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Original Article

Protective effects of recombinant *Brucella abortus* Omp28 against infection with a virulent strain of *Brucella abortus* 544 in mice

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The outer membrane proteins (OMPs) of *Brucella* (*B.*) *abortus* have been extensively studied, but their immunogenicity and protective ability against *B. abortus* infection are still unclear. In the present study, *B. abortus* Omp28, a group 3 antigen, was amplified by PCR and cloned into a maltose fusion protein expression system. Recombinant Omp28 (rOmp28) was expressed in *Escherichia coli* and was then purified. Immunogenicity of rOmp28 was confirmed by Western blot analysis with *Brucella*-positive mouse serum. Furthermore, humoral- or cell-mediated immune responses measured by the production of IgG1 or IgG2a in rOmp28-immunized mice and the ability of rOmp28 immunization to protect against *B. abortus* infection were evaluated in a mouse model. In the immunogenicity analysis, the mean titers of IgG1 and IgG2a produced by rOmp28-immunized mice were 20-fold higher than those of PBS-treated mice throughout the entire experimental period. Furthermore, spleen proliferation and bacterial burden in the spleen of rOmp28-immunized mice were approximately 1.5-fold lower than those of PBS-treated mice when challenged with virulent *B. abortus*. These findings suggest that rOmp28 from *B. abortus* is a good candidate for manufacturing an effective subunit vaccine against *B. abortus* infection in animals.

Keywords: *Brucella abortus*, immunization, rOmp28, vaccine

Introduction

Brucella spp. are facultative intracellular Gram-negative bacteria and an important etiological agent that causes zoonotic disease. *Brucella* spp. are able to evade the host bactericidal phagocyte functions and proliferate within macrophages, thereby leading to the establishment of chronic infection [9,10]. *Brucella* spp. may occur as either smooth or rough, expressing smooth lipopolysaccharide (S-LPS) or rough LPS (R-LPS) as major surface antigen (Ag). S-LPS expressed by *Brucella* is the strongest Ag compared to other antigenic molecules that are involved in the immune response against brucellosis [16]. Currently, *Brucella* (*B.*) *abortus* S19 is used to immunize cattle whereas *B. melitensis* Rev 1 is used to immunize goats and sheep [28]. In general, the use of live attenuated organisms as vaccines is associated with safety concerns during vaccine production, and attenuated *Brucella* vaccines have many specific disadvantages, including abortion in animals administered during pregnancy [26,28]. For these reasons, different strategies are being sought for the production of safe, non-replicating vaccines that are easy to reproduce with consistent quality [20].

Recently, outer membrane proteins (OMPs) of *Brucella* have been evaluated as a non-LPS group of immunogens and vaccine [6,8,16]. *Brucella* OMP Ag are categorized according to their molecular weight into three groups: group 1, 2, and 3. Group 1, 2, and 3 Ags have approximate molecular masses of 94, 41 to 43, and 25 to 30 kDa, respectively [31]. All *Brucella* OMPs Ags, especially those in group 3, are also known as important factors that affect *Brucella* virulence [22]. These OMPs are major components of the sodium dodecyl sulfate (SDS)-insoluble cell wall fraction, and confer important vaccinal properties against *Brucella* infection [13,14]. The role of the two major members of OMPs, Omp25/Omp31 family, in protective immunity against *Brucella* infection is being studied by several groups [4,12,31]. Although *Brucella* OMPs Ags have important roles in immunogenicity and

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virulence, vaccines using OMPs Ags have not been fully evaluated.

We performed the present study to develop a subunit vaccine against *Brucella* infection in a mouse model. The gene encoding *B. abortus* Omp28 was cloned and expressed using a maltose fusion protein (pMAL) expression system. The ability of this recombinant protein (rOmp28) to protect against challenge with virulent *B. abortus* was evaluated along with the mouse response to immunization.

Materials and Methods

Bacterial strains and growth condition

A smooth virulent *B. abortus* biovar 1 strain of *B. abortus* 544 was kindly provided by Animal, Plant and Fisheries Quarantine and Inspection Agency in Korea and *Escherichia* (*E.*) *coli* DH5α cells was purchased from Invitrogen (USA). *B. abortus* was routinely cultured overnight in *Brucella* broth (BD Biosciences, USA) at 37°C in a gyratory shaker (Waver Digital Platform; VWR International, USA) at 10 × g. When needed, solid medium was made by supplementing Brucella or Luria-Bertani (LB) broth (Becton Dickinson, USA) with 1.5% (w/v) agar (Takara, Japan). *E. coli* DH5α cells were used for producing the necessary plasmid constructs. *E. coli* cultures were routinely grown at 37°C in LB broth or agar supplemented with 100 μg/mL of ampicillin (Sigma, USA).

rOMP expression

Total genomic DNA was prepared from *B. abortus*. Briefly, *B. abortus* cells were cultured at 37°C overnight in Brucella broth with shaking. Next, 5 mL of the culture were collected and genomic DNA from the cells was recovered with a bacteria genomic DNA purification kit (iNtRON, Korea). The *B. abortus* Omp28 gene was amplified by PCR with the following primer pair: 5′-GATC GGA TCC AAC ACT CGT GCT AGC AAT TTT-3′ (*Bam*HI site underlined) and 5′-GATC AAG CTT TTA CTT GAT TTC AAA AAC GAC-3′ (*Hin*dIII site underlined). The amplified DNA was digested with appropriate restriction enzymes (*Bam*HI and *Hin*dIII; Takara, Japan) and ligated into a pMAL vector (New England Biolabs, USA). The recombinant plasmid was then used to transform the *E. coli* DH5α host cells. An exponential-phase culture of a rOmp28 clone confirmed in ampicillin-containing media was spread onto LB agar plates containing isopropyl β-D-1-thiogalactopyranoside (IPTG) (1 mM; Amresco, USA) and ampicillin (100 g/mL).

Purification of rOMP protein

One liter of LB broth supplemented with 100 μg/mL of ampicillin was inoculated with 10 mL of an overnight culture of bacteria expressing the fusion plasmid. IPTG was then added at a final concentration of 0.3 mM and the culture was further incubated at 37°C for 2 h. Bacterial cells were harvested by centrifugation at 2,560 × g for 20 min. The supernatant was then discarded, and the cells were resuspended in 50 mL column buffer (20 mM Tris HCl, 200 mM NaCl, and 1 mM EDTA, pH 7.4). The sample was frozen at −70°C and thawed in cold water three times. The cells were then was 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 Laboratories, USA) according to the manufacturer's instructions. The purified protein was stored at −20°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

SDS-PAGE was performed according to previously described methods [11]. The purified rOmp28 was boiled for 10 min at 100°C and diluted in 2× Laemmli sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl, pH 6.8). After electrophoresis, the separated proteins were transferred onto Immobilon-P membranes (Milipore, USA) in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) with a constant current of 2 mA/cm^2 for 30 min using a semi-dry electroblot assembly (Bio-Rad, USA). The membrane was blocked with 1% bovine serum albumin (Sigma, USA) for 1 h at 4°C, washed three times with 0.05% PBS-Tween 20, and incubated with *Brucella*-positive mouse sera (produced from mouse immunized by virulent *B. abortus*, 1 : 1,000 dilution) in blocking buffer at 4°C for 2 h. The membrane was then washed with 0.05% PBS-Tween 20 and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (1 : 10,000 dilution; Sigma, USA) in blocking buffer for 1 h at 4°C, and finally washed with 0.05% PBS-Tween 20. The proteins were detected with a luminol-coumaric acid-H_2O_2 detection solution (iNtRON, Korea) and exposure to X-ray film (Fuji, Japan).

Immunization and bacterial challenge in mice

Six-week-old female BALB/c mice (Japan SLC, Japan), two groups of five BALB/c mice each were immunized intraperitoneally with rOmp28 (100 μg/mL) or PBS in incomplete Freund's adjuvant (IFA) (Sigma, USA) on days 0, 14, and 35. Serum samples were obtained from all animals 14, 35, and 49 days after the first immunization *via* retro orbital bleeding. Serum titers of immunoglobulin G1 and IgG2a isotypes specific for rOmp28 were determined with an ELISA (BioTek, USA) according to the manufacturer's instructions. The cutoff value for the assay was calculated as the mean specific optical density for samples from nonimmunized mice diluted 1 : 100 as

previously described [27].

To test the protective effects of rOmp28, mice were challenged with approximately 10^4 colony forming unit (CFU) of *B. abortus* 14 days after the last immunization. Ten days post-infection, all mice were scarified and the spleens were removed, weighed, and homogenized (Wisd homogenizer; Witeg, Germany) in saline. The homogenates were serially diluted (1 : 100 ~ 1,000) with PBS and plated on *Brucella* agar and then incubated for 3 days at 37°C. The number of CFUs for each spleen sample was determined to assess the virulence of the bacteria in each group of animals.

Statistical analysis

The results for each of experiment are expressed as the mean ± SD. Student's *t*-test was used to compare the groups. *p*-values < 0.05 were considered significantly different.

Results

PCR amplification and immunoreactivity of rOmp28

A specific band (753 bp) corresponding to *B. abortus* Omp28 was amplified by PCR. The PCR-amplified gene fragment encoding Omp28 was integrated into the pMAL expression vector and used to transform competent *E. coli* cells. The cloned product sequence was confirmed and found to completely match that of the reported Omp28 gene sequence for *B. abortus* (Genbank accession No. NC_007618.1). *E. coli* harboring the Omp28 plasmid were grown in LB medium, and 1 mM IPTG was used to induce Omp28 protein expression.

The protein product of the cloned Omp28 gene in the pMAL expression system was purified and was approximately 28 kDa (Fig. 1A). To evaluate rOmp28 protein immunoreactivity, Western blotting was performed (Fig. 1B). Our results showed that the purified rOmp28 strongly reacted with *Brucella*-positive mouse serum (1 : 1,000 dilution).

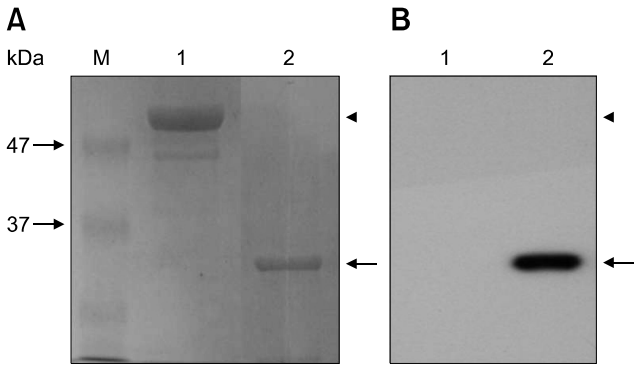

Fig. 1. Expression of recombinant *Brucella* (*B.*) *abortus* Omp28 (rOmp28) *via* a maltose fusion protein (pMAL) expression system. Total bacterial cell lysate proteins were separated by 10% SDS-PAGE and stained with Coomassie Brilliant Blue (A). The proteins were then transferred to a membrane and incubated with *Brucella*-positive mouse serum (B). pMAL (arrowheads) and rOmp28 (arrows) are indicated. M: marker, Lane 1: pMAL, Lane 2: rOmp28.

IgG1 and IgG2a responses to rOmp28 immunization

To test the protective effect of rOmp28, two groups of mice were immunized with purified rOmp28 protein or PBS with IFA on days 0, 14, and 35. Blood was collected from both groups 14, 35, and 49 days after the first immunization, and the IgG1 and IgG2a titers were determined. Throughout the entire experiment, the mean log titers of both IgG1 and IgG2a were significantly higher for the rOmp28-immunized mice compared to the animals injected with PBS (Figs. 2A and B). Our results clearly indicated that administration of rOmp28 increased both

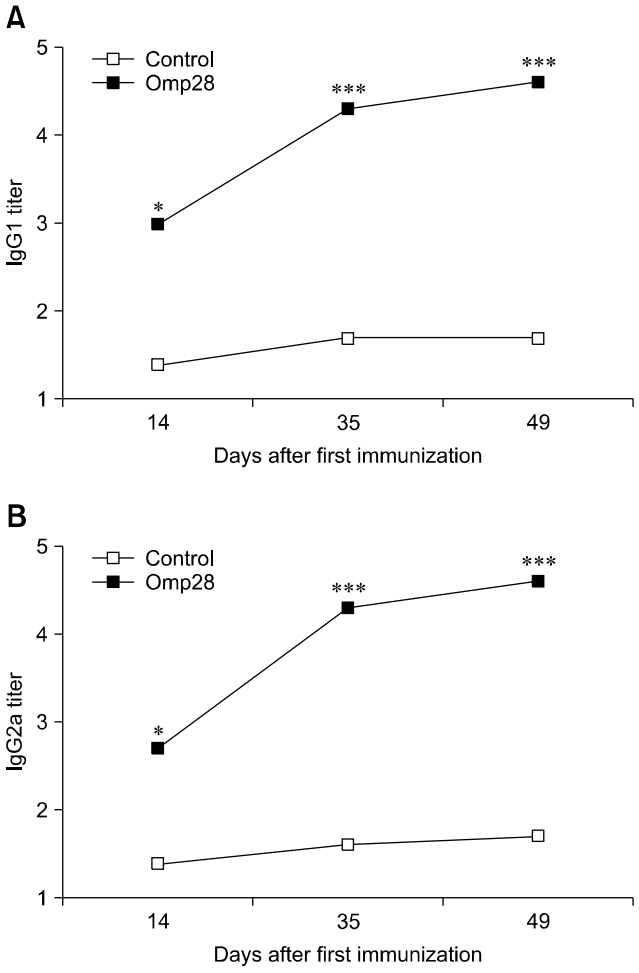

Fig. 2. Kinetics of the immune response elicited by immunization with rOmp28. The IgG1 (A) and IgG2a (B) log titers were determined by ELISA. Significant differences between the rOmp28-immunized and control (PBS-treated) mice are indicated by asterisks (*$p < 0.05$ and ***$p < 0.001$).

humoral and cell mediated immune response by inducing IgG1 and IgG2a production.

Protection of rOmp28 against *B. abortus* challenge

To analyze the ability of rOmp28 to protect against infection with virulent *B. abortus*, an *in vivo* protection study was performed in BALB/c mice (two groups with five mice each). In this experiment, protection was defined as a significant reduction ($p < 0.05$) in the mice receiving the vaccine when compared to animals injected with PBS. Mice treated with PBS developed splenomegaly that was enlarged 2 ~ 3 times its normal size as a consequence of the host inflammatory response, but this was not observed in the rOmp28-immunized group (Fig. 3). Vaccine efficacy was calculated by comparing the log units of bacterial burden in the spleens of the PBS-treated or rOmp28-immunized mice after *B. abortus* challenge. The mean log titers were 5.49 ± 0.49 for the PBS-treated animals and 2.89 ± 0.17 for the rOmp28-immunized (Fig. 4). These finding showed that immunization with rOmp28 conferred protection against challenge with virulent *B. abortus*.

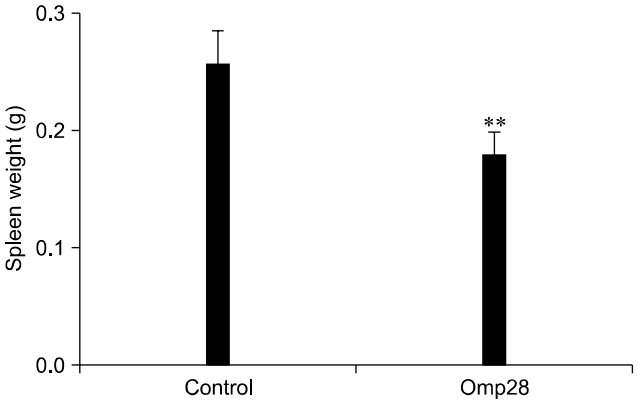

Fig. 3. Spleen weight of mice treated with PBS or immunized with rOmp28 and infected intraperitoneally with *B. abortus*. Spleen weights of the mice 10 days post-infection are shown. Data are presented as the mean ± SD (n = 5 per group). Spleen weight differed significantly between the rOmp28-immunized group and control (PBS-treated) animals as indicated by asterisks (**$p < 0.01$).

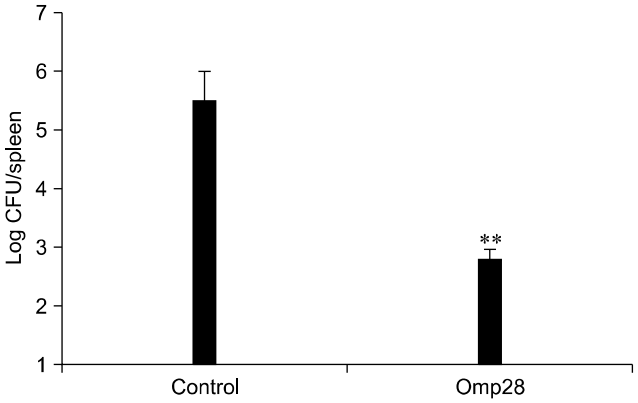

Fig. 4. Bacterial proliferation in the spleen of mice treated with PBS or immunized with rOmp28 and infected intraperitoneally with *B. abortus*. The numbers of viable bacteria recovered from spleens of the mice 10 days post-infections are shown. Data are presented as the mean ± SD (n = 5 per group). Bacteria proliferation differed significantly between the rOmp28-immunized group and control (PBS-treated) animals as indicated by asterisks (**$p < 0.01$).

Discussion

In a previous study, groups of *Brucella* OMPs were originally grouped according to their molecular weights and presences in outer membrane preparations [30]. OMPs play a vital role in maintaining the integrity and selective permeability of membranes [24]. In addition, expression of these proteins is often regulated by environmental signals; this characteristic is important for bacterial pathogenesis by enhancing adaptability to various environmental conditions [4,21].

Live attenuated *Brucella* vaccines interfere with serologic diagnosis of brucellosis because these vaccines induce a high-titer antibody response against the LPS O-polysaccharide. Antibody response is also limited to animals that develop an active case of brucellosis which is often delayed compared to a response against smooth LPS [19]. Accordingly, identification of *Brucella* protein components that can elicit an antibody response in the majority of infected animals would improve serological diagnostic methods and vaccinal strategies for treating brucellosis.

Development of a safe and effective vaccine against brucellosis has proven to be difficult. Indeed, numerous cell surface and intracellular components were examined as potential factors that could protect against *Brucella* infection. However, only low or intermediate levels of protection were achieved [1,5,29]. Regardless of the vaccine composition, a successful vaccination strategy would probably need to evoke a vigorous broad-based immune response. OMPs of *Brucella* spp. were characterized as immunogenic and protective Ags. Yet previous studies [4,7,12] were focused on the major OMPs (Omp25 and Omp31), and investigations on the protective capacity of minor OMPs were rarely performed. A previous report demonstrated that inoculation with *B. abortus* rOmp18 induces a Th1 response *in vitro* and the production of rOmp18-specific antibodies in BALB/c mice [30]. However, rOmp18 preparations were found to not protect against challenge with a virulent *B. abortus* strain [30]. Since *Brucella* species are facultative intracellular pathogens that reside mainly in macrophages, cellular immune responses are considered central for

immunization [30].

Host immunity against *B. abortus* infection involves both Th1- and Th2-specific immune responses. Th1 responses are characterized by cellular immunity and the production of IgG2a antibodies. Th2 responses are characterized by humoral immunity, specifically the production of IgG1 [23]. The present study demonstrated that immunization with purified rOmp28 elicits protection against *B. abortus* infection in mice. Injection with rOmp28 produced *in vivo* Th1 and Th2 immune responses in the BALB/c mice (as shown by the production of IgG1 and IgG2a), and conferred a significant level of protection against challenge with virulent *B. abortus* 544. Because rOmp28 provided protection against *B. abortus* infection, this recombinant protein may be an important candidate for producing a vaccine against *Brucella*.

B. abortus infection results in a Th1 cellular immune response that promotes bacterial clearance [2]. Th1-isotype antibodies, such as IgG2a, are preferentially generated in humoral immune response by B cells against intracellular microorganisms, and may also opsonize the pathogen to facilitate phagocytosis [18]. During the course of infection, *B. abortus* is mainly cell-associated. However, this pathogen can be accessed through other mechanisms for clearance, such as ones mediated by IgG2a antibodies [15]. Presumably, these antibodies may have a greater ability than other isotypes to recognize microbial Ags on the surface of infected cells. The current study demonstrated that IgG2a and IgG1 responses were robust in *B. abortus*-infected mice vaccinated with rOmp28. It has been demonstrated that rOmp28 of *B. melitensis* can also protect against *B. abortus* infection [17]. In addition, rOmp28 of *B. melitensis* induced a humoral immune response in humans infected with both *B. abortus* and *B. melitensis* [22]. According to gene sequence data (Genbank accession no. NC_007618.1), the Omp28 homology between *B. melitensis* and *B. abortus* is almost 99% (NC_010742.1). These findings suggest that Omp28 is highly conserved among *B. melitensis* and *B. abortus*, and rOmp28 of both *B. melitensis* and *B. abortus* offers significant protection against virulent *B. abortus* infection.

The development of subcellular protein-based vaccines to prevent brucellosis has several advantages. One major advantage is the possible ability to differentiate vaccinated animals from infected animals. The current live attenuated vaccines against brucellosis for animals induce the high-titer production of antibodies against the O-polysaccharide of LPS, which interferes with ability to serologically diagnose the disease [3]. It has been reported that the *B. abortus* strain RB51, which is used for a general live attenuated vaccine, expresses the LPS O-side chain. Some cattle vaccinated with this strain were found to produce positive results for the card or buffered acid plate agglutination tests as well as the standard tube agglutination test [25].

In summary, the results from our study suggested that rOmp28 of *B. abortus* is a good vaccine candidate for protecting against *B. abortus* infection. However, the protective effects of rOmp28 in mice and the role of Omp28 in *B. abortus* virulence are still unclear. Consequently, further characterization of group 3 OMP genes of *B. abortus* including Omp28 will be required to understand the immunological and pathological aspects of brucellosis.

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