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Research Article

RESEARCH ARTICLE

Protective efficacy by various doses of a new brucellosis vaccine candidate based on *Salmonella* strains expressing *Brucella abortus* BSCP31, Omp3b and superoxide dismutase against brucellosis in murine model

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One sentence summary: This report suggests that intraperitoneal immunization with the mixture of the delivery strains can effectively protect mice against brucellosis.

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ABSTRACT

Brucella species are important etiological agents of zoonotic diseases. Attenuated *Salmonella* strains expressing *Brucella abortus* BCSP31, Omp3b and superoxide dismutase proteins were tested as vaccine candidates in this study. In order to determine the optimal dose for intraperitoneal (IP) inoculation required to obtain effective protection against brucellosis, mice were immunized with various doses of a mixture of the three vaccine strains. Fifty BALB/c mice were divided into five equal groups (groups A–E). Group A mice were intraperitoneally inoculated with 100 μL of sterile phosphate-buffered saline. Group B, C, D and E mice were intraperitoneally immunized with approximately 1.2×10^5 colony-forming units (CFU) mL^{-1} of *Salmonella* containing pMMP65 in 100 μL and with 1.2×10^4 CFU mL^{-1}, 1.2×10^5 CFU mL^{-1} and 1.2×10^6 CFU mL^{-1} of the mixture of the three strains in 100 μL, respectively. Serum IgG, tumor necrosis factor alpha and interferon gamma concentrations were significantly higher in group E than in groups A–D. Following challenge with *B. abortus* 544, the challenge strain was not detected in the spleen of any mouse from group E. Thus, IP immunization with 1.2×10^6 CFU mL^{-1} of the mixture of the three vaccine strains induced immune responses and provided effective protection against brucellosis in mice.

Keywords: *Brucella abortus*; brucellosis; immunization; public health; attenuated *Salmonella* Typhimurium

INTRODUCTION

Brucella species are important etiological agents of zoonotic diseases (Avila-Calderon *et al.* 2013; Olsen 2013). Brucellosis is caused by *Brucella* and leads to abortion in domestic ruminants, thereby making it a major concern. *Brucella* is a facultative intracellular pathogen, which invades and multiplies intracellularly in macrophages within the host system (Bowden *et al.* 1995; Baldwin and Goenka 2006). Humoral and cell-

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mediated immune responses can change the course of *Brucella* infection but cell-mediated immune responses are necessary to clear *Brucella* from the host organism (Schurig, Sriranganathan and Corbel 2002). In particular, the Th1-type immune response mediated by interferon gamma (IFN-γ) helps to combat *Brucella* infections (Zhan, Kelso and Cheers 1993). Strong Th2-type humoral immune responses, such as those involving serum IgG, are also necessary for protection against infection with intracellular pathogens (Roesler *et al.* 2006; Brumme *et al.* 2007; Adone *et al.* 2012) because systemic antibodies eliminate pathogens from the blood and promote their phagocytosis by opsonization (Mastroeni *et al.* 2001; Brumme *et al.* 2007). Many vaccines have been designed to combat brucellosis, but the commercially available vaccines comprise live and attenuated *Brucella* strains. Notably, these strains can revert into pathogenic strains and may also interfere with the diagnosis (Avila-Calderon *et al.* 2013; Olsen 2013). Because of these limitations, there is an urgent need for better and safer vaccines.

Live attenuated *Salmonella* strains have been proposed as a useful vector for carrying protective antigens of other pathogens (Hur and Lee 2011; Hur, Byeon and Lee 2014). The gastrointestinal tract is the entry portal common for both *Brucella* and *Salmonella*. Furthermore, most *Salmonella* vaccine strains retain a limited ability to invade the internal lymphoid organs, and thus, adequate induction of cell-mediated immunity can be expected (Martinoli, Chiavelli and Rescigno 2007).

Recombinant proteins of *Brucella abortus* may be suitable vaccine candidates because they can never cause infections and the adjuvant or immunization route employed to obtain antigen-specific immune responses can also be modified (Zhao *et al.* 2009). Reportedly, a protein purified from strain 19 designated as the cell surface 31-kDa protein (or BCSP31) can serve as a protective subunit vaccine in rodents (Tabatabai and Deyoe 1983; Tabatabai, Deyoe and Patterson 1989). BCSP31 is either a glycosylated protein or may have a tight association with a *Brucella* polysaccharide moiety (Bricker *et al.* 1988). Outer membrane proteins (Omps) are the protective antigens of *Brucella* species (Verstreate *et al.* 1982). Omp3b, also known as Omp22, belongs to group 3 of the *Brucella* Omps (Vizcaino *et al.* 2004), which is a highly conserved family comprising up to seven members, including some of the most abundant and immunogenic *Brucella* proteins (Cloeckaert *et al.* 2002). The *sodC* gene encodes a Cu/Zn superoxide dismutase (SOD), which catalyzes the dismutation of oxygen radicals (Bricker *et al.* 1990). SOD might act as a virulence factor by scavenging harmful oxygen radicals produced during phagocytosis by macrophages (Tabatabai and Pugh 1994).

In a previous study (Kim *et al.* 2016), *Salmonella* Typhimurium strains expressing the BCSP31, Omp3b and SOD proteins of *B. abortus* were used as vaccine candidates to determine the optimal inoculation route in a murine model. Intraperitoneal (IP) immunization with a mixture of the *Salmonella* strains induced adequate immune responses and provided effective protection against brucellosis in mice. The present study aimed to determine the optimal dose for IP immunization using a mixture of the *Salmonella* strains to achieve protection against brucellosis in a murine model. The humoral immune responses and protective efficacy obtained after IP inoculation with various doses of the mixture of three vaccine strains were studied in mice. Mice intraperitoneally inoculated with the *Salmonella*-based *Brucella* vaccine candidate exhibited robust humoral and cell-mediated immune responses, thereby yielding effective protection against virulent *B. abortus*.

MATERIALS AND METHODS

Animals and ethics statement

Fifty 5-week-old female BALB/c mice were distributed into five equal groups (groups A–E). All mice were maintained according to the previously described method (Hur and Lee 2011). All animal experiments performed in this study received ethical approval (CBU 2015-052) from the Chonbuk National University Animal Ethics Committee in accordance with the guidelines of the Korean council on Animal Care.

Bacterial strains, plasmids and growth conditions

Attenuated *Salmonella* vector-mediated strains for delivering the recombinant BCSP31, Omp3b and SOD antigens, which have been constructed and used as vaccine strains in the previous study, were used as vaccine candidates in the present study (Table 1) (Kim *et al.* 2016). *Brucella abortus* strain 544 (HJL254) was used as the virulent challenge strain (Lee *et al.* 2014). *Escherichia coli* BL21(DE3) bacteria were used as host cells for overexpressing the recombinant BCSP31, Omp3b and SOD antigens. pET28a and pET32a plasmids were used as vectors for overexpressing the individual antigens. Except for *B. abortus* strain 544, all strains were grown in Luria-Bertani broth (LB; Becton, Dickinson and Company, Sparks, MD, USA) or on LB agar. *Brucella abortus* strain 544 was grown on Brucella agar (Becton, Dickinson and Company, Sparks, MD, USA). All strains were cultured at 37°C.

Preparation of individual recombinant proteins

The overexpressed recombinant proteins comprising BCSP31 from HJL906, Omp31 from HJL904 and SOD from HJL908 have been conducted and used in a previous study (Kim *et al.* 2016). The recombinant proteins were also used as coating antigens for an enzyme-linked immunosorbent assay (ELISA) and as re-stimulating proteins for splenocytes in the present study.

Preparation of *Salmonella* delivery strain formulation

The previously described vaccine candidate strains comprising HJL228, HJL219 and HJL213 were also used as vaccine candidates in the present study (Table 1) (Kim *et al.* 2016).

Immunization of mice and sample collection

Fifty 5-week-old female BALB/c mice were divided into five equal groups (n = 10 mice per group). All mice were immunized at 6 weeks of age. Group A mice were intraperitoneally inoculated with 100 μL of sterile phosphate-buffered saline (PBS) as the control. Group B mice were intraperitoneally immunized with approximately 1.2×10^5 colony-forming units (CFU) mL^{-1} of HJL229 in 100 μL as the vector control. Groups C, D and E mice were intraperitoneally immunized with approximately 1.2×10^4 CFU mL^{-1}, 1.2×10^5 CFU mL^{-1} and 1.2×10^6 CFU mL^{-1} of a mixture (equal CFU counts of each strain) of the three delivery strains in 100 μL, respectively. Blood samples were collected before immunization [0 weeks post-immunization (WPI)] and again at 3 WPI to evaluate the serum IgG levels. All samples were stored at −70°C until use.

Immune response measurement by ELISA

Standard ELISA was performed to evaluate the immune responses against the BCSP31, Omp3b and SOD antigens in serum

Table 1. Bacterial strains and plasmids used in this study.

Strain/plasmid		Description	Source
Strains			
Escherichia coli			
	BL21(DE3)	F^-, *ompT*, *hsd*S_B(r_B^-,m_B^-), *dcm*, *gal*, λ(DE3)	Lab stock
	HJL206	BL21 with pET32a-BCSP31	This study
	HJL204	BL21 with pET28a-Omp3b	This study
	HJL208	BL21 with pET28a-SOD	This study
Salmonella Typhimurium			
	JOL401	*Salmonella* Typhimurium wild type	Lab stock
	JOL911	*S. Typhimurium* JOL401derivave Δ*lon*Δ*cpxR*	Lab stock
	JOL912	S. Typhimurium JOL911 derivative Δ*asd*	[20]
	HJL229	JOL912 with pMMP65	[21]
	HJL228	JOL912 with pMMP65-BCSP31	[21]
	HJL219	JOL912 with pMMP65-Omp3b	[21]
	HJL213	JOL912 with pMMP65-SOD	[21]
Brucella abortus			
	Biotype 1	*B. abortus* biotype 1 isolate from cow in Korea	Lab stock
	Strain 544	*Brucella abortus* strain 544 (ATCC23448)	[20]
Plasmids			
	pET28a	IPTG-inducible expression vector; Km^r	Novagen
	pET32a	IPTG-inducible expression vector; Amp^r	Novagen
	pMMP65	Asd^+, pBR *ori*, β-lactamase signal sequence-based periplasmic secretion plasmid, 6× His tag	[21]

samples obtained from mice according to the previously described method (Kim *et al.* 2016). The ELISA results are expressed as mean ± standard deviation.

Collection of cytokines from splenocytes

At 3 WPI, five mice from each group were sacrificed and their spleens were aseptically removed. Single-cell suspensions were obtained by homogenizing the spleens. Splenocytes were collected by centrifugation at 1000 rpm for 5 min. Red blood cells were lysed with 0.9% NH_4Cl and then subjected to three washes with complete RPMI medium to remove NH_4Cl. Spleen cells were checked for viability using the Trypan blue exclusion method and were seeded into 24-well tissue culture plates at 2×10^6 cells per well (Velikovsky *et al.* 2003). Splenocytes were stimulated *in vitro* using BCSP31, Omp3b and SOD antigens (4 μg $well^{-1}$), with concanavalin A (0.5 μg $well^{-1}$) as the positive control or media as the unstimulated control, and incubated at 37°C under 5% CO_2 and 95% humidity (Adone *et al.* 2012). Supernatants were collected from the media after restimulation for 48 h and were stored at −70°C until use for quantifying cytokines (Adone *et al.* 2012).

Measurement of cytokines by ELISA

ELISA was employed to measure the concentrations of cytokines, including IFN-γ and tumor necrosis factor alpha (TNF-α), in the supernatants using a mouse cytokine ELISA Ready-SET-GO reagent set according to the manufacturer's instructions (eBioscience Inc., San Diego, CA, USA).

Challenge experiments

Strain 544 was prepared for use in the challenge experiments. Briefly, the strain was grown in *Brucella* broth at 37°C for 24 h and then resuspended to obtain approximately 1×10^5 CFU mL^{-1}. Five mice from each group were intraperitoneally challenged at 3 WPI with 100 μL of the challenge strain. Two weeks after the challenge, spleen weights were measured and the numbers of viable strain 544 cells in the spleens were counted (Kim *et al.* 2016). Vaccine and challenge strains were confirmed by polymerase chain reaction (PCR) using the *B. abortus*-specific primer (5′-GACGAACGGAATTTTTCCAATCCC-3′) and IS*711*-specific primer (5′-TGCCGATCACTTAAGGGCCTTCAT-3′) described in previous study (Bricker and Halling 1994, 1995).

Statistical analysis

CFU data were normalized by log transformation and evaluated by one-way analysis of variance, followed by Dunnett's post-hoc test using GraphPad software (GraphPad Software, Inc., La Jolla, CA, USA). The cellular responses were compared between the groups using one-way analysis of variance and Tukey's Multiple Comparisons test. The analyses were performed with SPSS version 16.0 software (SPSS, Chicago, IL, USA). The antibody responses were compared between the groups using two-way matching by rows (RM) analysis of variance and matching by rows test. The analyses were performed with SPSS version 16.0 software (SPSS, Chicago, IL, USA).

RESULTS

Humoral immune responses in the vaccinated mice

The antibody responses against each antigen in the sera are shown in Fig. 1. Serum IgG concentrations against all antigens were significantly higher in groups D and E than in the control group ($P \leq 0.05$). In addition, serum IgG concentrations against the individual antigens were significantly higher in group E than in groups B, C and D ($P \leq 0.05$) (Fig. 1).

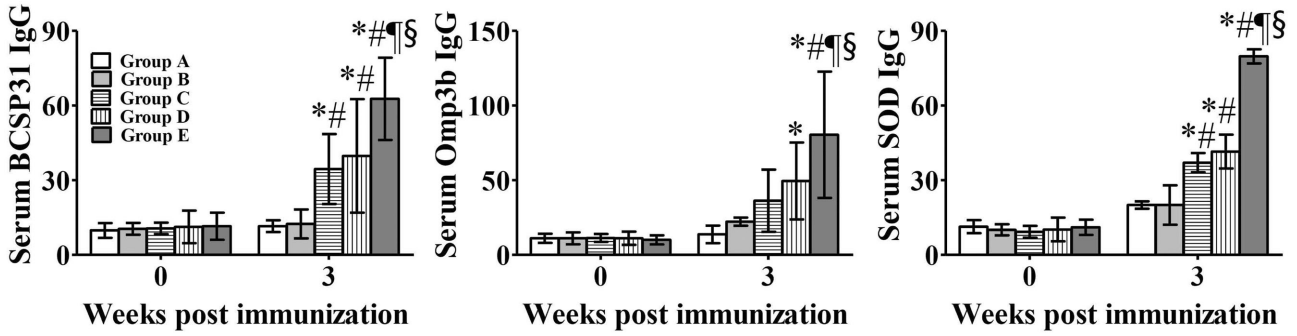

Figure 1. Antibody responses (ng mL^{-1}) against BCSP31, Omp3b and SOD antigens. Group A mice were inoculated with sterile phosphate-buffered saline as a control. Group B mice were immunized with *Salmonella Typhimurium* containing pMMP65 only as a vector control. Group C, D and E mice were inoculated with approximately 1.2×10^4 CFU mL^{-1}, 1.2×10^5 CFU mL^{-1} and 1.2×10^6 CFU mL^{-1} of the mixture of the three delivery strains in 100 μL, respectively. Data represent the means of all mice in each group, and error bars represent the standard deviations. $*$ $P < 0.05$ vs Group A; # $P < 0.05$ vs Group B; ¶ $P < 0.05$ vs Group C; § $P < 0.05$ vs Group D.

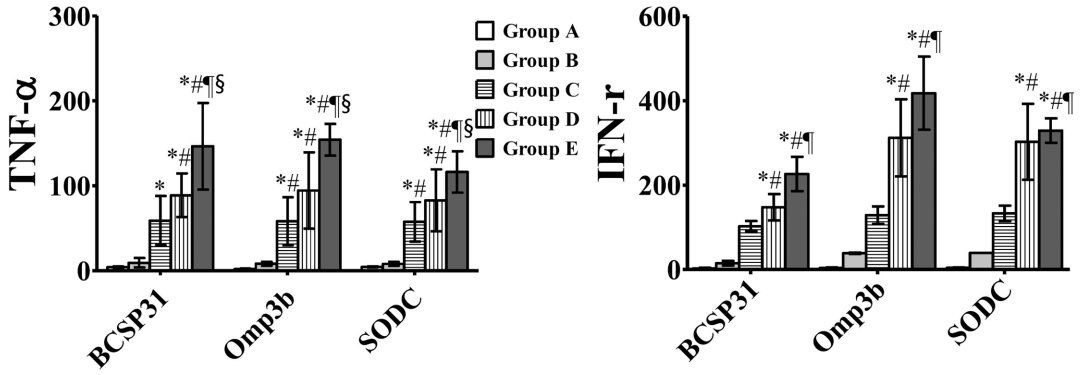

Figure 2. Cytokine concentrations (pg mL^{-1}) in splenocytes at 3 WPI. Groups A, B, C, D and E are indicated as in Fig. 1. Data represent the means of all mice in each group, and error bars indicate the standard deviations. $*$ $P < 0.05$ vs Group A; # $P < 0.05$ vs Group B; ¶ $P < 0.05$ vs Group C; § $P < 0.05$ vs Group D.

Cytokine analysis

TNF-α and IFN-γ concentrations in splenocytes in response to BCSP31, Omp31 and SOD after re-stimulation with the individual antigens were measured using ELISA at 3 WPI. TNF-α concentrations were significantly higher in groups C, D and E than in groups A and B ($P < 0.05$) (Fig. 2). In addition, compared with group C, TNF-α concentrations in response to all antigens in groups D and E were significantly increased. IFN-γ concentrations in response to all antigens were also significantly higher in groups D and E than in groups A and B. In addition, compared with group C, IFN-γ concentrations in response to all antigens were significantly increased in group E ($P < 0.05$) (Fig. 2).

Protection of mice against virulent challenge

Five mice from each group were intraperitoneally challenged with approximately 4×10^4 CFU of the challenge strain at 3 WPI. Fourteen days after the challenge, the infection level was evaluated by determining the CFU counts in the spleens. As shown in Fig. 3, group D and E mice exhibited significantly greater protection than group A, B and C mice. The challenge strain was isolated from all group A, B and C mice. By contrast, the challenge strain was isolated from only two-fifth of the group D mice and wild-type *Brucella abortus* was not detected in the spleen of any group E mouse.

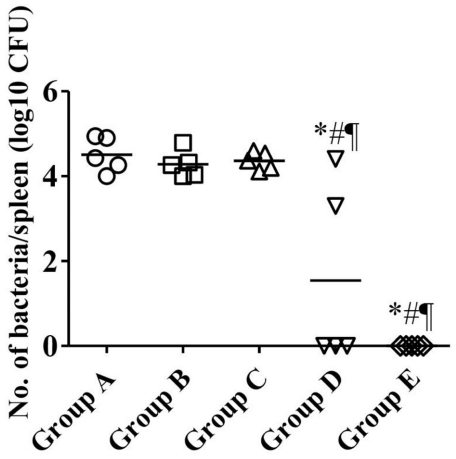

Figure 3. Bacterial proliferation in the spleens of mice challenged with wild-type *Brucella abortus* strain 544. Groups A, B, C, D and E are indicated as in Fig. 1. All mice in each group were intraperitoneally challenged with 1×10^4 CFU of virulent wild-type *B. abortus* 544 at 3 WPI. The numbers of viable bacteria recovered from the spleens of mice on day 14 post-challenge are shown. The lines represent the means (n = 5 mice per group). $*$ $P < 0.05$ vs Group A; # $P < 0.05$ vs Group B; ¶ $P < 0.05$ vs Group C; § $P < 0.05$ vs Group D.

DISCUSSION

Brucellosis is a globally distributed zoonotic disease, which causes severe detrimental effects to health and economic losses in endemic areas (Carvalho Neta *et al.* 2010). *Brucella abortus* is a facultative intracellular bacterium that can invade hosts through the mucosa and survive inside phagocytes by evading the endocytic pathway. The gastrointestinal tract is one of the major entry portals for *B. abortus* (Gorvel, Moreno and Moriyon 2009). The resistance of hosts to *Brucella* infection mainly depends on mucosal immunity and acquired cell-mediated immunity. The two main components of the host protective systemic immune responses are $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes secreting IFN-γ and the lysis of *Brucella*-infected cells (Paranavitana *et al.* 2005).

Attenuated *Salmonella* has many advantages as a vector for carrying protective *Brucella* antigens; these include the delivery of the antigen to the host, a simple preparation procedure and convenient inoculation (Chen and Schifferli 2003; Hur, Byeon and Lee 2014). In the present study, attenuated *Salmonella* Typhimurium host cells and the pMMP65 plasmid delivery system were used to deliver *B. abortus* BCSP31, Omp3b and SOD antigens, as described in a previous study (Kim *et al.* 2016). Mice were immunized with three different doses of a mixture of the *Salmonella*-based *Brucella* vaccine candidates. Compared with control group mice, group D (immunized with approximately 1×10^5 CFU) and E (immunized with approximately 1×10^6 CFU) mice produced significantly higher amounts of serum IgG in response to the three antigens. In addition, serum IgG concentrations in group E were significantly higher than those in groups B (immunized with the *Salmonella* containing vector only), C (immunized with approximately 1×10^4 CFU) and D (immunized with approximately 1×10^5 CFU). However, serum IgG concentrations in group B were only slightly higher than those in the control group. Thus, IP immunization with approximately 1.0×10^6 CFU significantly induced a protective IgG response.

The Th1-type immune response is considered to be desirable for protection against brucellosis (Zhan, Liu and Cheers 1996; Baldwin and Goenka 2006). The role of TNF-α in controlling *Brucella* infection in mice is important because of the activation of macrophages, which are responsible for killing *Brucella* in the absence of IFN-γ (Zhan, Liu and Cheers 1996). IFN-γ plays a crucial role in combating *Brucella* infection because of its ability to activate the antibacterial functions of infected macrophages (Paranavitana *et al.* 2005; Baldwin and Goenka 2006). In this study, we investigated the cell-mediated immune response generated by IP immunization with *Salmonella* vaccine candidates expressing *B. abortus* BSCP31, Omp3b and SOD antigens. Cytokine concentrations were evaluated in the supernatants obtained from the splenocytes of mice immunized with the vaccine candidates after re-stimulation with the heat-inactivated *B. abortus* whole antigens. TNF-α and IFN-γ concentrations were significantly higher in groups D and E than in groups A (immunization with PBS) and B (immunization with *Salmonella* containing pBP244 only). These results indicated that IP immunization with approximately 1×10^5 CFU of the mixture of *Salmonella* vaccine candidates could induce strong Th1-type immune responses. Furthermore, significantly higher IFN-γ and TNF-α concentrations were found in the culture supernatants of splenocytes obtained from group E mice than in those obtained from group C. Thus, it is logical to conclude that IFN-γ produced by IP immunization with approximately 1×10^6 CFU of the mixture might have contributed to the increased protection in this group.

A major difficulty that has prevented the development of a vaccine against *Brucella* is the lack of the definite proof of efficient protection. We found that group C, D and E mice exhibited significantly greater protection than group A and B mice, demonstrating that IP immunization with the *Salmonella* vaccine candidates could protect mice from infection with the virulent *B. abortus* strain 544. Furthermore, group E mice exhibited the best protection against infection with the virulent *B. abortus* strain 544. It is possible that IP immunization with approximately 1×10^6 CFU of the *Salmonella* mixture made larger amounts of antigens available to antigen-presenting cells, which induced the strongest mucosal and cell-mediated immune responses.

In summary, the results of this study demonstrated that IP immunization with approximately 1×10^6 CFU of *Salmonella*-based *Brucella* vaccine induced robust antibody and cell-mediated immune responses in mice. The vaccine conferred protection against infection with *B. abortus*, which was comparable to that by immunization with approximately 1×10^6 CFU of the *Salmonella*-based *Brucella* vaccine. Thus, we suggest that IP immunization with approximately 1×10^6 CFU of the *Salmonella* mixture is a good candidate for vaccine development to combat brucellosis.

FUNDING

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Conflict of interest. None declared.

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