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The Shiga toxin 2 B subunit inhibits net fluid absorption in human colon and elicits fluid accumulation in rat colon loops

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Abstract

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) colonizes the large intestine causing a spectrum of disorders, including watery diarrhea, bloody diarrhea (hemorrhagic colitis), and hemolytic-uremic syndrome. It is estimated that hemolytic-uremic syndrome is the most common cause of acute renal failure in infants in Argentina. Stx is a multimeric toxin composed of one A subunit and five B subunits. In this study we demonstrate that the Stx2 B subunit inhibits the water absorption (Jw) across the human and rat colonic mucosa without altering the electrical parameters measured as transepithelial potential difference and short circuit current. The time-course Jw inhibition by 400 ng/ml purified Stx2 B subunit was similar to that obtained using 12 ng/ml Stx2 holotoxin suggesting that both, A and B subunits of Stx2 contributed to inhibit the Jw. Moreover, non-hemorrhagic fluid accumulation was observed in rat colon loops after 16 h of treatment with 3 and 30 ng/ml Stx2 B subunit. These changes indicate that Stx2 B subunit induces fluid accumulation independently of A subunit activity by altering the usual balance of intestinal absorption and secretion toward net secretion. In conclusion, our results suggest that the Stx2 B subunit, which is non-toxic for Vero cells, may contribute to the watery diarrhea observed in STEC infection. Further studies will be necessary to determine whether the toxicity of Stx2 B subunit may have pathogenic consequences when it is used as a component in an acellular STEC vaccine or as a vector in cancer vaccines.

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Key words
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- Diarrhea
- Shiga toxin 2
- B subunit
- Water transport

Introduction

Shiga toxin-producing *Escherichia coli* (STEC), an important and emergent pathogen with various serotypes including O157:H7, has been linked to a spectrum of disorders, including watery diarrhea, bloody diarrhea (hemorrhagic colitis), and hemolytic-uremic syndrome (HUS) (1,2). The virulence of these strains is determined to a large extent by the production of Shiga toxin type 1 (Stx1) and/or Shiga toxin type 2 (Stx2).

In Argentina, *E. coli* serogroup O157 bacteria expressing both Stx1 and Stx2 are

the microorganisms most frequently isolated from children with HUS, although strains that express only Stx2 are also highly prevalent (3,4). Both Stx1 and Stx2 are multimeric toxins composed of an approximately 32-kDa A subunit protein in noncovalent association with a pentameric ring of identical B subunit proteins, each with a molecular mass of approximately 7.7 kDa. Toxin binding to cells is mediated by B subunits, which bind to the globotriaosylceramide (Gb3) glycolipid receptor present on the plasma membrane of certain eukaryotic cells (5).

After binding to Gb3, the holotoxin is endocytosed via a clathrin-dependent mechanism (6) and trafficking to the endoplasmic reticulum, leading to translocation of the enzymatic A subunit to the cytosol where it catalyzes depurination of a single adenine residue in the 28S rRNA of 60S ribosomes, resulting in inhibition of protein synthesis (7). If protein synthesis is completely inhibited, cell death eventually occurs, and Stx may cross the intestinal barrier to mediate the vascular complications of disease including hemorrhagic colitis and HUS. Such cellular destruction may occur either through the effects of the Stx on protein synthesis or as consequence of other pathological processes, such as apoptosis. Recently, it has been shown that Stx1 and Stx2 trigger signaling events that lead to apoptosis or programmed cell death in intestinal cells (8,9) which decreases barrier function (10) and could provide a mechanism for Stx to enter the bloodstream. However, there are other possible routes that Stx may take, including translocation across intestinal epithelial cells in an energy-dependent and saturated manner (11). Therefore, the variation in disease in response to Stx could be a consequence of different pathways and mechanisms involved in its movement through the intestinal epithelial cell barrier.

Studies on intestinal models have suggested that Stx can modulate disease severity, including the production of diarrhea and hemorrhagic colitis. Intraintestinal inoculation of rabbits with Stx and STEC has been used as a model of both hemorrhagic colitis and HUS (12). Inoculation of purified Stx1 from *E. coli* O157:H7 into ligated ileum segments of adult rabbits induced fluid accumulation in association with the presence of apoptotic intestinal villous epithelial cells (13). Crude and purified Stx2 holotoxin also significantly inhibited the absorptive water transport across human colon *in vitro* (14) and evoked fluid accumulation in a rat colon loop *in vivo*, events associated with damage to the colonic epithelial cells (15). Although the contribution of the Stx A subunit to the induction of these events has been demonstrated using the entire holotoxin, it is not clear if the Stx B subunit may be able to exert any effect on enterocyte function. The B subunit possesses signaling properties in its own right that can cause apoptosis of cells expressing the Gb3 receptor. In 1993 it was discovered that the B pentamer of Stx1 triggers apoptosis in Burkitt's lymphoma B cells, albeit at a much higher concentration than did the holotoxin (16), whereas the Stx2 B subunit triggers apoptosis in the Ramos but not in the Daudi Burkitt's lymphoma B-cell line (17). Other reports also showed that the Stx1 B subunit is non-toxic when applied to HeLa (18), Vero (19) or monocytic THP-1 (20) cells. However, to date, no studies have determined a biological action of the Stx2 B subunit on human colonic mucosa.

The purpose of the present study was to examine the effects of the Stx2 B subunit on water and ion transport in colonic epithelial cells in order to better understand the mechanism by which STEC induces diarrhea. We report here that the Stx2 B subunit inhibited water absorption in human colonic mucosa *in vitro* and elicited fluid accumulation in rat colon loops *in vivo*. We therefore suggest that the Stx2 B subunit is able to induce watery diarrhea and to contribute to the dysenteric syndrome independent of the presence of the active A subunit.

Material and Methods

Plasmid construction

Standard techniques were used to construct different expression plasmids (21). Plasmids were isolated from the bacteria using the Wizard Plus SV miniprep DNA purification system (Promega Corp., Madison, WI, USA). All constructs were sequenced by the dideoxy chain-termination method using an Applied Biosystem automatic sequencer.

The *stx2a* and *b* genes were obtained from chromosomal DNA of *E. coli* C600 containing the 933W bacteriophage (22) (a gift from M. Rivas, Instituto Nacional de Enfermedades Infecciosas "Dr. Carlos G. Malbrán", Buenos Aires, Argentina). A fragment of 1422 bp was amplified by the polymerase chain reaction (PCR) from chromosomal DNA using two specific primers (upstream: 5'-GAA TTC ATT ATG CGT TGT TAG-3' and downstream: 5'-GAA TTC TCA GTC ATT ATT AAA CTG-3', both containing an *Eco*RI restriction site) flanking both the *stx2a* and *b* genes. The PCR product was cloned into pGEM-T Easy (Promega). Competent *E. coli* DH5α cells (Life Technologies, Rockville, MD, USA) were transformed and the isolated plasmid was designated pStx2. The *stx2b* gene, including its native leader peptide coding sequences, was amplified by PCR from *E. coli* C600 (933W) by using two primers (upstream: 5'-GAA TTC ATG AAG AA ATG TTT ATG GCG-3' and downstream, same as above). The 282-bp fragment was ligated into pGEM-T Easy and *E. coli* DH5α was transformed. The isolated plasmid was named pStx2B. A recombinant *E. coli* containing a mutation in Stx2 was obtained by deleting 90% of the Stx2 A gene. Briefly, pStx2 was hydrolyzed by *Sma*I and *Stu*I (corresponding to positions 305 and 1102 bp, respectively) and religated. The plasmid thus obtained (pStx2ΔA) was introduced into *E. coli* DH5α. Sequence analysis of this construct showed deletion of the *stx2a* gene, while the whole *stx2b* gene was left intact. *E. coli* containing pGEM-T carrying a non-related sequence was used as a negative control (*E. coli* control).

Cultures of recombinant *E. coli* (pStx2), *E. coli* (pStx2B), *E. coli* (pStx2ΔA) and *E. coli* (control) were obtained by incubation overnight at 37ºC with shaking at 200 rpm in 5 ml of Luria-Bertani (LB) broth (Difco Laboratories) supplemented with 100 µg/ml ampicillin (Sigma Aldrich Co.). Bacterial cells were then removed by centrifugation and the resultant supernatants were filter-sterilized through 0.22-µm pore size filter units (Millipore Corp.) and assay by *stx2* expression.

In order to purify the Stx2 B subunit, the *stx2b* gene was amplified by PCR using pStx2B as template and specific primers containing *Sph*1 and *Bam*H1 restriction enzyme sites. The *stx2b* gene was then fused in frame with the 6XHis tag gene using the pQE-70 vector (QIAGEN) to create an Stx2 B-His fusion protein. The plasmid thus obtained (pStx2B-His) was introduced into *E. coli* DH5α.

Purification of the Stx2 B subunit

The Stx2 B subunit was purified by affinity chromatography under native conditions. Briefly, recombinant *E. coli* containing pStx2B-His were cultured by incubation overnight at 37ºC with shaking at 200 rpm in 20 ml of LB supplemented with 100 µg/ml ampicillin. On the subsequent day, 5 ml overnight culture was inoculated into 200 ml fresh medium containing ampicillin and grown until an OD_{600} of 0.6 was reached. The bacterial pellet was obtained by centrifugation at 6500 rpm for 10 min, resuspended in 4 ml of lysis buffer (50 mM NaH_2PO_4 and 300 mM NaCl) with lysozyme (1 mg/ml) and incubated on ice for 30 min. The suspension was then sonicated with six 10-s bursts at 200-300 W with a 10-s cooling

period between bursts and centrifuged at 10,000 *g* for 25 min at 4ºC. The supernatant was added to a 50% Ni-NTA slurry and shaken at 4ºC for 60 min. The lysate/Ni-NTA mixture was washed twice with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole, pH 8) and eluted four times with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250-500 mM imidazole, pH 8).

To determine the purity of the Stx2 B subunit, all fractions were diluted (1:1) in loading buffer (125 mM Tris-HCl, 4% SDS, 20% glycerol (v/v), 200 mM DTT, and 0.02% bromophenol blue, pH 6.8) and separated on a 12.5% polyacrylamide gel. One gel was stained with Coomassie blue and the other was electrotransferred to nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech). Membranes were blocked for 30 min with 1% (w/v) bovine serum albumin in phosphate-buffered solution (PBS) at room temperature, and incubated overnight at 4ºC with the mouse monoclonal antibody (mAb) 13C4 (10 µg/ml) (ATCC, kindly provided by Dr. Ludger Johannes) directed against the B-fragment of Stx1. Membranes were washed with PBS-0.1% Tween and incubated for 2 h at room temperature with goat anti-mouse IgG (Promega; 1:1000) conjugated with alkaline phosphatase. Finally, membranes were washed again as described above and antigen-antibody complexes were visualized with a BCIP/NBT color development substrate according to the manufacturer instructions (Promega).

Stx2 and Stx2 B subunit cytotoxicity to Vero cells

Culture supernatants from recombinant *E. coli* containing the Stx2 and Stx2 B subunits were assayed for toxicity to Vero cells (23) at Instituto Nacional de Enfermedades Infecciosas, ANLIS, "Dr. C.G. Malbrán", Buenos Aires. Purified Stx2 holotoxin (Denka Seiken Co., Ltd., Chuo-Ku, Tokyo, Japan) was also evaluated. The 50% cytotoxic dose (CD_{50}) corresponds to the dilution required to kill 50% of Vero cells.

Specimen collection and preparation

After obtaining informed consent, human colon specimens were obtained from organs surgically extracted from adult cancer patients. Immediately after ablation, sections of macroscopically unaffected regions were placed in ice-cold high K^+-Ringer solution (120 mM KCl, 10 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 1.2 mM K_2HPO_4, 0.2 mM KH_2PO_4, and 25 mM glucose) to preserve the transport functions (14). The mucosa and submucosa layers were then dissected from underlying tissue (kept at 4ºC) and mounted as a diaphragm in a modified Ussing chamber (1.76 cm^2). Rat colon specimens were surgically obtained from male Sprague Dawley rats weighing approximately 100 g and were immediately mounted on the Ussing chamber (0.94 cm^2).

Both sides of the tissue were then bathed with standard Ringer solution and bubbled with 95% O_2-5% CO_2. The bathing solution was maintained at 37ºC with water-jacketed reservoirs connected to a constant-temperature circulating pump. The Ussing chamber model used consisted in fact of two chambers, each with a mucosal and serosal compartment divided by the mounted tissue.

Net water flux and electrical measurements

Transepithelial net water flux (Jw) was recorded automatically with an Ussing chamber connected to a special electro-optical device. Briefly, the tissue was held against a nylon mesh by applying a hydrostatic pressure of 10 cm of H_2O. Water movement across the tissue was measured by displacement of a photo-opaque solution inside a glass capillary tube connected to the mucosal side of the chamber via an intermediate chamber. The liquid meniscus movement in

the glass capillary was detected using the electro-optical device connected to a computer. Data are obtained with an accuracy of 50 nl.

The spontaneous potential difference was recorded in the other chamber across the calomel electrodes, via agar bridges placed adjacent to the epithelium under open-circuit conditions. The short circuit current was measured with an automatic voltage clamp system that kept the potential difference at zero mV.

Once the tissue reached steady values, 200 µl of culture supernatant from different *E. coli* recombinants or different concentrations of purified Stx2 B subunit was added to the mucosal bath (time 0). Variations in Jw and short circuit current were continuously measured for at least 60 min. Because of tissue variability, data are presented as ΔJw, where ΔJw = Jw at time t - Jw at time 0.

For the inhibition test, 200 µl of each culture supernatant containing the Stx2 or Stx2 B subunit was mixed with an equal volume of mAb 13C4 (20 µg/ml) and incubated for 30 min at 37ºC before being added to the Ussing chamber.

Animal experimentation

A total of 21 male Sprague Dawley rats weighing 100-150 g were used in this study. The rats were fasted overnight and then anesthetized for surgery with an intramuscular injection of 100 µg ketamine/g body weight and 10 µg diazepam/g body weight. Body temperature was kept at 37ºC by a lamp. The abdominal cavity was opened by a midline incision and three 2-cm long colon segments (loops) separated by 1-cm interloops were prepared in each rat. Colon loops were then inoculated intraluminally with either 300 µl of culture supernatants from *E. coli* (control) and from *E. coli* (pStx2) or *E. coli* (pStx2B) containing approximately 24 ng of the B subunit. In selected experiments, colon loops were inoculated with either buffered saline (control loops) and 3 ng/ml of commercially purified Stx2 holotoxin or 3 and 30 ng/ml of Stx2 B subunit purified by us. The order of inoculation of the colon loops was randomized for each rat and the animals were killed by cervical dislocation 16 h after inoculation. Colon loops were rapidly removed, measured for length and fluid content, and prepared for histochemistry.

Light microscopy, fixation and histochemistry

Fragments of human and rat colon recovered 60 min after toxin addition were prepared for histochemistry. Tissues were fixed for at least 24 h in cold fixative (4ºC) containing 4% formaldehyde in PBS (145 mM NaCl/10 mM NaH_2PO_4, pH 7.2). After fixation, longitudinal 6-8-µm thick sections were cut, dehydrated, and carefully embedded in paraffin to provide sections perpendicular to the mucosa. Sections were then stained with hematoxylin-eosin and examined by light microscopy.

The colon loops were opened along their anti-mesenteric margins, pinned to a paraffin plate, fixed and stained as described above. Each tissue section was examined by an observer unaware of the treatment status.

Statistical analysis

Results are reported as means ± SEM. The statistical significance between two mean values obtained for two experimental conditions was calculated by the Student *t*-test. All data from the curves were analyzed by one-way analysis of variance (ANOVA). P values < 0.05 were taken as significant.

Results

Stx2 B subunit expression and purification

The Stx2 B subunit was purified by metal-affinity chromatography and in SDS-PAGE.

Two bands of approximately 7.5 kDa and 38 kDa were observed in the third eluted fraction (Figure 1, left). These bands correspond to the more stable conformations, i.e., monomeric and pentameric, described for StxB (24). The identity of the Stx2 B subunits was confirmed by immunoblotting (Figure 1, right). The quantity of the Stx2 B subunit was determined by densitometry of the protein bands on the nitrocellulose membrane using ß-actin as a reference standard. This purification procedure routinely resulted in approximately 150 µg Stx2 B subunit purified to at least 97% homogeneity from a 1-liter culture.

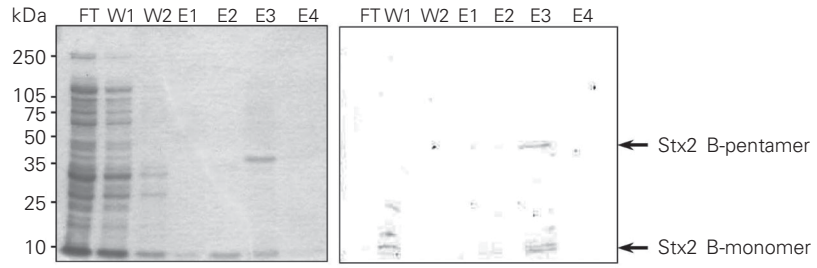

Figure 1. SDS-PAGE analysis of the Stx2 B subunit purified by affinity chromatography under native conditions. The Stx2 B subunit was expressed from the pQE-70 vector in DH5α *E. coli* cells and purified on Ni-NTA agarose with the indicated imidazole concentrations in the wash and elution steps. Proteins were visualized by Coomassie blue staining (left side) and developed by Western blots (right side) using the mouse mAb 13C4 (10 µg/ml). FT = flow-through; W1 and W2 = 10 mM imidazole wash; E1, E2 and E3 = 250 mM imidazole elution; E4 = 500 mM imidazole elution.

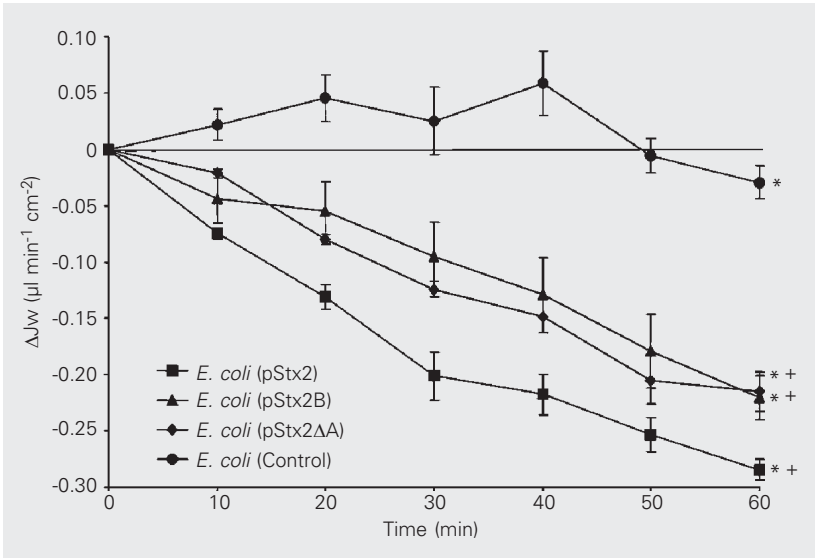

Figure 2. Effect of *E. coli* (pStx2), *E. coli* (pStx2B), *E. coli* (pStx2ΔA), and *E. coli* (control) on transepithelial net water flux (ΔJw) in human colon. At time zero, 200 µl of supernatants from *E. coli* (pStx2), *E. coli* (pStx2B), *E. coli* (pStx2ΔA), and *E. coli* (control) were added to the mucosal bath. Each point indicates the mean ± SEM of 3 to 8 experiments. *P < 0.001 for *E. coli* (pStx2), *E. coli* (pStx2B) and *E. coli* (pStx2ΔA) *vs* control. $^{+}$P < 0.05 for *E. coli* (pStx2B) and *E. coli* (pStx2ΔA) *vs E. coli* (pStx2) (ANOVA).

The average yield of B subunit in overnight cultures from recombinant *E. coli* (pStx2), *E. coli* (pStx2B) and *E. coli* (pStx2ΔA), calculated by comparison with known amounts of Stx2 B subunit, was approximately 80 ng/ml.

Cytotoxicity of the Stx2 and Stx2 B subunits to Vero cells

The cytotoxicity of overnight culture supernatants from *E. coli* containing the Stx2 or Stx2 B subunit was assayed on Vero cells. The CD_{50} of crude Stx2 was 0.4 pg/ml, which is consistent with previous values obtained with culture supernatants from *E. coli* C600 (933W) (14). In contrast, the crude Stx2 B subunit was non-toxic to Vero cells at ≤1 µg/ml. These results are consistent with data for the cloned Stx2 B subunit reported by Marcato et al. (19).

Effect of the Stx2 B subunit on colonic mucosa mounted on an Ussing chamber

Under basal conditions, a net absorptive Jw was observed in human intestine placed between two identical Ringer solutions in the Ussing chamber. After an initial stabilization period, 200 µl of culture supernatant from *E. coli* (pStx2) containing Stx2 was added to the mucosal bath (t = 0). Stx2 induced a significant Jw inhibition (Figure 2), whereas short circuit current remained unchanged during at least 60 min of incubation (data not shown).

The absorptive Jw was also reduced by supernatants from *E. coli* (pStx2B) and *E. coli* (pStx2ΔA) which contain the Stx2 B subunit at the same concentration (4 ng/ml) as that present in the supernatants from *E.*

coli (pStx2) (Figure 2). However, these effects were less marked, showing the contribution of the Stx2 A subunits to the mechanisms involved in water transport inhibition.

An absorptive Jw inhibition was also observed when rat colon mounted on an Ussing chamber was treated with supernatants from *E. coli* (pStx2) and *E. coli* (pStx2B) (Figure 3). However, this inhibition was dramatically reduced when culture supernatants were pretreated with mAb 13C4 (20 µg/ml) (Figure 3). mAb 13C4, by itself, had no effect on the absorptive Jw and was included as a negative control (Figure 3).

Jw inhibition depended on Stx2 B subunit concentration (Figure 4). The time-course of inhibition by 400 ng/ml of the Stx2 B subunit was similar to that obtained using 12 ng/ml of purified Stx2 holotoxin, suggesting that both the A and B subunits of Stx2 contribute to the inhibition of the absorptive Jw by independent mechanisms.

Compared with the data obtained for *E. coli* (pStx2B) supernatants, a 50-fold higher dose of the purified Stx2 B subunit was required to inhibit the absorptive Jw to the same level as that observed using supernatants expressing the Stx2 B subunit (compare Figures 3 and 4). These differences may be attributed to other endotoxins present in the bacterial supernatants that might act synergistically with the Stx2 B subunit to inhibit water absorption.

Histopathological analysis of the colonic mucosa

Colonic mucosa treated *in vitro* with crude and purified Stx2 (12 ng/ml) for 60 min revealed a moderate lesion in human (N = 3) and rat (N = 4) colon. Purified Stx2 holotoxin caused a lesion of colonic mucosa with destruction of surface epithelium, marked mucin depletion, and moderate neutrophil infiltration, in agreement with previous data (14). In contrast, at 400 ng/ml, the crude and purified Stx2 B subunits had no effect on the macro- or microscopic appearance of human colon (N = 4) or rat colon (N = 5), as did the PBS control on human colon (N = 4) or rat colon (N = 8).

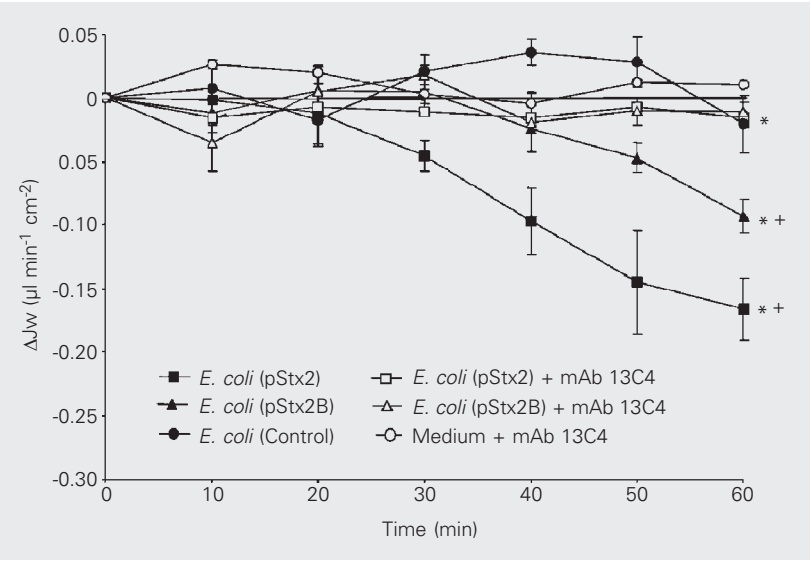

Figure 3. Inhibition of pStx2- and pStx2B-induced transepithelial net water flux (Jw) on rat colon by the mouse mAb 13C4. *E. coli* (pStx2) and *E. coli* (pStx2B) supernatants were preincubated with an equal volume of mAb 13C4 (20 µg/ml) for 30 min at 37ºC. The antigen-antibody complex was then added to the mucosal bath. Control consisted of non-inoculated culture medium plus mAb 13C4 (20 µg/ml). Each point indicates the mean ± SEM of 3 to 8 experiments. *P < 0.001 for *E. coli* (pStx2) and *E. coli* (pStx2B) *vs E. coli* (control). +P < 0.001 for *E. coli* (pStx2) *vs E. coli* (pStx2B) (ANOVA)

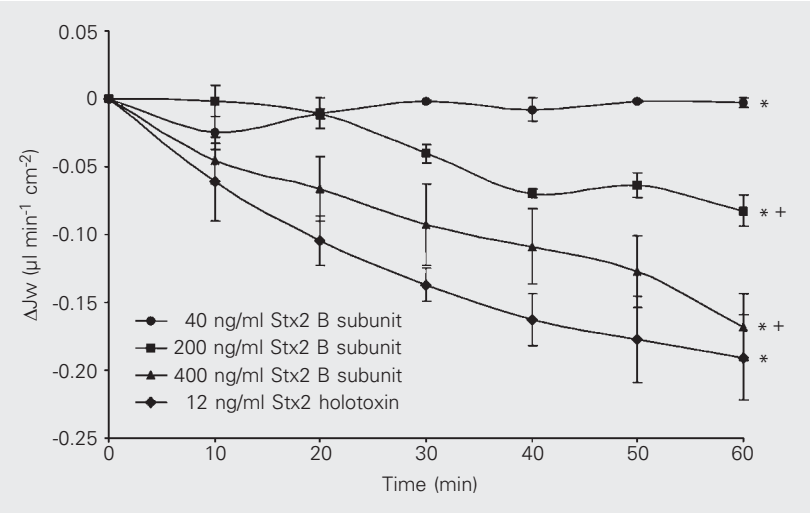

Figure 4. Effect of Stx2 B subunit and Stx2 holotoxin on transepithelial net water flux (ΔJw) in rat colon. Colonic mucosa was incubated at 37ºC for 60 min with the indicated concentrations of the Stx2 B subunit or 12 ng/ml of Stx2 holotoxin. Data are reported as means ± SEM of at least 3 experiments. *P < 0.001 for Stx2 holotoxin, 12 ng/ml, and Stx2 B subunit, 200 and 400 ng/ml *vs* Stx2 B 40 ng/ml (ANOVA). +P < 0.001 for Stx2 B subunit, 200 ng/ml *vs* 400 ng/ml (ANOVA).

Analysis of the effects of the Stx2 and Stx2 B subunits using rat colon loops

The effects of either culture supernatants from *E. coli* (pStx2) and *E. coli* (pStx2B) or purified Stx2 holotoxin and Stx2 B subunit were studied in colon loops 16 h after inoculation. The most severe lesions were observed with the use of Stx2. Grossly, after 16 h of treatment, the loops were distended and filled with abundant hemorrhagic fluid (Table 1). The serosal surfaces of these loops were hemorrhagic. In turn, the crude and purified Stx2 B subunit produced a non-hemorrhagic fluid accumulation in a concentration-dependent manner. The purified Stx2 B subunit caused a more significant fluid accumulation at 30 ng/ml than at 3 ng/ml (Table 1).

Table 1. Effects of the crude (culture) and purified Stx2 and Stx2 B subunits on rat colon loops.

16-h incubation with culture supernatants from	Fluid accumulation (µl/cm)	16-h incubation with purified Stx2 or vehicle		Fluid accumulation (µl/ cm)
E. coli (control)	IF[a] (6)	PBS (control)		IF[a] (8)
E. coli (pStx2)	645 ± 23 (2)	Holotoxin	3 ng/ml	553 ± 22 (3)
E. coli (pStx2B)	254 ± 16* (5)	B subunit	3 ng/ml	173 ± 13* (3)
			30 ng/ml	222 ± 15* (2)

Data are reported as means ± SEM for fluid accumulation by colon loops treated with the crude or purified Stx2 and Stx2 B subunits. The number of experiments is indicated in parentheses. [a]Insignificant amount of fluid present.
*P < 0.001 compared to Stx2 (Student *t*-test).

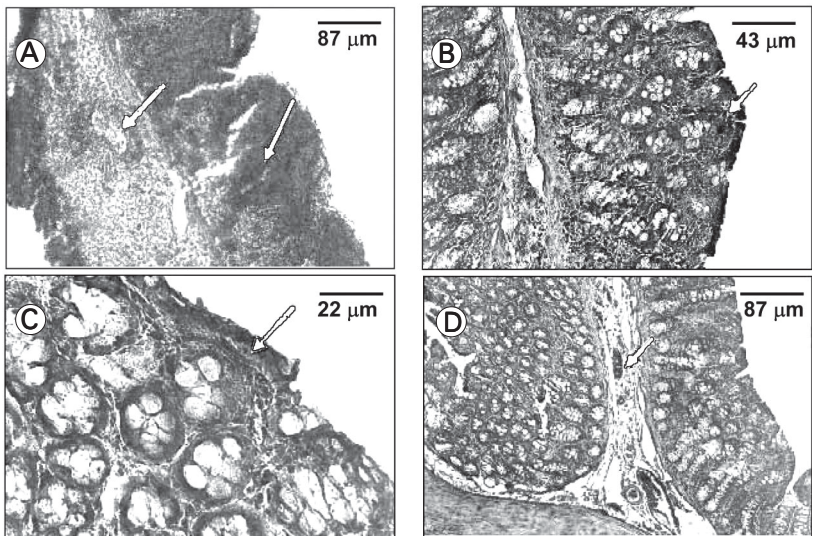

Figure 5. Rat colon loops treated with the Stx2 (A) or Stx2 B subunit (B and C) at 16 h of incubation (hematoxylin-eosin). The Stx2 B subunit produced subapical vascular congestion (yellow arrows) compared to control (D). In contrast, Stx2 produced distortion of the histoarchitecture because of extensive necrotic and hemorrhagic areas (white arrow) and marked edema and vascular congestion in the submucosa (green arrow)

Histologically, the Stx2 B subunit produced marked vascular congestion and a micro-hemorrhagic focus in the mucosa (Figure 5B and C) compared to control (Figure 5D), whereas Stx2 produced extensive necrotic areas containing hematic material (Figure 5A). Moreover, many of the degenerating absorptive cells were being extruded from the villi, and the intestinal lumen was filled with numerous dead and degenerating epithelial cells including erythrocytes (Figure 5A).

Discussion

Two features of the clinical syndrome caused by STEC suggest the importance of the effects of Stx on the epithelial cells of the distal small intestine and colon. STEC infection can result in fever, malaise, abdominal cramps, and hemorrhagic diarrhea (25,26).

The purpose of the present investigation was to study the ability of the Stx2 B subunit to produce physiological and morphological changes in human and rat colon.

We detected the biological effects of the Stx2 B subunit on colonic mucosa both *in vitro* and *in vivo*. The Stx2 B subunit inhibited water absorption across the human and rat colonic mucosa mounted on an Ussing chamber, while Stx2 showed highly significant water absorption inhibition concomitant with morphological damage in colonic surface cells including an inflammatory response. These findings contrast with those published by Li et al. (27), which showed that the effects of *E. coli* O157:H7, using rabbit colon, on ion and water fluxes were independent of Stx production. In the present experiments, however, the effects were

dramatically reduced by neutralization with mAb 13C4, indicating a specific action of the Shiga toxin on colonic mucosa. Histological alterations in colonic epithelial cells caused by the Stx2 but not the Stx2 B subunit at 1 h of incubation confirm the important role of the Stx2 A subunit in cell necrosis as a result of protein synthesis inhibition. The dose of purified Stx2 B subunit (200 ng/ml) necessary to inhibit water absorption across the rat colon at the same level as the culture supernatant containing the Stx2 B subunit (4 ng/ml) was 50-fold higher. These results suggest that additional bacterial factors present in the culture supernatants may potentiate the biological action of the Stx2 B subunit.

The Stx2 and Stx2 B subunits inhibit water absorption with no effect on the short circuit current. NaCl-coupled absorption has been described in the human colon and a water movement associated with an electroneutral absorption of Na^+ has also been reported (28). Our finding that the Stx2 B subunit inhibits water absorption without altering the electrical parameters is consistent with the hypothesis that an electrogenically silent transport system coupled to water absorption could be affected by the Stx2 B subunit, causing the watery diarrhea sometimes seen in STEC infections. However, we cannot rule out the possibility that these effects are due to apoptosis since Stx1B and Stx2B have been reported to trigger apoptosis, albeit after more than 1 h, in Burkitt's lymphoma B cells (16,17). Further experiments will be necessary to identify the membrane transporter specifically affected by the Stx2 B subunit, presumably in absorptive epithelial cells.

The results obtained here with rat colon loops also provide evidence for a role of the Stx2 B subunit in causing watery diarrhea. Stx2 produced hemorrhagic fluid accumulation and histological damage in colon loops while the Stx2 B subunit caused a non-hemorrhagic fluid accumulation after 16 h of incubation. One can therefore propose that the Stx2 B subunit induces fluid accumulation by altering the usual balance of intestinal absorption and secretion toward net secretion. The fact that the secretory activity of Stx2 is hemorrhagic and by far exceeds that of the Stx2 B subunit could be a consequence of the vascular damage associated with Stx2 (26).

In view of the reported immunoprophylactic potential of the Stx2 B subunit and of its proposed use as a component in an acellular STEC vaccine (19) or as a vector in DNA (29) and cancer vaccines (30,31), further studies will be necessary to determine whether the biological activity of the Stx2 B subunit may have pathogenic consequences when it is used as a vaccine.

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