

## *Clostridium perfringens* Enterotoxin Damages the Human Intestine In Vitro

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**In vitro, *Clostridium perfringens* enterotoxin (CPE) binds to human ileal epithelium and induces morphological damage concurrently with reduced short-circuit current, transepithelial resistance, and net water absorption. CPE also binds to the human colon in vitro but causes only slight morphological and transport changes that are not statistically significant.**

*Clostridium perfringens* enterotoxin (CPE) is produced by  $\leq 5\%$  of *C. perfringens* isolates. Epidemiological evidence suggests that CPE plays an important role in the pathogenesis of both food-borne and non-food-borne human gastrointestinal (GI) illnesses caused by *cpe*-positive *C. perfringens* type A isolates (9). Specifically, feces from patients suffering from GI illnesses caused by *cpe*-positive isolates contain CPE at concentrations that cause GI effects in animal models (1, 2). Moreover, ingestion of highly purified CPE by human volunteers induces diarrhea (24), a predominant symptom of GI illnesses involving *cpe*-positive *C. perfringens* type A isolates. Finally, inactivation of the *cpe* gene abrogates the ability of *cpe*-positive *C. perfringens* human GI disease isolates to cause GI disease in experimental animals (20).

Although CPE intestinal activity in animal models has been demonstrated (12, 14–21) and the cytotoxicity mechanism is relatively well characterized in cell lines (4, 6–11, 22, 23, 26), how those results correspond to CPE effects on the human intestine remains uncertain. Therefore, we examined whether CPE induces physiological and morphological changes to the human small and large intestine in vitro.

To study CPE effects on human intestinal water and ion transport, net water flux and electrophysiological parameters were measured in parallel using modified Ussing chambers. After informed consent was obtained, sections of macroscopically unaffected margins of human ileum and colon specimens were obtained from organs surgically removed from eight adult intestinal-cancer patients. The study protocol was approved by the Institutional Review Board of the University of Buenos Aires (Argentina) and the University of California Committee for Research with Human Tissues (no. 200513194-1).

Transepithelial net water flux ( $J_w$ ) was recorded automat-

ically in a modified Ussing chamber connected to a special electro-optical device (5). The spontaneous potential difference (PD) was recorded in the other chamber across Ag/AgCl electrodes, via agar bridges placed adjacent to the epithelium under open-circuit conditions. The short-circuit current ( $I_{sc}$ ) was measured with an automatic voltage clamp system that kept the potential difference at 0 mV. Transepithelial resistance ( $R_t$ ) was calculated using Ohm's law from the open-circuit PD and  $I_{sc}$ . Variations in electric parameters were continuously measured for a minimum of 60 min. The effects of CPE on intestinal electrophysiology were compared using PD,  $\Delta I_{sc}$ , and  $\Delta R_t$ , where  $\Delta I_{sc} = (I_{sc} \text{ at time } t) - (I_{sc} \text{ at time zero})$  and where  $\Delta R_t = (R_t \text{ at time } t) - (R_t \text{ at time zero})$ . Samples of each experimental specimen were obtained immediately after collection from the patients and also after treatment in the Ussing chambers, fixed by immersion in 10% buffered (pH 7.2) formalin for 24 h, embedded in paraffin wax, sectioned at 4  $\mu\text{m}$ , stained with hematoxylin and eosin, and examined by use of light microscopy. Sections from selected cases were also processed by an indirect immunoperoxidase technique to detect CPE (3). This technique was performed using a monoclonal antibody against CPE (25) and the Dako EnVision kit (Dako Corporation, Carpinteria, CA). CPE was purified to homogeneity from *C. perfringens* strain NCTC8239 (13).

Under basal conditions, a net absorptive  $J_w$  was observed when human ileum or colon was placed between two identical Ringer solutions in the Ussing chamber. Table 1 shows the mean  $J_w$  values and electric parameters measured simultaneously before toxin treatment. Normal ileal (Fig. 1) and colonic (not shown) mucosa segments incubated for 60 min with Ringer solution alone showed stable  $J_w$  and electrophysiological values. Addition of 10  $\mu\text{g/ml}$  of purified CPE to the mucosal side ( $t = 0$ ) of the human ileum inhibited  $J_w$  (Fig. 1A), concomitant with a decreased  $I_{sc}$  (Fig. 1B) and  $R_t$  (Fig. 1C). These effects depended upon the incubation time and became statistically significant 20 min after toxin exposure, continuing to decrease throughout the 60-min obser-

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TABLE 1. Net absorptive water flux and electric parameters in human intestine under basal conditions

Parameter	Value	
	Ileum	Colon
$J_w$ ( $\mu\text{l}/\text{min}/\text{cm}^2$ )	$0.28 \pm 0.09$	$0.35 \pm 0.10$
PD (mV)	$-1.5 \pm 0.3$	$-3.5 \pm 1.0$
$I_{sc}$ ( $\mu\text{A}/\text{cm}^2$ )	$10.3 \pm 2.1$	$13.4 \pm 3.4$
$R_t$ ( $\Omega \cdot \text{cm}^2$ )	$180 \pm 23$	$257 \pm 38$

vation period. In the colon, purified CPE (10  $\mu\text{g}/\text{ml}$ ) altered both  $J_w$  and electrophysiological parameters ( $I_{sc}$  and  $R_t$ ), although the changes were not statistically significant after 60 min of toxin treatment (Table 2).

TABLE 2. Effects of purified CPE (10  $\mu\text{g}/\text{ml}$ ) on large human intestine

Parameter	Value <sup>a</sup>	
	Control	CPE
$\Delta J_w$ ( $\mu\text{l}/\text{min}/\text{cm}^2$ )	$-0.025 \pm 0.02$	$-0.03 \pm 0.01$
$\Delta I_{sc}$ ( $\mu\text{A}/\text{cm}^2$ )	$1.9 \pm 1.4$	$-1.9 \pm 1.0$
$\Delta R_t$ ( $\Omega \cdot \text{cm}^2$ )	$-10 \pm 6$	$-23 \pm 7$

<sup>a</sup> Data are reported as means  $\pm$  standard errors of the mean ( $n = 4$ ).

Without CPE treatment, the human colonic and ileal tissue showed no significant histological changes (Fig. 2A). Ileal tissues ( $n = 4$ ) treated for 60 min in vitro with purified CPE (10  $\mu\text{g}/\text{ml}$ ) showed similar morphological changes, including shortening and blunting of villi. The villus tips showed enterocyte necrosis, with disruption of the basement membrane and extrusion of epithelial, goblet, and lamina propria cells into the intestinal lumen. The necrotic enterocytes showed pyknotic, condensed nuclei or figures of karyorrhexis and karyolysis. These cells had a rounded, homogeneous, and acidophilic cytoplasm, which was frequently vacuolated and/or disrupted. A basophilic layer of mucus, mixed with desquamated epithelial cells, covered the intestinal mucosa (Fig. 2B). Colonic mucosa from all patients ( $n = 4$ ) similarly treated with purified CPE showed mild effects, consisting only of flattening of superficial epithelial cells, individual epithelial cell necrosis, and sloughing of a few enterocytes.

Immunohistochemistry showed intense CPE staining along the brush border of the ileal epithelium, which sometimes extended to the intercellular lateral borders of the enterocytes. Desquamated epithelial cells in the intestinal lumen were surrounded by thin CPE staining (Fig. 3A). Clearly positive CPE staining was observed in only one colonic segment after CPE treatment and consisted of a fine brown line over the apical and basolateral enterocyte surface (Fig. 3C). Two other colonic segments gave similar but weaker staining. No CPE staining was observed in any tissues treated with CPE and stained using normal rabbit serum (negative control) (Fig. 3B and C).

Previous rabbit studies (15, 21) suggested that tissue damage is important for triggering fluid and electrolyte transport changes in CPE-treated ileum. Similarly, our results indicate that purified CPE also impairs ion and water transport in human ileum and that those effects are related to ileal tissue damage, i.e., villus damage was concurrent with decreases in ileal transepithelial net water absorption and  $R_t$ .

Caco-2 cell experiments suggested that CPE interactions with enterocytes disrupt intercellular tight junctions, which could alter paracellular permeability (23). Tight junctions of the small intestine are the low-resistance type, and the paracellular route is considered the main route for transport-associated water transfer (19). Therefore, CPE-induced tight-junction disruptions could explain the changes in  $J_w$ ,  $I_{sc}$ , and  $R_t$  observed in human ileum. However, the histological damage to villus cells caused by 60-min treatment with 10  $\mu\text{g}/\text{ml}$  CPE was very severe, indicating the occurrence of more than tight-junction disruption and suggesting that CPE induces diarrhea by altering the normal intestinal absorption-secretion balance toward net secretion. Since tight junctions of villus enterocytes

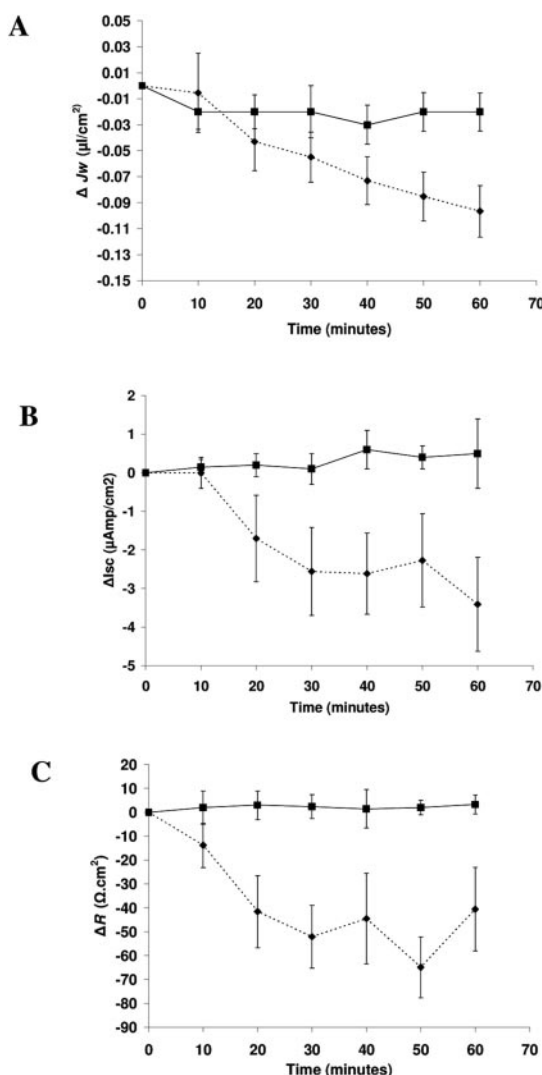


FIG. 1. Time course effect of CPE on (A) transepithelial net water flux ( $\Delta J_w$ ), (B) short-circuit current ( $\Delta I_{sc}$ ), and (C) transepithelial resistance ( $\Delta R_t$ ) in human ileum. After an equilibration period, intestinal mucosal sheets placed in Ussing chambers were incubated at the mucosal side with either Ringer solution alone (solid line) or Ringer solution containing 10  $\mu\text{g}/\text{ml}$  of CPE (dotted line) and monitored for 60 min. Each point indicates the mean  $\pm$  standard error of the mean of four experiments.

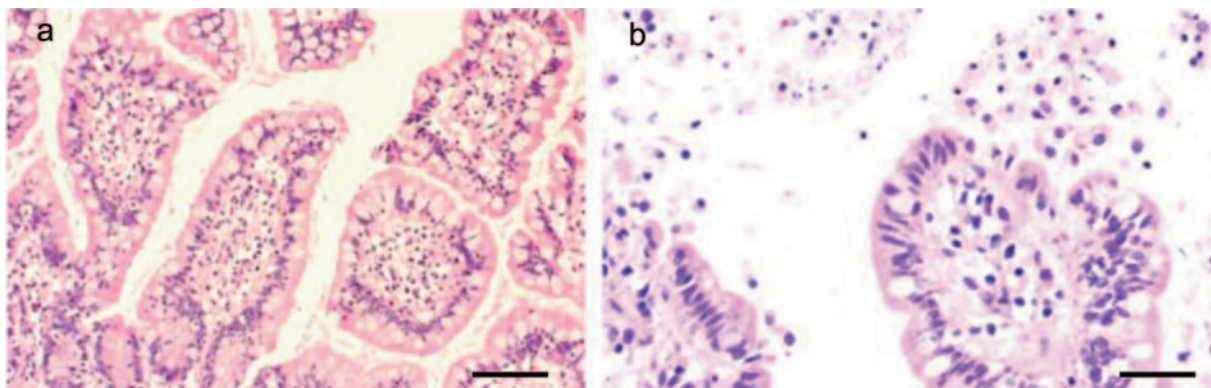


FIG. 2. Morphological effect of CPE on human ileal mucosa. (a) Human ileum after 60 min of incubation with Ringer solution (control) in Ussing chamber. No significant histological abnormalities were observed. Hematoxylin and eosin, 400 $\times$ . (b) Human ileum treated with CPE in an Ussing chamber for 60 min. Observe the necrosis and desquamation of the superficial epithelium at the tips of the villi. Hematoxylin and eosin, 400 $\times$ . Bars, 40  $\mu$ m.

have higher resistance than those of the crypts, tight-junction damage at the villi could fundamentally impact total mucosal electrical resistance.

The diarrhea observed in humans infected with CPE-

producing *C. perfringens* could result (at least partially) from impairment of small-intestine water absorption, which leads to a decreased intestinal absorptive capacity and increased stool mass.

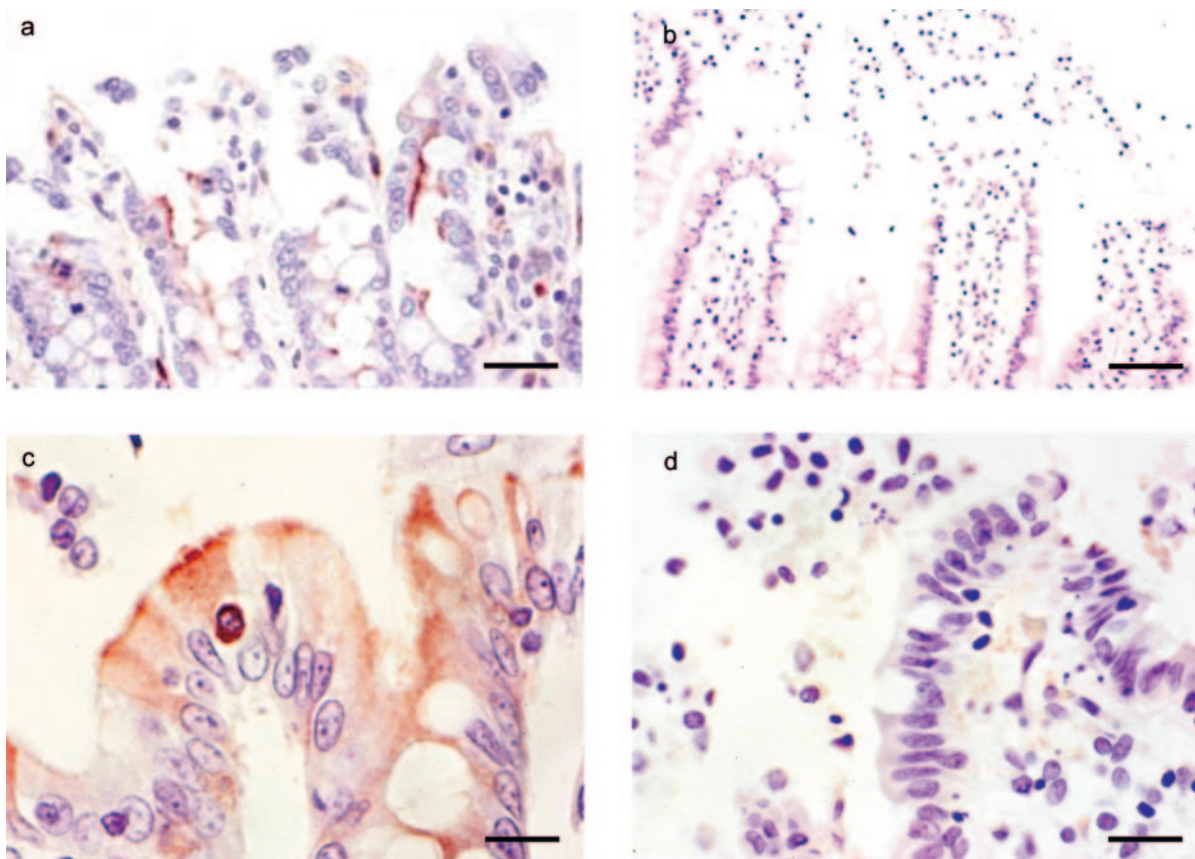


FIG. 3. Binding of CPE on human intestine. (a) Human ileum treated with CPE in an Ussing chamber for 60 min. Observe positively stained CPE on the apical borders of the enterocytes. Avidin biotin peroxidase, anti-CPE primary antibody, 400 $\times$ . (b) Human ileum treated with CPE in an Ussing chamber for 60 min. Avidin biotin peroxidase, normal rabbit serum (negative control), 400 $\times$ . (c) Human colon treated with CPE in an Ussing chamber for 60 min. Observe positively stained CPE on the apical and basolateral borders of the enterocytes. Avidin biotin peroxidase, anti-CPE primary antibody, 400 $\times$ . (d) Human colon treated with CPE in an Ussing chamber for 60 min. Avidin biotin peroxidase, normal rabbit serum (negative control), 400 $\times$ . Bars, 40  $\mu$ m (a and b) and 20  $\mu$ m (c and d).

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