Characterization of the receptor for cholera toxin and *Escherichia coli* heat-labile toxin in rabbit intestinal brush borders

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¹²⁵I-labelled heat-labile toxin (from *Escherichia coli*) and ¹²⁵I-labelled cholera toxin bound to immobilized ganglioside G_{M1} and Balb/c 3T3 cell membranes with identical specificities, i.e. each toxin inhibited binding of the other. Binding of both toxins to Balb/c 3T3 cell membranes was saturable, with 50% of maximal binding occurring at 0.3 nM for cholera toxin and 1.1 nM for heat-labile toxin, and the number of sites for each toxin was similar. The results suggest that both toxins recognize the same receptor, namely ganglioside G_{M1}. In contrast, binding of ¹²⁵I-heat-labile toxin to rabbit intestinal brush borders at 0 °C was not inhibited by cholera toxin, although heat-labile toxin inhibited ¹²⁵I-cholera toxin binding. In addition, there were 3–10-fold more binding sites for heat-labile toxin than for cholera toxin. At 37 °C cholera toxin, but more particularly its B-subunit, did significantly inhibit ¹²⁵I-heat-labile toxin binding. Binding of ¹²⁵I-cholera toxin was saturable, with 50% maximal of binding occurring at 1–2 nM, and was quantitatively inhibited by 10^{−8} M unlabelled toxin or B-subunit. By contrast, binding of ¹²⁵I-heat-labile toxin was non-saturable (up to 5 nM), and 2 × 10^{−7} M unlabelled B-subunit was required to quantitatively inhibit binding. Neuraminidase treatment of brush borders increased ¹²⁵I-cholera toxin but not heat-labile toxin binding. Extensive digestion of membranes with *Streptomyces griseus* proteinase or papain did not decrease the binding of either toxin. The additional binding sites for heat-labile toxin are not gangliosides. Thin-layer chromatograms of gangliosides which were overlaid with ¹²⁵I-labelled toxins showed that binding of both toxins was largely restricted to ganglioside G_{M1}. However, ¹²⁵I-heat-labile toxin was able to bind to brush-border galactoproteins resolved by SDS/polyacrylamide-gel electrophoresis and transferred to nitrocellulose.

INTRODUCTION

The heat-labile enterotoxin produced by *Escherichia coli* pathogenic for humans has many structural and functional characteristics in common with cholera toxin produced by *Vibrio cholerae*. The toxins share extensive immunological cross-reactivity (Clements & Finkelstein, 1978; Lindholm *et al.*, 1983) and are composed of five B-subunits that mediate binding to a cell-surface receptor and one A-subunit which is responsible for the biological effects of the toxin (for a review, see Eidels *et al.*, 1983). The B-subunits are made up of 103 amino acid residues and display 80% amino acid sequence homology (Yamamoto & Yokota, 1983). The A-subunits share less homology (Spicer *et al.*, 1981), but both have ADP-ribosyltransferase activity and irreversibly activate adenylate cyclase through covalent modification of the GTP-binding regulatory subunit (Gill & Richardson, 1980). In the small intestine the end result of the elevated cyclic AMP levels is a net fluid secretion into the gut lumen and the characteristic massive diarrhoea associated with cholera or the less-severe traveller's diarrhoea caused by heat-labile toxin.

The nature of the receptor for cholera toxin in a variety of cell types including the small-intestinal epithelial cell would appear to be well established as the ganglioside G_{M1} (Fishman, 1982; Eidels *et al.*, 1983). However, cholera toxin will bind to the galactoproteins

of rat intestinal brush borders (Morita *et al.*, 1981), although they represent only a minor species of receptor (Critchley *et al.*, 1981, 1982a), and also to gastric mucins (Strombeck & Harrold, 1974). Mucins may be responsible for the resistance of some species, such as rat, to the effects of the toxin. Much less is known about the receptor for *Escherichia coli* heat-labile toxin, although there is no doubt that it can also bind to ganglioside G_{M1} (Svennerholm & Holmgren, 1978; Moss *et al.*, 1979, 1981). In rabbit small intestine, however, the non-toxic B-subunit of cholera toxin was unable to block the fluid secretion induced by heat-labile toxin, although it was an effective inhibitor of the effects of cholera toxin itself (Holmgren, 1973; Pierce, 1973; Holmgren *et al.*, 1982). In other species, e.g. the dog, the B-subunits of either toxin inhibited the secretion induced by both heat-labile toxin and cholera toxin (Nalin & McLaughlin, 1978). The results suggest that in some species there are additional receptors recognized by heat-labile toxin to which cholera toxin cannot bind. An indication that the two toxins show subtle differences in their binding characteristics also comes from the observation that heat-labile toxin has a relatively high affinity for galactose polymers and can be purified by affinity chromatography on agarose columns, whereas cholera toxin binds only weakly to such columns (Clements & Finkelstein, 1979; Clements *et al.*, 1980). In the present study we have therefore investigated the nature of the receptor for

Abbreviations used: BSA, bovine serum albumin; PMSF, phenylmethanesulphonyl fluoride; PBS, 137 mM-NaCl/2.79 mM-KCl/8 mM-Na₂HPO₄/1.53 mM-KH₂PO₄, adjusted to pH 7.2; ganglioside nomenclature follows that of Svennerholm (1963).

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heat-labile toxin in rabbit small intestinal brush borders by using techniques previously successfully used to characterize the cholera-toxin receptor in a number of systems as the ganglioside G_{M1} (Critchley *et al.*, 1981, 1982b).

MATERIALS AND METHODS

Heat-labile enterotoxin and its B-subunit were isolated from genetically engineered recombinant strains MM 294 (pWD 600) and MM 294 (pJL 10) respectively, which were cloned for us by Dr. W. S. Dallas (Wellcome Research Laboratories, Research Triangle Park, NC, U.S.A.) from a human enterotoxigenic strain of *Escherichia coli* (H74-114). The proteins were purified as described by Geary *et al.* (1982) by using slight modifications of the procedures developed previously by Clements & Finkelstein (1979) and Clements *et al.* (1980). Cholera toxin and its B-subunit were purchased from List Biologicals (Campbell, CA, U.S.A.) for initial studies, but were subsequently purified as described by Finkelstein *et al.* (1971). The toxins were iodinated by a choramine- τ procedure. Briefly, 10 μ g of the toxin in 10 μ l of 0.2 M-sodium phosphate buffer, pH 7.4, was incubated with 0.5 mCi of $Na^{125}I$ (Amersham International) and 0.6 μ g of choramine- τ for 5 min at room temperature. Protein iodination was terminated by addition of 50 μ l of a saturated tyrosine solution and the labelled toxin purified on a Sephadex G-25 column equilibrated and eluted with 0.1 M-Tris buffer, pH 7.4, containing 0.2% gelatin.

Assay of ^{125}I -labelled-toxin binding

Brush borders were prepared from the small intestines of New Zealand White rabbits (more than 8 weeks old) by the method of Miller & Crane (1961), and portions were stored at $-20^{\circ}C$. A crude membrane preparation was isolated from Balb/c 3T3 cells as previously described (Critchley *et al.*, 1982b). Brush-border preparations were homogenized in 0.15 M-NaCl/1 mM-EDTA/25 mM-Tris, pH 7.4, containing 0.2 mM-PMSF and 0.1% BSA. Appropriate portions were incubated for 60 min at $0^{\circ}C$ with ^{125}I -labelled toxin in a total volume of 200 μ l with frequent mixing. Brush borders or membranes were sedimented at 11000 g for 4 min in a Microfuge, and pellets washed once with 500 μ l of buffer before samples were counted in a Beckman 5500 γ -radiation counter. Non-specific binding was determined in the presence of 0.1–0.2 μ M unlabelled toxin or toxin B-subunit. Binding of ^{125}I -labelled heat-labile toxin was linear up to 4 μ g of brush-border protein, and 2 μ g was used for routine assay. Binding of ^{125}I -labelled cholera toxin was linear up to 40 μ g of brush-border protein, and 10–20 μ g was used for routine assay. Binding of ^{125}I -labelled toxin to Balb/c 3T3 cell membranes (1 μ g) was assayed exactly as described for brush border, except that incubations were for 30 min and the buffer used was PBS containing 0.2 mM-PMSF and 0.1% BSA. Pilot studies showed that the results obtained using the centrifugation assay were essentially identical with those obtained in the filtration-binding assay used previously (Critchley *et al.*, 1981).

Treatment of brush borders with enzymes

Rabbit intestinal brush borders (1 mg) were incubated with or without: (i) *Streptomyces griseus* 'protease' (Sigma) (8 mg) in 1 ml of 0.15 M-NaCl/0.01 M- $CaCl_2$ /

50 mM-Tris, pH 7.4, for 90 min at $37^{\circ}C$; (ii) papain (Sigma) (10 μ g) in 1 ml of 3 mM-EDTA/3 mM-cysteine/12.5 mM-potassium phosphate, pH 7.0, for 90 min at $37^{\circ}C$ (Forstner, 1971) (papain was activated for 30 min before use in 67 μ M- β -mercaptoethanol/5.6 mM-cysteine/1.1 mM-EDTA, pH 6.2); (iii) 0.3 unit of *Vibrio cholerae* neuraminidase (Behringwerke) in 1 ml of 50 mM-sodium acetate/6.8 mM- $CaCl_2$ /0.15 M-NaCl, pH 5.5, for 60 min at $37^{\circ}C$. Treated and control membranes were washed three times with 15 ml of binding buffer without BSA.

Detection of toxin-binding gangliosides using a t.l.c./ ^{125}I -labelled toxin overlay procedure

Gangliosides, extracted from intestinal brush borders and Balb/c 3T3 cell membrane with chloroform/methanol (Critchley *et al.*, 1981), were separated on plastic-backed silica-gel-G plates (Merck) by using as solvent chloroform/methanol/0.2% $CaCl_2$ (5:4:1, by vol.). Chromatograms were immersed for 1 min in 0.1% (w/v) poly(isobutyl methacrylate) (Polysciences, Warrington, PA, U.S.A.) in hexane, dried, sprayed, then soaked for 1 h in 0.15 M-NaCl/50 mM-Tris, pH 7.4, containing 0.2% gelatin (Magnani *et al.*, 1982). Plates were overlaid with ^{125}I -labelled toxin (0.5 $\times 10^5$ c.p.m./ml) in the same buffer for 2 h at $4^{\circ}C$, washed five times over a 15 min period, dried and autoradiographed. Ganglioside standards were purified from human brain by the method of Svennerholm & Fredman (1980).

Detection of toxin-binding glycoproteins

Brush-border or Balb/c 3T3 cell membrane components were separated on SDS/7%-(w/v)-polyacrylamide gels and transferred to nitrocellulose sheets as described by Towbin *et al.* (1979), but omitting methanol from the transfer buffer. Sheets were overlaid with ^{125}I -labelled toxin (0.1 nM, 7×10^5 c.p.m./ml) in 0.15 M-NaCl/50 mM-Tris, pH 7.4, containing 0.05% (v/v) Tween 20 for 3 h at $4^{\circ}C$. Sheets were then washed with five changes of 200 ml each of buffer over a period of 5 min, dried, and bound toxin detected by autoradiography. Brush-border galactoproteins labelled by the galactose oxidase/ NaB^3H_4 procedure (Critchley *et al.*, 1981) were resolved by SDS/polyacrylamide-gel electrophoresis and detected by fluorography.

RESULTS

Binding of ^{125}I -cholera toxin and ^{125}I -heat-labile toxin to plastic-adsorbed ganglioside G_{M1} and Balb/c 3T3 cell membranes

Both cholera toxin and heat-labile toxin have previously been shown to bind to ganglioside G_{M1} (Moss *et al.*, 1981). To characterize the iodinated preparation of the toxins, we therefore investigated their ability to bind specifically to ganglioside G_{M1} adsorbed to plastic microtitre wells. Binding of ^{125}I -labelled cholera toxin to G_{M1} was quantitatively inhibited by pre-incubation of wells with both unlabelled cholera toxin and heat-labile toxin, and binding of ^{125}I -labelled heat-labile toxin was quantitatively inhibited by both unlabelled heat-labile toxin and cholera toxin (Table 1). The results show that both iodinated toxins can bind to ganglioside G_{M1} and that binding is specific, i.e. inhibited by excess unlabelled toxin. The cholera toxin receptor in Balb/c 3T3 cell membranes has previously been shown to be exclusively

Table 1. Specificity of binding of ^{125}I -cholera toxin and ^{125}I -heat-labile toxin to (a) plastic-adsorbed ganglioside G_{M1} and (b) Balb/c 3T3 cell membranes

(a) Binding to ganglioside G_{M1} was determined by using micro-c.l.i.s.a. plates (Dynatech) coated with G_{M1} ($50\ \mu\text{l}$, $0.1\ \mu\text{M}$ - G_{M1} in phosphate-buffered saline) overnight at $4\ ^\circ\text{C}$. The plates were washed with phosphate-buffered saline and incubated with 1% BSA in phosphate-buffered saline for 30 min at $37\ ^\circ\text{C}$. The wells were then pre-incubated with or without unlabelled toxins in phosphate-buffered saline/ 0.1% BSA for 30 min at $37\ ^\circ\text{C}$, ^{125}I -labelled cholera toxin ($0.22\ \text{nM}$, $2046\ \text{c.p.m./fmol}$) or ^{125}I -labelled heat-labile toxin ($0.24\ \text{nM}$, $1546\ \text{c.p.m./fmol}$) added, and the incubation continued for a further 60 min. The wells were rinsed with phosphate-buffered saline before counting radioactivity. Results are means of duplicate determinations differing by $< 10\%$ and have been corrected for binding to the wells in the absence of ganglioside. The experiment was performed twice with identical results. Abbreviation used: N.D., not done. (b) Balb/c 3T3 cell membranes ($1\ \mu\text{g}$) were pre-incubated for 30 min at $4\ ^\circ\text{C}$ with or without unlabelled toxins. ^{125}I -labelled heat-labile toxin ($0.08\ \text{nM}$, $4647\ \text{c.p.m./fmol}$) or ^{125}I -cholera toxin ($0.07\ \text{nM}$, $3775\ \text{c.p.m./fmol}$) was added and the incubation continued for 30 min. In some cases the ^{125}I -labelled toxins were pre-incubated with ganglioside G_{M1} for 30 min before addition of membranes. Binding was determined using the centrifugation assay described in the Materials and methods section, and the results have been corrected for binding to tubes in the absence of membranes. Results are means of duplicate determinations (which differed by $< 10\%$) from a single experiment, and the experiment was performed twice.

Incubation conditions	(a) Binding to ganglioside G_{M1} (% of control)		(b) Binding to Balb/c 3T3 cell membranes (% of control)	
	^{125}I -cholera toxin	^{125}I -heat-labile toxin	^{125}I -cholera toxin	^{125}I -heat-labile toxin
Control	100 (10.8 fmol bound)	100 (12.2 fmol bound)	100 (4.53 fmol/ μg)	100 (3.96 fmol/ μg)
$0.1\ \mu\text{M}$ Unlabelled heat-labile toxin	3.8	0	0	0
$0.1\ \mu\text{M}$ Unlabelled cholera toxin	2.4	1.7	0.9	1.9
$66\ \mu\text{M}$ -Ganglioside G_{M1}	N.D.	N.D.	9.9	6.2

Fig. 1. Binding of ^{125}I -cholera toxin and ^{125}I -heat-labile toxin to Balb/c 3T3 cell membranes

(a) Binding of ^{125}I -labelled cholera toxin (○) ($161\ \text{c.p.m./fmol}$) and ^{125}I -labelled heat-labile toxin (●) ($505\ \text{c.p.m./fmol}$) to Balb/c 3T3 cell membranes ($1\ \mu\text{g}$) as a function of toxin concentration. (b) Binding of ^{125}I -labelled cholera toxin (○) and ^{125}I -labelled heat-labile toxin (●) (both at approx. $0.1\ \text{nM}$) to membranes ($1\ \mu\text{g}$), after pre-incubation with increasing concentrations of the appropriate unlabelled toxin. Binding was assayed by centrifugation as described in the Materials and methods section, and the results have been corrected for non-specific binding determined in the presence of $0.1\ \mu\text{M}$ unlabelled toxin. Each point represents the mean of duplicates differing by $< 10\%$, and each experiment was performed twice.

ganglioside G_{M1} (Critchley *et al.*, 1982b). We therefore investigated the ability of the toxins to bind specifically to membranes using the above system. Both toxins were capable of binding specifically, and each toxin was able to quantitatively inhibit binding of the other toxin (Table

1), as had been previously found using plastic-adsorbed ganglioside G_{M1} . Binding of both toxins was saturable, with 50% maximal binding occurring at $0.3\ \text{nM}$ for cholera toxin and $1.1\ \text{nM}$ for heat-labile toxin (Fig. 1a), and the amount of toxin bound at saturation was

approximately the same (50 pmol/mg of membrane protein for cholera toxin, and 60 pmol/mg for heat-labile toxin). Pre-incubation of membranes with increasing amounts of unlabelled toxin before addition of the ^{125}I -labelled toxin produced a single-phase inhibition curve for both toxins, with complete inhibition of binding at 10^{-8} M unlabelled toxin (Fig. 1b). There was no evidence of heterogeneity in the binding interaction. Overall, the results are consistent with the conclusion that the receptor for both cholera toxin and heat-labile toxin in Balb/c 3T3 cell membranes is ganglioside G_{M1} .

Binding of ^{125}I -cholera toxin and ^{125}I -heat-labile toxin in rabbit intestinal brush borders

Time-course studies showed that binding of both toxins to rabbit intestinal brush borders was near maximal after 30 min at 0°C , and was complete by 60 min. Binding of both toxins was largely irreversible, with little or no displacement of bound ^{125}I -labelled toxin after addition of excess unlabelled toxin. Binding of ^{125}I -labelled cholera toxin was similar at 0°C , 20°C and 37°C , but binding of ^{125}I -labelled heat-labile toxin was consistently decreased at the higher temperature (Table 2).

The binding of ^{125}I -labelled cholera toxin at all temperatures tested was markedly inhibited by pre-incubation of brush borders with unlabelled cholera toxin, heat-labile toxin and their respective B-subunits (Table 2), essentially as previously observed with Balb/c 3T3 membranes. At 0°C , binding of ^{125}I -heat-labile toxin was inhibited as expected by both unlabelled heat-labile toxin and its B-subunit, whereas unlabelled cholera toxin or its B-subunit consistently produced an increase in ^{125}I -labelled heat-labile toxin binding, although

the level of the increase was variable (1.1–1.6-fold). At higher temperatures, binding of ^{125}I -labelled heat-labile toxin was inhibited by unlabelled cholera toxin, but the extent of the inhibition was again variable, ranging from 15 to 50%. The cholera toxin B-subunit was somewhat more effective in this respect. Because of the decreased binding of heat-labile toxin at 37°C and the altered specificity, binding studies were subsequently carried out at 0°C to avoid possible loss of a protein receptor. This general pattern of binding specificity was found in six preparations of brush borders from different rabbits. There was, however, some variation in the level of non-specific binding of cholera toxin between preparations. Non-specific binding of heat-labile toxin was generally less than 10% of the total radioactivity bound. At sub-saturating levels of toxin and at 0°C , rabbit intestinal brush borders specifically bound 3–10-fold greater amounts of heat-labile toxin than of cholera toxin, although the absolute levels of binding differed somewhat between brush-border preparations. The results clearly demonstrate that *E. coli* heat-labile toxin binds to a receptor(s) in rabbit intestinal brush borders not recognized by cholera toxin, although heat-labile toxin can also bind to the receptor recognized by cholera toxin and presumed to be ganglioside G_{M1} .

Binding of ^{125}I -labelled cholera toxin approached saturation at 2–3 nM, with 50% of maximal binding occurring between 1 and 2 nM (Fig. 2a). At saturation, rabbit brush borders bound approx. 7 pmol of toxin/ μg of protein, which is similar to the levels bound by rat intestinal brush borders (Critchley *et al.*, 1981). At higher toxin concentrations (> 5 nM) there was some additional binding. Binding of ^{125}I -labelled cholera toxin to brush borders in the presence of increasing amounts of

Table 2. Binding of ^{125}I -cholera toxin and ^{125}I -heat-labile toxin to rabbit intestinal brush borders

Rabbit intestinal brush borders were incubated in the presence and absence of unlabelled toxin or toxin B-subunit for 60 min, either on ice (0°C), at room temperature (20°C) or at 37°C . ^{125}I -labelled cholera toxin (0.3 nM, 2843 c.p.m./fmol) or ^{125}I -labelled heat-labile toxin (0.3 nM, 2897 c.p.m./fmol) were then added and the incubation continued for a further 60 min. A 15 μg and a 4 μg portion of brush-border protein were used for binding of ^{125}I -labelled cholera toxin and ^{125}I -labelled heat-labile toxin respectively. Binding was determined by the centrifugation assay described in the Materials and methods section, and the results have been corrected for binding to tubes in the absence of membranes. Results are means of duplicate determinations (which differed by $< 10\%$) from a single experiment, and the experiment was performed twice on the same brush-border preparation. Essentially similar results were obtained on three separate preparations of brush borders.

Incubation conditions Temp. ($^\circ\text{C}$) . . .	Binding (% of control)					
	^{125}I -cholera toxin			^{125}I -heat-labile toxin		
	0	20	37	0	20	37
Control	100 (0.23 fmol)*	100 (0.25 fmol)	100 (0.21 fmol)	100 (1.26 fmol)	100 (1.61 fmol)	100 (0.75 fmol)
0.2 μM Unlabelled heat-labile toxin	14.9	12.6	18.8	14.1	5.7	0
0.2 μM Unlabelled heat-labile toxin B-subunit	15.8	9.9	20.3	8.2	3.4	0
0.2 μM Unlabelled cholera toxin	9.8	7.8	22.6	155	88.8	74.6
0.2 μM Unlabelled cholera toxin B-subunit	6.6	5.8	21.1	141	84.7	41.7

*fmol bound/ μg of membrane protein.

Fig. 2. Binding of ^{125}I -cholera toxin to rabbit intestinal brush borders

(a) Binding of ^{125}I -labelled cholera toxin (226 c.p.m./fmol) to brush borders (10 μg) was determined as a function of toxin concentration. (b) Binding of ^{125}I -labelled cholera toxin (0.17 nm) after pre-incubation of brush borders (10 μg) with increasing amounts of unlabelled toxin. Binding was assayed by centrifugation as described in the Materials and methods section, and the results have been corrected for non-specific binding determined in the presence of 0.2 μM unlabelled toxin. Each point represents the mean of duplicates differing by < 10%, and each experiment was performed twice. Similar results were obtained on two other preparations of brush borders.

unlabelled toxin produced a single-phase inhibition curve, with complete inhibition of binding at 10^{-8} M unlabelled toxin (Fig. 2b). Binding of ^{125}I -labelled heat-labile toxin to this same preparation of brush borders showed markedly different characteristics. There was no evidence of saturable binding, with up to 5 nm toxin (Fig. 3a), and the inhibition of ^{125}I -labelled toxin binding by increasing amounts of unlabelled B-subunit proceeded through two distinct phases (Fig. 3b). There was a small, but reproducible, decrease in ^{125}I -labelled toxin binding in the range $(1-5) \times 10^{-10}$ M unlabelled B-subunit, but most of the binding was only inhibited at higher B-subunit concentrations (2×10^{-7} M). However, in all other brush-border preparations there was some slight indication of a saturable component to ^{125}I -labelled heat-labile toxin binding (Fig. 3c), and the inhibition of ^{125}I -labelled toxin binding by unlabelled B-subunit proceeded through a single phase, and was complete at 2×10^{-7} M unlabelled B-subunit (Fig. 3d).

In an attempt to characterize the nature of the additional binding sites recognized by heat-labile toxin, rabbit brush borders were treated with a variety of enzymes before assay of toxin binding. In agreement with previous studies, neuraminidase increased ^{125}I -labelled cholera toxin binding, presumably due to conversion of more complex gangliosides containing disialosyl linkages into the ganglioside G_{M1} . In contrast, neuraminidase had little or no effect on ^{125}I -labelled heat-labile toxin binding (Table 3). The results are consistent with the view that the major receptor for heat-labile toxin in rabbit brush borders is not ganglioside G_{M1} . However, no evidence was obtained that the receptor sites were sensitive to proteolysis (Table 3). Thus, despite extensive treatment of brush borders with *S. griseus* proteinase and papain, there was no decrease in heat-labile-toxin

binding. To rule out the possibility that a decrease in heat-labile toxin binding due to loss of a protein receptor had been offset by increased binding to ganglioside G_{M1} , the binding specificity of heat-labile toxin for papain-treated brush borders was investigated. As with untreated membranes, ^{125}I -labelled heat-labile-toxin binding at 0 $^{\circ}\text{C}$ was inhibited by unlabelled heat-labile toxin, but not cholera toxin (results not shown). The results suggest that the receptors for heat-labile toxin in rabbit intestinal brush borders are resistant to release by proteolytic enzymes.

To investigate the possibility that the additional binding sites for heat-labile toxin were gangliosides other than G_{M1} , thin-layer chromatograms of brush-border gangliosides were incubated with ^{125}I -labelled toxins, then washed and autoradiographed. Both toxins bound predominantly to a ganglioside with a mobility identical with that of G_{M1} (Fig. 4) and to a much lesser extent with a more complex unidentified ganglioside. Cholera toxin has previously been shown to show some affinity for ganglioside G_{D1b} (Moss *et al.*, 1981). There was no evidence for any novel ganglioside or neutral glycosphingolipid able to bind heat-labile toxin but not cholera toxin. Experiments in which the ^{125}I -labelled toxins were pre-incubated with ganglioside G_{M1} before addition of membranes showed that both toxins bound to G_{M1} with similar affinity (Fig. 5).

Previous experiments have shown that many of the membrane glycoproteins of rat intestinal brush borders have terminal galactose residues (Morita *et al.*, 1980; Critchley *et al.*, 1981). Given the known affinity of heat-labile toxin for galactose-containing polymers (Clements & Finkelstein, 1979), we therefore investigated the binding of the toxin to rabbit intestinal brush-border galactoproteins. Brush-border proteins were separated

Fig. 3. Binding of ^{125}I -heat-labile toxin to rabbit intestinal brush borders

(a, c) Binding of ^{125}I -labelled heat-labile toxin (246 and 697 c.p.m./fmol respectively) to brush borders ($2\ \mu\text{g}$) was determined as a function of toxin concentration. (b, d) Binding of ^{125}I -heat-labile toxin (approx. 0.15 nM) after pre-incubation of brush borders ($2\ \mu\text{g}$) with increasing concentration of unlabelled toxin. (a) and (b) represent data on a preparation of brush borders different from that in (c) and (d). The results have been corrected for non-specific binding determined in the presence of $0.2\ \mu\text{M}$ unlabelled toxin, and are representative of data obtained on five different brush-border preparations.

by SDS/polyacrylamide-gel electrophoresis and the proteins transferred to nitrocellulose sheets, which were then overlaid with ^{125}I -labelled toxin. ^{125}I -labelled heat-labile toxin bound to proteins with mobilities identical with those of the major galactoproteins detected by galactose oxidase/ NaB^3H_4 labelling (Figs. 6b and 6h). In contrast there was no detectable binding of the toxin to Balb/c 3T3 cell proteins, and binding was restricted to dye-front material (Fig. 6j), previously shown to represent binding to ganglioside $\text{G}_{\text{M}1}$ (Critchley *et al.*, 1982b). However, whereas binding of the ^{125}I -labelled toxin to the ganglioside $\text{G}_{\text{M}1}$ of Balb/c 3T3 cells was quantitatively inhibited by pre-incubation with 100 nM unlabelled toxin B-subunit (Fig. 6k), binding to

the brush-border galactoproteins was only partially inhibited under the same conditions (Figs. 6h and 6i). Interestingly, ^{125}I -labelled cholera toxin bound to these same galactoproteins, but binding was much less than with ^{125}I -labelled heat-labile toxin (results not shown). Brush-border galactoproteins may thus represent the additional binding sites recognized by heat-labile toxin but not cholera toxin, although there is some doubt as to the specificity of the interaction. The finding that prior treatment of membranes with neuraminidase does not markedly increase labelling of these galactoproteins by the galactose oxidase/ NaB^3H_4 method (Figs. 6b and 6c) suggests that brush-border glycoproteins are not heavily sialylated. The lack of effect of neuraminidase on

Table 3. Binding of ^{125}I -cholera toxin and ^{125}I -heat-labile toxin to rabbit intestinal brush borders treated with neuraminidase and proteolytic enzymes

Rabbit intestinal brush borders (1 mg) were treated with neuraminidase, *S. griseus* proteinase and papain as described in the Materials and methods section. Brush borders were resuspended in 1 ml, and aliquots assayed for ^{125}I -labelled cholera toxin (0.3 nM, 1162 c.p.m./fmol) or ^{125}I -labelled heat-labile toxin (0.3 nM; 3366 c.p.m./fmol) binding. The results were corrected for non-specific binding determined in the presence of 0.2 μM -cholera toxin or heat-labile toxin B-subunit respectively and represent the mean of duplicates. The results are from a single experiment, but similar results were obtained in two additional experiments.

Enzyme treatment	Binding (% control)	
	^{125}I -cholera toxin	^{125}I -heat-labile toxin
None*	100	100
Neuraminidase	206	94.7
<i>S. griseus</i> proteinase	163	140
Papain	109	128.6

* Binding to treated membranes is expressed as a percentage of binding to membranes treated with buffer but without enzyme.

Fig. 4. Binding of ^{125}I -cholera toxin and ^{125}I -heat-labile toxin to gangliosides separated by t.l.c.

(a) Bovine brain gangliosides (10 μg) plus ganglioside $\text{G}_{\text{M}3}$ (2 μg) and the gangliosides purified from (b) intestinal brush border (1 mg of protein) and (c) Balb/c 3T3 cells (2×10^6 cells) were chromatographed on silica-gel G t.l.c. plates as described in the Materials and methods section, and the gangliosides detected by spraying with the resorcinol reagent (Svennerholm, 1957). The two major bands in the brush-border track (b) did not give the characteristic purple colour with the resorcinol reagent and may not be gangliosides. Chromatograms of smaller amounts of the same samples [d, g, brain gangliosides (40 ng); e, h, rabbit intestinal brush borders (from 50 μg of protein); f, i, Balb/c 3T3 cells (1×10^5 cells)] were overlaid with ^{125}I -labelled heat-labile toxin (d-f) or ^{125}I -labelled cholera toxin (g-i), and the toxin-binding gangliosides detected by autoradiography as described in the Materials and methods section.

Fig. 5. Inhibition of ^{125}I -cholera toxin and ^{125}I -heat-labile toxin binding to rabbit intestinal brush borders by ganglioside $\text{G}_{\text{M}1}$

^{125}I -labelled heat-labile toxin (●) (0.33 nM, 2735 c.p.m./fmol) or ^{125}I -labelled cholera toxin (○) (0.38 nM, 2165 c.p.m./fmol) were preincubated with increasing concentrations of ganglioside $\text{G}_{\text{M}1}$ for 60 min at 0 °C, followed by the addition of brush borders (12 and 10 μg respectively) and the incubation continued for a further 60 min. Binding of labelled toxin was determined by centrifugation as described in the Materials and methods section, and the results have been corrected for non-specific binding in the presence of 0.2 μM unlabelled toxin. Each point represents the mean of duplicates differing by < 10%, and the experiment was performed twice.

heat-labile toxin binding is therefore consistent with these galactoproteins acting as receptors. However, most of the brush-border galactoproteins were released from the membrane by papain or *S. griseus* proteinase (Figs. 6d-6f), yet neither significantly affected ^{125}I -labelled toxin binding (Table 3).

DISCUSSION

Binding of cholera toxin and *Escherichia coli* heat-labile toxin to membranes isolated from Balb/c 3T3 cells was essentially similar. Both toxins showed identical binding specificities, the number of binding sites were similar and binding was saturable, with 50% maximal binding occurring at 0.3 nM for cholera toxin and 1.1 nM for heat-labile toxin. Increasing amounts of unlabelled toxin or B-subunit inhibited ^{125}I -labelled toxin binding in a single phase in both cases. Because both toxins are multivalent, and binding is essentially irreversible, Scatchard analysis of the data is inappropriate. However, the smooth shapes of the binding curves are consistent with a single class of binding site. We have previously provided unequivocal evidence that the cholera-toxin receptor in Balb/c 3T3 cells is ganglioside $\text{G}_{\text{M}1}$ (Critchley *et al.*, 1982b), and we therefore conclude that the receptor for heat-labile toxin in these cells is also $\text{G}_{\text{M}1}$. The results are in agreement with those of Donta *et al.*

Fig. 6. Binding of ^{125}I -heat-labile toxin to rabbit intestinal brush-border galactoproteins separated by SDS/polyacrylamide-gel electrophoresis

In (a)–(f), rabbit intestinal brush-border proteins labelled by the galactose oxidase/ NaB^3H_4 method were separated by SDS/7%-(w/v)-polyacrylamide-gel electrophoresis and detected by fluorography. (a) Proteins stained by Coomassie Blue; (b) plus galactose oxidase; (c) plus neuraminidase and galactose oxidase; (d) brush borders labelled by the galactose oxidase/ NaB^3H_4 method and (e) treated with papain; (f) papain supernatant. In (g)–(k) unlabelled brush-border components resolved by SDS/polyacrylamide-gel electrophoresis were transferred to nitrocellulose sheets, and then (g) stained for protein with Amido Black (h) overlaid with ^{125}I -labelled heat-labile toxin and autoradiographed; (i) as for (g), except sheets were pre-incubated with 5 μg of unlabelled heat-labile toxin B-subunit/ml. In (j) and (k), Balb/c 3T3 cell components resolved by SDS/polyacrylamide-gel electrophoresis were transferred to nitrocellulose sheets and then (j) overlaid with ^{125}I -labelled heat-labile toxin and autoradiographed; (k), as for (j), except sheets were pre-incubated with 5 μg of unlabelled heat-labile toxin B-subunit/ml. Relative-molecular-mass standards are shown to the left of the Figures. Each lane contains the equivalent of 100 μg of protein, apart from (b,c), which contains 10 μg of protein.

(1982), who found that binding of the two toxins to Y1 adrenal cells showed similar characteristics. That ganglioside G_{M1} can act as a functional receptor for both cholera toxin and heat-labile toxin has been shown by Moss *et al.* (1979). Addition of ganglioside G_{M1} to the culture medium of the ganglioside-deficient cell line N.C.T.C. 2071 A promoted binding of heat-labile toxin by the cells and the subsequent activation of adenylate cyclase.

In rabbit small-intestinal brush borders, however, it is clear that heat-labile toxin binds to additional sites not recognized by cholera toxin. Thus, whereas heat-labile toxin was able to inhibit ^{125}I -labelled cholera toxin binding quantitatively, cholera toxin actually increased ^{125}I -labelled heat-labile toxin binding at 0 °C, although there was some variable inhibition at 37 °C. Binding of cholera toxin was saturable, with 50% of maximal binding occurring between 1- and 2 nM-toxin, whereas binding of heat-labile toxin showed no clear evidence of saturable binding up to 5 nM-toxin, and there were also substantially more sites for heat-labile toxin than for cholera toxin. In addition, binding of ^{125}I -labelled cholera toxin was quantitatively inhibited by 10^{-8} M unlabelled toxin or its B-subunit, whereas binding of ^{125}I -labelled heat-labile toxin was quantitatively inhibited only at 2×10^{-7} M unlabelled B-subunit. Because binding of both toxins is essentially irreversible, we are unable to analyse the data in terms of affinity constants or the

number of classes of binding site. Overall the results suggest that, in rabbit small intestine, heat-labile toxin can bind to the same receptor as cholera toxin, presumed to be ganglioside G_{M1} . The affinity of the toxins for ganglioside G_{M1} would appear to be similar, if not identical. However heat-labile toxin also binds to a large number of additional sites which may mask binding of the toxin to ganglioside G_{M1} .

The results are in agreement with observations on the effects of the two toxins on rabbit intestinal loops. Whereas heat-labile toxin B-subunit blocked the fluid secretogenic action of both heat-labile toxin and cholera toxin, cholera toxin B-subunit did not inhibit the response to heat-labile toxin (Holmgren, 1973; Pierce, 1973; Holmgren *et al.*, 1982). In dogs, however, cholera toxin B-subunit has been reported to inhibit the secretogenic effect of heat-labile toxin (Nalin & McLaughlin, 1978), indicating that the nature of the heat-labile toxin receptor might vary between species. We have also found that the number of additional sites for heat-labile toxin varies between brush-border preparations. It is presently unclear whether this represents variations in the number of receptors between individual rabbits or whether there is variable loss of receptors from brush borders during the isolation procedure.

Our results also suggest that there is some alteration in heat-labile toxin binding specificity at elevated temperatures. At 37 °C, cholera toxin was able to

produce partial inhibition of heat-labile toxin binding, although the extent of the inhibition was variable. At 0 °C, no inhibition was seen. The results suggests that heat-labile toxin can switch, to some extent, from a site not recognized by cholera toxin to the receptor ganglioside G_{M1}. The observation may be explained by loss of a heat-labile toxin receptor from the membrane during incubation at 37 °C, although other experiments suggest that the additional receptor sites for heat-labile toxin are not proteinase-sensitive. Alternatively, heat-labile toxin may adopt a conformation at 37 °C that decreases its binding affinity for the additional receptor sites.

The additional sites recognized by heat-labile toxin do not represent binding to novel gangliosides or neutral glycosphingolipids. Experiments in which thin-layer chromatograms of brush-border gangliosides were overlaid with ¹²⁵I-labelled heat-labile toxin showed binding was largely restricted to ganglioside G_{M1}. Similarly, Moss *et al.* (1979, 1981) found that gangliosides other than G_{M1} were substantially less effective in promoting toxin binding to cells deficient in complex gangliosides. We have, however, found that heat-labile toxin binds to the major galactoproteins present in rabbit brush borders. Interestingly the galactose residues in these molecules are not masked by sialic acid residues, in agreement with the lack of effect of neuraminidase treatment of brush borders on heat-labile-toxin binding. Previous studies have shown that cholera toxin will also bind to the major galactoproteins in rat intestinal brush borders (Morita *et al.*, 1980), although they only represent a minor percentage of the total receptor population (Critchley *et al.*, 1981, 1982a). The greater level of binding of heat-labile toxin to these galactoproteins is in agreement with the well-documented high affinity of heat-labile toxin for galactose-containing polymers (Clements & Finkelstein, 1979). It is unclear, however, whether these galactoproteins represent the additional binding sites recognized by heat-labile toxin. Whereas the galactoproteins were quantitatively released from the brush borders by papain or *S. griseus* proteinase, these same proteinases did not decrease heat-labile-toxin binding. Holmgren *et al.* (1982) have also previously noted that Pronase did not decrease the binding of heat-labile toxin by rabbit intestinal brush borders. In addition, binding of ¹²⁵I-labelled heat-labile toxin to the galactoproteins could not be quantitatively inhibited by pre-incubation with unlabelled toxins, although binding to ganglioside G_{M1} was abolished. Similarly we have found that the weak interaction of ¹²⁵I-cholera toxin with galactoproteins in rat intestinal brush borders is not totally inhibited by unlabelled cholera toxin (Critchley *et al.*, 1982a). Although there is no doubt that heat-labile toxin binds to brush-border galactoproteins, there must remain some doubt about the specificity of the interaction.

The ability of heat-labile toxin to bind to additional sites not recognized by cholera toxin is interesting in relation to the extensive homology between the B-subunits of the two toxins. The amino acid residues in the B-subunit involved in toxin binding have yet to be precisely defined. Evidence suggesting the importance of residues 35 (Arg) (Duffy & Lai, 1979) and 88 (Trp) have been presented (De Wolf *et al.*, 1981; Ludwig *et al.*, 1985), and a mutant heat-labile toxin with substitution of glycine at position 33 was unable to bind to ganglioside G_{M1} (Tsuji *et al.*, 1985). All these residues are conserved

in both cholera toxin and human and porcine isolates of *Escherichia coli* heat-labile toxin. Antibodies raised against a synthetic peptide equivalent to residues 50–64 of cholera toxin were partially neutralizing *in vivo*, suggesting that these residues may also be involved in binding (Jacob *et al.*, 1983, 1984). Again these residues are conserved in the heat-labile toxins (Yamamoto & Yokota, 1983). The molecular basis for the difference in receptor specificities between the two toxins may be resolved through analysis of the binding characteristics of genetically engineered toxins.

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