

## Induction of a systemic IgG and secretory IgA responses in mice by peroral immunization with uropathogenic *Escherichia coli* adhesin protein coupled to cholera toxin A2B subunits

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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 *ctxa2b* gene and cloned into a pMAL-p2E expression vector. The chimeric construction of pMAL-fimH/ctxa2b 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 cholera toxin subunit B (CTXB), plus the N-terminal amino acid sequence was analyzed. The orderly-assembled chimeric protein was confirmed by a modified G<sub>M1</sub>-ganglioside ELISA using antibodies to adhesin. The results indicate that the purified chimeric protein was an Adhesin/CTXA2B protein containing UPEC adhesin and the G<sub>M1</sub>-ganglioside binding activity of CTXB. This study also demonstrate that peroral administration of this chimeric immunogen in mice elicited high level of secretory IgA and serum IgG Abs to the UPEC adhesin. The results suggest that the genetically linked CTXA2B acts as a useful mucosal adjuvant, and that the adhesin/CTXA2B chimeric protein might be a potential antigen for oral immunization against UPEC.

**Keywords** □ uropathogenic *E. coli* adhesin, Adhesin/CTXA2B, CTXA2B, 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 (Langermann *et al.*, 1997). This evi-

dence 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 (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 (Mckenzie *et al.*, 1984). A notable exception is the cholera toxin (CTX), whose strong enteric immunogenicity depends in part on its affinity for the G<sub>M1</sub> ganglioside and consequent tropism for gut-associated lymphoid tissue (Hajishengallis *et al.*, 1995; Kim *et al.*, 2001; Wu *et al.*, 1992). CTX (87 kDa) is composed of toxic A1 subunits of CTX (23 kDa), A2 subunits (5 kDa), and five identical subunits of CTXB (11.8 kDa each) that bind to the G<sub>M1</sub>-ganglioside receptors of the intestinal cells. The A2B subunit of CTX has adjuvant activity, and serves as a carrier protein for orally administered vaccine (Menge *et al.*, 1993; Nashar *et al.*, 1993). At the present time, the cholera toxin (CTX) and the *E.*

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*coli* heat-labile toxin (LTX) have both been used as mucosal adjuvants (Pizza *et al.*, 2001; Haan *et al.*, 1996). A protein antigen that is linked genetically or chemically to CTXA2B confers on them the ability to bind the G<sub>M1</sub> ganglioside and to elicits the secretory (s)-IgA antibodies while it provides protection for mucosal surfaces by interfering with microbial adherence, colonization, and invasion (Harokopakis *et al.*, 1998; Tochikubo *et al.*, 1998; Verweij *et al.*, 1998).

Accordingly, to produce a possible vaccine antigen against urinary tract infections, the current paper reports on the cloning and sequencing of the *fimH* gene and a fusion gene of *fimH* genetically linked to the *ctxa2b* gene. The expression, purification, and characterization of a recombinant protein containing Adhesin/CTXA2B is described. This study also demonstrates that peroral administration of this chimeric immunogen in mice elicited 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 DH5 [*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. *Ctxa2b* genes were amplified from plasmid pRIT10814 (clone 39052 containing the *ctx* gene: American Type Culture Collection, Rockville, U.S.A) by a PCR. The plasmid pMAL-p2E (New England Biolabs, Inc) was used for cloning, amplifying, and expressing the Adhesin/*Ctxa2b*, *FimH*, and *Ctxa2b* 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 MBP*fimH*CTXA2B chimera

The primers used to amplified the *fimH* gene were 5-CGAAGGATCCAT-TGTAATGAAACGAGTTATTACC-3 (underline containing HindIII site) and 5-TGTGAAGCTTT-TATTGATAAACAAAAGTCACG-3 (underline containing BamH site). The primers used to amplify the *ctxa2b* gene were 5-AGCTGGATCCGAAGAGCCGTGGATT-3 (underline containing HindIII site) and 5-TTTTAAGCTTTAATTTGCCAT-ACTAATTGC-3 (underline containing BamHI). The amplified *fimH* and *ctxa2b* genes digested with BamHI and HindIII and inserted into the expression vector pMAL-p2E to construct the pMAL*fimH* and pMAL*ctxa2b*, respectively. pMAL*ctxa2b* was digested with HindIII and BamHI, then amplified 576 bp DNA fragment was ligated into the pMAL*fimH* vector. The resulting construct was named pMAL*fimH/ctxa2b*.

*E. coli* TB1 was transformed with pMAL*fimH/ctxa2b* 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<sub>600</sub> 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 and Heppel, 1965; Slos *et al.*, 1994). 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 BALB/c mice in each group were immunized three times at 10-day intervals by oral administration with 100 µg and 25 µg of the purified adhesin/CTXA2B chimeric protein, 30 µg of the purified adhesin alone, and 25 µg of purified CTXA2B 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. Secretory IgA 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  $-20^{\circ}\text{C}$  until assayed.

#### Enzyme-Linked Immunosorbent Assay (ELISA)

Ninety-six-well microtiter plates (MaxiSorp U16j Nunc, Roskilde, Denmark) were coated with  $30\ \mu\text{g}$  of purified antigens per well for 12 h at  $4^{\circ}\text{C}$  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  $37^{\circ}\text{C}$  with appropriate dilutions of the sera and vaginal washes. The dilutions for the detection of serum IgG, serum IgA, serum 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  $37^{\circ}\text{C}$ . The plates were then washed three times and incubated with a substrate (paran-

itrophenyl phosphate; pNPP) for 30 min at room temperature. The absorbance read at 405 nm using a microplate reader.

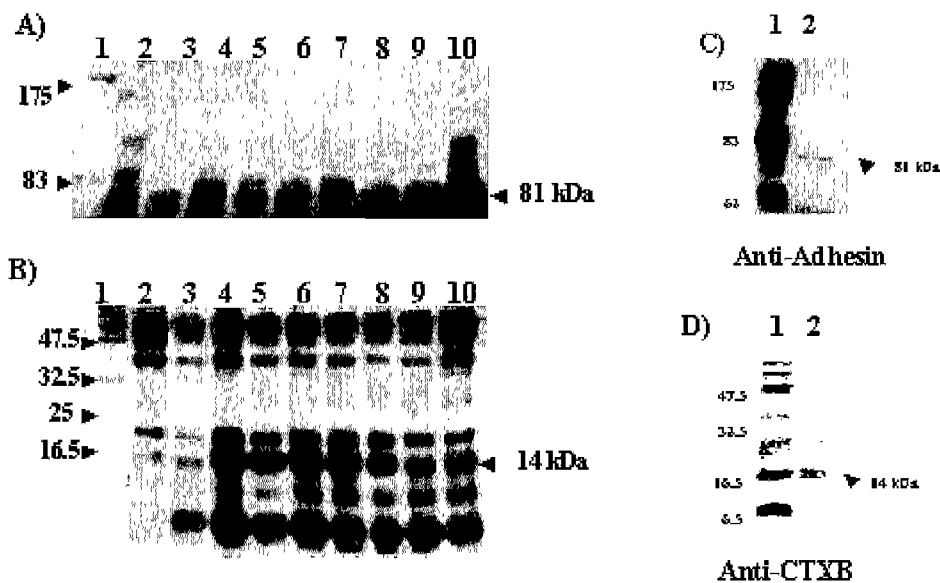
#### Statistical Analysis

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/ctxa2b* showed that large quantities of the MBPFimHCtxA2 fusion protein and CtxB were produced as insoluble aggregates following induction with 0.01 mM isopropyl- $\beta$ -D-thiogalactoside. Solubilization of the aggregates with 6 M urea following by dialysis resulted in a very low yield of the active MBPFimHCtxA2B chimera, as indicated by  $G_{M1}$ -binding enzyme-linked immunosorbent assay (ELISA) using anti-*fimH* as detecting antibody. The high yield of the active



**Fig. 1.** SDS/PAGE and Western blotting of purified adhesin-CTXA2B. A, B) Lane 1, prestained molecular weight marker. SDS/PAGE analysis of crude extract from recombinant *E. coli* TB1 carrying pMAL*fimH/ctxa2b*. The cell lysate proteins (lane 2-10) from *E. coli* TB1 carrying pMAL*fimH/ctxa2b* 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% (B) polyacrylamide gels. The gels were stained with Coomassie blue (A, B). Purified Adhesin/CTXA2B resolved by resolved by electrophoresis on 7% and 15% polyacrylamide gels and immunoblotted on nitrocellulose (C) 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 CTXB. In all cases, the samples applied to the gel were equivalent to 10  $\mu\text{g}$  of cell lysates. Right arrowheads indicate Adhesin/CTXA2 (A, C) and CTXB (B, D).

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, Ill.) according to the manufacturer's suggestions. The yield of the chimera was 800 µg per liter of culture.

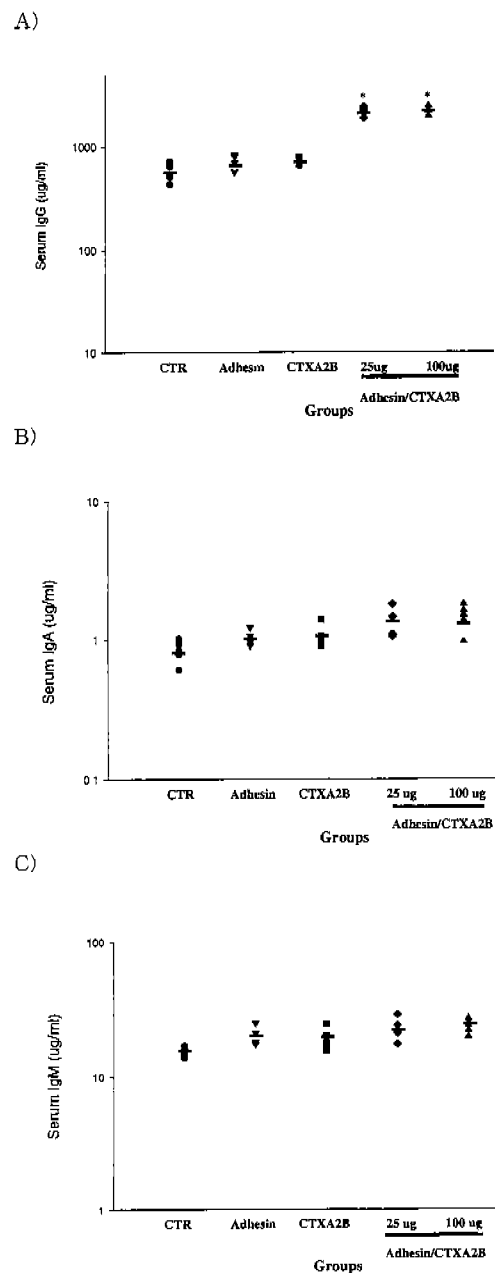
The composition of purified adhesin-CTXA2B was verified by SDS/PAGE and Western blot analysis using antibodies to the maltose binding protein and CTXB. An unheated sample of the affinity isolated chimera showed a single band with a mass of ca. 110 kDa on the SDS-PAGE gel, suggesting purity. The boiled sample showed the disappearance of the 110 kDa band and appearance of two bands of 79 and 14 kDa (Fig. 1A and 1B). Western immunoblots showed that the 110- and 79-kDa band were recognized by the monoclonal anti-fimH antibody. The 110-kDa band and other bands from the unheated sample were recognized by the anti-CtxB antibody (1/1,000; List Biological Laboratories, Campbell, Calif.). The multiple banding pattern is likely due to the different aggregated forms of the chimera (Gril, 1976; Mekalanos *et al.*, 1983). In the heated sample, only the 14-kDa band was recognized by the anti-CtxB antibody.

The molecular mass of the native chimera protein was estimated 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-CtxB antibodies has molecular weight of 127,000 as estimated by comparisons to known protein standards (Sigma). The results suggests that the chimera is the expected MBPFimHA2(CtxB)<sub>5</sub>. It is interesting that the MBPFimHCtxA2B chimera is considerably larger than the holocholera toxin (84 kDa) and the SBRCtxA2B chimera (107.9 kDa) (Hajishengallis *et al.*, 1995). Our results indicate that the A2 fragment can direct a polypeptide as large as 79 kDa for association with the CtxB pentamer.

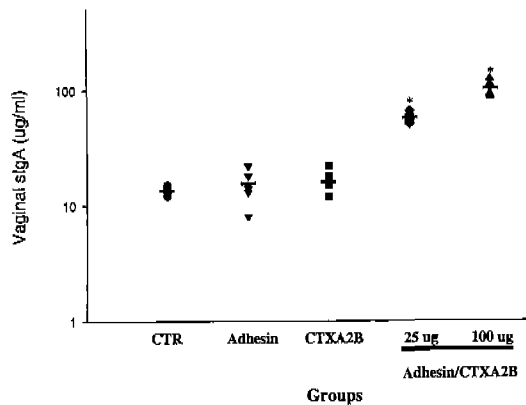
### Immunogenicity of the MBPFimHCtxA2B chimera

Antibody responses against *E. coli* adhesin and CTXB in vaginal wash and serum samples taken from individual vaccinated mice were measured by ELISA. Levels of the secretory IgA antibody response to *E. coli* adhesin with adhesin/CTXA2B were greater than those found with adhesin alone or CTXA2B alone ( $P < 0.05$  by Student's *t* test)(Fig. 3). A minimal effect was observed when adhesin alone was used. But no secretory IgG and IgM antibodies were detected in mice

immunized with adhesin/CTXA2B, adhesin or CTXA2B alone (data not shown).



**Fig. 2.** Serum IgG(A), IgA(B) and IgM(C) antibody responses in SPF BALB/c mice orally immunized with Adhesin/CTXA2B, adhesin and CTXA2B. The data are expressed as mean IgG, IgA, and IgM antibody levels achieved in response to adhesin and CTXB, measured as described in the Materials and Methods section. Individual animals are represented by different symbols: ●, control; ▼, immunized with Adhesin; ■, immunized with CTXA2B; ◆, immunized with Adhesin/CTXA2B (25 µg); ▲, immunized with Adhesin/CTXA2B (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/CTXA2B, Adhesin and CTXA2B. Data are shown as the mean antibody (Ab) levels to adhesin and CTXB, measured as described in the Materials and Methods section. Individual animals are represented by different symbols: ●, control; ▼, immunized with Adhesin; ■, immunized with CTXA2B; ◆, immunized with Adhesin/CTXA2B (25 µg); ▲, immunized with Adhesin/CTXA2B (100 µg). Horizontal bars represent median values. Significance of differences: \*  $P < 0.01$  compared with control group.

The induction of serum antibody responses against *E. coli* adhesin and CTXB was observed that higher serum IgG antibody responses to *E. coli* adhesin and CTXB were elicited in mice immunized with adhesin/CTXA2B than in those immunized with adhesin or CTXA2B alone ( $P < 0.05$  by Student's *t* test) (Fig. 2-A). Serum IgA antibody responses to *E. coli* adhesin and CTXB was also induced in mice immunized with adhesin/CTXA2B than in those immunized with adhesin or CTXA2B alone (Fig. 2-B). But serum IgM antibody was not induced in mice immunized with adhesin/CTXA2B, adhesin or CTXA2B alone (Fig. 2-C). Our present results demonstrate that it is possible to induce high levels of antibodies against cloned antigens in serum and mucosal secretions after peroral immunization. Thus mucosal immunization with *E. coli* adhesin coupled to CTXA2B conferred significant protection against *E. coli* infection in a mouse model.

The present studies show that oral administration of adhesin fused to CTXA2B elicited higher levels of mucosal IgA and serum IgG antibodies to the *E. coli* adhesin molecule in mice than did oral administration of adhesin alone. This finding provides evidence that CTXA2B 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 immunogenic by the mucosal route and induces antibody responses (Hajishen-

gallis *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 CTXA2B induces T cell responses are currently under way.

Taken together, the results indicate that the genetically linked CTXA2B acts as a useful mucosal adjuvant, and the adhesin/CTXA2B chimeric protein could be a potential component in future UPEC vaccine development.

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