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A nasal vaccine comprising B-subunit derivative of Shiga toxin 2 for cross-protection against Shiga toxin types 1 and 2

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Summary Enterohemorrhagic *Escherichia coli* (EHEC) produces Stx1 and Stx2 causing severe diseases. Their B-subunits (StxBs) exhibit low immunogenicity and the anti-StxB antibodies neutralizing both Stxs has not been prepared yet. By intranasal vaccination with His-tagged-StxB (Stx1B-His or Stx2B-His) plus a mutant heat-labile enterotoxin (mLT) in mice, their serum and lung fluid reacted with appropriate StxB. Mice vaccinated with Stx2B-His plus mLT had antibodies reacting Stx1B and showed the resistance to toxemia of Stx1 and Stx2. This is the first demonstration to get anti-Stx2B serum neutralizing both Stxs. These suggest that the nasal vaccination with Stx2B-His and mLT is effective for preventing toxemias by EHEC.

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) is also known as Shiga toxin-producing *E. coli* (STEC) or Verotoxin-producing *E. coli* [1]. EHEC causes hemorrhagic colitis and, in some cases, hemolytic-uremic syndrome (HUS), a life-threatening complication, in children [2,3]. HUS is characterized by hemolytic anemia, thrombocytopenia, and renal failure, and occurs as a consequence of the action of the Shiga toxin family [4,5].

EHEC has been reported to produce six types of Shiga toxins (Stxs), comprising one from *Shigella dysenteriae*, the closely related Stx1 of EHEC, and the more distantly related

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Stx2, Stx2c, Stx2d, and Stx2e of EHEC. Although all 6 Stx variants differ to some degree at the amino acid sequence level [6], Stxs consist of an A subunit (StxA), which exhibits toxic activity, and five B subunits (StxB). StxA is non-covalently associated with a pentameric StxB that binds to the widely distributed globotriaosyl ceramide (Gb_3) cellular receptor [7,8]. After receptor binding of StxB, the toxin enters the host cell, StxA and StxB dissociate, and StxA's *N*-glycanase activity appears. Then StxA removes adenine from position 4328 of eukaryotic 28s rRNA [9—11]. The resulting StxA-mediated inhibition of protein biosynthesis is cytotoxic for the target cell.

Although many serotypes of EHEC have been isolated from HUS cases [12,13], they produce two major types of Stxs, Stx1 and Stx2, in the intestine [5]. 94% of EHEC isolates from HUS cases produce Stx2 alone or a combination of Stx2 and Stx1. Only 6% of EHEC isolates produce Stx1 alone [14]. Finally, Stx2 is associated with the most severe disease in humans and Stx1 aggravates the disease [15].

Stx1A and Stx2A exhibit only 55% homology in amino acid sequence. Stx1B and Stx2B Exhibit 57% homology in amino acid sequence. Immunologically, Stx1 and Stx2 are different from each other and antibodies to each toxin do not neutralize the toxicity of both Stx1 and Stx2, as judged on in vivo assaying [2,16—18]. In contrast, in numerous studies on animals immunized with the holotoxoid of Stx1 or Stx2 [14,15], the animals were cross-protected against Stx1 and Stx2. It was likely that, this cross-protection was primarily due to the production of StxA-specific antibodies and not StxB-specific ones [14,19,20]. As the antigenicity of both StxBs is very low, antibodies raised against StxB are very weak [21—23]. It has been reported that antibodies to Stx1B or Stx2B administered i.m. or i.n. neutralize Stx1 or Stx2, respectively, but are not cross-protective against Stx1- and Stx2-toxemia [24—26]. Only one report suggested that a monoclonal antibody against Stx2B recognizes and neutralizes both Stx1 and Stx2 in HeLa cells, as judged on cytotoxicity assaying [27]. Therefore, there has been no paper showing that derivatives of Stx1B or Stx2B induce anti-StxB antibodies that neutralize the toxicities of both Stx1 and Stx2 and protect mice from toxemias of both toxins.

Then, we focused our efforts on the development of an intranasal (i.n.) vaccine comprising StxB derivative, which induces anti-StxB antibodies neutralizing Stx1 and Stx2.

Materials and methods

Mice

All experiments were performed using ICR or ddY female mice (Shizuoka Animal Co., Ltd., Shizuoka, Japan) aged 8—9 weeks at the onset of the priming immunization. The controls were age-matched female mice.

According to Guidelines for the Management of Laboratory Animals in Fujita Health University, all experiments using animals were performed with the permission of Law for the Humans treatment and Management and Animals in Japan. All animals were kept according to Standards Relating to the Care and Management of Laboratory Animals in Japan.

Construction and purification of Stx1B-His and Stx2B-His

To construct plasmids expressing histidine-tagged Stx1B (Stx1B-His) and histidine-tagged Stx2B (Stx2B-His) a two-step PCR-based strategy was adopted [28]. The Stx1B gene and the 3′-non-coding region of the Stx1B gene were amplified by PCR with the EHEC TS26 strain using primer pairs (P1 and P2: GGCCATGGGTCTGATGCGCAGAACTATTA and GTCGACGCTACCGCGGCCGCTACGAAAAATAACTTCGC; and P3 and P4: GTCGACGCTACCGCGGCCGCTACGAAAAATAACTTCGC and CGGAATTCCTCCGCCTGCTATTTTC, respectively. The resulting fragment mixture was reamplified using P1 and P4. The Stx2B gene and the 3′-non-coding region of the Stx2B gene were amplified by PCR from genomic DNA of the EHEC 86-24 strain using primer pairs (P5 and P6: GGCCATGGGCAACGGGTAAATAAAGGAG and GTCGACGCTACCGCGGCCGCTGTCATTATTAAACTGCACTT; and P7 and P8: GGCCGCGGTAGCGTCGACTGAGGCATAACCTGATTC and CGGAATTCATTACACTTGTTACCCACA, respectively. The resulting fragment mixture was used as a template for reamplification using P5 and P8. Then, PCR product for preparation of Stx1B-His was constructed by the genes of Stx1B containing upstream (35 bases), spacer (Sal*I* site) and 6 histidines plus stop codon. One for Stx2B-His was constructed by the genes of upstream (35 bases) and signal sequence of Stx1B, mature protein of Stx2B, spacer (Sal*I* site) and 6 histidines plus stop codon. Each PCR was digested with *Nco*I plus *Eco*RI, and inserted into pTrcHis2A to yield Stx1B and Stx2B.

Stx1B-His and Stx2B-His were purified with affinity chromatography on a Ni^{2+}-loaded HiTrap Chelating HP column (Amersham Biosciences K.K., Tokyo, Japan), as described previously [28]. The peak fractions of each StxB-His were dialyzed against TEAN buffer (pH 6.8) [29]. The degree of purification was determined by SDS-PAGE and the protein concentration was determined from the absorbance at 280 nm.

Preparation of a mucosal adjuvant

The mutant heat-labile enterotoxin of *E. coli* (mLT), in which a part of A subunit covering from Arg192 to Ile194 is deleted, was used as a mucosal adjuvant [30]. mLT was purified, using a galactose-immobilized column (Pierce Chemicals, Rockford, IL), as described previously [30]. The purity of the toxins was assessed by SDS-PAGE and no contaminating proteins bands were noted.

Nasal immunization of mice

mLT (10 μg/dose) was mixed with Stx1B-His or Stx2B-His (10 μg/dose) in phosphate-buffered saline (PBS). 20 μl of the mixture was i.n. administered to mice under light ketaral (Sankyou Co., Ltd.) and xylamine (Bayer Co., Ltd.) anesthesia three times on days 0, 7, and 14, as described previously [31]. Four weeks after the last administration, peripheral blood and bronchial alveolar lavage fluid (BALF) were obtained from the mice. BALF was prepared by washing each lung with 1ml PBS. After centrifugation of each sample, the supernatant was stored at −20 °C until assayed.