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Intranasal immunization of cocktail/fusion protein containing Tir along with ΔG active fragment of Zot as mucosal adjuvant confers enhanced immunogenicity and reduces *E. coli* O157:H7 shedding in mice

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ABSTRACT

Ruminants are the major reservoirs of *Escherichia coli* O157:H7 and its fecal shedding mainly act as a source of entry of this pathogen into the human food chain. In humans, *E. coli* O157:H7 infection causes diarrhea, hemorrhagic colitis and hemolytic uremic syndrome. Intimate adherence of *E. coli* O157:H7 is mediated by Translocated intimin receptor (Tir) to which intimin binds in the host cell. Since *E. coli* O157:H7 colonizes intestinal epithelium, the mucosal vaccine has a potential to prevent its colonization. Zonula occludens toxin (Zot) of *Vibrio cholerae* transiently, reversibly alters epithelial tight junction structure to increase mucosal permeability of macromolecules via paracellular route. The C-terminal region of Zot (ΔG) responsible for this function could be used for mucosal antigen delivery. Therefore, we employed individual (Tir), cocktail (ΔG + Tir), fusion protein (ΔG-Tir) and assessed the efficacy of its intranasal immunization on immunogenicity and fecal shedding of *E. coli* O157:H7 in streptomycin treated mouse model. Compared to control, ΔG + Tir, ΔG-Tir immunized mice elicited significant antigen specific antibody titers in serum (IgG, IgA) and feces (IgA), whereas Tir immunized mice induced only serum IgG titer. Cytokine analysis revealed mixed Th1/Th2 type immune response in case of ΔG + Tir, ΔG-Tir group while that of Tir group was solely Th2 type. Tir, ΔG + Tir and ΔG-Tir immunized mice showed reduction in shedding of *E. coli* O157:H7 compared to control group. However, ΔG-Tir immunized group performed better than ΔG + Tir, Tir group in reducing fecal shedding. Overall, our results demonstrate that intranasal immunization of ΔG-Tir induces effective systemic, mucosal, cellular immune responses and represents a promising mucosal subunit vaccine to prevent *E. coli* O157:H7 colonization.

1. Introduction

E. coli O157:H7 is an enteric pathogen of zoonotic origin causing a wide array of diseases including diarrhea, hemorrhagic colitis (HC), thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS) in humans. Ruminants are the asymptomatic carrier which shed these bacteria in their feces [1]. The use of antibiotics poses a threat of developing multiple antibiotic resistance strains in case of ruminants [2] and enhancement of pathogenesis in the case of humans [3]. Therefore, vaccination is an attractive strategy to prevent infection in ruminants as well as humans. Moreover, a recent report estimates that vaccination of cattle may reduce animal shedding by 50% and human cases by 85% [4].

The key step in *E. coli* O157:H7 colonization involves the formation of attaching and effacing (A/E) lesions with the aid of type III secretion system [5]. Type III secretion system is composed of a basal apparatus and a needle structure, which consists of polymers of the EscF and EspA proteins. The translocon proteins EspD and EspB in the tip of the structure form a pore in the epithelial cell, through which translocated proteins are delivered [6]. Tir is the first translocated protein which localizes to host plasma membrane and act as a receptor for bacterial outer membrane adhesin called intimin [7]. Tir-intimin interaction is necessary for bacterial adherence as well as for triggering cytoskeletal rearrangements required for actin pedestal formation [8]. In fact, *E. coli* O157:H7 lacking Tir gene was shown to be deficient in effective colonization of animals than the wild type strain [9]. Moreover, strong immune response to Tir was observed in both human and bovine serum during infection [10]. All these attributes make Tir one of the potential vaccine candidates. Several T3SS based *E. coli* O157:H7 recombinant vaccines containing Tir were previously evaluated in various animal

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models with variable efficacy [11–16]. However, with few exceptions, most of these studies rely solely on systemic immune responses to attribute protection without much focus on the role of mucosal, cellular immune responses.

Since *E. coli* O157:H7 colonizes intestinal epithelium, an effective strategy would be to induce mucosal immunity in the intestinal tract along with systemic immune responses following immunization. Despite the fact that contrary reports exist on necessity of *E. coli* O157:H7 candidate vaccine to elicit IgA for protection, previous report [17] on induction of strong IgA response against T3SS proteins following infection in terminal rectal mucosa of cattle accentuates its importance in counteracting this pathogen. In addition to this, a recent study in cattle [18] propounds the role of cellular immune responses during the colonization of *E. coli* O157:H7 which has been almost unanalyzed in many previous vaccine studies based on Tir. There are some previous studies that have employed different approaches such as route of delivery [19], adjuvants [15,16,20,21] or delivery vehicle [22] to achieve mucosal immunity in conferring protection against *E. coli* O157:H7. Nevertheless, very few vaccine studies concerning Tir have explored adjuvants such as cholera toxin [16,21], H7 flagellin [15] to elicit mucosal immunity. Additionally, both these adjuvants, though effective, seems to suffer from their inherent drawbacks such as adverse effects owing to its binding to GM1 receptors in case of cholera toxin [23], impairment of innate and/or adaptive immunity through inhibition of TLR5 signalling [24] in case of H7 flagellin.

In this regard, Zonula occludens toxin (Zot), a 44.8 kDa protein produced by *Vibrio cholerae* is a novel molecule that acts reversibly on epithelial tight junctions to transiently increase permeability of macromolecules via paracellular route [25]. Its properties like reversible action and not causing tissue damage [26] make it a safe and promising mucosal adjuvant. Moreover, previous studies have proven its mucosal adjuvant potential when administered via various mucosal routes [27,28]. In addition, earlier works have also shown that the intranasal immunization of Zot containing formulations have performed better in reducing fecal shedding of *E. coli* O157:H7 in both mice [29] as well as goats [30]. However, these studies have not characterized its influence on cellular immune response which is unravelling to be important during *E. coli* O157:H7 intestinal colonization in cattle. The functional analysis of different regions of Zot has identified the C-terminal region called ΔG [25] which is responsible for mediating mucosal permeability. Though there are reports of the efficacy of ΔG in oral drug delivery [31], its ability to act as a mucosal adjuvant when delivered via intranasal route has not been extensively explored. Hence, we postulated that ΔG responsible for tight junction permeability could be used for mucosal antigen delivery. Therefore, the purpose of this study was to construct fusion protein (ΔG-Tir) containing C-terminal active fragment of Zot (ΔG) fused with extracellular, C-terminal region of Tir and characterize its systemic, mucosal and cellular immune responses as well as determine its efficacy on fecal shedding of *E. coli* O157:H7 in mice compared to Tir, ΔG + Tir administered via intranasal route.

2. Materials and methods

2.1. Bacterial strains, cell line, plasmids and media

Plasmids, host strains and bacteria used in this study are listed in Table 1. Bacterial culture media, supplements as well as antibiotics were purchased from Himedia laboratories, India. The bacteria were grown in Luria-Bertani (LB) broth or on LB agar at 37 °C and were supplemented, when required, with necessary antibiotics. Caco-2 cell line was procured from National Centre for Cell Science, Pune, India. All the cell culture media, reagents, and chemicals were purchased from Sigma–Aldrich (India).

2.2. Design and construction of recombinant ΔG, tir, ΔG-tir

The list of primer sequences utilized in the present study is given in Table 2. Nucleotide sequences of *Vibrio cholerae ΔG* active fragment of *zot* encoding amino acid region 287–398 (accession no. AAA27582.1) and *E. coli* O157:H7 *tir* encoding amino acid region 252–551 (accession no. AAC31506.1) were PCR amplified from their corresponding genomic DNA using their specific primers with incorporated restriction sites. PCR conditions for both individual and overlap extension PCR were as described previously by [32]. *ΔG*, *tir* amplicon was digested with *Bam*HI/*Xho*I, *Xho*I/*Hin*dIII, respectively. Then, the digested *ΔG*, *tir* amplicon was ligated in-frame with pRSET B, pRSET A vector cut with the compatible set of enzymes, respectively. The fusion gene *ΔG-tir* was digested with *Bam*HI/*Hin*dIII and ligated in-frame with pRSET B vector cut with the same set of enzymes. *ΔG*-pRSET B, *tir*-pRSET A and *ΔG-tir*-pRSET B plasmids were transformed into DH5α and selected on LB agar with ampicillin (100 μg/mL). Recombinant clones harbouring these plasmids were confirmed by PCR using a T7 universal primer as well as sequencing. Recombinant plasmids containing inserts were extracted and transformed individually into *E. coli* BL21 (DE3) pLysS for expression studies.

2.3. Expression and purification of recombinant proteins

Transformed bacteria harbouring *ΔG*-pRSET B, *tir*-pRSET A, and *ΔG-tir*-pRSET B were plated on LB agar containing ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL). The PCR positive clones were inoculated in LB broth supplemented with ampicillin (100 μg/mL), chloramphenicol (34 μg/mL) and cultured in shaker incubator at 37 °C till 0.6–0.8 O.D. (600 nm) was reached. The cultures were then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma, India) and grown for 5 h in shaker incubator. The recombinant protein expression was analyzed using SDS-PAGE and expressed proteins were purified under denaturing condition (buffers containing 8 M urea) from 500 mL of induced culture using affinity chromatography based Ni-NTA (Qiagen, Germany) column as per manufacturer's protocol. Step dialysis in gradient concentrations of urea followed by PBS (pH - 7.4) was done to remove urea and refold the purified recombinant proteins.

2.4. Immunization

Four to six week old female BALB/c mice were obtained from Central Animal Facility, Defence Food Research Laboratory, Mysuru, India. All animal experimental procedures in this study have been approved by Committee For The Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India (code- IAEC-2016/15) and performed in accordance with guidelines of Institutional Animal Ethical Committee, DFRL, Mysuru. Forty eight BALB/c mice (female, 6 week old, central animal facility, DFRL) were randomly divided into four groups of 12 mice each, of which 6 mice were allotted for cellular immune response characterization. All the mice in four groups were immunized via intranasal route and the dose of recombinant protein for experimental groups were 30 μg administered in a final volume of 20 μL (10 μL/nostril). Group I was intranasally administered with sterile PBS (Control group), group II was immunized with 30 μg of purified Tir, group III was immunized with 30 μg of purified ΔG + Tir and group IV was immunized with 30 μg of purified ΔG-Tir. Subsequent doses were administered on day 7, 21 and 35. Blood and fecal samples were collected one day prior to immunization and one week after the final booster.

2.5. Analysis of serum IgG, IgA and fecal IgA antibody responses

The Tir specific serum IgG, IgA titer in immunized mice groups (Tir, ΔG + Tir and ΔG-Tir) and serum IgG titer against ΔG in sham, Tir, ΔG + Tir and ΔG-Tir group sera were measured as described earlier