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Comparison of potential protection conferred by three immunization strategies (protein/protein, DNA/DNA, and DNA/protein) against *Brucella* infection using Omp2b in BALB/c Mice

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ABSTRACT

In the present study, immunogenicity and protective efficacy of the *Brucella* outer membrane protein 2b (Omp2b) was evaluated in BALB/c mice using Protein/Protein, DNA/DNA and DNA/Protein vaccine strategies. Immunization of mice with three vaccine regimens elicited a strong specific IgG response (higher IgG2a titers over IgG1 titers) and provided Th1-oriented immune response. Vaccination of BALB/c mice with the DNA/Pro regimen induced higher levels of IFN-γ/IL-2 and conferred more protection levels against *B. melitenisis* and *B. abortus* challenge than did the protein or DNA alone. In conclusion, Omp2b is able to stimulate specific immune responses and to confer cross protection against *B. melitensis* and *B. abortus* infection. Therefore, it could be introduced as a new potential candidate for the development of a subunit vaccine against *Brucella* infection.

1. Introduction

Brucellosis is the world's most common bacterial zoonosis. Annually, global incidence of Brucellosis is more than half million human cases and in some countries, the prevalence rate exceeds ten cases per 100,000 population (Supriya et al., 2010; Cultuer et al., 2005). Despite restricted geographic distribution, the disease still remains major public health challenge in most countries (Gwida et al., 2010; Lopes et al., 2010). *B. melitensis*, *B. abortus*, and *B. suis* are considered as the most pathogenic species, worldwide (Sung et al., 2014). Brucellosis is known as a potentially life-threatening multi-system disease in human. Animal brucellosis causes significant economic losses by abortion and decreased milk production (Seleem et al., 2010). Although vaccination is probably the most economic control strategy, administration of current commercially available vaccines alone is not adequate for elimination of brucellosis. Therefore, complete elimination of the disease depends on the development of safe and protective vaccines for different hosts of *Brucella* [7].

Subunit vaccines are attractive for vaccination purposes due to their safety profile. Subunit DNA vaccine is safe and efficacious strategy offering the best approach to activate both T helper1 (Th1) and CTL cellular response. However, DNA vaccines have limitation of conferring less levels of protection than the available live attenuated *Brucella* vaccines (Golshani et al., 2015a). One of the most promising strategies to improve DNA vaccines is the prime-boost strategy. Results from previous studies have demonstrated that the combination of a DNA priming step and the homologous recombinant protein boosting resulted in improved immune responses and superior levels of protection against *Brucella* infection (Golshani et al., 2015b; Cassataro et al., 2007; Ramshaw and Ramsay, 2000).

Since representing the initial point of contact between the pathogen and the host immune system, bacterial surface exposed antigens are prime vaccine targets. Several *Brucella* immunogenic antigens have been identified in the outer membrane of this pathogen (Avila-Calderón et al., 2013; Salhi et al., 2003; Cloeckaert et al., 2002). Group 2 major outer membrane proteins (Omps) of *Brucella* includes two closely related *omp2a* and *omp2b* genes encoding the 33 kDa Omp2a and 36 kDa Omp2b porins, respectively (Salhi et al., 2003; Cloeckaert et al., 2002; Golshani et al., 2016). Recent studies focusing on the identification of *Brucella*

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protective antigens demonstrated that Omp2b is a priority antigen for designing a *Brucella* subunit vaccine (He and Xiang, 2010). Our previous study indicated that Omp2b protein is conserved in all *Brucella* pathogens with 99–100% sequence similarity among strains. Prediction of T cell and B cell epitopes demonstrated that Omp2b protein contains epitopes with high affinity for MHC molecules and antibodies (Golshani et al., 2016). Moreover, Om2b may be a potential protein for developing *Brucella* molecular diagnostic tests (Sung et al., 2014). Identification of four main *Brucella* pathogenic species: *B. melitensis*, *B. suis*, *B. abortus* and *B. canis* has been performed using combination of primer pairs for amplification of *bcsp*31, *Omp2b*, *Omp2a*, *Omp31* genes (Smirnova et al., 2013; López-Goñi and O'Callaghan, 2012; Yu and Nielsen, 2010). In the study done by Sung, stimulation of RAW 264.7 cells by Omp2b significantly enhanced the levels of IFN-γ and IL-4 produced by mice splenocytes (Sung et al., 2014). Additionally, *B. melitensis* Omp2b protein has been identified as anti-apoptotic effector candidate that essentially prevents Bax-induced cell death in yeast (Laloux et al., 2010).

Since *B. melitensis* and *B. abortus* are the main causes of human and animal brucellosis, designing a vaccine candidate conferring protection against both pathogens would be ideal. Our bioinformatics analysis of Omp2b showed that the protein is conserved among *B. melitensis* and *B. abortus* pathogen strains (Golshani et al., 2016). Therefore, Omp2b protein may be able to confer cross protection against *B. melitensis* and *B. abortus* infection. Based on this hypothesis, the current study aimed to evaluate the immunogenicity and protective efficacy of the recombinant Omp2b (rOmp2b) protein administered as protein/protein (Pro/Pro), DNA/DNA and DNA/Pro vaccine regimens.

2. Materials and methods

2.1. Mice

Six- to eight-week-old female BALB/c mice (obtained from the animal center of the Pasteur Institute of Iran) were randomly distributed into seven experimental groups (20 mice/group). The mice were kept in conventional animal facilities and received water and food at libitum. All animal care and procedures were in accordance with institutional policies for animal health and well-being.

2.2. Bacterial strains

The heat inactivated *B. abortus* strain 544 was obtained from the Microbial collection, Pasteur Institute of Iran, Karaj, Iran. The live attenuated *B. melitensis* Rev1 and *B. abortus* RB51 vaccines were obtained from the Iran Veterinary Organization, Tehran, Iran.

2.3. In silico analysis and recombinant proteins production

In silico analysis of Omp2b protein and *in vitro* production of recombinant Omp2b (rOmp2b) was performed as described previously (Golshani et al., 2016). Briefly, PCR amplified Omp2b (KP071938) coding sequence was cloned in pET28a vector and expressed in *E. coli* BL21 (DE3) upon induction with 0.2 mM IPTG. Identity of the purified 36.6 kDa recombinant protein was tested by SDS-PAGE and Western Blotting assay.

2.4. Construction and preparation of omp2b DNA vaccine

The PCR product of Omp2b coding sequence was digested with Fast digest *NheI* and *XhoI* restriction enzymes (Fermentas, USA) and then ligated to the pcDNA3.1 (+) vector, digested by the same restriction enzymes, using T4 DNA ligase (Fermentas, USA) at 4° C over night. *E. coli* TOP10 competent cells were used for transformation of pcDNA-Omp2b plasmid. The transformed bacteria were cultured on LB agar containing 100 μg/ml of ampicillin and then selected by screening the colonies on the media containing antibiotic. Some suspected colonies were further analyzed by PCR, restriction enzyme digestion and sequencing. Large-scale plasmid DNA isolation was performed by using an EndoFree Plasmid Mega Kit (QIAGEN, USA) according to the manufacturer's instructions. Transient expression of pEGFP-Omp2b construct in Cos-7 cells was performed as described previously (Golshani et al., 2015b).

2.5. Immunization

Mice group Pro/Pro were immunized subcutaneously with 40 μg of rOmp2b plus 50 μg of CpG ODN 1826 (MWG company, Germany) and Montanide ISA 70VG (SEPPIC company, France) at days 0 and 21. The sequence of phosphorothioate CpG 1826 motif was as follows: TCCATGACGTTCCTGA CGTT (Golshani et al., 2015a; Chu et al., 1997). Mice groups DNA/DNA, DNA/Pro and pcDNA were anesthetized intraperitoneally with a mixture of Xylazine 2% (7.5 μl), Ketamine 10% (30 μl) and normal saline solution (260 μl) per mouse. Mice group DNA/DNA were immunized by the footpad route with 50 μg of pcDNA-Omp2b plasmid followed by *in vivo* electroporation (EP) with electro square porator (ECM 830, BTX, Harvard apparatus, USA) mode: LV, 63 V, P.lengh: 20.6 ms, at days 0 and 21. Mice group DNA/Pro were primed by the footpad route with 50 μg of pcDNA-Omp2b plasmid following by *in vivo* EP at day 0, and then boosted subcutaneously with 40 μg of rOmp2b plus adjuvants at day 21. Mice group pcDNA were immunized by the footpad route with 50 μg of pcDNA vector plasmid followed by *in vivo* EP. Mice group PBS were immunized subcutaneously with 150 μl PBS. As positive controls, two other groups were immunized once intraperitoneally (i.p.) on day 0 with 2×10^8 CFU *B. abortus* RB51 and 2×10^8 CFU *B. melitensis* Rev.1 in 100 μl PBS, respectively. Sera were obtained at 21, 42, 63 and 84 days post first immunization.

2.6. ELISA

To determine serum reactivity against rOmp2b indirect enzyme-linked immunosorbent assay (ELISA) was used to measure immune serum immunoglobulin G (IgG), IgG1 and IgG2a levels as described previously (Golshani et al., 2015a). The cutoff value for the assay was calculated as the mean specific optical density plus 3standard deviation (SD) for 20 sera from non-immunized mice assayed at a dilution of 1:100. 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.

2.7. Stimulation of spleen cells

Four weeks after the final immunization, spleen cells from immunized or control mice were removed aseptically (4 mice/group) and individually cultured at 3.5×10^6 cells per well in 48-well flat-bottom plates (Greiner Bio-One, Germany) with rOmp2b (10 μg/ml), concanavalin A (ConA) (5 μg/ml), or medium alone. RPMI 1640 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, USA) and penicillin-streptomycin (Gibco, USA) 1× was used for culturing the splenocytes. Cultures were incubated for 72 h at 37 °C in a humidified atmosphere (5% CO2), and at the end of the incubation, supernatants were aliquoted and stored at −80 °C. Gamma interferon (IFN-γ), interleukin 2 (IL-2), IL-4 and IL-10 were quantified by a sandwich ELISA using mouse IFN-γ, IL-2, IL-4 or