The effects of *Clostridium difficile* crude toxins and purified toxin A on stripped rabbit ileal mucosa in Ussing chambers

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**Summary.** *Clostridium difficile* crude toxins and purified toxin A had similar effects on stripped rabbit ileal mucosa in Ussing chambers. Both toxin preparations caused secretion of sodium and chloride ions by increasing serosa to mucosa \((s\rightarrow m)\) fluxes. Transmural potential difference and resistance decreased after toxin treatment. Onset of changes in electrical measurements and ion fluxes coincided with onset of histological changes. The response to theophylline was greatly reduced in toxin-treated tissue compared with control tissue.

**Introduction**

*Clostridium difficile* is the primary aetiological agent of antibiotic-associated pseudomembranous colitis and some cases of antibiotic-associated diarrhoea (Bartlett et al., 1978; George et al., 1978). In some patients *C. difficile* induces watery diarrhoea with no apparent mucosal damage (Mogg et al., 1979). In this paper, we consider possible mechanisms and determinants of this watery diarrhoea against a background (briefly summarised below) of current knowledge on *C. difficile* toxinology.

We recently discussed (Mitchell et al., 1986) the range and properties of known toxins produced by *C. difficile* that are of possible relevance in the causation of diarrhoeal disease. These include (1) toxin A (enterotoxin), and (2) toxin B (cytotoxin), on the nature and properties of which there is a considerable body of evidence from several laboratories. In addition, from individual laboratories there exist largely unconfirmed reports of (3) a motility-altering factor, (4) a labile toxin that induces a clear fluid accumulation in rabbit ileal loops, and (5) a low-mol.-wt subunit toxin “active in rabbit ileal loops” (but uncharacterised in terms of the nature of the exsorbed fluid and induction of tissue damage). The current controversy concerning *C. difficile* toxins arising from the use of a latex test for *C. difficile* toxin A (Lyerly and Wilkins, 1986) is as yet unresolved.

There are at least three possibilities to explain watery fluid secretion by *C. difficile*. First, Lonnroth and Lange (1983) claimed that toxin A induces a watery secretion in mouse ileal loops and that a combination of toxins A and B produces a haemorrhagic response in this model. This is certainly not the case in rabbit ileum and hamster caecum—the two most widely used models in this field—in which toxin A on its own induces tissue damage and a haemorrhagic fluid response; at present it is not clear whether this reflects genuine differences in response by different species to toxin A.

Second, Banno et al. (1984) reported a labile toxin that produced a watery fluid response in the rabbit ileal loop test. However, in our laboratory, only one of numerous attempts was successful in repeating the findings of Banno and co-workers. The relevance of this factor therefore remains unresolved.

Third, it is our view that toxin A, which we have shown to be of overriding importance in the causation of tissue damage and fluid accumulation in rabbit experimental systems (Mitchell et al., 1986) could be responsible. Such a view is confirmed by Lyerly et al. (1985) who showed that toxin A was responsible for the enteric effects of toxic crude culture filtrates administered orogastrically to hamsters. The apparently high mol. wt of toxin A (Taylor et al., 1981) makes the concept of a multifunctional molecule capable of inducing fluid secretion, tissue damage, or both, inherently possi-
ble. The watery diarrhoea without tissue damage could ensue when, for whatever reason, only one aspect of its biological activity was expressed. Alternatively, toxin A may be a monofunctional entity that acts in a progressive manner on susceptible tissues, inducing first a watery secretory diarrhoea which is then masked or swamped by the later effects of gross tissue damage. If, for any reason, this progression is impeded early in the attack upon susceptible tissue, watery diarrhoea could ensue.

There are at least two studies that bear on the possibility of a *C. difficile*-induced secretory diarrhoea. Vesely *et al.* (1983) showed that both toxins A and B elevated levels of guanylate cyclase in hamster colon, ileum and duodenum; cyclic GMP is a known secretogogue. Hughes *et al.* (1983) claimed that crude *C. difficile* culture filtrate blocked sodium absorption and stimulated chloride secretion. This response was not associated with a change in transmural potential difference. Cyclic nucleotide levels were similar to those in control tissues but the response was found to be dependent on Ca$^{2+}$. Changes in ion transport occurred in the absence of any overt tissue damage. These workers proposed that a *C. difficile* factor was the first known example of an enterotoxin that caused secretion by a Ca$^{2+}$-mediated mechanism.

The aim of the present study was to determine whether, within the first few hours of exposure, *C. difficile* crude culture filtrate or purified toxin A affected intestinal transport in rabbit ileum with or without the tissue damage we have previously observed in vivo (Mitchell *et al.*, 1986) and in vitro (Stephen *et al.*, 1984). To maximise the possibility of dissecting primary from subsequent events, we have examined the effect of toxin A on stripped rabbit ileal mucosa.

**Materials and methods**

**Toxin production and purification**

*C. difficile* strain B-1 (supplied by Dr S. P. Borriello, Clinical Research Centre, Harrow) was grown in 1 L of saline inside a dialysis bag suspended in 7 L of the medium previously described by Stephen *et al.* (1984). After growth at 37°C for 4 days the culture was clarified by centrifugation (25 000 g, 30 min, 4°C). This crude material was sterilised by passage through a 0.22 µm pore size membrane filter (Millipore) and stored at 4°C.

Toxin A was purified from crude material as previously described (Stephen *et al.*, 1984) by ammonium sulphate precipitation, preparative electrophoresis and ion exchange chromatography.

**ELISA**

An ELISA procedure, as described by Redmond *et al.* (1985) was used to enable crude and purified preparations with similar toxin A contents to be compared.

**Ion transport studies**

Unidirectional ion fluxes were determined by the modified method of Isaacs *et al.* (1976). Male New Zealand white rabbits were killed by administration of a sodium pentobarbital overdose (60 mg/kg); 30 cm of distal ileum was removed, opened along its mesenteric border and placed in oxygenated Ringer solution (Na$^+$ 148 mM, Cl$^-$ 125 mM, K$^+$ 4.3 mM, HCO$_3^-$ 26 mM, Ca$^{2+}$ 1.2 mM, H$_2$PO$_4^-$ 1.2 mM, Mg$^{2+}$ 1.2 mM and glucose 10 mM). The mucosa from segments of ileum was stripped from the muscle layers by blunt dissection and mounted between two halves of a perspex tissue chamber (Ussing chamber). The tissue was bathed on both sides by 10 ml of Ringer solution. The buffer was circulated and oxygenated by a gas lift mechanism (Schultz and Zalusky, 1964) with CO$_2$ 5% in oxygen. The system was maintained at 37°C by a constant-temperature water jacket.

All electrical measurements and manipulations were done with an automatic voltage clamp. The electrical apparatus was connected to the tissue chamber with salt-agar bridges (agar 4% in Ringer solution). Potential difference (PD) measurements were made with matched calomel electrodes (Chemetric Ltd, Sherborne, Dorset) immersed in 3 M KCl. Short-circuit conditions were achieved by applying current via silver-silver chloride electrodes (Clark Electrochemical Instruments Ltd, Reading) immersed in 0.15 M NaCl, and the short-circuit conditions were maintained by an electronic feedback circuit. Corrections were made for the drop in PD between the PD electrodes caused by the resistance of the fluid between the tips of the measuring bridges and the tissue. This resistance was determined before mounting the tissue. The automatic voltage clamp contained a special circuit for making the correction based on the calculated value for fluid resistance. Tissue resistance was expressed as the quotient obtained by dividing the open circuit PD by the short circuit current (SCC).

Sodium and chloride fluxes were measured with paired tissues from the same segment of ileum. Tissues were not paired unless their electrical resistances were comparable and differed by less than 25%. One hour after mounting the tissue, crude toxin containing 100 µg of toxin A (determined by ELISA), or 100 µg of purified toxin A, was introduced into the mucosal side of the chambers. In control experiments, no toxin was added. $^{22}$Na 2 µCi and $^{36}$Cl 2.3 µCi (Amersham International) were then introduced on the mucosal side of one tissue of a pair and on the serosal side of the other. Samples of the Ringer solution from the labelled and unlabelled sides of the chambers were taken every 20 min for 2 h. At the time of sampling, the open circuit PD was recorded. Two separate 1-ml samples were taken at each time point, one for gamma counting and the other for liquid scintillation counting. Sample volumes were replaced with unlabelled...
buffer. $^{22}$Na in each sample was determined by gamma counting and total radioactivity by liquid scintillation counting with ACSII liquid scintillation cocktail (Amersham International). The activity for $^{22}$Na multiplied by a factor for the relative efficiency of the two counters for $^{22}$Na was subtracted from the total radioactivity to yield the counts due to $^{36}$Cl. Unidirectional and net ion fluxes were calculated by the method of Isaacs et al. (1976).

Statistical analysis of the flux data was by a paired t-test. All fluxes from tissues treated with either crude or pure toxin were compared with fluxes at their equivalent control time points. For example, Na$^+$ M→S flux at 100 min from control tissue was used for comparison of flux data from tissue treated with either crude or purified toxin at 100 min. Thus, significance is ascribed to values that are significantly different from controls at the given time points, but not necessarily different from zero.

**Theophylline response**

At the end of experiments, 1 ml of 100 mM theophylline was added (giving a final concentration of 10 mM) to the serosal side of the chambers and the change in open circuit PD noted.

**Histology**

After various incubation times, tissue was removed from the chambers and fixed in formaldehyde (4% in saline). Samples were embedded in paraffin wax, sectioned and stained with haematoxylin and eosin for examination by light microscopy.

**Results**

**Electrical response**

Addition of either crude toxins or purified toxin A had similar effects. After a lag period of approximately 80 min each preparation caused a steady decline in transmural PD and tissue resistance (fig. 1). Short-circuit current declined slightly but was not significantly different from that of the control (data not shown).

**Ion fluxes**

In control tissue, net sodium absorption occurred at all time points (fig. 2). Exposure to either crude or purified toxin A caused net secretion of sodium ions beginning after approximately 80 min and increasing with time of incubation. Statistical analysis indicated that sodium fluxes were significantly different from control values after incubation for 100 min with toxin.

Analysis of unidirectional sodium fluxes showed that m → s transport was not significantly different from that of controls. Net ion outflow was due to an increase in the s → m flux.
Toxin treatment also caused a reversal of chloride transport from net absorption in controls to net secretion beginning at approximately 80 min in toxin-treated tissue (fig. 3). Secretion of chloride was also due to an increase in the s→m flux; the m→s flux was not significantly different from that of controls. Statistical analysis indicated that chloride fluxes were significantly different from control values after incubation for 120 min with the toxin. The net rate of chloride secretion was always exceeded by the rate of sodium secretion in toxin-treated tissue.

**Theophylline response**

The response of tissue to theophylline was reduced after 2-h exposure to the toxin (table).

**Histology**

Examination of toxin-treated tissue showed that histological changes had occurred by 80 min. The changes included denudation of villus tips by desquamation of enterocytes and extrusion of cellular components from the lamina propria. Crypt areas appeared to be unaffected by the toxin (see Ketley et al., 1987).

**Discussion**

In this study, toxin A caused an apparent secretion of electrolytes but this was found to be associated with histological changes. Changes in transmural potential difference (PD) and short circuit current (SCC) can only be confidently interpreted in terms of altered active transport when tissue resistance remains fairly constant; tissue resistance is the electrical measurement reflecting tissue integrity. In our experiments, tissue resistance always fell; the onset of the fall correlated with the onset of histological changes to the architecture of the villus tips. In a preliminary study (Stephen et al., 1984), we reported a rise in SCC when toxin A was added to the mucosal side of stripped rabbit ileal mucosa mounted in Ussing chambers in which the tissue was bathed in salt solutions without glucose. Subsequent work showed that the rise in SCC was most likely to be due to a change in tissue resistance during a period when the transmural PD generated under such conditions was reasonably constant and comparatively low (0.8 mV).

Examination of ion fluxes indicated that crude or purified toxin A caused a net secretion of both sodium and chloride beginning at approximately 80 min. The secretion of both ions was due to increased s→m flux. The rate of sodium secretion exceeded the rate of chloride secretion at all time points.

There are two possible mechanisms by which toxin A could induce an increase in the s→m flux resulting in a net outflow of sodium ions. First, the toxin could stimulate an active secretory pathway; or second, the toxin could induce changes in ion fluxes that are primarily due to toxin-induced

**Table.** Effect of *C. difficile* toxins on the response of isolated rabbit ileum to 10 mM theophylline after incubation for 2 h

<table>
<thead>
<tr>
<th>Test preparation*</th>
<th>Response to 10 mM theophylline (mV)</th>
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</thead>
<tbody>
<tr>
<td>Control (N = 20)</td>
<td>2.15 ± 0.20</td>
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<tr>
<td>Crude toxins: final concentrations—</td>
<td></td>
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<tr>
<td>toxin A 10 μg/ml, toxin B 10⁵ units/ml (N = 20)</td>
<td>0.74 ± 0.08</td>
</tr>
<tr>
<td>Purified toxin A: final concentration 10 μg/ml (N = 8)</td>
<td>0.41 ± 0.16</td>
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</tbody>
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*After incubation for 2 h in the Ussing chamber, theophylline was added to the serosal reservoir to a final concentration of 10 mM. The change in open circuit potential difference (mV) was noted. N = number of observations. Errors are expressed as SEM.
changes in the structure, i.e., in the permeability of the tissue. The total resistance of ileal tissue is determined mainly by the low-resistance paracellular pathway (Frizzell and Schultz, 1972). Hence, in the absence of evidence to the contrary, any changes in tissue resistance would be likely to reflect changes in this pathway. If the net secretion of sodium is due to changes in the paracellular pathway (affecting the diffusion of sodium) then there must be a mechanism to impose directionality on this transport process. This is discussed in the following paragraphs.

An effect of toxin A on the paracellular pathway could lead to net sodium secretion as follows. The current model for normal sodium transport involves absorption of sodium by villus cells across their apical membranes. The sodium is then extruded into the intercellular spaces by Na⁺K⁺ ATPase. This would result in high levels of sodium ions within the intercellular spaces. Normally, sodium exit into the lumen is restricted by the tight junctions and sodium ions are retained on the tissue side. Toxin-induced damage to the tight junctions could change the permeability to sodium and allow movement of sodium down its electrochemical gradient to the mucosal side of the tissue. This electrochemical gradient exists within, rather than across the tissue and would not necessarily be eliminated by short-circuit conditions. The existence of electrochemical gradients within the tissue (even under short-circuit conditions) is often overlooked in the interpretation of data from Ussing chamber experiments.

It is not possible to state dogmatically from the available data whether sodium secretion induced by toxin A is due to stimulation of an active mechanism or to changes in tissue structure. However, it seems highly likely that it is due to the latter because it occurs after histological changes are apparent; these changes are also probably responsible for the fall in transmural PD.

Chloride ions are also secreted in response to toxin A. Again it is not clear whether the toxin is activating an active secretory mechanism or chloride ions are moving passively with sodium ions. The fact that chloride fluxes do not become significantly different from control values until 20 min after sodium fluxes suggests that chloride may be moving passively; this is the preferred explanation.

Several other cytotoxic enterotoxins cause changes in ion transport similar to those induced by toxin A. Rabbit ileum exposed to Shiga toxin for 5 h in vivo and then mounted in Ussing chambers had decreased transmural PD and tissue resistance and secreted sodium due to an increased s → m flux. The change in m → s sodium transport was not significant, even though glucose transport was reduced (Donowitz et al., 1975). C. perfringens enterotoxin also caused secretion of sodium ions in the perfused rat ileum due to an increase in s → m flux (McDonel and Asano, 1975); there was no change in the m → s flux, even though the toxin had previously been shown to inhibit glucose uptake (McDonel, 1974). As both of these toxins cause histological changes and perturb ion transport in a manner similar to that of toxin A, it appears that structural changes to the intestinal tissue may be responsible for the secretion induced by these toxins.

Tissue exposed to toxin A in the Ussing chamber was less responsive to theophylline than control tissues; the response was reduced by approximately 80% by treatment with purified toxin A for 2 h. Theophylline normally causes a rise in transmural PD by raising the level of cAMP in the tissue. There are several possible reasons for the lack of theophylline response in toxin A-treated tissue. First, cAMP levels could already be raised in the tissue. This is the reason proposed for the lack of response of tissue which has been exposed to cholera toxin (Field et al., 1972). Second, adenylate cyclase could be inactivated by the toxin with the consequent inability of the tissue to synthesise cAMP (Vesely et al., 1981). Third, tissue damage may have decreased tissue viability and prevented the physiological response to theophylline. However, Shiga toxin caused marked histological damage but the tissue remained responsive to theophylline (Donowitz et al., 1975). The reason for the lack of theophylline response in tissue treated with toxin A is unclear and requires further study.

C. difficile does not appear to produce or induce a true secretagogue as judged by the electrical and flux data presented here. This contrasts with the data of Hughes et al. (1983) who claimed that crude culture filtrates of C. difficile induced changes in ion transport across stripped rabbit ileum in the absence of histological damage.

The interaction of toxin A with ileum thus seems to have several stages. First, the toxin disrupts the epithelial cell layer, leading to a transient secretion of electrolytes which we think depends on the existence of paracellular electrochemical gradients; this could give rise to watery diarrhoea. Ion secretion would stop when these gradients were dissipated and hence watery fluid secretion would be transient and rapidly swamped by the subsequent bloody fluid secretion characteristic of toxin A-treated ileal loops (Mitchell et al., 1986).
This work raises at least two further questions. First, how does toxin A affect enterocytes, because their perturbation or removal seems to be an essential prerequisite for fluid secretion? Attempts to elucidate this point are described by Mitchell et al. (1987a). Second, since no mechanism has yet been found that would explain a sustained (as opposed to a transient) watery diarrhoea induced by toxin A in distal rabbit ileum, should more work be done to explain the watery fluid secretion in toxin-treated colon previously described by Mitchell et al. (1986)? Such an attempt has been made by Mitchell et al. (1987b).

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REFERENCES


