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Immunization with a Genetically Engineered Uropathogenic *Escherichia coli* Adhesin-*Escherichia coli* Enterotoxin Subunit A2B Chimeric Protein

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Abstract – The generation of secretory IgA antibodies (Abs) for specific immune protection of mucosal surfaces depends on stimulation of the mucosal immune system, but this is not effectively achieved by parenteral or even oral administration of most soluble antigens. Thus, to produce a possible vaccine antigen against urinary tract infections, the uropathogenic *E. coli* (UPEC) adhesin was genetically coupled to the heat-labile *Escherichia coli* enterotoxin A2B (*ltxa2b*) gene and cloned into a pMAL-p2E expression vector. The chimeric construction of pMAL*fimH*/*ltxa2b* was then transformed into *E. coli* K-12 TB1 and its nucleotide sequence was verified. The chimeric protein was then purified by applying the affinity chromatography. The purified chimeric protein was confirmed by SDS-PAGE and western blotting using antibodies to the maltose binding protein (MBP) or the heat labile *E. coli* subunit B (LTXB), plus the N-terminal amino acid sequence was analyzed. The orderly-assembled chimeric protein was confirmed by a modified G_{M1}-ganglioside ELISA using antibodies to adhesin. The results indicate that the purified chimeric protein was an Adhesin/LTXA2B protein containing UPEC adhesin and the G_{M1} -ganglioside binding activity of LTXB. This study also demonstrate that peroral administration of this chimeric immunogen in mice elicited high level of secretory IgA (sIgA) and serum IgG Abs to the UPEC adhesin. The results suggest that the genetically linked LTXA2B acts as a useful mucosal adjuvant, and that the adhesin/LTXA2B chimeric protein might be a potential antigen for oral immunization against UPEC.

Keywords □ Uropathogenic *E. coli* adhesin, adhesin/LTXA2B, LTXA2B, FimH

INTRODUCTION

The enterobacteria *Escherichia coli* are the main causative agents of the urinary tract infections (UTIs) and account for the occurrence of over 85% of acute cystitis and pyelonephritis, 60 % or more of recurrent cystitis, and at least 35% of recurrent pyelonephritis (Uehling *et al.*, 1994). The reservoir for uropathogenic *E. coli* (UPEC) is fecal flora, from which the bacteria spread to the urogenital mucosa, ascend into the bladder, and adhere to the bladder epithelium. Once established in the bladder, the bacteria multiply and establish a local infection (cystitis) and can further ascend to involve the ureters and kidneys (pyelonephritis) (Sauer *et al.*, 2000).

Recent in vivo studies in mice demonstrated that colonization of the bladder by UPEC requires the mannose-sensitive binding of FimH, the adhesin present at the tip of type 1 pili, to the bladder epithelium (Abraham *et al.*, 1988; Langermann *et al.*, 1997). This evidence suggests that the determinant in *E. coli* type 1 fimbriae responsible for mediating mannose-specific adherence is its FimH subunits and that the presence of this fimbrial moiety is important for initiating bacteria infections in the urinary tract (Beachey *et al.*, 1988; Knudsen *et al.*, 1998).

In general, protein antigens that may be highly immunogenic by parenteral routes are usually ineffective when administered orally, probably because of intestinal digestion and lack of uptake by the gut-associated lymphoid tissue. A notable exception is the heat-labile *Eshcherichia coli* enterotoxin (LTX), whose strong enteric immunogenicity depends in part on its affinity for the G_{M1} ganglioside and consequent tropism for gut-associated lymphoid tissue (Douce *et al.*, 1999; Hajishengallis *et al.*, 1995; Kim *et al.*, 2001). LTX (83 kDa) is composed of toxic A1 subunits of LTX (24 kDa), A2 subunits (6 kDa), and five identical subunits of LTXB (12 kDa each) that bind to the G_{M1}-ganglioside receptors of the intestinal cells. The A2B subunit of LTX has adjuvant activity, and serves as a

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carrier protein for orally administered vaccine (Dallas *et al.*, 1980; Nashar *et al.*, 1993; Rappuoli *et al.*, 1999). At the present time, the cholera toxin (CTX) and the *E. coli* heat-labile toxin (LTX) have both been used as mucosal adjuvants (Pizza *et al.*, 2001; Haan *et al.*, 1996; Rodighiero *et al.*, 1999). A protein antigen that is linked genetically or chemically to LTXA2B confers on them the ability to bind the G_{M1} ganglioside and to elicits the sIgA antibodies while it provides protection for mucosal surfaces by interfering with microbial adherence, colonization, and invasion (Elson *et al.*, 1984; Verweij *et al.*, 1998).

To produce a possible vaccine antigen against urinary tract infections, the current paper reports on the cloning and sequencning of the *fimH* gene and a fusion gene of *fimH* genetically linked to the *ltxa2b* gene. The expression, purification, and characterization of a recombinant protein containing Adhesin/LTXA2B is described. This study also demonstrates that peroral administration of this chimeric immunogen in mice elicited a high level of secretory IgA and serum IgG antibodies to the UPEC adhesin.

MATERIALS AND METHODS

Bacterial strains

The uropathogenic strain *Escherichia coli* (UPEC) J96 (O4:K6) was used as the source of chromosome DNA for constructing the recombinant plasmid; *E. coli* K12 strain DH5α[*F-lacZ_M15 hsdR17*(*r-m-*)*gyrA36*] was used as the host for maintaining the plasmid clones; *E. coli* K12 TB1 [*ara* (*lac proAB*) *rpsL thi*[80 *dlac*]q (*lacZ*) *M15*] *hsdR* (rk-mk+) (New England Biolabs, Inc, MA., U.S.A)] was used for the gene expression of the cloned DNA. The plasmid pMAL-p2E (New England Biolabs, Inc) was used for cloning, amplifying, and expressing the adhesin/*ltxa2b*, *fimH*, and *ltxa2b* gene.

Animals

Specific-pathogen-free female BALB/c mice at 6~8 weeks of age were purchased from Japan SLC, Inc., Shizuoka, Japan. Animals were housed under barrier-sustained conditions, with temperature (20~22°C), humidity (55±5%), and light (14 h of light and 10 h of darkness) automatically controlled, and were kept in polypropylene cages. All mice were fed commercial pellet-form food and given tap water *ad libitum*.

Construction and purification of the MBPfimHLtxA2b chimera

The primers used to amplified the *fimH* gene were 5-CGAAGGATCCATTGTAATGAAACGAGTTATTACC-3 (underline containing *Bam*HI site) and 5-TGTGAAGCTTTTATTGATAAACAAAAGTCACG-3 (underline containing *Hind*III site). The primers used to amplify the *ltxa2b* gene were 5-AGCTGGATCCGAAGAGCCGTGGATT-3 (underline containing *Bam*HI site) and 5-CCCCAAGCTTCTAGTTTTCCATACTGATTGC-3 (underline containing *Hind*III). The amplified *fimH* and *ltxa2b* genes digested with *Bam*H1 and *Hind*III and inserted into the expression vector pMAL-p2E to construct the pMAL*fimH* and pMAL*ltxa2b*, respectively. pMAL*ltxa2b* was digested with *Hind*III and *Bam*H1, then amplified 599 bp DNA fragment was ligated into the pMAL*fimH* vector. The resulting construct was named pMAL*fimH/ltxa2b*.

E. coli TB1 was transformed with pMAL*fimH/ltxa2b* and the transformant was cultured in a Luria-Bertani broth supplemented with 0.2% of glucose containing 100 μg/ml of ampicillin at 37°C with vigorous shaking. When the OD_{600} of the culture reached 0.5~0.6, IPTG was added to a final concentration of 0.01 mM. The supernatant containing the periplasmic peptide was released by osmotic shock procedure and purified by affinity chromatography on amylose resin in a 2.5 × 10 cm column (Neu *et al.*, 1965). The purification was carried out using a pMAL™ Protein Fusion and Purification System according to the manufactures directions (New England Biolabs).

Mice and immunization

Five female BABL/c mice in each group were immunized three times at 10-day intervals by oral adminstration with 100 μg and 25 μg of the purified adhesin/LTXA2B chimeric protein, 30 μg of the purified adhesin alone, and 25 μg of purified LTXA2B alone. The control mice were only given sterile PBS.

Collection of samples

Blood and vaginal washing were obtained at the termination of the experiment. Mice were anaesthetized with pentobarbital. sIgA was taken from vaginal washes by pipetting 50 μl of PBS into the vaginal tract using a Gilson pipette. For 3 days before the mice were killed, vaginal washes were obtained by withdrawn and reintroduced 10 times of the vagina with same volume and pooled for each mouse. After collection, the samples were centrifuged (1,000 × g, 10 min), the supernatants used for determining the antibody level. Sera were prepared from the blood and used for determining the serum IgG, IgA, and IgM levels. Sera and vaginal washes were stored at -20°C until assayed.

Enzyme-linked immunosorbent assay (ELISA)

Ninety-six-well microtiter plates (MaxiSorp U16j Nunc, Roskilde, Denmark) were coated with 30 μg of purified antigens per well for 12 h at 4°C and blocked with PBS containing 1% BSA for 120 min at room temperature. This blocking solution was then used as the diluent for the sera and vaginal washes, and conjugates for all subsequent steps. After blocking, the plates were incubated for 120 min at 37°C with appropriate dilutions of the sera and vaginal washes. The dilutions for the detection of serum IgG, IgA, IgM, and secretory IgA were 1:10, 1:5, 1:20, and 1:3, respectively. After washing three times with PBS/Tween (PBS containing 0.05% Tween-20), alkaline phosphatase-conjugated anti-mouse IgG (1:1,000), IgA (1;1,000), and IgM (1;1,000) (Sigma) was added and the plates incubated for 120 min at 37°C. The plates were then washed three times and incubated with a substrate (paranitrophenyl phosphate; pNPP) for 30 min at room temperature. The absorbance read at 405 nm using an microplate reader.

Statistical analysis

The differences in the antibody levels in immunized and non-immunized mice were evaluated using the Student's *t* test. The differences were considered significant for $P \leq 0.05$.

RESULTS AND DISCUSSION

Expression, purification, and characterization of chimeric protein

Initial expression studies with *E. coli* TB1 carrying pMAL *fimH/ltxa2b* showed that large quantities of the MBPFimHLtxA2 fusion protein and LtxB were produced as insoluble aggregates following induction with 0.01 mM isopropyl-β-D-thiogalactoside. IptG Solubilization of the aggregates with 6 M urea following by dialysis resulted in a very low yield of the active MBPFimHLtxA2B chimera, as indicated by G_{M1}-binding enzyme-linked immunosorbent assay (ELISA) using anti-fimH as detecting antibody. The high yield of the active form of the chimera was from noninduced cultures at 37°C. Therefore, batches of noninduced cultures were used as the source for the isolation of the chimera by affinity chromatography using amylose column (20 ml; New England Biolabs) following with a D-galactose column (15 ml; Pierce, Rockford III.) according to the manufacturer's suggestions. The yield of the chimera was 900 μg per liter of culture.

The composition of purified adhesin-LTXA2B was verified by SDS/PAGE and Western blot analysis using antibodies to the maltose binding protein and LTXB. An unheated sample of the affinity isolated chimera showed a single band with a mass of ca. 120 kDa on the SDS-PAGE gel, suggesting purity (data not shown). The boiled sample showed the disappearance of the 120 kDa band and appearance of two bands of 81 and 14 kDa (Fig. 1A and 1C). Western immunoblots showed that the 120- and 81-kDa band were recognized by the monoclonal anti-fimH antibody (Fig. 1B). The 120-kDa band and other bands from the unheated sample were recognized by the anti-LtxB antibody (1/1,000; List Biological Laboratories, Campbell, Calif.) (data not shown). The multiple banding pattern is likely due to the different aggregated forms of the chimera. In the heated sample, only the 14-kDa band was recognized by the anti-LtxB antibody (Fig. 1D).

The molecular mass of the native chimera protein was esti-

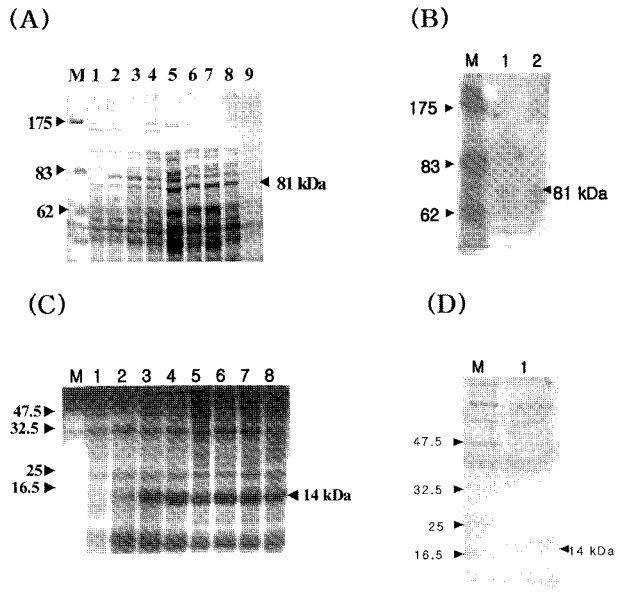

Fig. 1. SDS/PAGE and Western blotting of purified adhesin-LTXA2B. (A, B) Lane 1 (M), prestained molecular weight marker. SDS/PAGE analysis of crude extract from recombinant *E. coli* TB1 carrying pMAL*fimH/ltxa2b*. The cell lysate proteins (lane 2-10) from *E. coli* TB1 carrying pMAL*fimH/ltxa2b* after 0, 2, 4, 6, 8, 10, 12, 24, and 48 hours of induction with 0.01 mM of IPTG were resolved by electrophoresis on 7% (A) and 15% (C) polyacrylamide gels. The gels were stained with Coomassie blue. Purified Adhesin/LTXA2B resolved by resolved by electrophoresis on 7% and 15% polyacrylamide gels and immunoblotted on nitrocellulose(B) and PVDF membranes(D), respectively. The nitrocellulose membrane was exposed to a 1:10,000 dilution of a polyclonal antibody to MBP. The PVDF membrane was exposed to a 1:1,000 dilution of a polyclonal antibody to LTXB. In all cases, the samples applied to the gel were equivalent to 10 μg of cell lysates. Right arrowheads indicate Adhesin/LTXA2 (A, B) and LTXB (C, D).

mated by gel permeation chromatography on a Sephacryl S-200HR column (2.5 by 96 cm). The eluted protein was assayed by G_{M1}-binding ELISA. The single peak that immunoreactive to both anti-FimH and anti-LTXB antibodies has molecular weight of 131,000 as estimated by comparisons to known protein standards (Sigma). The results suggest that the chimera is the expected MBPfimHA2 $(ltxB)_5$. It is interesting that the MBPfimHltxA2B chimera is considerably larger than the holo-heat-labile toxin (84 kDa) and the SBRLtxA2B chimera (107.9 kDa) (Hajishengallis *et al.*, 1995). Our results indicate that the A2 fragment can direct a polypeptide as large as 81 kDa for association with the LtxB pentamer.

Immunogenicity of the MBPFimHLtxA2B chimera

Antibody responses against *E. coli* adhesin and LTXB in vaginal wash and serum samples taken from individual vaccinated mice were measured by ELISA. Levels of the sIgA antibody response to *E. coli* adhesin with adhesin/LTXA2B were greater than those found with adhesin alone or LTXA2B alone ($P<0.05$ by Student's *t* test) (Fig. 3). A minimal effect was observed when adhesin alone was used. However, no secretory IgG and IgM antibodies were detected in mice immunized with adhesin/LTXA2B, adhesin or LTXA2B alone (data not shown).

The induction of serum antibody responses against *E. coli* adhesin and LTXB was observed that higher serum IgG antibody responses to *E. coli* adhesin and LTXB were elicited in mice immunized with adhesin/LTXA2B than in those immunized with adhesin or LTXA2B alone ($P<0.05$ by Student's *t* test) (Fig. 2A). Serum IgA antibody responses to *E. coli* adhesin and LTXB was also induced in mice immunized with adhesin/LTXA2B than in those immunized with adhesin or LTXA2B alone (Fig. 2B). But serum IgM antibody was not induced in mice immunized with adhesin/LTXA2B, adhesin or LTXA2B alone (Fig. 2C). Higher vaginal sIgA antibody responses to *E. coli* adhesin and LTXB was also elicited in mice immunized with adhesin/LTXA2B than in those immunized with adhesin or LTXA2B alone (Fig. 3). Our present results demonstrate that it is possible to induce high levels of antibodies against cloned heterologous antigens in serum and vaginal secretions after peroral immunization. Thus mucosal immunization with *E. coli* adhesin coupled to LTXA2B may confer significant protection against *E. coli* infection in a mouse model.

The present studies show that oral administration of adhesin fused to LTXA2B elicited higher levels of mucosal sIgA and serum IgG antibodies to the *E. coli* adhesin molecule in mice

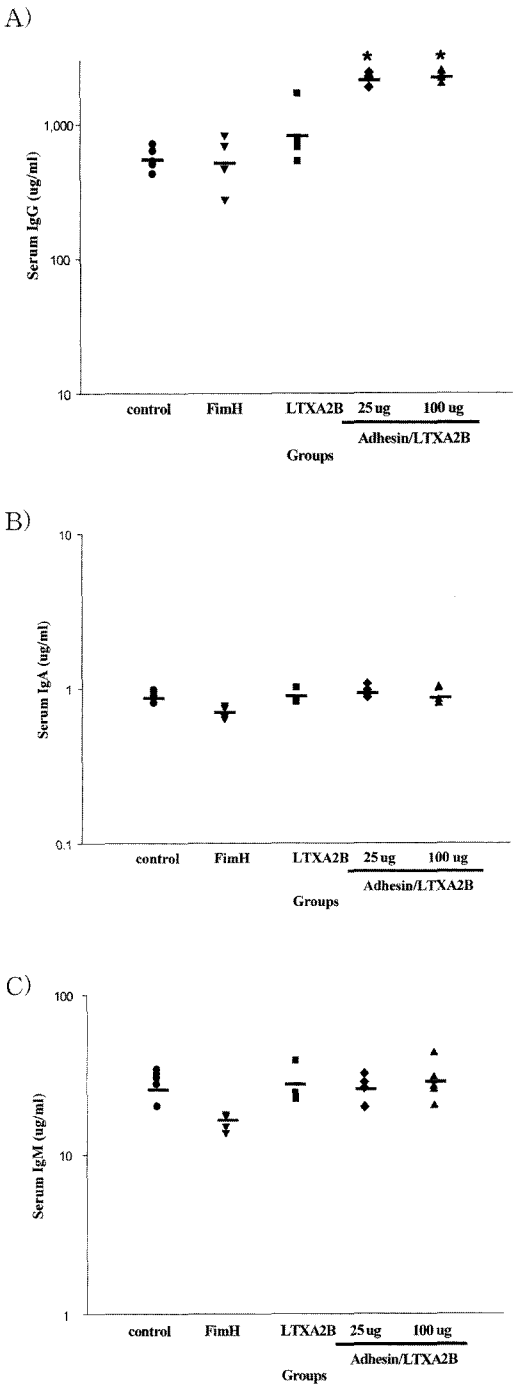

Fig. 2. Serum IgG(A), IgA(B) and IgM(C) antibody responses in SPF BALB/c mice orally immunized with Adhesin/LTXA2B, FimH, and LTXA2B. The data are expressed as mean IgG, IgA, and IgM antibody levels achieved in response to adhesin and LTXB, measured as described in the Materials and Methods section. Individual animals are represented by different symbols: ●, control; ▼, immunized with FimH; ■, immunized with LTXA2B; ◆, immunized with Adhesin/LTXA2B (25 μg); ▲, immunized with Adhesin/LTXA2B (100 μg). Horizontal bars represent median values. Significance of differences: * $P<0.01$ compared with control group.

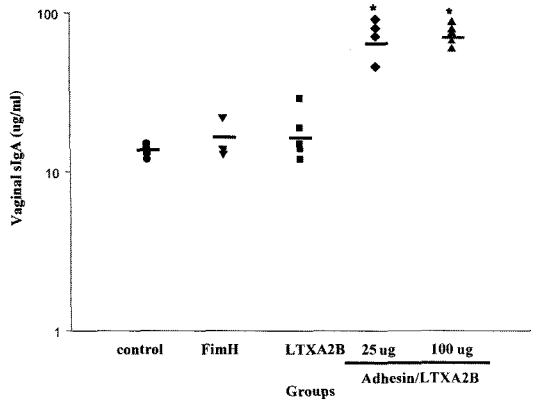

Fig. 3. Mucosal sIgA antibody responses in BALB/c mice orally immunized with Adhesin/LTXA2B, FimH, and LTXA2B. Data are shown as the mean antibody (Ab) levels to adhesin and LTXB, measured as described in the Materials and Methods section. Individual animals are represented by different symbols: ●, control; ▼, immunized with FimH; ■, immunized with LTXA2B; ◆, immunized with Adhesin/LTXA2B (25 μg); ▲, immunized with Adhesin/LTXA2B (100 μg). Horizontal bars represent median values. Significance of differences: * $P<0.01$ compared with control group.

than did oral administration of adhesin alone. This finding provides evidence that LTXA2B can serve as a mucosal adjuvant for the enhancement of an antibody response to *E. coli* adhesin (Nashar *et al.*, 1993). The present data are also in keeping with the previous finding that a chimeric protein is immnogenic by the mucosal route and induces antibody responses (Hajishengallis *et al.*, 1995). However, our data do not totally rule out the possibility that other immune cells, including T cells, may be important for the generation of a protective immune response against UPEC, because the antibodies generated during UPEC infections would be insufficient for bacterial clearance. It is also known that the immunoregulatory and pro-inflammatory cytokines play a major role in selecting the isotype of antibodies produced during the immune response and influence the nature of the local T cell response. Additional studies to elucidate whether peroral immunization of recombinant adhesin linked to LTXA2B induces T cell responses and the production of cytokines are currently under way.

Taken together, the results indicate that the genetically linked LTXA2B acts as a useful mucosal adjuvant, and the adhesin/LTXA2B chimeric protein could be a potential component in future UPEC vaccine development.

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