The effects of *Clostridium difficile* toxins A and B on membrane integrity and protein synthesis in intestinal cells *in vivo* and *in vitro* and in McCoy cells *in vitro*

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Summary. *Clostridium difficile* toxins A and B inhibited protein synthesis in McCoy tissue-culture cells but not in intestinal cells *in vitro* or *in vivo*. Toxins A and B had no effect on membrane permeability of either intestinal cells or McCoy cells.

Introduction

In this paper, we describe attempts to identify the biochemical basis of the action of *Clostridium difficile* toxin A on intestinal epithelial cells without which subsequent fluid secretion does not occur (Mitchell *et al.*, 1986). Crude culture filtrates of *C. difficile* have been shown to cause cytopathic effects (CPE) in tissue-culture cells. Associated with CPE were decreased uptake and incorporation of macromolecular precursors (Rothman and Brown, 1981), and decreased mitosis and decreased cloning efficiency in HeLa cells (Gurwith *et al.*, 1982).

Partially purified toxin B has been reported to cause an elevation of guanylate cyclase and a decrease in adenylate cyclase activity in hamster gut homogenates (Vesely *et al.*, 1981). It has also been shown to induce CPE associated with a disruption of the microfilament bundles in HeLa cells (Thelestam and Bronnegard, 1980). This was confirmed by Wedel *et al.* (1983) in studies with smooth muscle cells and fibroblasts. All other organelles within cells intoxicated with toxin B appeared normal (Wedel *et al.*, 1983).

Purified toxins A and B increased guanylate cyclase activity in hamster intestinal tissue (Veseley *et al.*, 1983), but the effects of the toxins on membrane permeability are controversial. Rothman *et al.* (1984) reported that toxin A caused leakage of potassium ions from, and inhibition of protein synthesis in, HeLa cells within 1 h of exposure and that toxin B produced similar effects on HeLa cells. On this basis, these workers proposed

that the primary event caused by both toxins is a change in membrane permeability leading to a loss of potassium ions and thus a shutdown of protein synthesis. In contrast, Gurwith *et al.* (1982) used ⁵¹Cr release as a marker and found no evidence of membrane damage by crude toxins in HeLa cells after exposure for 24 h. Thelestam and Bronnegard (1980) found no leakage of ³H-uridine from HeLa cells after exposure for 24 h to partially purified toxin B, even though CPE appeared at 3 h, and concluded that the toxin does not cause membrane damage.

The aims of the present study were to define the biochemical effects of these toxins on McCoy tissue-culture cells and to determine whether such effects could be reproduced in relevant cells, *i.e.*, enterocytes, *in vivo* and *in vitro*. In particular, changes in membrane permeability or protein synthesis, or both, induced by these toxins were sought, with a view to gaining some insight into the role of toxin A in epithelial cell detachment.

Materials and methods

Toxin production and purification

Toxin A was produced and purified as previously described (Mitchell *et al.*, 1987a).

Toxin B fractions from the flat-bed electrophoresis system used in the isolation of toxin A were identified by cytotoxin assay, pooled and subjected to the same ion exchange procedure as described for toxin A (Mitchell *et al.*, 1987a) to remove residual toxin A. Fractions containing toxin B eluted at a higher salt concentration than toxin A, and these were pooled. The resulting preparation contained several polypeptides as judged by silver stained polyacrylamide gels and is therefore referred to as partially purified toxin B; it was free from

Received 12 May 1986; accepted 13 Jun. 1986.

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toxin A as determined by ELISA (limit of detection, toxin A 5 ng/ml; Redmond *et al.*, 1985).

Cytotoxin assay

This was performed with McCoy cells as described by Chang *et al.* (1979). One cytotoxic unit was defined as the dilution (n) that caused CPE in 50% of the cells of a monolayer. Such a solution would be said to contain n units/ml.

Effect of crude toxins on protein synthesis in vivo

A rabbit (New Zealand White; c. 2–2.5 kg; Regal Rabbits, Surrey) was anaesthetised with Saffan (18 mg/kg; Glaxovet, Willington Medical, Shrewsbury, England) administered via a needle in the ear vein. Ileal loops were constructed as previously described (Mitchell *et al.*, 1986) inoculated at intervals with concentrated culture filtrate containing 20 µg of toxin A and 10⁵ cytotoxic units of toxin B. One loop served as a negative control and received an inoculum of phosphate buffered saline (PBS) pH 7.0 (which contained, /L, NaCl 4.2 g, KH₂PO₄ 2.44 g, and Na₂PO₄ 8.09 g), at the same time as toxin was inoculated into the first loop.

In one experiment, radiolabelled amino acids were administered via the intestinal lumen. On addition of toxin to the final loop, 20 µCi of ¹⁴C-labelled whole protein hydrolysate (Amersham International; 56 µCi/mAtom) was added to each loop and incubation continued for a further 1 h. In a second experiment, radiolabelled amino acids in saline were administered intravenously (50 µCi/kg body weight) 1 h before the animal was killed. The two routes of administration of label were used because the localisation of the label within the tissue is affected by the route of administration (Alpers, 1972).

After the 1-h labelling period, animals were killed by sodium pentobarbital overdose and the loops removed. Intestinal cells were isolated from the loops by the method of Jarmund and Telle (1982). The loops were washed out with ice cold PBS, filled with isolation solution A (PBS containing bovine serum albumin 0.2% w/v; BSA) and incubated at 37°C in 250 ml of the same buffer on a rotary shaker at 170 rpm for 15 min. This solution was discarded and the loops filled with isolation solution B (PBS containing BSA 0.2%, 1.5 mM ethylenediamine tetraacetic acid and 0.5 mM dithiothreitol). The loops were incubated in 250 ml of PBS in a flask on the rotary shaker (170 rpm) for 30 min at 37°C. The cell suspension was removed from each loop and the cells pelleted by centrifugation (1000g, 10 min, room temperature). The cells were solubilised in 5 ml of Triton-X100 0.1% v/v (30 min, room temperature). Samples (0.1 ml) were taken for total protein determination and alkaline phosphatase assay. Protein was precipitated by adding 0.1 ml of trichloroacetic acid 100% w/v (TCA). Precipitates were collected by centrifugation (2000g, 20 min, room temperature). Supernatant fractions were removed and radioactivity measured (1 ml of supernate in 9 ml of aqueous

counting scintillant; ACSII; Amersham International) to determine uptake of amino acids into the acid-soluble pool. Precipitates were resuspended in 5 ml of 0.1M sodium hydroxide and counted in ACSII. All counts were expressed as counts/min (cpm)/mg cell protein. All liquid scintillation counting was done with a Beckman L 7500 liquid scintillation counter.

Effect of toxins on protein synthesis in intestinal cells in vitro

Intestinal cells were isolated from a 20-cm length of normal rabbit ileum by the method described above. Cells were isolated as clumps or sheets in which condition they remained viable as judged by trypan blue dye exclusion for up to 3 h. Many attempts in this laboratory to produce viable individual enterocytes failed. The cells were resuspended in 20 ml of amino acid-deficient medium—Hanks's Balanced Salts Solution (HBSS) + dialysed fetal bovine serum 25% v/v—supplemented with ¹⁴C-labelled whole protein hydrolysate 10 µCi/ml. The cell suspension was divided into 1-ml volumes and challenged with toxin (10 µg of toxin A with or without 10⁵ units of partially purified toxin B) or saline. Suspensions were maintained in a humid atmosphere of CO₂ 5% in air. After various periods of incubation, incorporation was stopped by cooling in an ice water slurry. Cells were pelleted by centrifugation (1000g, 10 min, room temperature) and washed three times in HBSS.

Cells were solubilised in 5 ml of Triton-X100 0.1% v/v (30 min at room temperature) and the amount of radiolabel incorporated was determined as described above.

Effect of toxins on protein synthesis in McCoy cells

Wells (each of area 28.2 cm²) of 4-well cell-culture plates were each seeded with 1 × 10⁶ cells. The cells were incubated overnight at 37°C in air with CO₂ 5% to establish confluent monolayers. The culture medium was aspirated and replaced with amino acid-deficient medium (HBSS + dialysed fetal calf serum 5%) supplemented with ¹⁴C-labelled whole protein hydrolysate 10 µCi/ml. Monolayers were then challenged with toxin or saline (negative control). After incubation for a range of different times, incorporation was stopped by cooling the test preparation on an ice-water slurry. The radioactive medium was aspirated and the monolayers washed three times in HBSS. Cells were solubilised and the amount of radioactivity incorporated was determined as described above.

Measurement of membrane damage

The ³H-uridine assay used was that of Thelestam and Möllby (1975). McCoy cell monolayers and intestinal cell suspensions were prepared as described above. Fresh medium containing [5,6-³H]-uridine 10 µCi/ml was added

to the cells which were then incubated for 2 h. This was followed by a further incubation for 2 h with unlabelled medium. The labelled cells were washed three times in HBSS and medium containing toxin or saline was added. Reactions were stopped after various times by cooling on an ice-water slurry. Culture medium was aspirated. The cells were washed three times in HBSS and solubilised with Triton X-100 0.1%. Radiolabel released into the medium and that associated with the cells was counted in ACSII scintillation cocktail. Cellular membrane damage as measured by ^3H -uridine leakage was calculated on the basis of recorded cpm from the equation:

$$\text{Percentage release} = \frac{\text{cpm released by toxin} - \text{cpm released by control}}{\text{Total cpm} - \text{cpm released by control}}$$

Alkaline phosphatase assay

Alkaline phosphatase levels were used in the study of protein synthesis *in vivo* to monitor the proportions of the intestinal-cell preparations that were derived from villus epithelium. Alkaline phosphatase is a relatively specific marker for villus cells; only low amounts are found in the crypts (Moog and Grey, 1967). The assay was performed on 0.1 ml of solubilised enterocytes in 0.9 ml of assay mixture (0.5M Tris-hydrochloride at pH 9.3, 0.3 mM ZnCl_2 , 10 mM MgCl_2 , 0.23 mM *p*-nitrophenyl phosphate). After incubation for 15 min at 37°C, 0.5 ml of 1M NaOH was added to stop the reaction and absorbance at 405 nm (1 cm light path) was measured. Alkaline phosphatase activity was determined by reference to standards and expressed as arbitrary units/mg of cell protein.

Total protein assay

This was performed by the method of Bradford (1976) with the kit supplied by Bio-Rad Laboratories.

Results

Effects of toxins on protein synthesis in intestinal tissue *in vivo*

The effect of crude concentrated culture filtrate on amino acid incorporation into intestinal cells was tested over 6 h *in vivo* (fig. 1). When amino acids were administered via the intestinal lumen there was little effect over the first 2 h; levels of incorporation were similar to control values. At times of incubation greater than 2 h the incorporation of amino acids in toxin-treated loops declined below the control level until at 6 h incorporation was inhibited by 70%. Toxin-induced tissue damage had occurred in these loops by 2 h (data not shown but similar to those previously reported by Mitchell

Fig. 1. Effect of concentrated crude culture filtrate on amino acid incorporation in intestinal cells *in vivo*. Ileal loops were incubated with crude toxins containing toxin A 10 μg and 10^5 units of toxin B for various periods. The amount of radiolabelled amino acids incorporated per mg cell protein into acid-insoluble material in 1 h was measured for each loop after toxin exposure and was expressed as a percentage amount of the label incorporated by control loops. Two routes of administration of labelled amino acids were used:

- Amino acids administered via the intestinal lumen;
- Amino acids administered intravenously.

et al., 1986). Associated with the decrease in protein synthesis and the appearance of histological damage was a decrease in the amount of cells (measured as total protein; fig. 2) isolated from the loops. This presumably reflected the loss of cells from the mucosa as a result of tissue damage.

When amino acids were administered via the vascular system there was an apparent increase in incorporation of amino acids per mg of cell protein (fig. 1).

Alkaline phosphatase levels were monitored to determine the ratio of villus to crypt cells in cell populations isolated from loops. Fig. 3 shows the levels of alkaline phosphatase expressed in terms of total cells isolated (measured as total cell protein). The data show that as the levels of protein isolated from loops fall, the proportion of cells containing alkaline phosphatase also falls; this indicates that cells lost due to tissue damage are derived mainly from the tips of the villi, a suggestion supported by histological examination (Mitchell *et al.*, 1986).

Fig. 2. Amounts of protein isolated from toxin-treated ileal loops. Data were derived from the two experiments described in fig. 1. The level of cellular protein isolated decreases with increasing time of incubation, indicating that toxin is inducing loss of cells from the mucosa.

Effect of toxins on protein synthesis in intestinal tissue *in vitro*

The *in-vivo* studies described above suggested that the observed changes in protein synthesis may be due to villus cell loss from the mucosa rather than inhibition of protein synthesis *per se* (see *Discussion*). To test this hypothesis, protein synthesis assays were performed on isolated intestinal cells *in vitro*. No inhibition of incorporation of

Fig. 3. Activity of alkaline phosphatase isolated from ileal loops exposed to toxin (units/mg of cell protein). Data were derived from the two experiments described in fig. 1. The proportion of cells isolated containing alkaline phosphatase decreased with increasing time of incubation with toxin. The data indicate that the proportion of villus tip cells in isolated cell populations decreased with time.

amino acids was observed in intestinal cells tested with crude toxins, purified toxin A or partially purified toxin B over a 3-h period (fig. 4). Three hours was the longest incubation period tested because the viability, as judged by trypan blue dye exclusion, of the isolated cells declined after this period.

Since the toxin preparations used had no effect on protein synthesis in isolated intestinal cells the preparations were tested for those activities reported in tissue culture cells.

Effect of toxins on protein synthesis in McCoy tissue culture cells

The effects of toxin preparations on incorporation of amino acids into acid-insoluble material in McCoy cells are shown in fig. 5b. After incubation for 1 h with 20 μ g of purified toxin A, a reduction in amino-acid incorporation occurred which preceded the observed onset of CPE at 3 h.

Toxin B (10^5 cytotoxic units) caused a greater and more rapid inhibition of protein synthesis than toxin A, again preceding the onset of CPE. With

Fig. 4. The effect of *C. difficile* toxins on incorporation of amino acids in isolated intestinal cells. Isolated cells were incubated with toxin or saline in the presence of radiolabelled amino acids for various times. The amount of radiolabel incorporated into acid-insoluble material was measured as an indication of rate of protein synthesis. ●—● Control; ○—○ crude toxins containing toxin A 10 μ g/ml and 10^5 units of toxin B; ■—■ purified toxin A 10 μ g/ml; □—□ 10^5 units partially purified toxin B.

Data are the means of two experiments in each case.

Fig. 5. The effect of *C. difficile* toxins on (a) uptake and (b) incorporation of amino acids in McCoy cells. Cells were incubated with toxin or saline in the presence of radiolabelled amino acids for various times. The amount of radiolabel incorporated into acid-insoluble material was measured as an indication of the rate of protein synthesis. Acid-soluble radioactive material associated with the cells was measured as an indication of the size of the intracellular amino acid pool. ●—● Control; ○—○ crude toxins containing toxin A 10 μ g/ml and 10⁵ units/ml of toxin B; ■—■ purified toxin A 10 μ g/ml; □—□ 10⁵ units of partially purified toxin B. Data are the means of two experiments.

crude concentrated culture filtrate containing toxin A 20 μ g and 10⁵ cytotoxic units of toxin B, the effects of protein synthesis were more marked. With crude material, CPE appeared at c. 45 min. All toxin preparations tested inhibited protein synthesis to varying degrees in McCoy cells.

The uptake of amino acids (measured as soluble radioactivity) was also reduced in toxin-treated cells (fig. 5a). Protein synthesis was significantly depressed before the reduction in amino acid uptake. Thus, inhibition of protein synthesis is not a result of lowered amino acid uptake.

Effect of toxins on membrane integrity of intestinal and McCoy cells

None of the toxin preparations tested (crude concentrated culture filtrate, purified toxin A and partially purified toxin B) had any effect on membrane integrity of either McCoy cells or isolated intestinal cells as judged by the leakage of labelled uridine. The rate of leakage of nucleotide from isolated intestinal cells was greater than from McCoy cells but toxin-treated cells showed similar rates of leakage to controls. The higher rate of leakage from intestinal cells may reflect some membrane damage induced by the isolation procedure. Experimental data were all negative and are not shown.

Discussion

Toxin A, toxin B or crude toxin preparations had no effect on membrane integrity of either intestinal cells or McCoy cells as judged by nucleotide leakage. This finding is in agreement with the studies of Gurwith *et al.* (1982) and Thelestam and Bronnegard (1980) but not those of Rothman *et al.* (1984).

An apparent decrease in the rate of protein synthesis in intestinal tissue exposed to crude toxins *in vivo* was observed when labelled amino acids were administered via the intestinal lumen. This decrease was shown to be due to loss of protein-synthesising cells from the mucosa rather than a loss of protein synthetic capacity on the basis of the following evidence. First, lower numbers of cells were isolated from toxin-treated loops. Second, the proportion of isolated cells containing alkaline phosphatase decreased in toxin-treated cell preparations indicating a lower number of villus tip cells in these preparations. Since villus tip cells incorporate the majority of luminal amino acids (Alpers, 1972), a loss of tip cells would cause an apparent decrease in incorporation rates. Third, when experiments were repeated with the amino acids given intravenously, crude toxins caused an apparent increase in the rate of amino acid incorporation. This would be expected because intravenous amino acids are incorporated mainly into crypt cells (Alpers, 1972) and cell populations isolated from toxin-treated loops contain a higher proportion of crypt cells. Finally, toxins A and B, on their own or together in crude preparations, had no effect on the rate of protein synthesis in isolated intestinal cells *in vitro*.

Rothman *et al.* (1984) stated that toxins A and B inhibited protein synthesis in HeLa cells. The results presented in this paper show that both toxins

inhibit protein synthesis in McCoy cells, but toxin A had a much higher specific activity than that reported in the Rothman study. The latter showed that 1 mg/ml was necessary to inhibit protein synthesis in HeLa cells whereas toxin A (20 µg/ml, used in this study) would inhibit protein synthesis in McCoy cells. The inhibition of protein synthesis in McCoy cells began before the appearance of CPE, suggesting that inhibition of protein synthesis could be a causative factor in the development of CPE. Again it is significant that toxin B inhibited protein synthesis in tissue culture cells but had no observable effect when administered into the intestinal lumen (Mitchell *et al.*, 1986).

The inhibition of protein synthesis in tissue culture cells by toxin A appears to be unrelated to the primary mechanism of epithelial cell removal from mucosal tissue. No membrane damage was induced by the toxin as judged by nucleotide leakage. It is just possible that toxin A causes leakage of material of lower mol. wt, e.g., potassium ions from cells, as reported by Rothman *et al.* (1984) with respect to HeLa cells. In our experiments only a nucleotide marker (mol. wt > 2000) was used in our assessment studies of membrane permeability.

However, if the toxin had caused a leakage of potassium ions from intestinal cells, the loss of these ions would presumably halt macromolecular synthesis. No such shut-down was detected.

In summary, toxins A and B had no demonstrable effect on membrane integrity in tissue culture cells or rabbit intestinal cells in our studies. The toxins inhibited protein synthesis in McCoy cells but not in intestinal cells; this illustrates the dangers of using tissue culture cells for the investigation of toxin action on gut epithelial cells. The toxins caused an apparent alteration in protein synthesis *in vivo*, but this was not a direct metabolic effect and was a consequence of toxin-induced loss of cells from the mucosa.

The mechanism for toxin A-mediated removal of epithelial cells has not yet been elucidated but the data of Mitchell *et al.* (1987b) indicate that the primary point of attack could be somewhere at the basement membrane-lamina propria interface.

JS, DWB and DCAC thank Pfizer Central Research and the Science and Engineering Research Council for a CASE studentship to TJM, and the Medical Research Council for a research studentship to JMK.

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