Characterization of the cholera toxin receptor on Balb/c 3T3 cells as a ganglioside similar to, or identical with, ganglioside G_{M1}

No evidence for galactoproteins with receptor activity

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Balb/c 3T3 cells contain a large number $[(0.8-1.6) \times 10^6]$ of high-affinity (half-maximal binding at 0.2 nM) binding sites for cholera toxin that are resistant to proteolysis, but are quantitatively extracted with chloroform/methanol. The following evidence rigorously establishes that the receptor is a ganglioside similar to, or identical with, ganglioside G_{M1}. (1) Labelling of ganglioside G_{M1} by the galactose oxidase/NaB³H₄ technique on intact cells was inhibited by cholera toxin. (2) Ganglioside G_{M1} was specifically adsorbed from Nonidet P40 extracts of both surface- (galactose oxidase/NaB³H₄ technique) and metabolically ([1-¹⁴C]palmitate) labelled cells in the presence of cholera toxin, anti-toxin and *Staphylococcus aureus*. (3) Ganglioside G_{M1} was the only ganglioside labelled when total cellular gangliosides separated on silica-gel sheets were overlaid with ¹²⁵I-labelled cholera toxin, although G_{M3} and G_{D1a} were the major gangliosides present. In contrast no evidence for a galactoprotein with receptor activity was obtained. Cholera toxin did not protect the terminal galactose residues of cell-surface glycoproteins from labelling by the galactose oxidase/NaB³H₄ technique. No toxin-binding proteins could be identified in Nonidet P40 extracts of [³⁵S]-methionine-labelled cells by immunochemical means. After sodium dodecyl sulphate/polyacrylamide-gel electrophoresis none of the major cellular galactoproteins identified by overlaying gels with ¹²⁵I-labelled ricin were able to bind ¹²⁵I-labelled cholera toxin. It is concluded that the cholera toxin receptor on Balb/c 3T3 cells is exclusively ganglioside G_{M1} (or a related species), and that cholera toxin can therefore be used to probe the function and organisation of gangliosides in these cells as previously outlined [Critchley, Ansell, Perkins, Dilks & Ingram (1979) *J. Supramol. Struct.* 12, 273–291].

Amongst the many changes that accompany tumour-virus transformation of animal cells in culture is loss of the ability to synthesize the more complex glycosphingolipids. Although the observation is well documented, little headway has been made in clarifying its significance with respect to other characteristics of the transformed phenotype, but a role for glycosphingolipids in cellular interaction has been proposed (for reviews see Critchley & Vicker, 1977; Hakomori, 1981). The demonstration that ganglioside G_{M1} (ganglioside nomen-

clature is according to Svennerholm, 1963) can act as a cell-surface receptor for cholera toxin (Fishman *et al.*, 1976), and that a variety of other ligands can bind to glycosphingolipids (Kohn, 1978; Critchley *et al.*, 1979a; Hakomori, 1981) strongly suggests that the diverse carbohydrate structures associated with these molecules may indeed have important receptor functions.

One approach to establishing the significance of loss of the more complex glycosphingolipids from transformed cells would be to select mutants defective in glycosphingolipid biosynthesis. Such mutants would be expected to show only those characteristics of the transformed phenotype (if any) associated with loss of complex glycosphingolipids. If cholera toxin receptor activity was shown to be restricted to a ganglioside (G_{M1}), it might prove a useful selective agent in isolation of cells lacking

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; phosphate-buffered saline, 137 mM-NaCl/2.79 mM-KCl/8 mM-Na₂HPO₄/1.53 mM-KH₂PO₄, adjusted to pH 7.2; phosphate-buffered saline (Ca²⁺, Mg²⁺), as for phosphate-buffered saline, but containing in addition 0.9 mM-CaCl₂·2H₂O and 0.5 mM-MgCl₂·6H₂O.

complex gangliosides as we have previously outlined (Critchley *et al.*, 1979b). To investigate this approach we have chosen a cell line Balb/c 3T3, which has a large number of receptors for cholera toxin, and which on transformation by SV40 or polyoma virus is reported to show reduced synthesis of complex gangliosides, including ganglioside G_{M1} (Brady & Mora, 1970). However, before undertaking the mutant selection programme we felt it necessary to establish directly that the cholera toxin receptor in these cells was exclusively ganglioside (G_{M1}) for the following reasons. (a) Although there is no doubt that cholera toxin binds to ganglioside G_{M1} (Cuatrecasas, 1973; Holmgren *et al.*, 1973), the evidence that ganglioside G_{M1} acts as the cell-surface receptor is almost entirely indirect (Fishman *et al.*, 1976, 1978; Moss *et al.*, 1977) and the possibility that the receptor activity might be carried on a glycoprotein has not been rigorously excluded. (b) The cholera toxin receptor on rat intestinal brush borders has indeed been reported to be a heterogeneous collection of galactoproteins (Morita *et al.*, 1980). (c) Anti-ganglioside (G_{M1}) antibodies reportedly cross-react with galactoproteins on the surface of Balb/c 3T3 cells (Tonegawa & Hakomori, 1977). (d) It is known that antigenic determinants of the ABH blood group system (Fukuda *et al.*, 1979) and Forssman activity (Slomiany *et al.*, 1981) can be found on both glycosphingolipids and glycoproteins. (e) Some evidence that similar carbohydrate sequences are frequently present on both types of molecule has been presented (Rauvala & Finne, 1979). (f) Evidence that the glycoprotein hormone receptors involve both glycosphingolipids and glycoproteins has been reported (Mullin *et al.*, 1976; Kohn, 1978), although this has been disputed (Pacuszka *et al.*, 1978).

In this paper we now provide definitive evidence that the cholera toxin receptor on Balb/c 3T3 cells is a ganglioside with characteristics compatible with ganglioside G_{M1} , and that galactoproteins do not significantly contribute to receptor activity.

Materials and methods

Cells

Balb/c 3T3 cells were maintained at 37°C in Dulbecco's modified Eagle's medium (Gibco Bio-Cult) containing 10% heat-inactivated calf serum.

Iodination of cholera toxin and ricin

Cholera toxin (Schwarz-Mann, Orangeburg, NY, U.S.A.) was iodinated as previously outlined (Streuli *et al.*, 1981). Ricin (RCA₆₀; Miles Biochemicals) was iodinated as described by Burrige (1978).

Binding of ^{125} I-labelled cholera toxin

(a) *To cells.* Subconfluent monolayers were scraped into phosphate-buffered saline containing 1 mM-PMSF and washed once with the same buffer. Cells (6×10^4) were incubated with ^{125} I-labelled cholera toxin in 200 μ l of phosphate-buffered saline/PMSF/0.1% bovine serum albumin for 30 min at 20°C. The samples were filtered under vacuum on 1.0 μ m Millipore EAWP filters. Non-specific binding was determined in the presence of unlabelled 0.1 μ M toxin, and represented <10% of total radioactivity bound. Binding was proportional to cell concentration over the range 0.5×10^4 – 1×10^5 cells/assay.

(b) *To crude membranes.* Membranes were incubated with 300 000 c.p.m. of ^{125} I-labelled cholera toxin (3 nM) in 200 μ l of Tris-buffered saline (25 mM-Tris/135 mM-NaCl/1 mM-EDTA, pH 7.4) containing 0.1% bovine serum albumin for 20 min at 20°C. The samples were filtered under vacuum on 0.2 μ m Millipore EGWP filters. Each sample was assayed in duplicate at two membrane protein concentrations (15 μ g and 30 μ g) to ensure that the assay was quantitative. Non-specific binding was determined in the presence of unlabelled 0.1 μ M toxin. Binding was proportional to membrane protein concentration over the range 10–40 μ g.

Preparation of a crude membrane fraction from Balb/c 3T3 cells

Cell pellets stored at –20°C were thawed in phosphate-buffered saline/1 mM-PMSF, sonicated for 30 s on ice and undisrupted cells and nuclei were pelleted for 5 min at 2°C and 500 g (r_{av} , 12 cm). For delipidation and neuraminidase studies the supernatant was then centrifuged for 30 min at 2°C and 100 000 g (r_{av} , 5.35 cm) to pellet particulate material. For trypsin- and proteinase-treatment studies the supernatants were spun in a Beckman microfuge for 10 min at 4°C to pellet only the larger particulate material. Trypsin or proteinase treatment of membranes leads to a reduction in size of the particles to an extent where they are not quantitatively retained by 0.2 μ m filters. Unless only those membranes that can be pelleted in the microfuge are used, trypsin (50 and 200 μ g/ml) produces an apparent 20 and 30% reduction respectively in the ability of the membranes to bind ^{125} I-labelled cholera toxin.

Surface-labelling procedures

Terminal galactose or *N*-acetylgalactosamine residues in cell-surface glycosphingolipids and glycoproteins were labelled with ^{3} H by incubating confluent cell monolayers with galactose oxidase (Worthington Biochemicals) followed by reduction with NaB^3H_4 (Critchley, 1974). Cell-surface sialic acid residues were similarly labelled with ^{3} H after

incubation of cell monolayers with 5 mM-NaIO₄ in phosphate-buffered saline/PMSF (Critchley *et al.*, 1976).

Metabolic labelling

Proteins were labelled by incubating sub-confluent monolayers overnight in methionine-free medium (plus 10% calf serum) containing 1–5 μ Ci of [³⁵S]methionine/ml (sp. radioactivity > 600 Ci/mmol). Monolayers were washed twice with phosphate-buffered saline/PMSF and harvested by scraping. Specific radioactivities of 2×10^7 c.p.m./mg of cell protein were routinely achieved by using this procedure. Phospholipids and glycosphingolipids were labelled by growing cells in the presence of 1 μ Ci of [1-¹⁴C]palmitate/ml (sp. radioactivity 50–60 mCi/mol) for 48 h as described previously (Critchley & Macpherson, 1973).

Isolation of the cholera toxin receptor by immune adsorption

The cholera-toxin receptor was isolated from cells labelled by one of these procedures as previously described (Critchley *et al.*, 1979b). In brief, cholera toxin was bound to labelled cells, which were then lysed (1–3 mg of cell protein/ml) in 1% (v/v) Nonidet P40 in NET buffer (150 mM-NaCl/5 mM-EDTA/50 mM-Tris, pH 8.0)/PMSF, and the suspension centrifuged for 1 h at 2°C and 100 000 g (r_{av} . 5.35 cm). Portions of the Nonidet P40 extract were incubated with an excess of rabbit anti-toxin or pre-immune serum for 60 min at 0°C, and for a further 30 min after addition of a 10% suspension of a protein A-containing strain of *Staphylococcus aureus* (Kessler, 1975). The bacteria were pelleted in a microfuge for 2 min, and the pellet was subsequently washed three to five times either with 0.05% (v/v) Nonidet P40/NET buffer/0.2% (w/v) gelatin ([1-¹⁴C]palmitate or galactose oxidase/NaB³H₄ experiments) or with 1% Triton/1% deoxycholate/0.1% sodium dodecyl sulphate in NET buffer (Rohrschneider *et al.*, 1979) ([³⁵S]methionine experiments). Proteins adsorbed to the bacterial pellets were solubilized by boiling for 3 min with 9 M-urea/1% sodium dodecyl sulphate/1% mercaptoethanol in 0.15 M-Tris, pH 6.8. Labelled proteins were separated in sodium dodecyl sulphate/polyacrylamide gels (Laemmli, 1970) and detected by fluorography (Laskey & Mills, 1975). Bound lipids were extracted into chloroform/methanol and the gangliosides purified as described below.

Ganglioside purification

Lipids were extracted from cells using 20 vol. of chloroform/methanol (2:1, v/v) and the residue was re-extracted twice with chloroform/methanol (1:2, v/v). The extract was reduced to dryness under N₂, redissolved in chloroform/methanol (2:1, v/v) and

partitioned against 0.2 vol. of water. The organic phase was washed three times with theoretical upper-phase chloroform/methanol/water (3:48:47, by vol.), and the combined upper phases were dried and saponified with 0.1 M-NaOH in methanol for 4 h at 20°C. Samples were neutralized with acetic acid, and desalted by passage through Sephadex G-25 (superfine), equilibrated and eluted with chloroform/methanol/water (4:2:3, by vol.) (Fishman *et al.*, 1977). Gangliosides were separated by t.l.c. on pre-coated Silica gel G plates (Merck) with the solvent chloroform/methanol/water (60:35:8, by vol.) and detected by autoradiography. Standards [mixed brain gangliosides (Sigma Chemical Co.) and purified gangliosides (Supelco, Bellefonte, PA, U.S.A.)] were detected visually with the resorcinol reagent (Svennerholm, 1957).

Binding of ¹²⁵I-labelled cholera toxin to gangliosides of Balb/c 3T3 cells

Total cellular lipids or more usually a purified ganglioside fraction were separated on plastic-backed silica gel chromatography sheets (10 cm × 10 cm; Eastman Kodak) with chloroform/methanol/water (60:35:8, by vol.) as solvent. After drying, the sheets were overlaid with 5–7 ml of phosphate-buffered saline containing 1% polyvinylpyrrolidone [poly(vinylpyrrolidin-2-one)] followed by ¹²⁵I-labelled cholera toxin (2×10^6 c.p.m./ml) in the same buffer, as described by Magnani *et al.* (1980). The sheets were incubated for between 40 and 120 min at 4°C, washed three times with phosphate-buffered saline containing 1% polyvinylpyrrolidone, dried and autoradiographed. The specificity of ¹²⁵I-labelled toxin binding was established by pre-incubating sheets with 5 μ g of unlabelled toxin/ml.

Visual detection of cholera toxin and ricin-binding molecules after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Cholera toxin and ricin-binding molecules of Balb/c 3T3 cells were detected in sodium dodecyl sulphate polyacrylamide gels essentially as described by Burridge (1978). Gel slices were overlaid with Tris/saline buffer (0.15 M-NaCl/50 mM-Tris/0.05% NaN₃, at pH 7.5)/0.1% bovine serum albumin containing either 2×10^6 c.p.m./ml of cholera toxin (sp. radioactivity approx. 10 Ci/g) or 8×10^6 c.p.m./ml of ¹²⁵I-labelled ricin (sp. radioactivity 4 Ci/g).

Results

Binding of cholera toxin to Balb/c 3T3 cells

Studies of the binding of ¹²⁵I-labelled cholera toxin to Balb/c 3T3 cells suggested that there was a single class of high-affinity receptor site (Figs. 1a and 1b). Saturation of binding was observed at 1 nM and there

Fig. 1. Binding of ¹²⁵I-labelled cholera toxin to Balb/c 3T3 cells

(a) 6×10^4 cells were incubated with increasing concentrations of ¹²⁵I-labelled cholera toxin (95 c.p.m./fmol) in the presence or absence of unlabelled 0.1 μ M toxin. (b) Cells were incubated with 0.34 nM-¹²⁵I-labelled cholera toxin (510 c.p.m./fmol) in the presence of increasing concentrations of unlabelled toxin. In both (a) and (b) binding was determined as described in the Materials and methods section and each point represents the mean of triplicate determinations, the bars representing the range.

was no evidence for a gradual increase in toxin binding above this concentration (Fig. 1a). Half-maximal binding occurred at 0.2 nM. In addition binding of ¹²⁵I-labelled cholera toxin (0.34 nM) was inhibited >90% by unlabelled 10 nM toxin (Fig. 1b). The data does not support the idea of a second class of receptor site for cholera toxin, present in large numbers, but with lower affinity. At saturation $(0.8-1.6) \times 10^6$ molecules bound per cell or 2-4 pmol/mg of protein. Treatment of a crude membrane fraction from Balb/c 3T3 cells with trypsin (20 or 100 μ g/ml) or proteinase (20 μ g/ml) for 30 min at 37°C actually increased toxin binding, as did neuraminidase (Table 1). In contrast delipidation quantitatively abolished the ability of the membrane to bind toxin. The data lend support to the concept that the cholera toxin receptor is exclusively lipid in nature, and does not support the idea of a significant number of receptors that are proteinaceous.

Effect of cholera toxin on labelling of ganglioside G_{M1} by the galactose oxidase/NaB³H₄ method

To obtain more direct evidence that ganglioside G_{M1} is the cholera toxin receptor, the ability to label the terminal galactose residue of cell-surface ganglioside G_{M1} using galactose oxidase/NaB³H₄ was studied in the presence and absence of cholera toxin. Fig. 2(b) shows that a ganglioside with chromatographic properties of G_{M1} can be labelled at the surface of Balb/c 3T3 cells in the presence of galactose oxidase. Labelling in the absence of enzyme was largely restricted to a component that travelled close to the solvent front (Fig. 2a). Labelling of ganglioside G_{M1} by galactose oxidase/NaB³H₄ was quantitatively abolished if the cells were first pre-incubated with 60 nM cholera toxin (Fig. 2c). Although the results strongly support the idea that the toxin receptor in Balb/c 3T3 cells is ganglioside G_{M1}, the possibility that the above result was due to steric hindrance cannot be excluded.

Identification of the cholera toxin receptor by immunoadsorption

To provide more direct evidence concerning the involvement of ganglioside G_{M1} in toxin binding we have attempted to isolate the toxin receptor using immunochemical means. In brief, cells labelled by the galactose oxidase/NaB³H₄ method were incubated with cholera toxin, putative toxin-receptor complexes solubilized in 1% Nonidet P40 and, after addition of toxin antibodies, the immune complexes were adsorbed on to protein A-containing strains of *S. aureus*. To increase the amount of ganglioside G_{M1} available to galactose oxidase and cholera toxin, cells were preincubated with neuraminidase. Figs. 3(b) and 3(c) show that ganglioside G_{M1} was labelled in the presence but not in the absence of galactose

Table 1. *Effect of various treatments on the binding of ^{125}I -labelled cholera toxin to a crude membrane fraction from Balb/c 3T3 cells*

Crude membranes (1 mg) were incubated in 1 ml of 0.1 M-Tris, pH 7.8, containing 11 mM- Ca^{2+} with trypsin (twice-crystallized; bovine pancreas; Sigma Chemical Co.) or proteinase (from *Streptomyces griseus*; Sigma) for 30 min at 37°C. Soya-bean trypsin inhibitor (100 µg) plus 1 mM-PMSF were added to all tubes, and the membranes were pelleted in a Beckman microfuge for 10 min at 4°C. The membranes were washed twice with Tris-buffered saline and resuspended in 200 µl of the same buffer. Portions were assayed for ^{125}I -labelled cholera toxin binding and protein as described in the Materials and methods section. Membranes (0.5 mg) were treated with 0.01 unit of neuraminidase (Behringwerke, Marburg, Germany) in 0.5 ml of phosphate-buffered saline (Ca^{2+} , Mg^{2+}) for 60 min at 37°C, and assayed for ^{125}I -labelled cholera toxin binding without subsequent washing. Membranes (2 mg) were delipidated by extraction three times with chloroform/methanol (1:2, v/v), the second and third extractions being at 37°C and 45°C respectively. The residue was dispersed by sonication in Tris-buffered saline before assay for protein and ^{125}I -labelled toxin binding. Results are means $\pm$ S.D.

Treatment	^{125}I -labelled cholera toxin binding	
	(fmol/µg of protein)	(% of control)
(a) Control	3.16 $\pm$ 0.34 (4)	100
Trypsin (20 µg/ml)	4.70 $\pm$ 0.40 (4)	149
Trypsin (100 µg/ml)	4.50 $\pm$ 0.22 (4)	142
Proteinase (20 µg/ml)	5.40 $\pm$ 0.74 (4)	171
(b) Control	4.99 $\pm$ 0.41 (4)	100
Neuraminidase (0.02 units/ml)	10.05 $\pm$ 1.0 (4)	201
Delipidation	0.00 (4)	0

oxidase, and that [^3H]ganglioside G_{M1} could be isolated from a detergent extract of such cells by using the immunoadsorption procedure outlined above. [^3H]Ganglioside G_{M1} did not adsorb to *S. aureus* if cholera toxin was omitted from the isolation scheme (results not shown) or if rabbit anti-(cholera toxin) was replaced with pre-immune serum (Fig. 3c). This approach provides direct evidence that ganglioside G_{M1} acts as a cholera toxin receptor at the surface of Balb/c 3T3 cells. Comparison of the labelling profile using the galactose oxidase/ NaB^3H_4 methodology with $\text{IO}_4/\text{B}^3\text{H}_4$ labelling of sialic acid residues shows ganglioside G_{M1} to be the only ganglioside labelled extensively by the former technique (Figs. 3a and 3b). Therefore, although the immunoadsorption experiments using cells labelled by the galactose oxidase/ NaB^3H_4 method establish that ganglioside G_{M1} can act as a cell-surface receptor for cholera toxin, they do not establish whether other gangliosides might act in a similar capacity.

To clarify this point immunoadsorption experiments were carried out on cells metabolically labelled with [$1\text{-}^{14}\text{C}$]palmitate. Analysis of the total ganglioside profile of Balb/c 3T3 cells showed G_{M3} and G_{D1a} to be the major ganglioside species, G_{M1} being a relatively minor component (Fig. 4a). Cholera toxin was bound to the surface of such metabolically labelled cells and toxin receptors isolated from a 1% Nonidet P40 cell extract using immunoadsorption. Again ganglioside G_{M1} was the

only ganglioside specifically adsorbed using this procedure (Figs. 4d and 4e), the amount isolated being greater if the cells were first incubated with neuraminidase (Figs. 4b and 4c). There was no adsorption of ganglioside G_{M1} if pre-immune rabbit serum was used, or if cells were not exposed to cholera toxin. Although ganglioside G_{M3} adsorbed non-specifically to *S. aureus*, the levels present did increase under conditions where ganglioside G_{M1} was specifically adsorbed, although the extent of the increase was somewhat variable.

Specificity of cholera toxin-ganglioside G_{M1} interaction

To clarify more precisely the specificity of binding of cholera toxin to various gangliosides, lipid extracts of Balb/c 3T3 cells were chromatographed on silica-gel-coated plastic sheets, which were then overlaid with ^{125}I -labelled cholera toxin as described by Magnani *et al.* (1980). After washing, bound ^{125}I -labelled toxin was detected by autoradiography. Toxin was restricted to a component with a similar mobility to ganglioside G_{M1} (Figs. 5a-5f). There was no labelling of G_{M3} or G_{D1a} , quantitatively the major gangliosides of Balb/c 3T3 cells, and there was no significant labelling at the origin, as would be expected if a toxin-binding protein had been extracted by organic solvents. Labelling of ganglioside G_{M1} by ^{125}I -labelled toxin appeared to be specific in that it was abolished by pre-incubation of the silica gel sheet with unlabelled

Fig. 2. Effect of cholera toxin on labelling of cell-surface ganglioside G_{M1} on Balb/c 3T3 cells by the galactose oxidase/ NaB^3H_4 method

Confluent monolayers of Balb/c 3T3 cells were incubated with or without cholera toxin (60 nM) for 30 min at 37°C in phosphate-buffered saline (Ca^{2+} , Mg^{2+})/1 mM-PMSF, followed by a 3 h incubation after the addition of galactose oxidase (20 units/ml). The cells were labelled with ^3H by addition of NaB^3H_4 in suspension, and the gangliosides were purified and separated by t.l.c. as described in the Materials and methods section. After visual detection of the ganglioside standards with I_2 vapour, 0.25 cm divisions were marked on the t.l.c. plate starting below the origin (O), and the silica gel

toxin (Fig. 5g). There thus seems no doubt that ganglioside G_{M1} does act as a cholera toxin receptor on the surface of Balb/c 3T3 cells, and we therefore turned our attention to establishing whether or not galactoproteins also bind cholera toxin in these cells.

Immunochemical approach to the identification of proteins that bind cholera toxin

By using the immunochemical approach outlined, we had previously found that a galactoprotein (mol.wt. 84 000) could be isolated from Balb/c 3T3 cells labelled by the galactose oxidase/ NaB^3H_4 method under conditions where ganglioside G_{M1} was specifically adsorbed (Critchley *et al.*, 1979b). However, the galactoprotein contained <10% of the label specifically adsorbed. In an attempt to extend this observation further we used cells labelled with [^{35}S]methionine because of the ease of labelling total cellular protein to high specific activity. We obtained no evidence that a protein of mol.wt. 84 000, or indeed any other cellular protein, could be specifically adsorbed to *S. aureus* from Nonidet P40 extracts of [^{35}S]methionine-labelled cells in the presence of cholera toxin and antitoxin. The result was identical whether the toxin was bound to the cell before Nonidet P40 extraction, or the toxin added directly to the detergent extract. However, the approach is dependent on the ability to solubilize putative toxin-receptors in detergent, and during the course of this work it became clear that much of the toxin bound to cells before detergent extraction remained associated with material that could be sedimented at 100 000g (Streuli *et al.*, 1981). The possibility that this material contained toxin receptor activity, possibly galactoprotein in nature, had therefore to be considered.

Effect of cholera toxin on labelling of galactoproteins by the galactose oxidase/ NaB^3H_4 method

In an attempt to provide definitive evidence for or against such a galactoprotein able to bind cholera toxin, we used two additional approaches that did not rely on the ability to solubilize toxin-receptor complexes in neutral detergents. First as cholera toxin effectively blocked labelling of cell surface G_{M1} by the galactose oxidase/ NaB^3H_4 procedure (Fig. 2), we used the delipidated residues from the same experiments to look for evidence for a similar protection of galactoprotein labelling. Fig. 6(c) shows that a number of cell-surface galactoproteins

transferred to vials for scintillation counting. (a) Labelling by B^3H_4 in the absence of galactose oxidase; (b) labelling in the presence of galactose oxidase; (c) labelling as in (b), but the cells were first incubated with cholera toxin.

Fig. 3. Isolation of the cholera toxin receptor from Balb/c 3T3 cells labelled by the galactose oxidase/ NaB^3H_4 method by immune adsorption

Cells ($10\text{cm} \times 10\text{cm}$ dishes; approx. 2×10^7 cells) incubated in monolayer with neuraminidase (0.02 units/ml) and galactose oxidase (10 units/ml) for 3 h at 37°C were labelled with ^3H by reduction with NaB^3H_4 . Labelled cells were incubated for 30 min at 20°C in 1 ml of phosphate-buffered saline/PMSF/0.1% bovine serum albumin containing $0.6\text{ }\mu\text{M}$ cholera toxin. ^3H -labelled receptor-toxin complexes were isolated from 1% Nonidet P40 cell extracts by immune adsorption as described in the Materials and methods section. Bacterial pellets were extracted with chloroform/methanol, and the purified gangliosides were separated by t.l.c. on Silica gel G with chloroform/methanol/water (60:35:8, by vol.) as solvent. 1 cm divisions were marked on the t.l.c. plate starting below the origin (left-hand side), and the silica gel transferred to vials for scintillation counting. Cells (neuraminidase-treated) labelled either by the galactose oxidase or $\text{IO}_4^-/\text{NaB}^3\text{H}_4$ methods were also directly analysed to give the ganglioside-labelling patterns of the whole

were also labelled by the galactose oxidase/ NaB^3H_4 procedure, and that in the absence of enzyme, labelling was restricted to a protein with a mol.wt. of 55 000 (Fig. 6b). Although labelling of ganglioside $\text{G}_{\text{M}1}$ was inhibited in the presence of cholera toxin (Fig. 2), the same preparations showed no inhibition of labelling of cell-surface galactoproteins (Fig. 6d). That delipidation did not lead to a significant alteration in galactoprotein profile compared with that of the whole cell is shown in Fig. 6(e). The only major difference between the two preparations was the presence of a band below the dye front in unextracted cells, which probably represented labelled lipid.

Identification of the cholera toxin receptor in Balb/c 3T3 cells using sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and a ^{125}I -labelled toxin overlay technique

Our second approach was to separate total cell protein by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and to overlay the gels with ^{125}I -labelled cholera toxin. After extensive washing, toxin-binding components were identified by autoradiography. Fig. 7(a) shows that all of the bound toxin was localized in a region around the marker-dye front, and that the labelling was almost completely abolished by pre-incubating the gel with an excess of unlabelled toxin Fig. 7(b). The major cellular galactoproteins localized by overlaying with ^{125}I -labelled ricin (mol.wt. 95 000–110 000; Fig. 7c) were similar in size to the major galactoproteins labelled by the galactose oxidase/ NaB^3H_4 procedure. None of these galactoproteins were able to bind cholera toxin. Labelling of the component at the dye front by ^{125}I -labelled toxin was decreased by 99% if the cells were first delipidated (Fig. 7d), whereas labelling of galactoproteins by ^{125}I -labelled ricin was unaffected (results not shown). Analysis of the chloroform/methanol extract by the sodium dodecyl sulphate/polyacrylamide-gel electrophoresis ^{125}I -labelled toxin overlay technique showed all the toxin binding capacity again localized at the dye front (results not shown), the region of the gel

cell. (a) Gangliosides of neuraminidase-treated cells labelled by NaB^3H_4 in the presence (●) and absence (▲) of IO_4^- . (b) Gangliosides of neuraminidase-treated cells labelled with ^3H by addition of NaB^3H_4 with (●) or without (▲) exposure to galactose oxidase. (c) Analysis of gangliosides isolated by the immune adsorption method outlined in the text, from cells labelled as in (b) (●, in the presence of rabbit anti-toxin; ▲, with pre-immune serum from the same animal).

where ganglioside G_{M1} has previously been shown to migrate (Critchley *et al.*, 1979b).

Fig. 4. Isolation of the cholera toxin receptor from $[1-^{14}\text{C}]$ palmitate-labelled Balb/c 3T3 cells

$[1-^{14}\text{C}]$ Palmitate-labelled cells (2.4×10^7) were incubated in suspension (0.5 ml of phosphate-buffered saline/PMSF/0.1% bovine serum albumin) with $0.6 \mu\text{M}$ -cholera toxin (20 min at 20°C) and unbound toxin was removed by washing. The cholera toxin receptor was isolated by immune adsorption (see the Materials and methods section). Labelled cells (1.2×10^7) were also treated in monolayer with neuraminidase (0.02 units/ml) for 60 min, and then processed as above. Lipids were extracted from the bacterial pellets and the gangliosides were purified, separated and detected as described in the Materials and methods section. (a) Gangliosides from $[1-^{14}\text{C}]$ palmitate-labelled cells. (b and c) Gangliosides from Nonidet P40 extracts of neuraminidase-treated $[1-^{14}\text{C}]$ palmitate-labelled cells adsorbed on to bacterial pellets in the presence of pre-immune rabbit serum (b) and rabbit anti-toxin (c). (d and e) Gangliosides from Nonidet P40 extracts of $[1-^{14}\text{C}]$ palmitate-labelled cells adsorbed on to bacterial pellets in the presence of pre-immune rabbit serum (d) or rabbit anti-toxin (e). Note that twice as many cells were used for (d) and (e) compared with (b) and (c). It should be noted that gangliosides typically resolve into doublets on t.l.c., probably owing to heterogeneity in the ceramide moiety and/or the presence of the *N*-acetyl and *N*-glycolyl forms of neuraminic acid in the carbohydrate moiety.

Fig. 5. Demonstration of the specificity of cholera toxin for a ganglioside from Balb/c 3T3 cells with the mobility of ganglioside G_{M1}

Total lipid extracts or purified gangliosides from Balb/c 3T3 cells were chromatographed on plastic-backed silica gel G plates and overlaid with ^{125}I -labelled cholera toxin as described in the Materials and methods section. After washing, the sheets were air-dried and autoradiographed. Standards were visually detected by spraying with the resorcinol reagent. (a) Ganglioside G_{M1} (10 ng); (b)–(e) purified gangliosides from 2×10^4 , 1×10^5 , 2×10^5 and 4×10^5 cells respectively; (f) total lipid extract from 10^4 cells; (g) gangliosides from 4×10^5 cells (equivalent to e), but pre-incubated with $10 \mu\text{g}$ of unlabelled toxin before overlay with ^{125}I -labelled cholera toxin.

Finally the possibility that potential cholera toxin receptors exist on the surface of Balb/c 3T3 but are masked by sialic acid residues was considered. Treatment of cells with neuraminidase before sodium dodecyl sulphate/polyacrylamide-gel electrophoresis increased the levels of ^{125}I -labelled cholera toxin binding to the dye front approx. 2-fold (Figs. 7e and 7f). (In this experiment multiple bands of toxin binding activity appeared just behind the dye front, which we believe to be an artefact due to sample overloading. Such banding was quantitatively abolished by lipid extraction.) Neuraminidase treatment resulted in an even greater increase in the labelling of galactoproteins by ^{125}I -labelled ricin (Figs. 7g and 7h), but none of these were able to bind cholera toxin.

Discussion

The binding characteristics of ^{125}I -labelled cholera toxin to Balb/c 3T3 cells clearly show that there is a single class of high-affinity receptor that is resistant to proteolysis but quantitatively extracted by chloroform/methanol. This result provides immediate evidence that the toxin receptor is predominantly lipid in nature. The possibility that the extraction procedure has denatured receptor activity asso-

Fig. 6. Analysis of the effect of cholera toxin on galactose oxidase/ NaB^3H_4 labelling of galactoproteins on the surface of Balb/c 3T3 cells

Cells were labelled under the conditions specified in the legend to Fig. 2. Proteins were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (7% gels) and labelled proteins detected by fluorography. (a) Coomassie Blue stain of total cell protein; (b) NaB^3H_4 labelling in the absence of galactose oxidase; (c) galactose oxidase/ NaB^3H_4 labelling of Balb/c 3T3 cells; (d) as for (c), except cells were pre-incubated with cholera toxin. Tracks (b)–(d) show results for the delipidated protein residues from the experiment shown in Fig. 2. Specific radioactivities were as follows: (b), 2.35×10^5 c.p.m./mg; (c), 1.42×10^6 c.p.m./mg; (d), 1.46×10^6 c.p.m./mg of cell protein. (e) was as for (c) except cells were not delipidated before analysis of galactoprotein labelling profile. (f) ^{14}C -labelled proteins molecular-weight markers. For tracks (a)–(e) 100 μg of cell protein was applied.

Fig. 7. Separation of the cholera toxin and ricin binding molecules of Balb/c 3T3 cells by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

After sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (7%) of Balb/c 3T3 cell proteins and lipids (50 μg of protein equivalents per track), gels were overlaid with ^{125}I -labelled cholera toxin or ^{125}I -labelled ricin, washed, dried and autoradiographed as described in the Materials and methods section. (a) Overlaid with ^{125}I -labelled cholera toxin; (b) as (a), except gel track first incubated with 5 μg of unlabelled cholera toxin/ml; (c) overlaid with ^{125}I -labelled ricin; (d) 50 μg of delipidated Balb/c 3T3 cell protein overlaid with ^{125}I -labelled cholera toxin; (e) overlaid with ^{125}I -labelled cholera toxin; (f) same as (e) except cells pretreated with neuraminidase (0.02 units/ml) for 60 min at 37°C ; (g and h) equivalent to (e) and (f) except gel overlaid with ^{125}I -labelled ricin. The numbers refer to molecular weight ($\times 10^{-3}$). Those on the left refer to tracks (a)–(d), those on the right to tracks (e)–(h).

ciated with the carbohydrate moiety of a glycoprotein is considered unlikely, as such treatment has previously been found to have no effect on binding of ^{125}I -labelled ricin to membrane glycoproteins (Critchley *et al.*, 1981). The observed increase in binding of cholera toxin to trypsin- and proteinase-treated membranes expressed on a per μg of protein basis would also be expected if the receptor is mainly a lipid, because of the substantial release of protein from the membranes. Further, the increase in binding of toxin after neuraminidase treatment can readily be explained by conversion of ganglioside G_{D1a} into G_{M1} , the putative toxin receptor, an event previously shown to occur when intact

Balb/c 3T3 cells are incubated with neuraminidase (Critchley & Vicker, 1977). However, it might also be due to exposure of a similar carbohydrate sequence in glycoproteins.

The following evidence strongly supports the conclusion that the major receptor for cholera toxin in Balb/c 3T3 cells is indeed ganglioside G_{M1} . First, labelling by the galactose oxidase/ NaB^3H_4 method of a cell-surface ganglioside with chromatographic properties similar to G_{M1} is inhibited if the cells are first pre-incubated with cholera toxin. The data confirm the previous observation of Fishman and his co-workers in other cell systems (Moss *et al.*, 1977). Secondly, analysis of the cholera toxin receptor isolated by immune adsorption from cells labelled

both by the galactose oxidase/ NaB^3H_4 method and the $[1\text{-}^{14}\text{C}]$ palmitate method provides direct evidence that a ganglioside similar to, or identical with, G_{M1} acts as the cholera toxin receptor at the surface of Balb/c 3T3 cells. Finally, the specificity of the toxin for ganglioside G_{M1} compared with quantitatively the major ganglioside species of these cells, G_{M3} and G_{D1a} , was confirmed by overlaying the gangliosides separated on t.l.c. plates with ^{125}I -labelled toxin.

In the present study we have found no evidence that cell-surface galactoproteins constitute even a minor species of the cholera-toxin receptor. Although cholera toxin protected ganglioside G_{M1} from labelling by the galactose oxidase/ NaB^3H_4 method, there was no evidence that galactoproteins were similarly protected. Analysis of the data is required using two-dimensional sodium dodecyl sulphate/polyacrylamide-gel electrophoresis to improve the limited resolution of the one-dimensional separation. An additional problem with such experiments is that heterogeneity amongst the carbohydrate residues might conceivably generate glycoproteins in which only one of the oligosaccharide units contains the correct sequence specifying toxin receptor activity, although other units may end in terminal galactose. Such a glycoprotein would still be heavily labelled by the galactose oxidase/ NaB^3H_4 procedure, even though it might act as a toxin receptor. However, we have two pieces of evidence that tend to exclude this possibility. First, no toxin-binding glycoprotein could be detected when Balb/c 3T3 cell proteins were separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and the gel overlayed with ^{125}I -labelled cholera toxin. Binding of the ^{125}I -labelled toxin was restricted to the marker dye front, a pattern that was quantitatively abolished if the cells were first delipidated before electrophoresis. In contrast the delipidation procedure did not significantly affect the labelling of the major cellular galactoproteins by ^{125}I -labelled ricin. In addition there was no evidence that potential toxin-binding sites on cell-surface glycoproteins were masked by sialic acid residues. Whereas neuraminidase treatment of intact cells led to a major increase in levels of glycoproteins with terminal galactose residues as detected by labelling with ^{125}I -labelled ricin, the increase in ^{125}I -labelled cholera toxin-binding was again restricted to the dye front.

Secondly no such toxin-binding glycoproteins could be identified in immune adsorption experiments with $[^{35}\text{S}]$ methionine-labelled cells. However, using cells labelled by the galactose oxidase/ NaB^3H_4 procedure, we have previously reported the apparent specific immune adsorption of low levels of the major galactoproteins from Nonidet P40 extracts of Balb/c 3T3 cells and a mouse lymphoblastoid cell line in the presence of cholera toxin and anti-toxin

(Critchley *et al.*, 1979b). One explanation for this discrepancy is that labelling specifically cell-surface galactoproteins, rather than total cellular proteins, increases the sensitivity of the method such that lower levels of proteins with toxin-binding activity can be detected. The fact that the same toxin-binding glycoproteins were not detected by the ^{125}I -labelled toxin/gel overlay technique may also be due to the limited sensitivity of the method. An alternative explanation previously considered in some detail (Critchley *et al.*, 1979b) is that mixed micelles containing ganglioside G_{M1} -toxin complexes plus other contaminating components may be formed during the solubilization process. Such components would also be adsorbed on to the *S. aureus* in the presence of anti-toxin, along with the true toxin receptor. It might also account for (1) the increase in adsorption of ganglioside G_{M3} from Nonidet P40 extracts of $[1\text{-}^{14}\text{C}]$ palmitate-labelled cells in the presence of anti-toxin, as ganglioside G_{M3} clearly does not bind cholera toxin, and (2) the apparent cross-reactivity of anti-(ganglioside G_{M1}) antibodies with galactoproteins of Balb/c 3T3 cells (Tonegawa & Hakomori, 1977). In experiments with $[^{35}\text{S}]$ -methionine-labelled cells we therefore washed the adsorbed immune complexes more rigorously (see the Materials and methods section) in an attempt to extract non-specifically adsorbed material, and there was no evidence for the specific adsorption of any labelled protein in the presence of toxin and anti-toxin.

In summary, although the ability of cholera toxin to bind to ganglioside G_{M1} in solution (Schwarzmann *et al.*, 1978) and to G_{M1} inserted into cells (Moss *et al.*, 1976, 1977) and liposomes (Fishman *et al.*, 1979) has previously been clearly demonstrated, the chemical nature of the endogenous receptor has never been rigorously investigated. In the present study we have provided direct evidence that the major receptor for cholera toxin in Balb/c 3T3 cells is indeed a ganglioside with chromatographic and labelling properties compatible with G_{M1} . We have found no evidence that galactoproteins contribute in any substantial way to the toxin-binding capacity of Balb/c 3T3 cells, although we cannot, and do not, exclude the possibility that a minor population of galactoproteins with toxin-binding activity exist in these cells. By using techniques similar to those described here, we have also recently presented strong evidence that the predominant species of toxin receptor in rat intestinal brush borders is again ganglioside G_{M1} (Critchley *et al.*, 1981). Although we were able to identify putative toxin-binding galactoproteins by immunochemical means, confirming in part a previous report by Morita *et al.* (1980), our results do not suggest that they contribute in a major way (if at all) to the toxin-binding capacity of this system.

However, the possibility that receptor activity might be carried by glycoproteins in other cell types should not be ignored (Hakomori, 1981).

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