FULL PAPER *Bacteriology*

Protection efficacy of the *Brucella abortus* ghost vaccine candidate lysed by the N-terminal 24-amino acid fragment (GI24) of the 36-amino acid peptide PMAP-36 (porcine myeloid antimicrobial peptide 36) in murine models

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ABSTRACT. *Brucella abortus* cells were lysed by the N-terminal 24-amino acid fragment (GI24) of the 36-amino acid peptide PMAP-36 (porcine myeloid antimicrobial peptide 36). Next, the protection efficacy of the lysed fragment as a vaccine candidate was evaluated. Group A mice were immunized with sterile PBS, group B mice were intraperitoneally (ip) immunized with 3×10^8 colony-forming units (CFUs) of *B. abortus* strain RB51, group C mice were immunized ip with 3×10^8 cells of the *B. abortus* vaccine candidate, and group D mice were orally immunized with 3×10^9 cells of the *B. abortus* vaccine candidate. *Brucella* lipopolysaccharide (LPS)-specific serum IgG titers were considerably higher in groups C and D than in group A. The levels of interleukin (IL)-4, IL-10, tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) were significantly higher in groups B–D than in group A. After an ip challenge with *B. abortus* 544, only group C mice showed a significant level of protection as compared to group A. Overall, these results show that ip immunization with a vaccine candidate lysed by GI24 can effectively protect mice from systemic infection with virulent *B. abortus*.
KEY WORDS: antimicrobial peptide, *Brucella abortus*, brucellosis, vaccination

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Bovine brucellosis, caused by a gram-negative facultative intracellular pathogen, *Brucella abortus*, is an important zoonotic disease worldwide [2, 26, 34]. Brucellosis in cattle causes infertility, abortion, and production of contagious milk, thus resulting in major economic losses [5, 15]. It also affects humans and causes headache, fever, arthritis, and chronic fatigue; however, this infection does not spread among humans [16, 17, 50].

A number of strategies, including promotion of awareness, improvement of hygiene standards and the use of vaccines, have been implemented to prevent the spread of bovine brucellosis, because of its high socioeconomic impact [2, 34]. Currently, only live attenuated vaccines are available for prevention of brucellosis in livestock. Among them, *B. abortus* strain S19 and *B. abortus* strain RB51 (strain RB51) are widely used [2, 34]. *B. abortus* strain 19 has been effective in preventing abortion and controlling brucellosis in adult cattle. It also helped to decrease the prevalence of the disease in a herd [2, 35]. However, *B. abortus* strain 19 does not discriminate between infected and vaccinated animals. In addition, there is a low risk of abortion in livestock [2, 34, 35]. The live attenuated strain RB51 is an alternative to the *B. abortus* strain 19 vaccine. The strain RB51 vaccine is less abortifacient and virulent. Furthermore, it does not induce an antibody response in the standard serological diagnostic tests. It is also safe to use in calves elder than 3 months [2, 34, 35]. Nevertheless, vaccination of pregnant cows with strain RB51 carries a low risk of abortion or premature birth. Thus, it is recommended to be used with caution in pregnant cattle [2, 26, 29, 34, 35]. Although a live attenuated vaccine is a common practice for prevention of brucellosis, it also poses high risks due to the potential ability to revert to virulence and to cause abortion and because of shedding in milk, urine, semen or fecal matter, thus infecting the humans coming into contact with the animals. Hence, many different approaches, such as killed vaccines, subunit vaccines, recombinant proteins and vector vaccines, have been tried against brucellosis with varying degrees of success [2, 29].

In the past few years, bacterial lysates have emerged as an effective inactivated nonliving vaccine against a wide variety of gram-negative bacteria. Bacterial cell lysates constitute empty, nonliving bacterial envelops of gram-negative bacteria with intact cellular morphology, including cell surface structures, but lacking cytoplasmic content [27]. Host defense peptides (HDPs) or antimicrobial peptides (AMPs) are a part of the innate immune system [8, 9]. These peptides have a diverse range of activities against gram-positive as well as gram-negative bacteria [45], parasites [19], and enveloped viruses [12]. The mechanism of action of HDPs is disruption of membrane barrier function by pore formation or induction of membrane permeabilization, without disturbing integrity of the membrane [18, 20, 46, 47]. Until now, 11 porcine AMPs have been reported [40]. Porcine myeloid

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antimicrobial peptide 36 (PMAP-36) has the highest positive charge among all the porcine cathelicidins. It may be advantageous, because PMAP-36's binding to the bacterial cell membrane is mediated by the positive charge of the peptide and the negatively charged molecules at the surface of the bacterial cell membrane through electrostatic interactions [3]. In particular, in the 36-amino acid (aa) sequence of PMAP-36, the N-terminal α-helical domain consists of 24 aa (GI24), and GI24 can also penetrate the bacterial membrane like PMAP-36 can [3, 12, 19]. The aim of the present study was to compare the ability of bacteria lysed by GI24 to induce a cellular immune response and a humoral immune response between mice immunized orally and mice immunized intraperitoneally (ip). Another objective of this study was to compare the protection efficacy of the *B. abortus* vaccine candidate constructed via lysis of *B. abortus* biotype 1 isolate from Korean cattle by means of GI24 with that of *Brucella abortus* strain RB51 vaccine in a mouse model.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and the peptide: *B. abortus* biotype 1 isolate from Korean cattle was used for the construction of a vaccine by means of GI24. *B. abortus* strain RB51 served as the comparative vaccine (versus the vaccine candidate). *B. abortus* strain 544 (ATCC 23448)—smooth, virulent bacteria of the *B. abortus* biovar 1 strain—served as the virulent challenge strain (Table 1) [28]. These strains were grown in Brucella broth and on Brucella agar (Becton Dickinson, Sparks, MD, U.S.A.) at 37°C. The GI24 (GRFRRLRKKTRKRLKKIGKVLKWI-NH_2) peptide was chemically synthesized by Peptron (Daejeon, South Korea). The *B. abortus* biotype 1 isolate was kindly supplied by the National Veterinary Research and Quarantine Service (NVRQS), Anyang, Korea, after identification of the phenotypic characteristics of the *B. abortus* biotype 1 isolate by typical biochemical tests and *Brucella* biotyping using the CO_2 requirement for primary isolation, H_2S production, urease activity, growth in the presence of dyes, agglutination with monospecific sera, phage typing and oxidative metabolic rates.

Construction of a B. abortus *vaccine candidate*: A single colony (round with smooth margins and approximately 2 mm in diameter) of *B. abortus* biotype 1 isolate was individually inoculated into 200 m*l* of Brucella broth, and the cultures were incubated at 37°C with slow agitation to obtain optical density (OD) of 0.3 at 600 nm. GI24 was added into the culture broth at 40 μg/m*l* and was incubated at 37°C to lyse the isolate. After 24 hr, lysis induction was determined by counting the number of viable cells after incubation on Brucella agar for 72 hr at 37°C. After the lysis process, the cell lysates were harvested by centrifugation at 4,000 × *g* for 10 min. Finally, the harvested cells were washed three times with sterile phosphate-buffered saline (PBS), then resuspended in PBS at the concentration of approximately 3 × 10^9 cells/m*l* and stored at –20°C.

Preparation of a B. abortus *strain RB51 formulation*: Strain RB51 was cultured in Brucella broth for 48 hr at 37°C. The bacteria were washed with sterile PBS three times and resuspended in sterile PBS at approximately 3 × 10^9 colony-forming units (CFUs)/m*l*. Mice were immunized with the live vaccine on the day of the preparation.

Transmission electron microscopy (TEM): The lysate samples for TEM to examine the intracellular alterations in the *B. abortus* cells before and after treatment with peptide GI24 were prepared in the same manner as the *B. abortus* lysates. TEM was performed according to the method described by Lv *et al.* [30].

Immunization and sample collection: BALB/c mice were subdivided into four groups, each group containing 10 mice. All the mice were inoculated at 6 weeks of age [0 week post-inoculation (WPI)]. Ten mice of group A were inoculated ip with sterile PBS, as the control group was. Mice of groups B and C were also vaccinated ip with approximately 3 × 10^8 CFUs of *B. abortus* strain RB51 [25] and approximately 3 × 10^8 cells of the *Brucella* vaccine candidate. Group D was orally immunized with approximately 3 × 10^9 cells of the vaccine candidate. Blood and vaginal washing samples were collected 0, 2, 4 and 6 WPI for evaluation of the immune response according to the method described in another study [49]. The animal experiments described in this study were conducted with approval (CBU 2015-052) of the Chonbuk National University Animal Ethics Committee in accordance with the guidelines of the Korean Council on Animal Care.

Immune responses evaluated by an enzyme-linked immunosorbent assay (ELISA): A modified ELISA was performed to assess the *Brucella* lipopolysaccharide (LPS)-specific IgG and IgA titers in serum and vaginal washing samples, using the B. Brucella Ab ELISA 2.0 Kit (BioNote, Hwaseongsi, Gyeonggi-do, Republic of Korea). Briefly, serum samples and vaginal washing samples were diluted 1:50 and 1:3 with PBS, respectively. The plates were incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG or IgA antibody (Southern Biotechnology Associates, Birmingham, AL, U.S.A.). Enzymatic reactions were carried out by addition of a substrate solution containing *o*-phenylenediamine (Sigma-Aldrich, St. Louis, MO, U.S.A.) and were analyzed on an automated ELISA spectrophotometer (Thermo Scientific Multiskan GO, Thermo Fisher Scientific Oy, Ratastie, Vantaa, Finland) at 492 nm. Results of the ELISA are expressed as the mean optical density (OD) ± standard deviation.

Quantitation of cytokines from splenocytes: From each group, five mice were euthanized, and spleens were removed aseptically 4 WPI [39]. The splenocytes were prepared according to the method described elsewhere [1, 38]. The prepared spleen cells were seeded in 24-well tissue culture plates at 2 × 10^6/well [1, 38]. The splenocytes were stimulated *in vitro* with heat-inactivated bacteria of *B. abortus* strain 544 (10^8 cells/well), concanavalin A (0.5 μg/well) as a positive control or the medium as an unstimulated control, and incubated at 37°C, 5% CO_2 and 95% humidity [1, 52]. Culture supernatants were collected after 72 hr of restimulation and stored at –70°C until use for cytokine quantification [1, 52].

Quantification of cytokines by an ELISA: ELISA was used to measure the concentration of interleukin (IL)-4,

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Description	Source or reference
Strains		
B. abortus		
Biotype 1	Isolate from Korean cattle	Lab stock
Strain RB51	Commercial vaccine strain	Lab stock
Strain 544	ATCC23448, a smooth virulent *B. abortus* biovar 1 strain	[29]

IL-10, interferon-gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α) in the supernatants using the mouse cytokine ELISA Ready-SET-GO reagent kit (eBioscience Inc., San Diego, CA, U.S.A.) [6, 7, 42]. Results of the ELISA were expressed as the mean concentration ± standard deviation.

Challenge experiments: For challenge experiments, the challenge strain, strain 544 was prepared. Briefly, the strain was grown in Brucella broth at 37°C for 24 hr and was resuspended at approximately 10^5 CFUs/m*l*. All mice were challenged ip 6 WPI with 100 *μl* of the challenge strain. Two weeks after the challenge, the spleen weights of all mice were measured, and each spleen was diluted 1:100 using Brucella broth. A total of 100 *μl* of the diluted media was spread on blood agar to count the number of viable strain 544 cells from the spleens at 2 weeks after the challenge. If no colony was detected on the blood agar, then the number of viable cells of challenge strains from the spleen of the mice corresponded to $<10^3$ CFUs. Briefly, blended spleen samples were plated on Brucella agar with or without rifampicin (50 *μ*g/m*l)*, because strain RB51 is resistant to rifampicin [41]. The vaccine and challenge strains were confirmed by PCR using a *B. abortus*-specific primer (5′-GAC GAA CGG AAT TTT TCC AAT CCC), RB51/2308 primer (5′-CCC CGG AAG ATA TGC TTC GAT CC) and IS*711*-specific primer (5′-TGC CGA TCA CTT AAG GGC CTT CAT) with enhanced *Brucella* AMOS PCR primers [10, 11].

Statistical analysis: To evaluate the differences among various vaccinated groups, absorbance data from ELISAs were subjected to analysis of variance with *post hoc* Tukey's test for pairwise comparison in the SPSS software, version 16.0 (SPSS Inc., Chicago, IL, U.S.A.). The Kruskal-Wallis test followed by Dunn's procedure for multiple comparisons between groups was carried out to compare the log-transformed CFU values in organs obtained from each vaccinated group of mice with the values in the respective control group in the bacterial challenge experiments. Statistical significance was assumed at $P<0.05$.

RESULTS

TEM: As shown in Fig. 1, in the untreated *B. abortus* cells, full intracellular contents and an intact cell membrane were observed (Fig. 1A). On the other hand (Fig. 1B), the bacteria treated with GI24 showed clear cytoplasmic spaces, and a disrupted membrane of *B. abortus* (with visible pores) was observed.

The humoral immune response in immunized mice: Serum IgG titers against *B. abortus* LPS in groups C and D were 0.07 ± 0.009 and 0.08 ± 0.025 before immunization and increased gradually to 1.78 ± 0.484 and 1.66 ± 0.407 at 6 WPI (Fig. 2; $P<0.01$). In addition, the vaginal IgA titers against the *B. abortus* LPS in groups B, C and D were 0.13 ± 0.037, 0.14 ± 0.033 and 0.11 ± 0.025, respectively, before immunization and 0.2 ± 0.061, 0.7 ± 0.239 and 0.36 ± 0.185, respectively, 2 WPI. Four WPI, the IgA titers of groups B, C and D were 0.44 ± 0.093, 0.66 ± 0.195 and 0.45 ± 0.108, respectively ($P<0.01$). The IgA titers of groups B, C and D were 0.23 ± 0.036, 0.87 ± 0.089 and 0.36 ± 0.103, respectively, 6 WPI (Fig. 2; $P<0.01$).

Cytokine analysis: The mean concentrations of IL-4 in response to heat-inactivated *B. abortus* cells from splenocytes of mice from groups A, B, C and D were 25 ± 8.477 *p*g/m*l*, 112.1 ± 21.129 *p*g/m*l*, 168.7 ± 36.53 *p*g/m*l* and 199 ± 63.116 *p*g/m*l*, respectively ($P<0.05$) (Fig. 3). The IL-10 concentrations of the mice from groups A, B, C and D were 17.4 ± 10.644 *p*g/m*l*, 72.1 ± 21.829 *p*g/m*l*, 101.5 ± 39.049 *p*g/m*l* and 92.5 ± 48.855 *p*g/m*l*, respectively ($P<0.05$). The TNF-α concentration of mouse groups A, B, C and D were 162.5 ± 18.208 *p*g/m*l*, 375.8 ± 76.996 *p*g/m*l*, 276.3 ± 61.266 *p*g/m*l* and 316 ± 87.429 *p*g/m*l*, respectively ($P<0.05$). In addition, the levels of IFN-γ in groups A, B, C and D were 1 ± 0.385 *n*g/m*l*, 3.7 ± 1.29 *n*g/m*l*, 2.4 ± 0.274 *n*g/m*l* and 1.6 ± 0.091 *n*g/m*l*, respectively ($P<0.01$).

Protection of mice against a virulent challenge: All mice were challenged ip 6 WPI with approximately 4×10^4 CFUs of the challenge strain. Among the five mice of group A, the challenge strain was isolated from all mice, and the number of isolates was 115,296 ± 15,446 CFUs/spleen. The challenge strain was isolated from all mice of group B, and the number was 19,243 ± 9,229 CFUs/spleen. In contrast, in group C, the challenge strain was isolated from three out of five mice, and the number was only 4,267 ± 2,512 CFUs/spleen. Among four of five mice in group D, the wild-type *B. abortus* strain was isolated, and the number was 13,118 ± 1,005 CFUs/spleen (Fig. 4).

DISCUSSION

A novel method for production of an inactivated *B. abortus* vaccine candidate is based on *B. abortus* lysis using one of the AMPs leading to formation of empty cell envelops, termed "*Brucella* lysate vaccine". The peptides bearing a net positive charge get attached to the negatively charged phospholipids in the cell membranes of gram-negative and gram-positive bacteria [23, 48], followed by insertion of the peptide into to the bacterial cell membrane [20], resulting

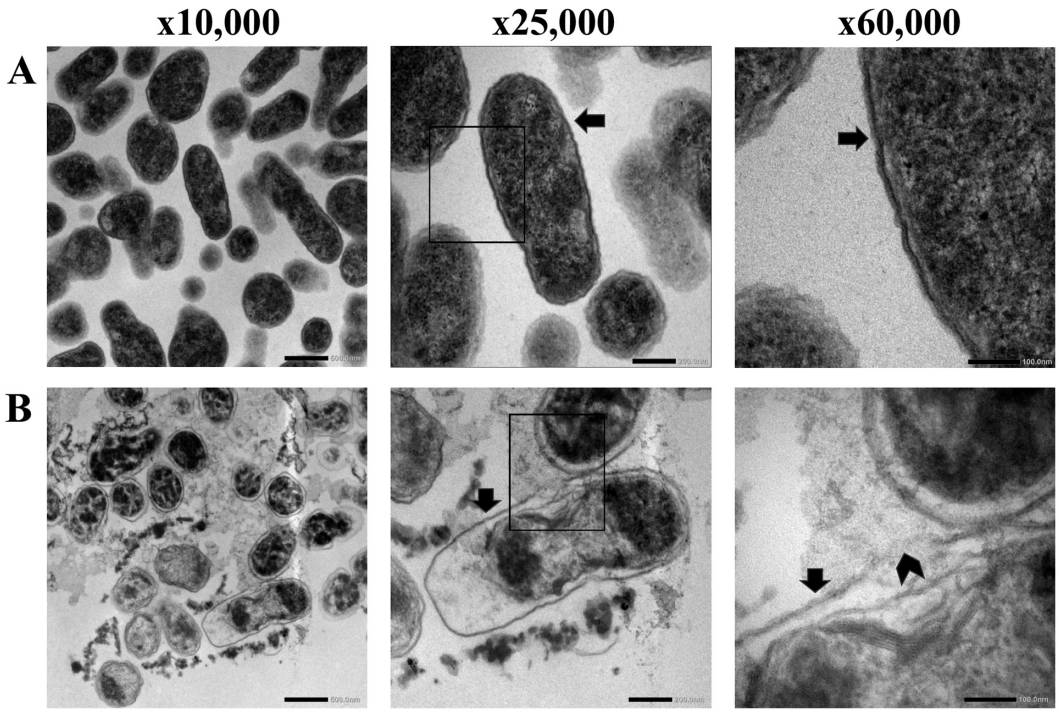

Fig. 1. Transmission electron micrographs of *Brucella abortus* biotype 1 treated with GI24. (A) The untreated *B. abortus* cells. (B) The bacterial cells treated with GI24. The bacterial cells were incubated with 40 μg/m*l* of GI24 for 30 hr at 37°C.

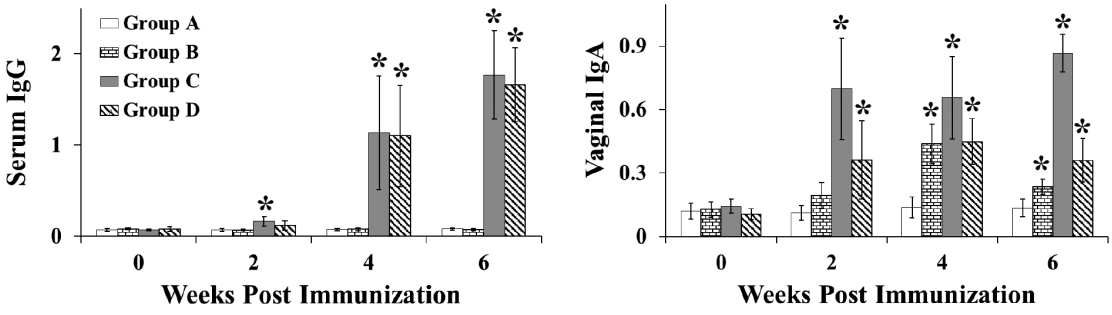

Fig. 2. Serum IgG (μg/m*l*) and vaginal IgA (μg/m*l*) titers against *Brucella abortus* 1119-3 LPS in mice intraperitoneally or orally immunized with each vaccine candidate. Group A mice were immunized with sterile PBS; group B mice were intraperitoneally immunized with 3×10^8 CFUs of *B. abortus* strain RB51; group C mice were intraperitoneally immunized with 3×10^8 cells of the *B. abortus* lysates; group D mice were orally immunized with 3×10^9 cells of the *B. abortus* lysates. Data shown are the means of all mice in each group, and error bars show the standard deviations (SD). Asterisks indicate a significant difference between the values of the groups immunized with the vaccine candidate (*$P \leq 0.05$) and those of the control group.

in formation of a transmembrane "pore" [20, 37]. As soon as pores form, they disrupt and destabilize the bacterial cell membrane, and then all cytoplasmic content comes out leading to bacterial lysis [20, 37], with intact natural outer membrane LPS structures that have highly immunogenic properties.

Vaccination via the mucosal route is a feasible method to induce acquired immune responses against infectious agents in humans and animals. In most animals, brucellosis is transmitted via mucosal routes. Therefore, an effective mucosal *Brucella* vaccine should induce a systemic immune response. Live attenuated *Brucella* strains were used as mucosal vaccine candidates in several studies [14, 21, 36]. However, the inherent safety risks, such as bacterial replication, may prevent the use of live attenuated *Brucella* strains as vaccines. Other studies showed that *Salmonella* Gallinarum cell lysates can generate protective antibody-mediated as well as cell-mediated immunity (CMI) when used as a vaccine via various routes in chickens [13, 22]. Bacterial lysates represent a relatively new concept for improvement of the vaccine technology, but this novel approach has been seldom tested on *Brucella*. As reported in this study, we

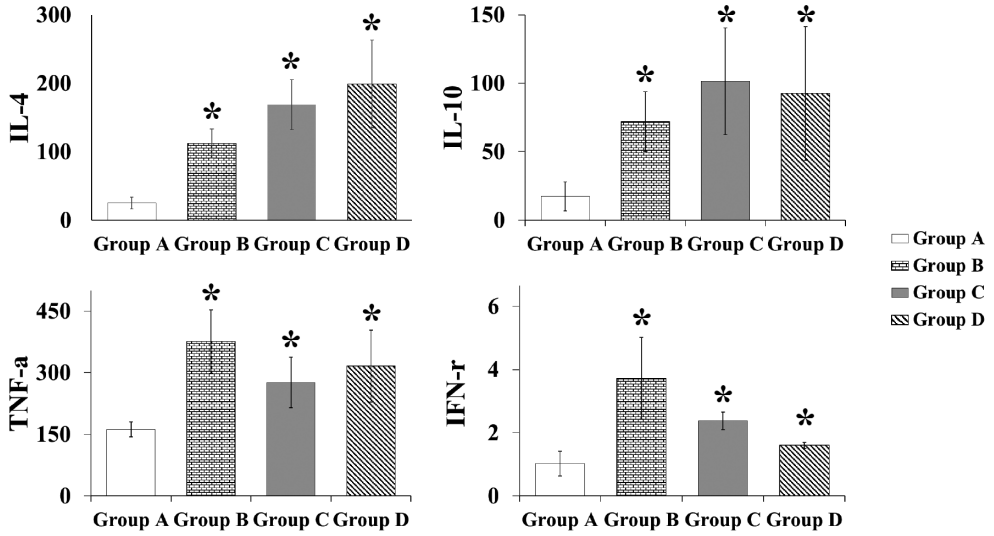

Fig. 3. IL-4 (*p*g/m*l*), IL-10 (*p*g/m*l*), TNF-a (*p*g/m*l*) and IFN-γ (*n*g/m*l*) concentrations in the supernatants of the splenocytes stimulated with heat-inactivated *Brucella abortus* strain 544 at 4 WPI. Group A mice were immunized with sterile PBS; group B mice were intraperitoneally immunized with 3×10^8 CFUs of *B. abortus* strain RB51; group C mice were intraperitoneally immunized with 3×10^8 cells of the *B. abortus* lysates; group D mice were orally immunized with 3×10^9 cells of the *B. abortus* lysates. Data are presented as the mean of all mice in each group; error bars show SD. Asterisks indicate a significant difference between the values of mouse groups B, C and D (**P*<0.05) and those of the control group.

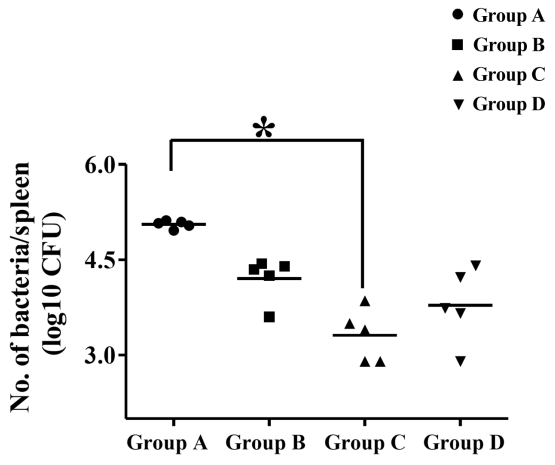

Fig. 4. Protection against *B. abortus* strain 544 in mice immunized with *B. abortus* strain RB51 or *B. abortus* ghost cells. Group A mice were immunized with sterile PBS; group B mice were intraperitoneally immunized with 3×10^8 CFUs of *B. abortus* strain RB51; group C mice were intraperitoneally immunized with 3×10^8 cells of the *B. abortus* lysates; and group D mice were orally immunized with 3×10^9 cells of the *B. abortus* lysates. Six weeks postinoculation (WPI), the mice were challenged with *B. abortus* strain 544. Two weeks later, the number of viable *Brucella* cells recovered from the spleen was determined. Data represent the mean of all mice in each group; error bars show SD. Asterisks indicate a significant difference between the values of mouse groups B, C and D (**P*<0.05) and those of the control group.

constructed a *Brucella* vaccine candidate using GI24, one of AMPs. In this study, we tested whether the *Brucella* vaccine candidate can be administered orally as well as ip to protect mice from a challenge infection with wild-type *B. abortus*. We investigated the efficacy of the *Brucella* vaccine candidate, constructed using GI24, for protection against brucellosis caused by strain RB51 in murine models. We first examined the effect of the *Brucella* vaccine candidate (constructed using GI24) on induction of CMI and protection. In line with our initial hypothesis, ip or oral immunization with the *Brucella* vaccine candidate significantly increased antibody titers and CMI responses in comparison with the unimmunized group.

In the present study, we evaluated humoral immunity as well as CMI induced by immunization with the *Brucella* vaccine candidate and its protective efficacy against challenge infection with *B. abortus* strain 544. The route of immunization alters the course of the immune response being induced by the vaccine candidate. Therefore, we evaluated two routes of immunization, ip and oral, to find the optimal immunization route required for an effective immune response. Approximately 3×10^8 and 3×10^9 cells of the *Brucella* vaccine candidate were used as the dose for immunization via ip and oral routes, respectively. A vaccine inducing protective immune responses, such as secretory IgA, at the mucosal surface would be an ideal vaccine, because it would prevent the entry of (and colonization by) pathogens and consequently would prevent the disease [24]. In the present study, immune responses, such as serum IgG and mucosal IgA, were analyzed in mice vaccinated ip or orally with the candidate vaccine. The serum IgG and vaginal IgA titers in the vaccine

candidate-inoculated groups irrespective of the immunization route were significantly increased as compared to the unimmunized group. Furthermore, induction of cytokines in splenocytes collected from all mice—immunized with the vaccine candidate or strain RB51 and restimulated *in vitro* with heat-inactivated bacteria of *B. abortus* strain 544—showed powerful Th2 type immunity (IL-4 levels represent the Th2 type of immunity). Generally, induction of mucosal IgA is strongly dependent on a type Th2 immune response. Major cytokines that enhance IgA responses are IL-5, IL-6 and IL-10 [33, 44]. In this study, IL-10 levels were significantly higher in all the mice immunized the *Brucella* vaccine candidate than in control mice. These results show that the production of cytokines that are associated with enhancement of an IgG and IgA response was also significantly increased by the *Brucella* vaccine candidate.

It is well known that *Brucella* is a facultative intracellular pathogen. For clearance of an intracellular pathogen, CMI is crucial [4, 51]. Therefore, in this study, we evaluated cellular immune responses using ELISA kits for cytokines. The results revealed strong secretion of TNF-α and IFN-γ into the culture supernatant when splenocytes were restimulated *in vitro*, giving an indication of the Th1 type of immune response; this result is in agreement with the previously shown protective response. For efficient macrophage functioning, such as killing and clearance of an intracellular pathogen, IFN-γ is an essential effector cytokine [32, 43]. In the present study, a strong Th1 response (TNF-α and IFN-γ) was induced by ip immunization with strain RB51 or by ip or oral vaccination with the *Brucella* vaccine candidate. These results showed that immunization with the *Brucella* vaccine candidate irrespective of the immunization route effectively induces the cytokines that are related to CMI as well as humoral immunity.

In mouse brucellosis models, the vaccine-induced protective immune responses are analyzed by comparing a reduction in a bacterial load in the spleen, liver or both between the vaccinated mice and unvaccinated animals [31]. On the basis of that study, mice vaccinated with the vaccine candidate and strain RB51 showed a decrease in the splenic counts of *Brucella* after an ip challenge with wild-type *B. abortus* strain 544. Nevertheless, the notable difference between mice immunized with the vaccine candidate and with strain RB51 was the number of bacterial cells isolated from the mice after the challenge with virulent *B. abortus* strain 544. Among the 5 mice immunized ip with the *Brucella* vaccine candidate, the challenge strains were isolated from the spleens of only 3 mice. The challenge strains were isolated from the spleens of only 4 mice among the 5 mice orally immunized with the vaccine candidate. In contrast, the strains were detected in spleens of all mice immunized ip with strain RB51. Furthermore, in this study, mice immunized ip with the vaccine candidate showed the best protection against infection with virulent *B. abortus* strain 544. This observation suggests that ip immunization with the *Brucella* vaccine candidate involves intact LPS, which is a potent natural immunogen. Therefore, the *Brucella* vaccine candidate elicits antibodies to LPS, which are effective at protecting the animals in mouse models of brucellosis.

CONFLICT OF INTEREST. The authors do not have competing interests.

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