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Effect of Shiga Toxin 2 on Water and Ion Transport in Human Colon *In Vitro*

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Shiga toxin-producing *Escherichia coli* (STEC) colonize the lower segments of the human gastrointestinal tract, causing gastrointestinal and systemic diseases. In this study, the effects of Shiga toxin 2 (Stx2) on fluid absorption and ion transport in the human colon were examined. Net water movement (Jw) and short-circuit current (Isc) were simultaneously measured across the colonic mucosa incubated with crude or purified Stx2. Stx2 significantly inhibited the absorptive J_w with no effect on the basal I_{sc} after 60 min of exposure. These effects may be due to the inhibition of a nonelectrogenic transport system present in the surface colonic villus cells. Morphological studies of the colonic mucosa treated with crude or purified Stx2 demonstrated a selective damage in the absorptive villus epithelial cells. These findings suggest that Stx2 inhibits water absorption across the human colon by acting on a specific cell population: the mature, differentiated absorptive villus epithelium.

KEY WORDS: Shiga toxin; *Escherichia coli*; hemolytic uremic syndrome; human colon; water transport; ion transport.

Shiga toxin-producing *Escherichia coli* (STEC) are responsible for a variety of clinical syndromes including bloody and nonbloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS). Although multiple serotypes of STEC have been isolated from hemorrhagic colitis cases, *E. coli* O157:H7 is the predominant STEC serotype isolated in outbreaks of hemorrhagic colitis in many countries, including Argentina (1, 2). A common finding in patients with HUS is the destruction of endothelial cells lining small blood vessels in the colon, kidney, and central nervous system (3). This destruction is produced when Shiga toxin 1 or 2 (Stx1 or Stx2) gains access to the systemic circulation and reaches the target cells (3). The precise way in which these toxins traverse the intestinal epithelial barrier is not clear. There are several possible routes the toxin may take, including transcellular or paracellular routes or via "holes" in the mucosa epithelial cell layer resulting from the destruction of the epithelial cells. Experiments *in vitro* with cell-free systems have demonstrated that Stx binds to a specific glycolipid globotriaosylceramide (Gb3) receptor, is then endocytosed, and finally the active subunit A inhibits eukaryotic protein synthesis, in a manner similar to 28S rRNA *N*-glycosidases (4). If protein synthesis is completely inhibited, cell death eventually occurs, and the free toxin crosses the mucosal barrier. Recently it has been also demonstrated that Stx1 is capable of moving across an intestinal barrier without apparent cellular disruption, probably via a transcellular pathway (5).

Two features of the clinical syndrome caused by

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STEC suggest the importance of Stx effects on the epithelial cells of the distal small intestine and colon. STEC infection can result in fever, malaise, abdominal cramps, and dysentery characterized by the passage of stool containing blood (6). While epithelial cell invasiveness is clearly the predominant virulence property responsible for the dysenteric syndrome, cytotoxin effects may in part dictate the severity of the dysenteric symptoms. More importantly, watery diarrhea typically precedes dysentery in STEC infection (7). The ability of Shiga toxin (purified from *S. dysenteriae* type 1) and Stx1 (purified from an *E. coli* O157:H7 strain) to cause fluid accumulation and histologic damage in ligated rabbit ileal loops was established some time ago (8, 9). In contrast, purified Stx fed orally to infant rabbits often resulted in grossly bloody diarrhea and histologic changes primarily in the colon (eg, apoptotic surface epithelial cells) (10). Predominantly right-sided colonic lesions were also described in humans (11). However, no studies to date have determined Stx activity on colonic transport. The purpose of the present study was to examine the effects of Stx2 on water and ion transport in the human colon, to better understand the mechanism by which STEC induces diarrhea.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. Reference *E. coli* C600 (933W) strain (12) and two *E. coli* O157:H7 strains, 20/96 and 26/96, isolated from HUS cases in Argentina were used. All three strains were characterized as Stx2 producers. Strains were maintained at −70°C in tryptic soy broth (Difco Laboratories, Detroit, Michigan) supplemented with 20% glycerol. Stx2 preparations were obtained according to the method described by Karmali et al (13). Briefly, strains were grown in Penassay broth (Antibiotic Medium No. 3, Difco) for 18 hr at 37°C with shaking (200 rpm), the supernatant was collected after centrifugation at 400 *g* for 10 min, followed by filtration through a 0.22-μm filter (Millipore). Stx2 production was assayed by cytotoxic activity on Vero cells as previously described (13, 14). The cytotoxic dose at 50% (CD_{50}) corresponded to the dilution required to kill 50% of the Vero cells per well. In selected experiments, purified Stx2 (Denka Seiken Co., Ltd.) was added at different concentrations to the mucosal side of colonic mucosa.

Specimen Collection and Preparation. Colon specimens were obtained from surgically extracted organs from adult patients with cancer (informed consent was given). Immediately after ablation, sections of macroscopically unaffected regions were placed in ice-cold high K^+-Ringer solution (in mM: 120 KCl, 10 $NaHCO_3$, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 1.2 K_2HPO_4, 0.2 KH_2PO_4, 25 glucose) to preserve the transport functions (15). The mucosa and submucosa layers were then dissected from the underlying tissue (kept at 4°C) and mounted as a diaphragm in a modified Ussing chamber (1.76 cm^2). Both sides of the tissue were immediately bathed with the standard Ringer solution and bubbled with 95% O_2–5% CO_2. The bathing solution was maintained at 37°C with water-jacketed reservoirs connected to a constant temperature circulating pump. This model of the Ussing chamber had two chambers, each with a mucosal and serosal compartment divided by the mounted tissue.

Net Water Flux and Electrical Measurements. Transep-

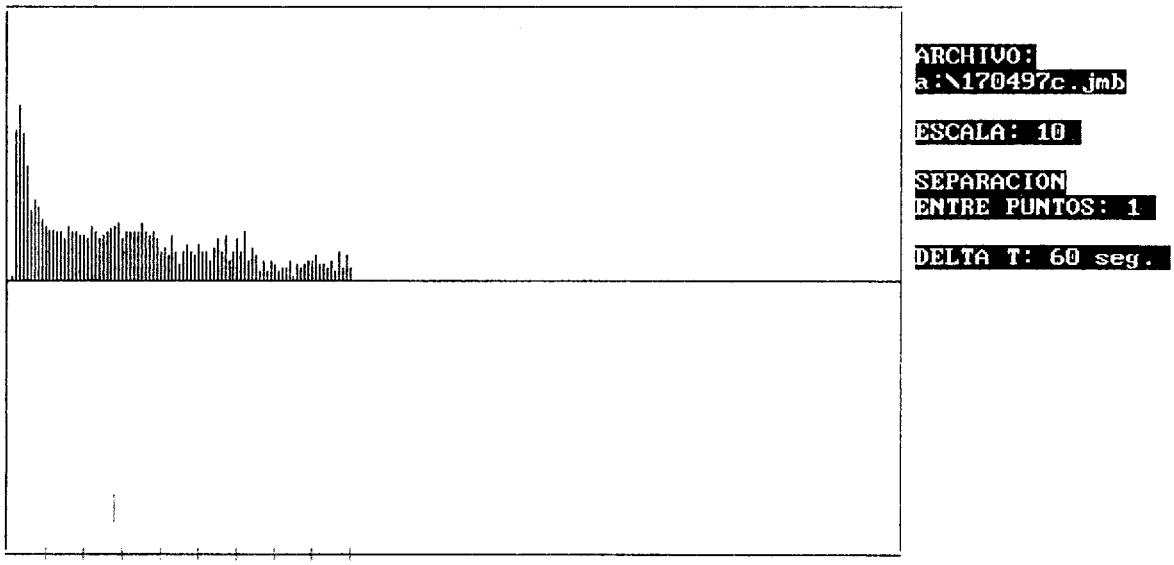

Fig 1. Screen of a typical experiment, showing an absorptive J_w across human colon tissue mounted between two identical Ringer solutions under a hydrostatic pressure gradient of 10 cm H_2O (mucosal side positive). The height of each line is proportional to the amount of water crossing per minute. At the mark line, culture supernatant from *E. coli* C600 (933W) was added to the mucosal bath.

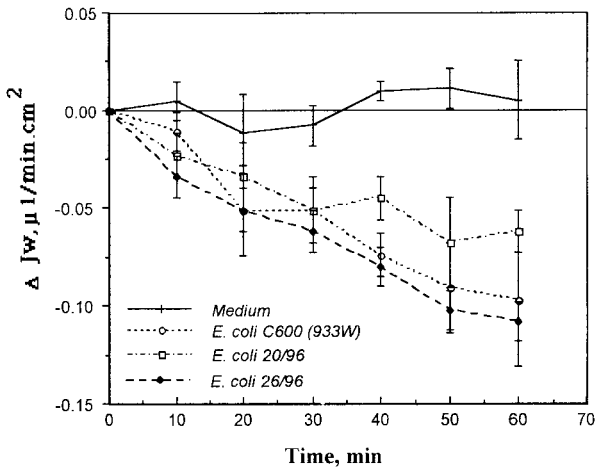

Fig 2. Time course of ΔJ_w in human colon after the addition of supernatant from different STEC strains. Curves represent the means ± 1 SE of four experiments each for *E. coli* strains C600 (933W), 20/96, and 26/96. Control medium consisted of noninoculated culture medium.

ithelial net water flux (Jw) was measured automatically by a modified Ussing chamber connected to a special electrooptical device (16). Briefly, the tissue was held against a nylon mesh by a hydrostatic pressure of 10 cm of H_2O. Water movement across the tissue was measured by displacement of a photoopaque 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 electrooptical device connected to a computer. The sensitivity of this instrument is on the order of 50 nl.

The spontaneous potential difference (PD) 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 (I_{sc}) was measured by an automatic voltage clamp system that kept the PD at 0 mV. Pulses of 2 mV, each lasting 10 min across the intestinal tissue under short-circuit conditions, allowed the calculation of the tissue conductance (G_t) according to Ohm's law ($I_{sc} = PD \times G_t$).

Once the tissue reached steady values, 200 μl of culture supernatant from different STEC strains or noninoculated medium (control) was added to the mucosal bath (time 0). Variations in J_w, I_{sc}, PD, or G_t were continuously measured during at least 60 min. Because of tissue variability, data are presented at ΔJ_w, ΔI_{sc}, and ΔG_t, where $\Delta J_w = J_w$ at time $t - J_w$ at time 0, $\Delta I_{sc} = I_{sc}$ at time $t - I_{sc}$ at time 0, and $\Delta G_t = G_t$ at time $t - G_t$ at time 0. At the end of every experiment, 10^{-4} M ouabain was added to the serosal bath of each chamber. Only those tissues which showed a decrease in response to ouabain (indicating tissue viability) were included in the data analysis.

Light Microscopy, Fixation, and Histochemistry. Fragments of human colon recovered at different intervals of time after the addition of culture supernatant or purified Stx2 were prepared for histochemistry. For this purpose, tissues were fixed at least 24 hr in cold fixative (4°C) containing 4% formaldehyde in PBS (145 mM NaCl/10 mM NaH_2PO_4, pH 7.2). After fixation, longitudinal sections were cut, dehydrated, and carefully embedded in paraffin to provide sections perpendicular to the mucosa. Sections were cut at 6–8 μm thick, stained with hematoxylin–eosin and examined by light microscopy. Each tissue section was examined by an observer unaware of the treatment status.

RESULTS

Effects of STEC Culture Supernatant on Water and Ion Transport. Under basal conditions, a net absorptive J_w was observed in human intestine placed between two identical Ringer solutions in the Ussing chamber. Figure 1 shows a screen captured from a typical experiment where the height of each vertical stroke indicates the amount of water moving from the mucosal to the serosal bath per minute. It is apparent that, after an initial stabilization period, the net water movement (Jw) was clearly absorptive. At the mark line, a culture supernatant from *E. coli* C600 (933W) containing Stx2 at a final titer of 4×10^4 CD_{50}/ml, was added to the mucosal bath. Under these conditions, the absorptive J_w was reduced, whereas I_{sc} and G_t remained unchanged during the 60 min of incubation. This effect was also observed with the culture supernatants from the *E. coli* 20/96 and *E. coli* 26/96 (Figure 2). Table 1 summarizes the mean differences in J_w, I_{sc}, and G_t obtained. The absence of I_{sc} and G_t effects could not attributed to cell death, since they were then inhibited by addition of ouabain (10^{-4} M) to the serosal bath (Figure 3A and B).

TABLE 1. EFFECTS OF CRUDE Stx2 FROM STEC STRAINS ON HUMAN COLON WATER TRANSPORT

Incubation with E. coli *culture supernatant*	ΔJ_w ($\mu l/min \cdot cm^2$)	ΔI_{sc} ($\mu A/cm^2$)	ΔG_t (mS/cm^2)	*Microscopic lesions**	N
C600 (933W)	−0.10 ± 0.03	+1.99 ± 0.90	+0.87 ± 0.71	2–3	4
4×10^4 CD_{50}/ml	$P < 0.05$	NS	NS		
20/96,	−0.06 ± 0.01	+1.93 ± 1.50	+0.51 ± 0.42	2–3	4
2×10^4 CD_{50}/ml	$P < 0.05$	NS	NS		
26/96,	−0.10 ± 0.03	+0.71 ± 2.16	+0.29 ± 0.29	2–3	4
2×10^4 CD_{50}/ml	$P < 0.05$	NS	NS		

*Average severity grades: 0 no change from control, +1 mild, +2 moderate, +3 marked, +4 severe.

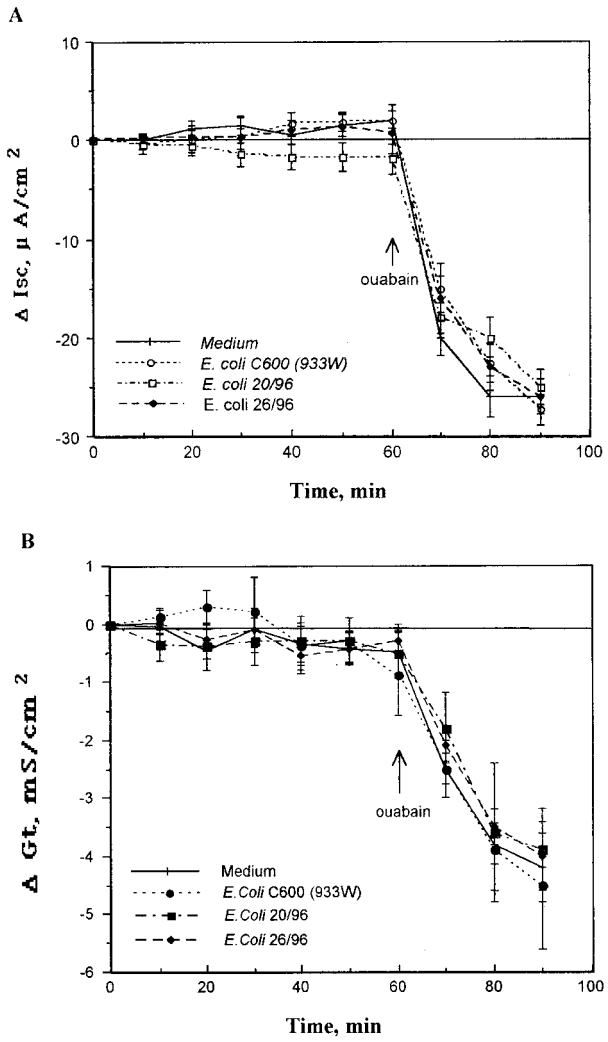

Fig 3. Effects of STEC culture supernatants on I_{sc} and G_t in human colon. Values are the means of four experiments for each time point. Error bars indicate ± 1 SE. Time 0: time at which the supernatant was added to the mucosal bath. (A) Effects of STEC strains on I_{sc}. (B) Effects of STEC supernatants on G_t. Arrow indicates time at which 10^{-4} M ouabain was added to the serosal side of both STEC- and medium-exposed tissue.

Comparison of J_w Inhibition by Crude and Purified Stx2. To examine whether the inhibition of J_w absorption could be attributed to the presence of Stx2 in the cultures, human colonic mucosa was exposed to different doses of purified Stx2. A significant J_w inhibition was observed with 2×10^7 CD_{50}/ml of purified Stx2, and this degree of inhibition was not modified at 10-fold-higher doses (Table 2). Compared with the results obtained with crude Stx2, a dose equivalent to 2000 times the CD_{50} per milliliter was required to inhibit the colonic J_w absorption to the same level. These differences in cytotoxic activity could be attributed to other endotoxins released by the STEC into the supernatant that could act synergistically with Stx2 to induce the inhibition of water absorption.

Histopathological Studies of Human Colonic Mucosa Incubated with Crude and Purified Stx2. Histopathology of colonic mucosa treated 60 min with culture supernatant from *E. coli* C600 (933W) containing Stx2 at a final titer of 4×10^4 CD_{50}/ml revealed destruction of surface epithelium, marked mucin depletion, and moderate infiltration of neutrophils with a minor effect on the crypt cells, compared to the control (Figures 4A and B, Table 1). Similar effects were observed with the crude preparations of *E. coli* 20/96 and 26/96 at a concentration of 2×10^4 CD_{50}/ml (Table 1). Purified Stx2 at 2×10^7 CD_{50}/ml did not affect the crypt cells, while a minor mucine depletion, and a mild infiltration of neutrophyils could be observed (Figure 4C, Table 2). Using 2×10^8 CD_{50}/ml of purified Stx2, marked changes occurred in the upper third of the colonic mucosa (Figure 4D, Table 2). Incubation of colonic mucosa with purified Stx2 at 1×10^7 CD_{50}/ml for up to 60 min showed effects indistinguible from the controls (data not shown).

DISCUSSION

A clear relationship between water and ion movements in the rat cecum and in the human colon has been previously reported (17, 18). In addition, we have determined the existence of a net absorptive water flux in the absence of any osmotic, chemical, or

TABLE 2. EFFECT OF PURIFIED Stx2 ON HUMAN COLON WATER TRANSPORT

Incubation with purified Stx2	ΔJ_w ($\mu l/min/cm^2$)	ΔI_{sc} ($\mu A/cm^2$)	ΔG_t (mS/cm^2)	*Microscopic lesions**	N
1×10^7 CD_{50}/ml	0.05 ± 0.02	+2.00 ± 0.90	+0.90 ± 0.70	0	4
	NS	NS	NS		
2×10^7 CD_{50}/ml	−0.05 ± 0.02	+0.29 ± 0.29	+0.26 ± 0.20	2	5
	$P < 0.05$	NS	NS		
2×10^8 CD_{50}/ml	−0.06 ± 0.01	+3.40 ± 1.60	+0.40 ± 0.30	3	3
	$P < 0.05$	NS	NS		

*Average severity grades: 0 no change from control, +1 mild, +2 moderate, +3 marked, +4 severe.

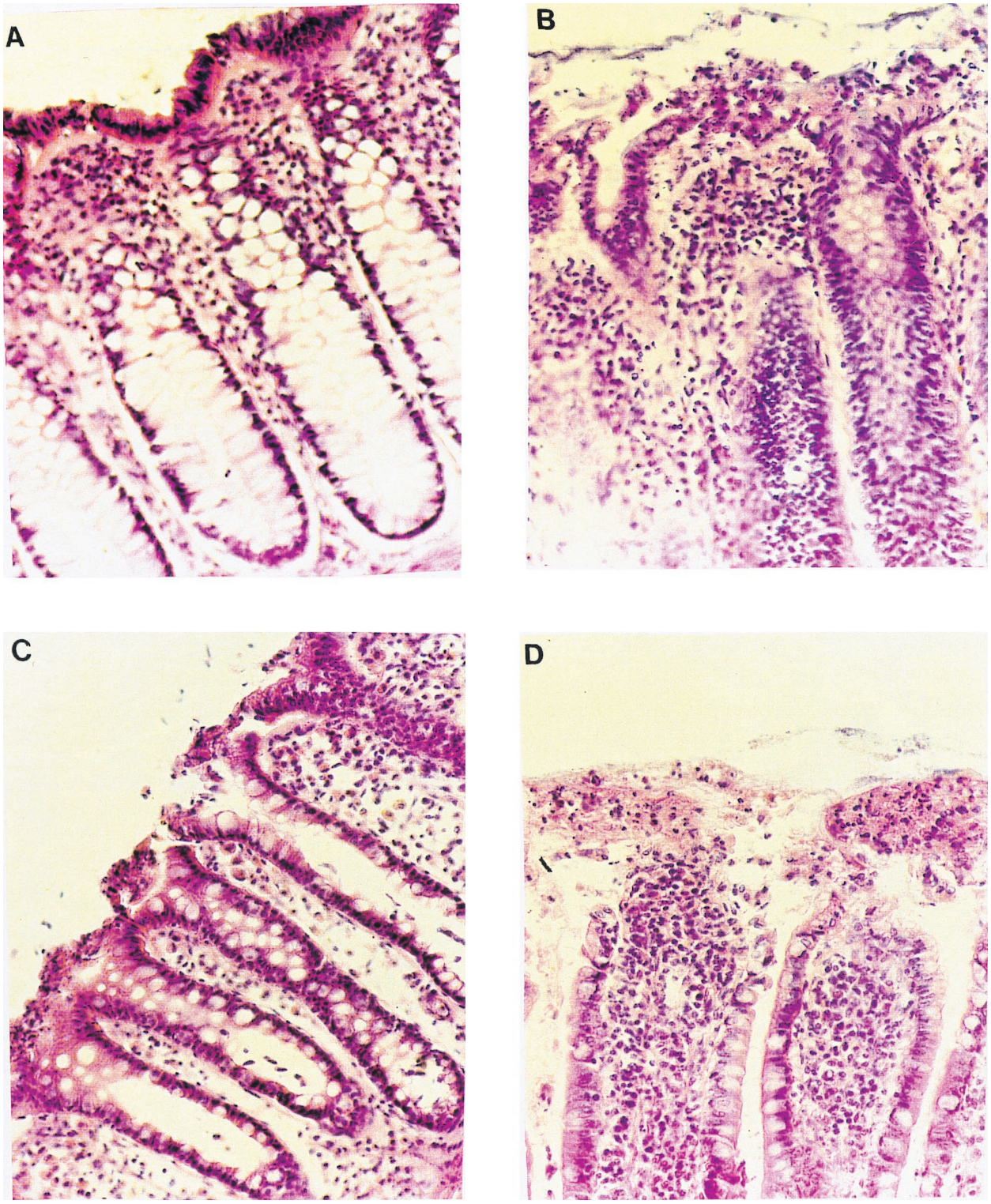

Fig 4. Hematoxylin-and-eosin-stained sections of untreated human colonic mucosa (A) and mucosa treated for 60 min with crude or purified Stx2 (B–D). Crude Stx2 preparation from *E. coli* C600 (933W) at a dose of 4×10^4 DC_{50}/ml produced destruction of surface epithelium, marked mucine depletion, and moderate neutrophil leukocytes (B, ×212) compared with normal colonic mucosa (A, ×240). Purified Stx2 at a dose of 2×10^7 DC_{50}/ml also caused a destruction of surface cells without affecting crypt cells, minor mucine depletion, and mild infiltration of neutrophyls (C, ×220). At purified Stx2 doses 10 times higher, marked changes occurred in the upper third of the colonic mucosa (D, ×193).

hydrosmotic gradient, which probably represents the water flux associated with the ionic active transport in the rat cecum (17). Recently, we have reported a reduction of the absorptive water transport coupled to electrically silent ionic transport in the human small intestine incubated with *E. coli* heat-stable enterotoxin (19). The present study shows an inhibition of water absorption across the human colon, after 60 min of incubation with culture supernatants from different STEC strains. These effects could be attributed to the presence of Stx2 in the cultures, since purified Stx2 caused a similar inhibition of water movement. We found that I_{sc} and G_t remained unchanged, indicating that the effect on water absorption could be a consequence of a nonelectrogenic transport inhibition by Stx2.

Kandel et al (8) observed an inhibition of fluid absorption in the rabbit jejunum exposed to Shiga toxin with no effect on the I_{sc}. They suggested that Shiga toxin produces a selective inhibition of NaCl absorption without altering active anion secretion. A NaCl-coupled absorption has been described in the human colon (20), and a water movement associated with an electroneutral absorption of Na was also reported in rat cecum and colon (17–22). The present results might indicate that an electrogenically silent transport system coupled to water absorption could be affected by Stx2. However, the histological alterations reported here do not allow for separate changes in colonic water absorption due to specific effects of Stx2 on electrolyte transport, and to a general cytotoxic effect resulting from protein synthesis inhibition. The present results indicate that Stx2 can act directly on the surface epithelial cells of the human colon, with only minor effects on the crypt cells. Selective apoptosis in the absorptive epithelial cells was also reported in most of the studies examining the effects of Shiga toxin *in vivo* (23), suggesting that these cells are a specific target for Stx2.

Kandel et al (8) have shown that the first step in the gut cell-toxin interaction is the binding of Shiga toxin to Gb3 on the villus cell brush border membrane. These authors speculate that the toxin is subsequently internalized, probably through receptor-mediated endocytosis, as shown for HeLa and Vero cells (24, 25) in order to reach its ribosomal site of action and cause protein synthesis inhibition. This inhibition leads to impaired villus cell absorptive function, and eventually to morphological changes and cell death. Crypt cells are affected in a minor degree, possibly because they lack sufficient quantities of the glycolipid receptor Gb3, as reported for the rabbit intestine (8). The dose of purified Stx2 necessary to inhibit water absorption across the human colon at the same level as crude Stx2, was 2000-fold greater. These results suggest that additional bacterial factors present in the crude Stx2 preparation contribute to the cytotoxic action of the toxin. Endotoxin constituents of the bacterial outer membrane may be released into the culture supernatant and participate in the Stx2 action by triggering proinflammatory cytokine liberation (26).

In summary, we have shown that crude and purified Stx2 are able to inhibit water absorption across the human colonic mucosa without affecting the electrical parameters. Morphological studies of the colonic mucosa treated with purified Stx2 showed selective damage to the absorptive villus epithelial cells that extended to the crypt cells, when Stx2 was used at higher doses. Similar effects were observed with the crude Stx2 preparations. Studies with recombinant Stx2 are in progress to improve our understanding of how Stx2 could contribute to water transport changes in the human colon, in the absence of other possible toxins.

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