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Subcutaneous immunization with a novel immunogenic candidate (urease) confers protection against *Brucella abortus* and *Brucella melitensis* infections

MORTEZA ABKAR,[1] JAFAR AMANI,[2] ABBAS SAHEBGHADAM LOTFI,[3] GHOLAMREZA NIKBAKHT BRUJENI,[4] SAEED ALAMIAN[5] and MEHDI KAMALI[6]

[1]Department of Molecular Genetics, Faculty of Basic Sciences, Tarbiat Modares University, Tehran; [2]Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran; [3]Department of Clinical Biochemistry, Faculty of Medicine, Tarbiat Modares University, Tehran; [4]Department of Microbiology and Immunology, Faculty of Veterinary Medicine, University of Tehran, Tehran; [5]Brucellosis Department, Razi Vaccine and Serum Research Institute, Karaj; and [6]Nanobiotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

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Brucellosis is a world prevalent endemic illness that is transmitted from domestic animals to humans. *Brucella* spp. exploits urease for survival in the harsh conditions of stomach during the gastrointestinal infection. In this study, we examined the immune response and the protection elicited by using recombinant *Brucella* urease (rUrease) vaccination in BALB/c mice. The *urease* gene was cloned in pET28a and the resulting recombinant protein was employed as subunit vaccine. Recombinant protein was administered subcutaneously and intraperitoneally. Dosage reduction was observed with subcutaneous (SC) vaccination when compared with intraperitoneal (IP) vaccination. rUrease induced mixed Th1–Th2 immune responses with high titers of specific IgG1 and IgG2a. In lymphocyte proliferation assay, splenocytes from IP and SC-vaccinated mice displayed a strong recall proliferative response with high amounts of IL-4, IL-12 and IFN-γ production. Vaccinated mice were challenged with virulent *Brucella melitensis*, *B. abortus* and *B. suis*. The SC vaccination route exhibited a higher degree of protection than IP vaccination (p value ≤ 0.05). Altogether, our results indicated that rUrease could be a useful antigen candidate for the development of subunit vaccines against brucellosis.

Key words: Brucellosis; urease; subcutaneous; intraperitoneal; subunit vaccine.

Jafar Amani, Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Vanak Square, Molasadra Street, P.O. Box 19395-5487, Tehran, Iran. e-mail: jafar.amani@gmail.com
Abbas Sahebghadam Lotfi, Department of Clinical Biochemistry, Faculty of Medicine, Tarbiat Modares University, P.O. Box: 14155-6343, Tehran, Iran. e-mail: lotfi_ab@modares.ac.ir

Brucellosis is a world prevalent endemic disease that is transmitted from domestic animals to humans. It is caused by *Brucella*, a gram-negative, facultative intracellular pathogen (1). Owing to the chronic nature of the infection and lack of impressive control measures, brucellosis remains a permanent risk in developing countries. It can result in abortion in cattle, sheep and goats and Malta fever in humans. Hence, it is an economic and public health problem (2, 3). Previous studies have demonstrated that the stealthy spread of brucellosis among animals can only be prevented or diminished by the application of vaccines. The prevention of human brucellosis involves dairy products pasteurization, appropriate sanitation and vaccination of domestic animals (4). At present, there is no available vaccine for application in humans and commercially available domestic animals' vaccines are confined to live-attenuated strains of *Brucella*. Despite their effectiveness, these vaccines display a number of disadvantages.

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They are considered very virulent or unsafe for human application and they can cause abortion in pregnant domestic animals. In recent years, many studies have been conducted with the aim of developing improved vaccines against brucellosis. As recombinant vaccines are free of the disadvantages of live-attenuated vaccines, they can be promising vaccine candidates (5–7).

Human brucellosis generally does not have any particular clinical symptoms. It is mostly associated with periodic fever, pain, weakness, anorexia and inflammation in different organs. Manifestations of the disease include arthritis, epididymo-orchitis, spondylitis and osteomyelitis. Mucosal contact, breakage in the skin or inhalation of aerosols containing *Brucella* can cause human infection (8). The most common route of *Brucella* entry into the human body is gastrointestinal. Gastrointestinal transmission of pathogenic *Brucella* (*B. abortus*, *B. melitensis*, *B. suis* and *B. canis*) is crucial to pathogenesis in humans, whereas this route is not important for pathogenesis of *Brucella ovis* in domestic animals. The common route of *B. ovis* transmission is the sexual route. Previous studies have shown that most *B. ovis* isolates are urease negative (9).

Infectious bacteria use urease activity to advantage in various ways along pathogenesis. *Brucella* spp., as well as *Helicobacter pylori*, *Yersinia* and *Klebsiella* exploit the enzyme for survival in the harsh conditions of stomach during the gastrointestinal infection (10–13). Urease is a multi-subunit, nickel-containing enzyme that catalyzes the urea molecule into ammonia and carbon dioxide. The hydrochloric acid in the intestinal tract is considered a strong barrier to many pathogens and the consequence of urease activity is neutralization of the stomach's acidic conditions and the creation of assimilable nitrogen (14). Hence, it is considered a crucial virulence factor for different human pathogens. In addition to the resistance developed to low gastrointestinal pH associated with urease, the enzyme plays a key role in pathogenesis independent of its intrinsic enzymatic activity (13). Urease is one of the few enzymes that contain nickel ions in their active sites. Hence, the bacteria must have a nickel uptake system as well as a mechanism for incorporating the ion into the active site of urease. The nickel atom is toxic and cannot be free in the bacterial cytoplasm. Transport systems deliver the nickel to chaperons and the metal is stored by these proteins until assembly to the enzyme (15). Because of the importance of urease in *Brucella* spp. (except *B. ovis*), this virulence factor was investigated as a subunit vaccine candidate against brucellosis. In this study, we examined the immune responses and protection elicited in BALB/c mice by subcutaneous (SC) and intraperitoneal (IP) vaccinations using the recombinant urease (rUrease).

MATERIALS AND METHODS

Mice and ethics statement

The 4–6 week old female BALB/c mice (obtained from Pasteur Institute of Iran) were acclimated and randomly divided into experimental groups. All experimental procedures on animals were approved by the ethical committee of Razi Vaccine and Serum Research Institute. After Rev.1 inoculation, mice were kept in biosafety level 3 animal facilities.

Bacterial strains and plasmid

Brucella abortus 544, *B. melitensis* 16 M and *B. suis* 1330 were used in the protection assay. *Brucella abortus* S19 and *B. melitensis* Rev.1 were used as vaccine controls. All strains were obtained from Razi Vaccine and Serum Research Institute, Karaj, Iran. Bacterial growth was performed as previously demonstrated (16). *Escherichia coli* BL21 (DE3) and pET28a (Novagen, Madison, WI, USA) were employed for recombinant protein expression.

Polymerase chain reaction amplification and cloning

A 482 bp long open reading frame of the middle part of *urease* alpha subunit gene (Ala_{201} to Leu_{350}) of the synthetic gene (GenBank accession number: JQ965699) was amplified by polymerase chain reaction (PCR) using a gene-specific forward primer (5′-ATATTCGAAGCCACCATGGCCTCGTTAC-3′) and a reverse primer (5′-GCTACAAGCTTTTACAAAATATCTTCAGCAGCG-3′). The underlined parts of the primer sequences above represent the restriction sites for *EcoRI* and *HindIII* in the forward and reverse primers, respectively. The following method was applied for amplification: hot start at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, an extension at 72 °C for 1 min, concluded by another final extension step of 10 min duration at 72 °C. The PCR product was digested with *EcoRI* and *HindIII* and then purified by High Pure PCR Cleanup micro kit (Roche Applied Science, Penzberg, Upper Bavaria, Germany). The pET28a vector, too, was digested by the same enzymes and the purified PCR product ligated to the digested vector (17). The competent *E. coli* strains of DH5α were transformed by the recombinant pET28a-urease. The host was grown on the LB agar plate containing 50 μg/mL kanamycin. For validation of recombinant colonies, a colony PCR was performed. Plasmid was extracted from positive colonies. The plasmids were subjected to double digestion by *EcoRI* and *HindIII* enzymes. After analysis on agarose gel, the purified plasmids were sequenced (Seq Lab, Göttingen, Germany).

Expression and purification of recombinant urease

In order to express the rUrease protein, *E. coli* BL21 (DE3) cells were transformed using purified recombinant

plasmids. Three colonies were incubated in LB broth containing kanamycin (50 μg/mL) and grown overnight at 37 °C with shaking at 150 rpm. Five hundred micro litre of overnight cultures were then diluted inside 4.5 mL of LB broth containing kanamycin (50 μg/mL). Induction of protein synthesis was performed with 1 mM of IPTG (isopropyl β-D-thiogalactoside) in a culture of bacteria with an OD_{600} measuring 1. Bacteria were incubated at 37 °C for 16 h with shaking at 150 rpm. The recombinant protein expression was evaluated using 12% SDS-PAGE gel.

Urease was expressed as an inclusion body. The inclusion bodies, containing rUrease, were resuspended in a mixture of 5.0 mM of EDTA, 50 mM of Tris, and 8.0 M of urea (pH 8.0) for 18 h. After centrifuging (18 000 *g*, 30 min at 4 °C), the soluble proteins were purified and refolded by affinity chromatography on Ni-agarose beads (Qiagen, Dorking, UK). The purified rUrease was analyzed for purity by SDS-PAGE and its concentration was determined by the Bradford method (17). The purified protein was approved by western blotting using the anti-His antibody. The recombinant protein was loaded on to SDS-PAGE gel before the proteins were transferred to a polyvinylidene difluoride membrane (Immobilon PTM; Millipore, Germany). Blocking was carried out overnight using a PBS buffer containing 5% BSA. After washing, the membrane was incubated with anti-histidine antibody followed by anti-mouse IgG horseradish peroxidase-conjugated antibody. After a 1-h incubation at room temperature followed by washing, diaminobenzidine was used as chromogen for visualization.

Immunization of mice with different doses of urease via different routes

Twenty female mice in each group administered 10, 20, 30 and 40 μg of rUrease by the SC route and a corresponding number in the IP groups. The recombinant protein was mixed with Complete Freund's Adjuvants (CFA; Sigma, St. Louis, MO, USA) on day 0 and with Incomplete Freund's adjuvants (IFA; Sigma) on day 15. The negative control groups (n = 20) were immunized with PBS emulsified in IFA in SC and IP routes. The positive groups (n = 5) were immunized intraperitoneally on day 15 with 10^5 CFU of *B. abortus* S19 and *B. melitensis* Rev.1.

Specific urease enzyme-linked immunosorbent assay

Sera were obtained 15, 30, 45 days after the first immunization. Anti-urease IgG, IgG1 and IgG2a specific antibody titers in the sera of immunized mice were determined by specific indirect enzyme-linked immunosorbent assay (ELISA). During this process, 96-well plates were coated with 10 μg of rUrease in 100 μL PBS (pH 7.4) at 37 °C for 1 h and blocked with 100 μL of 1% (m/v) bovine serum albumin at 37 °C for 30 min. After washing twice with PBS-Tween (PBS containing 0.05% Tween 20, pH 7.4), the samples were diluted to 1:100 with PBS and applied to the plates. The plates were incubated at 37 °C for 1 h and washed with PBS/T20 five times. They were then incubated with 100 μL/well of Horseradish peroxidase-conjugated IgG, IgG1 and IgG2a antibodies (diluted 1:5000) at 37 °C for 1 h. After washing them with PBS/T20 five times over, and following the addition of 100 μL of o-phenylenediamine dichloride (Sigma) in phosphate-citrate buffer (pH 5.5) and H_2O_2 as a substrate, the plates were again incubated at 37 °C for 15 min. Finally, color development was stopped by the addition of 50 μL of 1N H_2SO_4 and each well was measured at A_{450} nm by using a microtiter plate reader (Eurogenetics, Torino, Italy). The results of total IgG, IgG1 and IgG2a are presented as the mean A_{450} at a serum dilution of 1:250.

Determination of cytokine production

One month after the last immunization, five mice from each group were sacrificed and their spleens were separated under aseptic conditions. Spleen cells were homogenized and suspended in RPMI 1640 medium (NUNC; Thermo Fisher Scientific Inc., Roskilde, Denmark) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal calf serum (HyClone, Logan, UT, USA) and 1% of antibiotic–antimicotic solution (Invitrogen™, Auckland, New Zeland). The cells were cultured at 4×10^6 m/L in duplicate wells with 10 μg of purified rUrease, 2.5 μg of Concanavalin A (ConA) (Sigma) or with culture medium alone. The cultures were incubated on a 96-well flat-bottom plate kept at 37 °C, and with 5% Co_2, for 48 h. IL-4, IL-12 and IFN-γ in culture supernatants were measured using mouse ELISA kits in accordance with the manufacturer's instructions (R & D Systems, Minneapolis, MN, USA).

Lymphocyte proliferation assay

The mice were sacrificed one month after the last immunization. The cells from both SC and IP vaccinated mice (2×10^5 cells/well) were cultured in quadruplicate well. The spleen cells were co-stimulated with purified rUrease (0.1 μg/well) and incubated at 37 °C with 5% Co_2 for 72 h. MTT assay was employed for lymphocyte proliferation determination. 1 mL of 5 μg/mL MTT was introduced to incomplete media, then 5 μL of this preparation were added to each well and the result incubated in the dark at 37 °C for 2 h in conditions of 5% Co_2 and 95% humidity. After removing the media from each well, formazane crystals were solubilized using 90% acidified isopropanol (0.5% w/v SDS and 25 mM HCl in 90% isopropanol). Finally, the absorbance (OD) was measured at 540 nm.

Protection assay

The mice immunized with different doses of rUrease were challenged intraperitoneally with 2×10^7 CFU of virulent strains of *B. abortus* 544, *B. melitensis* 16 M and *B. suis* 1330 after 1 month of the last immunization (five mice from each group). After 1 month of challenge, the infected mice were sacrificed and protection was ascertained by plating the spleen cells on Brucella Agar. The number of CFUs per spleen was counted after incubation for 4 days at 37 °C. The results were represented as the mean of the standard deviation of the common logarithm of CFU± per group. Units of protection were obtained by calculating the difference between the common logarithm of CFU obtained from control mice and the common logarithm of CFU obtained from the corresponding experimental mice group (18).

Statistical analysis

Student's *t*-test was employed to analyze the data to evaluate the levels of lymphocyte proliferation, antibodies and protection test. The p values ≤ 0.05 were considered as statistically significant.

RESULTS

Expression and purification of recombinant urease

Expression of the recombinant protein was analyzed by SDS-PAGE and Western blotting using the anti-His-tag antibodies. Figure 1A shows the electrophoresed protein samples from the uninduced and induced cell lysates of *E. coli* cells.

Western blotting analysis of purified protein showed a single band, corresponding to the expected size of urease (Fig. 1B). Protein concentration was estimated by the Bradford protein assay while the average yield for recombinant urease was 1.25 mg/mL of culture.

Specific urease ELISA

In order to examine the immunogenic characteristics of rUrease, the production of antigen-specific antibodies was determined in the sera of the animals immunized via SC and IP routes by ELISA, with control mice as reference. The results showed that immunization with rUrease stimulated humoral immune response, with the response being stronger in IP immunization (Fig. 2A, B). The data shown in the following figures and tables in the remainder of this section represent the best protection which was raised based on SC (20 μg) and IP (30 μg). Comparison of antibody titers of SC (20 μg) and IP (30 μg) administered mice indicated that IP immunization results in a higher antibody titer (Fig. 2C). The titers of both IgG1 and IgG2a were significantly higher for the rUrease-immunized animals compared to the mice immunized with PBS (Fig. 3A). The results showed that administration of rUrease induced both humoral and cell-mediated immune responses by increasing IgG1 and IgG2a production. IgG2a:IgG1 ratio was found to be 1.08 and 1.12 for IP and SC vaccinated mice, respectively (Fig. 3B).

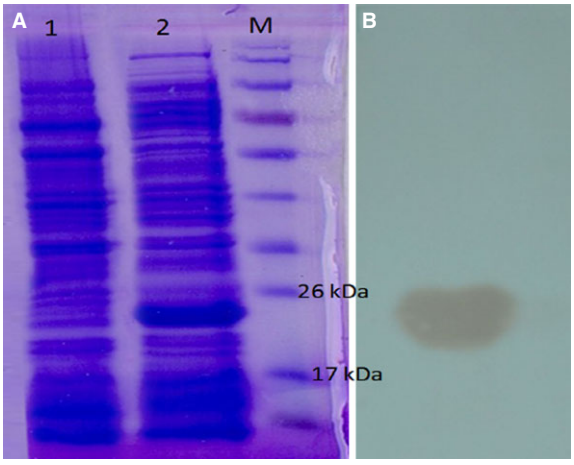

Fig. 1. Expression analysis of recombinant *Escherichia coli*. After induction with IPTG, the rUrease protein produced by recombinant cells was analyzed by SDS-PAGE (A) Lanes 1 and 2 show the uninduced and induced cell lysates of rUrease expressing *E. coli* cells, respectively. Western blot analysis of purified urease with anti-His tag monoclonal antibody (B).

Determination of cytokine production

According to the cytokine profile, supernatants of splenocyte cultures from IP and SC vaccinated mice contained significant levels of IFN-γ, IL-4 and IL-12 compared to the negative control groups (Fig. 4A–C). SC immunization with rUrease showed higher IL-12 and IFN-γ titers in comparison with IP immunization, whereas compared with IP vaccination, the mice that were immunized subcutaneously demonstrated lower IL-4 titers. The results show that IP and SC immunization with the rUrease elicited a mixed T helper1 (Th1)–T helper2 (Th2) immune response.

Lymphocyte proliferation assay

The results of MTT proliferation assay were shown as stimulation index (S.I.). As shown in Fig. 5, the S.I. for IP vaccinated mice was determined to be 1.14, whereas for that of SC vaccinated mice, it was found to be 1.32 when stimulated with rUrease. The S.I. values obtained from splenocytes collected from mice vaccinated with PBS did not show detectable proliferative response when primed with rUrease protein *in vitro*. Thus, this high S.I. value suggests cell stimulatory activity of rUrease and thus, may be one of the reasons behind a strong immune response.

Protection assay

The ability of rUrease to protect against infection with virulent *B. melitensis* 16 M, *B. abortus* 544 and *B. suis* 1330 was analyzed in BALB/c mice immunized with rUrease. The level of protection was evaluated by determining the number of CFUs in spleens at 1 month post-challenge.

The number of CFUs in spleens of vaccinated mice were significantly lower ($p \leq 0.05$) than in the

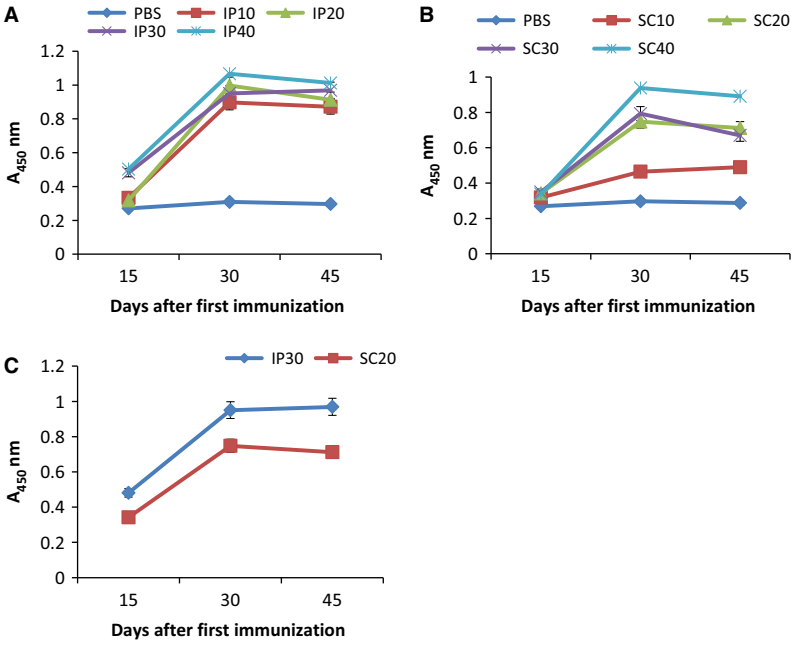

Fig. 2. Urease-specific serum IgG following IP (A) and SC (B) immunization. Mice were immunized with recombinant urease using complete and Incomplete Freund's adjuvants. The sera were assessed for rUrease IgG by enzyme-linked immunosorbent assay method. PBS-immunized mice sera were used as negative control ($p \leq 0.05$). (C) Comparison between SC (20 μg) and IP (30 μg) indicates that IP immunization results in a higher antibody titer. SC, subcutaneous and IP, intraperitoneal.

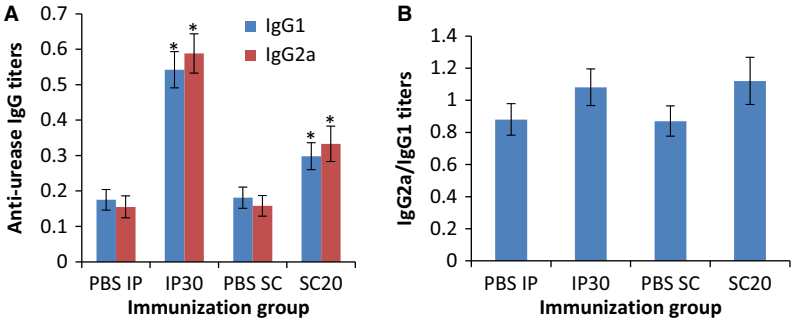

Fig. 3. Antibody isotyping: the isotype profile of urease specific antibodies in serum of intraperitoneally and subcutaneously (A) vaccinated mice were analyzed by enzyme-linked immunosorbent assay using HRP conjugated anti-mouse IgG1 and IgG2a (dilution 1:5000) antibodies. Ratio of IgG2a on IgG1 titers (B). Subcutaneous (SC) 20: subcutaneous (20 μg), intraperitoneal (IP) 30: intraperitoneal (30 μg), and PBS IP: negative control for IP immunization, PBS SC: negative control for SC immunization (*$p \leq 0.05$).

spleens of negative control mice (Table 1), showing that immunization with rUrease results in a high degree of protection against *B. abortus* challenge (1.83 in IP vaccination, 2.21 in the SC route). Furthermore, the results show that vaccination with rUrease confers a high degree of protection against *B. melitensis* challenge (1.71 and 1.88 in IP and SC routes, respectively) (Table 2). As expected, the Rev.1 and S19 vaccines showed high protection at 1 month post-challenge, with 1.95 and 2.17 log units of protection, respectively. Additionally, the protection was not only against *B. abortus* and *B. melitensis* infections, but also against *B. suis* infection (data not shown). Thus, the results indicate that rUrease, when injected subcutaneously, confers better protection. These data emphasize the vaccine potential of rUrease, particularly via SC route of immunization.

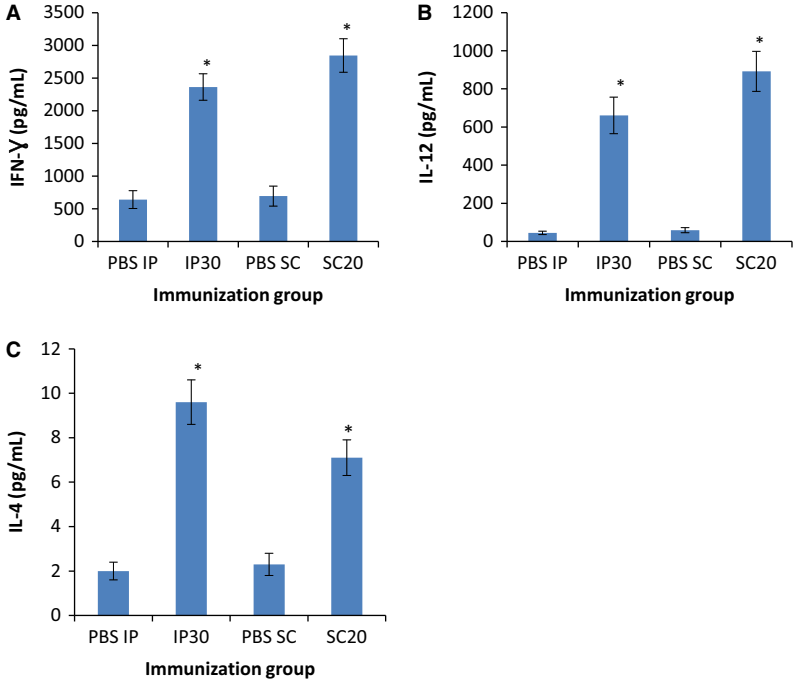

Fig. 4. IFN-γ (A), IL-12 (B) and IL-4 (C) levels in cell supernatants were determined (ρg/mL) by MAb capture enzyme-linked immunosorbent assay. The cells were cultured at 4×10^6 m/L in duplicate wells with 10 μg of rUrease for 48 h. Subcutaneous (SC) 20: subcutaneous (20 μg), intraperitoneal (IP) 30: intraperitoneal (30 μg), and PBS IP: negative control for IP immunization, PBS SC: negative control for SC immunization. (*p ≤ 0.05).

DISCUSSION

In regions with a high incidence of brucellosis, vaccination is one of the major strategies in controlling the spread of this illness. To overcome the disadvantages of live-attenuated vaccines such as *B. abortus* RB51 and *B. abortus* S19 against bovine brucellosis, and of *B. melitensis* Rev.1 against sheep and goat brucellosis, the development of new-generation vaccine systems is necessary (19).

The investigation of antigenicity of recombinant proteins and their application in combination with novel systems of vaccination is necessary for the design and development of improved vaccines. Among different immunization strategies, recombinant proteins are promising vaccine candidates, because these subunit vaccines can be produced at high yield and purity, and can also be manipulated to improve their desirable activities and reduce their undesirable ones. Subunit vaccines offer many advantages compared with attenuated vaccines. Notably, the subunit type of vaccine is safer, well defined, not infectious and unable to change to become virulent, unlike attenuated vaccines (19). Development of a safe and efficient vaccine against brucellosis has been a challenge to researchers all over the world. The identification of novel

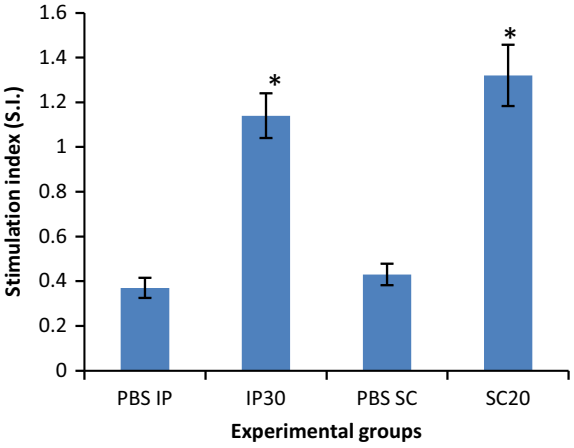

Fig. 5. Lymphocyte proliferation assay of spleen cells from mice immunized with rUrease. Mice immunized with PBS were used as controls. Splenocytes from vaccinated mice (2×10^5 cells/well) were stimulated with rUrease (0.1 μg/well) for 72 h and the proliferative response was determined by *in vitro*-MTT assay. The stimulation index corresponds to the count per minute of stimulated spleen cells divided by the count per minute of unstimulated spleen cells. The data are the mean SI ± SD of five individual mice from each group with three repeats. Subcutaneous (SC) 20: subcutaneous (20 μg), intraperitoneal (IP) 30: intraperitoneal (30 μg) and PBS IP: negative control for IP immunization, PBS SC: negative control for SC immunization. (*p ≤ 0.05).

Table 1. Level of protection against *Brucella abortus* 544 conferred by intraperitoneal (IP) and subcutaneous (SC) immunization with rUrease compared with the vaccine strain S19

Vaccine (n = 5)	Adjuvant	Log_{10} CFU of *Brucella* at spleen[1]	Protection units*
IP control	Freund's adjuvant	6.30 ± 0.23	0
rUrease (30 μg) IP	Freund's adjuvant	4.47 ± 0.17	1.83*
SC control	Freund's adjuvant	6.36 ± 0.31	0
rUrease (20 μg) SC	Freund's adjuvant	4.15 ± 0.29	2.21*
B. abortus S19	–	4.13 ± 0.18	2.17*

[1]The content of bacteria in spleens is represented as the mean log CFU ± SD per group.
*p value ≤ 0.05.

Table 2. Level of protection against *Brucella melitensis* 16 M conferred by intraperitoneal (IP) and subcutaneous (SC) immunization with rUrease compared with the vaccine strain Rev.1

Vaccine (n = 5)	Adjuvant	Log_{10} CFU of *Brucella* at spleen[1]	Protection units*
IP control	Freund's adjuvant	6.14 ± 0.25	0
rUrease (30 μg) IP	Freund's adjuvant	4.43 ± 0.19	1.71*
SC control	Freund's adjuvant	6.12 ± 0.17	0
rUrease (20 μg) SC	Freund's adjuvant	4.24 ± 0.26	1.88*
B. melitensis Rev.1	–	4.19 ± 0.23	1.95*

[1]The content of bacteria in spleens is represented as the mean log CFU ± SD per group.
*p value ≤ 0.05.

immunoreactive proteins as vaccine candidates aids the development of subunit vaccines that will completely inhibit *Brucella* at the various entry sites and protect the different hosts of this microorganism. In this study, we introduced and examined the potential role of rUrease, as an important virulence factor, against different *Brucella* spp.

It has been shown that production of the interferon gamma (IFN-γ) by Th1 and cytotoxic T lymphocyte responses are crucial defense components against *Brucella*, whereas Th2 responses have only a minor role in developing host resistance to this intracellular pathogen. An efficient vaccine against *Brucella* has to activate CD_4^+ and CD_8^+ T cells and stimulate IFN-γ production. Principally, the role of IFN-γ is to stimulate macrophages for more impressive killing and replication inhibition of intracellular pathogens such as *Brucella* (20). Despite its advantages, IP administration of rUrease showed a lower protection level compared with SC administration. After *in vitro* stimulation, splenocytes from IP and SC vaccinated mice produced high levels of IL-4, IL-12 and IFN-γ. Increased IL-4 levels suggested a Th2 bias of the immune response. Urease in IP immunization induced stronger IgG titers than SC immunization. Additionally, the isotype antibody response suggests that IP and SC administration of rUrease induced both humoral and cell-mediated immune responses by increasing IgG1 and IgG2a production. Hence, the results suggest that SC and IP immunization induced mixed Th1–Th2 immune responses. As SC immunization showed higher protection levels in comparison to IP immunization, SC administration of rUrease may be a peripheral route of vaccination.

Different intracellular and outer membrane proteins have been used as vaccine candidates protecting against *Brucella* spp. (21). A previous report has shown that IP immunization of S-adenosyl-L-homocysteine hydrolase (AdoHcyase) induces a Th1 response *in vitro* and causes a strong antibody response in BALB/c mice. The protection afforded by this recombinant antigen at 4 weeks post-challenge was similar to that of the live-attenuated *B. melitensis* Rev.1 (22). IP immunization of CobB and AsnC induced strong IFN-γ and antibody responses in BALB/c mice. Furthermore, these proteins could provide protection against virulent *B. abortus* 544 infection, similar to the licensed vaccine *B. abortus* S19 (23). The recombinant protein Omp25 was also evaluated by Goel et al. in mice. Intraperitoneal and Intradermal (ID) immunization of this protein elicited a mixed Th1–Th2 immune response. Intradermal immunization induced protection comparable to that of *B. abortus* S19 strain. Intradermal vaccination showed a high antibody titer with a low level of antigen, whereas the IP vaccination showed a lower antibody titer even with a high level of antigen. Low amounts of stimulating cytokines in the IP route of vaccination can be one of the reasons behind the low level of protection obtained against the *B. abortus* 544 challenge compared to the protection level attained in ID immunization. Altogether, these results indicate the importance of routes of immunization in protective efficiency of the recombinant protein Omp25 antigen (24). We studied the optimum amount of

antigen dose required for enhanced immune response via SC and IP routes of administration. Our result showed that 20 and 30 μg doses of rUrease were optimum dose for SC and IP routes of administration, respectively. Both SC and IP immunized mice that received rUrease showed high protection against virulent *B. abortus*, *B. melitensis* and *B. suis*. Goel et al. (24) reported that for vaccination with ID and IP routes of administration, 30 and 40 μg doses of rOmp25 were chosen, respectively. Additionally, ID administration of rOmp25 showed high protection against virulent *B. abortus* challenge as compared to IP immunization.

The selection of immunization route can be a key factor for success or failure of an antigen under development. An improper route of immunization may present an antigen ineffective or mask its potential efficiency, although the antigen within its formulation would be significantly efficient in another route (25). Based on protection units obtained in this study, when rUrease was administered subcutaneously confers more protection in comparison to IP route. When an antigen administered into skin, it has a good chance of capturing and transporting into the lymph nodes for induction of immune responses. As skin has immunologically properties, this tissue is considered an ideal tissue for vaccine injection (26, 27). The results showed that rUrease can induce protection against *B. abortus*, *B. melitensis* and *B. suis* infection. According to protein sequence data (Genbank accession no. AFK76472.1), the selected fragment of urease (Ala_{201} to Leu_{350}) has 100% identity in the amino acid composition among *B. suis*, *B. abortus* and *B. melitensis*. These findings show that this fragment of urease is completely conserved among these species. This is the first report of rUrease response against *B. abortus*, *B. melitensis* and *B. suis* with an optimization of the route of administration and of the dosage. We showed that SC vaccination with rUrease in low dose (20 μg/mL) induces high protection levels. The data can be further applied to exploring the various formulations of recombinant protein for better vaccine delivery, administration in different hosts with different adjuvants, and enhanced Th1 type of immune response for gaining higher protection levels. Taken together, our results show that rUrease could be a useful antigen candidate for the development of subunit vaccines against brucellosis, as it induces an antigen-specific cellular response.

CONFLICT OF INTEREST

The authors declare that they have no competing interest.

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