Improved Immunogenicity of a Vaccination Regimen Combining a DNA Vaccine Encoding *Brucella melitensis* Outer Membrane Protein 31 (Omp31) and Recombinant Omp31 Boosting

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In the present study, we report an attempt to improve the immunogenicity of the Omp31 antigen by a DNA prime-protein boost immunization regimen. We immunized BALB/c mice with an Omp31 DNA vaccine (pClOmp31) followed by boosting with recombinant Omp31 (rOmp31) in incomplete Freund’s adjuvant and characterized the resulting immune responses and the protective efficacy against *Brucella ovis* and *B. melitensis* infection. Immunoglobulin G1 (IgG1) and IgG2a titers were higher in sera from pClOmp31/rOmp31-immunized mice than in sera from mice immunized with pClOmp31 or rOmp31 alone. Splenocytes from pClOmp31/rOmp31-immunized mice produced significantly higher levels of gamma interferon than did those from mice given rOmp31 alone. In contrast, interleukin 2 (IL-2) production levels were comparable between the two groups of immunized mice. Cells from all immunized mice produced undetectable levels of IL-4. Notably, rOmp31 stimulated IL-10 production in the pClOmp31/rOmp31-immunized group but not in the pClOmp31- or rOmp31-immunized group. Although the prime-boost regimen induced specific cytotoxic responses, these responses could not reach the levels achieved by the pClOmp31 immunization. In conclusion, pClOmp31 priming followed by rOmp31 boosting led to moderately improved protection against a challenge with *B. ovis* or *B. melitensis.*

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Brucellosis remains endemic in many developing countries, where it undermines animal health and productivity, causing important economic losses (30), and takes a great toll on humans (34). *Brucella melitensis* causes abortions in sheep, goats, and cows, and it is considered the most pathogenic *Brucella* sp. for humans (34). *B. ovis* also infects sheep and, together with *B. melitensis*, causes ovine brucellosis, a disease characterized by epididymitis and a reduction in fertility for rams and by placenta and abortion for ewes (9). Vaccination is the only practical means of controlling the disease when its prevalence is high (3), and live attenuated *B. melitensis* Rev 1 is the strain most widely used for this (5, 23). Yet Rev 1 has many disadvantages (4, 5, 17, 20), which indicates that the need for a better vaccine exists for brucellosis eradication (2).

Immunization with plasmid DNAs expressing foreign proteins provokes both cellular and humoral immune responses and protective immunity against viruses, bacteria, parasites, and tumors (19). We have recently reported that *Brucella* outer membrane protein 31 (Omp31) DNA immunization induces partial protection against *B. ovis* and *B. melitensis* infection. Protection was associated with the induction of Omp31-specific CD8⁺ T cells that eliminate *Brucella*-infected cells via the perforin pathway, a weak humoral response, and an absent T helper 1 (Th1) response (12). On the other hand, vaccination with recombinant Omp31 (rOmp31) elicited a CD4⁺ Th1 response that provided partial protection against *B. ovis* and *B. melitensis* infections (10). A more potent immune response would be needed to afford full protection against brucellar challenge, but immunization regimens inducing such responses have not yet been described. Several vaccine strategies have been reported to greatly enhance the immunogenicity of plasmid-derived antigens (26). One of the most promising strategies is DNA priming followed by protein boosting (27). Results from several studies have demonstrated that the combination of a plasmid DNA priming step and a subsequent boost with the homologous protein resulted in improved cellular and humoral antigen-specific responses which led to superior levels of protection against infection (27, 32).

In the present study, we report an attempt to improve the immunogenicity of the Omp31 antigen by a DNA prime-protein boost immunization regimen. We immunized BALB/c mice with an Omp31 DNA vaccine, followed by boosting with rOmp31 in incomplete Freund’s adjuvant (IFA), and charac-
terized the resulting immune responses and the protective efficacy against *B. ovis* and *B. melitensis* infection. Here we present the results of this study.

**MATERIALS AND METHODS**

**Mice.** Six- to 8-week-old female BALB/c mice were acquired from the University of La Plata, Argentina, and were maintained under standard laboratory conditions.

**Bacterial strains.** For the propagation of plasmids, *Escherichia coli* strain JM109 (Promega, Madison, WI) was used. For the expression of the recombinant protein, strain BL21(DE3) (Stratagene, La Jolla, CA) was employed. Bacterial strains were routinely grown as described in references 10 and 12. *B. ovis* PA76250 and *B. melitensis* H38S (virulent strains) were cultured as described previously (15, 18).

**Antigen production.** rOmp31 was obtained and treated as described previously (11). Purity was determined by Coomassie blue staining as reported previously (11). The plasmid pCIOmp31 was obtained as described in reference 12.

**Immunization.** Anesthetized mice were primed intramuscularly with 100 µg of pCIOmp31 at days 0, 15, 30, and 45, and then the animals were boosted by the intraperitoneal route at days 60 and 75 with 10 µg of rOmp31 in IFA (Sigma, St. Louis, MO). This group was called the pCIOmp31/Omp31-immunized group. Other groups of mice were immunized intraperitoneally with 10 µg of rOmp31 or phosphate-buffered saline (PBS; as a control) in IFA on days 60 and 75. Another group of mice was injected intramuscularly with pCI or pCIOmp31 at days 30, 45, 60, and 75 as described previously (12). As a positive control (6), another group was immunized with *B. melitensis* H38S (H38) in IFA as described previously (12). Mice were bled at different days after the first immunization (eight mice per group), and sera were obtained. At the time of the last bleeding (30 days after the last antigen injection) mice were challenged intravenously with virulent *Boccella* organisms (eight mice per group) or were sacrificed to conduct the analysis of cellular immune responses (five mice per group). Serological, cellular, and challenge studies were conducted at least twice.

**Omp31 enzyme-linked immunosorbent assay (ELISA).** The reactivities of sera against rOmp31 were determined as described previously (11).

**Cytokine responses.** Spleen cells from immunized or control mice were cultured at 4 × 10^6 cells per well in 24-well flat-bottom plates (Nunc, Roskilde, Denmark) with rOmp31 (1 µg/ml), concanavalin A (ConA) (2.5 µg/ml), or medium alone. The concentration of stimulating antigens was found to be optimal for cytokine secretion in our previous reports (10, 12). Cultures were incubated for 48 h at 37°C in a humidified atmosphere (5% CO₂), and at the end of the incubation, supernatants were aliquoted and stored at −70°C. Gamma interferon (IFN-γ), interleukin 2 (IL-2), IL-4, and IL-10 were quantified by a sandwich ELISA as previously described (10, 12).

**Cr⁵ CTL assays.** Cytotoxic T lymphocyte (CTL) assays were performed as described previously (10, 12).

**Challenge studies.** rOmp31-, pCIOmp31-, pCIOmp31/Omp31-, pCI-, PBS-, or H38-immunized mice were challenged, by intravenous inoculation, with 1 × 10⁹ *B. melitensis* H38S organisms or 1 × 10⁹ *B. ovis* organisms. Mice were sacrificed 30 days after the bacterial challenge, and their spleens were removed, homogenized, plated, and incubated as described previously (10, 12, 15, 18). The number of CFU per spleen or liver was counted, and the results were given as the mean log number of CFU ± standard deviation (SD) per group.

**Statistical analysis.** Th and CTL responses were compared between the groups by using the nonparametric Mann-Whitney U test. The CFU data were normalized by log transformation, and a one-way analysis of variance followed by Dunnett’s post hoc test was conducted (Instat; GraphPad, CA).

**RESULTS**

Omp31 prime-boost immunization elicits higher IgG responses than rOmp31 alone. Omp31 is an excellent model to use to evaluate the role of the humoral response in protection, since it is well exposed in *B. ovis* but hidden in *B. melitensis*. To evaluate the humoral response elicited by the three different regimens of immunization, anti-Omp31 immunoglobulin G (IgG) titers were measured by ELISA in sera from immunized mice. As described previously (12), immunization with pCIOmp31 elicited a weak IgG response that was detectable only 30 days after the first immunization and that declined thereafter (Fig. 1A). IgG1 antibodies (Abs) were not detected throughout the experiment. Low titers of IgG2a were detected in only three out of eight mice (data not shown). A *B. ovis* challenge was unable to boost this response (Fig. 1A and C). In contrast, immunization with rOmp31 elicited a vigorous IgG response that was detectable after the first immunization, increased steadily, and reached a maximum after the second

![Fig. 1](http://cvl.asm.org)}
Omp31 prime-boost immunization induces stronger Th1 responses. To get further information on the type of immune response induced by the three immunization regimens at the time of the bacterial challenge, cytokine secretion in culture supernatants of spleen cells from immunized mice was evaluated by ELISA. rOmp31 significantly stimulated the production of IFN-γ and IL-2 in spleen cells from rOmp31-immunized animals but not from pCIOmp31-immunized animals (Fig. 2A and B), corroborating and extending our findings (10). Splenocytes from mice immunized by pCIOmp31 priming and rOmp31 boosting produced significantly (P < 0.05) higher levels of IFN-γ than did those from mice given rOmp31 alone (Fig. 2A). In contrast, IL-2 production levels were comparable between the two groups of immunized mice (Fig. 2B). Cells from all immunized mice produced undetectable levels of IL-4 (data not shown). Notably, rOmp31 stimulated IL-10 production in the pCIOmp31/rOmp31-immunized group but not in the pCIOmp31- or rOmp31-immunized groups. Spleen cells from all mice produced all investigated cytokines in response to ConA, with no significant differences observed among the groups (not shown). These results indicate not only that compared with rOmp31 or pCIOmp31 immunization, pCIOmp31/Omp31r immunization induced improved Th1 responses but also that the combined immunization regimen stimulated production of T cells that secrete IL-10, whereas individual immunizations did not.

Omp31 prime-boost immunization does not improve cytotoxic responses. Omp31 immunization, either as a DNA vaccine or as a recombinant protein, has been shown to be effective in inducing CTLs (10, 12). Thus, the induction of Omp31-specific CTLs in all immunized mice was examined at the time of the bacterial challenge. CTL responses were assessed by the 51Cr release assay after in vitro stimulation with mitomycin C-treated A20/Jmp31 stimulator cells. Target cells were A20/JpCI or A20/JOmp31 (control). Specific lysis of A20/JOmp31 cells was observed at an effector/target cell ratio of 25/1 or higher using CTLs from mice of all groups (Fig. 3). Maximal lysis was observed at a ratio of 100/1. The cytotoxicity level elicited by Omp31 DNA vaccination was significantly higher (P < 0.05) than the one achieved by either rOmp31 or prime-boost immunization (Fig. 3). Splenocytes from PBS- or pCI-immunized animals failed to lyse A20/JpCI or A20/JOmp31 cells. These results indicate that although the prime-boost regimen induced specific cytotoxic responses, these responses cannot reach the levels achieved by the pCIOmp31 immunization responses.

Prime-boost immunization slightly improves the protection conferred either by pCIOmp31 or by rOmp31 against B. melitensis and B. ovis infection. Protection experiments were carried out by challenging PBS-, pCI-, pCIOmp31-, rOmp31-, pCIOmp31/rOmp31-, or H38-vaccinated mice with B. ovis or B. melitensis, and the level of infection was evaluated by determining the numbers of CFU in spleens. Mice given rOmp31 exhibited a significant degree of protection against B. ovis and B. melitensis compared with that of controls receiving PBS or
Interestingly, these vaccines elicited different immune responses either as a recombinant protein or as a DNA vaccine (10, 12). This probably because of the higher bacterial burden used in these studies. Levels of protection were lower than those reported previously (12), despite statistically significant. The control vaccine H38 induced 2.25 log units of protection, but this increase was not significant from results for PBS-immunized mice (P < 0.01, estimated by Dunnett’s test).

We have recently reported that Omp31 is a promising candidate to be used as a subunit vaccine against brucellosis. It conferred partial protection against B. ovis and B. melitensis (1.21 and 1.03 log units of protection, respectively) (Table 1 and Table 2). The levels of protection were lower than those reported previously (12), probably because of the higher bacterial burden used in these experiments (10^6 versus 10^4 bacteria in spleen). pCIOmp31 priming followed by rOmp31 boosting enhanced the degree of protection conferred either by pCIOmp31 or rOmp31 against B. ovis and B. melitensis (1.42 and 1.45 log units of protection, respectively), but this increase was not statistically significant. The control vaccine H38 induced 2.25 log units of protection against B. ovis and 2.56 log units of protection against B. melitensis. The enhancing effect of priming with DNA was specific to pCIOmp31 because priming with the empty vector pCI failed to increase protection among mice boosted with rOmp31 (not shown).

## DISCUSSION

The development of a safe and effective vaccine against brucellosis has proved to be difficult. Indeed, numerous cell surface and intracellular components have been assessed as protective antigens against Brucella infection, although only low or intermediate levels of protection have been achieved with these isolated proteins (1, 12, 13, 25, 29, 31, 33). These results underscore the difficulties inherent in a single-protein-subunit approach to Brucella vaccination. Regardless of the vaccine composition, however, a successful vaccination strategy will probably need to evoke a vigorous broad-based immune response.

We have recently reported that Omp31 is a promising candidate to be used as a subunit vaccine against brucellosis. It conferred partial protection against B. ovis and B. melitensis, either as a recombinant protein or as a DNA vaccine (10, 12). Interestingly, these vaccines elicited different immune responses; specific humoral, Th1, and CTL responses were elicited after rOmp31 immunization, while only vigorous specific CTL responses were observed after pCIOmp31 immunization. Then, we sought to determine if these levels and types of Omp31-specific immune responses would be generated using a mixed protocol of immunization and if the elicited responses together would provide stronger protection against Brucella. The prime-boost strategy, which consists of the sequential delivery of a vaccine antigen by two fundamentally different methods, has been reported to generate extremely potent humoral and cellular immune responses (27).

In the present study, we demonstrate that a prime-boost immunization strategy that uses pCIOmp31 priming followed by rOmp31 boosting increases the Ab response and the production of IFN-γ by Omp31-stimulated splenocytes in vitro relative to that provided by two immunizations of rOmp31. This response led to moderately improved protection against a challenge with B. ovis or B. melitensis.

It has been demonstrated that Abs against Brucella Omps have little relevance in B. melitensis infections (10, 20), whereas these Abs (in particular the anti-Omp31 Abs) protect against B. ovis infection (7, 8). Thus, in terms of immune correlates of protection, the comparative advantage of the magnitude of the humoral response against Omp31 elicited by the prime-boost protocol of immunization would explain or might correlate in part with the enhanced protection elicited against B. ovis. After the B. ovis challenge, an increase in IgG2a titers was observed only in sera from pCIOmp31/rOmp31-immunized mice. Th1-type Ab isotypes such as IgG2a may also opsonize the pathogen to facilitate phagocytosis (22).

### TABLE 1. Protection induced by prime-boost immunization against B. ovis infection

<table>
<thead>
<tr>
<th>Vaccine or control</th>
<th>Adjuvant</th>
<th>Log_{10} no. of B. ovis bacteria in spleen</th>
<th>Log unit(s) of protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>IFA</td>
<td>5.76 ± 0.15</td>
<td>0</td>
</tr>
<tr>
<td>pCI</td>
<td>IFA</td>
<td>5.75 ± 0.16</td>
<td>0.01</td>
</tr>
<tr>
<td>pCIOmp31</td>
<td>IFA</td>
<td>4.73 ± 0.89</td>
<td>1.03</td>
</tr>
<tr>
<td>rOmp31</td>
<td>IFA</td>
<td>4.63 ± 0.79</td>
<td>1.13</td>
</tr>
<tr>
<td>pCIOmp31/rOmp31</td>
<td>IFA</td>
<td>4.31 ± 0.81**</td>
<td>1.45</td>
</tr>
<tr>
<td>H38</td>
<td>IFA</td>
<td>3.20 ± 0.40**</td>
<td>2.56</td>
</tr>
</tbody>
</table>

*Values are mean log numbers of CFU ± SD per group. **, significantly different from results for PBS-immunized mice (P < 0.01, estimated by Dunnett’s test).
TABLE 3. Omp31-specific immune response and protection against Brucella challenge in BALB/c mice

<table>
<thead>
<tr>
<th>Vaccine or control</th>
<th>Immune response*</th>
<th>Protection against:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antibody Th1 CTL</td>
<td>B. ovis B. melitensis</td>
</tr>
<tr>
<td>PBS</td>
<td>– – – –</td>
<td>– –</td>
</tr>
<tr>
<td>PCl</td>
<td>– – ++ ++ +</td>
<td>– –</td>
</tr>
<tr>
<td>pCIOmp31</td>
<td>+ + ++ ++ +</td>
<td>+ +</td>
</tr>
<tr>
<td>rOmp31</td>
<td>++ ++ ++ ++</td>
<td>+ +</td>
</tr>
<tr>
<td>pCIOmp31/rOmp31</td>
<td>+++ + + + + +</td>
<td>++ ++</td>
</tr>
</tbody>
</table>

*a, +, induce a weak response; +++, induce a strong response; –, no response.

In the case of smooth brucellae, like B. melitensis, it is well known that cellular immune responses characterized by the production of IFN-γ are crucial to conferring protection (24). The increased production of IFN-γ in Omp31-stimulated splenocytes from mice primed with pCIOmp31 and boosted with rOmp31 would correlate with the higher degree of protection against B. melitensis. pCIOmp31/Omp31 also induced IL-10 production in response to rOmp31. IL-10 has been shown to actively down-modulate Th1 responses in cases of brucellosis (16). Hence, that the prime-boost immunization moderately improved protection against the Brucella challenge could be due to the induction of IL-10, as has been seen for other antigens and microorganisms (28).

In addition, we have recently demonstrated that the lysis of infected cells and the subsequent killing of Brucella by Omp31-specific CTLs, including CD4+ and CD8+ T cells, are important in vaccine-mediated protection against B. ovis and B. melitensis (12). The present study also shows that the prime-boost protocol of immunization induces a specific cytotoxic response against Omp31 but at a lower magnitude than the one elicited by the DNA vaccine. Again, IL-10 production could, in this case, down-modulate the expected increase in CTL responses (14).

pCIOmp31 did not induce a detectable Th1 response and induced a very low humoral response (reference 12 and this study). Conversely, the prime-boost immunization strategy that uses pCIOmp31 priming followed by rOmp31 boosting increases the Ab response and the production of IFN-γ by Omp31-stimulated splenocytes in vitro relative to that provided by two immunizations of rOmp31. These results indicate that despite the inability of pCIOmp31 to induce a detectable Th1 response and to induce a very low humoral response, it generates a memory Th1 and humoral immune response that would be recalled by rOmp31 immunization. This response led to improved protection against a challenge with a high burden of B. ovis and B. melitensis.

Notably, our study demonstrates that different arms of the immune system can be equally effective against Brucella infection. Even though smooth and rough strains are handled differently by macrophages (21), the levels of protection achieved by a DNA vaccine that induces only CTLs, a recombinant protein that induced specific Abs, IFN-γ, and CTLs, and by a prime-boost immunization that enhanced these responses are the same (Table 3).

Finally, while the prime-boost immunization improves immunogenicity, the protection afforded did not reach a level similar to that provided by immunization with the whole bacterium. Future development of a subunit vaccine for brucellosis may require more-complex vaccine candidates. These include multiple brucellar antigens such that several stages of the interaction of the bacterium and the host can be targeted.

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