Clostridium difficile toxin-induced intestinal secretion in rabbit ileum *in vitro*

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SUMMARY In rabbit ileum *in vitro* *Clostridium difficile* toxin (200 μ l crude extract) almost abolished net Na absorption, by decreasing mucosa to serosa flux, and induced net Cl secretion by increasing the serosa to mucosa flux. These flux changes were induced when there was no visible histological damage to the mucosa. The toxin did not influence adenylate or guanylate cyclase activity in a plasma membrane fraction of isolated rabbit enterocytes nor did it affect cAMP concentrations in intact rabbit ileum pre-incubated with toxin. The flux responses to the toxin were prevented by removing calcium from the serosal medium, suggesting that the secretory process may be calcium dependent. These results indicate a possible mechanism by which this toxin could induce diarrhoea.

Clostridium difficile toxin has been implicated as an important cause of antibiotic-associated colitis.^{1,2} Although the toxin damages the colonic epithelium³ and has cytotoxic effects in tissue culture,⁴ watery diarrhoea is a common feature of the disease in many cases when mucosal damage is inapparent.⁵ We have, therefore, investigated the effect of a crude toxin on intestinal transport in isolated rabbit ileum to elucidate whether it can act as an intestinal secretagogue.

Methods

TOXIN PREPARATION

Toxigenic *Cl difficile* was grown in Oxoid cooked meat medium at 37°C for five days. The cultures were centrifuged and the supernatant filtered through a 0.22 μ membrane filter (Millipore Corporation, Bedford, Mass.) and stored at -70°C until used. The cytotoxicity of this material was confirmed using tissue culture methods.⁶ Other workers have recently separated *Cl difficile* toxin into fractions containing an enterotoxin and a cytotoxin.⁷ Thus it is likely that our crude extract contained more than one toxin. Control medium

was prepared as above using non-toxigenic *Cl difficile*.

ION TRANSPORT STUDIES

These were performed as previously described.⁸ Briefly, male New Zealand white rabbits (2-4 kg weight) were killed by air embolus and the distal ileum rapidly removed and bathed in oxygenated Ringer's bicarbonate buffer solution. Sections of the tissue were stripped of muscle layers and mounted in a modified Ussing chamber.⁹ The tissue was bathed on each side by 10 ml of isotonic buffer solution of composition Na 146, K 4.2, Cl 125.8, HCO₃ 26.6, Ca 1.2, H₂PO₄ 0.2, HPO₄ 1.2, Mg 1.2, glucose 10 mmol/l, and pH 7.4. When investigating the effect of calcium on secretion a calcium-free buffer solution with a calcium-chelating agent was placed on the serosal side (Na 146, K 4.2, Cl 125.8, HCO₃ 26.6, H₂PO₄ 0.2, HPO₄ 1.2, Mg 1.2, ethylene glycol-bis-(β -amino ethyl ether)N, N'-tetraacetic acid (EGTA) 0.2, glucose 10 mmol/l, and pH 7.4). The buffer solution was stirred and oxygenated via a bubble lift mechanism by a 95% O₂/5% CO₂ mixture and maintained at 37°C.

The transmucosal potential difference (PD) was measured as previously described⁸ and the short-circuit current (Isc) by means of a voltage clamp. Tissue resistance was calculated from PD and Isc. Sodium and chloride fluxes were measured using 0.5

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$\mu\text{Ci}^{22}\text{Na}$ and $2.5 \mu\text{Ci}^{36}\text{Cl}$ (Radiochemical Centre, Amersham) added to either mucosal or serosal reservoirs. After a 20 minute equilibration period serial 1 ml samples were taken at 20 minute intervals and this volume was replaced with unlabelled buffer solution. Eight tissues from one rabbit were mounted in each experiment and these were paired provided that their resistances differed by less than 25%. Unidirectional fluxes were used to calculate a net flux for each tissue pair and these fluxes and residual ion flux were calculated as described previously.⁸ After a 20 minute control flux period $200 \mu\text{l}$ of toxin-containing medium was added to the mucosal chamber and left in contact with the tissue for 40 minutes before three consecutive 20 minute flux determinations were carried out. Two hundred microlitres of control medium (without toxin) was added to paired control tissues and flux measurements carried out as for the toxin-treated tissues. After the experiments tissues were carefully removed, fixed with 2.5% glutaraldehyde in cacodylate buffer, and post-fixed with 2% osmium tetroxide. The tissue was dehydrated, embedded in epoxy resin, and 1μ sections were stained with toluidine blue for examination by light microscopy.

CYCLIC NUCLEOTIDE STUDIES

1 cAMP

Pieces of stripped rabbit ileal mucosa were placed in flasks containing 10 ml of buffer solution (as above) oxygenated with 95% O_2 5% CO_2 , and incubated at 37°C . After a 45-minute pre-incubation period *Cl difficile* toxin ($200 \mu\text{l}$) was added. Control flasks contained $200 \mu\text{l}$ of culture medium. At 45 and 90 minutes after addition the mucosal pieces were removed and placed immediately in absolute ethanol at -40°C containing 4 nCi (^3H) cAMP as a recovery marker. Tissues were homogenised with a motor driven Teflon pestle and centrifuged at 2500 g for 20 minutes. The resulting supernatants were dried under N_2 at 40°C and suspended in a buffer containing Tris-HCl 50 mmol/l and ethylene diamine tetraacetic acid (EDTA) 4 mmol/l pH 7.4. Aliquots were assayed for cAMP using a commercial kit (Radiochemical Centre, Amersham). The precipitate recovered after centrifugation was dissolved in 1N NaOH and assayed for protein.¹⁰ Results were expressed as picomoles of cAMP per milligram of protein.

2 Effect of *Clostridium* toxin on nucleotide cyclases

A semi-purified plasma membrane fraction was prepared from isolated rabbit small intestinal epithelial cells according to the method of Murer *et al.*¹¹ The final membrane pellet was resuspended in Ringer's bicarbonate buffer and stored at -70°C until use.

Adenylate cyclase assays were performed according to the method of Amiranoff *et al.*¹² The reaction mixture contained 0.8 mmol/l adenosine triphosphate (ATP); 10 mmol/l MgCl_2 ; 1 mmol/l EDTA; 0.2 mmol/l 3-isobutyl-1-methyl-xanthine (IBMX); 20 mmol/l creatinine phosphate; 25 mmol/l Tris; 4 mg/ml bovine serum albumin; 1 mg/ml phosphocreatine kinase and 50–100 μg membrane protein in a final volume of $250 \mu\text{l}$. Incubations were performed at 30°C for periods of up to one hour and the reaction stopped by heating to 100°C for three minutes. cAMP was determined with the assay kit. Guanylate cyclase was determined in a similar way except that the reaction mixture contained 1 mmol/l guanosine triphosphate (GTP); 10 mmol/l caffeine; 5.35 mmol/l MnCl_2 creatine phosphate; 1 mg/ml creatinine phosphokinase; 40 mmol/l Tris-HCl, pH 7.4; and 10–15 μg membrane protein. The cGMP produced was assayed by a specific radio-immunoassay (Radiochemical Centre, Amersham).

Both assays were started by the addition of membrane protein. Toxin and control medium were added to the incubations immediately before the addition of the membranes in a volume of $5 \mu\text{l}$. Results are expressed as picomoles of cyclic nucleotides formed per milligram of protein.

All results are expressed as the mean $\pm$ 1 SEM. Statistical comparisons were performed using Student's *t*-test for paired variables and considered significant if $p < 0.05$. An analysis of variance was also performed for some of the data comparisons.

Results

The addition of $200 \mu\text{l}$ of toxin-containing medium caused a slow but significant rise in potential difference (10.9% at 60 minutes and 22.9% at 100 minutes, $p < 0.02$) and short circuit current (21.2% at 60 minutes and 36.5% at 100 minutes, $p < 0.01$) and a fall in tissue resistance (*R*) (7.8% at 60 minutes and 9.5% at 100 minutes, $p < 0.02$, $n = 15$). The control medium, however, caused similar but smaller changes in short circuit current and tissue resistance and an analysis of variance showed that there was no significant difference between the responses to control and toxin-containing medium.

The control medium had no significant effect on unidirectional fluxes over a period of 100 minutes but the toxin-containing medium almost abolished net sodium absorption, mainly by decreasing the mucosa to serosa flux, and induced net chloride secretion mainly by increasing the serosa to mucosa flux. Significant changes were able to be seen at 40 to 60 minutes after exposure but were maximal at 80 to 100 minutes. The residual ion flux ($J^{\text{R}}_{\text{net}}$) was unaffected (Table 1). Analysis of variance

Table 1 Effect of *Cl difficile* toxin on unidirectional and net Na and Cl fluxes over 20 minute periods immediately before and after addition of toxin-free medium (A) or toxin-containing medium (B)

	Na			Cl			Isc	J ^R Net
	ms	sm	Net	ms	sm	Net		
A Control (n=6)	17.56±1.80	11.78±1.41	+5.78±0.85	11.87±1.22	9.69±0.68	+2.18±0.74	4.59±0.76	+1.00±0.77
Toxin-free medium (80–100 min)	14.43±0.90	10.34±0.58	+4.35±0.58	10.05±0.99	8.85±0.73	+1.20±0.65	5.81±0.60	+3.41±0.20
	NS	NS	NS	NS	NS	NS	p<0.01	NS
B Control (n=7)	14.00±0.88	9.71±0.94	+4.36±0.75	10.88±0.62	10.63±1.18	+0.26±1.04	3.52±0.42	-0.57±0.22
Toxin-containing medium (40–60 mins)	11.48±1.34	10.81±1.18	+1.21±1.26	11.16±0.51	12.54±1.13	-1.39±1.24	4.32±0.72	+0.94±0.55
	NS	NS	p<0.05	NS	p<0.05	NS	NS	NS
(80–100 mins)	10.72±0.96	10.50±0.91	+0.29±1.41	10.21±0.97	12.90±1.10	-2.43±0.98	4.80±0.42	+0.35±1.41
	p<0.02	NS	p<0.01	NS	p<0.05	p<0.01	p<0.01	NS

All fluxes expressed as $\mu\text{Eq cm}^{-2}\text{h}^{-1}$. ms = mucosa to serosa flux, sm = serosa to mucosa flux, + values signify absorption, - values signify secretion. p values refer to significance of difference from its control.

confirmed that all the significant flux responses to the toxin were significantly different from the toxin-free medium responses.

With a calcium-free buffer solution on the serosal side the inhibition of net sodium absorption and the net chloride secretion induced by the toxin was markedly reduced (Table 2). The addition of theophylline 10^{-2}M to the calcium-free serosal reservoir after 100 minutes' incubation with toxin induced a sharp rise in short circuit current (22.3% in two minutes, $p<0.01$) confirming viability of the ileal tissue ($n=3$).

CYCLIC NUCLEOTIDE STUDIES

Cl difficile toxin incubated for periods of up to 90 minutes with segments of stripped rabbit ileum had no effect on mucosal cAMP concentrations when compared with control tissues (Table 3) at a time when flux changes due to the toxin were observable. The ability of tissues incubated in this way to respond to cAMP stimulating agents was confirmed with prostaglandin E_2 (10^{-4}M)¹³ which produced a two-fold rise in cAMP concentration after 45 minutes (Table 3).

Similar results were obtained when the toxin was

incubated directly with a plasma membrane rich fraction prepared from isolated rabbit epithelial cells. Production of cAMP under control conditions increased linearly up to 30 minutes followed by a gradual 'levelling-off' over the final 30 minutes' incubation. Assays which included toxin closely followed this pattern with no statistically significant effect on cAMP generation ($n=5$) at a time when flux changes due to the toxin were observable. There was also no evidence for the involvement of cGMP in the action of this toxin. The time course of cGMP production in membranes was identical for both toxin-containing and control media ($n=5$). The responsiveness of the membrane preparation to stimulation was assessed for adenylate cyclase with VIP and guanylate cyclase with heat stable *E. coli* enterotoxin.¹⁴ In both instances marked stimulation occurred.

Histological examination by light microscopy showed that the tissue was undamaged after two hours' incubation (Figure).

Discussion

Antibiotic-associated colitis is now recognised to be

Table 2 Unidirectional and net Na and Cl fluxes over 20 minute periods immediately before and 80 to 100 minutes after addition of toxin-containing medium utilising Ca^{2+} free medium serosally.

	Na			Cl			Isc	J ^R Net	R
	ms	sm	Net	ms	sm	Net			
Control (Ca^{2+} free) (n=6)	14.72±1.23	8.22±0.86	+6.52±0.71	11.46±0.94	8.85±0.62	+2.61±0.46	3.71±0.23	-0.19±0.65	28.6±1.50
Toxin (Ca^{2+} free)	12.81±1.52	7.55±0.83	+4.6±1.30	10.39±0.72	8.96±0.71	+1.43±0.49	3.75±0.42	+0.36±1.40	33.1±3.00
	p<0.05	NS	NS	NS	NS	NS	NS	NS	NS

Mean tissue resistance (R) expressed in ohms cm^{-2} ($n=12$).

Table 3 cAMP concentration in mucosal pieces (n=9)

	45 minute	90 minute
Control medium (200 μ l)	5.47 $\pm$ 0.89	4.82 $\pm$ 0.51
Control medium + toxin (200 μ l)	5.01 $\pm$ 0.63	4.59 $\pm$ 0.577
PGE ₂ (10 ⁻⁴ M)	11.72 $\pm$ 1.72*	—

* p<0.01

caused by *Cl difficile* toxin. It is usually associated with damage to the colonic mucosa giving rise to the characteristic sigmoidoscopic appearances.³ These cytotoxic effects of the toxin have been shown in tissue culture.^{4,7} The clinical picture, however, is often one of watery diarrhoea without bleeding, commonly without visible mucosal damage⁵ and this is consistent with the idea that the toxin can provoke diarrhoea in the absence of mucosal damage, perhaps by acting as a secretagogue. In our study we have used a crude extract which probably contains both an enterotoxin and a cytotoxin. This crude *Cl difficile* toxin did, indeed, stimulate chloride secretion and reduced sodium absorption in isolated rabbit ileum. The observations that the ion flux responses to the toxin were selective and were abolished by removal of calcium from the serosal bathing medium suggest that the toxin activated a specific secretory process rather than simply exerting a non-specific damaging effect. The absence of histological evidence of tissue damage also tends to support this conclusion.

The observation that our control medium had

similar electrical effects to the toxin suggests that they were mainly because of components of the culture medium. The ionic basis for the electrical response is unclear; certainly no significant effects on Na and Cl fluxes were observed with control medium.

The cyclic nucleotide studies failed to reveal evidence for an action involving cAMP, adenylate cyclase, or guanylate cyclase and thus the action of this toxin is distinct from that of other toxins including cholera toxin, and *E. coli* heat labile toxin which activate adenylate cyclase^{13,15} and of *E. coli* heat stable toxin which activates guanylate cyclase.¹⁴ Vesely and co-workers¹⁶ recently showed that *Cl difficile* cytotoxin stimulated guanylate cyclase activity in cultured hamster fibroblasts and in hamster ileum and caecum. In these experiments, however, adenylate cyclase activity was stimulated in the supernatant obtained from homogenising whole gut tissue. Cyclase activity in the pellet fraction, presumably containing plasma membranes, was not stimulated in these experiments. Our studies were performed on purified plasma membrane fractions of epithelial cells, the site of the transport cyclases. It seems likely, therefore, that the guanylate cyclase activity stimulated in hamster ileum and caecum was that normally located in other sites, possibly the muscle coats and serosa.

The inhibition of toxin-mediated secretion, by removal of calcium from the serosal bathing medium, suggests that extracellular calcium is required for full activity of the toxin. The response to theophylline after toxin exposure in calcium-

Figure Well-preserved mucosa brush border of stripped rabbit ileum after 100 minutes' incubation with *Cl difficile* toxin (light micrograph, on g. magnification $\times 1500$.)

deprived tissues confirms their viability and potential to respond to other stimuli. The mechanism for the action of calcium in controlling intestinal secretion is unclear but the involvement of a calcium-dependent regulator protein (calmodulin) has been proposed.¹⁷ Furthermore, work recently reported in an abstract implicates calcium in the control of a neutral NaCl brush border uptake process.¹⁸ Our data are not incompatible with such an involvement of calcium in toxin-induced secretion. A full description of the role of calcium must, however, await further study.

Other secretagogues which require extracellular calcium for their action include 5-hydroxy tryptamine (5 HT),¹⁹ acetylcholine, and the calcium ionophore A23187.²⁰ It is conceivable, therefore, that *Cl difficile* toxin activates a similar calcium-‘gating’ process. Alternatively, it may act by liberating a local mediator, such as 5 HT or acetylcholine. Although the effects of the toxin are not identical with those of 5 HT – for example, 5 HT decreases M→S chloride flux, which the toxin does not – they are sufficiently similar to invite further study.

Whatever the underlying mechanism, it seems likely that these secretory effects of *Cl difficile* toxin contribute to the diarrhoea associated with this infection.

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