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Induction of Immune Responses by Two Recombinant Proteins of *Brucella abortus*, Outer Membrane Proteins 2b Porin and Cu/Zn Superoxide Dismutase, in Mouse Model

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The diagnosis of *Brucella abortus* is mainly based on serological methods using antibody against LPS, which has diagnostic problems. Therefore, to solve this problem, we evaluated two proteins of *B. abortus*, Cu/Zn superoxide dismutase (SodC) and outer membrane proteins 2b porin (Omp2b). The genes were cloned and expressed in a pMAL system, and the recombinant proteins, rOmp2b and rSodC, were purified as fusion forms with maltose-binding protein. The identity of the proteins was confirmed by SDS-PAGE and Western blot analysis with sera of mice infected with *B. abortus*. Production of cytokines and nitric oxide (NO) was investigated in RAW 264.7 cells and mouse splenocytes after stimulation with the proteins. Moreover, cellular and humoral immune responses were investigated in BALB/c mice after immunization with the proteins. TNF-α, IL-6, and NO were significantly inducible in RAW 264.7 cells. Splenocytes of naive mice produced IFN-γ and IL-4 significantly by stimulation. Moreover, number of IgG, IFN-γ, and IL-4 producing cells were increased in immunized mice with the two proteins. Production of IgG and IgM with rOmp2b was higher than those with rSodC in immunized mice. These results suggest that the two recombinant proteins of *B. abortus* may be potential LPS-free proteins for diagnosis.

Keywords: *Brucella abortus*, cytokine, immunogenicity, Omp2b, SodC

Introduction

Brucellosis is a major zoonotic disease with public health importance worldwide and causes huge economic loss on the livestock industry. The causative agent of brucellosis is the genus *Brucella*, which is a group of facultative intracellular bacteria [3]. The genus consists of six classical species according to primary preferred host and antigenic variation: *Brucella melitensis* (sheep and goats), *B. suis* (hogs), *B. abortus* (cattle), *B. ovis* (sheep), *B. canis* (dogs), and *B. neotomae* (rats) [17]. Recently, *B. ceti* and *B. pinnipedialis* isolated from marine mammals [10] were included in this genus. The zoonotic potential of *Brucella* species is variable: *B. melitensis*, *B. abortus*, and *B. suis* are considered as the most pathogenic species for humans, and *B. canis*, *B. ceti*, and *B. pinnipdialis* also cause human brucellosis but with comparatively low zoonotic potential.

Infections of *B. abortus* show different clinical signs. This zoonotic pathogen causes undulant fever, endocarditis, arthritis, and osteomyelitis in humans, and abortion and infertility in cattle [11]. *B. abortus* organisms are present in the reproductive tissue and fetal fluids, and also concentrate in the udders of animals that produce milk. Therefore, the predominant route of exposure for *B. abortus* between animals is through ingestion or inhalation of organisms that are present in the products of parturition. Human brucellosis is usually transmitted from infected animals by direct contact with the bacteria highly presented in tissues,

or by consumption of unpasteurized dairy products from infected animals. The best approaches to prevent and control both human and bovine brucellosis have been focused on eliminating infected animals and preventing the spread of the disease using diagnostic methods and vaccines.

Lipopolysaccharide (LPS) has been considered the most important antigen molecule during immune responses in brucellosis and can elicit long-lasting serological responses in both vaccinated and infected animals [2]. The diagnosis of *B. abortus* in the ruminant is mainly based on serological confirmation with LPS-based antigens. The most widely used standard serological tests are the standard tube agglutination test, the Rose Bengal plate agglutination test, and the complement fixation test. Although LPS elicits a strong humoral immune response, there are several problems using LPS-based diagnostic methods. It is impossible to differentiate infected and/or vaccinated animals [27]. *Brucella O*-polysaccharide of LPS is similar to that of various gram-negative bacteria, such as *Yersinia enterocolitica* O:9 [16], *Escherichia coli* O:166/O157 [23], *Salmonella urbana* [24], *Francisella tularensis*, *Pseudomonas maltophilia* [9], and *Vibrio cholera* [19], and the specificity of LPS-based diagnostic methods in brucellosis is low owing to cross-reactivity. For these reasons, many researchers have tried to develop LPS-free protein-based diagnostic reagents [1].

Immunoproteomics is the approach to identify specific immunogenic proteins in high resolution in the wide range of proteins expressed by *Brucella*. Several proteins have been identified as immune-reactive proteins in proteomic analysis of *B. abortus* [1, 8, 18]. Outer membrane proteins (Omps) have received the most attention as major immunoreactive components in the bacterial cells to develop new diagnostic methods or vaccines [5]. Although the role and size of each Omp are various, porin Omps as a bacterial component have important roles in the bacterial living [29]. Cellular proteins with catalytic activities also have important roles in the bacterial living [12]. Based on this knowledge, two proteins, Omps2b and Cu/Zn superoxide dismutase (SodC), were selected to analyze the possibility of using LPS-free protein antigens in the diagnosis of bovine brucellosis through the investigation of the *in vitro* and *in vivo* immunostimulating activities of the recombinant proteins.

Materials and Methods

Bacterial Strains and Growth Condition

The bacterial strains used in this study were *Brucella abortus* 544 (ATCC23448), a smooth virulent *B. abortus* biovar 1 strain and *E. coli* DH5α (Invitrogen, USA). *B. abortus* was cultured in *Brucella* broth (BD Bioscience, USA), overnight at 37°C in a gyratory shaker at 260 rpm. *E. coli* DH5α (producing the necessary plasmid constructs) cultures were routinely grown at 37°C in Luria-Bertani (LB) broth (Difco, USA) or agar supplemented with ampicillin (Sigma, USA). When solid medium and ampicillin were required, the above media were supplemented with 1.5% (w/v) agar (Takara, Japan) and 100 μg/ml of ampicillin (Sigma).

Cloning of Omp2b and SodC Genes

Total genomic DNA was prepared from *Brucella abortus* 544 culture using the G-spin Genomic DNA Extraction kit for bacteria (Intron, Korea). Genes encoding Omp2 and Sodc of *B. abortus* were amplified by PCR with primers pairs with appropriative restriction enzyme sites as previously described: the SodC forward primer was 5′-GAAGTGATGGAATTCTTATTTATTGC-3′, and the SodC reverse primer was 5′-AGCGCTGCAGGCTTATCGGAAT-3′ [24]; the Omp2b forward primer was 5′-GGCGGATCCCATATGGAC GCAATCGTCGCGCCA-3′, and the reverse primer was 5′-GGA TCCGGTCAGCATAAAAAGCAAGC-3′ [25]. The amplified DNAs were digested with appropriate restriction enzymes and ligated into a pMAL vector (New England Biolab, USA). The recombinant plasmids were then transformed into the *E. coli* DH5α host cell for expression. Conservation of the correct sequences of the insert in the expression vector was confirmed by nucleotide sequencing.

Expression and Purification of the Recombinant Omp2b and SodC

The expression and purification of recombinant proteins were performed as described previously [21]. Briefly, the *E. coli* containing fusion plasmid was cultured overnight and 10 ml of the bacteria was inoculated with 1 L of ampicillin-containing LB broth. Isopropyl β-D-1-thiogalactopyranoside (Amresco, USA) was added to a final concentration of 0.3 mM and further incubated at 37°C for 2 h. Bacterial cells were harvested by centrifugation at 4,400 ×*g* for 20 min. The supernatant was discarded and resuspended in 50 ml of column buffer (20 mM Tris HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4). The samples were sonicated at 10,000 Hz in an ice-water bath and centrifuged at 8,700 ×*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 proteins were stored at −20°C.

SDS-PAGE and Western Blot Analysis

SDS-PAGE and Western blotting were performed as previously described [22]. Briefly, the purified recombinant proteins were diluted with Laemmli sample buffer and boiled for 10 min at 100°C. After electrophoresis, samples were visualized by staining with Coomassie Brilliant Blue R-250 (Intron). Proteins resolved by SDS-PAGE were transferred to PVDF membrane (Millipore, USA) at 2 mA/cm^2 constant current for 30 min using a semidry electroblot

assembly (Bio-Rad) containing transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol). The membrane was blocked with 5% bovine serum albumin (BSA, Sigma) for 1 h at 4°C and washed three times with 0.05% PBS-Tween 20, and was incubated with *Brucella*-positive mouse sera (1:200 dilution) for 2 h at 4°C. The membrane was washed with 0.05% PBS-Tween 20 and incubated with horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (1:10,000 dilution; Sigma) at 4°C, overnight, with gentle shaking, and washed as mentioned above. The immunolabeling was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, USA), according to the manufacturer's instructions, and exposure to X-ray film (Fuji, Japan).

Cytokines and NO Measurement in Murine Macrophage Cell Line RAW 264. 7

A murine macrophage cell line, RAW 264.7, was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and penicillin (50 μg/ml) and streptomycin (50 μg/ml), at 37°C under 5% CO_2. After incubating the cells for 18 h in the 12-well plates containing 1 × 10^6 cells/ml, they were stimulated with 10 μg/ml of rOmp2b and rSodC. Activity of LPS contaminated in the recombinant proteins was inhibited by incubation with polymyxin B (100 μg/ml) for 30 min on ice before stimulation of macrophages and splenocytes. *E. coli* LPS (1 μg/ml; Sigma) was used as the positive control and maltose-binding protein (MBP), and RPMI 1640 media only were used as the negative control. The culture supernatants were collected at 24 h after stimulation. Amounts of TNF-α and IL-6 were measured using the enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instruction (eBioscience Inc., USA). Production of NO was estimated by measuring the nitrite accumulation with the Griess reaction as described previously [30]. Briefly, 100 μl aliquots of the culture supernatants were incubated with the same volume of the solution containing 1% sulfanilamide (Sigma) and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride (Sigma) in 2.5% phosphoric acid. Then, they were incubated for 10 min at room temperature and the absorbance was measured at 540 nm. Nitrite concentrations in each well were calculated based on the standard curve generated with sodium nitrite.

Cytokines Measurement in Splenocytes of Naive Mouse

For the IFN-γ and IL-4 measurement assay, splenocytes (1 × 10^6 cells/ml) isolated from healthy female, 5-week-old BALB/c mice (Orient-Bio, Korea) were stimulated with 10 μg/ml of rOmp2b, rSodC, and MBP. After 24 h, amounts of IFN-γ and IL-4 in the culture supernatants were measured using the ELISA according to the manufacturer's instruction (eBioscience Inc.). Concanavalin A (Sigma) and media were used as positive and negative controls, respectively. All stimuli were pretreated with polymyxin B to avoid the activity of LPS contamination with recombinant proteins. All care and handling of animals were performed with the approval of the Seoul National University Institutional Animal Care and Use Committee (IACUC, approval number SNU-121129-4-1).

Production of Immunoglobulins in Mice Immunized with the Recombinant Proteins

The 6-week-old BALB/c mice (approval by Seoul National University, SNU-121129-4-1) were immunized by intra-peritoneal injection of 30 mg of the purified recombinant proteins, rOmp2b and rSodC, mixed with complete Freund's adjuvant (Sigma) on day 0 and with incomplete Freund's adjuvant (Sigma) on day 14. Sera for antibody response detection were obtained at 0, 3, 7, 14, and 28 days after the first immunization. Production of antibody was determined by ELISA using purified recombinant proteins as coating antigens. Briefly, 96-well microplates (Greiner Bio One, Germany) were coated by incubation of the purified recombinant proteins, Omp2b and SodC (5 ng per well), in coating buffer (14.2 mM Na_2CO_3, 34.9 mM $NaHCO_3$, 3.1 mM NaN_3, and pH 9.6) overnight at 4°C. The plates were then blocked with 1% BSA (Sigma) in a solution of PBS containing 0.1% Triton X-100 (PBST) for 2 h at 37°C. After washing with PBST, 1/200 diluted serum samples were added to the wells and incubated for 1 h at 37°C. HRP-conjugated goat anti-mouse IgG (Bio-Rad) and IgM (Jackson Immuno, USA) diluted 1:2,000 in PBS containing 1% BSA were used to detect IgG and IgM, respectively. The color development was carried out using a 3,3′,5,5′-tetramethyl-benzidine (TMB) substrate (Sigma). The absorbance was measured using an automatic microplate reader (Molecular Device Co., USA) at 420 nm. All samples were in triplicate.

Measurement of IgG-, IFN-γ-, and IL-4-Secreting Cells in Mouse Splenocytes

Recombinant Omp2b and rSodC specific IgG memory B cell and IFN-γ- and IL-4-secreting T-cells from spleen of immunized mice were measured by the Enzyme-Linked ImmunoSpot (ELISpot^{PLUS}) assay kit according to the manufacturer's instruction (Mabtech AB, Sweden). For IgG memory B cells, 200 μl of rOmp2b and rSodC in PBS (50 μg/ml) were added to an ELISpot plate after pretreatment with 70% ethanol and coated by incubation overnight at 4°C. For IFN-γ- and IL-4-secreting T cells, 200 μl of monoclonal antibody against IFN-γ (AN18 15 μg/ml) and IL-4 (11B11, 15 μg/ml) was added to the plate and coated as described above. After the incubation, the plate was then extensively washed with PBS five times and blocked with RPMI 1640 with 10% FBS for 30 min at room temperature (RT). After removing the medium, 5 μg of rOmp2b and rSodC was added to the well and splenocytes isolated from mice at 28 days after first immunization were added into the wells at 1 × 10^5 cells/well concentration. The plate was incubated at 37°C under 5% CO_2 for 24 h for IgG or 48 h for IFN-γ and IL-4. After removing the cells, 100 μl of biotinylated anti-IgG, IL-4, and IFN-γ antibodies in PBS containing 0.5% FBS (PBS-0.5% FBS) was added to each well. After incubation for 2 h at RT, the plates were washed and streptavidin-HRP in PBS-0.5% FBS was added and incubated for 1 h at RT. IgG-, IFN-γ, and IL-4 secreting cells were visualized upon addition of ready-to-use TMB substrate solution after washing the wells with PBS. The numbers

of responding cells were counted using Eli.Scan+ (A.EL.VIS, Germany). All samples were in triplicate.

Statistics

Statistical significance (p-value) was calculated using Student's t-test with the Statistical Package for Social Science software ver. 4.0 (SPSS, USA). Differences were considered to be significant if a value of $p < 0.05$ was obtained. All experiments were repeated at least three times.

Results

Cloning, Expression, and Purification of Recombinant Omp2b and SodC

Cloning of the Omp2b and SodC genes in the pMAL expression system led to the expression of a MBP fusion protein and then they were purified on a maltose resin column. The SDS-PAGE profiles of purified rOmp2b and rSodC gave a size of approximately 78.5 kDa and 60 kDa, respectively (Fig. 1-A1, B1). To evaluate the immunoreactivity of the recombinant proteins, western blotting was performed. Both expected and unexpected size bands of rOmp2b and rSodC showed sero-reactivity to *B. abortus*-positive mouse serum but not to the purified MBP (Fig. 1-A2, B2).

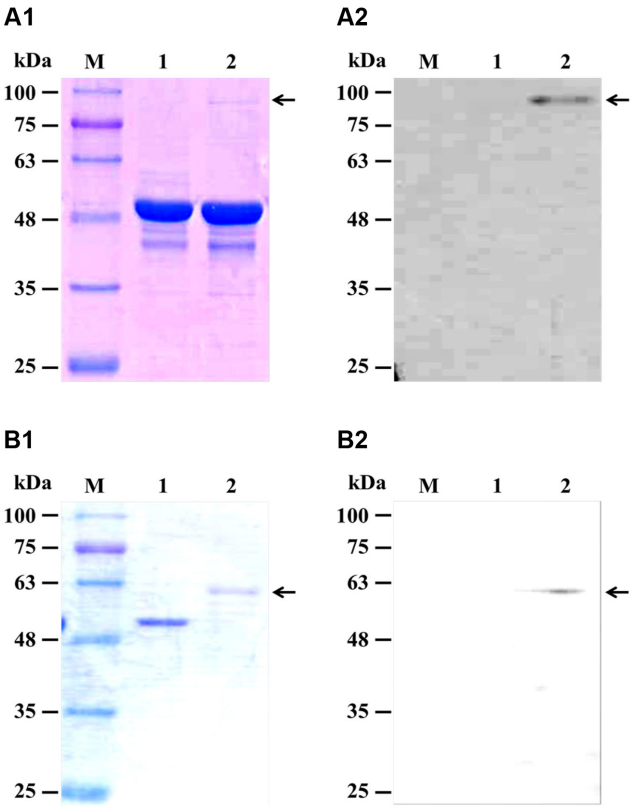

Fig. 1. Analysis of purified recombinant Omp2b and SodC of *Brucella abortus*.
SDS-PAGE (**A1**) and Western blot (**A2**) analysis of soluble protein fractions of pMAL-expressed Omp2b. *M*, molecular weight markers; *Lane 1*, Purified MBP protein; *Lane 2*, Purified MBP-fusion protein. SDS-PAGE (**B1**) and Western blot (**B2**) analysis of soluble protein fractions of pMAL-expressed SodC. *M*, molecular weight markers; *Lane 1*, Purified MBP protein; *Lane 2*, Purified MBP-fusion protein. The molecular mass of the MBP-fusion proteins were 78.5 kDa (Omp2b) and 60 kDa (SodC).

Production of TNF-α, IL-6, and NO in RAW 264.7 Cells

Culture supernatants of RAW 264.7 cells were assayed for TNF-α, IL-6, and NO production at 24 h after stimulation with rOmp2b and rSodC, by ELISA and Griess assay. The amounts of TNF-α from the stimulating groups with the two recombinants proteins were higher than those from the group stimulated with MBP ($p < 0.01$) (Fig. 2A). Similarly, a significant amount of IL-6 in the recombinant proteins exposured group was produced ($p < 0.01$) (Fig. 2B). Production of NO in the experimental groups stimulated with the two recombinant proteins was also significantly higher than the MBP control group ($p < 0.01$) (Fig. 2C). There was no detection of TNF-α, IL-6, and NO in the RPMI 1640-treated group. Thus, the two recombinant proteins were proven to have immune-stimulating activities in RAW 264.7 cells by production of TNF-α, IL-6, and NO.

Production of Cytokines in Naive Mouse Splenocytes

The amounts of IFN-γ and IL-4 in naive mouse splenocyte culture supernatants were measured at 24 h after stimulation with the two recombinant proteins, by ELISA. Production of IFN-γ and IL-4 from the mouse splenocytes stimulated with rOmp2b and rSodC was higher than those from the control group ($p < 0.01$) (Fig. 3A). However, production of IL-4 in the MBP-stimulated group was not detected ($p < 0.01$) (Fig. 3B). The two proteins have significant effect to induce the production of IFN-γ and IL-4 compared with MBP alone.

Analysis of Immune Cells in Splenocytes of Mice Immunized with rOmp2b and rSodC

The number of antigen-specific IgG-secreting B cells and IL-4- and IFN-γ-secreting T cells from the mice splenocytes at 28 day after immunization were analyzed by ELISpot. The number of antigen-specific IgG-secreting B cells and IFN-γ-secreting T cells were significantly increased in mice immunized with the recombinant proteins than in the negative control groups ($p < 0.05$) (Figs. 4A and 4B). However, the number of IL-4-secreting T cells was significantly

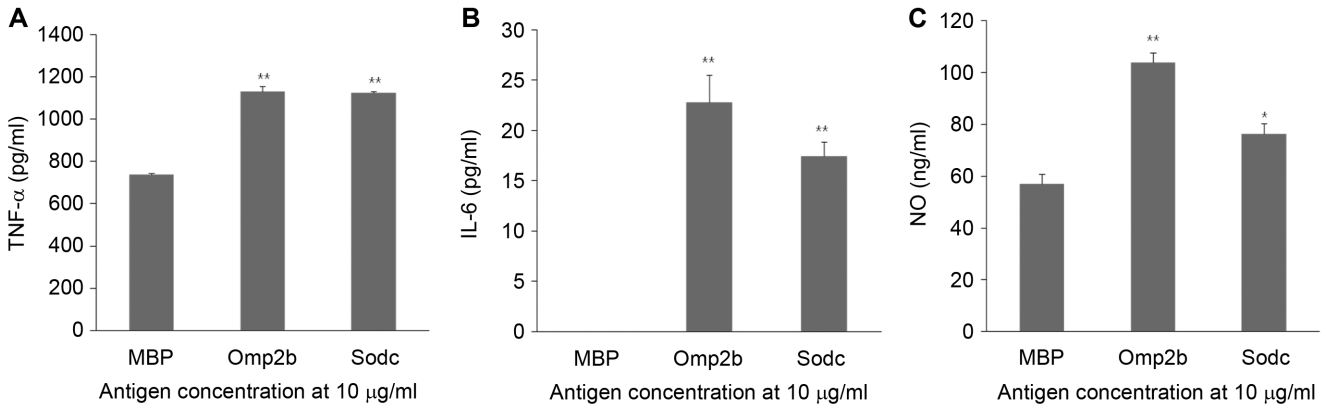

Fig. 2. Production of TNF-α (**A**), IL-6 (**B**), and NO (**C**) from murine macrophage RAW 264.7 cells stimulated with purified rOmp2b and rSodC of *Brucella abortus* at 24 h post stimulation (*, $p < 0.05$; **, $p < 0.01$).
Maltose-binding protein (MBP) was used as the vector control. The recombinant proteins were pretreated with polymyxin B to avoid possible contamination of LPS before stimulation of cells.

increased in only the Omp2b-immunized group compared with the non-immunized group (Fig. 4C).

Production of Antibodies in Mice Immunized with rOmp2b and rSodC

In the analysis of Ig production in mice by immunization with the two recombinant proteins, earlier and higher productions of IgG and IgM were observed in the groups immunized with Omp2b compared with the other groups (Fig. 5). Antibody production against SodC was slow in both IgG and IgM compared with the Omp2b group. However, the final amount of IgG production in the SodC group was similar to the Omp2b group, even though the response in the SodC group was slow and later (Fig. 5). The amount of IgM in the SodC group was significantly slower and lower than those in the Omp2b group (Fig. 5). The specificity of the IgM and IgG was confirmed by Western blot analysis with rOmp2b and rSodC (data not shown).

Discussion

B. abortus is an infectious disease of domestic cattle and wild animals, with serious zoonotic implication in humans [15]. In this study, we investigated the immunogenicities of recombinants Omp2b and SodC of *B. abortus* to discover new LPS-free proteins. The genes coding the proteins were

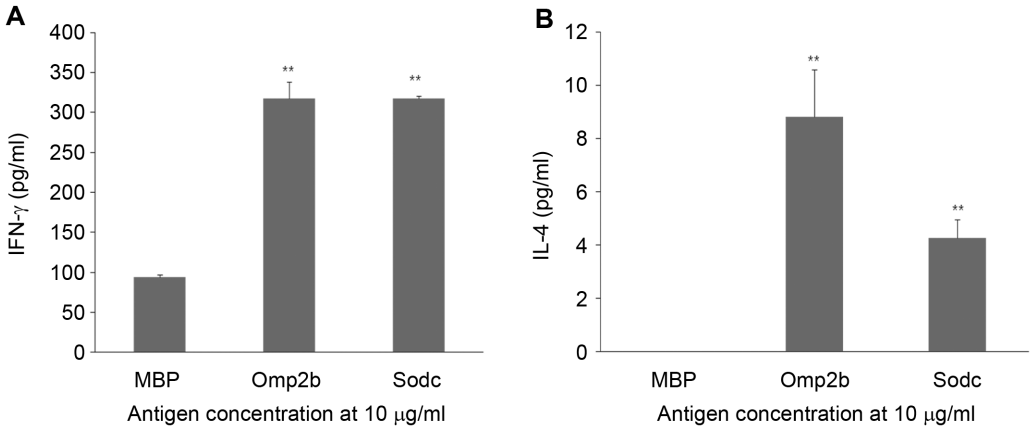

Fig. 3. Production of IFN-γ (**A**) and IL-4 (**B**) from naïve mice splenocytes stimulated with purified rOmp2b and rSodC at 24 h post stimulation (*, $p < 0.05$; **, $p < 0.01$).
Maltose-binding protein (MBP) was used as the vector control. The recombinant proteins were pretreated with polymyxin B to avoid possible contamination of LPS before stimulation of cells.

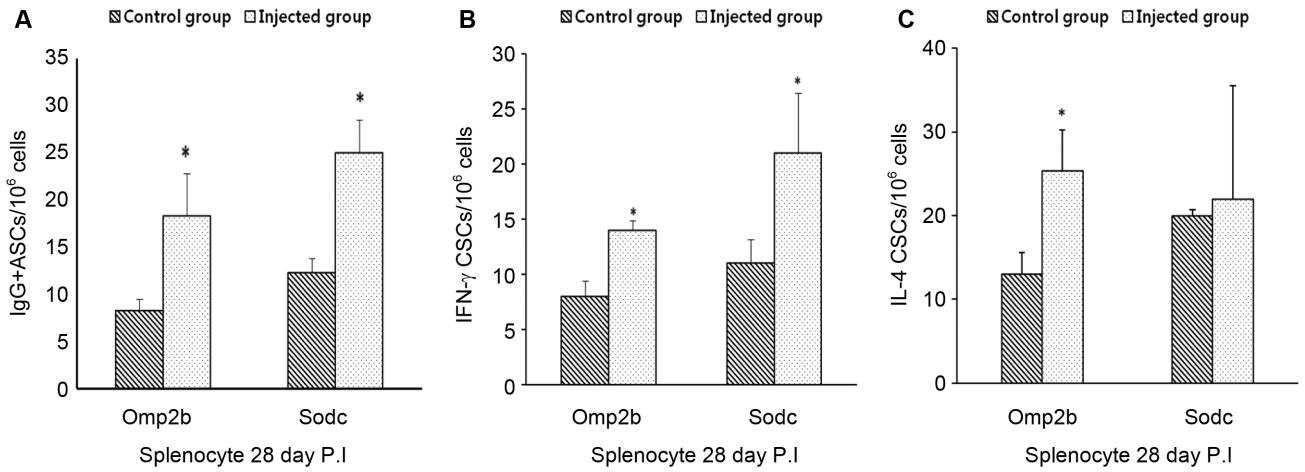

Fig. 4. Number of antibody-secreting cells (ASC) and cytokine-secreting cells (CSC) from splenocytes of mice immunized with rOmp2b and rSodC of *Brucella abortus* at 28 days after immunization.

Antigen-specific IgG (A), IFN-γ (B), and IL-4 (C) secreting cells were measured by ELISpot after restimulation of splenocytes from immunized mice with the homologous antigens. The recombinant proteins were pretreated with polymyxin B to avoid possible contamination of LPS before stimulation of cells.

cloned and recombinant proteins were expressed using the pMAL expression system. The purified MBP fusion recombinant proteins were identified by SDS-PAGE and Western blot assay at the expected size. However, unexpected proteins were also identified at 45 to 55 kDa size in both the rOmp2b and rSodC lanes. These were probably due to the protein of interest being in the wrong translational reading frame, and MBP-sized bands would be produced by translational termination at the first in-frame stop codon. Alternatively, MBP-sized breakdown products were produced as a result of the instability of target proteins [26].

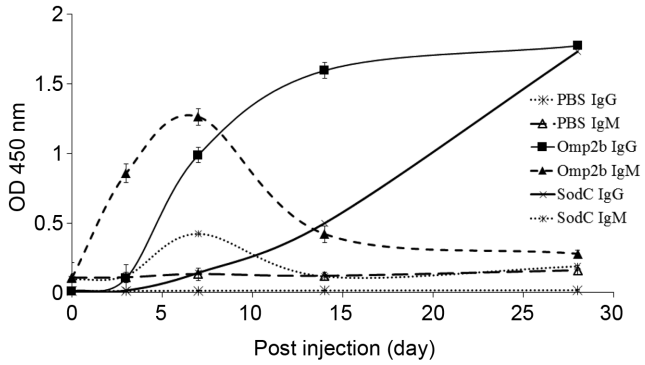

Fig. 5. Humoral immune responses induced in mice by immunization with rOmp2b and rSodC of *Brucella abortus*.

BALB/c mice (n = 5) were inoculated intraperitoneally with the purified recombinant proteins at two week interval. Serum samples were collected at different day points after the first immunization and antigen-specific IgG and IgM titers were measured by ELISA.

In the present study, rOmp2b and rSodC could elicit production of TNF-α and IL-6 from the RAW 264.7 cells after 24 h stimulation with recombinant proteins. These results are in agreement with previous studies that described that *B. abortus* can induce in a variety of cell types the release of pro-inflammatory cytokines such as IL-6 [31] and TNF-α [32]. In addition, heat-killed *B. abortus* induces the production of TNF-α, and IL-6 in human monocyte cell-line (THP-1) and mouse peritoneal macrophage of both C3H/HeJ C3HeN mice [14]. The weak production of NO in RAW 264.7 cells stimulated with rOmp2b and rSodC was in agreement with the result that live *B. abortus* is poor at inducing NO production by RAW 264.7 cells [30]. After rOmp2b and rSodC *in vitro* stimulation, spleen cells from naive mice produced high levels of IFN-γ and relatively low levels of IL-4. These results imply that rOmp2b and rSodC triggered T helper type 1 (Th1) cells to secrete IFN-γ, and could induce cell-mediated immunity. In addition, rOmp2b could elicit T helper type 2 cells to secrete IL-4, but lower than Th1 cell stimulation. These results agreed with the fact that *Brucella* Omps-inoculated mouse splenocytes showed high IFN-γ production [4].

Both rOmp2b- and rSodC-immunized mice produced antigen-specific IgM and IgG sufficiently. Interestingly, rOmp2b-specific antibodies were secreted more than the rSodC. Although rOmp2b has antigenicity in mouse model,

Omp2b as major antibody targets in animal species has been controversial in previous papers [7, 20]. Therefore, further studies about the antibody against Omp2b are needed. The rSodC antibodies production of immunized mice was in agreement with previous results [28]. Productions of antigen-specific IgG-secreting B cells and IFN-γ-secreting T cells in spleen collected from immunized mice with rOmp2b and rSodC were higher than from the negative mouse group. This result that rOmp2b and rSodC could induce memory T cells of the Th1 phenotype predominantly agreed with that *B. abortus* preferred Th1 stimulation [13]. Until now, the immunological properties of Omp2b recombinant proteins have been poorly studied [6]. Therefore, the present study results contribute to an understanding of the notable immunogenicity of Omp2b.

In conclusion, our recent study provides useful data about the immunogenicities of Omp2b and SodC of *B. abortus* in terms of various immune responses. These recombinant proteins showed immunogenicities *in vitro* and *in vivo*, especially in the mouse model. Consequently, it will lead to further investigation about Omp2b and SodC as new diagnostic candidates for *B. abortus*.

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