Vaccine xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Development of recombinant vaccine candidate molecule against *Shigella* infection

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ARTICLE INFO

Article history:
Received 10 February 2016
Received in revised form 28 July 2016
Accepted 12 August 2016
Available online xxxx

Keywords:
Shigella
S. Typhi
IpaB
GroEL
Fusion protein

ABSTRACT

Shigellosis is an acute bacillary diarrheal disease caused by the gram negative bacillus *Shigella.* The existence of multiple *Shigella* serotypes and their growing resistance to antibiotics stress the urgent need for the development of vaccine that is protective across all serotypes. *Shigella*'s IpaB antigen is involved in translocon pore formation, promotes bacterial invasion and induces apoptosis in macrophages. *S.* Typhi GroEL (Hsp 60) is the immunodominant antigen inducing both arms of immunity and has been explored as adjuvant in this study. The present study evaluates the immunogenicity and protective efficacy of recombinant IpaB domain-GroEL fusion protein in mice against lethal *Shigella* infection. The IpaB domain and GroEL genes were fused using overlap extension PCR and cloned in pRSETA expression vector. Fused gene was expressed in *Escherichia coli* BL-21 cells and the resulting 90 KDa fusion protein was purified by affinity chromatography. Intranasal (i.n.) immunization of mice with fusion protein increased the IgG and IgA antibody titers as compared to the group immunized with IpaB and GroEL and control PBS immunized group. Also IgG1 and IgG2a antibodies induced in fusion protein immunized mice were higher than co-immunized group. Significant increase in lymphocyte proliferation and cytokine levels (IFN-γ, IL-4 and IL-10), indicates induction of both Th1 and Th2 immune responses in both immunized groups. Immunization with fusion protein protected 90–95% of mice whereas 80–85% survivability was observed in co-immunized group against lethal challenge with *S. flexneri*, *S. boydii* and *S. sonnei*. Passive immunization conferred 60–70% protection in mice against all these *Shigella* species. Organ burden and histopathology studies also revealed significant decrease in lung infection as compared to the co-immunized group. Since IpaB is the conserved dominant molecule in all *Shigella* species, this study will lead to an ideal platform for the development of safe, efficacious and cost-effective recombinant vaccine against *Shigella* serotypes.

1. Introduction

Shigellosis, is an acute inflammatory gastrointestinal disease caused by the enterobacteria *Shigella*. It is transmitted through fecal-oral route. Only 10–100 organisms are sufficient to cause the infection. It is manifested by the passage of loose stools mixed with mucous and blood, accompanied by fever, abdominal cramps and tenesmus [1]. It is one of the pathogens responsible for moderate to severe diarrhea in infants and children below 5 years of age and repeated bouts of diarrheal disease during early development can impair physical growth and cognitive abilities [2,3]. Tourists, pilgrims and military personnel are also susceptible to infection. Despite the progress made in recent years, no licensed vaccine is currently available to prevent Shigellosis. There are more than 50 serotypes of *Shigella* based on the diversity in protective O-antigen, a polysaccharide unit of their outer membrane lipopolysaccharide (LPS), thereby increasing their antigenic variability. The existence of multiple Shigella serotypes and emergence of antibiotic resistant strains accentuate the urgent need of development of vaccine protective against all serotypes [4–8].

To meet this huge challenge, researchers are adopting different strategies to develop O antigen based vaccines including live, subunit and conjugate vaccine candidates. Although they were found effective against major serotypes, *S. sonnei* and *S. flexneri* 2a, 3a, 6 but no protection was observed in children under 2 years of age. Further, this serotype based strategy would require developing vaccines against all 50 serotypes which is a humongous task [7,8]. Another novel concept is conserved antigen vaccine where

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http://dx.doi.org/10.1016/j.vaccine.2016.08.034
0264-410X/

key protein antigens are being targeted that are conserved in all *Shigella* serotypes. *Shigella* contains 220-Kb virulence plasmid which encodes 30 Kb Mxi-Spa type III secretion system (TTSS). The TTSS mainly consists of the invasion plasmid antigens (IpaA, IpaB, IpaC, IpaD) which are required for the invasion of epithelial cells. These Ipa genes code for primarily effector/translocator proteins directly responsible for the release of the bacteria into the host cell cytosol by inducing cytoskeletal rearrangements, membrane ruffling and pathogen uptake [9–11]. Ipa protein antigens which are antigenically similar in different *Shigella* spp. were reported as the dominant antigens inducing humoral immune response during infection [12]. Macrophages are the primary cells that *S. flexneri* meets after transcytosis through M cells of the intestine which leads to apoptosis [13]. This macrophage death is mediated by the Mxi-Spa secreted effector protein IpaB required to induce phagocyte apoptosis in host macrophages and dentritic cells leading to inflammation [10,14–16]. IpaB protein antigens have been shown to be the major immunogens in the convalescent sera from the infected monkeys or humans [12]. IpaB and IpaD when administered with double mutant of *E. coli* heat labile toxin (dmLT) as mucosal adjuvant conferred protection against *Shigella* infections in mouse model [17–20]. Recently another unique approach of use of non-living *Lactococcus lactis* bacterium like particles (BLP) as an adjuvant and vaccine display system for mucosal delivery of IpaB and IpaD has been reported to be effective in mice [21,22].

Heat Shock Proteins (HSPs) are known to be the immunodominant antigens for the host immune response to a variety of pathogens. They are highly conserved, ubiquitous molecules and also contribute to cell survival under stressful conditions by facilitating the proper folding of denatured proteins [23]. As Hsps share a high degree of sequence homology across species, we previously reported *in vitro* and *in vivo* cross-protective efficacy of recombinant GroEL of *S.* Typhi (rGroEL) against different pathogens [24]. HSPs have also been reported as effective adjuvant molecules [25]. The immunodominant domain of *Shigella* IpaB (44–310 aminoacids) that we have explored in the present study, is necessary for the process of invasion [26–28]. Thus, exploiting the properties of GroEL as adjuvant and IpaB antigen, we have recently reported that co-administration of these two molecules elicited immune responses and protective efficacy which were higher than those obtained with rIpaB domain or rGroEL individually against *Shigella* infection in mice [29]. As the children in developing countries are the main target of *Shigella* vaccine, the cost of final vaccine formulation is also very important in addition to its efficacy. Therefore, to reduce the cost of vaccine and simplify manufacturing process and formulation, we have developed a fusion protein that includes both of these previously tested molecules, rIpaB domain region of *Shigella* genetically fused with rGroEL of *S.* Typhi. The protective efficacy and immune responses elicited by fusion protein were comparable to or better than that elicited by rIpaB and rGroEL combination vaccine against lethal challenge with *S. flexneri, S. boydii and S. sonnei* in mice.

2. Materials and methods

2.1. Mice

Four to six-week old female BALB/c mice were used in all the experiments and maintained in the Experimental Animal Facility, Defence Institute of Physiology and Allied Sciences, Delhi, India under standard laboratory conditions. Food and water were given *ad libitum*. All animal protocols were approved by CPCSEA (Committee for the purpose of control and supervision of experiments on animals), Government of India.

2.2. Growth conditions and maintenance of bacterial strains, vectors

Shigella flexneri 2a, *Shigella boydii*, *Shigella sonnei* were clinically isolated pathogens collected from All India Institute of Medical Sciences, New Delhi, India. All *Shigella* strains were grown in tryptic soy broth at 37 °C. *Shigella* colonies bearing virulent plasmid were isolated by growing the strain regularly in Tryptic soy agar with 0.02% Congo red. *E. coli* DH5α cells were maintained in LB medium, *E. coli* BL21(DE3) pLysS cells were maintained in LB medium with 35 μg/ml chloramphenicol and pRSET A vector in LB medium with 50 μg/ml ampicillin at 37 °C.

2.3. Cloning of IpaB domain-GroEL fusion gene in pRSETA vector

Plasmid DNA was isolated by Gene elute plasmid DNA isolation kit (Sigma,USA) and stored at −20 °C.

IpaB domain gene was amplified from 220 Kb invasion plasmid of *S. flexneri* and GroEL gene was amplified from the genomic DNA of *S.* Typhi as template using the designed primers. Fusion gene was constructed by overlap extension PCR and used as template to amplify the fusion gene using the IpaB forward and GroEL reverse primers (Supplementary data 1).

pRSETA vector and fusion gene were restricted with XhoI and HindIII, gel eluted and purified (Sigma) followed by ligation. *E. coli* BL21 pLysS cells were then transformed with the ligated product and colonies were screened for the recombinant fusion gene.

2.4. Isolation, expression, purification of recombinant fusion protein

Transformed *E. coli* BL21 colony bearing recombinant fusion gene was inoculated into LB broth and incubated until OD_{600} reached 0.5. The cells were then induced with 1 mM isopropylthiogalactoside (IPTG) for 4 h at 37 °C followed by centrifugation at 5000*g*. The pellet was suspended in the lysis buffer containing 8 M urea, 200 mM NaCl, 2 mM imidazole and 50 mM Tris-Cl and sonicated under cold conditions. The sample was purified by Ni-NTA chromatography as per manufacturer's instruction (Qiagen). The purified fusion protein was refolded *in vitro* using 1 M arginine at 4 °C, dialysed in buffer containing 50 mM Tris and 1 mM EDTA for 48 h. Fusion protein was concentrated by the Amicon filters (Millipore) and observed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The expressed fusion protein was confirmed by western blot by probing with IpaB/GroEL antibody (Santacruz, USA). LPS content in the purified protein as determined by Limulus amoebocyte lysate (LAL, Sigma) was found to be negligible (<1 EU/mg protein).

2.5. Immunization of mice

Three groups of four to six-week old female BALB/c mice (n = 10/group) were taken. Group I was immunized i.n. with rIpaB domain (40 μg/mouse) combined with rGroEL (40 μg/mouse) [29], group II with the fusion protein (50 μg/mouse). Subsequent booster doses were given on the 7th day and 28th day. Mice that received Phosphate buffer saline alone were included as controls (group III).

2.6. Determination of antibody titers

Blood was drawn seven days after the last booster dose (35th day) from all the three groups. Serum was isolated to measure the IgG/IgA/IgG1/IgG2a levels and BAL fluid (BALF) was collected on day 35 for IgA and IgG levels by Enzyme linked immunosorbent assay (ELISA). Briefly, 200 μl of the coating buffer (0.1 M bicarbonate, pH 9.3) containing 1 μg of recombinant fusion protein was added to 96 well micro- titer plates (Grenier) and incubated

Please cite this article in press as: Chitradevi STS et al. Development of recombinant vaccine candidate molecule against *Shigella* infection. Vaccine (2016), http://dx.doi.org/10.1016/j.vaccine.2016.08.034