Resistance to Brucella abortus 544 challenge in BALB/c mice by inoculation of highly immunogenic multiple B. abortus antigens

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Abstract: In this study, we examined the protective immunity of a combination of seven Brucella abortus recombinant proteins; superoxide dismutase (rSodC), riboflavin synthase subunit beta (rRibH), 50S ribosomal protein (50s rL7/L12), nucleoside diphosphate kinase (rNdk), malate dehydrogenase (rMDH), arginase (rRocF), and elongation factor (rTsf) cloned in a pMal vector system and expressed in DH5α. Mice groups were immunized thrice with a combined subunit vaccine (CSV-7) at 0, 2, and 5 weeks and subsequently challenged with B. abortus at 5 × 10^4 CFU at 6 weeks. At four weeks post-infection, the mice were sacrificed and the bacterial burden in their spleens was quantified. Results revealed bacterial log reductions of 0.63 and 0.34 in comparison to PBS and maltose-binding protein (MBP), respectively. Cytokine profiling revealed a marked increase in IFN-γ (interferon-gamma), MCP-1 (macrophage chemoattractant protein-1) and IL-6 (interleukin 6) cytokines at 5-weeks post-immunization. On the other hand, only TNF was heightened at 7-weeks post-immunization. In general, this cytokine profile is consistently reflective of a Th1 immune response, which is beneficial for host immunoresistance.

Key words: B. abortus, cytokines, protection, recombinant proteins, immunization

INTRODUCTION

Brucella is a non-spore forming, non-motile, Gram-negative coccobacillus and is the main the causative agent of brucellosis [10]. Brucella infections affect a variety of domestic and wild animals, and more importantly cattle and small ruminants in particular [5]. In humans, it leads to a chronic and debilitating illness called Malta fever manifesting as intermittent fevers, anorexia, headache, and myalgia [12]. The intracellular nature of this bacteria is a major challenge in its treatment protocol, as there are only very limited antibiotics to combat the infection. The most effective control measure remains vaccination of host animals, however, this poses an additional challenge since current vaccines available for animals have their own drawbacks. In addition, licensed vaccines for humans are still unavailable, thus the need to optimize vaccine protocols in animals to prevent transmission in humans [9]. Previously, we characterized the immunogenicity and protective effect of eight B. abortus recombinant proteins namely rSodC, rRibH, 50S rL7/L12, rDps, rNdk, rMDH, rRocF, and rTsf. The current B. abortus recombinant proteins were cloned into a pMal vector system [7, 11] and inoculated into BALB/c mice to determine whether they can induce comparatively better protection among other recombinant protein combinations. Based on those results, we eliminated antigen rDps and increased the individual concentrations of each of the other seven proteins.

Results revealed a lower protective efficacy of a log10 reduction of 0.63 despite the presence of higher concentrations of individual antigens. However, cytokine analysis results were still consistently reflective of a cell-mediated immune response enhancing IFN-γ and TNF, which are key elements in the eradication of a Brucella infection. To enhance the protective effect, the obtained combination of individual antigens can still be considered for further modification.

MATERIALS AND METHODS

Bacterial strains and growth conditions

B. abortus 544 (ATCC 23448), a smooth wild-type strain was cultured in Brucella agar and grown in Brucella broth (Becton Dickinson, MD). The B. abortus 544 were cultured...
Expression and purification of recombinant protein

The seven genes under investigation in this study (SodC, ribH, L7/L12, Ndk, Mdh, rocF and tsf) were cloned and expressed through a pMal vector system as previously described. The system’s expression and immunogenicity were characterized in our previous study [2]. Briefly, each of the recombinant proteins was expressed in E. coli DH5α induced with different concentrations of IPTG (0.1–0.5 mM) along with 100 µg/mL of ampicillin and 0.2% glucose for 4 h at 37°C. The cells were then harvested at 5,000 × g for 15 min while submerged on ice. The supernatant was centrifuged at 45,000 × g for 10 min. The pellet was collected and suspended in column buffer (20 mM Tris HCl, 1 mM EDTA, 200 mM NaCl, 4% glycerol, pH 7.4) in a total volume of 25 mL. Then, the proteins were characterized by performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting as described previously [2]. After purification, recombinant proteins were boiled for 5 min in 2× SDS buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.125 M Tris HCl, and 0.004% bromophenol blue, pH 6.8). Proteins were transferred onto Immobilon-P membranes (Millipore, USA) after electrophoresis by using a transfer buffer (25 mM Tris, 20% methanol, and 190 mM glycine, pH 8.3) and an ATTO semi-dry transfer machine (WSE-7210, Japan) which ran for 30 min. Subsequently, membranes were blocked with 5% skim milk (Difco, USA) and washed with 0.05% Tween-20 (PBS-T). Overnight, the membranes were incubated with virulent Brucella-infected mouse sera or uninfected mouse sera at a dilution of 1:10000. The membranes were then washed and further incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody for an hour at a dilution of (1:1000) (Sigma Aldrich). Finally, the membranes were washed and analyzed with the aid of a molecular imager (Chemidoc XR System Machine, Bio-Rad Laboratories).

Mice vaccination and B. abortus 544 challenge

Seven purified B. abortus recombinant proteins (rSodC, rRibH, 50s rL7/L12, rNdk, rMDH, rRocF, and rTsf) were combined to form combined subunit vaccine (CSV-7) and administered to groups of mice to evaluate its protective efficacy. Sixteen female BALB/c mice at ten-weeks-old were assigned to four groups. The vaccinated groups were inoculated with a 1:1 volume ratio of incomplete Freund’s adjuvant (Sigma, USA) and 28.5 µg of each recombinant protein in a final volume of 300 µL. The total concentration of the proteins was 200 µg. The control groups included groups injected with PBS (negative control) or maltose-binding protein (MBP, 100 µg) (positive control). A group of mice was inoculated with RB51. B. abortus strain RB51 vaccine (RB51), rough mutant from virulent strain B. abortus 2308, (1 × 10⁶ CFU) at 0, 2, and 5 weeks. Serum was collected from mouse tails at weeks 5 and 7 after the first vaccination. The mice were infected with B. abortus 544 (5 ×10⁶) in 100 µL after a week of the last vaccination. The mice were sacrificed one month after infection by cervical dislocation.

Cytokine Quantification

Serum cytokine levels were analyzed at 5 and 7 weeks after the first vaccination by using a cytometric bead array (BD CBA Mouse Inflammation Kit, USA) and the results run through a FACS Calibur Flow cytometer (BD Biosciences, CA, USA). Cytokines evaluated included IL-12p70, IL-10, IFN-γ, TNF, MCP-1, and IL-6.

Protective Experiments

Bacterial clearance efficiency in the spleens of infected mice was quantified to determine the induction of protective immunity as previously described [8]. Four weeks after infection, mice were sacrificed by cervical dislocation. Spleens were collected, weighed, homogenized in PBS, and serially diluted. The log₁₀ CFUs were analyzed and log protection values were calculated for the experimental groups.

Statistical analysis

All experiments were analyzed using a one-way ANOVA program and data are expressed as mean ± SD values.
RESULTS

Purification and immunogenicity of multiple recombinant proteins

Identification of target molecular weights and immunogenicity of each of the recombinant proteins in this study have been described in our previous study [2].

Vaccination of multiple recombinant proteins protects mice against \textit{Brucella} infection

In this study, bacterial proliferation in the spleens of the vaccinated group produced a reduction of 0.63 log\textsubscript{10} CFU in comparison to that in the PBS control group ($p < 0.05$) and a reduction of 0.34 log\textsubscript{10} CFU in comparison to the MBP control group ($p < 0.05$). The log\textsubscript{10} CFU of the PBS and MBP groups were not significantly different from each other, and the log\textsubscript{10} CFU of the RBS1 group significantly induced immunity and protection of up to 1.5 log\textsubscript{10} CFU reduction (Fig. 1). Similarly, the weight of the spleens in the vaccinated group was lower than those in the PBS ($p < 0.05$) and MBP ($p < 0.01$) groups.

Determination of immune responses

Vaccination with the CSV-7 multiple antigen vaccine induced significant elevations of essential cytokines, including IFN-\gamma, TNF, MCP-1, and IL-6. At 5 weeks after the first vaccination, there was an enhancement of IFN-\gamma with an up to 3.4-fold increase over that in the PBS group ($p < 0.05$) and up to a 4.6-fold increase in comparison to the MBP group ($p < 0.05$). Similarly, TNF was also increased by up to 14.7-fold over that of the PBS group ($p < 0.001$) and 5.32-fold over that of the MBP group ($p < 0.01$). At 7 weeks after the first vaccination, the TNF level showed a 1.5-fold increase compared to that of the PBS group ($p < 0.05$) and a 2.9-fold increase over that of the MBP group ($p < 0.05$). The level of MCP-1 was also intensified at 5 weeks after the first immunization and showed a 13-fold increase over that of the PBS group ($p < 0.01$) and a 5.1-fold increase compared to the MBP group ($p < 0.01$). At 5 weeks after the first immunization, the IL-6 level showed an 11.2-fold increase over that of the PBS group ($p < 0.01$) and a 2.8-fold increase over that of the MBP group ($p < 0.01$). Lastly, IL-12p70 and IL-10 were not significantly different between the treatment and control groups.

DISCUSSION

In this study, a combination of multiple antigens, formulated as the CSV-7 vaccine, conferred significant resistance to \textit{B. abortus} infection in mice. Previously, all of the selected proteins were established to have the ability to induce protective immunity in a murine model of brucellosis whether individually or in groups having different combinations of the same set of proteins. The current study indicates that the addition of more proteins in a vaccine combination does not always lead to greater protective immunity. In comparison to a five-antigen combination by Arayan \textit{et al.}, 2019 [3], the addition of two more recombinant proteins resulted in a lower level of protective immunity.
Fig. 2. Concentration of IFN-γ (pg/ml) (A), TNF (B), MCP-1 (C) and IL-6 (D) in the sera of mice infected with B. abortus infection at 5- and 7-week time point from first vaccination. Serum cytokines were quantified by flow cytometry. Asterisks indicate significant difference: * P<0.05, ** P< 0.01, *** P< 0.001, **** P< 0.0001.

In earlier study by Arayan et al., 2018 [2], the addition of rDps to the antigen combination yielded higher protection than that produced by the present seven antigen combination. Antigen-specific responses with significant protection have been demonstrated in our previous recombinant protein combinations. Interestingly, unlike a previous combination of eight proteins, the current seven protein combination triggered the production of INF-γ, which is considered as paramount in the control of brucellosis. Antigen-specific T lymphocytes are activated to secrete INF-γ and promote bactericidal activities of macrophages, thereby promoting the death of infected macrophages via apoptosis, as well as stimulating the expression of antigen-presenting molecules among antigen-presenting cells [4, 15]. In this study, TNF was also elevated. TNF is another cytokine that has a pivotal role in the eradication of Brucella. Together with INF-γ, these two are important determinants in the final outcome of a Brucella infection, particularly in a mouse model but even in humans [6, 13, 14]. MCP-1 and IL-6 are proinflammatory cytokines that are indicative of early defense against intracellular bacteria. However, adjuvants could also induce the production of these cytokines [1].

Although the log protection conferred by this seven-member vaccination group was lower than that of previous combinations, the cytokine profile it produces is desirable as IFN-γ and TNF are key factors in the control of brucellosis. More modifications to the proteins used in this study should be undertaken in an effort to enhance its protective effect.

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REFERENCES


