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Immune response variations to *Salmonella enterica* serovar Typhi recombinant porin proteins in mice

Hoda Toobak [a], Iraj Rasooli [a,*], Daryush Talei [b], Abolfazl Jahangiri [c], Parviz Owlia [d], Shakiba Darvish Alipour Astaneh [a]

[a] Department of Biology, Shahed University, Tehran-Qom Express Way, Opposite Imam Khomeini's Shrine, Tehran 3319118651, Iran
[b] Medicinal Plant Research Center, Shahed University, Tehran, Iran
[c] Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
[d] School of Medicine, and Molecular Microbiology Research Center, Shahed University, Tehran Iran

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ABSTRACT

Objectives: Typhoid fever is caused by *Salmonella enterica* serovar Typhi. OmpC, OmpF and OmpA, the three major outer membrane proteins (OMPs), could serve as vaccine candidates.
Methods: The porins antigenicity was predicted *in silico*. The OMP genes were amplified, cloned and expressed. Sero-reactivities of the recombinant proteins purified by denaturing method were assayed by ELISA. BALB/c mice were immunized with the recombinant porins followed by bacterial challenge.
Results: Bacterial challenge of the animal model brought about antibody triggering efficacy of the antigen in OmpF > OmpC > OmpA order. Experimental findings validated the *in silico* results. None of the antigens had synergic or antagonistic effects on each other from immune system induction points of view. Despite their high immunogenicity, none of the antigens was protective. However, administration of two or three antigens simultaneously resulted in retardation of lethal effect. Porins, in addition to their specific functions, share common functions. Hence, they can compensate for each other's functions.
Conclusions: The produced antibodies could not eliminate the pathogenicity by blockade of one or some of the antigens. Porin antigens are not suitable vaccine candidates alone or in denatured forms. Native forms of the antigens maybe studied for protective immunogenicity.

1. Introduction

Salmonella enterica serovar Typhi is a Gram-negative bacterium, the causative agent of human typhoid fever. Typhoid fever spreads by fecal-oral route via contaminated food and water [1,2]. A recent study estimated approximately 22 million cases of typhoid each year with at least 200,000 deaths [3,4]. Gram-negative bacteria have an outer membrane layer in their cell wall structures. Several proteins including porins present in outer membrane form water filled channels on it that allow small hydrophilic solutes to pass through the pore [5,6]. Furthermore these proteins involve in a variety of the pathogen functions [7,8]. *S. enterica* serovar Typhi synthesizes three major outer membrane proteins (OMPs): OmpC and OmpF with homotrimers structures, and OmpA with monomeric structure [9]. Expression of OmpC and OmpF genes in *S. enterica* serovar Typhi is controlled by *ompR–envZ*, members of a two-component signal transduction system. Shifting of osmolarity conditions in *S. enterica* serovar Typhi only affects the OmpF expression. OmpF is expressed primarily in low osmolarity conditions, and OmpC levels remain constant and expressed under low and high osmolarity condition [10]. Several researches have shown that OmpA, like porins and LPS, is also a target of the host immune response but its role in immuno-protection is not clearly demonstrated [11].

The OmpA folding is in majority and minority conformers. The majority conformers fold in to a structure with two large domains, the N-terminal domain and the C-terminal domain but the minority conformer forms channels allowing the diffusion of solutes up to several hundred daltons in size [12]. It was also shown that *Salmonella* porins play a role in pathogenesis and are important antigens against which the host immune response is directed to induce both humoral and cell mediated immunity [13]. The OMPs

* Corresponding author. Department of Biology, and Molecular Microbiology Research Center, Shahed University, Tehran-Qom Express Way, Opposite Imam Khomeini's Shrine, Tehran 3319118651, Iran. Tel.: +98 (21)51212600; fax: +98 (21) 51212601.
E-mail address: rasooli@shahed.ac.ir (I. Rasooli).

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of *S. enterica* serovar Typhi and many other Gram-negative bacteria are widely recognized as important immunogens that contribute to the pathogenesis of the disease and to protection against challenge [14]. Antibodies to porin antigens of *S. enterica* serovar Typhi induced during typhoid infection in human maybe of diagnostic value in typhoid infections [2]. Protection generated by ant-Vi Abs is induced by immunization of plain Vi polysaccharide or a Vi PS conjugate vaccine. This is currently the only licensed and efficacious subunit vaccine for typhoid [3]. Immunization with OMPs from other gram-negative bacteria also induces a protective status in experimental animals [15]. Since the purification of native porin proteins by routine methods is a lengthy procedure associated with contaminations particularly with lipopolysaccharide (LPS) [2]. Here, we report cloning, expression and purification of OmpA, OmpC and OmpF from *S. enterica* serovar Typhi PTCC 1609. Their immunogenicity and potential protective efficacy were studied in BALB/c mice. The current study was undertaken to define the role of OmpA, OmpC and OmpF of *S. enterica* serovar Typhi in the induction of protective immunity against a challenge with live bacteria.

2. Materials and methods

2.1. In silico analyses

2.1.1. Similarity studies

Protein sequences of OmpC (Acc. NP_804453.1), OmpF (Acc. NP_805701.1) and OmpA (Acc. NP_805619.1) served as queries for BLAST against all protein sequences of *S. enterica* ssp I serovars at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The first sequences of the OMP hits with E-value of 0.0 and the highest total score were retrieved in FASTA format to more precise similarity analyses. Therefore, alignment of these sequences was performed by PRALINE at http://www.ibi.vu.nl/programs/pralinewww/ for each type of the tree OMPs.

2.2. Prediction of antigen probability

The OMP sequences were submitted to VaxiJen v.2.0 at http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html in order to determine their antigenic probability.

VaxiJen is the first server developed to allow antigen classification based on the physicochemical properties of proteins without recourse to sequence alignment. Accuracy of its predictions is 70%–89% [16].

2.3. Chemicals and enzymes

T4DNA ligase and restriction endonucleases were purchased from Fermentas (Vilnius, Lithuania). The gel purification and plasmid extraction kits were from Bioneer (Daejeon, Korea). Nickel–nitrilotriacetic acid (Ni–NTA) agarose was from Qiagen (Valencia, USA). Other chemical reagents were procured from Merck (Darmstadt, Germany). The primers were synthesized by Bioneer.

2.4. Bacterial strains, plasmids, culture media and human sera

S. enterica serovar Typhi PTCC (Persian Type Culture Collection) 1609 and *Escherichia coli* BL21 (DE3) were from our research laboratory. The plasmid pET28a (+) was a Novagen product (USA), Luria–Bertani (LB) broth or LB agar and Salmonella–Shigella (SS) agar were of Hi-media products. Serum samples were collected from 10 individuals with acute typhoid fever and positive Widal test (15–40 years old) referred to Razi laboratory of Andimeshk city of Iran. Sera from 10 healthy volunteers served as a control.

2.5. PCR amplification and cloning of OmpA, OmpC and OmpF genes

Heterologous expression in *E. coli* BL21 (DE3) along with its signal peptide is toxic to *E. coli*. While the removal of signal peptide leads to the formation of cytoplasmic inclusion bodies (IBs) [6], so the signal peptides from 5′-terminal were omitted in primer designing. The genes coding for OMPs were amplified from genomic DNA. The amplification of the OmpA, OmpF and OmpC genes was carried out using the following primers:

Forward (OmpC): 5′-GCAGCGAATCATATGGAAATTTATAATAAA-GAC-3′
Reverse (OmpC) [6]: 5′-AACATCTTTGGATCCTTAGAACTGGTAA AC-3′
Forward (OmpA): 5′-CGCTGAATTCATGCCGAAAGATAACACCTG-3′
Reverse (OmpA): 5′-CAAAAAGCTTTTAAGCCTGCGGCTGAGTTAC-3′
Forward (OmpF): 5′-CGCAGAATTCATGGCAGAAATTTATAATAA AGA-3′
Reverse (OmpF): 5′-AGTCAAGCTTTCAGAACTGGTAAGTAATACCG AC-3′

PCR reactions of 25 μl each for OmpA, OmpC and OmpF contained 2 μl of each primer (20 pM), 250 μM each dNTP. 1 μl DNA (50 ng/ml), 2 μl $MgSO_4$ (50 mM) and 0.5 μl of pfu DNA polymerase in a final volume of 25 μl was put in a thermal cycler (Techne Gradient). 30 PCR cycles were run as follows: initial denaturation at 95 °C for 5 min, denaturation at 94 °C for 1 min, primer annealing at 57 °C (OmpC), 60 °C (OmpA) and 66 °C (OmpF) for 1 min, and extension at 72 °C for 1 min, finally extended for 5 min at 72 °C. The amplified DNA products were electrophoresed in a 0.8% (*w*/*v*) agarose gel. PCR products were purified using a PCR purification kit as per the manufacturer's instructions. The PCR product (OmpC) was digested with BamHI and NdeI and OmpA, OmpF were digested with HindIII and EcoRI. They were cloned into pET28a (+) vector digested with the relevant endonucleases. The new constructs were named pET28a–OmpA, pET28a–OmpC and pET28a–OmpF. The ligated products were transformed into *E. coli* BL21 (DE3) as the expression host. The recombinant clones were selected on LB plates containing ampicillin (50 μg/ml). After mini-scale isolation of the plasmid DNA using the plasmid extraction kit, the presence of the open reading frame (ORF) was confirmed by restriction analysis and by sequencing.

2.6. Expression and purification of recombinant porins (OmpA, OmpC and OmpF)

The transformed *E. coli* BL21 cells were grown in LB medium (500 ml) and were then induced with 0.5 mM iospropyl b-D-thiogalactoside (IPTG). The expressed protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). *E. coli* BL21 cells harboring the OmpA–pET28a, OmpC–pET28a and OmpF–pET28a constructs were grown overnight at 37 °C in 10 ml of LB medium containing 50 μg/ml ampicillin under constant shaking (200 rpm). This culture was then used for inoculating 500 ml of LB medium. 0.5 mM isopropyl b-D-thiogalactoside (IPTG) was added at the optical density of 0.6 at 600 nm to induce expression. The expressed proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Cells were further incubated at 37 °C for 6 h and were then harvested by centrifugation at 14,000× *g* for 10 min at 4 °C. The cell pellet was re-suspended in lysis buffer (100 mM NaH_2PO_4, 10 mM Tris Cl, 8 M urea) with addition of lysozyme at 1 mg/ml, and was sonicated 5 times for 1 min at intervals of 1 min. Protein was purified from the supernatant by affinity chromatography using Ni^{2+}–NTA agarose (Qiagen, CA). The supernatant was separated from