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Cell mediated immune response after challenge in Omp25 liposome immunized mice contributes to protection against virulent *Brucella abortus* 544

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ABSTRACT

Brucellosis is a disease affecting various domestic and wild life species, and is caused by a bacterium *Brucella*. Keeping in view the serious economic and medical consequences of brucellosis, efforts have been made to prevent the infection through the use of vaccines. Cell-mediated immune responses [CMI] involving interferon gamma and cytotoxic $CD4^+$ and $CD8^+$ T cells are required for removal of intracellular *Brucella*. Omp25 has been reported to be involved in virulence of *Brucella melitensis*, *Brucella abortus* and *Brucella ovis*. In our previous study, we have shown the protective efficacy of recombinant Omp25, when administered intradermally. In this study, the recombinant Omp25 was formulated in PC–PE liposomes and PLGA microparticles, to enhance the protective immunity generated by it. Significant protection was seen with prime and booster liposome immunization in Balb/c mice against virulent *B. abortus* 544 as it was comparable to *B. abortus* S-19 vaccine strain. However, microparticle prime and booster immunization failed to give better protection when compared to *B. abortus* S-19 vaccine strain. This difference can be attributed to the stimulation of cell mediated immune response in PC–PE liposome immunized mice even after challenge which converted to cytotoxicity seen in $CD4^+$ and $CD8^+$ enriched lymphocytes. However, in PLGA microparticle immunized mice, cell mediated immunity was not generated after challenge as observed by decreased cytotoxicity of $CD4^+$ and $CD8^+$ enriched lymphocytes. Our study emphasizes on the importance of liposome encapsulating Omp25 immunization in conferring protection against *B. abortus* 544 challenge in Balb/c mice with a single dose immunization regimen.

1. Introduction

Brucellosis accounts for almost 500,000 cases annually, thus, emerging as one of the most common zoonosis [1,2]. Because of the serious economic consequences of brucellosis, efforts have been made to prevent the infection via the use of vaccines [3]. *Brucella* has been known to infect and multiply inside host macrophages. IFN-γ is one of the Th1 stimulatory cytokine that helps in clearance of intracellular pathogen and hence, may aid in *Brucella* clearance [4–6].

The major outer membrane proteins [OMPs] of *Brucella* spp. were initially identified in the early 1980s and characterized as potential immunogenic and protective antigens [7–9]. PCR–RFLP analysis of *omp25* suggested that the *omp25* gene is highly conserved in *Brucella* species, biovars and strains [10]. *Brucella* species lacking Omp25 have been shown to be attenuated in mice as well as cattle [11–13]. There are reports which have shown that DNA vaccine of Omp25 of *Brucella meltensis* is protective against the virulent *B. melitensis* challenge in mice [14]. This makes the Omp25 a viable vaccine target.

Both the humoral antibody response and $CD4^+$ and $CD8^+$ T cells mediate the acquired immunity against *B. abortus* infection in murine model [9,10] but the latter are essential for clearance of intracellular bacteria [15]. For better stimulation of cell mediated immune response, different controlled release formulations of Omp25 were studied. Non-toxic, biodegradable and biocompatible polymers like PLGA have been used as controlled release delivery vehicles for many years [16]. Liposomes have been shown to induce cell mediated immune response [17] and to possess adjuvant properties against bacteria [18–27].

Abbreviations: ELISA, enzyme linked immunosorbent assay; HRP, horse radish peroxidase; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffer saline; TMB, 3,3′,5,5′ tetramethyl benzidine; LB, Luria Bertani; DMEM, Dulbecco's modified eagle medium; PMSF, phenyl methane sulfonyl fluoride; CTAB, cetyl trimethyl ammonium bromide; OMP, outer membrane protein; NH_4Cl, ammonium chloride; PC–PE, phosphatidylcholine–phosphatidylehtanolamine; PLGA, poly-L-co-glycolic acid; CD4, cluster of differentiation 4; CD8, cluster of differentiation 8.

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We have earlier reported that recombinant Omp25 (rOmp25) when administered intradermally, protects against virulent *Brucella abortus* 544 challenge [28]. In order to further improvise upon the efficacy of rOmp25, we investigated the vaccine potential of phosphatidylcholine–phosphatidylethanolamine (PC–PE) liposomal and poly-L-co-glycolic acid (PLGA) microparticle formulation of rOmp25 with a single immunization dose.

2. Materials and methods

2.1. Bacterial strains and plasmids

B. abortus S-19 and *B. abortus* 544 bacterial strains were obtained from Indian Veterinary Research Institute, Bareilly, India. Biosafety level 3 facilities were used to carry out any *Brucella* strain related experiments.

2.2. Purification of rOmp25

rOmp25 was purified using urea denaturation method as described previously [28]. Purified protein was dialyzed against PBS pH 7.4 and its concentration was estimated using Bradford's reagent. The concentration of LPS in purified protein was determined by Limulus amoebocyte lysate (LAL; Sigma). LPS concentration was found within permissible limit of the current standard of the European pharmacopeia for a vaccine dose. The endotoxin content was found to be 0.02 pg of LPS equivalent/μg of rOmp25.

2.3. Liposome preparation

Egg-PC (Sigma–Aldrich) and Egg-PE (Sigma–Aldrich) in a molar ratio of 8:2 were used. In a round bottom flask, 50 μmol of lipids dissolved in chloroform were added and lipid film was formed at 37 °C under vacuum by using a rotary evaporator (Wheaton) and desiccated for 2 h. 1 ml of PBS as control and 1 ml of rOmp25 (2.0 mg/ml) were used for hydration of lipid film which was suspended under N_2 atmosphere by hand shaken method and kept at 4 °C overnight. Liposomes were sonicated in a bath sonicator (Branson) at 25 °C for 30 min. Then they were ultracentrifuged 3 times in 1XPBS at 1,00,000 × *g* for 1 h. Finally liposomes were suspended in 1 ml of 1XPBS. The encapsulation efficiency was checked by lysis with Triton-X-100 and then estimating the protein content by Lowry's method. The size and zeta potential of the liposomes was determined on Zeta-sizer (Malvern Instruments).

2.4. Microparticle formulation

PLGA (lactide:glycolide::50:50) (Sigma–Aldrich) microparticle were prepared by double emulsion (W1/O/W2) solvent evaporation method. Briefly, 200 mg of PLGA was dissolved in 4 ml of Dichloromethane (DCM). Then 200 μl of the rOmp25 (5 mg/ml) was added drop wise to it during sonication at 30% energy for 1 min on ice bath (Sonics Vibra Cell). This emulsion was added drop wise to 1% PVA while homogenizing it for 10 min (IKA homogenizer) on ice bath. The emulsion was then kept on magnetic stirrer for organic phase evaporation. Then microspheres were separated by centrifugation at 3100 × *g* for 15 min and washed with MilliQ water to remove residual PVA. Final pellet of microspheres was suspended in 5 ml water and flash frozen in liquid nitrogen for freeze drying. The amount of protein encapsulated was estimated by dissolving the PLGA and precipitating the protein in acetonitrile. The pellet of protein was dissolved in 1% SDS and protein concentration was estimated as μg of protein/mg of microsphere using micro BCA kit (Pierce, Thermo fisher). The size of the microparticle was measured on Meta-sizer (Malvern Instruments) and morphology and shape was examined by Scanning electron microscopy (SEM).

2.5. Immunization of mice

The 6–8 weeks old female Balb/c mice were used for immunization. Six groups of six Balb/c mice namely, liposome control, liposome prime (liposome S), liposome booster (liposome B), microparticle control, microparticle prime (MP S), microparticle booster (MP B) were formed for immunization studies. Liposome control and microparticle control groups were injected on day 0 and 15 with empty liposomes and microparticles respectively. Liposome S and MP S groups of mice were injected subcutaneously with liposome/microparticle containing 50 μg of rOmp25 as a prime immunization on day 0 however; the liposome B and MP B groups of mice were injected subcutaneously with liposome/microparticle containing 50 μg of rOmp25 on day 0 and 15. Mice of each group were bled for serum collection on day 0, 7 and 15 after last immunization. Pre-immune control serum was obtained from unimmunized mice. Serum was separated from blood and stored at −80 °C till further analysis. The regulations of Institutional Animal Ethics Committee (IAEC) were followed in all mice experiments. BSL-3 animal facilities were used for keeping mice that were injected with *B. abortus* 544.

2.6. End-point antibody titer and isotyping

Antibody titer and isotypes of IgG, namely IgG1, IgG2a and IgG2b, in liposome and microparticle immunized mice sera were determined by Enzyme linked immunosorbent assay (ELISA) as described previously [28]. Different dilutions of anti-rOmp25 sera, of each time point from each immunized group, were prepared. The threshold value for titer determination was taken as the absorbance plus 3 times the standard deviation obtained at 1:100 dilution of pre-immune sera. Isotypes of IgG were determined by using anti-mouse IgG1–HRP, anti-mouse IgG2a–HRP and anti-mouse IgG2b–HRP conjugated antibodies (Santa cruz Biotechnology). The highest titer dilution of anti-rOmp25 antisera was used for isotyping, i.e. 1.0×10^5 for both liposomes and microparticle immunized mice sera. Dilution of anti-mouse IgG1–HRP, IgG2a–HRP and IgG2b–HRP used was 1:10,000.

2.7. In vitro cytokine measurement

Fourteen days after last immunization with PC–PE liposomes/PLGA microparticles of rOmp25 in mice, the control and experimental mice from each group were sacrificed and spleen was aseptically removed and spleen cells were isolated as described [28]. The spleen cells were isolated from two mice of each group for all the three times the experiment was repeated. Media supernatants of splenocyte culture were collected after 24, 48, and 72 h of stimulation and stored at −80 °C, till used for cytokine measurement. The cytokine levels were measured using BD OptEIA™ kits for IL-4, TNF-α and IFN-γ according to the manufacturer's protocol. The absorbance was read at 450 nm. Cytokine concentrations in the supernatants were calculated using linear regression equation obtained from absorbance values of standards provided by the manufacturer.

2.8. Protective efficacy

The mice immunized with liposome/microparticle of rOmp25, were challenged intraperitoneally with 2×10^7 CFU of virulent strain of *B. abortus* 544 after 14 days of last immunization in each group (6 mice in each group). A positive control of *B. abortus* S-19 mice group was intraperitoneally immunized with 10^5 CFU of