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Intradermal immunization with outer membrane protein 25 protects Balb/c mice from virulent *B. abortus* 544

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ABSTRACT

Brucella abortus is a causative agent of brucellosis, a zoonosis affecting the endemic areas, which infects domestic animals as well as humans, thus, posing a potential bioterror threat. Outer membrane protein 25 is conserved among the *Brucella* species. Omp25 mutant strain of *Brucella* is shown to be attenuated in mice emphasizing on the role of Omp25 in *Brucella* virulence. Moreover, Omp25 has been shown to inhibit TNF-α production in human macrophages, thereby, abrogating cell mediated immunity. In this study, we evaluated the immunogenic potential of recombinant Omp25 and its protective efficacy against virulent *B. abortus* challenge in Balb/c mice. Recombinant Omp25 was administered *via* two routes of immunization: intraperitoneal and intradermal. Dosage reduction was observed with intradermal immunization when compared with intraperitoneal immunization. A higher IgG1:IgG2b ratio suggested a strong Th2 bias of immune response in both the routes of immunization. *In vitro* stimulation of splenocytes from immunized mice resulted in high level of IL-4 along with increasing levels of IL-12 and IFN-γ indicating a mixed Th1 and Th2 type of immune response. Immunized mice were challenged with virulent *B. abortus* and splenic colonization of *B. abortus* reduced significantly in intradermally immunized mice. Intradermal immunization gave protection comparable to that of *B. abortus* S-19 strain. Cytokine levels in spleen homogenate after challenge revealed a cell mediated immune response with elevated levels of IL-12 and IFN-γ but no detectable amount of IL-4. This can be a possible reason behind the protection observed in mice after rOmp25 immunization. Thus, our study proposes recombinant Omp25 to be a potential subunit vaccine candidate against brucellosis.

1. Introduction

Brucellosis is an infectious disease that can be transmitted from animals to humans. Brucellosis has been a major cause of economic concern and bioterror threat in developing countries (Yagupsky and Baron, 2005). Brucellosis causes abortion in domestic ruminants, thus, becoming a principal concern. *Brucella*, the causative agent of brucellosis, is a gram negative, non-motile and non-spore forming bacterium. *Brucella* is a facultative pathogen that invades and multiplies intracellularly in macrophages inside the host system (Baldwin and Goenka, 2006; Bowden et al., 1995a,b; Godfroid et al., 2005). *Brucella suis* has been shown to prevent the programmed cell death of human monocytes, thus, multiplying intracellularly (Gross et al., 1999). Both humoral and cell mediated immune responses can change the course of infection of *Brucella* but cell mediated response is necessary for clearance of *Brucella* from host organism (Schurig et al., 2002). Th1 type of immune response mediated by IFN-γ helps in evasion of *Brucella* infection (Zhan et al., 1993). Many vaccines have been designed against brucellosis but commercially available vaccines are live and attenuated *Brucella* strains. These strains can revert back to pathogenic strain and may also interfere with the diagnosis. With these limitations, there is an urgent need for better vaccines which are safer to use.

Recombinant proteins are a wise choice for vaccine as they can never cause infection and moreover, can be modified in terms of adjuvant or route of immunization for antigen specific immune response. Outer membrane proteins are known to be protective antigens of the *Brucella* spp. (Verstreate et al., 1982) as monoclonal antibodies directed against 25–27 kDa Omps of *Brucella*, reduce the splenic counts of *Brucella* (Cloeckaert et al., 1996a; Jacques et al., 1992). Omp25 of *Brucella* is found to be conserved among the

Abbreviations: IP, intraperitoneal; ID, intradermal; 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; IFA, incomplete Freund's adjuvant; NH_4Cl, ammonium chloride.

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species, biovars and strains (Cloeckaert et al., 1995). Omp25 is an outer membrane protein of *Brucella* which is bound to the peptidoglycan layer of *Brucella* (Cloeckaert et al., 1990, 1992, 2002). Outer membrane protein Omp25 is one of the virulent factors and major antigens involved in survival of *Brucella* as *Brucella* species lacking Omp25 gene are attenuated in mice (Edmonds et al., 2001, 2002a,b; Cloeckaert et al., 1996b). Omp25 has been shown to inhibit TNF-α production in *B. suis* infected human macrophages (Jubier-Maurin et al., 2001). TNF-α is an important component of the cytotoxic immune response which is needed for the *Brucella* clearance from macrophages. TNF-α stimulates a cytokine cascade that involves IFN-γ which in turn stimulates Natural killer cells for cytotoxicity. TNF-α also stimulates phagocytic activity of macrophages and induces apoptosis in infected macrophages (Zhan et al., 1996; Zhan and Cheers, 1998). Based on the significance of Omp25 in *Brucella* virulence, we investigated the Omp25 of *Brucella abortus* as a subunit vaccine candidate against brucellosis. Subunit vaccines are inert, can be better controlled as they are of higher homogeneity and their composition is completely known. Therefore, subunit vaccines are considered a better and safer option for new generation vaccines. The route of administration can change the course of immunity generated by the recombinant protein, thus, it is one of the important aspects for study of a vaccine formulation. Intraperitoneal route of immunization gives a larger absorptive surface area for vaccine that is being administered. Intradermal route of immunization has been studied due to increased probability of high antibody response and generation of Th1 stimulatory cytokines. Intradermal route of immunization also helps in dosage reduction due to the ability of dermal dendritic cells (DCs) to generate strong immune response (Lambert and Laurent, 2008).

Recombinant Omp25 (rOmp25) was administered in Balb/c mice for protection studies and to ascertain the type of immune response generated through intradermal (ID) and intraperitoneal (IP) routes of immunization. ID immunization results in high antibody titre with low amount of antigen and a single booster dose while the IP immunization resulted in lower antibody titre even with high amount of antigen and a second booster dose. The IgG1/IgG2a ratio suggested a strong Th2 bent of response in both forms of immunization. However, cytokine levels indicated a mixed Th1 and Th2 type of immune response. Cytokine profile of immunized mice after challenge with *B. abortus* 544 skewed towards Th1 type of immune response which may be the reason behind clearance of *Brucella* from spleen after challenge. Reduced splenic counts of *Brucella* were observed when mice were immunized intraperitoneally and intradermally with rOmp25.

2. Materials and methods

2.1. Bacterial strains and plasmids

Bacterial strains of *B. abortus* S-19 and *B. abortus* 544 were obtained from Indian Veterinary Research Institute, Izatnagar, Bareilly, India. *E. coli* strains of DH5α and C43 cells were used for propagation and expression of plasmids. *E. coli* strains were grown in LB medium whereas all *Brucella* strains were maintained in Tryptic Soy medium. All *Brucella* strain related experiments were carried out in Biosafety level 3 facilities.

2.2. Mice

4–6 weeks of inbred female Balb/c mice were procured from National Institute of Nutrition, Hyderabad, India. The regulations of Indian Animal Ethics Committee (IAEC) of Jawaharlal Nehru University were followed in all mice experiments. After Balb/c mice were injected with *B. abortus*, they were kept in Biosafety level 3 animal facilities.

2.3. Cloning of Omp25 gene of B. abortus

B. abortus S-19 strain was used for genomic DNA isolation by CTAB method. Full length Omp25 gene was amplified using genomic DNA as template with Taq polymerase enzyme. Following set of primers were used during PCR reaction: Omp fwd – 5′ GGAGGGATCCATGCGCACTCTTAAGTCTCTCG 3′ and Omp rev – 5′ GCGCGTCGACGAACTTGTAGCCGATGCC 3′. The PCR reaction was carried out at specified conditions: initial denaturation at 95 °C for 5 min; thermal cycle repeated 30 times includes: denaturation at 95 °C for 30 s; annealing at 65 °C for 45 s and extension at 72 °C for 1 min. Final extension was carried out at 72 °C for 7 min. The amplified product 642 bp Omp25 was digested with *BamHI* and *SalI* restrictions enzymes (NEB) and the fragment was ligated into pET28a vector double digested with same enzymes, in frame with 6×-His tag at N as well C terminal, to generate a plasmid pETOmp. The plasmid was then transformed into DH5α and the transformants were checked for Omp25 gene insertion. The clone was confirmed by DNA sequencing.

2.4. Recombinant Omp25 expression

E. coli C43 cells were used for rOmp25 protein expression. The transformed cells were grown in LB media at 37 °C till mid log phase. The protein was induced with 1 mM isopropylthiogalactoside (IPTG) at $O.D._{600}$ of 0.6–0.8 for 5 h. The expression of recombinant protein was analysed by SDS-PAGE and then confirmed by western blotting using anti-His antibody. The reactivity of the protein using anti-Omp25 sera was also checked by western blotting. Uninduced and induced cell lysates of *E. coli* C43 expressing recombinant Omp25 and purified rOmp25 were incubated with anti-Omp25 sera. Then, the western blot was developed using anti-mouse IgG AP conjugated antibody.

2.5. Purification of recombinant Omp25

rOmp25 was purified using urea denaturation method as rOmp25 was found to be present in inclusion bodies. One litre culture pellet was suspended in 50 ml of buffer containing 0.1 M KPO_4, 150 mM NaCl and 8 M urea. The pellet was kept for lysis at 37 °C for 1 h and then centrifuged at 13,000 rpm for 30 min. The supernatant obtained was kept for batch binding with Ni^{2+}-NTA agarose for 2 h. After collecting the flow through of unbound proteins, the protein was refolded on column with decreasing gradient of urea concentration from 8 M to 0 M. Then the His-tagged rOmp25 was recovered by eluting the protein at 200 mM imidazole concentration pH 8.0. The fractions of eluted protein were analysed on SDS-PAGE wherein rOmp25 showed a single band of 25 kDa. Purified protein was dialysed 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).

2.6. Immunization of mice with different doses of rOmp25 via different routes

The 6–8 weeks old Balb/c mice were used for immunization. Groups of 6 female mice were injected intraperitoneally and intradermally with different concentrations of rOmp25 10, 20 30 and 40 μg, respectively, on day 0. Before injecting the mice on day 0, the protein was emulsified in Complete Freund's Adjuvant (CFA) in a final volume of 100 μl. On 14th and 28th day booster doses were