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Immunization of BALB/c mice with a combination of four recombinant *Brucella abortus* proteins, AspC, Dps, InpB and Ndk, confers a marked protection against a virulent strain of *Brucella abortus*

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ABSTRACT

In this study, we assessed the protective efficacy of single subunit vaccines, encoded by the *B. abortus* 544 genes *aspC*, *dps*, *yaeC* and *inpB*, against *B. abortus* infection in mice. First, immunization with these antigens, with the exception of the YaeC protein, was found to elicit both humoral and cellular immune responses with IgG2a being dominant over IgG1. In addition, a massive production of IFN-γ but lower degree of IL-10 was observed, suggesting that all three antigens were able to induce predominantly cell-mediated immunity in response to *B. abortus* infection. Further investigation of a combined subunit vaccine (CSV) consisting of purified AspC, Dps, InpB and Ndk proteins showed a superior protective effect in mice against brucellosis. The intraperitoneal injection of this combination was shown to induce a remarkable production of IFN-γ and IL-2, which occurred in conjunction with an increase of blood $CD4^+$ and $CD8^+$ T cell proportions. In addition, the higher titer of IgG2a compared to IgG1 elicited by this CSV was obtained, suggesting that this CSV induced a typical T-helper-1-dominated immune response *in vivo*. Furthermore, the protection level induced by this combination was significantly higher than that induced by single antigens and was not significantly different compared to a group immunized with a live attenuated vaccine (RB51). Altogether, our findings suggest that the combination of different immunogenic antigens could be a useful approach for the development of a new, effective and safe brucellosis vaccine that can replace current vaccine strains.

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1. Introduction

Brucella species are intracellular facultative zoonotic pathogens that causes brucellosis, a major disease in both humans and domestic animals with an increasing public health burden. Brucellosis is considered to be one of the most important zoonoses worldwide, which makes the eradication, control and prevention of brucellosis in animals essential for the eradication of the disease in humans [1]. Since *Brucella* is an intracellular pathogen, the host resistance primarily depends on acquired cell-mediated immunity (CMI), and live attenuated vaccines that can stimulate strong CMI responses are usually effective against brucellosis [2]. Although vaccination is the most economical control measure, current available live attenuated vaccines, such as *B. abortus* S19 (smooth) and strain RB51 (rough), are not sufficient by themselves to eliminate brucellosis in any host species [3]. Furthermore, live attenuated vaccines also possess several disadvantages, including the abortion and excretion of the vaccine strain in milk [4,5]. Therefore, different approaches are necessary for the development of efficient, safe and effective vaccines for the control of the disease, and a promising candidate addressing these concerns is immunization with recombinant proteins.

To date, numerous studies have reported the protective effect of different recombinant *Brucella* proteins against virulent *Brucella* infections, including the L7/L12 ribosomal protein [6], Cu-Zn superoxide dismutase [7], a 22.9-kDa protein [8], lumazine synthase [9], outer membrane protein Omp31 [10], outer membrane proteins Omp16 and Omp19 [11], and nucleoside diphosphate

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kinase [5]. However, the administration of single antigens could not induce an efficient immune response to successfully protect mice from infection. Thus, a combination of different antigens in this context is expected to confer a higher protective effect than single antigens.

According to proteomic analysis studies [12,13], the invasion protein B (InpB), DNA starvation/stationary phase protection protein (Dps) and aspartate aminotransferase (AspC) were recognized as potential immunogenic antigens of *Brucella* during infection. Another study showed that InpB is required for bacterial effector protein delivery into target eukaryotic cells in *Salmonella* and may contribute to the reproductive pathology of *Brucella* [14]. On the other hand, Dps was found to form extremely stable complexes with DNA during starvation to develop starvation-induced resistance to hydrogen peroxide, an agent that can cause oxidative damage to DNA [15]. Moreover, AspC is responsible for the synthesis of aspartate and is essential for *Brucella* replication in HeLa cells [16]. Furthermore, YaeC belongs to a lipoprotein group that plays an important role in the regulation of *Brucella*-host interactions, cell invasion and intracellular survival [17]. Thus, in this study, we assessed the ability of purified recombinant proteins, including InpB, AspC, Dps and nucleoside diphosphate kinase (Ndk, a proven immunogenic antigen) [5], to prevent brucellosis when administered to BALB/c mice alone or in combination.

2. Materials and methods

2.1. Bacterial strains and growth condition

A smooth, virulent *B. abortus* 544 biovar 1 strain was kindly provided by the Animal, Plant and Fisheries Quarantine and Inspection Agency of Korea, and *Escherichia* (*E.*) *coli* DH5α cells were purchased from Invitrogen (MA, USA). *B. abortus* was cultured in Brucella broth (BD Biosciences, USA) at 37 °C until stationary phase. *E. coli* DH5α was used to produce the necessary plasmid constructs. *E. coli* cultures were grown at 37 °C in LB broth or agar supplemented with 100 μg/mL of ampicillin (Sigma, Missouri, USA).

2.2. Preparation of plasmids and purified proteins

The cloning of *B. abortus aspC*, *inpB*, *yaeC*, *dps* and *ndk* genes and purified protein were obtained following the methods of a previous study [5]. Briefly, the respective primer pairs were used to clone full-length *B. abortus* genes into pMAL vectors. The subsequent production of recombinant proteins was induced in LB broth supplemented with 100 μg/mL ampicillin, 0.2% glucose and different IPTG concentrations at 37 °C (Table 5). After centrifugation at 3000×*g* for 10 min, *E. coli* cells were resuspended in 25 mL of column buffer and then were subjected to three freeze-thaw cycles at −70 and 4 °C, respectively, prior to sonication (Bandelin electronic, Germany) at 10,000 Hz on ice. After centrifugation at 5000×*g* for 20 min, the supernatants were carefully collected and purified with maltose resin column (Bio-Rad, CA, USA) using 10 mM maltose in column buffer as the elution solution, according to the manufacturer's instructions.

2.3. SDS-PAGE and western blot assays

The lysates of induced cells and the purified proteins were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot assays as previously described [5]. *B. abortus*-negative and positive mouse sera were used as primary antibodies.

2.4. Mice immunization and bacterial challenge

Following the protocol of a previous study [5], to evaluate the protective effect of single antigens, thirty-five six-week-old female BALB/c mice (Japan SLC, Japan) were distributed into seven groups. Each mouse was immunized intraperitoneally with 10 μg of either purified Maltose-binding protein (MBP), *B. abortus* proteins or PBS in 100 μL incomplete Freund's adjuvant (IFA) (Sigma, Missouri, USA) at weeks 0, 2, 4 and 6. At week 8, serum samples were obtained from the tail vein and mice were intraperitoneally challenged with approximately 5×10^5 CFU of *B. abortus* in 100 μL PBS.

To assess the protective effect of CSV, twenty mice were randomly distributed into four groups. Each mouse was immunized intraperitoneally with 20 μg of four *B. abortus* proteins (rDps, rNdk, rAspC and rInpB at a ratio of 1:1:1:1) or PBS in 100 μL incomplete Freund's adjuvant (IFA) (Sigma, Missouri, USA) at weeks 0, 2, 4 and 6. Mice used as positive controls were intraperitoneally injected with 5×10^6 CFU of RB51 in 100 μL PBS at day 0 [2]. At week 8, blood samples were obtained from the tail vein and mice were intraperitoneally challenged with approximately 5×10^5 CFU of *B. abortus* in 100 μL PBS.

2.5. Quantitation of peripheral blood $CD4^+$ and $CD8^+$ T cells

Cellular populations in peripheral blood from the experimental mice were examined as previously described [28]. At the indicated times, 100 μL of blood collected from each mouse was added to 75 μL of FITC-conjugated rat anti-mouse CD4 and PE-conjugated rat anti-mouse CD8 monoclonal antibodies (Santa Cruz Biotechnology, Texas, USA) and incubated at room temperature for 30 min in the dark. One milliliter of red blood cell lysis buffer was added to each tube and incubated at 37 °C for 10 min. The reactions were terminated by the addition of 2 mL of PBS. After centrifugation (380 ×*g* at 4 °C for 5 min), the pelleted cells were washed twice with PBS, resuspended in 0.5 mL of PBS buffer and analyzed using a FACS Calibur flow cytometer (BD Biosciences, CA, USA).

2.6. ELISA and cytokine quantitation

The serum titers of IgG1 and IgG2a isotypes specific for recombinant proteins were determined by ELISA as previously described [2,11]. The cutoff 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 that yielded an OD two fold higher than the cutoff value.

The levels of IL-10, IL-12p70 and INF-γ in sera were determined using a cytometric bead array (BD CBA Mouse Inflammation Kit, CA, USA).

2.7. Protective experiments

The protective study followed previously described methods [2,5] with a few modifications. Four weeks post-infection, mice were scarified, and the spleens were removed, weighed, and homogenized in PBS. The homogenates were serially diluted 10-fold with PBS and plated on Brucella agar, which were then incubated for 3 days at 37 °C. The $\log_{10}$ CFUs in spleens were determined. Log protection was calculated as the mean $\log_{10}$ CFU of the PBS group minus the $\log_{10}$ CFU of the experimental group. All of the performed procedures were approved by the Animal Ethical Committee of Gyeongsang National University (Authorization Number GNU-170331-M0017).

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