The effects of *Clostridium difficile* crude toxins and toxin A on ileal and colonic loops in immune and non-immune rabbits

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Summary. Rabbits were solidly immunised by parenteral injection of purified *Clostridium difficile* toxin A such that they resisted an intravenous challenge with a normally lethal dose of toxin A. Ileal and colonic loops constructed in non-immune and immune animals received challenge injections of crude culture filtrate or purified toxin A of *C. difficile*. Protection of ileum was manifest after sufficient initial mucosal damage resulted in release of high levels of antitoxin A into the loop lumen of immune animals. There was less fluid accumulation in ligated ileal loops of immune than of non-immune rabbits. Less protection was observed when loops were challenged with crude culture filtrate containing toxins A and B than when challenged with purified toxin A. In-vitro studies with Ussing chambers yielded no evidence for tissue-localised immunity as judged by electrical responses and histology of toxin-treated tissue from non-immune and immune animals. No differences were found in the degree of epithelial damage, or volume or composition of fluid accumulating in colonic loops of non-immune and immune rabbits challenged with toxin A or crude culture filtrate. However, in colonic loops of immune rabbits there was no overt tissue-localised haemorrhage, whereas in those of non-immune rabbits tissue-localised haemorrhage was marked. In contrast to our findings with ileal loops, fluid accumulating in colonic loops was watery and contained substantially less total protein and (in immune animals) antitoxin A.

Introduction

An important primary role has been assigned to toxin A in *Clostridium difficile*-associated ileocaecitis in hamsters and by extrapolation in *C. difficile*-associated disease in man, which includes antibiotic-associated diarrhoea (AAD) and pseudomembranous colitis (PMC) (Libby *et al.*, 1982; Lyerly *et al.*, 1985; Mitchell *et al.*, 1986; Borriello *et al.*, 1987). Toxin B is thought to have a secondary role (Lyerly *et al.*, 1985; Mitchell *et al.*, 1986). Assignment of an important role in the causation of disease to an antigenic toxin presents the possibility of protection by means of a toxoid vaccine. The aims of the present work were to answer questions arising from the work of Libby *et al.* (1982) on hamsters as to the anatomical site of

toxoid-induced immunity to *C. difficile* toxins A and B and to re-examine the question of whether antitoxin B would be necessary in addition to antitoxin A for protection in a rabbit model system.

Libby *et al.* (1982) investigated the protection of clindamycin-treated hamsters (i.e., a presumed *C. difficile* infection) by parenteral immunisation with toxoids of partially purified toxins A and B. Protection was only achieved by immunisation against both toxins. Neither caecal damage nor toxin-neutralising activity in caecal contents was evident in surviving hamsters.

Elucidation of the role of immunity in *C. difficile*-induced disease is particularly important in view of the common occurrence of relapses following treatment. Multiple relapses suggest that protective immunity has not developed in these patients.

There has been much work in protecting the small bowel against toxin-induced secretory diarrhoeas such as cholera (Levine *et al.*, 1983). Examples of immune protection of small intestine also exist in the veterinary field. Immunity can be

induced by parenteral injections of toxoids that protect sheep against several clostridial small bowel diseases (briefly summarised by Stephen and Pietrowski, 1986). Although the mechanisms of such immune protection are unknown, almost all of the diseases are associated with varying degrees of damage to parts of the small bowel though not the colon. In contrast, much less well controlled work has been done on infectious colitis. In this paper, we describe the responses of ileal and colonic loops constructed in non-immune and immune rabbits to crude culture filtrate (CCF) and toxin A. Additionally, ileal tissues from immune and non-immune animals were challenged in vitro in Ussing-chamber systems to determine whether parenterally administered toxoid induces a localised mucosal immunity.

From the detailed studies of our group (Mitchell et al., 1986, 1987a,b) on the biological mode of action of C. difficile toxins A and B in ligated rabbit ileal and colonic loops, it was predicted that parenterally-induced antitoxin A would protect the colon less well than the ileum; the reasons were as follows. Initial toxin-induced ileal tissue damage would release serum antitoxin A into the gut lumen resulting in neutralisation of toxin A. In contrast, in the colon, there is a failure to release significant amounts of vascular protein into the lumen following toxin A-mediated tissue damage, thus antitoxin A would not enter the colonic lumen. The results would have potentially important clinical implications and would influence evolving concepts on the mode of action of toxin A (Mitchell et al., 1987b).

Materials and methods

Animals

Male New Zealand white rabbits (2·5–3·0 kg; Regal Rabbits, Surrey) were used in all studies. Mice were BK:W outbred mice, 12–16 weeks of age (Bantin and Kingsmans, Hull).

Toxin production and purification

This was as described by Mitchell et al. (1987a).

Toxin assays

Toxins A and B were assayed as described by Mitchell et al. (1986).

Immunisation schedule

Rabbits were immunised (see table I) with pure toxin A which, when necessary, was neutralised by antitoxin A. Neutralisation with antitoxin A was required in the early injections because experience showed some rabbits to be very sensitive to small doses of toxin. For reasons unconnected with the work described in this paper, neutralisation with antitoxin was chosen rather than chemical toxoiding to render the initial antigenic preparation non-lethal. Neutralisation with antitoxin A was possible, because it had been found in earlier attempts to raise antitoxin A that some partially immune animals became moribund after intravenous injection of purified toxin A (Redmond et al., 1985). Such animals were bled out before death; the sera thus obtained were used in the work described in this paper to neutralise small doses of toxin.

Animals were sham-immunised according to the above schedule with toxin A replaced by 0·05M Tris-HCl buffer (Trisma base, Sigma, 6·06 g/L adjusted to pH 7·5 with HCl). Non-immune rabbits were bled (10 ml), but not challenged with a lethal dose of toxin A before use.

A total of eight rabbits was immunised and another eight (randomly selected from the same batch) were sham-immunised for non-immune controls.

Antitoxin A determination by neutralisation of lethal activity

This was tested by inhibition of toxin-A lethality in mice. Toxin A was tested in mice for lethality by intraperitoneal (ip) injections of a range of dilutions of toxin A. Each dilution of toxin A had been incubated at room temperature for 30 min with 0·2 ml of normal rabbit serum (NRS) before inoculation. In the presence of NRS, 110 ng of toxin A was lethal to all mice within 24 h. Neutralising activity was determined by incubating 0·2 ml of serum with 220 ng of toxin A (i.e., 2 LD100) before ip inoculation into mice. Each serum sample was tested undiluted and diluted ten-fold; one mouse was used per sample. Because this test was used mainly as a preliminary screen before challenging immune rabbits with a lethal dose of toxin A and because in all cases mice were passively protected against toxin A (see Results), no attempt was made to quantitate the protective potency of sera by using large numbers of animals.

Antitoxin A determination by ELISA

A direct sandwich ELISA to detect antitoxin A was developed from the ELISA for detecting toxin A (Redmond et al., 1985). A 0·1-ml volume of crude culture filtrate (CCF) containing toxin A 10 µg/ml in coating buffer (Na_2CO_3, 1·59 g/L; $NaHCO_3$, 2·93 g/L; pH 9·6) was dispensed into each of a series of wells in a microtitration plate and incubated at 37°C for 2 h. Without emptying the wells, 0·02 ml of coating buffer containing bovine serum albumin (BSA; Sigma) 0·5 g/L and normal goat serum (Nordic Immunological Laboratories Ltd, Maidenhead) 2% v/v was added to each well and the plate incubated for a further 30 min. The plate was washed with phosphate buffered saline (PBS)

Table I. Immunisation schedule for rabbits used in immunity experiments

Times of injection (weeks + days)	Composition of "antigen"			Adjuvant and ratio of antigen: adjuvant (v:v)		Route of injection
	Toxin A (μg)	mixed with	Antitoxin A* (μl)			
0	1·5		10	FCA	1:1	s.c.
1	3–5		10–15	FICA	1:1	s.c.
2	1·5		8	FICA	1:1	s.c.
5	1·0		10	—		i.v.
6	0·3		—	—		i.v.

Animals were rested for 20 weeks then given booster doses at stated times

26 weeks	1–4		5–10	—		i.v.
+2 days	1		5	—		i.v.
+4 days	1		5	—		i.v.
+6 days	1		5	—		i.v.

At 26 weeks + 8 days, all animals were bled (10 ml) from ear veins and sera were used in the mouse protection and ELISA assays.

At 26 weeks + 10 days, all immune animals were challenged (i.v.) with toxin A 0·85 μg/kg body weight. This dose renders non-immune rabbits symptomatic in 24 h and is lethal by 48 h.

* Antitoxin A: see Redmond *et al.* (1985).
FCA = Freund's complete adjuvant.
FICA = Freund's incomplete adjuvant.
s.c. = subcutaneous.
i.v. = intravenous.
— = not used.

containing Tween (PBST) (NaCl, 8·0 g/L; KH_2PO_4, 0·2 g/L; Na_2HPO_4, 2·8 g/L; KCl, 0·2 g/L; Tween 20, 0·05% v/v; *p*H 7·4) before 0·1 ml of each test sample, diluted in PBST, was added and the plate incubated for 2 h at 37°C. After four washes with PBST, 0·1 ml of goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Miles-Yeda, Slough), diluted 1 in 5000 in PBST, was applied to the plate and incubated at 37°C for 1 h. After four washes with PBST, 0·1 ml of substrate solution (citric phosphate buffer, *p*H 5·0, containing ortho-phenylene diamine 0·34 g/L and H_2O_2 0·04% v/v) was added to each well. After 10 min, the reaction was stopped with 0·05 ml of H_2SO_4 12·5% v/v. All incubations at 37°C were in humid conditions. Each plate had one well that contained no horseradish conjugate to provide the blank value, another that contained no coating antigen, and a third that contained no test sample; the latter two acted as specificity controls. One unit of antitoxin A immunoglobulin was arbitrarily defined as the amount that gave a mid-point value between a maximum measurable absorbance of 2 units (Titertek Multiskan; Flow Laboratories Ltd, Irvine, Scotland) and the threshold of positivity (defined as an absorbance value of three standard deviations above the blank).

Rabbit ileal and colonic loops

These were constructed as described by Mitchell *et al.* (1987*b*).

Ussing chambers

Ussing-chamber studies were performed as described by Mitchell *et al.* (1987*a*). Tissue was treated with CCF which was added to the mucosal bathing solution to a final concentration of toxin A 20 μg/ml.

Histology

See Mitchell *et al.* (1986, 1987*a*, *b*).

Total protein determination

Total protein concentrations of loop contents were determined with the BioRad protein assay kit (BioRad Laboratories Ltd, Watford.

Experimental protocol

Six immune and six non-immune rabbits were tested by the rabbit ileal loop test (RILT) before analysis of the tissue by the Ussing-chamber technique. Each of the 12 animals was challenged with CCF, or purified toxin A, or both, as described below but in every animal one loop received PBS and another Tris–HCl (*p*H 7·5) as negative controls; a third loop received cholera toxin (Sigma; 1·5 μg) as a positive control. The data for PBS, Tris–HCl and cholera toxin (figs. 1 and 2) were compiled from all of these tests.

Two immune animals were used to titrate CCF and toxin A respectively to establish realistic challenge doses for the main experiment. One non-immune animal was similarly used with toxin A. The remaining four immune and five non-immune animals were treated identically and only the data from these four immune and five non-immune toxin-treated animals were used to compile figs. 1 and 2. In each rabbit, three loops in addition to the controls were constructed and challenged with CCF at low (LCCF) or high (HCCF) concentrations (LCCF, toxin A 36 μg and toxin B 5×10^3 units; HCCF, toxin A 60 μg and units toxin B 8×10^3), or purified toxin A (37 μg). After 12 h, 30 cm of ileum adjacent to the ligated loops was removed and placed in oxygenated Ringer's solution, while the ligated ileal loops were sampled. Volume/length (V/L) ratios for distended loops were then calculated and loop contents saved for analyses of total protein content and antitoxin-A titre. Tissue samples were taken from each loop for histological examination. Ileal tissue which had been maintained in Ringer's solution was then stripped and mounted for analysis in the Ussing chamber. Statistical comparisons between the non-immune and immune ileal loop responses to CCF or purified toxin A were done with Student's *t* test.

The remaining two immune and two non-immune animals were investigated for their respective responses to CCF and toxin A in colonic-loop studies. In each animal, five loops were constructed and each received one of the following samples: two negative control loops received either PBS or Tris–HCl buffer; one loop received LCCF; one received purified toxin A at high concentration (HA; 75 μg) and one at low concentration (LA; 36 μg). After 12 h the V/L ratios of colonic loops were calculated. The loop contents were saved for later analyses of protein content and antitoxin A titre. Tissue samples were taken for histological examination.

Results

Immunisation

All animals immunised with toxin A had high levels of antitoxin-A activity (96 000–192 000 units/ml). In contrast, sham-immunised (non-immune) rabbits had very low levels (< 100–550 units/ml). All sera from immune animals protected whereas sera from non-immune animals did not protect mice against toxin A. All immune rabbits were challenged and survived a normally lethal dose of toxin A given intravenously.

Effect of CCF and toxin A on ileal mucosa in vivo

Fluid accumulation. None of the 12 negative control loops contained fluid (V/L ratio = 0) in immune and non-immune animals. Positive control loops (cholera toxin 1–2 μg/loop) gave positive V/L ratios in immune and non-immune animals. Loops treated with cholera toxin in non-immune animals

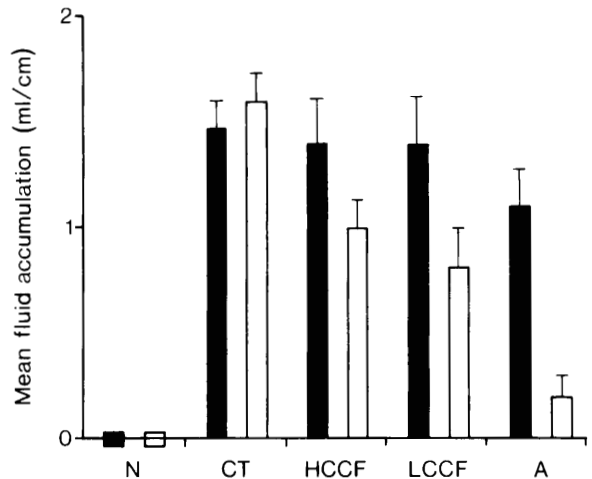**Fig. 1.** Mean fluid accumulation ($+$SEM) in non-immune ($\blacksquare$) and immune ($\square$) rabbit ileal loops treated with the following. N = Negative controls, PBS and Tris–HCl (n = 12); CT = Cholera toxin (non-immune n = 5, immune n = 6; p > 0·05); HCCF = CCF containing toxin A 60 μg/loop and 8×10^3 units toxin B (non-immune n = 5, immune n = 4; 0·05 > p > 0·01); LCCF = CCF containing toxin A 36 μg/loop and 5×10^3 units toxin B (non-immune n = 5, immune n = 4; p < 0·01); A = Toxin A (37 μg/loop) (non-immune n = 5, immune n = 4, p < 0·01).

showed a mean lower response when compared with loops in immune rabbits, but this difference was biased by one non-immune rabbit which (as sometimes happens) was relatively unresponsive to cholera toxin (V/L = 0·2). If this atypical result is discounted and n becomes 5, the difference between the responses of immune and non-immune rabbits to cholera toxin became statistically insignificant (p > 0·05; fig. 1).

HCCF gave an average V/L ratio of 1·4 in non-immune animals, whereas immune animals were less responsive, giving an average V/L ratio of 1; this difference was only just significant (0·05 > p > 0·01). In non-immune rabbits, loops treated with LCCF gave average V/L ratios similar to those of loops treated with HCCF. This suggested that maximum V/L ratios were being attained in these animals. In ileal loops treated with LCCF, fluid accumulation was significantly inhibited (p < 0·01) in immune rabbits compared to that found in non-immune rabbits. The difference in response to HCCF and LCCF in immune animals was not significantly different (p > 0·05). Loops treated with 36 μg of toxin A produced an average V/L ratio of 1·1 in non-immune animals, which was not significantly different from the response to LCCF in non-immune animals. However, the response to toxin A was significantly inhibited in immune animals, with an average V/L ratio of 0·2 (p < 0·01).

Thus, the inhibition of fluid accumulation in immune rabbits was just evident when they were challenged with HCCF, significantly different when challenged with LCCF, and highly significantly different when challenged with pure toxin A.

Total protein and antitoxin-A contents of ileal-loop fluids. Protein contents (mg/cm of loop length) of loops containing > 1 ml of fluid are shown in fig. 2. Cholera-positive control loops showed very low total protein contents in non-immune or immune animals (< 10 mg/cm), a finding in agreement with those of Mitchell *et al.* (1986).

Both HCCF- and LCCF-treated loops contained much protein (60–65 mg/cm) with no significant difference observed between the two groups. In addition, there was no significant difference between the amounts of protein in loops in non-immune and immune rabbits treated with LCCF or HCCF. In non-immune rabbits the amount of protein measured in loops treated with purified toxin A (36 μg) was not significantly different (p > 0·05) from that found in those loops treated with LCCF. However, the amount of protein in loops

treated with purified toxin A was significantly (p < 0·01) less in immune rabbits than in non-immune animals.

In fig. 2, n = 2 for immune ileal loops treated with toxin A instead of 4. The reason is that in two of the ileal loops the fluid response to toxin A was too small to obtain a sample for meaningful analysis without washing. The latter was not done, because of the risk of removing cells (and hence protein) from the mucosa.

Luminal contents were also analysed for antitoxin A (table II). LCCF, HCCF, toxin A and cholera toxin-treated loops from two non-immune rabbits contained low amounts of antitoxin A activity which was in the same range as that found in the serum of non-immune animals. LCCF, HCCF and toxin A-treated loops of immune rabbits contained high levels of antitoxin A; in the same animals, cholera toxin-treated loops contained 10–30 fold less antitoxin A activity. This pattern of antitoxin-A levels was similar to that found for total protein contents in immune animals (see fig. 2). Thus, antitoxin A almost certainly entered loops as a result of protein leakage from the vascular system.

The fluid removed from cholera toxin-treated loops of both immune and non-immune treated animals was virtually free of blood. The fluid removed from LCCF, HCCF and toxin-A treated loops of both animal groups was bloody.

Ileal loop histology. Ileal loops treated with PBS and Tris–HCl (*p*H 7·5) buffers mostly showed good structural integrity (not shown). Most cholera toxin-treated loops showed intact villus structure; a slight
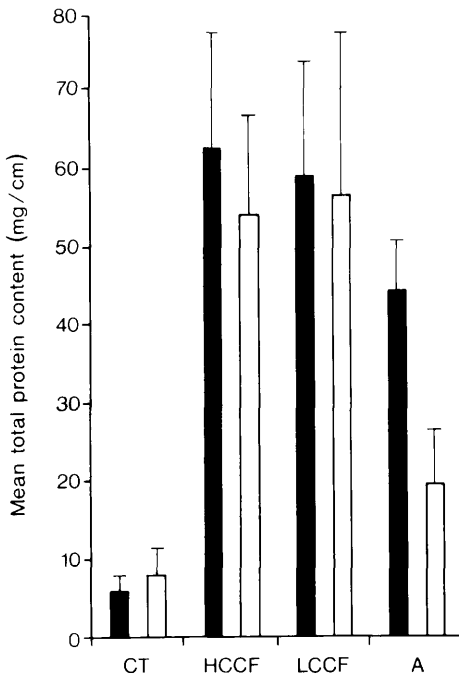

Fig. 2. Mean total protein content (+SEM) of the accumulated fluid from non-immune (■) and immune (□) rabbit ileal loops treated with the following: CT = Cholera toxin (non-immune n = 5, immune n = 6, p > 0·05); HCCF = CCF containing toxin A 60 μg/loop and 8×10^3 units toxin B (non-immune n = 5, immune n = 4, p > 0·05); LCCF = CCF containing toxin A 36 μg/loop and 5×10^3 units toxin B (non-immune n = 5, immune n = 4, p > 0·05); A = Toxin A (37 μg/loop) (non-immune n = 5, immune n = 2, p < 0·01).

Table II. Presence of antitoxin-A activity in luminal fluid from ligated ileal loops treated with cholera toxin, crude culture filtrates or toxin A in non-immune and immune rabbits

Immune status of rabbits	Serum antitoxin A (units/ml)	Luminal fluid antitoxin A (units/ml) after loop treatment*			
		CT	HCCF	LCCF	A
Non-immune					
A	100	<100†	<100	100	500
B	100	<100	400	300	300
Immune					
C	192000	5500	153600	115200	57600
D	176000	4200	41600	67200	35200

* Ligated ileal loops were treated with: cholera toxin (CT), two concentrations of CCF—HCCF (toxin A 60 μg/loop; 8×10^3 units toxin B) and LCCF (toxin A 36 μg/loop; 5×10^3 units toxin B)—and toxin A (A, 37 μg/loop).
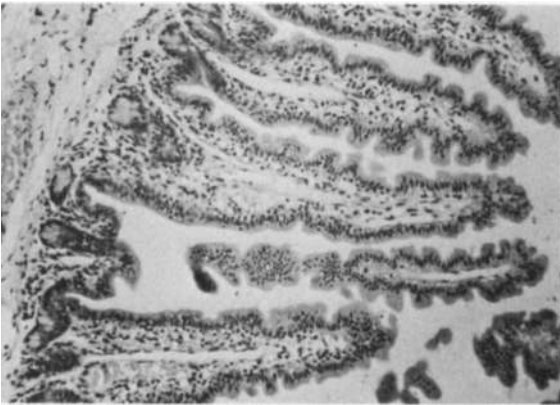† Below level of detection of 100 units/ml.

Fig. 3. Rabbit ileum treated with cholera toxin. Note feathered appearance of villi. (Haematoxylin and eosin, HE; original magnification × 277).

Fig. 4. Immune rabbit ileum treated with HCCF. Haemorrhage (H) is mainly localised to the lamina propria; sloughed off epithelial cells, polymorphonuclear cells and some red blood cells overlie damaged villi. Crypt regions are still intact. In general, many villi show a layer of flattened cells overlying damaged villi. The degree of damage is significantly reduced compared with none-immune ileum. (HE × 144).

"feathering" appearance was often seen with some villi (fig. 3); some showed macroscopic focal subepithelial haemorrhage thought to be caused by pressure-induced damage. Fluid from cholera toxin-treated loops contained a small amount of protein (< 10 mg/cm) which correlates with the histological picture of sub-epithelial damage. No histological differences were found between loops from non-immune or immune animals treated with cholera toxin.

Non-immune rabbit ileal loops treated with HCCF responded as already described by Mitchell *et al.* (1986, 1987*b*). There was extensive necrosis of the whole mucosa, with only a few crypt remnants remaining. Severe haemorrhage was present throughout the mucosa and also in large areas of the sub-mucosa, including the muscularis propria. Polymorphonuclear leukocytes (PMN) and cellular debris were present in the necrotic mucosa. In immune rabbits, ileal loops treated with HCCF (fig. 4) showed markedly less damage. Tissue necrosis predominantly involved the upper half of villi with detached villus cores often overlying the mucosa (not shown). Sloughed epithelial cells and PMN cells, but few erythrocytes, occurred on the mucosa. PMN were also present in the lamina propria and sub-mucosa. The degree of haemorrhage in immune tissue (fig. 4) was significantly less marked than in non-immune tissue. Many villi showed a layer of flattened cells overlying damaged villi (fig. 4). This was thought to reflect villus resealing (i.e., repair of the integrity of the epithelial layer) and was not evident in similarly treated loops in non-immune animals.

In non-immune rabbits, ileal loops treated with LCCF showed similar but less severe amounts of structural damage compared with the effects of
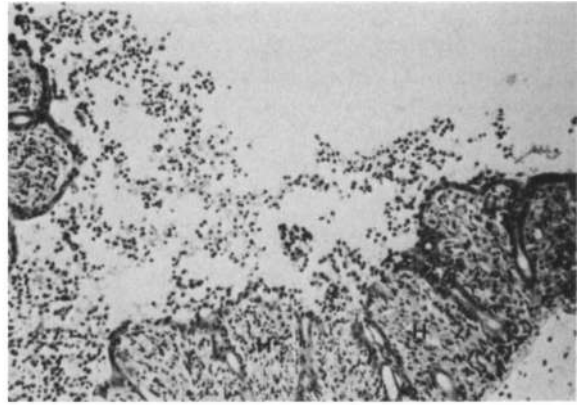
HCCF. Though villi were damaged, many crypts remained apparently intact (fig. 5a). The lamina propria was haemorrhagic and focal areas of haemorrhage were present in the sub-mucosa. Sloughed epithelial cells, PMN and erythrocytes were present overlying the tissue. PMN were also present throughout the lamina propria and sub-mucosa. In immune rabbits, ileal loops treated with LCCF were less damaged (fig. 5b) than similarly
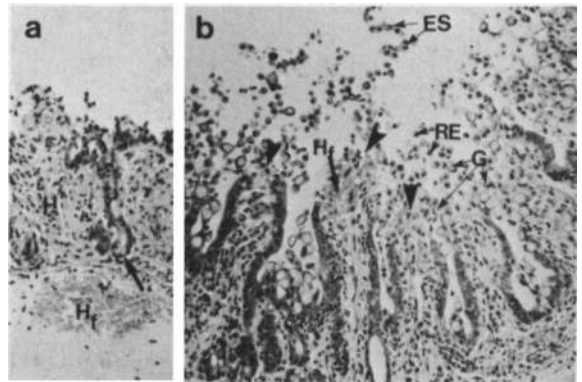

Fig. 5. (a) Non-immune rabbit ileum treated with LCCF. Note villi are shortened with a disrupted epithelial layer and are uniformly haemorrhagic (H); focal haemorrhage (H$_f$) was also observed in the submucosa. Some crypts (arrow) were intact. The short section shown was typical of large stretches of tissue (see Mitchell *et al.*, 1986). (HE × 144). (b) Immune rabbit ileum treated with LCCF. Note, in general, less severe damage to mucosa. Focal haemorrhage (H$_f$) was observed in villus tips but not in submucosa. Villus cores remained reasonably intact (arrow heads) with epithelial cells removed as if peeled off from apical regions. Many detached cells remained seemingly intact in the lumen and can be identified as rounded enterocytes (RE), goblet cells (G), or epithelial sheets (ES). (HE × 144).

treated loops in non-immune animals (fig. 5a). Villus tips showed loss of epithelial cells with many villus cores remaining attached and reasonably intact; many damaged villi had apparently resealed (not shown, but see fig. 6). Villus tips were surrounded with detached epithelial cells with few PMN and erythrocytes. Only slight focal haemorrhages were present in the lamina propria.

In non-immune rabbits, ileal loops treated with toxin A showed severe damage (not shown but see fig. 5a). The top halves of villi were damaged, but crypts remained intact. Haemorrhage was noted throughout the mucosa with focal haemorrhage also present in the sub-mucosa. Sloughed epithelial cells, PMN but few erythrocytes were seen overlying the necrotic mucosa. PMN were observed throughout the mucosa and sub-mucosa. In immune rabbits, ileal loops treated with toxin A (fig. 6) generally showed damage only to villus tips. Villus cores remained attached and apparently intact in a large number of villi that showed only loss of the epithelial cell layer. Large numbers of sloughed epithelial cells were present with few PMN and almost no erythrocytes. Small foci of haemorrhage were observed. Some rabbits showed areas with little or no mucosal necrosis. In immune animals villus resealing was much more pronounced in ileum treated with toxin A than in ileum treated with LCCF.

The effect of CCF and toxin A on colonic mucosa of immune and non-immune rabbits in vivo

Animals immunised with toxin A or sham-immunised with Tris–HCl (*p*H 7·5) were used to compare the response of colon with that of ileum
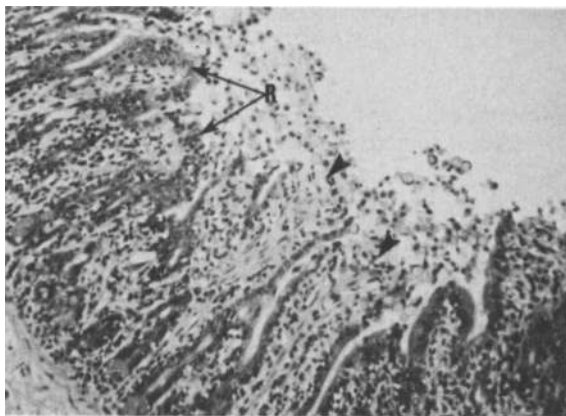

Fig. 6. Immune rabbit ileum treated with toxin A. Tissue damage was almost entirely confined to villus-tip regions and consisted mainly of removal of apical epithelial cells. Villus cores remained largely intact (arrow heads) with clear evidence of resealing (R) of naked cores. (HE × 144).

challenged with toxin A or CCF. Two animals from each group were used and hence a Student's *t* test was not done.

Fluid accumulation. V/L ratios of ligated colonic loops treated with PBS and Tris–HCl buffers, CCF (toxin A: 36 μg; toxin B 5 × 10³ units) or toxin A at two concentrations (HA, 70 μg; LA, 36 μg) are shown in table III. Colonic loops of non-immune animals treated with PBS and Tris–HCl buffers were consistently negative. However, the PBS control loops in both immune animals were found to contain fluid (V/L ratios of 1 and 0·7), whereas the Tris–HCl (*p*H 7·5) negative control loops did not contain fluid in either animal. The presence of fluid in these PBS-treated loops was almost certainly due to the technical procedure used to construct colonic loops in rabbits. In these particular animals, PBS-treated loops were those constructed nearest to the caecum and were therefore in the area most handled during the movement of colonic contents into the caecum before loop construction. It is probable that fluid secretion resulted from excessive handling of the colon.

In non-immune animals, colonic loops treated with LCCF produced an average V/L of 2·1 ml/cm which was similar to the average level of fluid accumulation in colonic loops of immune animals (1·9 ml/cm). There was insufficient toxin A available to treat all colonic loops with HA and LA; therefore HA was not used in one non-immune animal. The average amount of fluid accumulation in immune animal colonic loops treated with HA was 3·3 ml/cm, almost identical to the amount of fluid found in the non-immune animal loop (3·1 ml/cm). There was only a slight difference between the amount of loop fluid produced in immune (1·7 ml/cm) and non-immune (2·0 ml/cm) animals treated with the lower concentration of toxin A (LA).

Total protein and antitoxin A contents in colonic loop fluid. Total protein contents of colonic loop fluids are summarised in table III. Two negative control loops contained some fluid, but only very low levels of protein (an average of 1·3 mg/cm). Total protein contents of luminal fluid, from colonic loops treated with LCCF in non-immune and immune animals, were found to be similar to each other and to the level of protein found in cholera toxin-treated ileal loops, but very different from ileal loops treated with *C. difficile* toxins. This confirms the lower protein levels of colonic loop fluid observed by Mitchell *et al.* (1986). The level of protein found in toxin A-treated loops at both concentrations was very similar in both groups of animals and also similar to that found with CCF.

Table III. Colonic loop fluid accumulation, fluid total protein content and fluid antitoxin-A activity in immune and non-immune animals

Observa-tion	Immune status of rabbits	Values obtained* after treatment of colonic loop with			
		Buffers	LCCF	HA	LA
Fluid accumula-tion (ml/cm)	Non-immune	0	2·1 (0·1)	3·1	2·0 (0·3)
	Immune	0·4 (0·3)	1·9 (0·9)	3·3 (0·5)	1·7 (0·2)
Protein content (mg/ml)	Non-immune	ND†	9·3 (1·2)	8·6	5·8
	Immune	1·3 (0·7)	13·3 (7·2)	7·5 (2·3)	5·9 (0·3)
Loop anti-toxin A (units/ml)	Non-immune	<100‡	<100	<100	<100
	Immune	ND	4300 (1111)	1050 (51)	1550 (550)

* Fluid accumulation, protein content and antitoxin A titre were all measured for the same group of 2 non-immune and 2 immune animals in colonic loops treated with: negative control buffers (PBS and Tris–HCl), LCCF (toxin A 36 μg/loop; 5×10^3 units toxin B) and two concentrations of toxin A (HA, 70 μg/loop; LA, 37 μg/loop). The data are the means and SEM (in parentheses) of two observations except for negative controls (n = 4) and HA-treated non-immune animal loops (n = 1).
† Not done.
‡ Below level of detection of 100 units/ml.

Detectable antitoxin A was not found in non-immune rabbit colonic loops (i.e., <100 units/ml; table III). Small amounts of antitoxin A were observed in colonic loops of immune animals. Those loops treated with LCCF showed higher concentrations of antitoxin A (4300 units/ml) than those treated with toxin A (1050–1550 units/ml). However, the concentration of antitoxin A found in *C. difficile* toxin-treated colonic loops of immune animals, was very much lower than that found in ileal loops (table II), but similar to that found in cholera toxin-treated ileal loops of immune animals. Thus, in immune animals, the parallel pattern of antitoxin A and total protein strongly implicates the vascular system as the source of the antitoxin.

Colonic loop histology. Ligated colonic loops from non-immune and immune animals treated with either PBS or Tris–HCl buffers showed normal structure with no damage.

The damage produced by CCF or toxin A in colonic loops from non-immune animals was markedly less severe than that seen in similarly treated ileum. Colonic loops from immune and non-immune animals challenged with LCCF showed equivalent degrees of tissue damage. This involved approximately half the depth of the mucosa with varying degrees of haemorrhage which always remained tissue-localised; crypts remained structurally intact.

Loops treated with HA showed approximately the same amount of epithelial damage in tissue derived from immune and non-immune animals (figs. 7a, b). However, haemorrhage in the lamina propria was present in tissue from non-immune animals, but not evident in tissue from immune animals. Similar results were obtained in colonic loops treated with LA.

The effect of CCF on ileal mucosa of immune and non-immune rabbits in vitro

Electrical responses. Open circuit potential difference (PD), short circuit current (SCC), theophylline response, tissue resistance and unidirectional Na and Cl ion fluxes of untreated and toxin-treated ileal tissue from both non-immune and immune rabbits were measured (PD, theophylline response, SCC and ion fluxes) and calculated (tissue resistance). There was no obvious difference between the CCF-induced changes in ileal tissue from immune and non-immune animals *in vitro*. Because these data were almost identical to those reported by Mitchell *et al.* (1987*a*) they are not reproduced here.

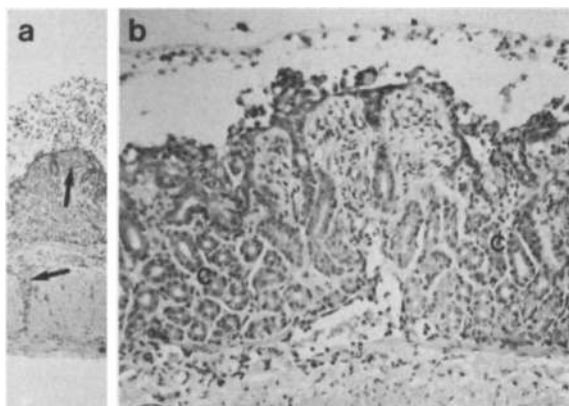*Histology.* Untreated ileal tissue from both

Fig. 7. (a) Non-immune rabbit colon treated with toxin A. Note tissue-localised haemorrhage (arrows) in mucosa and submucosa. Crypt regions remain intact. Sloughed epithelial cells and PMN overlie damaged colonic mucosa. (HE × 63). **(b)** Immune rabbit colon treated with toxin A. Note tissue damage almost completely confined to the patchy removal of epithelial cells. No tissue haemorrhage was evident; no red blood cells or PMN observed in lumen. Crypt regions (C) remained intact. (HE × 250).

immune and non-immune animals remained structurally intact after maintenance for 120 min in Ussing chambers (fig. 8a). CCF treatment of ileal tissue in Ussing chambers resulted in tissue damage to ileum, derived from both non-immune and immune animals. Tissue damage was characterised by extensive villus "feathering" and damage to villus tips (fig. 8b). There was no significant difference between the responses of ileum from non-immune and immune rabbits to CCF *in vitro*.

Discussion

Seven important points emerge from this study and the related work of Mitchell *et al.* (1986, 1987*a*, *b*), the significance of which may be extrapolated to *C. difficile* infections in man: 1. *C. difficile* toxin A induces fluid secretion in rabbit ileal and colonic loops; a common feature is the removal of epithelial cells. 2. The histological picture of toxin A-treated ileum and colon is different; secondary tissue damage is much more extensive in the ileum than in the colon. 3. The rate of penetration of toxin A to sub-mucosal ileal tissues is much greater in ileum than in colon. 4. The basement membrane-lamina propria interface is breached in ileum, but not in colon; this gives rise to an ultrafiltered exudate into the colonic lumen. 5. The volumes of fluid induced by toxin A in ileum and colon are similar. 6. Although their ionic compositions are similar, ileal fluid is haemorrhagic and contains much more protein than corresponding colonic

fluid. 7. In immune animals, fluid secretion is diminished in ileum, but not in colon. This last point can be seen as the consequence of the previous points and fulfils the major prediction tested in this paper. Possible implications of this result in man are discussed below, particularly in relation to circulating antitoxin immunity and PMC.

At first sight, the amounts (36–70 μg) of toxin A used in individual loops in these experiments seem inordinately high in relation to the quantities required (<10 μg) to initiate extensive tissue damage and fluid responses (Mitchell *et al.*, 1986). This work was executed in parallel with that described by Mitchell *et al.* (1987*b*) but before the full significance of that work was appreciated. It was logistically impracticable to titrate toxin A in the small number of immune animals available, hence two immune animals and one non-immune animal were challenged with arbitrarily selected high levels of toxin A. The hope was that the
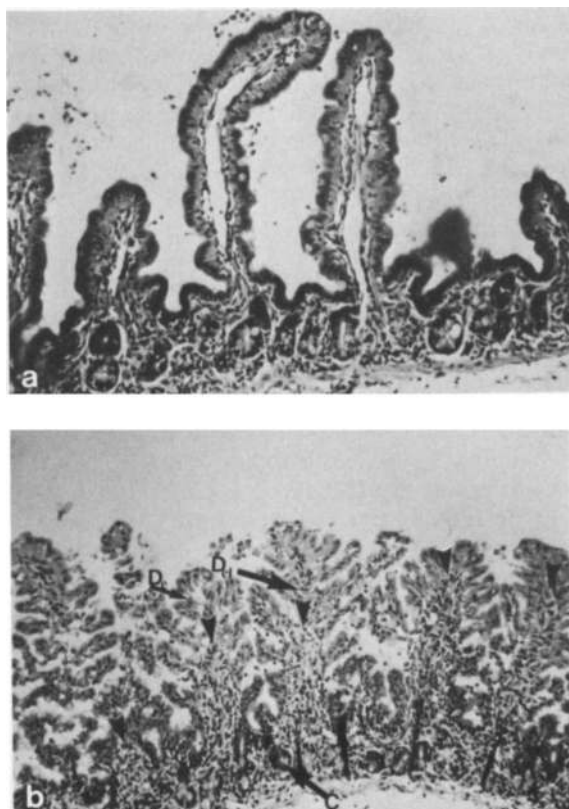

Fig. 8 a and **b**. Control (**a**), and toxin-treated immune (**b**), stripped rabbit ileum after 120 min in Ussing chamber. Note the feathered appearance of villi associated with detaching invaginated epithelium from villus cores (apices marked by arrow heads); detachment of enterocytes (D_e) or enterocytes and lamina propria cells (D_{el}); crypt regions (C) remain intact. (HE × 144).

immune ones would not die; the fear was that non-immune animals would die. However, all animals survived for the 12-h duration of the experiment and independently confirmed the data of Mitchell *et al.* (1987*b*) on the effects of administering a large bolus of toxin to a comparatively short length of ligated gut.

It is clear that parenterally-induced circulating antitoxin A will significantly protect the ileum from structural damage and fluid secretion induced by toxin A. Moreover, protection occurred only after incipient damage sufficient to cause controlled haemorrhage releasing antitoxin A into the lumen of the gut. No protection of colonic loops was achieved as judged by fluid secretion or loss of colonocytes. However, there was no overt haemorrhage in immune colonic tissue.

There was no evidence from either histology or the Ussing-chamber experiments to suggest the induction of a tissue-localised protective response in these parenterally-immunised animals. In fact, the histology of toxin A-treated immune ileum (fig. 6) is highly instructive and confirms aspects of the mode of action proposed for toxin A by Mitchell *et al.* (1986, 1987*b*). Toxin A causes removal of epithelial cells and subsequently facilitates access of more toxin A to the vascular endothelium; the mechanism is unclear. The action on the vascular endothelium results in the release of blood which in ileum reaches the lumen but in colon is largely retained in the tissue. When the ileum of immune rabbits is treated with toxin A, the process stops at stripping epithelial cells with retention of denuded cores of lamina propria and production of only minute foci of haemorrhage (fig. 6) presumed sufficient to leak neutralising doses of antitoxin A into the area, hence stopping the progression towards more extensive tissue necrosis. In colon of immune rabbits, epithelial cells are removed but overt haemorrhage into the tissue does not occur. We presume that sufficient alteration in capillary permeability occurs to release plasma that provides both the source of neutralising antibody for toxin in the lamina propria and the "pool" of fluid which is ultrafiltered into the lumen as described by Mitchell *et al.* (1987*b*).

What is the possible significance of these findings in relation to human PMC? There are two possible scenarios between which the data in this paper cannot discriminate. First, in an unobstructed colon, the fluid secreted would be released and could result in the flushing away of organisms and toxins; toxin neutralisation in the lamina propria and vascular repair would also occur, which would explain the absence of gross haemorrhage in immune colon. Thus, protective immunity would be evident following an initial limited diarrhoea. Alternatively, the removal (by the initially secreted fluid) of organisms and toxin may not be complete. Tissue repair could still take place, but a fresh cycle of toxin binding and epithelial and vascular damage would occur, leading to further fluid release. If the latter situation occurred, serum antitoxin alone would not prevent initial disease or subsequent relapses.

It has been formally proven that the origin of the fluid in ileal loops is vascular and it has been argued that this is the case with colonic loops (Mitchell *et al.*, 1986, 1987*b*). However, the results in this paper allow an alternative explanation for colonic secretion. It is possible that, in the colon, toxin A produces fluid accumulation by somehow stimulating active transport from the crypts. In unprotected animals, there is significant haemorrhage induced in colon which remains tissue-localised (Mitchell *et al.*, 1987*b*). Colonic haemorrhage could be a "secondary" phenomenon which, if prevented (in this case by active immunization), does not result in inhibition of secretion, but rather unmasks the real site of origin of the secretion. In ileum, because of the breach of the basement membrane-lamina propria interface, haemorrhage becomes the dominating lesion and the source of proteinaceous bloody fluid. Even if this alternative mechanism were correct, circulating antitoxin predictably would still not protect the colon against *C. difficile*-induced diarrhoea. A fuller discussion of the basis and wider significance of these phenomena is provided by Stephen and Pietrowski (1986).

In a study of patients convalescing from *C. difficile*-associated disease, Aronsson *et al.* (1985) found that 13 of 61 patients had serum antitoxin A (levels not measured) and 28 of 61 patients had serum antitoxin B (levels determined by ELISA). They claimed a correlation between late seroconversion to antitoxin B with lower levels of antitoxin B and relapse in patients after treatment. These authors imply that non-relapse correlates with higher levels of antitoxin immunity and that relapse correlates with low levels of antitoxin immunity. However, two constructive criticisms of the Swedish group's work can be made. First, no quantitative statements were made regarding antitoxin A levels in any (including relapsing) patients. This is clearly an important consideration because antitoxin-A levels are important in relation to toxin A-initiated disease. Second, no statements were made regarding the patients' mucosal immune status—something that might be considered important in patients recovering from an actual infection. For

this reason, it would be difficult to assess the significance of any conclusions related only to levels of circulating antitoxin in animals or human subjects experiencing intestinal antigenic stimulation.

A speculative synthesis might explain the age-related pattern and severity of disease caused by *C. difficile*. Infants comprise one of the biggest groups colonised with *C. difficile*, some strains of which are toxigenic (see Borriello *et al.*, 1987). However, the majority remain asymptomatic. This could be explained in terms of their intestinal mucosae being inherently less susceptible (for whatever reason) to toxin A, and the turnover rate of mucosal epithelia in relation to the rate of production of toxin A *in vivo*; this latter concept is developed more fully by Borriello *et al.* (1987). During such infections, both circulating antitoxic and mucosal immunity could develop which, because of the ubiquitous nature of the organism, could be stimulated during later life. Should mucosal immunity become impaired or wane in more elderly people, exposure to orally administered antibiotics could result in *C. difficile* infection with toxin A-mediated diarrhoeal sequelae in the colon, which would not be prevented by antitoxin A even though the degree of sub-mucosal damage might be diminished. Those patients who relapse may well do so through failure to develop or boost a protective mucosal immunity which would be necessary to prevent subsequent recolonisation with *C. difficile*.

The studies outlined in this paper help to explain the results of Libby *et al.* (1982) who investigated the protection of clindamycin-treated hamsters with parenteral immunisation with toxoids of partially-purified toxins A and B. Protection could be achieved only by parenteral immunisation with both toxoids which, at first sight, seems strange because toxin B on its own is now recognised as having no enterotoxic activity (Mitchell *et al.*, 1986). Surviving hamsters were found to have both toxins present in their caecal contents, but no caecal tissue damage was evident at least 4 days after post-clindamycin challenge. Most unimmunised hamsters died on days 2 and 3 after clindamycin treatment. The lack of caecal damage could be due to several factors. First, both toxins could have been neutralised in the lamina propria before the occurrence of significant tissue damage. We think that this is unlikely because fluid secretion is always preceded by tissue damage which we have shown to be necessary (in the rabbit model) for release of antitoxin A. Second, IgA-mediated immunity could have been elicited during infection. This seems unlikely because no toxin-neutralising activity was found in the caecal contents. Third, repair of the caecal epithelium within 24 h may have occurred. Resealing of villus tips was noted in toxin-treated immune rabbit ileal loops in this paper (fig. 4). To us, this seems the most likely explanation. It appears that hamster caecum responds to *C. difficile* toxin-mediated damage (see Borriello *et al.*, 1987) in a manner analogous to that described in this paper for rabbit ileum rather than colon. Thus, one could explain how serum antitoxin would protect hamsters from *C. difficile*-induced ileocaecitis. The fact that immunisation with both toxoids is necessary is supported by the evidence presented here. When ileum was challenged with CCF (which contained both toxins A and B) the damage induced, the volume of fluid excreted, and the total protein concentration in loops were always greater than when toxin A only was used. This is because toxin B can have an effect after primary damage induced by toxin A (Lyerly *et al.*, 1985) and hence would contribute to tissue damage and death even in animals immunised against toxin A. Because protection against toxin A occurred only in ilea which had undergone initial damage sufficient to release antitoxin A, toxin B could thus gain access to submucosal target sites.

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