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Immune Modulation of Recombinant OmpA against *Brucella abortus* 544 Infection in Mice

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Brucellosis affects a wide range of host species, including humans and many livestock animals. Chronic infections of the disease make antibiotic treatment costly, and the current vaccine used in livestock has not been approved for human use. This study investigated the possible use of the *Brucella abortus* outer membrane protein A (OmpA) as a candidate subunit vaccine in an infected mouse model. The *ompA* gene was cloned and overexpressed, and the recombinant OmpA (rOmpA) protein fused to maltose binding protein (MBP) was purified in *Escherichia coli*. Immunogenicity was verified through western blotting, and mice were immunized and challenged to evaluate its protective effect. Mice treated with rOmpA exhibited induced humoral and host cell-mediated responses, with a significant increase in immunoglobulin G (IgG1 and IgG2a) and cytokine levels, especially TNF-α and IL-12, compared with the control groups treated with either MBP or PBS. In conclusion, rOmpA should be highly considered as a future subunit vaccine for brucellosis, and further studies regarding rOmpA and its protective ability are suggested.

Keywords: *Brucella abortus*, outer membrane protein A, recombinant, vaccine

Introduction

Brucella abortus, a facultative intracellular bacterium, infects both humans and many domestic animals [27]. Brucellosis causative agents are gram-negative bacteria of the genus *Brucella*, which are non-spore-forming and non-encapsulated coccobacilli (short rods with rounded ends) [23]. Brucellosis is a zoonotic disease with a worldwide distribution [1]. In endemic areas, transmission is mainly from domestic animals to humans [8]. The organisms display a tropism to the reproductive system of domesticated animals, causing abortion and infertility that results in a serious economic crisis [10].

Antibiotic treatment of animals is systematically discouraged and highly regulated [12]. A study conducted in human brucellosis treated with doxycycline or ofloxacin with rifampin yielded several reoccurrences after 1–6 months, as well as side effects, including gastric irritation and skin rashes [2]. A retrospective evaluation of 480 patients treated for brucellosis noted several symptoms, including malaise in 432/480 patients (90%), sweating in 405/480 patients (84.4%), arthralgia in 393/480 patients (81.9%), fever in 383/480 patients (79.8%), back pain in 281/480 patients (58.5%), hepatomegaly in 102/480 patients (21.3%), osteoarticular involvement in 91 patients (91%), splenomegaly in 68 patients (14.2%) and nervous system involvement in 31 patients (6.4%) [5]. The limited number of effective antibiotics and the potential for accidental introduction of antibiotic resistance into the organism emphasize the need for alternative solutions [12].

Current vaccines that are widely accepted for brucellosis include the live attenuated Strain 19 and RB51 for cattle and Rev1 for sheep. The use of attenuated vaccines has always been associated with concerns regarding safety, especially for introduction into humans and pregnant/sick animals, due to the possible induction of symptoms similar

to the full-blown disease [12]. Strain 19 induced persistent cross-reactivity of immune responses, and in the late 1990s the use of this vaccine was discontinued [32]. Additionally, the RB51 vaccine did not present any significant protection levels in rams with *B. ovis* infection [15], possibly limiting its exclusive use to cattle brucellosis.

The most ideal vaccines are nonliving. However, killed bacterial preparations have been disregarded owing to poor efficacy, unsuitable and unwanted immune response generation, and poor standards [33]. There are many known subcellular vaccine candidates against *B. abortus*, and a few studies have focused on culture supernatant proteins. A previous study stated that recombinant SurA immunization induced humoral and mixed T helper (Th)1-Th2 responses, whereas recombinant DnaK immunization induced humoral, Th1, and CTL immune responses [10]. The administration of both recombinants in combination with an adjuvant protected BALB/c mice against *B. abortus* infection. Moreover, the immunogenicity and protective efficacy of a DNA vaccine encoding the GroEL heat-shock protein from *B. abortus* was tested; vaccination induced a Th0-type immune response that was characterized by a high IgG1/IgG2a ratio and IL-4 and interferon (IFN)-gamma production [17]. Other studies regarding recombinant vaccines used the recombinant L7/L12 ribosomal protein [28], bacterio-ferritin or P39 recombinant proteins with CpG oligodeoxynucleotides [3], and CuZn superoxide dismutase DNA vaccine to induce protective immunity [29]; all of these vaccines induced either or both humoral and cellular responses.

The major mechanisms of the outer membrane protein (Omp)-mediated bacterial responses to the host environment include iron uptake, antimicrobial peptide resistance, serum resistance, multidrug resistance, and bile resistance [20]. Omps play an important part in preserving the integrity and discriminatory permeability of membranes [26]. Many subunit vaccines based on Omps have been investigated, including Omp31 (or an Omp31-derived 27-amino-acid synthetic peptide) that elicited a CD4+ T helper 1 response [7], Omp16 and Omp19 that induced specific CD4+ and CD8+ T cells [30], and Omp28 adjuvanted with CpG oligonucleotides that induced both humoral and cellular responses [16]. OmpA was noted in our previous study to display strong immunoreactivity in serum with murine brucellosis [18]. OmpA has been mainly investigated in *E. coli* and *Klebsiella pneumoniae*, but its functions and purpose in *Brucella* vaccination and infection have not been studied.

Consequently, the purpose of this study was to evaluate the immune modulation of rOmpA against brucellosis using BALB/c mice as a model.

Materials and Methods

Bacterial Strains and Growth Conditions

The bacterial strains used in this study were *B. abortus* 544 (ATCC23448), a smooth virulent *B. abortus* biovar 1 strain, and *E. coli* DH5α. *B. abortus* and *E. coli* were cultured in *Brucella* broth and LB broth (Becton Dickinson, USA), respectively, overnight at 37°C in a gyratory shaker at 260 rpm. When solid medium and ampicillin were required, the above media were supplemented with 1.5% (w/v) agar and ampicillin (100 μg/ml).

rOmpA Expression and Purification

Total genomic DNA was prepared from *B. abortus*. Briefly, *B. abortus* was cultured in *Brucella* broth overnight with shaking incubation, and 5 ml was collected and lysed with the Dokdo-Prep Bacterial Genomic DNA Purification Kit (Elpis Biotech, Korea). The *B. abortus* OmpA coding gene was amplified by PCR with the primers 5′-ACGGATCCATGCTGAAGAAAACCGGTATTG-3′ (BamHI site underlined) and 5′-ATACTGCAGTCAGGCTCC GCGTAGCGGCGA-3′ (PstI site underlined). The amplified DNA was digested with the restriction enzymes and ligated into a pMal vector (New England Biolabs, USA). Then, the ligated products were used to transform the *E. coli* DH5α expression host. The bacteria containing the fusion plasmid were induced using isopropyl ß-D-1-thiogalactopyranoside (IPTG, 0.5 mM). Bacterial cells were harvested by centrifugation at 2,560 ×*g* for 20 min. The supernatant was discarded, and cells were resuspended in 50 ml of column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 0.1% Triton X100, and 10% glycerol, pH 7.4). Samples were frozen at −70°C and thawed in cold water three times. Then, cells were sonicated (Bandelin Electronic, Germany) at 10,000 Hz in an ice-water bath and centrifuged at 5,200 ×*g* for 30 min to collect the supernatant. The supernatant was diluted with column buffer (1:5) and loaded into maltose resin (Bio-Rad, USA) according to the manufacturer's instructions. The purified recombinant proteins were stored at −20°C for protein assays.

SDS-PAGE and Immunoblotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using previously described methods [19]. Two gels were run simultaneously for Coomassie brilliant blue staining and immunoblotting. After electrophoresis, the samples were transferred to Immobilon-P membranes (Millipore, USA) for 60 min using a semi-dry electroblot containing transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol). The membrane was blocked with 5% skimmed milk for 30 min at room temperature (RT), washed three times with Tris-buffered saline containing 0.1% Tween-20 (TBS-T), and incubated overnight at 4°C with *Brucella*-positive sera (TAT; 400, 1:1,000 dilution), which

was kindly provided by the Animal and Plant Quarantine Agency (Korea). The membrane was washed with TBS-T, incubated with horseradish peroxidase-labeled protein G (Santa Cruz Biotechnology, USA; 1:10,000 dilution) for 2 h at RT and washed with TBS-T. After adding ECL (Atto, Japan), immunoreactive protein spots were visualized using a ChemiDoc XRS camera equipped with Quantity Analysis software (Bio-Rad, USA).

Immunization of Mice

Immunization of mice with recombinant proteins was performed using previously described methods (Lim *et al*., 2012). Briefly, three groups of five 6-week-old female specific pathogen free BALB/c mice (Samtako, Korea) were immunized by intraperitoneal (IP) injection at days 0, 14, and 35 with 10 μg of rOmpA, and 10 μg of MBP (positive control) or PBS (negative control) in 100 μl of incomplete Freund's adjuvant (IFA; Sigma, USA). Fourteen days after the last immunization, the mice were challenged with approximately 2×10^4 colony forming units (CFU) of *B. abortus* 544 in 100 μl of PBS by IP injection. At 10 days post-infection, mice were sacrificed and the spleens were removed, weighed, and homogenized in PBS. Tissue homogenates were serially diluted with PBS and plated on *Brucella* agar to count the number of CFU in each spleen to assess the virulence in each group. All of the procedures described were reviewed and approved by the Animal Ethical Committee of Gyeongsang National University (Authorization No. GNU-130125-M0008).

Immune Response Analysis

The serum immunoglobulin G and cytokine levels of mice treated with PBS, MBP, or rOmpA were analyzed after the completion of immunization and blood collection. IgG1 and IgG2a were calculated by an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's procedure (Abcam, USA). The cut-off value was calculated as the mean specific OD plus standard deviation (SD) for non-immunized mice diluted 1:100. The titer was defined as the highest dilution of serum giving an OD twice higher than the cut-off value. Cytokines (TNF-α, IFN-γ, MCP, IL-12p70, IL-10, and IL-6) were evaluated by a mouse inflammation cytometric bead array (BD CBA Mouse Inflammation Kit, USA).

Statistical Analysis

The data were expressed as the mean ± standard deviation (SD) for replicate experiments. The Student's *t*-test, two-way ANOVA, and post-hoc test with the Bonferroni correction method were used to analyze all of the data in this study. Results with $P < 0.05$ were considered statistically significant.

Results

Cloning, Purification, and Immunoblotting of rOmpA

A defined band (663 bp) conforming to the *B. abortus* 544 *ompA* gene was amplified by PCR, and subsequently inserted into the pMAL expression vector (43.5 kDa) for transformation of competent *E. coli* DH5α cells. Transformed *E. coli* containing the rOmpA insert (66.5 kDa; Fig. 1A) was induced with IPTG and purified with a maltose resin column. Western blotting was performed to evaluate rOmpA protein immunoreactivity. Fig. 1B indicates that the purified rOmpA significantly displayed strong reactions with *Brucella*-positive mouse serum (1:1,000 dilution), whereas the reaction was not detected with *Brucella*-negative mouse serum (Fig. 1C). After sequencing, the cloned product was confirmed with 96% homology to the reported *ompA* gene sequence for *B. abortus* biovar strain 9-941 (GenBank Accession No. NC006932.1).

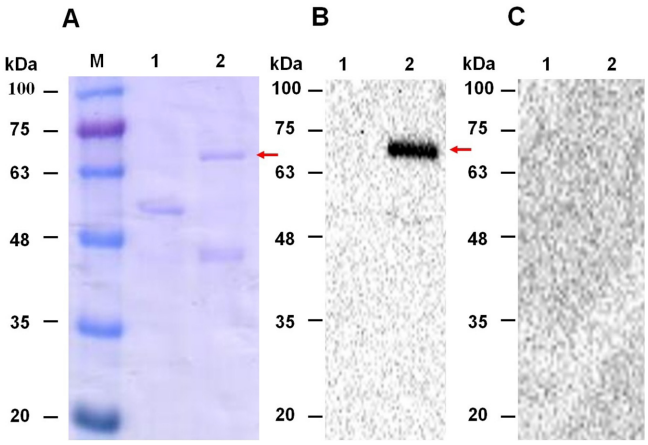

Fig. 1. Expression of recombinant *B. abortus* rOmpA.
MBP (43.5 kDa) and rOmpA (66.5 kDa) were separated with 10% SDS-PAGE, and then subjected to (**A**) Coomassie blue staining or transferred to a membrane and probed with *Brucella* positive (**B**) or negative (**C**) mouse serum. Line M: Marker; Lane 1: MBP; Lane 2: rOmpA (arrows).

Analysis of the Immune Response against rOmpA in Mice

To test the protective effect of rOmpA, three sets of mouse groups were immunized with purified rOmpA, MBP (positive control), or PBS (negative control) in IFA on 0, 14, and 35 days. Blood was collected from the groups 35 and 49 days after the first immunization to determine IgG1 and IgG2a titers. The mean log titers of both IgG1 and IgG2a were significantly higher for the rOmpA-immunized mice compared with the animals injected with either PBS or MBP throughout the experiment (Fig. 2). Our figures clearly indicate that after rOmpA vaccination, there were increases in both the humoral and cell-mediated immune responses observed by the induction of IgG1 and IgG2a

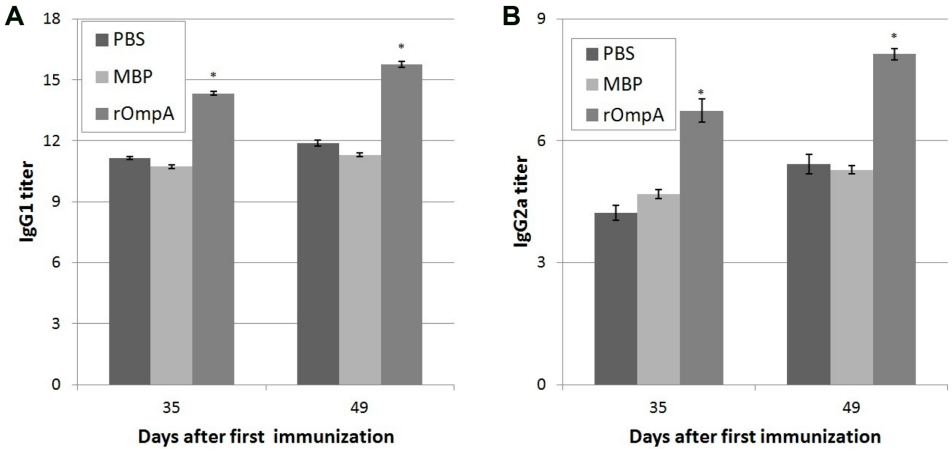

Fig. 2. Immunoglobulin G titers of mice treated with PBS, MBP, or rOmpA at 35 and 49 days after the first immunization.
Data are presented as the mean ± SD (n = 5), and asterisks indicate significant differences ($p < 0.05$).

production, respectively.

Because the cytokine content in the serum and spleen cells were significantly identical [4], we analyzed the cytokine content using mouse serum samples. In this study, serum samples were collected three weeks after the last immunization and one week after bacterial challenge for measurement. Mice treated with rOmpA exhibited an increased level of numerous cytokines, including TNF-α, IFN-γ, MCP, IL-12, IL-10, and IL-6, with increases in expression from days 35 to 49 after the first immunization that ranged from 1.93-fold to 7.69-fold. Additionally, an increase in the range of 1.01-fold to 3.39-fold was observed for all cytokines compared with the PBS- or MBP-treated mice (Figs. 3A–3F). The rOmpA-immunized mice displayed a significant increase in TNF-α and IL-12 levels ($p < 0.005$) compared with mice treated with either PBS or MBP 49 days after the first immunization (Figs. 3A and 3D).

Protection Efficiency of rOmpA in Mice

To scrutinize the ability of rOmpA to deliberately protect against virulent *B. abortus*, an in vivo protection study was performed using BALB/c mice (three sets of groups with

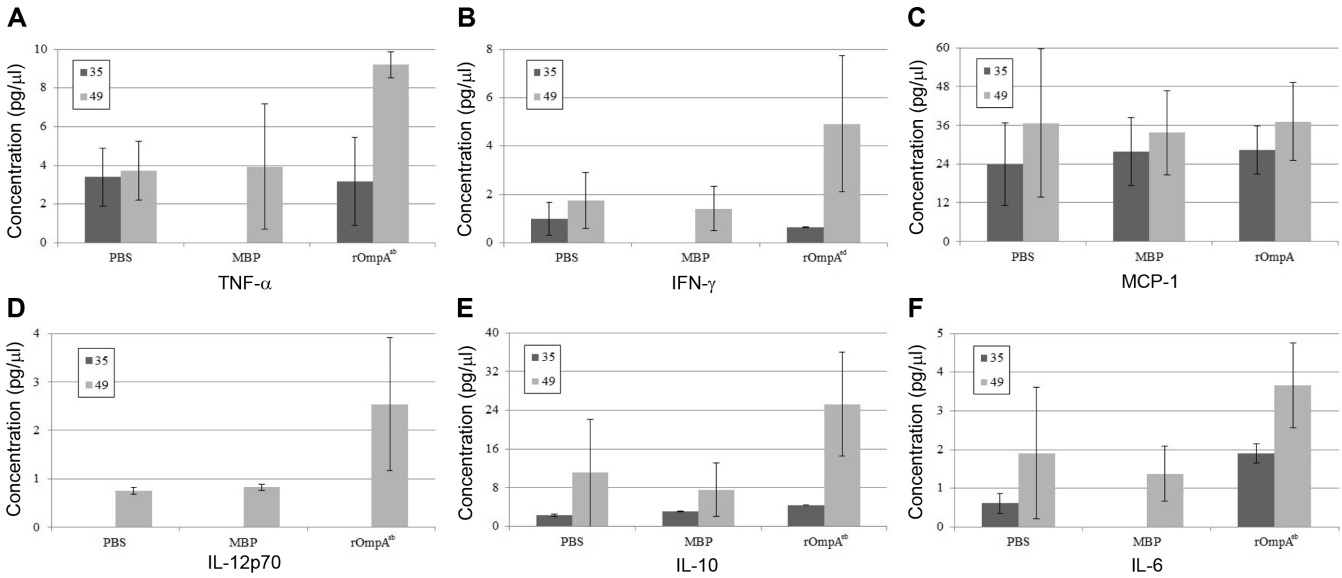

Fig. 3. Concentration of cytokines in mice vaccinated with PBS, MBP, or rOmpA after 35 and 49 days.
Significant difference from PBS-immunized group (**A**, $p < 0.005$; **C**, $p < 0.01$; **E**, $p < 0.05$) and MBP-immunized group (**B**, $p < 0.005$; **D**, $p < 0.01$; **F**, $p < 0.05$).

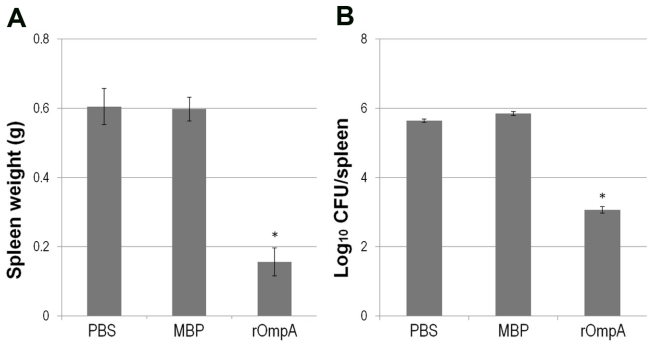

Fig. 4. Protection level of mice treated with PBS, MBP, or rOmpA after 10 days of infection with *B. abortus*.
Spleen weight (**A**) and bacterial proliferation in the spleen (**B**) are presented as the mean ± SD ($n = 5$), and asterisks indicate significant differences ($p < 0.05$).

five mice in each). In this experiment, protection was defined as a significant reduction in bacterial burdens ($p < 0.05$) in mice that received the vaccine compared with animals treated with PBS and MBP. Mice in both sets of the negative and positive controls developed splenomegaly, with enlargement up to 2–3 times its normal size, resulting in host inflammatory reactions; in contrast, no splenomegaly was detected in the rOmpA-immunized set (Fig. 4A). Vaccine efficacy was calculated by comparing the log units of bacterial burden in the spleens. The mean log titers of the rOmpA-immunized mice (3.068 ± 0.09) were approximately 1.8-fold lower than the PBS (5.643 ± 0.44) or MBP (5.852 ± 0.56) immunized mice groups (Fig. 4B).

Discussion

In this study, the *ompA* gene was successfully cloned and expressed in *E. coli* DH5α with pMal vectors. The *ompA* gene was highly reactive to *Brucella*-positive sera but not to *Brucella*-negative sera. The mean log titers of both IgG1 and IgG2a were significantly higher for the rOmpA-immunized mice compared with the animals injected with either the positive or negative control. These results clearly showed that the rOmpA vaccination increased the humoral immune response by inducing IgG production to protective levels compared with the control. Increases in both immunoglobulin titers compared with the controls implied that rOmpA induced both host cell-mediated (Th1) and humoral (Th2) immunity. Mice vaccinated with rOmpA developed OmpA-specific antibodies that exhibited a higher level of IgG1 over IgG2a (Fig. 2), which indicates the induction of strong Th2 responses. This result differed from other subcellular vaccines that were dominated by IgG2a responses [3, 10, 16, 17, 29]. The Omp31 subunit vaccine was one of the few *Brucella* vaccines that induced IgG1 over IgG2a [7]. Subunit vaccines (including rOmpA) have also been noted to induce IgG1 over IgG2a in other bacteria, including *Pasteurella multocida* [9], *Burkholderia cenocepacia* [25], and *Acinetobacter baumanni* [21]. A great deal of attention has been focused on the Omps as a non-LPS group of immunogens [22]. However, the roles of Omps in survival and virulence during infection remain unknown [11].

In the current study, mice treated with rOmpA exhibited an increased level of numerous cytokines, including TNF-α, IFN-γ, MCP, IL-12, IL-10, and IL-6, compared with the PBS- and MBP-treated mice. Furthermore, the rOmpA-vaccinated mice displayed a significant increase in the production of TNF-α (2.34–2.89-fold) and IL-12 (2.54–3.39-fold) compared with mice treated with either PBS or MBP 49 days after the first immunization.

TNF-α and IL-12 are produced early during intracellular bacterial infections and have been reported to contribute to resistance to *B. abortus* by different mechanisms [35]. The production of TNF-α in brucellosis is Toll-like receptor 2 (TLR2)-dependent, while both TNF-α and IL-12 are involved in the MyD88 pathway [13]. *B. abortus* survives inside macrophages by inhibiting the major histocompatibility complex class II (MHC II) via the TLR [6]. MyD88 is a common adaptor molecule that leads to host cell activation through TLR transducer signals [24]. In this study, both TNF-α and IL-12 were increased compared with the MBP-treated mice, and we can assume that macrophage MHC II via the TLR transducer signals and MyD88 adaptor molecules were not suppressed compared with the naturally infected mice.

The OmpA of *K. pneumoniae* was reported to induce IL-12 production and macrophage maturation [14] and was demonstrated to activate macrophages through the production of IL-12 and TNF-α [34]. The *Shigella flexneri* OmpA vaccine also rendered the highest TNF-α levels between the other cytokines reported compared with the control [31]. *B. abortus* OmpA had sequence identity or sequence similarity to *K. pneumoiae* (33–36%, average 33.94%) and *S. flexneri* (34–58%, average 36.36%), after all the sequences were compared with the National Center for Biotechnology Information - Basic Local Alignment Search Tool.

In conclusion, rOmpA displayed significant protection that was confirmed by the spleen weight and bacterial burden and supported by the IgG and cytokine levels in the sera. The results of this study showed that rOmpA

administration increased both the cell-mediated and humoral immune responses. The recombinant protein should be highly considered as a future single subunit vaccine and a candidate for protection against *B. abortus* infection. Further studies regarding the importance of OmpA in the pathogenesis of *Brucella* and its protective ability are recommended.

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