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Intranasal immunization with novel EspA-Tir-M fusion protein induces protective immunity against enterohemorrhagic *Escherichia coli* O157:H7 challenge in mice

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ABSTRACT

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 causes hemorrhagic colitis and hemolytic uremic syndrome in humans. Due to the risks associated with antibiotic treatment against EHEC O157:H7 infection, vaccines represent a promising method for prevention of EHEC O157:H7 infection. Therefore, we constructed the novel bivalent antigen EspA-Tir-M as a candidate EHEC O157:H7 subunit vaccine. We then evaluated the immunogenicity of this novel EHEC O157:H7 subunit vaccine. Immune responses to the fusion protein administered by intranasal and subcutaneous routes were compared in mice. Results showed higher levels of specific mucosal and systemic antibody responses induced by intranasal as compared to subcutaneous immunization. Intranasal immunization enhanced the concentration of interleukin-4, interleukin-10, and interferon-γ, while subcutaneous immunization enhanced only the latter two. In addition, intranasal immunization protected against EHEC O157:H7 colonization and infection in mice at a rate of 90%.Histopathological analysis revealed that vaccination reduced colon damage, especially when administered intranasally. In contrast, subcutaneous immunization elicited a weak immune response and exhibited a low protection rate. These findings demonstrate that intranasal immunization with the fusion protein induces both humoral and cellular immune (Th1/Th2) responses in mice. The novel EspA-Tir-M novel fusion protein therefore represents a promising subunit vaccine against EHEC O157:H7 infection.

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1. Introduction

Enterohemorrhagic *Escherichia coli* O157:H7 (EHEC O157:H7) is a food-borne pathogen that can cause diarrhea, hemorrhagic colitis, or life-threatening hemolytic uremic syndrome (HUS) [1,2], particularly in children under 5 years old [3]. Currently, there is no effective prophylactic or therapeutic treatment for the prevention of HUS [4]. Due to the risks associated with antibiotic treatment against EHEC O157:H7 infection, a vaccine that inhibits the bacteria from colonizing the intestinal epithelium represents the most promising method for the prevention of EHEC O157:H7 infection [5,6]. Presently, candidate vaccines that contain a variety of virulence factors against EHEC O157:H7 are being developed and have been tested in mouse models [7,8]. However, a vaccine that includes an antigen against adhesion factors is needed to inhibit the pathogen from adhering to the intestinal tract [9].

EHEC O157:H7 adherence to host cells is the first step in the infection process, leading to the formation of attaching and effacing (A/E) lesions. A number of virulence factors contribute to EHEC O157 colonization of the intestine, including type III secretion system (T3SS) proteins, fimbriae, flagella, auto-transporters, and outer membrane proteins [10,11]. In order to create A/E lesions, EHEC O157:H7 requires the T3SS and a filamentous tip, mainly composed of EspA, which delivers effectors [12]. Tir is one of the first proteins translocated during infection, and, as a receptor for the EHEC O157:H7 adhensin intimin, it plays an essential role in bacterial adherence to epithelial cells [13]. EspA and Tir represent

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promising immunogens, therefore, as they not only play key roles in EHEC O157:H7 colonization and adhesion but also can be detected in sera from convalescent individuals [14].

Candidate vaccines consisting of espA and Tir alone or fused with other virulence factors are under development [15–18]. The protective efficiency of a protective epitope of EspA was less than that of the full-length EspA protein, suggesting that there are additional protective epitopes in EspA [19]. Purified Tir can protect mice against EHEC O157:H7 challenge after intranasal immunization [16]. In our previous work, we predicted through bioinformatics analysis that the Tir central domain (Tir-M) may include a major antigen epitope. Therefore, a novel recombinant subunit vaccine consisting of EspA and Tir-M may hold the key to successful pre-harvest intervention against EHEC O157:H7. To test this hypothesis, we constructed the bivalent antigen EspA-Tir-M, which is composed of espA and the Tir-M domain, as a candidate EHEC O157:H7 subunit vaccine. We then evaluated the immunogenicity of this novel fusion protein and its ability to protect against EHEC O157:H7 challenge in mice.

2. Material and methods

2.1. Bacteria, plasmids, and cells

EHEC O157:H7 strain EDL933 (our laboratory stock) was grown in Luria–Bertani (LB) broth supplemented with 5 g/L streptomycin. The pET-28a (+)-based *E. coli* strains were grown in LB broth or on LB agar supplemented with 50 μg/mL kanamycin as needed for selection of recombinant plasmid. All strains were grown at 37 °C. The Ht-29 human colonic cancer cell line (our laboratory stock) was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Plasmid construction

The *espA* (GenBank accession no. KJ549678.1) and *Tir-M* (GenBank accession no. NC_002655.2) genes were PCR-amplified from EHEC O157:H7 EDL933 using primer pairs with the following sequences: P1 (forward), 5′-CG GAATTC ATG GAT ACA TCA AAT GCA-3′ and P2 (reverse), 5′-GCT ACC TCC TCC ACT TCC GCC TCC TTT ACC AAG GGA TAT TGC TG-3′ for*EspA*; P3 (forward), 5′-GGA GGC GGA AGT GGA GGA GGT AGC AGC CCA ACC ACG ACC GAC-3′ and P4 (reverse), 5′-CCC AAGCTT GGC TTG CTG TTT GGC CTC TT-3′ for *Tir-M* (*Eco*R I and *Hind*III sites are underlined). The EspA-Tir-M (et) construct was amplified by overlap extension PCR from EspA and Tir-M joined with a linker (GGA GGC GGA AGT GGA GGA GGT AGC) using primers P1/P4. The et fusion gene was inserted into plasmid pET-28a (+). Thereafter, the recombinant plasmid pET-28a (+)-et (28et) was transformed into *E. coli* DH5α cells (TIANGEN, Beijing, China) for amplification. The construct was identified by PCR amplification of et, restriction enzyme digestion, and sequencing. The 28et plasmid was transformed into *E. coli* BL21 (DE3) cells (TIANGEN, Beijing, China).

2.3. Expression, purification, and western blot analysis of et fusion protein

Cells containing 28et were induced with 1 mM IPTG and incubated for 4 h, as previously reported [20]. Concentrated bacterial suspensions were purified using a 6 × His-Tagged Protein Purification Kit (CWBIO, Beijing, China) according to the manufacturer's instructions. The protein samples were analyzed by 12% SDS-PAGE and purity was assessed by gel optical scanning. Furthermore, the purified protein was quantified with the BCA Protein Assay Kit (Bio Teke, Beijing, China) and analyzed by western blotting using an anti-EHEC O157:H7 polyclonal antibody (EterLife, Birmingham, UK) according to the manufacturer's instructions.

2.4. Immunization of mice with et

We maintained six-week-old female BALB/c mice at the Southern Medical University Laboratory Animal Center (Guangzhou, China) under pathogen-free conditions. All experiments were approved by the Institution Animal Care Committee of Southern Medical University (permit 44002100006397) and carried out in accordance with the regulations of the Chinese Council on Experimental Animal Care. Mice in each group were randomly divided into the following three subgroups: those receiving et subcutaneously (s.c.), those receiving et intranasally (i.n.), and those receiving PBS either subcutaneously or intranasally. The PBS group received 100 μL sterile PBS as a control; five mice received PBS intranasally and five mice received PBS subcutaneously. The s.c. group received a subcutaneous immunization of 100 μg et in PBS and was emulsified with the same volume of Freund's complete adjuvant (CFA, total volume 100 μL) for primary immunization and Freund's incomplete adjuvant (IFA) for boosting on days 7 and 21. The i.n. group was intranasally immunized with 100 μg et in PBS (total volume 100 μL) on days 0, 7, and 21. Serum samples were collected on days 0, 7, 21, and 35 and stored at −80 °C until use. Fecal extracts were collected on day 35 for secretory immunoglobulin A (SIgA) measurement as previously described [21].

2.5. Detection of antibody and cytokine levels by ELISA

Total serum IgG (total IgG) and fecal SIgA were measured by indirect enzyme-linked immunosorbent assay (ELISA) as previously described with minor changes [22]. ELISA plates were coated with 100 μL of 10 μg/mL purified et protein. Serum and fecal extracts were added to wells at a dilution of 1:50 and 1:10, respectively. Murine interferon (IFN)-γ and interleukin (IL)-4 and IL−10 were analyzed by quantitative ELISA at 1:20 dilutions using a mouse ELISA kit (Elabscience, Hubei, China) according to the manufacturer's instructions. All ELISA results were measured at OD_{450} using an EL9800 ELISA microplate reader (Bio-Tek, Winooski, VT, USA).

2.6. Survival analysis of immunized mice

Assessment of the protective effect of immunization against EHEC O157:H7 challenge was conducted 15 days after the last immunization. To clear intestinal flora and enhance EHEC O157:H7 colonization, mice received streptomycin (5 g/L) in drinking water three days before infection until the end of the experiment [23]. Mice were then challenged by intragastric administration of 1×10^{10} CFU EHEC O157:H7 EDL933 in PBS. Fecal shedding was detected at two-day intervals as previously described [21]. Surviving mice in each group were counted daily and sacrificed by cervical dislocation at the end of experiment.

2.7. Histopathology

Mouse tissue was obtained after infection and fixed by immersion in 10% neutral formalin, embedded in paraffin, and cut into sections that were stained with hematoxylin and eosin. Sections were evaluated under a light microscope by two board-certified pathologists who were blinded to the experimental groups.

2.8. Bacterial adherence to Ht-29 cells

To further evaluate the neutralization abilities of the antisera of

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