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DNA Vaccine Encoding the Omp31 Gene of *Brucella melitensis* Induces Protective Immunity in BALB/c Mice

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Abstract: Brucellosis is caused by different species of *Brucella*. Nowadays attenuated strains of *Brucella* are being used to control brucellosis in domestic animals and there is no safe and effective vaccine available for human. This study was designed to evaluate the immunogenicity and the protective efficacy of a DNA vaccine encoding the *Brucella melitensis* Omp31 protein designated Omp31-pcDNA3.1. Intramuscular injection of this DNA vaccine into BALB/c mice elicited markedly both humoral and cellular immune responses. The specific antibodies exhibited a dominance of immunoglobulin G2a over IgG1. In addition, the DNA vaccine elicited a T-cell-proliferative response and also induced the production of gamma interferon, but not IL-10 or IL-4, upon restimulation with either recombinant Omp31 or crude *Brucella* protein, suggesting the induction of a typical T-helper-1-dominated immune response in mice. The Omp31-pcDNA3.1 (but not the control vector) induced a strong, significant level of protection in BALB/c mice against challenge with *B. melitensis* virulent strain M16; the level of protection was similar to the one induced by *B. melitensis* vaccine strain Rev 1. Consequently, a genetic vaccine based on the Omp31 gene can elicit a strong cellular immune response and these data suggest that Omp31-pcDNA3.1 is a good candidate for use in future studies of vaccination against brucellosis.

Key words: Brucellosis, DNA vaccine, omp31, *B. melitensis*

INTRODUCTION

Brucellosis, an infectious disease affecting livestock and humans, is caused by different species of the genus *Brucella* (Delpino *et al*., 2007) and remains endemic in many developing countries, where it undermines animal health and productivity, causing important economic losses (Cassataro *et al*., 2007) and in Human is a potentially life-threatening multisystem disease (Sauret *et al*., 2001). However, this disease has a limited geographic distribution remains a major problem in the Mediterranean region, Western Asia and parts of Africa and Latin America (Abtahi *et al*., 2004). Bacteria of the genus *Brucella* are gram-negative intracellular parasites of both humans and animals (Cloeckaert *et al*., 1996). Six species are recognized within the genus *Brucella*: *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis* and *B. neotomae* (Morgan and Corbel, 1976). This classification is based mainly on differences in pathogenicity and host preference (Cloeckaert *et al*., 1996; Vizcaino *et al*., 1997). And differentiation between the biovar of these species is currently based on serotyping, phage typing, dyesensitivities, CO_2 requirement, H_2S production and metabolic properties (Alton *et al*., 1988). The main pathogenic species worldwide are *B. abortus* and *B. melitensis*, which are involved in bovine and ovine brucellosis, respectively and can cause abortions and Infertility in cattle and sheep, with resulting high economic losses (Cloeckaert *et al*., 1996). Vaccination is the only practical means of controlling the disease when its prevalence is high (Cassataro *et al*., 2007). The attenuated strains such as *Brucella melitensis* Rev 1 and *B. abortus* S19 and RB51 are used to control brucellosis in domestic animals. Unfortunately, these are less than ideal because of their limited efficacy and potential to cause disease in humans (Yang *et al*., 2005). Numerous attempts to develop killed vaccines that are as effective as Rev 1 have met with limited success (Cassataro *et al*., 2005a). Thus, the necessity of design and produce of a better vaccine is seemed (Cassataro *et al*., 2007). Among the immunodominant antigens of *Brucella* identified by

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Western Blot, some belong to the cell envelope and correspond to both major outer membrane proteins (OMPs) and minor OMPs (Letesson *et al*., 1997). The 31-34 kDa OMP has been identified as a minor OMP in *B. abortus* strains but as a major OMP in the other *Brucella* sp. (Vizcaino *et al*., 1997). The major OMPs of *Brucella* sp. were initially identified in the early 1980s (Dubray *et al*., 1980). And have been extensively characterized as potential immunogenic and protective Ags (Cassataro *et al*., 2005b).

Major OMP genes of *Brucella* have recently attracted attention as they exhibit sufficient polymorphism to allow differentiation between *Brucella* sp. and some of their biovars (Cloeckaert *et al*., 1996) Protection against *Brucella* 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 (Cassataro *et al*., 2005a). Polymorphism of the omp31 locus in *Brucella* sp. has been studied by Southern blot hybridization of HindIII digested genomic DNA and PCR-RFLP (Vizcaíno *et al*., 1996). Also, Southern blot hybridisation showed that the omp31 gene is present in all *Brucella* sp. except *B. abortus* (Cloeckaert *et al*., 2002). And this gene was firstly cloned and sequenced from *B. melitensis* reference strain 16 M in 1996 (Vizcaíno *et al*., 1996). Immunization with plasmid DNAs expressing foreign proteins provokes both cellular and humoral immune responses and protective immunity against viruses, bacteria, parasites and tumors (Gurunathan *et al*., 2000) and is a relatively novel and powerful method of immunization (Cassataro *et al*., 2005a). The aim of this study is based on evaluating the immunogenicity and protective efficacy of a DNA vaccine encoding the Omp31 protein of *Brucella melitensis* to evaluate it's protective capacity of immunization.

MATERIALS AND METHODS

Animals: Female BALB/c mice (5-6 weeks old; obtained from the Animal Center of Pasteur Institute of Iran, Tehran, Iran) were randomly distributed into experimental groups. The mice were kept in conventional animal facilities, where they received water and food *ad libitum*.

Bacterial strains and growth conditions: *B. melitensis* virulent strain 16 M and attenuated strain Rev 1 were obtained from the microbiology department of Islamic Azad University-Shahrekord Branch (Shahrekord, Iran). The bacterial cells were grown under aerobic conditions in tryptose-soy broth (Difco Laboratories, Detroit, Mich.) for 72 h at 37°C. For inoculation, the bacterial suspensions were adjusted spectrophotometrically to an OD600 corresponding to 10^4 CFU of *B. melitensis* strain 16 M and 2×10^8 CFU of *B. melitensis* Rev 1. All experiments with live brucellae were performed in biosafety level 2 facilities. *E. coli* strain DH5α (Life technology, Gaithersburg, Md.) was used for producing the necessary plasmid constructs. The *E. coli* cultures were routinely grown at 37°C in Luria-Bertani broth or agar supplemented, when required, with 100 μg of ampicillin per mL.

Construction and preparation of omp31 DNA vaccine: Full-length open reading frames of the omp31 gene were amplified with PCR from the g enome of attenuated *B. melitensis* strain Rev 1. The PCR primers were used that are: Omp-F (5'-ACCGGATCCACCACCATGAAATCCG AATTTTG-3') and Omp-R (5'-TGCTCGAGGAACTTGTA GTTCAGACCGAC-3'). The gene amplified with Omp31 primers (Omp-F and Omp-R) was inserted into pcDNA3.1(+) vector (Invitrogen, San Diego, Calif.) at the BamHI/XhoI sites to construct recombinant plasmids omp31-pcDNA3.1. This process kept the omp31 gene in one open reading frame. A colony of *E. coli* containing omp31-pcDNA3.1 was cultured in Luria-Bertani broth containing ampicillin (100 μg mL^{-1}). Large-scale plasmid DNA isolation was performed by using an EndoFree Plasmid Giga Kit (Sigma) according to the manufacturer's instructions. The plasmid DNA was finally resolved in phosphate-buffered saline (PBS; pH 7.0) at a concentration of mg/mL. DNA concentration and purity was determined by optical density and the A260/A280 ratio was typically greater than 1.8. The omp31-pcDNA3.1 plasmid construct was verified by restriction digestion and by sequencing.

Purification of rOmp31 protein: To prepare the recombinant omp31 (rOmp31) protein, the omp31 gene was excised from the above plasmid omp31-pcDNA3.1 by BamHI/XhoI digestion and inserted into the same restriction sites of prokaryotic expression vector pET32a(+) (Novagen). The recombinant plasmids were then transformed into *E. coli* BL21(DE3) (Novagen) and the positive clones were selected. The recombinant protein was expressed in successfully transformed bacteria by induction with Isopropyl-β-D-Thiogalactopy-ranoside (IPTG) in Luria-Bertani medium and then purified with a Ni^{2+}-HiTrap chelating 5 mL prepacked column (Amersham Pharmacia Biotech) by using imidazole as the elution reagent, according to the manufacturer's protocol. The lysates of transformed cells and the purified protein were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot assays. The purified protein was then stored

at -70°C until use for enzyme-linked immunosorbent assay (ELISA) or for *in vitro* stimulation of splenocytes.

SDS-PAGE and Western blot assays: The lysates of BL21 (DE3) bacteria transformed with pET32a(+)-omp31 were migrated in SDS-PAGE gels and the protein bands were stained with Coomassie brilliant blue. For Western blot assays, the protein bands in the gels were electrotransferred to a nitrocellulose filter and this step was followed by the antigen-antibody reactions. The rabbit anti-*B. melitensis* Rev 1 hyperimmune sera were used as the detecting antibody and horseradish peroxidase-goat anti-rabbit Immunoglobulin G (IgG) served as the secondary antibody. Following the addition of diaminobenzidine substrate, the antibody-specific protein band was revealed.

Immunization: Mice were anesthetized with inhaled halothane and injected with 50 μg of plasmid DNA (omp31-pcDNA3.1) in 50 μL of PBS in each tibialis anterior muscle (100 μg of DNA/mouse) by using an insulin syringe with a 28-gauge needle. Mice were vaccinated at weeks 0, 2 and 4 with omp31-pcDNA3.1. As negative controls, groups of mice were immunized with pcDNA3, with plasmid without omp31 insert, or with PBS only. In protection experiments, mice in the positive control group were vaccinated intraperitoneally with 2×10^8 CFU of *B. melitensis* strain Rev 1 in 0.2 mL of PBS.

ELISA: The presence of serum immunoglobulin G (IgG), IgG1 and IgG2a isotypes with specificity to rOmp31 was determined by indirect ELISA. The purified recombinant Omp31 was diluted to 3 μg mL^{-1} in carbonate buffer (pH 9.6) and used to coat the wells of a polystyrene plate (100 μL $well^{-1}$; Nunc-Immuno plate with MaxiSorp surface). After overnight incubation at 4°C, plates were washed 4 times in wash buffer (Tris-buffered saline (pH 7.4) with 0.05% Tween 20) and blocked with 5% gelatin in Tris-buffered saline for 2 h at 37°C and then incubated with serial dilution of the sera for 3 h at room temperature and washed 4 times. Isotype-specific rabbit anti-mouse horseradish peroxidase conjugates (ICN Biomedicals, Inc., Aurora, Ohio) were added (50 μL $well^{-1}$) at an appropriate dilution. After 30 min of incubation at room temperature, plates were washed 4 times and 100 μL of substrate solution (200 μmol of o-phenylenediamine and 0.04% H_2O_2) was added to each well. After 20 min of incubation at room temperature, the enzyme reaction was stopped by addition of 100 μL of 0.18 M sulfuric acid/well and the absorbance of the developed color was measured at 492 nm. The cutoff value for the assay was calculated as the mean specific optical density plus Standard Deviation (SD) for 10 sera from nonimmunized mice assayed at a dilution of 1:40. The titer of each serum was calculated as the reciprocal of the highest serum dilution yielding a specific optical density higher than the cutoff value.

Splenocyte cultures and lymphocyte proliferation: Two weeks after the last immunization, mice were sacrificed and their spleens were removed under aseptic conditions. Single-cell suspensions were prepared from the spleens and the red blood cells were lysed with ACK (150 mM NH4Cl, 1 mM $KHCO_3$, 0.1 mMNa2 · EDTA, pH 7.3) solution. Splenocytes were cultured at 37°C in 5% CO_2 in a 96-well flat-bottom plate at a concentration of 4×105 cells $well^{-1}$ in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal calf serum (Sigma), in the presence of 0.08 μg of purified rOmp31 protein or no additives (unstimulated control). The cells were cultured for 3 days and pulsed for 8 h with 0.4 μCi of (3H]thymidine (50 Ci $mmol^{-1}$; Amersham) per well. The radioactivity incorporated into the DNA was measured in a liquid scintillation counter. Cell proliferation was expressed as mean counts per minute (cpm) from five mice for each group. All assays were performed in triplicate and repeated 3 times.

Cytokine ELISAs: For detection of cytokines, culture supernatants of spleen cells were collected after 48 h of antigen stimulation and tested for the presence of the cytokines by antigen-capture ELISA using OptEIA Set Mouse IFN-γ, IL-4 and IL-10 (BD Biosciences, San Diego, Calif.). All assays were performed in triplicate. The concentration of IFN-γ, IL-4 and IL-10 in the culture supernatants was calculated by using a linear-regression equation obtained from the absorbance values of the standards.

Protection experiments: The protection experiments were performed as described previously (Onate *et al*., 1999). Briefly, 5 weeks after vaccination (day 60), 6 mice from each group were challenged by intraperitoneal injection of 10^4 CFU of *B. melitensis* 16 M. Two weeks later, the infected mice were sacrificed, their spleens were macerated and dilutions were plated to determine the number of *Brucella* CFU per spleen. This experiment was repeated 3 times and results from the pooled data are shown. Statistical analyses were performed with Student's paired t-test. Log10 units of protection were obtained by subtracting the mean log10 CFU for the experimental group from the mean log10 CFU of the corresponding control group.

RESULTS

Immune response of mice vaccinated with omp31-pcDNA3.1: Titers of anti-omp31 antibodies were measured by ELISA in serum from mice immunized with omp31-pcDNA3.1, with the expression vector alone (pcDNA3.1), or control PBS. Mice immunized with omp31-pcDNA3.1 gave a rapid IgG response by 2 week following vaccination (titers ranged from 80:160) and the peak titers (range, 2,560:5,120) were detected at 60 day postvaccination (end of experiment). None of the animals inoculated with pcDNA3.1 showed specific anti-Omp31 antibodies. Subisotype analysis of these antibodies (IgG1 and IgG2a) indicated that the anti-Omp31 antibodies detected in omp31-pcDNA3.1-immunized mice were predominantly IgG2a at 60 day postvaccination. The specific IgG2a titers (range, 640:1,280) were higher than the specific IgG1 titers (range, 80:160) (IgG2a/IgG1 ratio = 9.1). To examine the Cell-Mediated Immune (CMI) response to *Brucella* recombinant Omp31 (rOmp31) protein and crude *B. melitensis* proteins, the proliferation response and cytokine profile of spleen cells from mice immunized with omp31-pcDNA3.1, pcDNA3.1 and PBS were determined. The lymphocytes from mice immunized with omp31-pcDNA3.1 had a significant t-cell proliferation response to rOmp31 protein ($p<0.01$) with a stimulation index of 14.95. On the other hand, when splenocytes from mice immunized with omp31-pcDNA3.1 were stimulated *in vitro* with crude *Brucella* proteins they showed proliferation, but it was not statistically significant from control groups ($p>0.1$). With respect to cytokine profile, supernatants of spleen cell cultures from omp31-pcDNA3.1-immunized animals contained high levels of IFN-γ compared to the negative control groups. The rOmp31 protein and crude *Brucella* proteins significantly induced the production of IFN-γ in cells from omp31-pcDNA3.1-immunized mice ($p<0.05$ in both groups) and only a low level of IL-10 was detected in all groups. In addition, no IL-4 was detected in any of the culture supernatants of splenocytes stimulated with specific antigens. Splenocytes from all groups of mice, including the saline-inoculated group, produced a similar level of IFN-γ, IL-4 and IL-10 upon stimulation with concanavalin A. Taken together, our results indicate that immunization with omp31-pcDNA3.1 induces a specific Th1-type immune response in mice (Table 1).

Efficacy of omp31-pcDNA3.1 immunization in generating protective immunity against *B. melitensis*: Protection experiments were carried out by challenging vaccinated and control mice by intraperitoneal injection of virulent *B. melitensis* and the level of infection was evaluated by determining the CFU in their spleens. The results indicate that immunization with omp31-pcDNA3.1 resulted in a significantly higher degree of protection (2.16 log increases in protection) compared to the unimmunized control groups ($p<0.0005$). To compare the extent to which mice could be protected, vaccination with live *B. melitensis* strain Rev 1 induced 2.22 log protection. No significant difference in the number of CFU was seen between groups injected with pcDNA3.1 and PBS. These results indicate that omp31-pcDNA3.1 vaccine afforded a significant degree of protection against *Brucella* infection.

Table 1: Comparison of stimulation of omp31-pcDNA3, pcDNA3, PBS in Splenocytes culture from all groups of mice

	Anti-bodies		Cytokines		
Vaccine or control groups	IgG1	IgG2a	IFN-γ	IL10	IL-4
PBS	-	-	-	-	-
pcDNA3	-	-	-	-	-
omp31-pcDNA3	++	+++	+++	+	-

DISCUSSION

The first effective *Brucella* vaccine was based on live *Brucella abortus* strain 19, a laboratory derived strain attenuated by an unknown process during subculture. This vaccine induces reasonable protection against *B. abortus*, but at the expense of persistent serological responses. A similar problem occurs with the *B. melitensis* Rev 1 strain that is still the most effective vaccine against caprine and ovine brucellosis. After that developed a live attenuated and rifampicin-resistant mutant *B. abortus* RB51 strain. This strain has proved safe and effective in the field against bovine brucellosis and exhibits negligible interference with diagnostic serology (Schurig *et al*., 2002).

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 (Cassataro *et al*., 2007). In the case of smooth *Brucella*, like *B. melitensis*, it is well known that cellular immune responses characterized by the production of IFN-γ are crucial to conferring protection (Murphy *et al*., 2001). In this study, we evaluated the capacity of omp31-pcDNA3 to elicit an immune response and protective immunity in BALB/c mice. We have recently reported that Omp31 is a promising candidate to be used as a DNA vaccine against brucellosis.

Several previously described DNA vaccines to *Brucella* induced antibody titers lower than the omp31-pcDNA3.1 employed in this research (Al-Mariri *et al*., 2001; Leclero *et al*., 2002). This difference may be

attributed to several factors, including the amount of protein expressed or development of a preferential CMI (Vemulapalli *et al*., 2000).

CONCLUSION

In conclusion, we have shown that immunization of plasmid DNA containing of omp31 gene leads to the elicitation of both antibody and CMI responses of Th-1 type and confers protection against *B. melitensis* challenge.

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