BACTERIAL PATHOGENESIS

***Clostridium difficile* toxin A binding to human intestinal epithelial cells**

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Clostridium difficile radiolabelled toxin A ($[^3\text{H}]$ -toxin A) bound to human duodenal and colonic epithelial cells isolated from endoscopic biopsies. Binding was greater at 4°C than 37°C, consistent with the thermal binding characteristic of toxin A to a carbohydrate moiety. At 37°C colonic cells bound significantly more $[^3\text{H}]$ -toxin A than duodenal cells. The amount of $[^3\text{H}]$ -toxin A binding varied considerably between individuals. $[^3\text{H}]$ -toxin A was displaced by unlabelled toxin A by 50% for duodenal cells and 70% for colonic cells with 94.3 nM unlabelled toxin A. Low non-displacable binding was observed in some samples at 4°C and 37°C, suggesting that these cells came from individuals incapable of specifically binding toxin. Pre-treating cells with α - or β -galactosidases to cleave terminal α - and β -galactose residues reduced $[^3\text{H}]$ -toxin A binding. There was also a reduction in $[^3\text{H}]$ -toxin A binding after heat treating cells, which is suggestive of protein binding. The reduction in binding varied between individuals. The reduction of $[^3\text{H}]$ -toxin A binding, after the removal of β -linked galactose units, implicates these as components of the receptor and adds credence to the idea that the Lewis X, Y and I antigens may be involved in toxin A binding to human intestinal epithelial cells. However, because the Lewis antigens do not possess terminal α -galactose units, the reduction in binding after α -galactosidase treatment suggests that other receptors may be involved in toxin A binding to some human intestinal cells. These data are the first demonstration of direct toxin A binding to human intestinal epithelial cells.

Introduction

In the 1970s *Clostridium difficile* was found to be an aetiological agent of antibiotic-associated diarrhoea and pseudomembranous colitis. Intestinal infection with *C. difficile* is one of the most common causes of diarrhoea and colitis among hospitalised patients, particularly the elderly [1]. Interestingly, not all individuals colonised by toxigenic strains of *C. difficile* develop disease and human infants are not usually affected [2, 3].

C. difficile produces two toxins, A and B, which play a role in the pathogenesis of disease [1, 4]. Animal studies have shown toxin A to be an enterotoxin that elicits increased intestinal permeability, fluid secretion and inflammation and causes severe disruption of the intestinal epithelium [5–8]. It is a large, 520–540 kDa, multi-subunit protein. One of the major subunits contains repeating sequences in the C-terminal component of the molecule which are thought

to be involved in toxin A binding to carbohydrate-containing receptors [4].

In model systems, the carbohydrate component to which toxin A binds has been shown to contain the terminal trisaccharide sequence Gal α 1-3Gal β 1-4GlcNAc [9]. Bovine thyroglobulin [10], rabbit erythrocytes and rodent intestinal brush border membranes [9] possess this trisaccharide. It has been postulated that normal human intestinal cells do not express this sequence [11] and that the oligosaccharide blood group antigens Lewis X, Y and I may be responsible for toxin A binding to human intestine [12].

Studies on human colonic biopsy specimens have shown that toxin A causes epithelial cell rounding, detachment and apoptosis [13]. This study also identified individuals who were not susceptible to toxin A-induced cell damage, indicating a lack of toxin A-binding receptors.

Studies of direct toxin A binding to human intestinal

cells have not been done. In the present study, a previously described method of isolating intestinal epithelial cells from human endoscopic biopsies [14] was used to examine the binding characteristics of tritium-labelled purified toxin A to duodenal and colonic epithelial cells.

Materials and methods

Coffee bean α -galactosidase (EC 3.2.1.22) and *Escherichia coli* β -galactosidase (EC 3.2.1.23) were purchased from Boehringer Mannheim. The purity of these enzymes had been tested previously by Krivan *et al.* [9] and confirmed by us. Each preparation was free from contamination by the other enzyme. All other chemicals were from Sigma.

Patients and epithelial cell isolation

Human duodenal or colonic biopsy samples were collected from patients attending for endoscopy. Patients with suspected irritable bowel syndrome, diarrhoea, iron deficiency anaemia or colonic polyp surveillance had endoscopic biopsy samples taken as part of diagnostic investigations [14] and were asked to donate six additional samples for research purposes. No single patient had samples taken from both sites. Permission for the study was obtained from the Nottingham City Hospital Ethical Committee and patients gave informed written consent. Two further biopsy samples were taken for histological assessment and only data from patients subsequently found to have histologically normal mucosa are presented. Patients' age, sex and blood group (ABO) were recorded.

Epithelial cells were isolated from biopsy samples by a method described previously [14] with the following modifications. Biopsy samples were collected into 10 ml of citrate buffer (pre-warmed to 37°C) containing (mmol/L): KCl, 1.5; NaCl, 96; sodium citrate, 27; KH_2PO_4 , 8; Na_2HPO_4 , 5.6; and 200 IU of penicillin and 100 μg of streptomycin; (pH 7.3). After incubation for 10 min, the citrate buffer was replaced with 10 ml of EDTA buffer containing (mmol/L): $\text{Na}_2\text{-EDTA}$ 1.5; dithiothreitol, 0.5; NaH_2HPO_4 , 10; NaCl, 154; and 200 IU of penicillin and 100 μg of streptomycin (pH 7.3). The biopsy samples were then incubated for a further 30 min at 37°C with gentle agitation at intervals. The cell suspension was separated from the biopsy fragments and washed three times in HEPES-bicarbonate buffered Hanks's Balanced Salts Solution (HBH) containing (mmol/L): CaCl_2 , 1.26; KCl, 5.36; KH_2PO_4 , 0.44; MgSO_4 , 0.81; NaCl, 137; NaHCO_3 , 4.17; Na_2HPO_4 , 0.34; HEPES, 10; at 4°C and pH 7.4. The HBH buffer was gassed with CO_2 5%, O_2 95% (v/v). All buffers were supplemented with 5.5 mM glucose, 2 mM glutamine, bovine serum albumen (BSA) 2 mg/ml and soybean trypsin inhibitor 1 mg/ml [15].

A mean of 2.28×10^6 SD 0.51×10^6 cells/ml was isolated from six duodenal biopsy samples and a mean of 1.44×10^6 SD 0.18×10^6 cells/ml was isolated from six colonic samples; 1×10^6 cells were equivalent to 30 μg of DNA. More than 80% of isolated cells excluded trypan blue for at least 2 h (86 SEM 3% immediately after isolation and 84 SEM 5% 2 h later).

C. difficile toxin A purification and radiolabelling

Highly purified toxin A was used for this study as described previously [10, 16]. *C. difficile* strain VPI 10463 was used for toxin production. Briefly, separation and purification of toxin A from toxin B was by thermal elution of *C. difficile* culture filtrate from a bovine thyroglobulin affinity column. Further purification of toxin A was by anion-exchange gel-Q Sepharose FF (Pharmacia) followed by Mono Q (Pharmacia) incorporated into a fast protein liquid chromatography apparatus (Pharmacia) [17].

Toxin A was [^3H]-labelled with N-succinimidyl[2,3- ^3H] propionate (Bolton-Hunter reagent) as described by Pothoulakis *et al.* [18]. As found by others, no loss of cytotoxicity was exhibited by [^3H]-toxin A when compared to unlabelled toxin A tested in cultured Vero cells and Caco2 cells [10, 13, 18]. Binding of toxin A to bovine thyroglobulin [10] and haemagglutination of rabbit erythrocytes [9] were also unaffected by radiolabelling.

[^3H]-toxin A binding to isolated duodenal and colonic epithelial cells

Displacement curves were constructed by incubating isolated intestinal epithelial cells (200 μl) with 100 μl of [^3H]-toxin A (65 ng = 1.2 nM = 87 598 dpm) and 100 μl of increasing concentrations of unlabelled toxin A over the range 0–50 $\mu\text{g}/\text{ml}$ at 4°C and 37°C. Incubations were for 60 min because preliminary time course experiments showed that [^3H]-toxin A binding was maximal by this time.

Bound [^3H]-toxin A was separated from free toxin A by rapid filtration through glass fibre filters (25 mm) pre-wetted with 50 mM Tris-BSA 1%, pH 7.5 (T-BSA) mounted on a filtration manifold connected to a vacuum pump. Filters were washed with three 3-ml volumes of ice-cold T-BSA and liquid scintillation counted. Non-specific binding (NSB) to filters was < 2% of total radioactivity added. The amount of radioactivity bound to cells was calculated by subtracting NSB and expressed as dpm/ μg of DNA or as a percentage of [^3H]-toxin A bound in the absence of unlabelled toxin A.

The importance of carbohydrate (galactose residues) and protein to the binding of toxin A to human intestinal cells was investigated. The carbohydrate

dependency of [^3H]-toxin A binding was tested by pre-treating cells with α - or β -galactosidases [16]. These enzymes cleave terminal α - and β -galactose residues found in the known toxin A binding sequence Gal α 1-3Gal β 1-4GlcNAc and the Lewis X, Y and I antigens [9, 12, 19]; α - or β -galactosidase (1.5 U) was incubated with 200 μl of cells for 1 h at 22°C. The protein dependency of toxin A binding was tested by heating cells for 10 min at 100°C. The ability of pre-treated cells to bind toxin A was then tested by incubating cells at 4°C for 1 h in the presence of 100 μl of [^3H]-toxin A. Cell-bound [^3H]-toxin A was separated from free toxin as described above.

Statistical analysis

Data were analysed by the StatView 512+ statistics software package. Mean radioactivity counts were compared by Student's *t* test.

Results

Greater toxin A binding was observed at 4°C than at 37°C in both duodenal ($p = 0.05$) and colonic ($p = 0.02$) isolated epithelial cells. This is consistent

with the known thermal binding characteristic of toxin A to a carbohydrate moiety. At 4°C, duodenal and colonic epithelial cells bound similar amounts of [^3H]-toxin A. However, at 37°C (i.e., physiological temperature), colonic cells bound significantly more toxin A than duodenal cells ($p = 0.02$). The amount of [^3H]-toxin A binding to intestinal cells from different individuals varied considerably (Table 1). The amount of [^3H]-toxin A binding did not correlate with patient age, sex or blood group.

[^3H]-toxin A displacement curves (Fig. 1) constructed for epithelial cells isolated from duodenal and colonic biopsy samples showed that [^3H]-toxin A was

Table 1. [^3H]-Toxin A binding to isolated human intestinal epithelial cells at 4°C and 37°C after incubation for 1 h

Temperature	Mean (and SEM) dpm/ μg of DNA (range)	
	Duodenum (n = 9)	Colon (n = 10)
4°C	421.79 SEM 136.01 (61.80–1254.81)	439.29 SEM 131.56 (68.90–1248.74)
37°C	50.61 SEM 26.75 (12.48–154.85)	188.38 SEM 75.20 (17.40–814.35)

Fig. 1. Displacement of [^3H]-toxin A binding to isolated human duodenal (a) and colonic (b) epithelial cells at 4°C (■) and 37°C (●) after incubation for 1 h. [^3H]-toxin A was displaced by unlabelled toxin A by c. 50% and 70%, respectively, for duodenal (n = 5) and colonic (n = 6) cells at the maximum concentration of unlabelled toxin A tested (50 $\mu\text{g}/\text{ml}$ = 94.3 nM). Data are expressed as percentage of [^3H]-toxin A bound where maximum binding at 4°C in the absence of any unlabelled toxin A was taken to be 100% for experiments at 4°C and 37°C.

displaced by unlabelled toxin A by *c.* 50% and 70%, respectively, at 4°C at the maximum concentration of unlabelled toxin A tested (50 µg/ml = 94.3 nM). This was the maximum concentration available and was a 314-fold excess over the final labelled [³H]-toxin A concentration (0.3 nM). There was little displacement at 37°C. The displacement of [³H]-toxin A was more pronounced with colonic cells than duodenal cells.

Of the nine duodenal and 10 colonic samples tested, unlabelled toxin A was unable to displace labelled [³H]-toxin A from four duodenal and four colonic samples, suggesting that binding to these cells was non-specific. However, increased binding at 4°C compared to 37°C was still observed in these cell preparations (Fig. 2).

The effectiveness of reducing [³H]-toxin A binding by pre-treating cells with α-galactosidase varied considerably between individuals. Cleavage of terminal α-galactose residues had no effect on [³H]-toxin A binding to cells from three of the seven duodenal samples and reduced binding by 3%, 8%, 37% and 49% in the other four. In contrast, binding to all the colonic samples tested was reduced by an average of 80% (range 18–100%). Table 2 compares data from

Table 2. [³H]-toxin A binding to isolated human intestinal epithelial cells after pre-treatment with α- or β-galactosidases or heat compared to untreated cells

Site	[³ H]-toxin A bound (%) following pre-treatment with		
	Heat (100°C)	α-galactosidase	β-galactosidase
Duodenum	47	51	45*
	59	100	0†
	56	29	34*
	70	32	12*
Colon	39	37	0†
	100	0	0‡
	14	17	18*
	1	29	34*

Each datum point is the mean of duplicate determinations from cells isolated from duodenal or colonic biopsy samples.
*Cells in which all three parameters were important for toxin A binding.
†Cells in which terminal β-galactose residues were important for toxin A binding.
‡Cells in which toxin A binding was completely carbohydrate dependent. Heat treatment had no effect on toxin A binding.

cell preparations pre-treated with α- or β-galactosidase or heated. The significance of α- or β-galactose residues and the proportion of toxin A binding that was dependent on heat-stable protein was different for each individual tested. For all but one sample, an

Fig. 2. [³H]-toxin A displacement curves for isolated human duodenal (a) and colonic (b) epithelial cells at 4°C (■) and 37°C (●) after incubation for 1 h. Unlabelled toxin A was unable to displace [³H]-toxin A in four duodenal and four colonic cell samples over the unlabelled toxin A concentration range of 0.125–50 µg/ml. Data are expressed as percentage [³H]-toxin A bound where maximum binding at 4°C in the absence of any unlabelled toxin A was taken as 100% for experiments at 4°C and 37°C.

element of protein-dependent binding was present in all the cell preparations tested.

Discussion

Isolated human intestinal cells bound *C. difficile* toxin A. Binding was greater at 4°C than at 37°C. Greater binding at 4°C is a characteristic of toxin A [12] and is indicative of cold agglutination where increased cell surface receptor density at 4°C enhances binding of large multivalent proteins to carbohydrate receptors [20]. Toxin A is such a protein, with several repeating epitopes that facilitate multiple binding interactions between the toxin and its receptor [21]. Receptor mediated endocytosis (RME) is thought to be involved in toxin A internalisation and cellular intoxication [21]. Under normal circumstances, when a hormone-receptor complex is internalised, it is processed and the receptor is returned to the cell surface [22]. At 4°C RME is arrested and toxin A binding receptors remain on the cell surface. The lower toxin A binding at 37°C also suggests that toxin A-bound receptors may not be returned to the cell surface.

In man, the colon is the main site of *C. difficile* infection but there are reports that *C. difficile* has been isolated from the human small bowel [23] and can cause small bowel disease [24, 25]. To our knowledge, this study is the first to demonstrate that toxin A can bind to human small intestinal epithelial cells. Colonic cells bound significantly more [³H]-toxin A than duodenal cells at 37°C. This finding is indicative of a greater number of toxin A receptors in the colon than in the duodenum. An alternative explanation is that preparations of duodenal cells, which have been disorganised, permit brush border enzymes, such as β -galactosidase (lactase E.C. 3.2.1.108), access to brush border carbohydrate receptors, and thereby reduce the number of functional receptors that may be present. This would not be the case for colonic tissue.

The results of this study demonstrate several significant differences in the binding characteristics of *C. difficile* toxin A to intestinal epithelial cells from the human population compared to those demonstrated in laboratory animals.

First, human gut cells pre-treated with α - or β -galactosidase or heat denatured membrane receptors, revealed variation between individuals in the carbohydrate and possibly the protein component of receptors involved in binding toxin A. In contrast, Pathoulakis *et al.* [26] demonstrated a single class of membrane receptors, identified as Gal α 1-3Gal β 1-4GlcNAc (with an apparent dissociation constant of 54 nM), thought to be coupled to sucrase-isomaltase in New Zealand rabbit ileal brush border membranes. It is unlikely that toxin A binds to a single receptor in the human intestinal mucosa, given the variation in the

binding characteristics observed between individuals in this study. Therefore, it was considered inappropriate to calculate affinity constants from the displacement data. Cell-specific displaceable binding was observed predominantly at 4°C. The low non-displaceable binding observed in some samples at 4°C and 37°C suggests that these cells came from individuals incapable of specific binding of toxin A. At a physiological temperature specific receptor binding is greatly reduced and non-specific cell binding predominates. The pathological significance of this non-specific binding to cells is unknown. However, it may have relevance to disease because lack of the receptor binding repeating sequence of the toxin A molecule, or lack of the cellular carbohydrate receptor, did not completely abolish the cytotoxic effects of toxin A on cultured cells [21, 27, 28].

Secondly, the removal of terminal β -galactose residues by β -galactosidase reduced [³H]-toxin binding to human intestinal epithelial cells. However, similar experiments have shown that [³H]-toxin A binding was increased in rabbit ileal brush border membranes [18] and had no effect on binding to hamster brush border membranes or rabbit erythrocytes [9]. This latter finding is of interest because it suggests that *Escherichia coli* β -galactosidase is incapable of cleaving β -linked galactose of the known toxin A binding trisaccharide sequence Gal α 1-3Gal β 1-4GlcNAc in these tissues. The reduction of [³H]-toxin A binding to human gut cells, after the removal of β -linked galactose units, adds credence to the idea that the carbohydrates of the Lewis X antigen system may be involved in toxin A binding to human intestinal epithelial cells (Table 2). Similarly, because the Lewis antigens do not possess terminal α -galactose units, the reduction in binding after α -galactosidase treatment suggests that Gal α 1-3Gal β 1-4GlcNAc may also be involved in toxin A binding to some human intestinal cells. These findings do not concur with those of Galili *et al.* [11] who showed that Gal α 1-3Gal β 1-4GlcNAc was not present on the cells of man, apes and old world monkeys. This discrepancy needs further investigation. In contrast to our findings, heat treatment of hamster brush border membranes did not reduce toxin A binding and it was concluded that binding in hamster tissue was predominantly carbohydrate dependent [9].

Cell surface carbohydrates may be protein- or lipid-linked and they exhibit diverse structural polymorphisms that are tissue specific [29]. Protein glycosylation is important for post-translational protein folding and migration and most proteins expressed at the cell surface are glycosylated [30]. Therefore, the specificity of toxin A binding will be dependent not only on the carbohydrate component but also on the conjugate to which it is attached. Individual variation in a 'wild' population is inevitable and likely to account for differences in disease susceptibility. Therefore, further

clinically relevant studies on human intestinal epithelial cells are required to correlate disease susceptibility with toxin A binding characteristics.

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