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Enterotoxic Effect of Stool Supernatant of *Cryptosporidium*-Infected Calves on Human Jejunum

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Background/Aims: **The clinical pattern of cryptosporidial diarrhea suggests an enterotoxic mechanism. No evidence for this mechanism has been reported thus far. This study aimed to look for enterotoxic effect elaborated by *Cryptosporidium*. *Methods:* The effects on human intestinal transport of stool supernatant of diarrheal calves infected with *Cryptosporidium parvum* were examined. Aliquots of centrifuged and filtered stools were added to the mucosal or serosal side of human jejunum obtained from patients undergoing surgery and mounted in Ussing chambers. Electrical parameters were recorded. Stool supernatants of uninfected calves served as a control. *Results:* The mucosal addition of 2.5 mg protein of fecal supernatant from diarrheal calves induced a prompt and significant increase in short circuit current with no effects on tissue conductance. The serosal addition of this material and the addition of control supernatant to either side did not induce modifications of electrical parameters. The enterotoxic effect was dose-dependent and saturable. It was reversible by withdrawing the supernatant from the incubation medium. The electrical effect was chloride- and calcium-dependent and was sensitive to heating. *Conclusions:* An enterotoxic activity is present in the stools of *Cryptosporidium*-infected calves. This activity may be responsible for secretory diarrhea in humans.**

Enteric cryptosporidiosis is a frequent problem in both adults and children.[1,2] Diarrhea associated with *Cryptosporidum* infection may be profuse and watery and is often referred to as choleralike.[3] In patients affected by acquired immunodeficiency syndrome, enteric cryptosporidiosis may be devastating with an excessive loss of body fluid.[3] The pathophysiology of diarrhea induced by *Cryptosporidium* is not clear. A major problem in investigating its mechanism is the lack of convenient animal models capable of reproducing the features of enteric cryptosporidiosis in humans.[4] The need for such a model is considered a priority for developing effective therapeutic strategies for human immunodeficiency virus–infected patients.[5]

Diarrhea associated with *Cryptosporidium parvum* infection has been observed only in juvenile large animals such as foals,[6] lambs,[7] and calves.[8,9] Laboratory animals such as mice, rats, hamsters, and rabbits experimentally infected with *Cryptosporidium parvum* do not have diarrhea.[10–12] A number of cytopathic changes have been described using various animal models[11,13] and cell lines.[14,15] Impairment of glucose-stimulated Na^+ and water absorption has been reported in pigs experimentally infected with *Cryptosporidium parvum.*[16] However, the loss of large volumes of water and electrolytes, which is often observed in patients affected by enteric cryptosporidiosis, is more likely associated with an enterotoxic rather than a cytotoxic mechanism. There has been only one previous report (in preliminary form) describing an enterotoxic effect of cultured cryptosporidial oocysts in rabbit ileum.[17] More recently, Argenzio et al. showed that part of the diarrhea in experimental porcine cryptosporidiosis can be attributed to local prostanoid production, which inhibits Na^+ absorption.[18]

We have looked for enterotoxic activity in stool supernatant of diarrheal calves experimentally infected with *Cryptosporidium* oocysts. The investigation was performed using human jejunal specimens mounted in Ussing chambers because this is a sensitive and well-established method used to investigate the secretory effects of classical enterotoxins such as cholera toxin and *Escherichia coli* heat-stable toxin.[19,20]

Materials and Methods

Experimental Infection

A *Cryptosporidium parvum* isolate (code ISS1) from a naturally infected calf was used. The isolate was maintained by serial passages in calves every 2–3 months; oocysts were purified through Sheater's solution and discontinuous Percoll

Abbreviations used in this paper: Gt, tissue ionic conductance; Isc, short-circuit current.

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gradient and stored in 2% potassium dichromate at 4°C for 2–4 months.[9]

Holstein–Friesian male calves were infected orally at 2–4 days of age with 400×10^6 purified oocysts. Microbiological examination was performed before infecting the animals and was then performed every 3 days afterwards to confirm *Cryptosporidium* infection and rule out the presence of enteric pathogens other than *Cryptosporidium.* The pattern of cryptosporidial infection in calves has been previously described.[9]

When the number of shed oocysts was greater than 6×10^6/mL (usually 5–6 days after infection), diarrheal feces were collected directly from the rectum and immediately stored in aliquots at −80°C.

Stools collected immediately before infecting the same calves with *Cryptosporidium* oocysts were used as control.

Preparation of Fecal Supernatant

Stools from infected animals were liquid. Fecal filtrates from control animals were obtained by water dilution of solid stools. Dilution factor was considered in evaluating the chemical features. Fecal material was thawed at room temperature, and centrifuged at 3000 × *g* for 30 minutes at 4°C. Supernatant was filtered through 0.22-μm pore membrane filters and used. Stools from three different animals were used throughout the study.

Microbiological Analysis

Microbiological analysis was performed on both stools and filtered supernatant. *Cryptosporidium* oocysts were looked for by an immunofluorescence method with monoclonal antibody fluorescein–conjugated stain (Merifluor, *Cryptosporidium* kit; Meridian Diagnostic, Cincinnati, OH). Search for other enteric pathogens included *Salmonella, Shigella, Campylobacter,* enterotoxigenic (heat-labile and heat-stable toxin–producing) *E. coli, Yersinia enterocolitica, Clostridium perfringens, Pseudomonas, Candida albicans, Rotavirus, Coronavirus,* and parasites. Microbiological methods have been described elsewhere.[21]

Chemical Characterization of Fecal Supernatant

The chemical features of the stools of infected and control animals used in this study were determined. Data were interpreted according to Eherer and Fordtran.[22]

Protein concentration was determined by the method of Lowry et al.[23] Na^+, Cl^-, and K^+ concentrations were determined as previously reported.[24] Osmolality was determined using an Osmometer Automatic (Roebling, Berlin, Germany). Anion gap was determined by the equation: Stool Osmolality − ($[Na^+]$ + $[K^+]$) × 2. The presence of carbohydrates was estimated as previously reported.[25]

Heat Inactivation

In the experiments of heat inactivation, stool supernatant was heated at 100°C for 10 minutes before being added to intestinal tissue.

Human Jejunal Specimens

Specimens were obtained from 46 men (mean age, 58 ± 10 years) undergoing surgery because of intestinal tumors (19 cases), intussusception (3 cases), adhesion (6 cases), Crohn's disease (9 cases), vascular occlusion (7 cases), and volvulus (2 cases). The specimens were obtained from the edges of the resected margins and appeared normal. Eighteen specimens were discarded because histological analysis revealed moderate to severe inflammatory changes or because fragments were too small to be mounted in Ussing chambers. Intestinal tissue was kept in ice-cold saline solution and mounted in Ussing chambers within 30 minutes of surgical excision. The study protocol was approved by the Ethical Committee of the II School of Medicine, University Federico II, Naples.

Electrical Parameters of Intestinal Mucosa Mounted in Ussing Chambers

Two to four paired fragments of unstripped jejunal mucosa were mounted in Ussing chambers. In each experiment, one fragment served as control of baseline electrical parameters. The bathing Ringer's solution contained (in mmol/L) NaCl, 53; KCl, 5; Na_2SO_4, 30.5; mannitol, 30.5; Na_2HPO_4, 1.69; NaH_2PO_4, 0.3; $CaCl_2$, 1.25; $MgCl_2$, 1.1; and $NaHCO_3$, 26. The solution was maintained at 37°C with water-jacketed reservoirs connected to a thermostated circulating pump and constantly gassed with 95% O_2/5% CO_2. Each side of the Ussing chambers contained 10 mL of Ringer's solution. In experiments performed to investigate the role of Ca^{2+}, a modified Ringer's solution was used to bathe the mucosal side. The modified Ringer's solution had the following composition: $NaHPO_4$, 1.65; NaH_2PO_4, 0.3; $NaHCO_3$, 25; NaCl, 53; KCl, 5; Na_2SO_4, 30.5; $MgCl_2$, 2.35; and ethylenediaminetetraacetic acid, 0.5. Ca^{2+}-free experiments were performed as described by Fasano et al.[26] In experiments performed to investigate the role of Cl^- in the electrical response, SO_4^{2-} substituted Cl^- at an equimolar concentration.[27]

Transepithelial potential difference (PD), short-circuit current (Isc), and tissue ionic conductance (Gt) were measured as previously described.[28]

The viability of intestinal specimens mounted in each Ussing chamber was checked at the end of each experiment by adding 10 mmol/L glucose to the mucosal side to obtain an evident Isc response. If this was not seen, the result was discarded.

Histology

Fragments of small intestinal mucosa were fixed in 10% buffered formaldehyde and paraffin-embedded. Sections were stained with H&E, periodic acid-Schiff and periodic acid-Schiff–diastase stains and examined by light microscopy.

Chemicals

All chemicals were of reagent grade and were obtained from Sigma Chemical Co. (St. Louis, MO).

Statistical Analysis

Each electrical experiment was performed at least three times. Results are reported as means ± SE. The significance of the differences was calculated using Student's *t* test.

Results

Microbiological Studies

Cryptosporidial oocysts were detected in the fecal material from diarrheal calves but not in filtered stool supernatants. Search for other pathogens was consistently negative.

Chemical Characteristics of Fecal Supernatant

The chemical features of fecal supernatant from infected and uninfected calves are reported in Table 1. Osmolal gap of stools from diarrheal calves was close to 70 mOsm/kg, indicating a secretory pathway of *Cryptosporidium*-induced diarrhea. Also, the pH value and Cl^- concentration were consistent with a secretory mechanism.[22] Upon the addition of a standard dose of 2.5 mg protein in 750 μL of filtered fecal supernatant, the chemical composition of the bathing solution showed only slight modifications of pH and osmolality.

Histology

Histological analysis of intestinal specimens before mounting in Ussing chambers showed normal morphology in all 28 specimens used. Analysis of intestinal tissue exposed to stool supernatant from healthy calves showed no abnormalities. Human tissue used as baseline control in Ussing chambers showed normal morphology when examined after the experiment (Figure 1*A*). Only slight abnormalities were observed in intestinal specimens exposed to stool supernatant from diarrheal calves. These were represented by vacuolization of absorptive cells and by a sparse infiltrate of mononuclear cells in the lamina propria. The overall histological picture was consistent with a mild and nonspecific inflammatory response (Figure 1*B*).

Table 1. Chemical Features of Fecal Supernatant From Calves

	Diarrheal	Normal
Na^+ (*mmol/L*)	66.77 ± 12	31 ± 7[a]
K^+ (*mmol/L*)	31.67 ± 9	16.9 ± 2.2[a]
Cl^- (*mmol/L*)	40.37 ± 7.3	19.3 ± 3.3[a]
Anion gap	72 ± 4	193.2 ± 10[a]
Protein (*mg/mL*)	3.4 ± 0.4	3.4 ± 0.4
Osmolality (*mOsm/kg*)	267 ± 13	289 ± 14
pH	5.78 ± 0.09	5.76 ± 0.08
Carbohydrates	not detected	not detected

NOTE. Values are mean ± SD of fecal specimens obtained from three animals.
[a]$P < 0.01$.

Features of Enterotoxic Effect

A standard amount of approximately 2.5 mg protein in 750 μL of fecal supernatant was usually added to the mucosal side of tissue mounted in Ussing chambers. Preliminary experiments had shown an evident electrical response to this dose of fecal supernatant. The addition of filtered supernatant from healthy calves to either the mucosal or serosal side of jejunal mucosa did not induce modifications of electrical parameters.

The addition of filtered supernatant from diarrheal calves to the serosal side of human jejunum had no effect on transepithelial PD or Isc, but when the same amount of fecal protein was added to the mucosal side, a prompt increase in Isc was observed (Figure 2). This effect was entirely related to an increase of PD because no variations of Gt were recorded.

Isc increase was time-dependent, reaching its maximum 30 minutes after the addition of the filtrate supernatant and then slowly decreasing toward the baseline levels. Isc increase was observed within approximately 2 minutes of the addition of the supernatant.

The addition of a further aliquot of filtered stool supernatant induced a corresponding increase in Isc of approximately the same magnitude as for the first addition (Figure 3). Upon addition of a further dose of fecal supernatant, no increase in Isc was observed, indicating a saturation pattern of the enterotoxic effect (Figure 3).

To see whether the enterotoxic effect was reversible, fecal supernatant was removed after 20 minutes of incubation with jejunal mucosa and replaced with standard Ringer's solution. A rapid fall of Isc was observed (Figure 4), indicating that the electrical effect required the presence of enterotoxic activity.

Chloride Dependency of the Enterotoxic Effect

To prove that the Isc increase was caused by anion secretion rather than cation absorption, experiments were performed in Cl^--free Ringer's solution as described in the Materials and Methods section. No increase in Isc was observed upon the addition of stool supernatant in the absence of Cl^-. Gt values were not modified in the absence of Cl^- (Figure 5).

Calcium Dependency of the Enteroxic Effect

The absence of Ca^{2+} clearly blunted the Isc increase in response to fecal supernatant addition. Indeed,

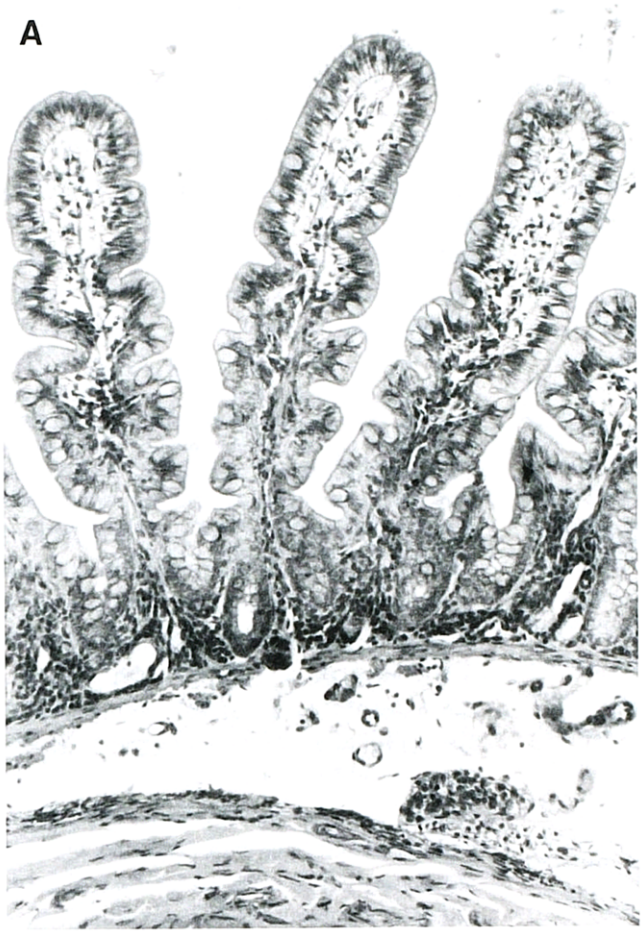

Figure 1. (*A*) Human jejunum exposed to fecal supernatant from healthy calves for 60 minutes at 37°C in Ussing chamber. Histology shows normal picture. (*B*) Human jejunum exposed to fecal supernatant from infected calves. Fecal supernatant was added to the mucosal side. There is a mild inflammatory infiltrate in the lamina propria and a mild and focal microvacuolization of absorptive cells (H&E; original magnification, ×106).

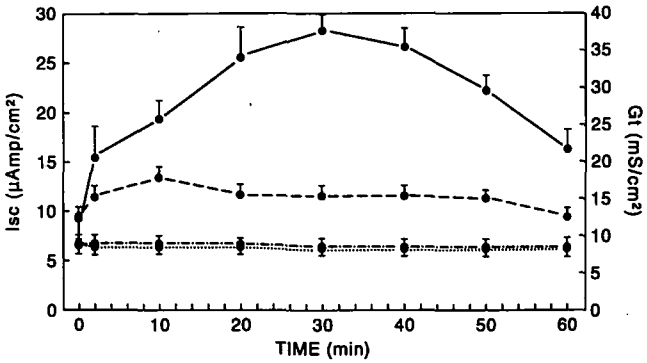

Figure 2. Time course of the effect of the mucosal addition of fecal filtered supernatant from diarrheal (——) and healthy (------) calves on Isc of human jejunum mounted in Ussing chambers. Gt values were not modified by the addition of fecal supernatant from diarrheal (– –) or healthy (· · ·) calves. For each Isc data point, the difference is statistically significant ($P < 0.01$). Gt did not change, indicating the stability of the tissue. Six tissue specimens were analyzed.

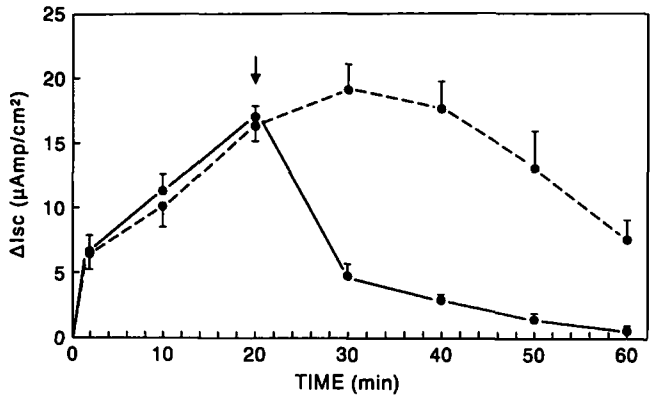

Figure 4. Isc modifications in response to the addition of fecal supernatant from *Cryptosporidium*-infected calves and its subsequent removal and replacement by standard Ringer's solution (*arrow*). The increase in Isc was sustained in the presence of supernatant, whereas it rapidly shifted toward basal values when this was removed. Eight tissue specimens were analyzed.

only approximately half of the Isc increase was observed in tissues exposed to Ca^{2+}-free Ringer's solution compared with that observed in paired tissues bathed with the standard Ca^{2+}-containing Ringer's solution. Gt values were not modified in the absence of Ca^{2+} (Figure 5).

Heat Sensitivity

Heating of stool supernatant virtually eliminated the electrical effect of *Cryptosporidium* supernatant (Figure 5), suggesting that the moiety responsible for the enterotoxic activity is proteic in nature.

Discussion

The pathophysiology of *Cryptosporidium*-associated diarrhea in humans is not clear. Most experimental data obtained in animals showed histological changes including villous atrophy, crypt hyperplasia, intracellular infiltration of the parasite, and inflammatory changes in the lamina propria.[7,16,29,30] However, histopathologic studies performed in humans affected by enteric cryptosporidiosis showed that intestinal mucosa is usually intact and enterocytes are well preserved.[31,32] Histological data obtained in this work are consistent with an absence of a significant cytotoxic effect (at least in the short-term) on human intestine. From a clinical standpoint, the loss of large volumes of watery stools associated with *Cryptosporidium*[3] and the efficacy of antisecretory drugs[33] suggest that the diarrhea may be related to an enterotoxic activity. The symptomatology observed in infected calves, used as the source of fecal supernatant in this work, was consistent with a secretory diarrhea. This was confirmed by the chemical features of diarrheal stools

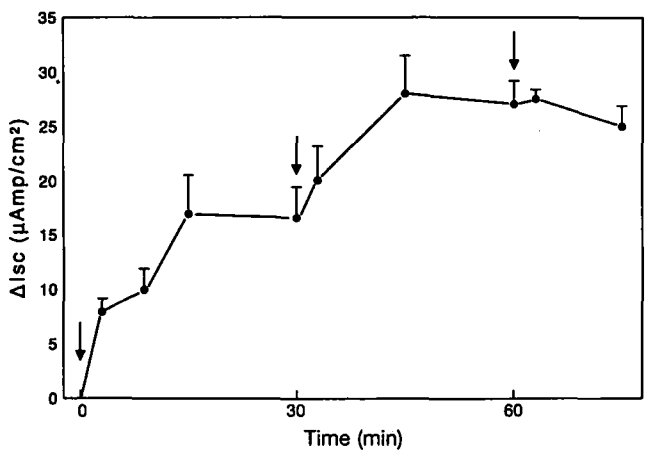

Figure 3. Isc increase in response to repeated additions of 2.5 mg protein of fecal supernatant from *Cryptosporidium*-infected calves to the mucosal side of human intestinal jejunum mounted in Ussing chambers. Aliquots of stool supernatant were added at 30-minute intervals (*arrows*), and modifications of transepithelial PD were monitored. Four tissue specimens were analyzed.

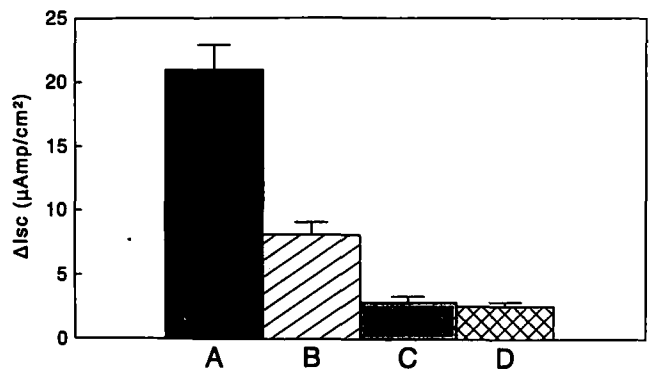

Figure 5. Effect of the addition of fecal supernatant from *Cryptosporidium*-infected calves on human jejunal mucosa in (*A*) standard Ringer's solution, (*B*) in the absence of Ca^{2+}, (*C*) in the absence of Cl^-, and (*D*) in standard Ringer's solution after heating the supernatant. Ten tissue specimens were analyzed.

obtained from *Cryptosporidium*-infected calves, which were consistent with a pattern of secretory diarrhea.[22]

Cryptosporidium parvum was used because this is reported to be one of the species responsible for enteric cryptosporidiosis in humans.[3] Furthermore, infected calves are a recognized source of enteric cryptosporidiosis in humans.[34]

Human jejunal specimens were used because it has been reported that the small intestine is a target site of *Cryptosporidium* infection.[3,16] Furthermore, the clinical pattern of watery diarrhea, often observed in patients with cryptosporidial diarrhea, suggests that a major role is played by the small rather than the large intestine.

We have provided evidence for an enterotoxic effect of fecal supernatant of diarrheal calves infected with *Cryptosporidium* on human jejunum. The enterotoxic effect was not related to the presence of the parasite because any form of the life cycle of *Cryptosporidium* was too large to pass through the pores of the membrane filter.[3] This was confirmed by the absence of oocysts and sporozoites in the filtrate material. Therefore, the enterotoxic effect was probably elaborated as an exotoxin resembling the prototypes of bacterial enterotoxins such as cholera toxin or *E. coli* heat-stable toxin.

Based on electrical parameters, the addition of stool supernatant from diarrheal calves induced a classical enterotoxic effect, i.e., an increase in Isc entirely related to an effect on transepithelial PD with no modification of Gt. The lack of electrical modifications in the tissue exposed to control supernatant indicates that the effect observed with diarrheal stool supernatant was related to *Cryptosporidium* infection. No effect was observed upon the addition of the toxin to the serosal side. These data are in contrast to data previously reported in a pig model and a rabbit model. In the first model, a complete impairment of glucose-stimulated Na^+ absorption was described in pigs with enteric cryptosporidiosis.[16] In the second model, an enterotoxic activity was found in cell-free medium of cultured *Cryptosporidium* oocysts added to the serosal side of rabbit ileum.[17] However, different responses in the species used, different receptor distribution, or differences in the experimental procedures may explain conflicting results.

The electrical effect seen in human intestine was prompt and sustained, resembling that observed with *E. coli* heat-stable toxin rather than cholera toxin or *E. coli* heat-labile toxin.[19,20] However, the enterotoxic effect produced by *Cryptosporidium* was heat-sensitive.

The enterotoxic effect was time dependent and saturable, as suggested by the trend to reach a plateau upon subsequent additions of fecal protein to the mucosal side. We do not have conclusive proof of a dose-related effect. However, two repeated additions of fecal supernatant induced corresponding increases in Isc. The effect was also rapidly reversible when supernatant was removed from the incubation medium.

The pathophysiology of the effect of *Cryptosporidium* toxic activity was partially elucidated in that it was Cl^- dependent, indicating that the effect on Isc was related to Cl^- secretion. Again, in this respect, *Cryptosporidium* toxin resembles the classical effect on ion transport induced by *Vibrio cholerae* and *E. coli* enterotoxins.

Interestingly, Isc response was reduced by lowering Ca^{2+} concentration. The finding of a hampered response of Isc to *Cryptosporidium* toxin in the absence of either Cl^- or Ca^{2+} strongly suggests that this toxin acts through a Ca^{2+}-mediated mechanism inducing Cl^- secretion. An increasing number of enterotoxins, including those produced by *Bordetella pertussis*,[35] *Clostridium difficile*,[36] and by the toxin responsible for *Ciguatera* fish poisoning,[27] are now known to act via Ca^{2+}-mediated mechanisms. The rapid but transient nature of Isc response to *Cryptosporidium* toxin strongly supports this hypothesis.

Finally, this is the first demonstration of enterotoxic effect induced by *Cryptosporidium* in human intestine. However, the possibility exists that the enterotoxic effect induced by *Cryptosporidium* is indirect. Argenzio et al. have recently found that prostaglandin E_2 is increased in the ileum of piglets experimentally infected with *Cryptosporidium* and that this increase is associated with an antiabsorptive pattern of intestinal transport.[18] The relative roles of secretion and malabsorption as well as their mechanisms in human enteric cryptosporidiosis need to be evaluated. The model developed by us offers this opportunity and may help in developing appropriate antisecretory therapy for the increasing number of patients, mostly immunodeficient patients who experience devastating diarrhea caused by enteric cryptosporidiosis.

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