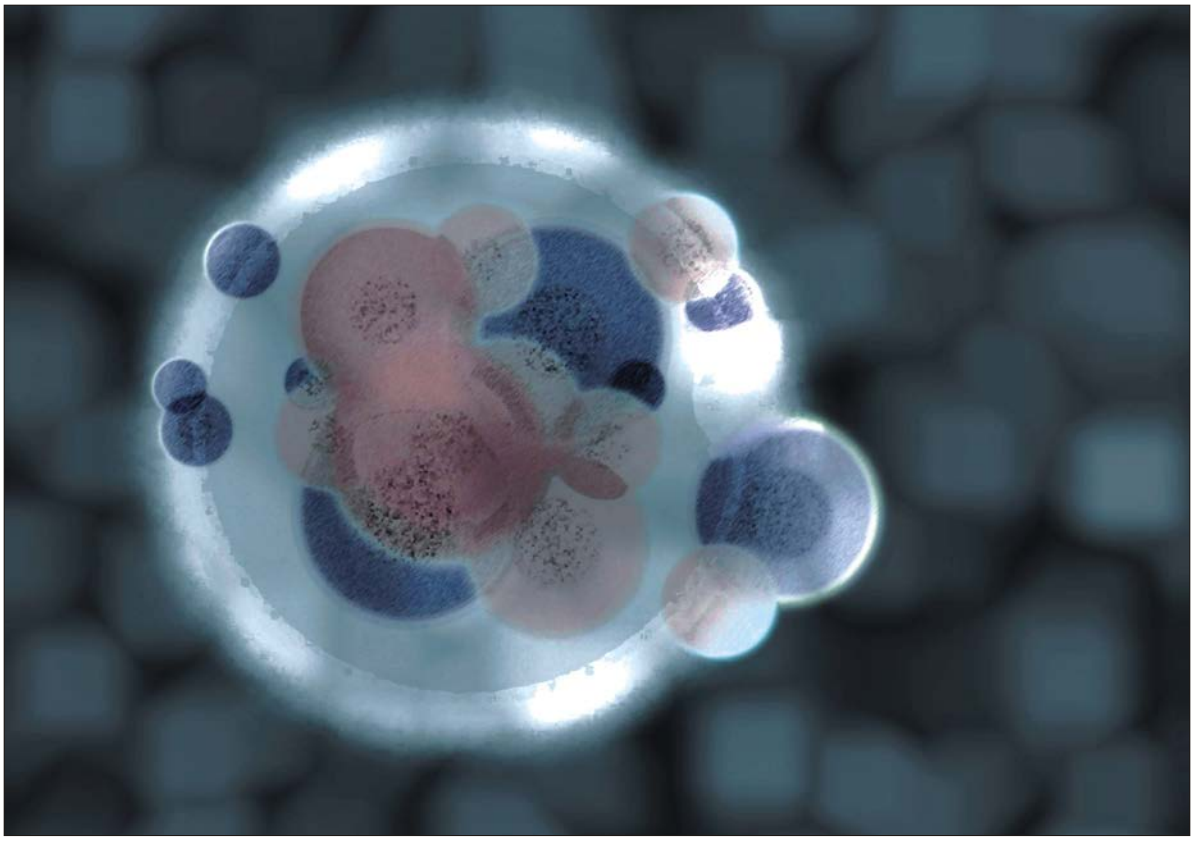

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Novel Approach of Vaccination against *Brucella abortus* 544 based on a Combination of Fusion Proteins, Human Serum Albumin and *Brucella abortus* Lipopolysaccharides

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Abstract: Lipopolysaccharide (LPS) of Brucella *abortus* is an essential component for developing the subunit vaccine against brucellosis. *B. abortus* LPS was extracted by *n*-butanol, purified by ultracentrifugation and detoxified by alkaline treatment. Pyrogenicity and toxicity of *B. abortus* LPS and detoxified–LPS (D-LPS) were analyzed and compared with LPS of *E. coli*. Different groups of mice were immunized intraperitoneally with purified *B. abortus* LPS, D-LPS, a combination of LPS with human serum albumin (LPS-HSA) and *B. abortus* S19 bacteria; besides, control mice were inoculated with sterile saline. Two doses of vaccine were given 4 weeks apart. Mice were challenged intraperitoneally with virulent *B. abortus* 544 strain 4 weeks after the second dose of vaccine. Sera and spleens of mice were harvested 4 weeks after challenge. LPS-*B. abortus* was 10,000-fold less potent in LAL test and 100-fold less potent in eliciting fever in rabbits than in *E. coli* LPS. And D-LPS was very less potent in LAL test and eliciting fever in rabbits ordinary LPS. The antibody titer of anti-LPS immunoglobulin G (IgG) was higher than D-LPS. However, mice immunized with either LPS, D-LPS or LPS-HSA vaccines showed a significant protection against infection of the spleen ($p<0.01$). There was no significant difference between mice immunized with LPS and D-LPS in terms of protection ($p<0.99$). Therefore, it was concluded that D-LPS and LPS-HSA for *B. abortus* can be used as safer and more potent vaccines than ordinary LPS-*B. abortus* vaccine.

Key words: *Brucella abortus*, lipopolysaccharide, subunit vaccine, fusion proteins, human serum albumin

INTRODUCTION

For the time being, there is no licensed vaccine against brucellosis in humans. Several live attenuated brucella vaccines have been tried in humans, but none was found to be satisfactory (Spink *et al*., 1962; Pappagianis *et al*., 1966). A number of genetically defined mutants that are attenuated for growth in macrophages or in animal models have been developed recently, but their suitability for human use has not been evaluated (Elzer *et al*., 1998; McQuiston *et al*., 1999; Edmonds *et al*., 2002).

It has been reported that a single vaccination with a complex consisting of Porins and smooth lipopolysaccharides from *B. abortus* strain 2308 provided significant protection against challenge with the same strain and this protection was found to be equivalent to the protection achieved by vaccination with live attenuated strain 19 (Winter *et al*., 1988). There is an evidence that mice immunized with a Brucella O-polysaccharide-bovine serum albumin conjugate were protected against challenge with B. melitensis strain H38 (Jacques *et al*., 1991). The brucella O-polysaccharide-specific monoclonal antibodies have been shown to provide protection against challenge with B. melitensis and *B. abortus* smooth strains (Cloeckaert *et al*., 1992; Cloeckaert *et al*., 1993). A recent report showed that mice immunized subcutaneously and intranasally with a *Brucella melitensis* Lipopolysaccharide subunit vaccine were protected against challenge with B. melitensis strain 16 M (Bhattacharjee *et al*., 2006). Moreover, several Brucella proteins such as L7/L12, Cu/Zn, superoxide dismutase, p39 have been tested as vaccines to provide protection against brucellosis (Oliveira and Splitter, 1996; Vemulapalli *et al*., 2000; Al-Mariri *et al*., 2001). Besides, the anionic and amphiphilic nature of lipid A of LPS was

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exploited to use LPS as conjugates with other substances for enhanced immunogenicity by binding LPS to numerous substances such as Human Serum Albumin, which are positively charged and possess amphipathic character (David, 1999).

Accordingly, it was conceived that binding LPS of Brucella with some Brucella immunodminant proteins might provide new approach of more effective vaccine against Brucellosis. Unfortunately, very few studies have been conducted to fulfill the newly hypothesized approach of LPS-based vaccination against Brucella infection. In order to prepare a combination of LPS with Brucella immunodominant proteins such as fusion proteins L7/L12 and P39 that are fused with human serum albumin (HAS), it was necessary to evaluate different combinations of LPS with HSA. Hence, we have developed a vaccine composed of purified LPS and detoxified LPS (D-LPS) from *B. abortus* S99 as well as a combination of LPS with HSA.

MATERIALS AND METHODS

Preparation of purified LPS: The LPS was extracted from killed *B. abortus* 99 cells and purified by a method described previously (Goldstein *et al*., 1992; Winter *et al*., 1996). This latter procedure is considered a mild extraction in which the bacterial cells are not disrupted. Briefly, 50 g of killed *B. abortus* organisms were extracted in 400 mL of water-saturated n-butanol at 4°C. The aqueous phase was obtained by using a separator funnel, centrifuged to remove insoluble material and then pooled. To precipitated LPS, 4 volumes of methanol were added. This precipitate was dissolved in 0.1 M Tris buffer (Merck, Germany) (pH 8) containing 2% Sodium Dodecyle Sulfate (SDS) (Merck, Germany) and 2% mercaptoethanol (Merck, Germany) and heated for 5 min at 100°C and for 90 min at 60°C with proteinase K (Invitrogen, USA). After overnight incubation at 4°C, LPS were precipitated with methanol (Merck, Germany), washed twice with cold methanol, dissolved in water and ultracentrifuged at 100000 ×g for 8 h. The pellet was dissolved in water and freeze-dried. LPS from *E.coli* O157:H7 was extracted by the phenol method and purified. The protein content was estimated by the Bradford method, with bovine serum albumin as a standard. Nucleic acid was estimated by measuring the A260 nm. The LPS content was determined by 1, 9 dimethyl methylene blue with standard LPS in A510 nm (Apicella *et al*., 1994). The SDS-PAGE was carried out by a recommended procedure (Tsai and Frasch, 1982).

Lipopolysaccharide detoxification: The purified LPS was treated by 0.1 N NaOH (Sigma, USA) at 100°C for 2 h and pH was adjusted to 3.5 by HCl (Sigma, USA). Upper phase was removed with great care (Poelstra *et al*., 1997).

LAL and Rabbit pyrogen test: The Limulus Amebocyte Lysate (LAL) test was performed by Gel Clot kit (Haemachem, USA) following the USP and FDA guidelines for LAL testing. The rabbit pyrogen test was also performed as previously described by Goldstein *et al*. (1992).

Vaccination and challenge of mice with *B. abortus*: Five groups of female 6-8 weeks old Balb/c mice (12 mice in each group) were injected intraperitoneally with PBS, LPS (10 μg), D-LPS (10 μg), LPS (10 μg) + HSA (5 μg), *B. abortus S19* (5×10^4 CFU). Injection volumes were 0.2 mL mouse^{-1}. A second dose was given 4 weeks after the first dose. Blood was collected from five killed mice in each group 4 weeks after the second dose of the vaccine. Sera were collected and stored at -20°C until they were analyzed for antibody by an enzyme-linked immunosorbent assay (ELISA). A group of immunized mice were challenged intraperitoneally 4 weeks after the second dose of immunization with 5×10^4 CFU of virulent *B. aborus* 544 strain (provided by the Pasteur Institute of Iran). Spleens were aseptically collected from killed mice 4 weeks post-challenge. The *Brucella* count in term of CFU was determined by dilution and culture on Brucella agar as described previously by Winter *et al*. (1996).

ELISA: The ELISA was performed in 96-well flat-bottom polystyrene microtiter plates (Nunc, UK) using a recommended method with slight modification (Engvall and Perlmann, 1972). Briefly, wells were coated with purified *B. abortus* LPS and D-LPS at a concentration of 10 μg mL^{-1} in PBS-azide (0.01 M sodium phosphate, 0.14 M sodium chloride, 0.02% sodium azide from Sigma, USA; pH 7.5) by adding 100 μL to each well incubating the plates for 3 h at 37°C. The wells were washed three times with PBS-azide and were blocked by adding 100 μL blocking buffer (1% casein in PBS-azide) and the plates were incubated for 1 h at 37°C. Serial 2-fold dilutions of primary antibodies (100 μL) were made on the plates and the plates were incubated at 37°C for 1-3 h. Then the plates were incubated with peroxidase-conjugated rabbit anti-mouse IgG at a concentration of 1 μg mL^{-1} (100 μL per well) for 1-2 h at 37°C. The substrate used was TMB (BioRad, UK) at a concentration of 1 mg mL^{-1} and incubated for 10 min at room temperature. The action of enzyme was stopped by adding 50 μL of 20% sulfuric acid solution. Absorbance at 450 nm was determined with an ELISA reader (Labsystems Multiskan MCC/340; Fisher Scientific, Pittsburg, PA). The titer, expressed in Optical Density (OD) units, was obtained by multiplying the reciprocal dilution of the serum by the OD (A450 nm) at that dilution (Winter *et al*., 1996).

Statistical analysis: The statistical analysis performed was achieved by using statistical software SPSS version 14.2.1. Antibody titers of the studied groups of mice were expressed as means±standard deviations. The intensities of bacterial infection in spleen were expressed as mean log CFU±standard deviation per infected organ. The lower limit for the detection of infected spleen was log 2 CFU. Moreover, the differences in ELISA titers and in log CFU per infected organ were evaluated using Student's t-test. p-values less than 0.05 were considered significant.

RESULTS

Characterization of LPS of *B. abortus*: The purified LPS from *B. abortus* by butanol extraction had less than 2% (w/w) contamination of protein and less than 1% (w/w) contamination of nucleic acids. LPS was added to 14% polyacrylamide SDS-PAGE gels containing 4 M urea (Fig. 1) stained with silver, resulting in patterns seen previously with LPS of *B. abortus* (Goldstein *et al*., 1992).

LAL test: In the LAL assay, 1 mg of LPS- *B. abortus* was found to contain 25×10^4 Endotoxin Units (EU), whereas 1 mg of LPS-*E. coli* was found to contain 12×10^6 EU. The increased reactivity of LPS-*E. coli* compared with that of LPS- *B. abortus* in the LAL assay is probably due to its different lipid structure (Goldstein *et al*., 1992). On the other hand, 10, 25, 50, 100, 200 and 400 ng mL^{-1} concentrations of D-LPS were negative in the LAL test; this showed that D-LPS-*B. abortus* was less toxic than LPS.

Rabbit pyrogen test: Three groups of three rabbits were inoculated with serial dilutions of *B. abortus* LPS, *B. abortus* D-LPS, or *E. coli* LPS; these groups were observed for changes in their body temperatures every hour for 3 h. The dose of LPS that induced 50% of the maximal increase in temperature (EC_{50}) for LPS-*B. abortus*, 7×10^{-2} mg mL^{-1}, was approximately 100 fold greater than the EC_{50} for LPS-*E. coli*, 5×10^{-4} mg mL^{-1}. It was indicated that LPS-B. abortus was less pyrogenic than LPS-*E.coli* and was less likely to produce endotoxic shock. On the other hand, 50, 100, 150, 200, 250, 300, 350 and 400 µg mL^{-1} concentrations of D-LPS in 3 mL kg^{-1} body weight were unable to increase the body temperature of the tested animals.

Immune response in mice: The intraperitoneal immunization of mice with *B. abortus* LPS, LPS-HSA and D-LPS induced antibody titers either 4 weeks after the first dose of vaccination or 4 weeks after the second dose of vaccination ($p<0.05$) (Table 1). Antibody titer of LPS was significantly higher than the titer of D-LPS (($p<0.05$).

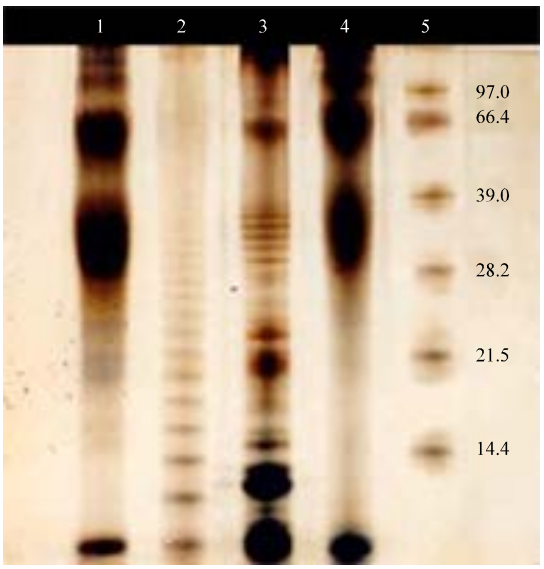

Fig. 1: SDS-PAGE of LPS of *B. abortus*, *E. coli* and Salmonella typhimurium: samples of 10 µg (lane 1) and 20 µg (lane 4) of LPS-*B. abortus* and 10 µg of LPS-*E. coli* (2) and *S. typhimurium* (3) were used. The slower-migrating smear in lanes 1 and 4 and the slower migrating set of bands in lanes 2 and 3 represent Intact LPS (i.e., lipid A, Core and o-linked sugars). The fast migrant bands in all lanes represent lipid A without O-linked sugars

Table 1: Anti-*B. abortus* LPS IgG ELISA titers of mouse sera[a]

		Anti-*B. abortus* LPS IgG ELISA titer (mean±SD)	
Groups	Vaccine	4 weeks after the first dose	4 weeks after the second dose
1	*B. abortus* LPS, i.p.	2.68±0.98	14.853±5.99
2	*B. abortus* D-LPS, i.p.	0.821±0.37	2.671±1.06
3	*B. abortus* LPS-HSA, i.p.	13.96±3.16	24.512±7.26
5	Saline control	<3	<4

[a]Mice were immunized i.p. Two doses of vaccine were given 4 weeks apart. Sera were collected from five of each group 4 weeks after the dose, 4 weeks after the second dose. The data are expressed in OD units

Table 2: Log number of CFU in Spleen 4 weeks after intraperitoneally challenge with 5×10^4 CFU of *B. abortus* 544[a]

Groups	Vaccine	Log CFU (mean±SD) spleens
1	*B. abortus* LPS, i.p.	4.271±0.02
2	*B. abortus* D-LPS, i.p.	4.22±0.03
3	*B. abortus* LPS-HSA, i.p.	3.96±0.86
4	*B. abortus* S19	2.146±0.18
5	Saline control	4.968±0.23

[a]Mice were immunized interaperitoneally with two doses 4 weeks apart and then challenged interaperitoneally 4 weeks after the second dose of immunization. Bacterial CFU were determined at the indicated times after challenge by plating organ homogenates on brucella agar. The limit of detection was 2 CFU per organ

Interestingly, the antibody titer obtained by LPS-HSA vaccine 4 weeks after the first dose of vaccination was five-fold higher than the titer obtained with the LPS vaccine ($p\leq0.05$).

Protection of mice after challenge: The protective efficacy of vaccines was measured by determining the clearance of spleens of mice against the challenge strain 4 weeks post challenge (Table 2). The protection conferred by LPS, LPS-HSA, D-LPS and S19 *B. abortus* strain inoculation was statistically significant and different from that obtained by PBS ($p<0.007$). The LPS-HSA vaccine provided significantly higher protection than that of LPS ($p<0.01$); however, the greatest protection provided was with the inoculation of *B. abortus* S19 in that significant differences between the protection obtained by S19 and that obtained by LPS, D-LPS and LPS-HSA were observed ($p<0.01$). However, the difference between the protection obtained by LPS and that by D-LPS was not statistically significant ($p<0.995$). Collectively, the results of the current study indicate that antibody titers against LPS are higher than that against D-LPS but the protection is the same.

DISCUSSION

LPS has been long seen as an ideal vaccination molecule because of its high molecular weight and strong immunogenicity. Nevertheless, adverse effects of LPS have been problematic; therefore, many studies have been exerted to assess the safer and more potent modailities of LPS in vaccination. In the current study, LPS-*B. abortus*, compared to LPS-*E. coli* in LAL and rabbit pyrogen tests, was less potent in toxicity by 10,000 folds and less potent in inducing fever in rabbits by about 100 folds, respectively. The toxicity of lipid A was also changed by alkaline treatment. The difference in toxicity between LPS-*B. abortus* and LPS-*E. coli* found in the present study is probably due to variations in their lipid A structures. In consistence with Goldstein study, we found that LPS-*B. abortus* was considerably less likely to induce endotoxic shock than LPS-*E.coli*. This property of LPS-*B. abortus* suggests that it might be considered as a candidate carrier for immunoconjugates in the development of vaccines (Goldstein *et al*., 1992; Pakzad *et al*., 2010).

The current study showed no similarity in toxicity and pyrogenicity between LPS-*B. abortus* and D-LPS-*B. abortus*. Different doses of D-LPS-*B. abortus* have shown that D-LPS was by far less toxic and pyrogenic, in rabbits fever and LAL tests, than ordinary LPS indicating that the D-LPS is safe and quite suitable for the use in vaccination. The decreasing antibody titer in D-LPS group compared to LPS group is probably due to decline of D-LPS immunogenicity. However, the protection test based on CFU number of target bacteria showed that both LPS and D-LPS yielded very close protection levels. This important finding indicates that difference in antibody titer can not influence the D-LPS-based protection. Moreover, this indicates that the protection gained by D-LPS or LPS was not solely attributed to the titer of the produced anti-*B. abortus* antibodies.

On the other hand, the highest titer response of anti-*B. abortus* antibodies in LPS-HSA group reflects an essential role of the HSA component in enhancing the potency of the currently used vaccine. These findings are consistent with some of recent reports that confirmed that LPS and LPS-GOMP in mouse induced decent protection against the corresponding virulent strains (Bhattacharjee *et al*., 2002; Jamalan *et al*., 2010). This might be explained that HSA, probably any other high molecular weight protein, can effectively increase the immunogenicity of LPS vaccine either by increasing the overall molecular weight of the immunogen or the combination of the two components can lead to remodeling of the 3-dimensional conformational structure of LPS allowing to expose more immunodominant epitopes to the immune system of the vaccinated animal. This phenomenon needs to be studied thoroughly in order to find the most suitable combination for LPS-based vaccines against *B. abortus* bacteria.

Nevertheless, the greatest protection provided in the current study was by *B. abortus* S19 suggesting that the cell-mediated immunity elicited by live vaccine induced much better protection than other forms of vaccination used in this study. This finding is supported by the results of other reports where live bacteria vaccines affected maximally the spleen of tested animals in

reducing properly the intensity of infection (Bhattacharjee *et al*., 2002). The mechanism of the immune response against *B. abortus* and vaccines protection against brucellosis have not been well described. The complement-mediated bacterial killing (Corbeil *et al*., 1988), antibody-dependent cytotoxicity by NK cells or macrophages and phagocytosis and subsequent killing by activated macrophages (Jones and Winter, 1992; Elzer *et al*., 1994) are potential mechanisms of protection in which antibody might play a role. However, the use of live vaccines and the use of real bacteria are hazardous when used in human beings. Therefore, LPS products-based vaccines are still the most suitable and accepted forms of vaccines against brucellosis.

CONCLUSIONS

Taken together, the current study showed that LPS and most interestingly D-LPS are potent vaccines against brucellosis. Above all, *B. abortus* LPS vaccines were found to be much safer than *E. coli* LPS vaccines. Most importantly, the D-LPS vaccine was found the safest at all and exerted no toxicity at all where it conferred the same level of protection of LPS vaccines. Therefore, it is recommended to conduct more vaccines' modifications caring out to elicit antibodies and to enhance cell-mediated immune responses. This might be achieved by the addition of protein antigens in fusion protein form with HSA (gene fusion) leading to inhibition of local, as well as disseminated infections.

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