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Inhibition of Water Absorption and Selective Damage to Human Colonic Mucosa Are Induced by Subtilase Cytotoxin Produced by *Escherichia coli* O113:H21

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Shiga toxin-producing *Escherichia coli* O157:H7 (STEC) is by far the most prevalent serotype associated with hemolytic uremic syndrome (HUS) although many non-O157 STEC strains have been also isolated from patients with HUS. The main virulence factor of STEC is the Shiga toxin type 2 (Stx2) present in O157 and non-O157 strains. Recently, another toxin, named subtilase cytotoxin (SubAB), has been isolated from several non-O157 strains and may contribute to the pathogenesis of HUS. Here, we have demonstrated that an O113:H21 STEC strain expressing SubAB and Stx2 inhibits normal water absorption across human colon and causes damage to the surface epithelium, necrosis, mononuclear inflammatory infiltration, edema, and marked mucin depletion. This damage was less marked, but nevertheless significant, when purified SubAB or *E. coli* O113:H21 expressing only SubAB was assayed. This is the first study showing that SubAB may directly participate in the mechanisms of diarrhea in children infected with non-O157 STEC strains.

Shiga toxin-producing *Escherichia coli* (STEC) strains colonize the human colon and may cause systemic complications such as hemolytic uremic syndrome (HUS) (1, 2). HUS develops in 5 to 10% of children several days after bloody diarrhea and is a systemic disease characterized by thrombotic microangiopathy, hemolytic anemia, thrombocytopenia, and acute renal failure. HUS is the most common cause of acute renal failure in children and the second leading cause of chronic renal failure in children younger than 5 years old (3, 4). STEC O157:H7 is by far the most prevalent serotype associated with HUS although non-O157 STEC strains have been also isolated from children with HUS (5, 6). The main virulence factor of STEC is the Shiga toxin type 2 (Stx2) present in O157 and non-O157 strains (7, 8). Unlike O157:H7 strains, some non-O157 STEC strains, among them O113:H21, lack the locus of enterocyte effacement (LEE) but encode additional proteins in order to adhere to intestinal epithelial cells (9). Recently, it has been reported that certain LEE-negative STEC strains produce another cytotoxin, named subtilase cytotoxin (SubAB), which may contribute to the pathogenesis of HUS. SubAB is toxic for eukaryotic cells, and its mechanism of action involves highly specific A-subunit-mediated proteolytic cleavage of the essential endoplasmic reticulum chaperone BiP (10, 11). To date, the *in vivo* effects of SubAB have only been examined in mice. Gut colonization with recombinant *E. coli* expressing *subAB* genes did not cause diarrhea but produced a dramatic weight loss over a 6-day period (11). Interestingly, intraperitoneal injection of purified SubAB caused microangiopathic hemolytic anemia, thrombocytopenia, and renal impairment, characteristics typical of Stx-induced HUS (12). These findings raise the possibility that SubAB directly contributes to pathology in humans infected with STEC strains that produce both Stx and SubAB.

The purpose of the present study was to examine the physiological and morphological effects of STEC strain O113:H21 on human colonic mucosa mounted in an Ussing chamber. A previous report has shown that Stx2 induces a significant inhibition of absorptive water transport across human colon concomitant with morphological damage in colonic surface cells, including an inflammatory response (13). Here, we report that SubAB also inhibits water absorption and causes histological damage in human colon independent of the presence of Stx2. These studies contribute to a better understanding of the mechanisms by which non-O157 STEC strains induce diarrhea.

MATERIALS AND METHODS

Cytotoxicity assay. Purified SubAB and an inactive mutant with a serine-to-alanine mutation at residue of 272 of SubA ($SubA_{A272}B$) (10) were assayed for cytotoxicity on Vero cells as previously described (1, 11). Briefly, Vero cell monolayers grown in 96-well plates were treated for 72 h with different concentrations of SubAB or the nontoxic mutant $SubA_{A272}B$ under growth-arrested conditions (serum-free medium). At the end of the incubation, plates were washed twice with phosphate-buffered saline (PBS; 145 mM NaCl, 10 mM NaH_2PO_4, pH 7.2) and incubated for 2 h with freshly diluted neutral red in PBS to a final concentration of 50 μg/ml. Cells were then washed with 1% $CaCl_2$ and 4% formaldehyde twice and then were solubilized in 1% acetic acid and 50% ethanol. Absorbance at 546 nm (A_{546}) was read in an automated plate spectrophotometer. Results were expressed as percent viability, with 100% represented by cells incubated under identical conditions but without SubAB or $SubA_{A272}B$ treatment. The 50% cytotoxic dose (CD_{50}) corresponded to the dilution required to kill 50% of Vero cells.

Bacterial strains and growth conditions. Properties of *E. coli* strains used in this study are listed in Table 1. *E. coli* O113:H21 wild-type strain 98NK2 (98NK2wt) (14) and derivatives of this strain with deletion mutations in either stx_2 (98NK2 Δ*stx2*) (15) or *subAB* genes (98NK2 Δ*subAB*)

Received 8 March 2013 Returned for modification 26 April 2013
Accepted 24 May 2013
Published ahead of print 3 June 2013
Editor: B. A. McCormick
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doi:10.1128/IAI.00287-13

TABLE 1 *E. coli* strains used in this study

Strain	Serotype	Sample source[a]	Gene profile[b]					Reference or source
			eae	stx_1	stx_2	*subAB*	*ehxA*	
98NK2wt	O113:H21	HUS	−	−	+	+	+	14
98NK2 Δ*stx2*	O113:H21	Lab	−	−	−	+	+	15
98NK2 Δ*subAB*	O113:H21	Lab	−	−	+	−	+	11
98NK2 Δ*stx2* Δ*subAB*	O113:H21	Lab	−	−	−	−	+	This study
Commensal *E. coli*	Not determined	Lab	−	−	−	−	−	This study

[a] STEC strains were originally isolated from feces of a patient with HUS.
[b] Presence or absence of a gene was determined by multiplex PCR. *eae* and *ehxA* are markers for the LEE pathogenicity island and the large STEC virulence plasmid, respectively.

(11) have been previously described. The double mutant 98NK2 Δ*stx2* Δ*subAB* was constructed by mutagenesis of the stx_2 gene in 98NK2 Δ*subAB* by the lambda red recombinase method, using the protocol and primers previously described for mutagenesis of stx_2 in 98NK2 (15). A commensal nonpathogenic wild-type *E. coli* strain designated MACI was isolated from the feces of a healthy human. This strain was used as a negative control. Bacterial strains were grown in LB medium for 18 h at 37°C with shaking at 150 rpm and then diluted 1/50 in Dulbecco's modified Eagle's medium (DMEM)-F12 medium and grown to exponential phase (optical density at 600 nm [OD_{600}] of 0.3 to 0.4) at 37°C with shaking at 50 rpm. Kanamycin was used at 50 μg/ml for 98NK2 Δ*stx2*, 98NK2 Δ*subAB*, and 98NK2 Δ*stx2* Δ*subAB* strains. In all cases bacterial supernatants were collected after centrifugation at 10,000 × *g* for 5 min, followed by filtration through a 0.22-μm-pore-size filter (Millipore Corp., USA). The titers of filter-sterilized supernatants were determined on Vero cells.

Human colonic mucosa collection and preparation. The colon fragments used in this study were obtained from surgeries performed on 21 adult patients with cancer (informed consent was obtained) at the División de Cirugía Gastroenterológica, Hospital de Clínicas José de San Martín, Buenos Aires, Argentina. The Ethics Committee of the Universidad de Buenos Aires approved the use of human tissues for research purposes. Colon fragments were removed immediately after ablation from macroscopically unaffected regions inside the "security zone" adjacent to the pathologically affected zone. Tissues were transported to the laboratory in oxygenated ice-cold high-potassium 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, 25 mM glucose) to preserve the transport functions. The mucosa and submucosa layers were then dissected from the underlying tissue (kept at 4°C) and cut into three to five fragments, depending on the size. Each mucosa fragment was mounted as a diaphragm on a modified Ussing chamber (1.76 cm^2) and used either for one of the treatments or for a control. Both sides of the tissue were washed and bathed with a standard Ringer solution (113 mM NaCl, 4.5 mM KCl, 25 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, 25 mM glucose) and bubbled with 95% O_2 and 5% CO_2. The bathing solution was maintained at 37°C with water-jacketed reservoirs connected to a constant-temperature circulation pump. This model of the Ussing chamber has mucosal and serosal compartments of 4 ml each, separated by the mounted tissue.

Net Jw and electrical measurements. Transepithelial net water flux (Jw) was recorded automatically across an Ussing chamber connected to a special electro-optical device (16, 17). Briefly, the tissue was held against a nylon mesh by a hydrostatic pressure of 13 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 an electro-optical device connected to a computer. The sensitivity of this instrument is approximately 50 nl.

The spontaneous potential difference (PD), expressed in mV, was periodically recorded in the other chamber across the microreference electrodes (Harvard Apparatus Inc., USA) placed adjacent to the epithelium under open-circuit conditions. The short-circuit current (Isc) expressed in μA/cm^2 was measured with an automatic voltage clamp system that kept the PD at 0 mV. The transepithelial resistance (Rt) expressed in ohm · cm^2 across the colonic mucosa was calculated from the Isc and open-circuit PD values, according to Ohm's law. When the parameters were stabilized, the following samples were added to the mucosal side of each colon tissue (time [*t*] zero): (i) purified Stx2, SubAB, or $SubA_{A272}B$; (ii) 200 μl (approximately 10^8 CFU) of bacterial culture of 98NK2wt, 98NK2 Δ*stx2*, 98NK2 Δ*subAB*, 98NK2 Δ*stx2* Δ*subAB*, or commensal *E. coli*; (iii) 200 μl of filtered culture supernatant from 98NK2wt, 98NK2 Δstx_2, or 98NK2 Δ*subAB*. Then, both Jw and Isc were simultaneously recorded for 90 min. Because of tissue variability, data are analyzed as ΔJw, calculated as the Jw at a given time *t* minus the Jw at time zero ($Jw_t - Jw_0$), and ΔIsc, calculated as $Isc_t - Isc_0$. Each assay was carried out three to six times with colon fragments obtained from different patients.

Histological studies. Human colonic mucosae were recovered after physiological experiments and fixed for at least 24 h in cold fixative (4°C) containing 4% formaldehyde in PBS. After fixation, longitudinal 2- to 4-μm-thick sections were cut, dehydrated, and carefully embedded in paraffin to provide sections perpendicular to the mucosa. Sections were then stained with hematoxylin and eosin (H&E). The slides were then blindly examined by light microscopy.

Statistical analysis. Results are reported as means ± standard errors of the means (SEM), and the significance of any differences was determined using a Mann-Whitney test. Statistical analysis was performed using GraphPad Prism software (San Diego, CA, USA). Statistical significance was set at a *P* value of <0.05.

RESULTS

Cytotoxicity on Vero cells. A significant cytotoxic effect was observed when monolayers of Vero cells were exposed to different concentrations of purified Stx2 and SubAB under growth-arrested conditions. The CD_{50} was maximal after 72 h of incubation and corresponded to 1 ng/ml for Stx2 and 3 ng/ml for SubAB (Fig. 1A). In contrast, $SubA_{A272}B$ was nontoxic to Vero cells at ≤1 μg/ml at 72 h of incubation (Fig. 1A).

Culture supernatants from either 98NK2wt or 98NK2 Δ*subAB* showed similar degrees of cytotoxicity on Vero cells, with a titer of approximately 900 CD_{50}s/ml, whereas supernatant from 98NK2 Δstx_2 exhibited a titer of 75 CD_{50}s/ml (Fig. 1B). Culture supernatants from 98NK2 Δ*stx2* Δ*subAB* or commensal *E. coli* were nontoxic (Fig. 1B). The average yield of SubAB in the filtered culture supernatant, estimated by comparison with the CD_{50} for the corresponding purified SubAB, was 225 ng/ml.

SubAB inhibits normal water absorption across the human colonic mucosa and causes necrosis of the surface epithelium. Under basal conditions, an absorptive Jw (0.21 ± 0.01 μl/min · cm^2; $n = 21$) was observed in the human colonic mucosa placed between two identical Ringer solutions in the Ussing chamber. The electrical parameters tested simultaneously with water fluxes

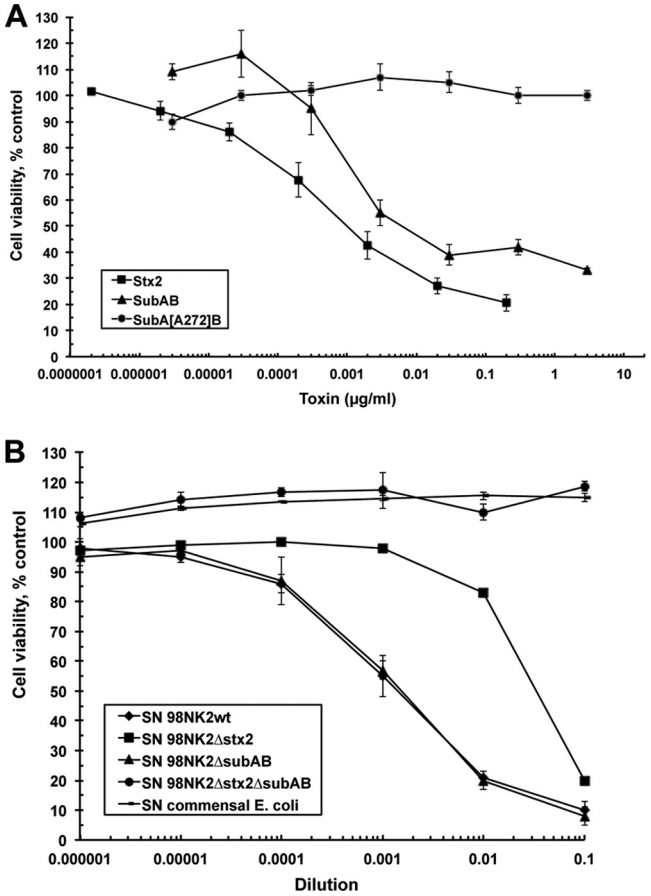

FIG 1 Cytotoxicity of Stx2, SubAB, and *E. coli* O113:H21 supernatants on Vero cells. Vero cell monolayers were plated in 96-well plates and grown to confluence in complete minimal essential medium. The cells were then exposed to different concentrations of purified Stx2, SubAB, or $SubA_{A272}B$ (A) or different dilutions of filtered culture supernatants (SN) from 98NK2wt and mutant strains (B) under growth-arrested conditions for 72 h. Cell viability was analyzed by neutral red uptake, and 100% represents cells incubated under identical conditions but without treatment.

showed a PD of 2.8 ± 0.4 mV, Isc of 18.6 ± 2.1 μA/cm^2, and Rt of 147 ± 11 Ω · cm^2. Addition of 10 ng/ml Stx2 or 0.4 μg/ml SubAB to the mucosal bath of the colonic mucosa ($t = 0$) resulted in a significant decrease in the absorptive Jw within 90 min of incubation (Fig. 2) ($P < 0.05$, $n = 5$). The time course of inhibition by SubAB was less marked than that for Stx2 even when SubAB was used at a 40-fold higher concentration, indicating that this toxin is a less potent inhibitor of water absorption across the human colonic mucosa. In contrast, 0.4 μg/ml $SubA_{A272}B$ had no significant effect on the Jw, indicating that the proteolytic activity of the toxin A subunit (SubA) was required to alter the water absorption (Fig. 2). No change in the Jw was detected at concentrations less than 10 ng/ml for Stx2 and 0.4 μg/ml for SubAB for 90 min. Under all experimental conditions, Isc remained unchanged for at least 90 min (data not shown). Histological analysis of colon sections after 90 min of incubation with 10 ng/ml Stx2 or 0.4 μg/ml SubAB (Fig. 3B and C, respectively) revealed detachment of the surface epithelium (black arrows), loss of goblet cells (black arrowheads) and moderate mononuclear inflammatory infiltrate (black squares) compared with tissues treated with PBS (Fig. 3A). In the case of Stx2, crypt epithelium was also affected (Fig. 3B, black asterisks).

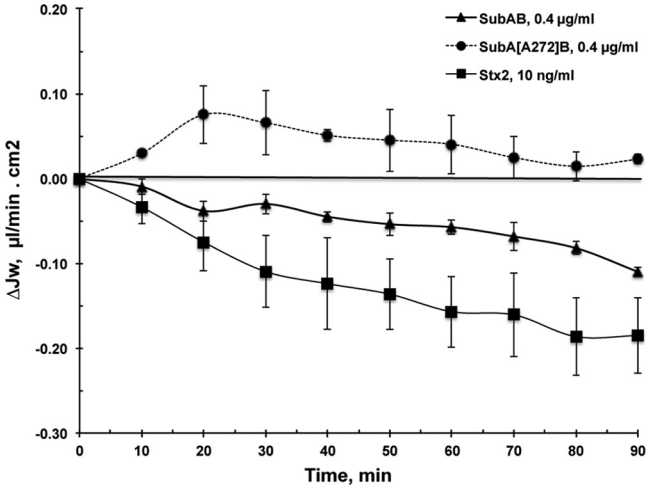

FIG 2 Inhibition of normal water absorption in human colon by Stx2 and SubAB. Data represent the time course of the transepithelial net water flux (ΔJw) across colonic mucosa incubated at 37°C with 10 ng/ml of Stx2, 0.4 μg/ml of SubAB, or 0.4 μg/ml of $SubA_{A272}B$. Data are reported as means ± SEM of at least three experiments. Significant differences in ΔJw values between Stx2, SubAB, and $SubA_{A272}B$ were observed (*, $P < 0.05$ for 10 ng/ml Stx2 versus 0.4 μg/ml SubAB and 0.4 μg/ml SubAB versus $SubA_{A272}B$).

***E. coli* O113:H21 inhibits normal water absorption across human colonic mucosa.** To establish the relative contribution of bacterial cells and their toxins to the cytotoxic effects, matched colonic mucosal samples obtained from the same patient were incubated with 98NK2wt, 98NK2 Δ*subAB*, and 98NK2 Δ*stx2*. Some of these samples were also incubated with 98NK2 Δ*stx2* Δ*subAB*. A commensal *E. coli* was used as a negative control. The wild-type and single mutant 98NK2 strains caused significant Jw inhibition compared with the commensal *E. coli* (Fig. 4A) ($P < 0.05$, $n = 6$). However, the level of Jw inhibition by the double mutant 98NK2 Δ*stx2* Δ*subAB* was very similar to that of commensal *E. coli* and was significantly lower than that induced by the other 98NK2 strains that expressed Stx2 and/or SubAB (Fig. 4A) ($P < 0.05$, $n = 4$). This indicates that both toxins significantly affect water absorption across human colonic mucosa. Jw remained unchanged when the corresponding filtered culture supernatants from 98NK2wt and single mutant strains were used, suggesting that the amount of toxin present in *in vitro*-grown bacterial supernatants is simply insufficient to mediate the absorptive water inhibition (Fig. 4B). Isc simultaneously measured in colonic mucosa incubated with bacterial culture or culture supernatant remained unchanged for at least 90 min (data not shown). The fact that the 98NK2wt strain did not cause a significantly greater effect on water absorption than the 98NK2 Δ*stx2* or 98NK2 Δ*subAB* strain appears to indicate that there are no synergistic effects between SubAB and Stx2 but, rather, that there is perhaps even a degree of redundancy.

***E. coli* O113:H21 expressing Stx2 and/or SubAB causes selective histopathological damage in human colonic mucosa.** The treatment of colonic mucosa for 90 min with bacterial culture of the 98NK2wt strain caused notable histological damage compared with tissues treated with the filtered culture supernatant from 98NK2wt or bacterial culture of commensal *E. coli* (Fig. 5). The 98NK2wt caused rupture of the surface epithelium, necrosis (based on presence of pyknotic nuclei and eosinophilic cyto-

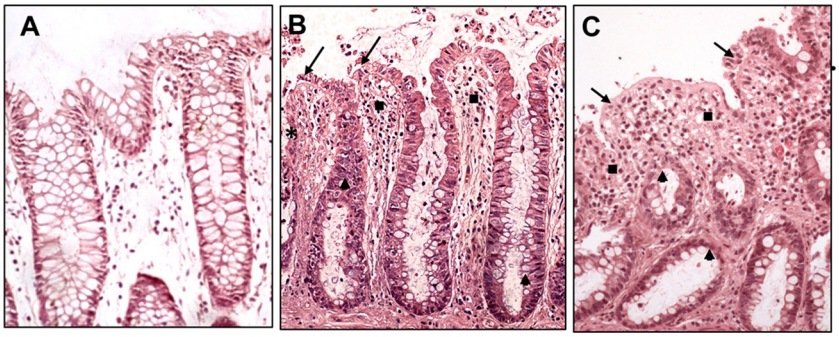

FIG 3 Selective destruction of human colonic mucosa by Stx2 and SubAB. Human colonic mucosa sections were treated with PBS (A) or treated with 10 ng/ml of Stx2 (B) or 0.4 μg/ml of SubAB (C) for 90 min, and samples were stained with H&E. Detachment of the surface epithelium (black arrows in panel B), marked mucin depletion (black arrowheads in panels B and C), and moderate mononuclear inflammatory infiltration (black squares in panels B and C) were observed compared with the controls (A). Original magnification, ×200. Data are representative of three independent experiments.

plasm), mononuclear inflammatory infiltration, areas of edema in the interglandular chorion, and marked mucin depletion (Fig. 5A). In contrast, the corresponding filtered culture supernatant did not affect the human colonic mucosa under the same experimental conditions (Fig. 5B), and tissues presented a normal histoarchitecture similar to that observed with commensal *E. coli* (Fig. 5C).

Furthermore, the incubation of colonic mucosa for 90 min with 98NK2 Δ*subAB* caused damage to the surface epithelium (Fig. 6A and B) similar to that found with purified Stx2 (Fig. 3B). In some cases, the presence of erythrocytes outside the vessels was also observed (Fig. 6B). On the other hand, the 98NK2 Δ*stx2* strain caused detachment of the surface epithelium, moderate mononuclear inflammatory infiltration, and marked mucin depletion (Fig. 6C and D) similar to the damage caused by purified SubAB (Fig. 3B).

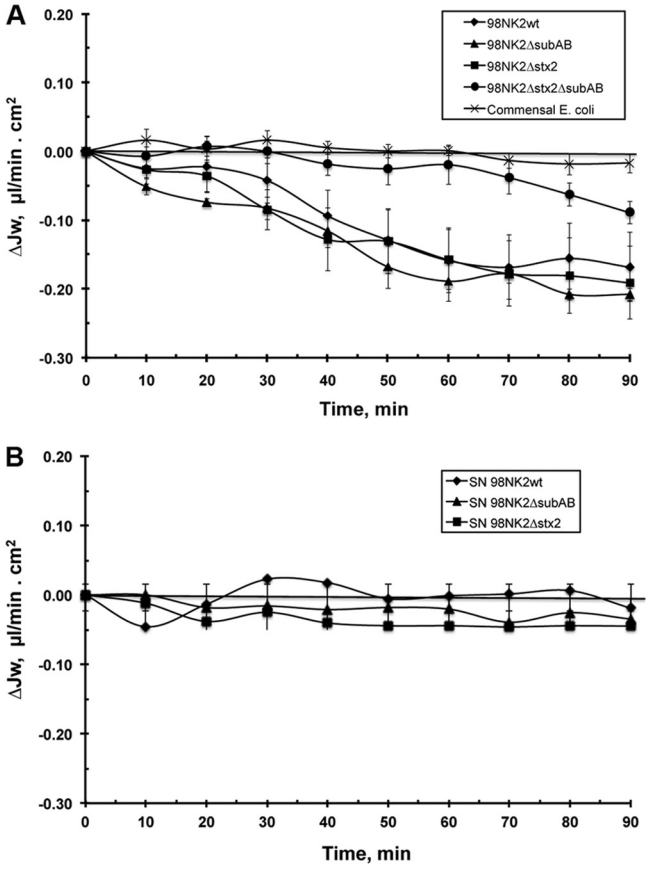

FIG 4 Inhibition of water absorption in human colon by *E. coli* O113:H21 expressing Stx2 and/or SubAB. Data represent the time course of the transepithelial net water flux (ΔJw) after addition of 98NK2wt or mutant strains (A) or of filtered culture supernatants (SN) from 98NK2wt or single mutant strains (B) on the mucosal side of colonic mucosa. A time-dependent Jw inhibition significantly different from that obtained with a commensal *E. coli* was observed in the case of tissues incubated with bacterial cells.

DISCUSSION

Although O157:H7 STEC strains are the predominant cause of intestinal disease associated with HUS in many parts of the world, other non-O157 STEC strains have also been associated with human disease. Unlike O157:H7, some of these STEC strains produce SubAB, which may also contribute to the development of diarrhea. However, assessment of its contribution to intestinal disease is complicated by the fact that, with the exception of one recent report (18), all *E. coli* strains that produce SubAB also produce Stx1 and/or Stx2. In the present work, we have shown that Stx2 alters the normal water absorption across human colon and causes irreversible histological damage, as previously demonstrated (19). Purified SubAB also produces a significant inhibition of the water absorption across human colonic mucosa although at much higher concentration than Stx2. It is generally accepted that water absorption in the human colon is associated with ionic active transport across colonic epithelium. The resulting diarrhea is an imbalance of absorption and secretion of ions and solutes, followed by the movement of water in an attempt to restore the appropriate ion concentrations (20). The present study provides evidence that SubAB was ineffective in modifying the Isc, as we have previously observed with Stx2 (13). These data are consistent with the hypothesis that an electrogenically silent ionic transport coupled to water absorption may be affected by the SubAB treatment. Electroneutral absorption of a coupled Na^+/H^+ and Cl^-/HCO_3^- exchange has been described in the human colon (21), and water movement associated with electroneutral absorption of Na^+ has been reported (22). However, histological observations of human colon incubated with purified SubAB have shown general damage to absorptive epithelial cells.

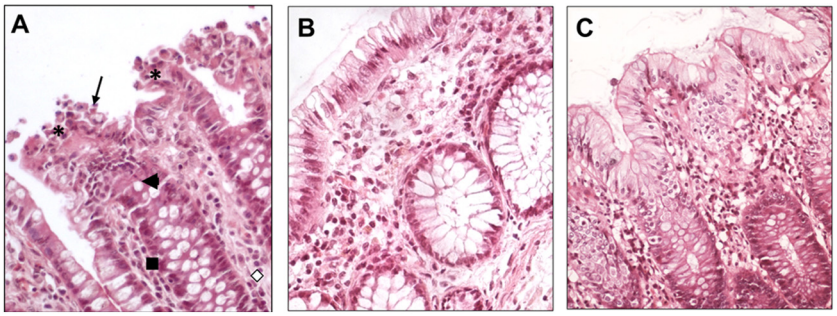

FIG 5 Destruction of human colonic mucosa by *E. coli* O113:H21. Human colonic mucosa was incubated for 90 min with 98NK2wt, culture supernatant from 98NK2wt, or commensal *E. coli* used as a negative control, and samples were stained with H&E. 98NK2wt bacterial cells (A) caused detachment of the surface epithelium (black arrow), necrosis (based on presence of pyknotic nuclei and eosinophilic cytoplasm; black asterisks), mononuclear inflammatory infiltration (black square), areas of edema in the interglandular chorion (white diamond), and marked mucin depletion (black arrowhead). In contrast, culture supernatant from 98NK2wt (B) presented a normal histoarchitecture similar to that of the control (C). Original magnification, ×400. Data are representative of six independent experiments.

Hence, changes in the water absorption may be due to the direct cytotoxic effect of SubAB on the absorptive function surface cells due to morphological changes and cell death. Crypt cells are not affected after 90 min of incubation with SubAB, possibly because these cells lack specific receptors for SubAB. The B subunit of the toxin has a high degree of specificity for glycans terminating in *N*-glycolylneuraminic acid, which is not synthesized by humans but is incorporated from

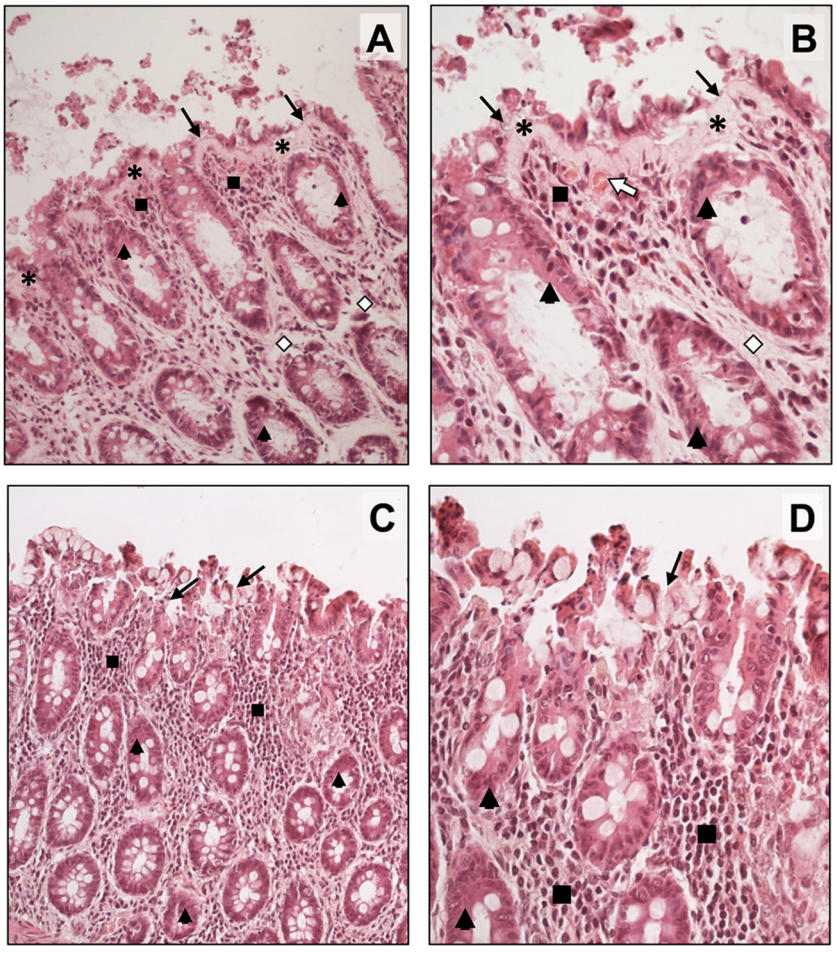

FIG 6 Histological changes in human colonic mucosa by *E. coli* O113:H21 expressing Stx2 and/or SubAB. Human colonic mucosa were incubated for 90 min with 98NK2 Δ*subAB* (A and B) or 98NK2 Δ*stx2* (C and D), and samples were stained with H&E. Both strains caused detachment of the surface epithelium (black arrows), moderate mononuclear inflammatory infiltration (black squares), and marked mucin depletion (black arrowheads). Treatment with 98NK2 Δ*subAB* caused a disorganization of the surface epithelium, subapical necrosis (black asterisks), areas of edema in the interglandular chorion (white diamonds), and presence of erythrocytes outside the vessels (white arrow). Original magnifications, ×200 (A and C) and ×400 (B and D). Data are representative of six independent experiments.

dietary sources into glycoconjugates expressed on the colonic epithelial surface more than on crypt cells (23, 24).

In this study, we have found that O113:H21 STEC strain 98NK2wt and its derivatives the 98NK2 Δ*subAB* and 98NK2 Δstx_2 strains inhibit the normal absorptive Jw and cause significant histological damage. Destruction of the surface epithelium similar to that observed using purified SubAB occurs in the presence of 98NK2 Δ*stx2*. Furthermore, extensive disorganization and damage of the apical surface of the colonic mucosa similar to that observed with purified Stx2 occur with the 98NK2 Δ*subAB* strain. The fact that all 98NK2 strains exhibit similar degrees of inhibition suggests that both Stx2 and SubAB are equally effective in altering water absorption and that they do not exert synergistic effects. Furthermore, the absence of cytotoxic effects using culture supernatants from 98NK2wt shows that the amount of toxin present in *in vitro*-grown bacterial supernatants is simply insufficient to mediate the absorptive water inhibition. However, the interaction of bacterial cells with the colonic mucosa may increase toxin expression and/or secretion into the intestinal lumen, reaching the necessary concentration to cause effects. It has been demonstrated that signaling occurs between epithelial cells and enterohemorrhagic *E. coli* that increases virulence, including Stx expression (25). Previous reports have shown that enhanced CXC chemokine responses in human colonic epithelial cells by the 98NK2 strain may be attributed to the presence of O113 lipopolysaccharide (LPS) (26) and H21 flagellin (15). These bacterial products may potentiate the activity of SubAB in the bacterial culture although further experiments will be necessary to clarify this point.

In summary, we present here evidence indicating that O113:H21 STEC strains expressing SubAB and Stx2 inhibit the normal water absorption across human colon mounted in a modified Ussing chamber and cause selective damage to the human colonic mucosa. These pathological effects are limited to the surface epithelial cells when purified SubAB or O113:H21 mutant lacking Stx2 is present in the lumen. This pattern is consistent with the expected distribution of the SubAB receptor.

This is the first study showing that SubAB produced by an O113:H21 STEC strain may directly participate in the diarrheagenic mechanism in children infected with STEC strains that produce both Stx2 and SubAB toxins.

ACKNOWLEDGMENTS

This work was supported by grants to C.I. from Buenos Aires University (UBA-M095), the National Council of Research of Argentina (CONICET-PIP 344), and the National Agency for Promotion of Science and Technology (ANPCYT-PICT 08-642). J.C.P. is a National Health and Medical Research Council Australia Fellow; A.W.P. is an Australian Research Council DORA Fellow.

We are grateful to Alejandro Moreira and Mariano Giménez from the Hospital de Clínicas José de San Martín for providing human colon fragments. We also thank to Marisa Almuzara for providing the commensal *E. coli* and Alicia Araoz for her technical assistance with histological samples.

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