Improved Immunogenicity of Tetanus Toxoid by *Brucella abortus* S19 LPS Adjuvant

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ABSTRACT

Background: Adjuvants are used to increase the immunogenicity of new generation vaccines, especially those based on recombinant proteins. Despite immunostimulatory properties, the use of bacterial lipopolysaccharide (LPS) as an adjuvant has been hampered due to its toxicity and pyrogenicity. *Brucella abortus* LPS is less toxic and has no pyrogenic properties compared to LPS from other gram negative bacteria. **Objectives:** To evaluate the adjuvant effect of *B. abortus* (vaccine strain, S19*)* LPS for tetanus toxoid antigen (TT) and to investigate the protective effect of different tetanus vaccine preparations. **Methods:** LPS was extracted and purified from *B. abortus* S19 and KDO, glycan, phosphate content, and protein contamination were measured. Adipic acid dihydrazide (ADH) was used as a linker for the conjugation of TT to LPS. Different amounts of *B. abortus* LPS, TT, TT conjugated with LPS, and TT mixed with LPS or complete Freund's adjuvant (CFA) were injected into mice and antibody production against TT was measured. The protective effect of induced antibodies was determined by LD50. **Results:** Immunization of mice with TT+LPS produced the highest anti-TT antibody titer in comparison to the group immunized with TT without any adjuvant or the groups immunized with TT-LPS or TT+CFA. Tetanus toxid-S19 LPS also produced a 100% protective effect against TT in immunized mice. **Conclusion:** These data indicate that *B. abortus* LPS enhances the immune responses to TT and suggest the possible use of *B. abortus* LPS as an adjuvant in vaccine preparations.

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INTRODUCTION

There is an increasing interest in the development and use of subunit vaccines because they contain the minimal antigenic structures necessary for generation of protective immunity and appear to be safer than conventional vaccines (1). A major problem in this context, however, is that the immunogenicity inherent in complex antigens is lost with simpler structures of lower molecular weight, such as peptides or proteins. One of the methods for enhancing the immunogenicity of these simpler antigens without introduction of significant chemical complexity is the use of adjuvants. Nowadays adjuvants are extensively used as immuno-stimulator and immuno-modulator compounds to design subunit vaccines. The chemical nature of adjuvants, their mode of action and the profile of their side effects are highly variable (2). Adjuvants can be classified into two groups based on their dominant mechanism of action: vaccine delivery systems and immunostimulatory adjuvants. Immunostimulatory adjuvants are derived from pathogens and represent pathogen associated molecular patterns (PAMPs) such as LPS, MPL and CpG DNA (3). Adjuvants can be used by two different methods; either conjugated to the antigen or mixed with the antigen.

The adjuvant effect of lipopolysaccharide (LPS) was first demonstrated in 1956 (4). LPS, a major amphiphilic molecule located at the outer membrane of gram-negative bacteria, is a classic immunostimulatory adjuvant composed of O-specific polysaccharide, core oligosaccharide and lipid A (5). The lipid A region of LPS contains most of the biological activities including adjuvanticity and toxicity (6,7). LPS induces immune responses through TLR2 and TLR4. LPS stimulates production and release of various growth factors, inflammatory mediators, and proinflammatory cytokines such as IL-6, tumor necrosis factor-α (TNF-α), IL-12 and IFN-γ, enhances the Th1/Th2 cytokine ratio and increases antigen uptake, processing and presentation (8). LPS has a diverse array of biological activities including pyrogenicity (human, rabbit), induction of cytokines, anti-tumor activity, activation of complement, macrophage and granulocytes, and B-cell mitogenicity (2). The adjuvanticity effect of LPS was demonstrated for both humoral (4) and cell mediated immunity (9). The mode of action of LPS as an adjuvant for protein antigens has been mainly through T-cell independent polyclonal B-cell mitogenicity (10). But the adjuvanticity of LPS for polysaccharide antigens has been linked to the regulation of T cells, particularly the T-suppressor cells (11). However, the practical use of LPS as an immunological carrier in vaccines has been hampered due to its highly toxic and pyrogenic nature, even at minute doses. This has led to a search for naturally occurring or modified forms of LPS with reduced endotoxicity but with strong adjuvant activity (12).

LPS extracted from *Brucella abortus* (LPS-BA) has less toxicity and no pyrogenic properties in comparison to other bacterial LPS and it is also a potent inducer of the Th1 pathway. Thus, it may be considered as a candidate carrier for immune conjugates in the development of vaccines (13,14).

The antigen selected for our study was tetanus toxoid (TT) which is a thymus-dependent antigen with excellent immunogenic properties. It stimulates development of anti-TT antibodies. TT has been used in vaccines for the prevention of tetanus which is a nervous system disease characterized by severe muscle spasms caused by the toxin tetanospasm produced by *Clostridium tetani* organisms (15). Measurement of anti-TT antibody levels in patients' sera has been used to assess the immunological response of patients subsequent to vaccination with TT.

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In the current study, we evaluated the immune stimulatory properties of *B. abortus* LPS as an adjuvant in combination with TT antigen. Immune responses to TT along with complete Freund's adjuvant (CFA) or *B. abortus* LPS were measured in BALB/c mice. The protective effect of different vaccine preparations was also assessed by challenging the immunized mice with a median lethal dose (LD_{50}) of TT and following the lethality.

MATERIALS AND METHODS

Animals and Bacterial Strains. Female BALB/c mice (6-8 weeks) were purchased from Pasteur Institute of Iran (Tehran, Iran) and were used for experimental purposes with the approval of the Animal Ethics Committee of the Ministry of Health and Medical Education (Tehran, Iran). Tetanus toxoid (TT) antigen was obtained from Razi Vaccine and Serum Research Institute (Karaj, Iran) and *B. abortus* S19 vaccine strain was purchased from Pasteur Institute of Iran.

Extraction and Characterization of *B. abortus* LPS. Extraction of LPS was performed by the hot-phenol method according to Leong (16). In brief, 1.5 g wet weight of cells was suspended in distilled water, followed by sonication for 90s. Lysed cells were added to phenol solution (90% w/v) and maintained at 67°C for 15 min. The mixture was centrifuged for 30 min at 6000 ×g and the phenol layer was removed. The LPS in the phenol phase was precipitated by adding three volumes of cold methanol solution (99% methanol, 1% sodium acetate) and resuspended in distilled water. For further enzymatic digestion, DNase (10 unit/ml), RNase (10 unit/ml), lysozyme (25 μg/ml) and proteinase K (100 μg/ml) were added to reduce nucleic acid and protein contamination of the extracted LPS samples. Finally, LPS solution was dialyzed against distilled H_2O and lyophilized. Lyophilized LPS was dissolved in 0.1 M NaCl and fractionated in a Sephadex G-50 column (30 by 1 cm) equilibrated with 0.1 M NaCl. Fractions of 2 ml volume were collected, and the presence of LPS was monitored by a glycan assay (17). The fractions that contained LPS were pooled and dialyzed against multiple changes of distilled H_2O at 4°C for 48 h, and finally lyophilized and stored at -20°C until used. Purified LPS was analyzed for protein component by the Bradford method and for 2-keto-3-deoxyoctulosonic acid (KDO, a unique component of LPS and the most popular marker of LPS from gram-negative bacteria), glycan, and phosphate content by the method described by Karkhanis *et al*. (18), Raff and Wheat (17) and Ames (19), respectively. To determine the quality of purified LPS, SDS-gel electrophoresis with LPS-specific silver staining was performed according to Tsai and Frasch (20).

Derivatization of LPS with ADH. Adipic acid dihydrazide (ADH) is a chemical substance used for cross-linking water-based emulsions. The purified LPS was derivatized with ADH as described previously (21-25). Briefly, purified LPS (5 mg in 1 ml of 50 mM NaCl) was brought to pH 10.5 to 11 with 0.1 M NaOH, and an equal amount of CNBr (1 g/ml of acetonitrile) was added. The reaction was carried out for 6 min on ice, and the pH was maintained at 10.5 to 11 with 0.1 M NaOH. An equal volume of 0.8 M ADH in 0.5 M $NaHCO_3$ was added, and the pH was adjusted to 8.5 with 0.1 M HCl. The reaction mixture was stirred at 3-8 ℃ overnight, dialyzed against 50 mM NaCl at 4°C for 24 h and then dialyzed against distilled H_2O at 4°C for 48 h. Finally, the mixture was lyophilized and stored at -20 °C until used.

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Conjugation of TT to LPS. ADH-derivatized LPS (5 mg) was dissolved in 2 ml of 50 mM NaCl solution. An equal weight of TT was added, and the pH was maintained at 5.1 to 5.5 with 0.1 M HCl. The reaction mixture was put on ice, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) was added to a final concentration of 0.05 M, and the pH was maintained at 5.1 to 5.5 with 0.1 M HCl for 4 h. The reaction mixtures were dialyzed against 0.1 M NaCl for 48 h with multiple changes of outer fluid and then were passed through a column (1 by 64 cm) of Sephadex G-150. Samples (1.5 ml) were collected, and the presence of conjugated product (TT-ADH-LPS) was monitored by measuring protein and glycan content in fractions using the Bradford method and glycan assay (17), respectively. The fractions that had protein and glycan were pooled and stored at 4°C. The conjugation reaction defined as the degree of derivatization of LPS and TT was 1:1.

Animal Immunization. Different amounts (5 or 10 μg) of *B. abortus* LPS alone, TT alone, TT conjugated with LPS or CFA [TT-LPS or TT-CFA] and TT mixed with LPS or CFA [TT+LPS or TT+CFA] were injected subcutaneously into twelve different groups of 6-8 week old female BALB/c mice (three mice per group) according to Table 1. The booster injections were carried out on days 14 and 28 after the first injection. The immunized animals were bled on days 0, 28 and 42 and the immune sera were separated, and stored at -20ºC until use.

Table 1. Different compounds that used for injection.

Groups/ Compound			
1	Control group (100 μl normal saline)	7	TT+CFA: 5 μg of TT dissolved in 100 μl of CFA
2	100 μl of complete Freund's adjuvant (CFA)	8	TT+CFA: 10 μg of TT dissolved in 100 μl of CFA
3	5 μg of TT without any adjuvant	9	TT+LPS : 5 μg of TT along with LPS
4	10 μg of TT without any adjuvant	10	TT+LPS : 10 μg of TT along with LPS
5	5 μg of LPS alone	11	LPS-TT: 5 μg of LPS conjugated with TT
6	10 μg of LPS alone	12	LPS-TT: 10 μg of LPS conjugated with TT

Measurement of TT Antibody Levels in Immunized Mice. An enzyme linked immunosorbent assay (ELISA) was used for determining TT antibody levels in immunized mice. The ELISA was performed in 96-well flat-bottom polystyrene microplates (Nunc, Denmark). The wells were coated with 5 μg of TT in 100 μl coating buffer (carbonate sodium-bicarbonate sodium; pH 9.6) and incubated overnight at 4°C. The wells were washed three times with washing solution (50 mM Tris, 0.1 M NaCl, 0.05% Tween-20; pH 8.0). The wells were blocked by adding a blocking buffer (50 mM Tris, 0.1 M NaCl and 1% BSA) and were incubated on a shaker at room temperature (RT) for 1 h. After five washes, serial two-fold dilutions of the sera were added to the plates, and the plates were shaken at RT for 1 h. The wells were washed five times, 100 μl biotinylated anti-mouse IgG was added to the wells at a 1/10000 dilution, and the plates were incubated at RT for 1 h. Peroxidase conjugated avidin at a 1/1000 dilution was then added to the wells, and the plates were incubated on the shaker at RT for 1 h. After five washes, 100 μl (1 mg/ml) of 3, 3', 5, 5' tetra methyl benzidine (TMB)

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substrate was added to the wells, and the plates were incubated at RT for 30 min. Finally, 100 μl stop solution (2 M H_2SO_4) was added to the wells, and absorbance was measured at 450 nm using an ELISA plate reader. The antibody titers were expressed in OD units and calculated by multiplying the reciprocal dilution of the serum by the OD at that dilution.

In the other test, serum samples from each group were added at different dilutions and then assayed by ELISA. Log titers were obtained from the $\log_{10}$ of the endpoint titration, using serial twofold dilutions of the sera. The endpoint titers represent the intercept of the linear part of the titration curve and the x axis. In all cases, the reported titers were obtained following the second injection.

Protective Effect of Different Vaccine Preparations Against TT. To determine median lethal dose (LD50) of TT in mice, groups of 5 BALB/c mice were intraperitoneally injected with increasing doses of TT (10^{-7}, 10^{-6} and 10^{-5} dilution). Then, the protective effect of different vaccine preparations was assayed in immunized mice injected with the TT LD50 by following the development of signs of toxicity and lethality for 48 h.

Statistical Analyses. Antibody titers of mouse groups were expressed as means ± standard deviations (SD). The significance of differences in ELISA titers was determined by Student's *t-test*.

RESULTS

Characterization of *B. abortus* LPS. As expected, purified LPS had a heterogeneous structure and appeared as a smear with two distinctive band zones (low and high molecular weight) on a silver-stained SDS gel (Figure 1) similar to the patterns previously reported for the LPS of *B. abortus* (26).


Figure 1. Silver stained SDS-PAGE profile of *B. abortus* S19 LPS.

The purified LPS had less than 2% (w/w) protein contamination, in agreement with values obtained previously for highly purified LPS-BA (27). Based on KDO standard

curve, 4 µg KDO was detected in one mg of purified LPS. The glycan and phosphate contents were 29.3% and 0.76%, respectively.

Measurement of TT Antibody Levels in Immunized Mice. Anti-TT antibody levels in all groups after the first and second booster injections are shown in Figure 2.
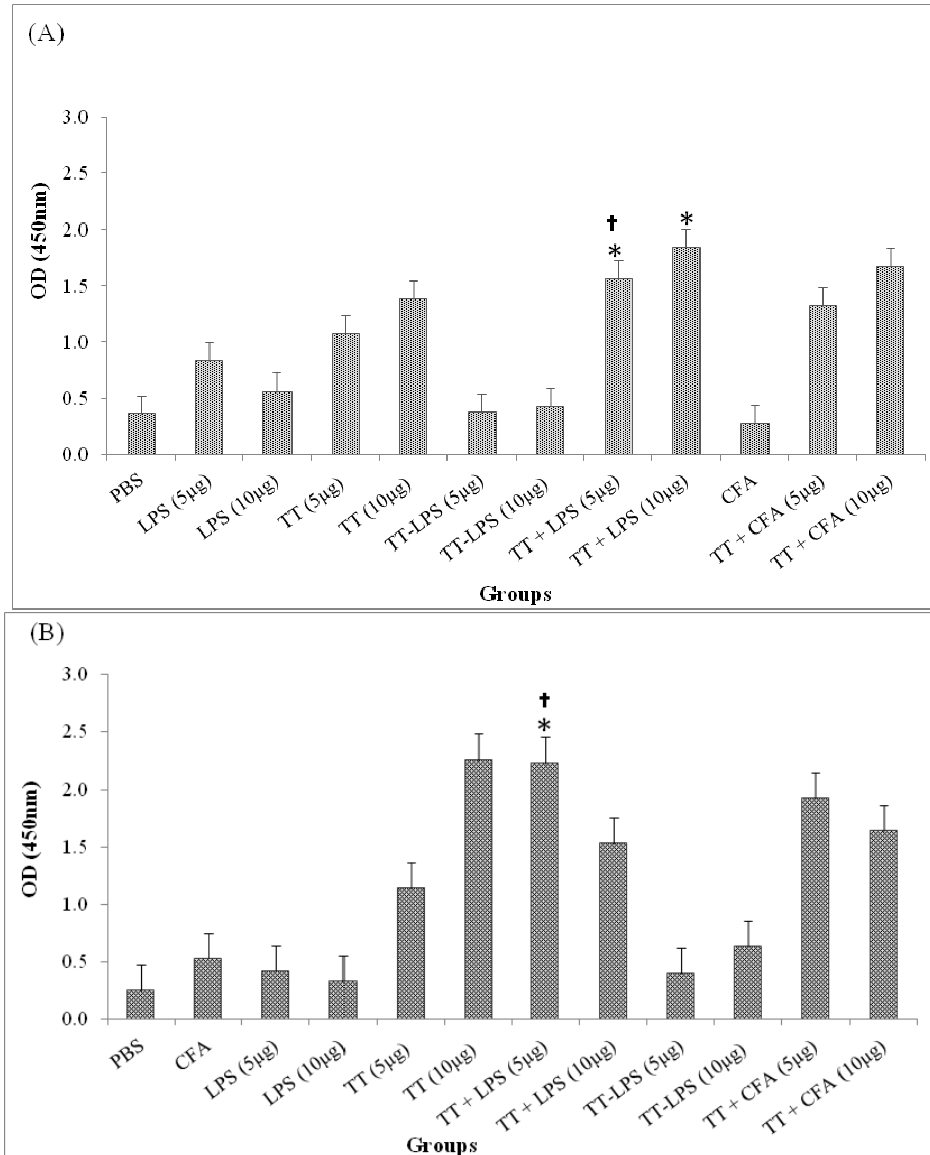
Figure 2. Induction of anti-TT antibody response by different compound injection. Antibody titers against TT in sera (diluted in 1:2) tested by ELISA, two week after the first booster (28 d) (A) and two week after the second booster injections (48 d) (B). BALB/c mice were immunized with TT antigen either alone or along with an adjuvant (*B. abortus* LPS or CFA). At two weeks post-immunization, mice were bled and sera of individual mice (diluted in 1:2), assayed by ELISA. Data are means ± SD OD in 450 nm. The levels of statistical significance for differences between test groups and the control untreated group were determined by the student's t-test [*$p < 0.05$, in comparison to group immunized with TT without any adjuvant; †$p < 0.05$, in comparison to group immunized with TT+CFA].
TT; tetanus toxoid antigen, CFA; Freund's adjuvant, TT-LPS; TT conjugated with LPS
TT+LPS; TT mixed with LPS

The group immunized with TT+LPS (5 and 10 μg) had the highest anti-TT antibody level in comparison to groups immunized with TT-LPS, TT+CFA, TT, or LPS alone. After the second booster, anti-TT antibody levels significantly increased in groups immunized with TT (10 μg), TT+LPS (5 μg) and TT+CFA (5 μg). The anti-TT antibody titers are displayed in Figure 3. Among the different injected compounds (TT, LPS and CFA), TT+LPS (5 μg) was the most immunogenic compound and produced the highest IgG titer compared to IgG titers elicited against TT-LPS, TT+CFA, TT or LPS alone.
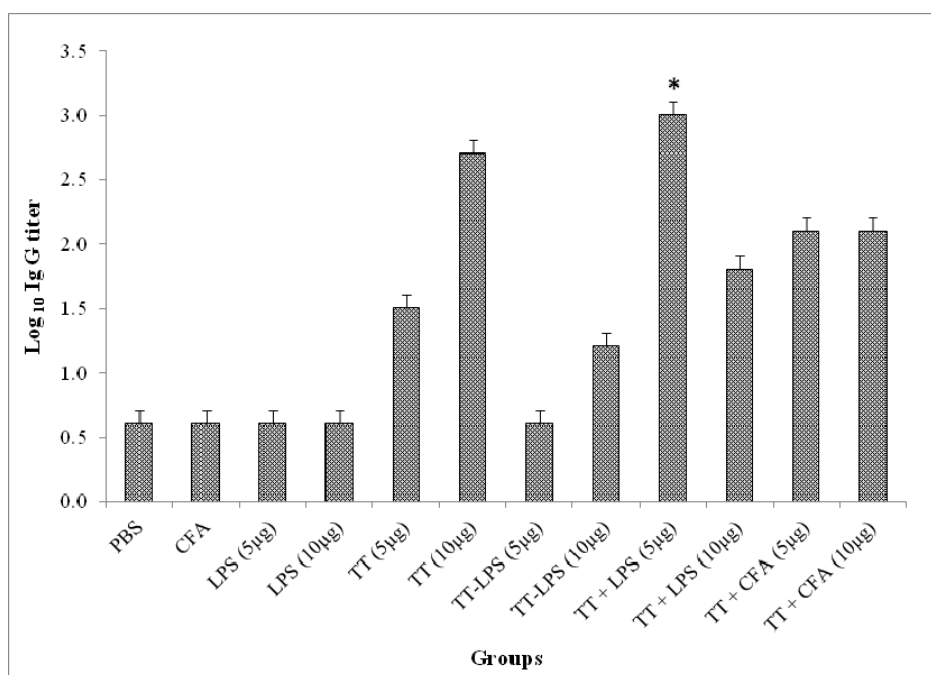
Figure 3. Anti-TT antibody titer assay. Serum samples from each group were added at different dilutions and then assayed by ELISA. Log titers were obtained from the $\log_{10}$ of the endpoint titration, using serial twofold dilutions of the sera. The endpoint titers represent the intercept of the linear part of the titration curve and the x axis. Results are expressed as the $\log_{10}$ of the ELISA titers. Group immunized with TT+LPS (5 g) produced significantly (*p < 0.05) the highest anti-TT antibody titer compared to with other groups that immunized with TT, TT+CFA or LPS alone.

Protective Effect of Different Vaccine Preparations Against TT. The dose that induced 50% mortality (LD50) for TT was determined to be 10^{-5} dilution per mouse. The protective effect of different vaccine preparations and lethality index results after injection of LD50 TT are presented in Table 2. The TT+LPS vaccine provided higher protection (100%) than other components such as TT+CFA.

Table. 2. The lethality data. The dose that induced 50% mortality (LD$_{50}$) for TT was determined to be 10^{-5} dilution per mouse. Groups of 5 or 3 BALB/c mice that were previously immunized by different vaccine preparation (Table 1) were intraperitoneally injected with of 10^{-5} dilution TT. The mice were observed for 48 h for the development of signs of toxicity and death. The TT+LPS vaccine provided higher protection than other components.

Groups	No. of mice	lethality
PBS	5	100%
CFA	5	100%
LPS (5μg)	3	100%
LPS (10μg)	3	100%
TT (5μg)	3	33%
TT (10μg)	3	33%
TT-LPS (5μg)	5	100%
TT-LPS (10μg)	5	100%
TT+LPS (5μg)	3	0%
TT+LPS (10μg)	3	0%
TT+CFA (5μg)	5	60%
TT+CFA (10μg)	5	40%

DISCUSSION

Recently, LPS has received extensive attention for application in several fields including i) design of new diagnostic methods (such as candidate antigens to develope new ELISA assay); ii) potential use of LPS as an inducer for the production of proinflammatory and immunosuppressive cytokines by tumor cells through TLR4 ligation (28); iii) use of LPS-BA as one of the major vaccine candidates for human brucellosis; and iv) as an adjuvant to boost immune responses against other components.

In several studies, it has been shown that *B. abortus* LPS has unique properties such as less toxicity and no pyrogenic properties in comparison to other bacterial LPS (13,14). In this context, Goldstein *et al*. (14) indicated that the LPS from *B. abortus* acts as a T-independent type 1 carrier in mice and is much less toxic than the LPS from *E. coli* in causing endotoxic shock. They suggested that it may be considered as a candidate carrier for immunoconjugates in the development of vaccines.

In a previous study (29), we evaluated IL-10 and IL-2 production in response to LPS extracted from *B. abortus* (a field isolate) and *B. melitensis* vaccine strain (Rev1). We observed that LPS from both *Brucella* strains are potent inducers of IL-10 in human peripheral blood mononuclear cell culture. Also, IFN-γ priming is able to significantly down-regulate IL-10 production and up-regulate IL-12 production in these cells. In addition, our results showed that the ability of *B. abortus* LPS to induce IL-12 in CD14$^+$ cells is more potent than *B. melitensis* (Rev1) LPS (29).

In another study, we showed that immunization of mice with the 17th tuberculin purified protein (PPD) fraction along with *B. abortus* LPS can induce a Th1-type cytokine response characterized by a high IFN-γ/IL-5 ratio, while immunization with PPD or the 23rd PPD fraction along with the same adjuvant resulted in a mixed Th1/Th2-type cytokine response (30). The titer of specific IgG in the PPD, 17th and 23rd PPD fraction test groups, was significantly higher in mice immunized with a combination of antigen and CFA compared with ones immunized with bare antigens or antigen + LPS. Therefore, *B. abortus* LPS could diminish IgG production by 17th PPD fraction but it had no effect on the IgG production by PPD, 23rd PPD fraction and also BCG.

Therefore, in the current study, we chose *B. abortus* vaccine strain (S19) LPS and investigated its potency as an adjuvant with TT antigen. The original part of this study was the application of *B. abortus* S19 LPS as an adjuvant and CFA as a classic adjuvant along with TT antigen and the evaluation of these adjuvants' potency to induce anti-TT IgG antibodies.

Different concentrations (5 or 10 μg) of *B. abortus* LPS alone, TT alone, TT conjugated with LPS [TT-LPS] and TT mixed with LPS or CFA [TT+LPS or TT+CFA] were injected subcutaneously into the different groups of mice. Schromm *et al.* have shown that the conjugation process has a better effect on enhancing antigenicity and immunogenicity of antigens (31) and causes higher antibody production in BALB/c mice. But incontrast to our expectation the conjugated product (TT-LPS) was not able to produce higher antibody titers in mice. One possibility would be that the conjugation has influenced the immunogenicity and have decrease exposure of the immunodominant epitopes of the two combined components (TT-LPS) to the immune system of the vaccinated animal either by remodeling of the 3-dimensional conformational structure of a component (LPS Lipid A) or by interfering with the binding of LPS to its particular receptor (TLR4) on the target cell surface.

To confirm adjuvanticity of LPS, Arora *et al.* showed that co-immunization with TT+LPS of *Pseudomonas aeruginosa* significantly elevated the anti-TT IgG concentrations in the plasma of vitamin A-sufficient and deficient rats as compared with identical rats immunized with TT alone. They also observed that the adjuvant effect of LPS on the antibody response toward TT is mediated at least in part through production of TNF-α. Although the basic response to TT was consistently low in the vitamin A-deficient condition, both vitamin A-sufficient and deficient rats produced equal anti-TT IgG concentrations after co-immunization with TT+LPS (32).

In agreement with other studies (14,33), our results indicated that *B. abortus* LPS is safe and quite suitable for use in vaccination. Two weeks after the second injections, the highest antibody titers were detected in the group immunized with 5 μg TT+LPS (endpoint 1/1024) followed by the group immunized with 10 μg TT (endpoint = 1/512). According to our results, the optimal amount of LPS-BA as adjuvant mixed with TT is 5 μg. Antibody production in mice has a direct relation with the TT intake of mice. The

increasing antibody titers in the TT+LPS group compared to TT+CFA indicated that LPS-BA has a better adjuvanticity effect than CFA. When the results in Figure 2 and 3 are compared, the outcome may be mixed for the TT+LPS combinations and TT on its own, which depending on the dose, can give a stronger IgG response. Thus, a combination of 5 μg LPS with 5 μg TT could significantly ($p < 0.05$) increase antibody titers compared with the same amount of TT antigen alone. Whereas, in a quoted study of Jamalan *et al*. (30) there is evidence that LPS can diminish IgG production by 17th PPD fraction but it has no effect on the IgG production by PPD, 23rd PPD fraction and also BCG. Therefore, it seems that the effect of *B. abortus* LPS on the IgG production was associated with the antigen preparations in use.

LPS-BA as adjuvant mixed with TT antigen provided the greatest protection against TT, such that the least lethality was found in the group immunized with TT+LPS compared to other groups.

Together, these data support the potential use of *B. abortus* LPS as an adjuvant. However, further researches using different antigens would be compliant in providing complementary and elucidative data about the potential of *B. abortus* LPS as an adjuvant which enhances both the innate and cell-mediated immune responses to these antigens.

ACKNOWLEDGEMENTS

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