Original article

Biological and immunological characteristics of *Brucella abortus* S99 major outer membrane proteins

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Abstract

Introduction and objective: Outer membrane proteins (OMPs) of *Brucella* are considered as immunogenic structures which can be used to design and develop a subunit vaccine for human brucellosis. *Brucella abortus* S99 OMPs promote the synthesis of high levels of specific anti-*Brucella* IgG molecules in rabbits when administrated with lipopolysaccharide (LPS). The objective of this study is evaluation of the efficacy of *B. abortus* major OMPs with LPS in the induction of immune response against brucellosis.
Materials and methods: OMPs were derived from *B. abortus* by sequential extraction of sonicated cells with ultracentrifugation and predigestion with lysozyme. Proteins could be separated by anion-exchange chromatography and gel-filtration. Based on SDS-PAGE profiles, porins have been dominantly purified among three different classes of *B. abortus* OMPs. Sera of immunized rabbits against *B. abortus* porins were analyzed by enzyme-linked immunosorbent assay (ELISA). LPS of *B. abortus* and complete Freud's adjuvant (CFA) were also applied to elicit higher levels of anti-*Brucella* antibodies.
Results: ELISA confirmed the potency of porins and porins combination with CFA and LPS to promote humoral specific response. Among the above-mentioned compounds, a combination of porins + LPS or porins + CFA has been the most potent immunogenic compound to induce higher titer of antibody against *B. abortus S99* in the animal model.
Conclusion: The application of a complex of *Brucella* LPS and porins as an effective method to elicit protective and long-lasting immunity against *Brucella* infection and would be studied to design and develop a subunit vaccine for human brucellosis.
Keywords: *Brucella abortus* S99; Outer membrane proteins; Porins; Lipopolysaccharide

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Introduction

Brucellosis is one of the most common bacterial zoonoses caused by organisms belonging to genus *Brucella*, Gram-negative, non-spore-forming, facultative intracellular bacteria [1-3]. The genus *Brucella* consists of seven species according to antigenic variation and primary host. *Brucella melitensis* (sheep and goats), *B. suis* (hogs), *B. abortus* (cattle), *B. ovis* (sheep), *B. canis* (dogs), *B. neotomae* (wood rats) and *B. maris* (marine mammals) [4,5].

Brucella abortus induces spontaneous abortion in cattle and causes economic and industrial loss. Although brucellosis has been a health hazard for man and domestic animals in many countries, a licensed human *Brucella* vaccine has not been designed and produced yet [6,7]. Currently, antigenic determinants of *Brucella* cell wall are considered as potential candidates to develop a subunit vaccine and substantial numbers of antigenic components of *Brucella* have been characterized [8]. However, one of the antigens that promotes the major antibody response is the lipopolysaccharide (LPS) [9,10].

Numerous cytoplasmic and periplasmic protein antigens contain outer and inner membrane, have also been characterized. Ribosomal proteins have been interested in vaccine studies as immunological components with both humoral and cellular responses against brucellosis. In current study, we focused on potency of major outer membrane protein (OMP) of *B. abortus* S99 as protective antigens. Lysozyme digestion is necessary to release of OMPs from peptidoglycan (PG). The apparent molecular mass of OMP_S of *Brucella* are used in their classification as group 1 (94 or 88KDa), group 2 (36-38 KDa, identified as porins) [11-15] and group three (31-34 and 25-27 KDa). Recently these proteins have been used in vaccine studies and intra-species classification.

We previously synthesized a subunit brucellosis vaccine composed of *B. abortus* S99 LPS with *Neisseria meningitidis* serogroup B outer membrane vesicle (GBMOMV) as a noncovalent complex and then evaluated specific antibody response against the LPS of *B. abortus* S99. We reported that our purified GBMOMV may be applied as a safe and potent subcutaneous adjuvant to induce high titres of specific anti-*B. abortus* S99 IgG and may be used in candidate vaccines for human brucellosis [16]. In the present study, we designed a subunit brucellosis vaccine composed of *B. abortus* S99 porins and LPS as a noncovalent complex and then evaluated specific antibody response against *B. abortus* S99.

Materials and methods

Bacterial strain

Brucella abortus S-99 (biovar 1) was obtained from the type bacteria collection of Pasteur Institute of Iran. The bacteria were routinely grown on *Brucella* agar medium (Merck, Germany) at 37±1°C for 72h. Then the bacteria propagated into a 60 liter industrial fermentor (Nova-paljas, contact-flow BV, Netherlands) with 40 liters working volume at 37±1°C. After 72h of incubation, the bacteria were inactivated with 10% phenol and biomass of *B. abortus* S99 harvested by centrifugation [17].

Extraction of OMPs of B. abortus S99

Cells were suspended at 1g (wet weight) per 20ml of 10mM tris-hydrochloride buffer (pH=7.5), and 1mg each of DNase and RNase (Sigma chemical co., St. louis, MO) was added per 100ml. Samples were chilled on ice and sonicated in a Bronson Sonifer 450, using a continuous cycle (seven pluses

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per 1min) with a microtip. The samples were centrifuged twice at 3000g for 15min at 4°C to remove unbroken cells. The supernatants were submitted to ultracentrifugation at 43500rpm for 90mins to pellet the crude membranes. Pellets were collected and resuspended in 50mM tris-Hcl (pH=7.6) with 2mM phenyl methane sulfonyl fluoride (PMSF) as a protease inhibitor [18].

Detergent extraction of cytoplasmic membranes was performed by using sodium N-lauroyl sarcosinate. Resultant soluble material was dialyzed against tris buffer at 4°C for 72h with repeated changes. Lysozyme (1mg/50mg of membrane protein) was added to isolate the PG from OMPs. This process was followed by ultracentrifugation at 100,000g for 20mins at 4°C. The supernatants were held at 4°C [19].

Anion-exchange chromatography

Solubilized membrane fractions were concentrated by lyophilization to 10 to 20 mg/ml, equilibrated with 10mM tris buffer containing 0.1% zwittergent and 0.25M NaCl and applied to a column of DEAE-sephacel (Pharmacia Fine chemicals, Inc., pis-cataway, NJ) equilibrated with the same buffer. Elution was performed at room tempreture with upward flow at a rate of $2ml.cm^{-2}.h^{-1}$. After the initial wash, a gradient of NaCl (0.25 to 0.75) was established and collected over a period of 24h. Protein samples from the ion exchange column, concentrated by lyophilization, were separated under the same conditions of flow on a column of sephacryl S-300 (Pharmacia Fine chemicals, Inc.) equilibrated with 10 mM tris buffer containing 0.1% zwittergent and 0.25M NaCl [20].

Protein content measurement

Protein concentration was determined using the Nanodrop ND-1000 (full-spectrum, 220-750nm) spectrophotometer which measures 1µl samples with high accuracy. The A280 method is applicable for purified proteins exhibiting absorbance at 280nm. This module displays the UV spectrum, measures the protein's absorbance at 280 nm (A280) and calculates the concentration (mg/ml). It automatically switches to the 0.2 nm at very high concentrations of protein. In brief, 1µl sample added on the end of a fiber optic cable and the protein concentaration was calculated.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on 12% polyacrylamide gel [21]. Prestained protein (Ferments Co. Finland) was used as marker. 20µl of extract was loaded per wells. Power supply was regulated in 150V for 1h. After electrophoresis, the gel was stained with coomassie blue.

Extraction of B. abortus S99 lipopolysaccharide

S-LPS extracted by hot phenol-water method [17,22]. In brief, 50g wet weight of cells was suspended in 170ml of distilled water followed by the addition of 190ml of 90% (v/v) hot phenol (66°C). After 30mins, the mixture was centrifuged and phenol layer was removed. The LPS in the resultant mixture precipitated by cold methanol (4°C) and dissolved in 0.1M tris buffer. Proteinase K (50µg per 10mg protein) and then both DNase and RNase (50µg per 1mg nucleic acid) added to extracted samples to reduce protein and nucleic acid contaminations.

Animal groups

Three groups of 4-month-old, 2.5 to 3kg white rabbits have been immunized with different compounds as will be described in the immunization procedure. Each group was consisted of three rabbits.

Immunization procedure

Animal models immunized intramuscularly with three different compounds: 1) porins of *B. abortus* S99: 120µg/ml of purified porins. 2) A combination of 120µg/ml of porins and 120µg/ml of LPS. 3) A combination of 120µg/ml of porins and the same volume of CFA [23]. Immunization carried out in 0, 7 and 14 days and immune sera collected in 0 (before the first injection and as the negative control), 7 (before the 2nd injection), 14 (before the 3rd injection) and 21 (one week after the 3rd injection) days and pooled at -20°C [23,24].

ELISA

Anti-*B. abortus* S99 IgG titer of immunized animals demonstrated by ELISA method. Microtiter plates were coated with *B. abortus* S99 antigen (100µl per well) and incubated for 1h at 37°C. Wells were saturated for 1h at 37°C in blocking buffer (phosphate-buffered saline containing 0.1% casein per ml). Antisera were diluted 1000, 10000 and 100000-fold and 100µl of each dilution added to each well and incubated for 1h.

Wells were washed three times with phosphate buffer saline-azide (PBS-azide). Anti-rabbit IgG-HRP conjugated was added (100µl per well) and incubated for 30mins and wells were washed again. Afterwards, the substrate of 3,3,5,5-tetramethylbenzidine (TMB) was added to the wells. Finally stop solution (50µl) was added to the wells and absorbance measured at 405nm using an automated ELISA plate reader (TECAN, Sunrise model, Switziland). The antibody titres expressed in OD units [19,20,25-27].

Statistical methods

Antibody titres of groups of rabbits were expressed as means ± standard deviations. The significance of differences in ELISA titers at different time points was determined by Student's T test.

Results

Extracted OMPs

OMPs concentration has been reported to be 6.27mg/ml by the Nanodrop ND-1000 (full-spectrum, 220-750nm) spectrophotometer (Fig. 1). SDS-PAGE analysis indicated a band (36-38KDa) which would be classified as *Brucella* porins (Fig. 2).

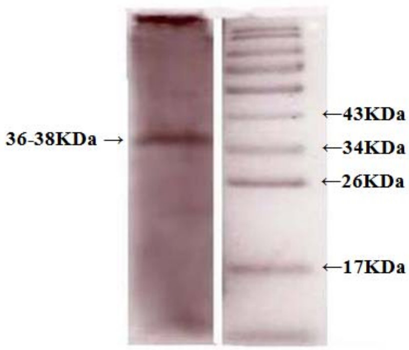

Fig. 1: Electrophoretic pattern of the extracted OMPs of *B. abortus* S99 (Left line) in comparison with protein marker (Righ line)

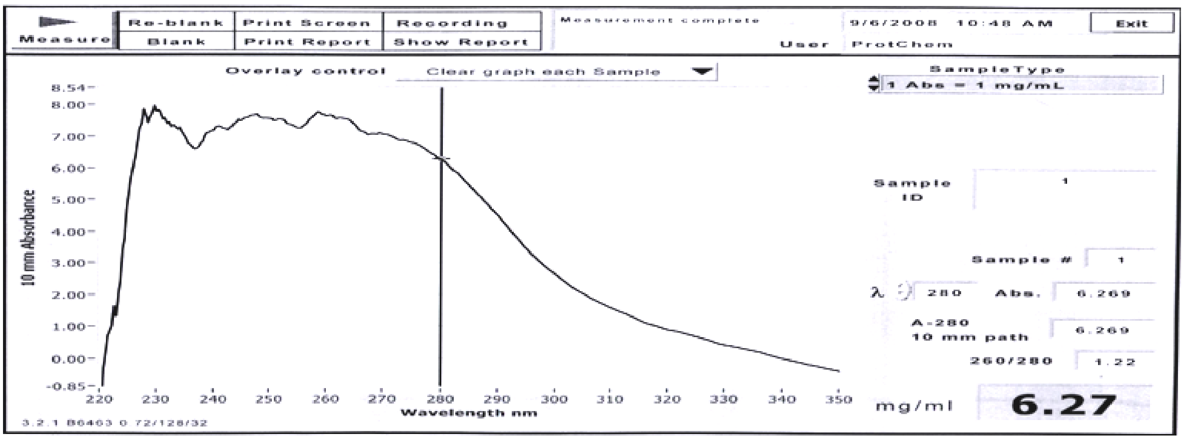

Fig. 2: The plot measured purified protein concentration at 280nm by ND-1000 spectrophotometer

LPS characterization

The LPS content of the extracted sample (measured by the chromogenic LAL assay) was 108 endotoxin units (EU)/ml. The KDO content came out to be 1.3%. Also the protein content of the sample (measured by Lowry method) was <2.0 and the nucleic acid content was <1%.

Specific antibody response against porins and LPS

IgG antibody titers were assayed and expressed in OD unit ± SD (Table 1). The IgG titers in all of the samples have been significantly higher than negative control (P<0.05). The combination of Porins+LPS of *B. abortus* S99 promoted the highest humoral response to *B. abortus* S99 assayed one week after the 3rd injection (Table 1). In addition, in rabbit immunized with the combination of porins+LPS and porins + CFA, the antibody titers were significantly higher than that obtained from porins. Although the differences between the IgG tiers of these two groups (porins+LPS and porins+CFA) has not been significant (P>0.05), both of these groups significantly increased the IgG titer in comparison to the animals immunized with porins without LPS or CFA (P<0.05).

Table 1: Anti-*Brucella* total IgG titers following immunization with three different compounds (Titers are expressed in OD unit)

Injected compounds	Anti *B. abortus* S99 IgG titer, one week after the 1st injection	Anti *B. abortus* S99 IgG titer, one week after the 2nd injection	Anti *B. abortus* S99 IgG titer, one week after the 3rd injection
B. abortus S99 Porins	68242 ± 606	76326 ± 923	124270 ± 2660
B. abortus S99 porins + CFA	122681 ± 3026	132896 ±1693	136706 ± 1300
B. abortus S99 Porins + B. *abortus* S99 LPS	123989 ±2026	134726 ±2980	155847 ± 2220
Negative Control	<3	<3	<3

LPS: Lipopolysaccharide, CFA: Complete Freund's adjuvant.

Discussion

As previously indicated, the applied OMP extraction method in the present study led to the purification of *B. abortus* S99 porins and would be recommended as a specific method to extract and isolate this group of OMPs. Furthermore, the yield of this extraction method has been satisfactory since the concentration of the extracted porins is considerable.

Anti-*B. abortus* S99 porins IgG titer exponentially and significantly increased in all of the groups as compared to the control group. The high levels of anti- *B. abortus* S99 IgG in comparison with the control group indicates the immunogenicity of *B. abortus* S99 porins and the accuracy of our Porin extraction procedure which does not interfere with the natural and immunogenic structure of Porins. Since porins + LPS and porins + CFA equally elicited the IgG titers, LPS of *B. abortus* S99 would be applied as a potent adjuvant (with the same effect of CFA) for porins of *B. abortus* S99.

As previously described [28] LPS of *B. abortus* S99 is 10,000 fold less pyrogenic than *E. coli* LPS and it would be a promising aspect of the application of *B. abortus* S99 LPS as a new adjuvant with microbial origin while the high pyrogenicity, the local and systemic hypersensitivity reactions of enterobacterial LPS and Freund's adjuvants has limited their application for human antigens and vaccines. Although the epitopes of O-polysaccharide (OPS) of LPS are strongly dominant over those of associated with outer membrane proteins and the majority of produced antibodies are specific for OPS, porins stimulate T-cell responses while LPS is a T-independent antigen and unable to promote immunological memory and memory T and B-Cells expansion [29].

Since porins are less accessible to antibodies because of the long OPS chains in the smooth strains of *Brucella* (most of the wild type *Brucella* strains) and LPS lacks the immunological characteristics of T-dependent antigens, a combination of these two structures of *B. abortus* S99 may efficiently induce both humoral and cellular mechanisms. Low pyrogenicity of LPS in parallel with its high potency to induce specific anti-*Brucella* antibodies and possible application of this cell wall component as an adjuvant for Brucella porins that induce T-cells responses are all the factors that highly recommend the application of a complex of *Brucella* LPS and porins as an effective method to elicit protective and long-lasting immunity against *Brucella* infection and would be studied to design and develop a subunit vaccine for human brucellosis [30,31].

Although there are a lot of proved efficient optimizations in the cultivation and fermentation conditions and also in the procedure of porins and LPS extraction [17,32], the main novelty of this study is the simultaneous application of one classic adjuvants along with LPS (as a candidate adjuvant) and the evaluation of these two adjuvants' potency to induce anti-*B. abortus* S99 LPS IgG antibodies. Bhattacharjee *et al*. [32] and Bhattacharjee *et al*. [33] have evaluated the outer membrane vesicle of *Neisseria meningitidis* serogroup B -*B. melitensis* strain 16M LPS non-covalent complex to elicit the immunity against brucellosis in mice, they have extracted the LPS from *B. melitensis* and applied outer membrane proteins of *N. meningitidis* as an adjuvant.

Conclusion

In the present study, we evaluated the efficacy of *B. abortus* Porins+LPS (LPS as a probable adjuvant) after intramuscular injections with two boosters (versus one booster) administrated two weeks (versus one week) after the previous immunizations. After both boosters, the induced anti-*B. abortus* S99 IgG titres increased significantly. Also we reached higher titres of IgG in comparison with previous studies. According to the results of this study, a combination of porins and LPS of *B. abortus* S99 are recommended as potential candidates to design a subunit vaccine.

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