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Identification of a new immunogenic candidate conferring protection against *Brucella melitensis* infection in Mice

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ABSTRACT

Identification of bacterial proteins that contribute to the replication and survival of the engulfed bacteria within phagolysosome is critical in the pathogenesis of intracellular bacteria. Heat shock proteins (HSPs) are molecular chaperones that prevent unwanted protein aggregation and protect the bacteria against cell stress. In order to study the potential of HspA for development of a *Brucella* subunit vaccine, immunogenicity and protective efficacy of recombinant HspA (rHspA) from *Brucella melitensis* was evaluated in BALB/c mice. The *hspA* gene was cloned in pDEST42 and the resulting recombinant protein was used as subunit vaccine. rHspA elicited mixed TH1/TH2 immune responses with higher titers of specific IgG1 than IgG2a. In lymphocyte transformation assay, splenocytes of immunized mice exhibited a strong recall proliferative response with high amounts of IFN-γ, IL-12, IL-10 and IL-6 and very low levels of IL-5 and IL-4 production. The protective effect of rHspA was evaluated by administering rHspA to mice that resulted in a significant reduction in bacterial load and high degree of protection against *B. melitensis* challenge compared to control mice ($p < 0.001$). These results suggest that rHspA may be a useful candidate for the development of subunit vaccine against brucellosis.

1. Introduction

Brucella species are Gram negative, facultative intracellular pathogens that cause abortion in domestic animals (sheep, cattle, and goats) and a severe illness in humans (Corbel, 1997). Brucellosis is mostly exhibited as an endemic disease, especially in developing countries and continues to impose significant health problems and important economic losses (Ko and Splitter, 2003). *Brucella melitensis* is the most common and pathogenic species that infects humans worldwide and the least species specific, infecting livestock (Young, 1995). For the time being, the live attenuated *B. melitensis*, Rev.1 strain, is the most widely used vaccine for controlling brucellosis in sheep and goat (Garin-Bastuji et al., 1998; Scharp et al., 1999). The Rev.1 vaccine was firstly obtained by using streptomycin as the selective agent to develop the attenuated streptomycin-resistant Rev.1 strain from streptomycin-dependent bacteria selected from a virulent strain of *B. melitensis* in the 1950s (Elberg and Faunce, 1957). However, it is less than ideal because Rev.1 elicits a long-lasting serological response against the O polysaccharide making it difficult to differentiate vaccinated animals from those naturally infected (Baldi et al., 1996; Zygmunt et al., 1994). Moreover, due to occasional problems caused by Rev.1, its use is prohibited in countries free of *B. melitensis* (Jimenez de Bagues et al., 1994). These problems include occasional induction of abortion when administered during pregnancy, pathogenicity for humans (Moriyon et al., 2004) and resistance to streptomycin which is one of the preferred antibiotics for treatment of brucellosis (Delpino et al., 2007). In order to avoid these drawbacks, alternative vaccination approaches like subunit vaccines are highly demanded and identification of *Brucella*-associated antigens is the subject of extensive research in many countries. In this regard, different components of *B. melitensis* have been proposed as candidates for subunit vaccines (Al-Mariri,

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2010; Cassataro et al., 2007; Hoover et al., 1999; Letesson et al., 1997; Yang et al., 2005).

One of the eminent approaches to discover effective vaccines against *Brucella* is to investigate the proteome of bacteria growing under harsh conditions usually present in phagosome. Bacteria respond to different stresses by synthesizing a limited set of stress proteins. Such stressful microenvironment within phagosome is characterized by decreased iron availability, low pH and high content of reactive oxygen intermediates (Contreras-Rodriguez et al., 2003). Effect of this harsh environment, especially low pH, on production of *Brucella*-associated proteins essential for intracellular survival has been reported (Rafie-Kolpin et al., 1996). Thus, identification of bacterial proteins that contribute to the replication and survival of the engulfed bacteria is critical in understanding the pathogenesis of the disease. We previously described that Rev.1-immunized rabbit sera strongly react with *B. melietensis* Heat shock protein A (Accession No. 1197813) (Ghasemi et al., 2013b), exists definitely in other species of *Brucella* as blasted in NCBI, a small heat shock protein produced at stress conditions (Haslbeck and Buchner, 2002). In the present study, we evaluated for the first time the immunogenicity and protective efficacy of the purified recombinant HspA to assess the usefulness of this antigen for the development of a new vaccine against brucellosis.

2. Materials and methods

2.1. Mice

Six to eight week-old female BALB/c mice were obtained from Pasteur Institute of Iran. Mice were kept under optimal conditions of hygiene, temperature, humidity and light (cycles of 12 h dark/light). All experimental procedures on animals were approved by the ethical committee of Avicenna Research Institute. After Rev.1 inoculation, mice were kept in biosafety level 3 animal facilities.

2.2. Bacterial strains

Escherichia coli TOP10 and BL21 (DE3) (gifted by Dr. Pourmand) were used for propagation and expression of the recombinant protein, respectively. Bacterial strains were routinely grown at 37 °C in LB broth or agar. *B. melitensis* 16 M (virulent strain) or *B. melitensis* Rev.1 (vaccine strain) were cultured in *Brucella* agar (HiMedia, Delhi, India) as described elsewhere (Delpino et al., 2007).

2.3. Production of recombinant protein

Methods for cloning and expression of recombinant HspA (rHspA) from *B. melitensis* in *E. coli* BL21 and its purification have been previously described (Ghasemi et al., 2013b). The whole *hspA* gene was amplified by specific primers and cloned using Gateway cloning system (Invitrogen, NY, USA). *B. melitensis* 16 M genomic DNA was used as a template for PCR reaction. The products were cloned into the pDEST-42 vector (Invitrogen), resulting in the plasmid pDEST-*hspA* containing the gene with a COOH-terminal six-histidine tag. The recombinant HspA was successfully expressed in *E. coli* BL21, solubilized with 8 M Urea and purified by Ni-NTA agarose. Contaminating endotoxin was eliminated during purification step by inclusion of 0.1% Triton X-114 in washing buffers (Aida and Pabst, 1990; Liu et al., 1996; Reichelt et al., 2006). The purity of the recombinant protein and its identity was monitored by SDS-PAGE, Coomassie blue staining and Western blotting, respectively (Ghasemi et al., 2013a). The purified recombinant protein that had an endotoxin content of less than 0.05 endotoxin units per mg of protein evaluated by *Limulus amebocyte* lysate analysis kit (Lonza, Basel, Switzerland) was considered for next steps. The concentration of recombinant protein was determined by Bradford method (Stoscheck, 1990).

2.4. Immunization

Three groups each containing 15 mice were investigated. Mice were anaesthetized with methoxyfuorane (Mallinckrodt, Phillipsburg, NJ, USA) and immunized intraperitoneally (i.p.) either with 30 μg of rHspA or PBS (negative control). Antigen or PBS was administered mixed with Complete Freund's Adjuvant (CFA) (Sigma, NY, USA) on day 0 and with incomplete Freund's Adjuvant (IFA) (Sigma) on day 15. Vaccine group was immunized by the subcutaneous route (s.c.) at day 0 with 8×10^8 formalin-killed Rev.1 in IFA (Delpino et al., 2007). Sera were obtained at 0, 15, 30, and 45 days after the first immunization. On day 45 after the first immunization, five mice from each group were challenged intraperitoneally with virulent *Brucella* organism, five mice were sacrificed to conduct the analysis of immune responses including cytokine production and proliferation assay and the remaining five mice were bled on day 75 to monitor persistence of immune responses.

2.5. Humoral immune responses

The titers of specific IgG1 and IgG2a antibodies against rHspA were investigated by ELISA in sera obtained in Section 2.4. The purified rHspA at the concentration of 5 μg/mL (in PBS) was used to coat of 96 well polystyrene plates (Greiner bio-one, Frickenhausen, Germany) (100 μL/well). After overnight coating, plates were washed four times with wash buffer (Tris-buffered saline, pH 7.4, containing 0.05% Tween 20, TBS-T) and blocked with 300 μL 10% fetal bovine serum (FBS) in PBS for 2 h at 37 °C. Plates were then incubated with serial dilutions of the mouse sera for 2 h at room temperature, and washed three times as above. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1or IgG2a antibodies (BD Pharmingen, USA) was added to the wells and incubated for further 90 min at 37 ° C. After final washing steps, the specific reactivity was determined by the addition of enzyme substrate, TMB (Pishtaz Teb, Tehran, Iran). The reaction was stopped after 10 min by adding 20% H2SO4 to the wells. Optical densities were read at 495 nm using an ELISA plate reader (Bio-Tek Instruments, Winooski, Vt., USA). In order to find a cut-off value for the tests, the mean specific OD+ three SD of sera from PBS-immunized mice evaluated at 1:100 dilution. Serum titers were obtained as the reciprocal of the last serum dilution giving an OD higher than the cut-off (Delpino et al., 2007).

2.6. XTT assay for lymphocyte proliferation

Thirty days after the last immunization, spleens were removed from the mice immunized with rHspA, PBS or Rev.1 and homogenized with a syringe in 10 ml PBS containing 5 mM ethylene diamine tetraacetic acid (PBS-EDTA). The resulting cell suspension was kept on ice. Total cell suspensions were washed twice with PBS-EDTA. Mononuclear cells (MNCs) were isolated by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) discontinuous gradient centrifugation. MNCs were then rinsed in cold PBS-EDTA and re-suspended in DMEM without phenol red (Sigma) containing 10% FBS. The cell concentration was adjusted to 2×10^6 cells/mL and 100 μL was added to each well of 96-well culture plates. Cells were then stimulated with different concentrations of purified rHspA ranging from 0.5 μg/ml to 15 μg/ml. Cell proliferation was determined in triplicates. The blank control wells contained cells treated with PBS and also wells that contained medium only. After 48 h incubation at 37° C in 5% CO2 atmosphere, 100 μL of 1 mg/ml 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-((phenylamino) carbonyl)-2H-tetrazolium hydroxide (XTT) (Sigma) containing 25 μl of 5 mM phenazine methosulfate (PMS) (Sigma) were added to each well. Plates were