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Immunization of mice with a novel recombinant molecular chaperon confers protection against *Brucella melitensis* infection

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ABSTRACT

Brucella spp. are zoonotic Gram-negative intracellular pathogens with the ability to survive and replicate in phagocytes. It has been shown that bacterial proteins expressed abundantly in this niche are stress-related proteins capable of triggering effective immune responses. BMEI1549 is a molecular chaperone designated DnaK that is expressed under stress conditions and helps to prevent formation of protein aggregates. In order to study the potential of DnaK as a prospective *Brucella* subunit vaccine, immunogenicity and protective efficacy of recombinant DnaK from *Brucella melitensis* was evaluated in BALB/c mice. The *dnak* gene was cloned, expressed in *Escherichia coli*, and the resulting recombinant protein used as subunit vaccine. DnaK-immunized mice showed a strong lymphocyte proliferative response to *in vitro* antigen stimulation. Although comparable levels of antigen-specific IgG2a and IgG1 were observed in immunized mice, high amounts of IFN-γ, IL-12 and IL-6, no detectable level of IL-4 and very low levels of IL-10 and IL-5 were produced by splenocytes of vaccinated mice suggesting induction of a Th1 dominant immune response by DnaK. Compared to control animals, mice vaccinated with DnaK exhibited a significant degree of protection against subsequent *Brucella* infection ($p < 0.001$), albeit this protection was less than the protection conferred by Rev.1 ($p < 0.05$). A further increase in protection was observed, when DnaK was combined with recombinant Omp31. Notably, this combination, as opposed to each component alone, induced statistically similar level of protection as induced by Rev.1 suggesting that DnaK could be viewed as a promising candidate for the development of a subunit vaccine against brucellosis.

1. Introduction

Brucella melitensis is a zoonotic Gram-negative pathogen that is an important etiological agent causing abortion and infertility in domestic animals, and undulant fever, migratory arthralgia, myalgia and osteomyelitis in humans [1,2]. Because of the severe economic and medical burden of brucellosis, vaccination of all vulnerable hosts and culling of infected animals is the only way of controlling the disease [3]. The live attenuated *B. melitensis* Rev.1 strain is the most broadly used vaccine in control programs against brucellosis in the livestock [4]. It has been shown that Rev.1 can be useful for eradicating this disease [5]. Thus, it is considered in widespread vaccination programs in many countries [6]. Nevertheless, availability of such vaccines as Rev.1 does not obviate the need for development of new vaccines due to some problems associated with application of this vaccine, included among them are eliciting long lasting immune responses against the O polysaccharide making it difficult to differentiate vaccinated animals from those naturally infected, induction of abortion when administered during pregnancy, pathogenicity for humans and resistance to streptomycin [7]. These problems have stimulated scientists to find alternative ways to protect the livestock from *Brucella* infection.

In order to increase safety, subunit vaccines have been developed but these depend on the identification of antigens able to confer protection against brucellosis. Numerous protein antigens are shown to stimulate protective immune response in mice model.

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The recent examples comprise HspA [8], ribosomal protein L9 [9], BLSOmp31 [10], rF278a [11], FlgJ and FliN [12], Omp31, Omp16 and BP26 expressed by invasive *Escherichia coli* vaccines [7], CobB and AsnC [13], Omp28 formulated with CpG oligonucleotides [14], P39 protein formulated with CpG oligodeoxynucleotides [15], Ado-Hcyase [16] and Rs-α [17], combination of Omp16 and Omp19 [18]. Although some of these antigens have been recently identified, the protection conferred is low in most settings. Thus, to develop efficient subunit vaccines, screening and assessment of new protective antigens is essential.

Brucella is able to infect macrophages and to persist and replicate in the intracellular environment [19]. Identifying those bacterial proteins that are necessary for intracellular survival of *Brucella* may provide new insights into mechanisms of pathogenesis and immune protection, and candidate antigens for vaccine design. We previously described that sera from Rev.1-immunized rabbits strongly reacted with the molecular chaperone DnaK of *B. melitensis*, which is also expressed in other strains [20]. The molecular chaperone DnaK (BMEI1549) is a member of the highly conserved 70-kilodalton heat-shock protein (hsp70) family [21]. Under stress conditions, DnaK assists in protein folding, translocation and interaction by binding to unfolded polypeptide domains [22]. However, No data about the immunological properties of BMEI1549 product has been reported yet. Importantly, the gene coding for this molecular chaperone is different from the previously described *Brucella* gene BMEI2002 that encodes a protein also designated DnaK. It was shown that the latter confers a partial protection against *Brucella abortus* infection in mice [23] and is necessary to resistant of *Brucella suis* to bacterial killing of macrophages [24]. In the present study, we evaluated for the first time the immunogenicity and protective efficacy of the purified recombinant DnaK (BMEI1549) in mice. Protection against subsequent infection was evaluated after vaccination with DnaK alone, or in combination two well-known protective antigens of *B. melitensis* recombinant outer membrane protein, Omp31 [20,25,26] and cytoplasmic protein, Trigger Factor (TF) [27–30]. We hypothesized that inclusion of such antigens in vaccine formulation could potentially augment the protective efficacy of each antigen alone. With this in mind, the combination of different panel of antigens was tested in our experiments.

2. Materials and methods

2.1. Bacterial strains

E. coli TOP10 and BL21 (DE3) (a gift from Dr. Pourmand, Tehran University of Medical Sciences) were used for expression of DnaK. 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 [31].

2.2. Production and purification of DnaK

Cloning, expression, and purification of DnaK from *B. melitensis* in *E. coli* BL21 and its purification have been described previously [20]. Briefly, the *dnak* gene was amplified by PCR from genomic DNA of *B. melitensis* 16 M (Forward: 5′ CATATGACACCTT CTG 3′, Reverse: 5′ GGATCCTACCGACCAGCG 3′). The amplified DNA fragment was directly inserted into pTZ57R (InsTAclone™ PCR Cloning Kit) (Fermentas, Vilnius, Lithuania) and then subcloned into the pET28a+ vector (Novagen, Madison, WI, USA). 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was used to induce expression of DnaK. Purification of DnaK was performed under denaturing condition as previously described [32]. Contaminating endotoxins were eliminated during the purification step by 0.1% Triton X-114 in washing buffers [33–35]. Vaccine protein should be in its native form as far as effective blocking immune responses are concerned. In our experiment we first solubilized the protein in 8 M urea for the purpose of purification and at the next step we re-folded the protein in a stepwise dialyzing process with decreasing gradient of urea. Finally purified protein was dialyzed against PBS.

2.3. The SDS-PAGE and Western blotting

The purity of the recombinant protein and its identity was assessed by SDS-PAGE, Coomassie blue staining and Western blotting [20]. Briefly, purified recombinant protein was size-separated by SDS-PAGE and the proteins transferred to a nitrocellulose membrane (BioRad, USA). Next, the membrane was incubated with anti-6-His peroxidase (Roche, Mannheim, Germany) (1/40,000) for 1 h. Finally, the bound conjugates were detected using diaminobenzidine (DAB) (Sigma, NY, USA). Only purified recombinant protein with 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 used. The concentration of recombinant protein was determined by the Bradford method [36].

2.4. Mice

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

2.5. Immunization

Mice were randomly divided into seven groups. Three groups with 15 mice each received DnaK, PBS or Rev.1 vaccine only to study immunogenicity and protective efficacy. Two groups including 10 mice each received Omp31 and TF to assess lymphocyte proliferation and conferred protection. The other groups consisting of five mice each were used to evaluate and protection induced by antigen cocktails. Mice were anaesthetized with methoxyfuor ane (Mallinckrodt, Phillipsburg, NJ, USA) and immunized intraperitoneally (i.p.) either with 30 μg of DnaK, TF or Omp31, 30 μg DnaK and 30 μg TF [28], or 30 μg DnaK and 30 μg Omp31 [20], or PBS (negative control) on day 0 and 15 as described previously [23]. Briefly, mice were injected with proteins or PBS in Complete Freund's Adjuvant (CFA) (Sigma) on day 0 and with incomplete Freund's adjuvant (IFA) (Sigma) on day 15. For comparison, a control group was immunized by the subcutaneous route (s.c.) at day 0 with 8×10^8 formalin-killed Rev.1 in IFA. Sera were obtained 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 *B. melitensis* 16 M, five mice were sacrificed to assess immune responses including cytokine production and proliferation assay, and the remaining five mice were bled on day 75 to monitor memory responses.

2.6. Humoral immune responses

The titers of DnaK-specific IgG1 and IgG2a antibodies in mouse sera were investigated by ELISA as previously reported [20]. In order to find a cut-off value for this test, the mean specific OD plus 3 S.D. from 20 sera from PBS-immunized mice at 1:100 dilutions was determined. Serum titers are denoted as the reciprocal of the last serum dilution giving an OD higher than the cut-off [23].

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