSOmp31	AH	Neg	+++
rBLSOmp31	IFA	Neg	++
rBLSOmp31	Montanide	Neg	++
rBLSOmp31	Quil A	Neg	+++
B. canis M- bacterin	IFA	++	+++
Live *B. ovis* PA76250	None	+	++

Neg, no agglutination, homogeneous mixture; +, mild agglutination in 2 to 3 min, fine clumping when observed carefully; ++, moderate agglutination in 1–2 min, visible small and homogeneous clumps; +++, heavy agglutination within 30 s to 1 min, big clumps on traslucid background.

3.2. Immunization with rBLSOmp31 based strategies did not induce antibodies against B. canis agglutination test

Sera from mice vaccinated with BLSOmp31 based strategies of immunization were negative in RSAT. In contrast, and as expected, sera from mice vaccinated with whole cellular vaccines were positive in *B. canis* agglutination test because this assay detects antibodies directed against outer membrane antigens. Furthermore, after challenge, all mice were seropositive using this technique (Table 2).

3.3. Specific cellular immune responses were induced by rBLSOmp31 based vaccines

To get further information on the type of cellular immune responses induced by rBLSOmp31 formulated in different adjuvants at the time of the bacterial challenge, cytokine secretion in culture supernatants of spleen cells from immunized mice was evaluated by ELISA. Recombinant BLSOmp31 significantly ($P < 0.01$) induced the production of IFN-γ in cells from rBLSOmp31-IFA-, rBLSOmp31-AH-, and rBLSOmp31-Quil A-immunized mice. In contrast, IL-4 production only was significantly higher on group immunized with rBLSOmp31-AH ($P < 0.01$), but not in the other two groups. In response to Con A, spleen cells from all of the animals studied produced IFN-γ and IL-4, with no significant differences among the groups (Fig. 3).

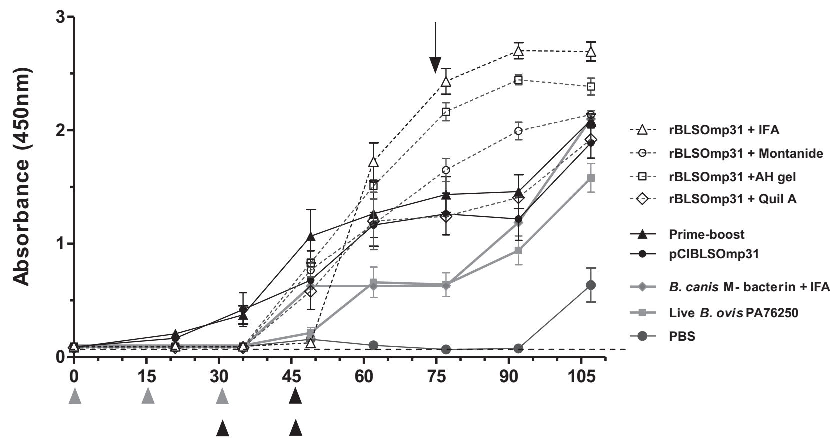

Fig. 1. Kinetics of the humoral immune response elicited after immunizations at days 0, 15, 30 and 45 in accordance to Table 1 (immunization with pCIBLSOmp31is indicated with ▲ and recombinant BLSOmp31 with ▲). The gray arrow (↓) indicate the time of challenge with virulent *B. canis* RM6/66. IgG specific antibodies against rBLSOmp31 were evaluated by specific indirect ELISA. Each symbol represents the mean ± S.D. of five mice.

3.4. Prime-boost and rBLSOmp31 based strategies protect BALB/c mice against B. canis infection

Protection experiments were carried out challenging vaccinated and control mice by i.p. inoculation of virulent *B. canis* RM6/66 and infection level was evaluated measuring CFU in spleens. Vaccination of BALB/c mice with rBLSOmp31 + IFA provided the best protection level against *B. canis* (4.02 log of protection) compared with control mice receiving PBS, as seen in Table 3. Similar results were elicited by the whole cell vaccines *B. canis* bacterin + IFA (4.38 log) and live *B. ovis* (4.12 log). Interestingly, in these groups four out of five mice did not develop *Brucella* growth in pure homogenate spleen cultures. Also, the mice vaccinated with those three strategies exhibited significantly higher degree of protection ($P < 0.01$) than the protection elicited by the prime-boost strategy (2.29 log), rBLSOmp31-Quil A (1.54 log) or rBLSOmp31-AH gel (1.37 log). Nevertheless, these last three groups achieved significantly higher protection compared with PBS group ($P < 0.01$),

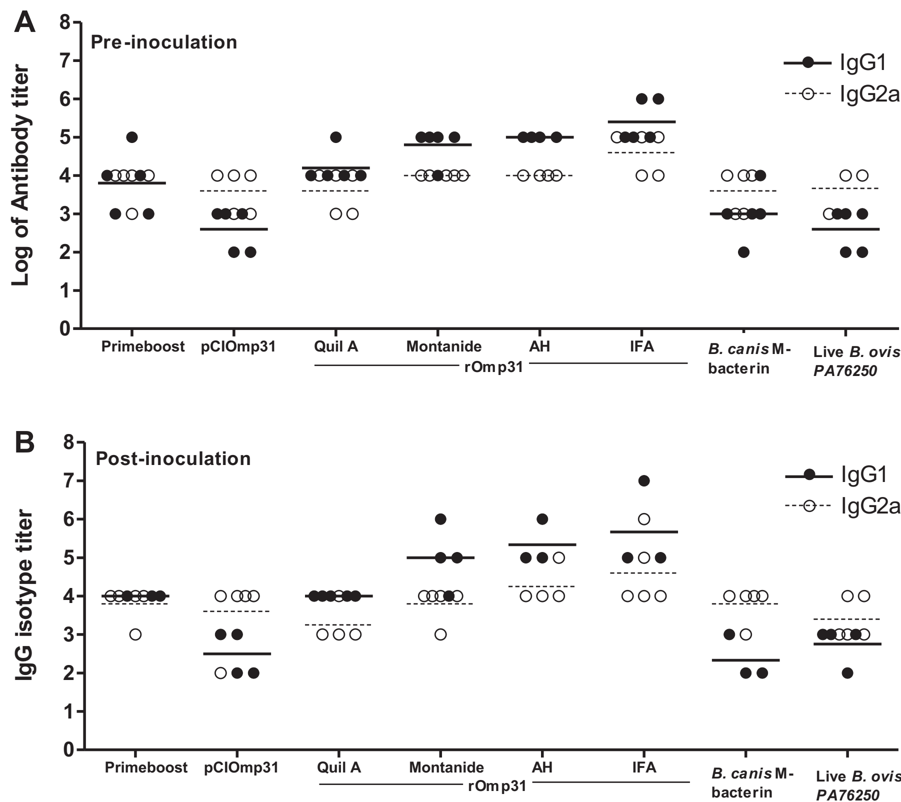

Fig. 2. Characterization of antibody isotype profiles against rBLSOmp31. Antibodies were determined by specific indirect ELISA in sera from mice immunized as indicated in Section 2 just before (A) and after the challenge with virulent *B. canis* RM6/66 (B). Each symbol represents every individual mouse value, and mean is displayed as a horizontal line. The titer is represented as the logarithm of the last serum dilution yielding a specific optical density higher than the cut off value.

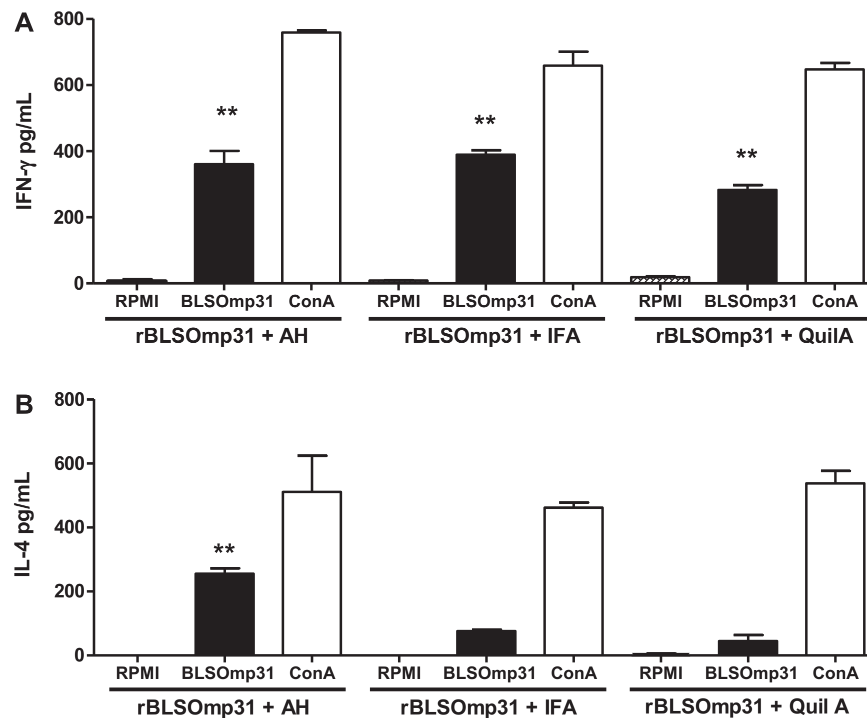

Fig. 3. Determination of IFN gamma and IL-4 production in spleen cells from mice immunized with rBLSOmp31-IFA, rBLSOmp31-AH or rBLSOmp31-QuilA. Cells (4×10^6 mL^{-1}) were stimulated with complete medium RPMI 1640, rBLSOmp31 (5 μg/mL) or Con A for 48 h. Levels of IFN-γ (upper panel), IL-4 (lower panel) in the cell supernatants were quantified (pg/mL) by MAb-capture ELISA. Each value represents the mean of duplicates ± S.D. of the responses of spleen cells from five individual mice. (**) Significantly different from the same stimulus in PBS-immunized mice ($P < 0.01$).

rBLSOmp31-Montanide (0.36 log) and pCIBLSOmp31 (0.47 log). It is worth noting that rBLSOmp31-Montanide was the only vaccine that failed to give any level of protection against *B. canis* infection.

Also, both whole vaccines used as positive control groups resulted in very high protection levels as expected from previous work [21].

4. Discussion

Canine brucellosis is a very common infectious disease that not only causes mortality and has detrimental effects on quality of life of canine population, but also it is considered contagious to humans [25]. Although there has been a great effort in improving diagnosis techniques, the evaluation of alternative means of prevention has not been fully explored [21]. In this matter, subunit vaccines such as recombinant proteins or DNA vaccines, are promising candidates because they are safer, well defined, not infectious and cannot revert to virulent as live attenuated vaccines [8]. Furthermore, a careful selection of the immunogen can avoid cross reaction in the diagnosis.

Table 3
Protection conferred by chimera BLSOmp31 using different strategies of immunization against *B. canis* in BALB/c mice.

Vaccine ($n = 5$)	Adjuvant	Log_{10} B. canis at spleen[a]	Log units of protection
PBS	–	6.23 ± 0.37	–
pCIBLSOmp31	None	5.76 ± 0.46	0.47
Prime-boost	IFA	3.95 ± 1.17	2.29[**]
rBLSOmp31	AH	4.86 ± 1.28	1.57[**]
rBLSOmp31	IFA	2.22 ± 1.20	4.02[***]
rBLSOmp31	Montanide	5.87 ± 0.17	0.36
rBLSOmp31	Quil A	4.69 ± 1.09	1.54[**]
B. canis M- bacterin	IFA	1.85 ± 0.07	4.38[***]
Live *B. ovis* PA76250	None	2.11 ± 0.95	4.12[***]

[a] The content of bacteria in spleens is represented as the mean log CFU ± S.D. per group.
[*] Significantly different from PBS-immunized mice $P < 0.05$ estimated by Dunnett's-test.
[**] Significantly different from PBS-immunized mice $P < 0.01$ estimated by Dunnett's-test.
[***] Significantly different from PBS-immunized mice $P < 0.001$ estimated by Dunnett's-test.

Most of the knowledge about pathogenicity and vaccine development in brucellosis is based on studies of the smooth species *Brucella abortus*, *Brucella suis* and *Brucella melitensis* [26]. As *B. ovis* and *B. canis* share the common feature of being pathogenic rough strains, it is tempting to extrapolate the knowledge and strategies used for the development of a vaccine for ovine brucellosis to the development of a vaccine for canine brucellosis. We have previously demonstrated that the chimera BLSOmp31 is able to develop strong humoral responses against *B. ovis* in mice and sheep [17,18,27]. Moreover, protection experiments against *B. ovis* in mice and subsequent studies in rams using BLSOmp31 by different strategies [18] strongly suggested that it is possible to develop a successful vaccine against rough *Brucella* species by using this immunogen. Thus, we decided to extend this approach to canine brucellosis. Importantly, vaccination with BLSOmp31 did not interfere with RSAT, a current serological test for diagnosis of canine brucellosis [1,28]. Thus, BLSOmp31 could be used as a vaccine against *B. canis* in a control program.

The addition of an adjuvant is often necessary in a subcellular vaccine formulation to enhance and elicit a certain type of immune response and to induce early, strong and long lasting immunity. Appropriate adjuvants should be carefully selected to induce a well balanced immune response [29]. However, practical acceptance of these preparations has been limited, mainly because of possible undesirable reactions. For this reason, on the selection of adjuvant

a risk/benefit analysis has to be carried out, taking into account the animal welfare, the protection generated and the impact of the disease [30–33].

Aluminum hydroxide gel, Montanide and Quil A adjuvants are frequently used in commercial veterinary vaccines, and were selected for this study because they are well tolerated and induce fast and sustainable humoral and cell-mediated immune responses, essential for protection against a rough *Brucella* [29]. In addition, we tested IFA because it is a classical adjuvant that induces potent immune responses. We administered these vaccines subcutaneously, since this route is commonly used in dogs. In addition, we tested previously used DNA and prime-boost strategies [19].

Why does BLSOmp31 protect better when it is emulsified in IFA? There is not a clear correlate with mechanisms of protection. In contrast to the predominant role of cell-mediated immunity in protection against smooth *Brucella* species, several studies showed that antibodies play a predominant role in protective immunity to *B. ovis* in mice and rams [9,18,34]. Immunization with detergent-extracted recombinant Omp31 induced bactericidal antibodies against outer membrane proteins and rough lipopolysaccharide which are able to kill *B. ovis* in the presence of complement [9,10,18,27]. In addition, this recombinant Omp31 extract formulated in IFA elicited serum antibodies capable of killing efficiently *B. ovis* in the presence of homologous o heterologous serum and induced a mucosal immune response [10]. Also, as previously reported [18], ram specific antibodies elicited by the chimera BLSOmp31 emulsified in IFA were able to produce complement mediated bacteriolysis of *B. ovis*. Therefore, antibodies induced by the BLSOmp31/IFA formulation may promote the killing of *B. canis* by complement activation, by enhancing opsonization and phagocytosis or by promoting antibody-dependent cellular toxicity by NK (or other killer cells) during the extracellular life of this pathogen in sera or in mucosal tissues [35].

Cellular studies showed that all BLSOmp31 formulations stimulated a mixed Th1–Th2 response, represented by IFN-γ and IL-4, respectively. As previously reported, cells from rams immunized with BLSOmp31 in IFA 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 [18,26].

For a potential subcellular vaccine against *B. canis* in dogs, particularly in pets, appropriate adjuvants giving a well-balanced immune response and avoiding local inflammatory reactions should be selected [31]. In the present study, recombinant BLSOmp31-IFA formulation was the only vaccine that induced abscesses in the site of injection (results not shown). Thus, in future protection studies conducted in dogs, it will be necessary to test different formulations in order to reach an adequate equilibrium between the degree of protection and the practical acceptance of a vaccine for pets.

Finally, the BLSOmp31 vaccine elicited an immune activation status that could be potentially compatible with effective control of the aethiological agent of canine brucellosis. Consequently, further investigations will focus on the safety and efficacy of rBLSOmp31-based subcutaneous vaccination in the natural host of this disease: the dog.

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