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Comparison of the glycolipid-binding specificities of cholera toxin and porcine *Escherichia coli* heat-labile enterotoxin: identification of a receptor-active non-ganglioside glycolipid for the heat-labile toxin in infant rabbit small intestine

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The binding specificities of cholera toxin and *Escherichia coli* heat-labile enterotoxin were investigated by binding of ^{125}I-labelled toxins to reference glycosphingolipids separated on thin-layer chromatograms and coated in microtitre wells. The binding of cholera toxin was restricted to the GM1 ganglioside. The heat-labile toxin showed the highest affinity for GM1 but also bound, though less strongly, to the GM2, GD2 and GD1b gangliosides and to the non-acid glycosphingolipids gangliotetraosylceramide and lactoneotetraosylceramide. The infant rabbit small intestine, a model system for diarrhoea induced by the toxins, was shown to contain two receptor-active glycosphingolipids for the heat-labile toxin, GM1 ganglioside and lactoneotetraosylceramide, whereas only the GM1 ganglioside was receptor-active for cholera toxin. Preliminary evidence was obtained, indicating that epithelial cells of human small intestine also contain lactoneotetraosylceramide and similar sequences. By computer-based molecular modelling, lactoneotetraosylceramide was docked into the active site of the heat-labile toxin, using the known crystal structure of the toxin in complex with lactose. Interactions which may explain the relatively high toxin affinity for this receptor were found.

Keywords: glycolipid receptor, cholera toxin, porcine *Escherichia coli* heat-labile enterotoxin, infant rabbit intestine

Abbreviations: CT, cholera toxin; CT-B, B-subunits of cholera toxin; LT, *Escherichia coli* heat-labile enterotoxin; hLT, human *Escherichia coli* heat-labile enterotoxin; pLT, porcine *Escherichia coli* heat-labile enterotoxin; EI, electron ionization

Introduction

It was more than 20 years ago that the GM1‡ ganglioside was first recognized as the receptor for cholera toxin, CT [1–5]. Subsequently, it was demonstrated that GM1 also served as the receptor for the related heat-labile enterotoxin (LT) of *Escherichia coli* [6–8] which causes diarrhoea in humans and farm animals. Since then a significant amount of information about the structure and function of these toxins has accumulated (reviewed in [9]). Both toxins are heterohexameric proteins consisting of one A-subunit and five B-subunits, which possess enzymatic and binding properties, respectively. The receptor-binding B-subunits of CT and LT are 80% homologous in their amino acid sequence, and the sequence of the B-subunits of the heat-labile toxins causing diarrhoea in humans (hLT) and in pigs (pLT) differ only by up to four amino acids. The binding properties of CT and LT are however not entirely identical, and it has been suggested that glycoproteins may function as additional receptor sites for LT, both in rabbit [10] and human small intestine [11]. The binding of LT, but not CT, to major galactoproteins present in rabbit small

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‡ The glycosphingolipid nomenclature follows the recommendations by the IUPAC-IUB Commission on Biochemical Nomenclature (CBN for Lipids: *Eur J Biochem* (1977) **79**:11–21, *J Biol Chem* (1982) **257**:3347–51, and *J Biol Chem* (1987) **262**:13–8). It is assumed that Gal, Glc, GlcNAc, GalNAc, and NeuAc are of the D-configuration, Fuc of the L-configuration, and all sugars present in the pyranose form.

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intestinal brush borders has been reported, although some doubt about the specificity of the interaction has been voiced [12].

In order to further investigate the differences in receptor recognition between CT and LT we compared their binding to gangliosides and non-acid glycosphingolipids, using ^{125}I-labelled CT B-subunits and porcine LT. A non-acid glycosphingolipid with LT-binding activity was isolated from infant rabbit small intestine, and structurally characterized.

Materials and methods

Glycosphingolipids

All gangliosides and non-acid glycosphingolipids used for the binding assays were isolated as described previously [13], and structurally characterized by mass spectrometry [14], gas chromatography after degradation [15, 16], and proton NMR spectroscopy [17].

Microtitre well assay

The microtitre well assay was done as previously described [18]. The wells of polyvinylchloride microtitre plates (Cooks M24, Nutacon, Holland) were coated with 50 μl of serial dilutions of pure glycosphingolipids in methanol (each dilution in triplicate). After the solvent had evaporated, the wells were incubated for 2 h at room temperature with 200 μl of PBS, pH 7.3, containing 2% bovine serum albumin, w/v, and 0.1% NaN_3, w/v (Sol. 1). After washing once with Sol. 1, 50 μl of Sol. 1 containing ^{125}I-labelled B-subunits of CT (List Biological Laboratories Inc., Campbell, CA) or porcine LT, isolated as described [19], was added to each well, and incubated at room temperature for 4 h. The toxins were labelled by the Iodogen method [20], and diluted to 5×10^6 cpm ml^{-1}. After six washings with Sol. 1 and drying, the wells were cut out and the radioactivity counted in a gamma counter.

Chromatogram binding assay

The binding of CT-B and LT to glycosphingolipids separated on thin-layer plates was performed as described elsewhere [21], with some modifications [18]. Mixtures of glycosphingolipids (40 μg) or pure compounds (1–2 μg) were separated on aluminium-backed silica gel 60 HPTLC plates (Merck, Darmstadt, Germany), using chloroform: methanol:water 60:35:8 (by vol) or chloroform:methanol: 0.02% $CaCl_2$ in water 60:40:9 (by vol) as solvent systems. Chemical detection was done by staining with anisaldehyde [22] or resorcinol reagent [23]. The chromatograms aimed for binding experiments were treated with 0.5% polyisobutylmethacrylate (Plexigum P28, Röhm, GmbH, Darmstadt, Germany) in diethylether, w/v, and subsequently soaked in Sol. 1. After 2 h at room temperature, the plates were covered with ^{125}I-labelled CT-B or LT diluted in Sol. 1 ($2–5 \times 10^6$ cpm ml^{-1}). Incubation was done for 2 h at room temperature, followed by six washings with PBS. The plates were exposed to XAR-5 X-ray films (Eastman Kodak, Rochester, NT) for 12–48 h, using an intensifying screen.

The binding of the lectin of *Erythrina cristagalli* (Vector Laboratories Inc., Burlingame, CA) was done by the same method, using ^{125}I-labelled lectin diluted to 2×10^6 cpm ml^{-1} in Sol. 1.

Isolation and characterization of the LT-binding non-acid glycosphingolipid from infant rabbit small intestine

From 32 mg of total non-acid glycosphingolipids isolated from a 5-day-old rabbit small intestine by standard methods [13], receptor-active tetraglycosylceramides were isolated by chromatography on a 40 g silicic acid column (Iatrobeads 6RS-8060, Iatron Laboratories Inc., Tokyo), eluted by stepwise increases of the concentration of methanol and water in chloroform. The LT-binding fractions were detected by the chromatogram binding assay. After pooling of binding-positive fractions, 4.9 mg were obtained, which were further separated on a 10 g Iatrobeads column, eluted with a linear gradient of chloroform:methanol:water (50:50:1 to 10:90:3, by vol). Pooling of LT-binding fractions gave 0.8 mg of pure tetraglycosylceramide.

The glycolipid was analysed by proton NMR spectroscopy [17]. Part of the sample was permethylated [24], followed by reduction with $LiAlH_4$ [25]. These derivatives were analysed by direct inlet electron ionization mass spectrometry [14], and by gas chromatography after degradation [15, 16].

For comparison, non-acid glycosphingolipids were isolated from mucosal scrapings of human small intestine, obtained at autopsy, from five individuals.

Molecular modelling

The minimum energy conformation of lactoneotetraosylceramide and the initial complex of the oligosaccharide part of this glycolipid, with a substructure of pLT consisting of fifty amino acids surrounding one of the lactose binding sites, were obtained within the Biograf molecular modelling program (Molecular Simulations Inc.) using the Dreiding-II force field [26] on a Silicon Graphics 4D/35TG workstation. The terminal 4-linked β-galactose unit was superimposed on the galactose in the LT-lactose crystal structure [27]. The docked structure was then transferred to QUANTA 3.3/CHARMm (Molecular Simulations Inc.) on a Silicon Graphics Indigo$^{2.2}$Extreme workstation. In order to allow the structure to assume a configuration with minimum strain, a molecular dynamics heating-cooling cycle was performed. However, before simulations of the dynamics were commenced, a water shell (outer cutoff 11 Å) covering the active site region was added using Biograf software. The water was first minimized to a root mean square deviation of 0.1 Å using CHARMm ($\varepsilon = 1$) while keeping the complex fixed, whereafter the whole structure including the water was minimized down to the same root mean square value.

For the dynamics run all bond lengths were kept constant using the SHAKE algorithm [28], allowing a time step of 2 fs. The system was thus heated to 300 K during 6 ps, equilibrated at this temperature for another 12 ps and finally cooled down to 10 K, which also took 12 ps, before a last minimization was performed using the same criterion as above for discontinuation. The overall root mean square deviation for the toxin substructure compared with the minimized starting structure was 1.8 Å and the corresponding value for the peptide backbone was 1.3 Å. In general, very small movements were observed for amino acids in the binding cavity and those covered by the water shell.

Results and discussion

The results from binding of ^{125}I-labelled toxins to glycosphingolipids coated in microtitre wells are shown in Fig. 1. CT-B showed the highest affinity for GM1 ganglioside, with a half-maximal binding at 0.5 ng per well (Fig. 1A), while all other gangliosides tested were negative. The GM1 ganglioside was also the most efficient receptor for LT, with a half-maximal binding occurring at 0.5–0.7 ng per well (Fig. 1B and C). In addition, a binding of LT to the GM2, GD2 and GT1b gangliosides was obtained, with half-maximal binding at 200, 120 and 150 ng per well, respectively (Fig. 1B). Furthermore, LT bound to two non-acid glycosphingolipids, gangliotetraosylceramide and lactoneotetraosylceramide, with half-maximal binding at 40 and 120 ng per well, respectively (Fig. 1C). This binding was completely abolished when the terminal galactose was substituted with an α-fucose in 2-position (fucosylgangliotetraosylceramide, Fucα2Galβ3GalNAcβ4Galβ4Glcβ1Cer, and H5 type 2 glycosphingolipid, Fucα2Galβ4GlcNAcβ3-Galβ4Glcβ1Cer).

Glycosphingolipids isolated from infant rabbit small intestine were examined for toxin-binding activity, using the chromatogram binding assay (Figs 2 and 3). Although only GM3 and slow-moving gangliosides were seen by resorcinol staining (Fig. 2A), a binding of both CT-B and LT to a compound with the mobility of GM1 ganglioside was obtained (Fig. 2B and C). LT, but not CT, also bound to a glycosphingolipid in the non-acid fraction, with mobility in the tetraglycosylceramide region (Fig. 3, C). In addition, a binding in the region of GM1 was obtained, which probably was due to residual GM1 ganglioside in these fractions.

The LT-binding non-acid tetraglycosylceramide was isolated by chromatography on Iatrobeads columns, and the identity was established as follows. The carbohydrate sequence and the composition of the lipophilic part were obtained by mass spectrometry of permethylated (Fig. 4), and permethylated and reduced (not shown) derivatives. Carbohydrate sequence ions from the permethylated glycolipid, corresponding to a hexose-*N*-acetylhexosamine-hexose-hexose sequence, were seen at *m/z* 219 and 187

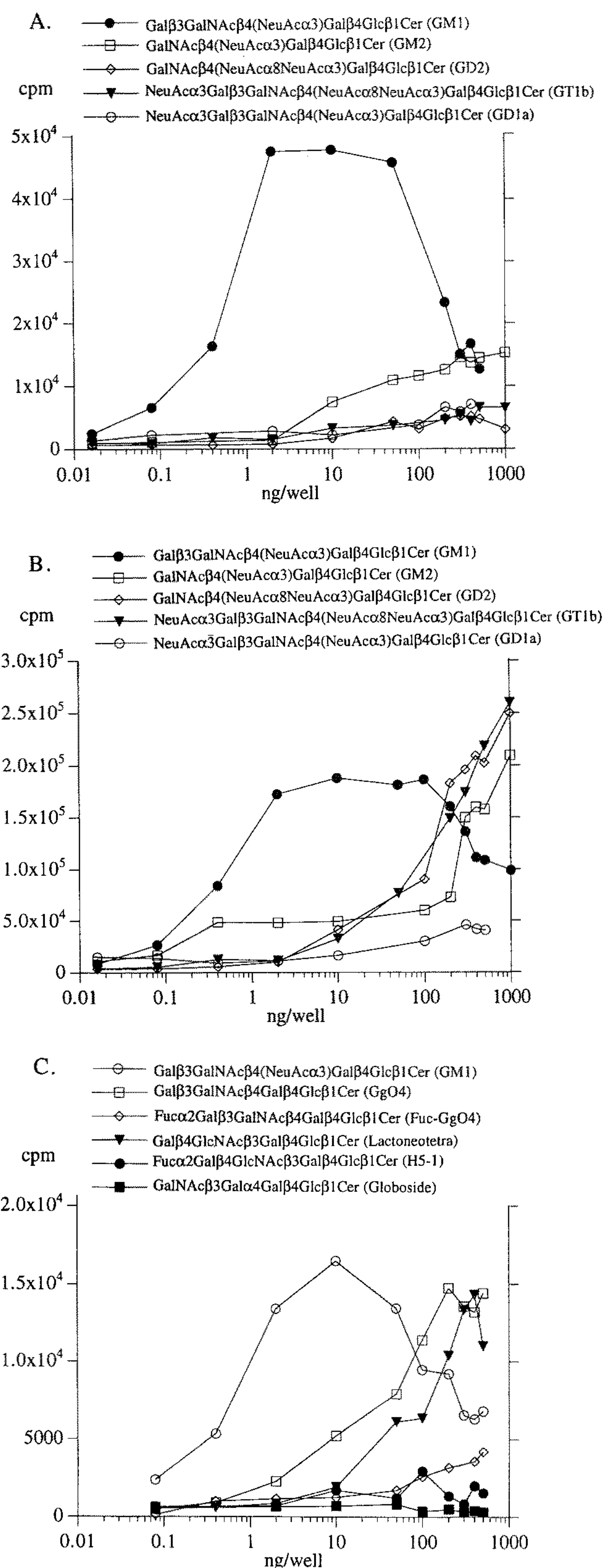

Figure 1. Binding of ^{125}I-labelled cholera toxin B-subunits (A) and porcine *E. coli* heat-labile enterotoxin (B and C) to glycosphingolipids coated in microtitre wells. The assays were performed as described in Materials and methods. Data are expressed as mean values of triplicate determinations.

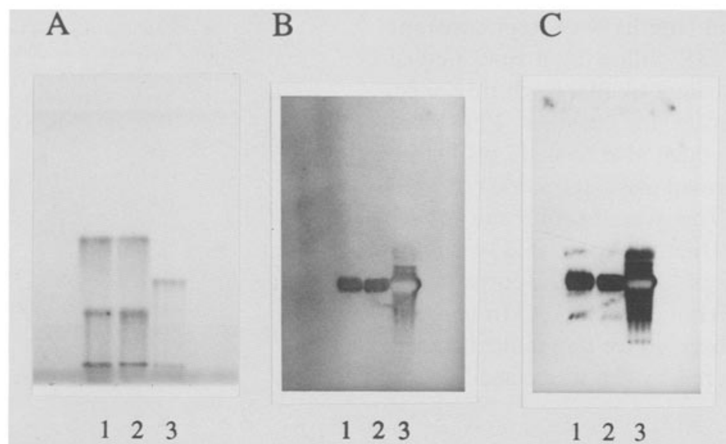

Figure 2. Thin-layer chromatogram of glycosphingolipids stained with resorcinol (A) and autoradiograms after binding of ^{125}I-labelled cholera toxin B-subunits (B) and porcine *E. coli* heat-labile toxin (C). The assay was done as described in Materials and methods. Autoradiography was done for 12 h. The lanes contained the following glycosphingolipids: 1) acid glycosphingolipids from a 5-day-old rabbit small intestine, 20 μg; 2) acid glycosphingolipids from a 10-day-old rabbit small intestine, 20 μg; 3) reference GM1, 2 μg. The solvent system was chloroform:methanol:0.02% $CaCl_2$ in water 60:40:9 (by vol).

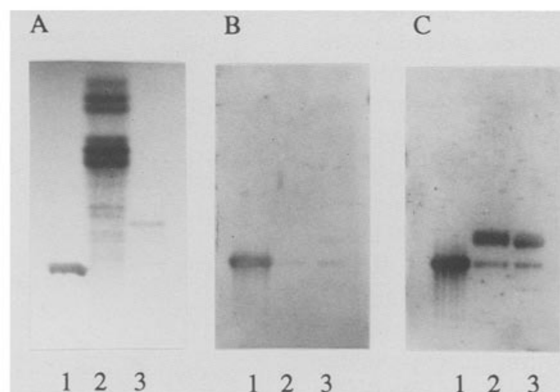

Figure 3. Thin-layer chromatogram of glycosphingolipids detected with anisaldehyde (A) and autoradiograms after binding of ^{125}I-labelled cholera toxin B-subunits (B) and porcine *E. coli* heat-labile enterotoxin (C). The assay was done as described in Materials and methods. The lanes contained the following glycosphingolipids: 1) reference GM1, 2 μg; 2) non-acid glycosphingolipids from a 10-day-old rabbit small intestine, 20 μg; 3) isolated receptor-active tetraglycosylceramide from 5-day-old rabbit small intestine, 2 μg. Chloroform:methanol:water 60:35:8 (by vol) was used as solvent system. Autoradiography was done for 12 h.

(219 minus 32), *m/z* 464 and 432 (464 minus 32), *m/z* 668 and *m/z* 872, together with a rearrangement ion at *m/z* 945 (944 + 1), containing the whole carbohydrate chain and part of the fatty acid. The ion at *m/z* 182 indicated a type 2 carbohydrate chain, (hexose-β4-*N*-acetylhexosamine) [29, 30]. The ions at *m/z* 548–722 came from the ceramide part, and demonstrated a mixture of sphingosine and phytosphingosine long-chain bases, combined with both hydroxy and non-hydroxy 16:0–24:0 fatty acids. Fragments containing the whole carbohydrate chain and the fatty acid appeared at *m/z* 1183 (non-hydroxy 16:0) and as a series of ions at *m/z* 1213–1325 (hydroxy 16:0–24:0). The series of ions at *m/z* 1257–1369 indicated the presence of phytosphingosine in combination with hydroxy 16:0–24:0 fatty acids. In mass spectra recorded at higher temperatures (not shown) a weak series of molecular ions appeared at *m/z* 1498 (phytosphingosine with hydroxy 16:0 fatty acid)–1610 (phytosphingosine with hydroxy 24:0 fatty acid).

From the relative intensities of the ceramide ions and the immonium ions it was concluded that the dominating ceramide species was phytosphingosine combined with hydroxy 22:0 fatty acid.

The mass spectrum of the permethylated and reduced glycosphingolipid (not shown) supported the presence of a hexose-*N*-acetylhexosamine-hexose-hexose structure, with phytosphingosine and hydroxy 22:0 fatty acid as major ceramide species.

The binding positions between the carbohydrate residues were obtained by degradation studies. Figure 5 shows the gas chromatogram after degradation of the permethylated derivative, with peaks corresponding to acetates of 2,3,4,6-tetramethyl-galactitol (terminal galactose), 2,4,6-trimethyl-galactitol (3-substituted galactose), 2,3,6-trimethyl-glucitol

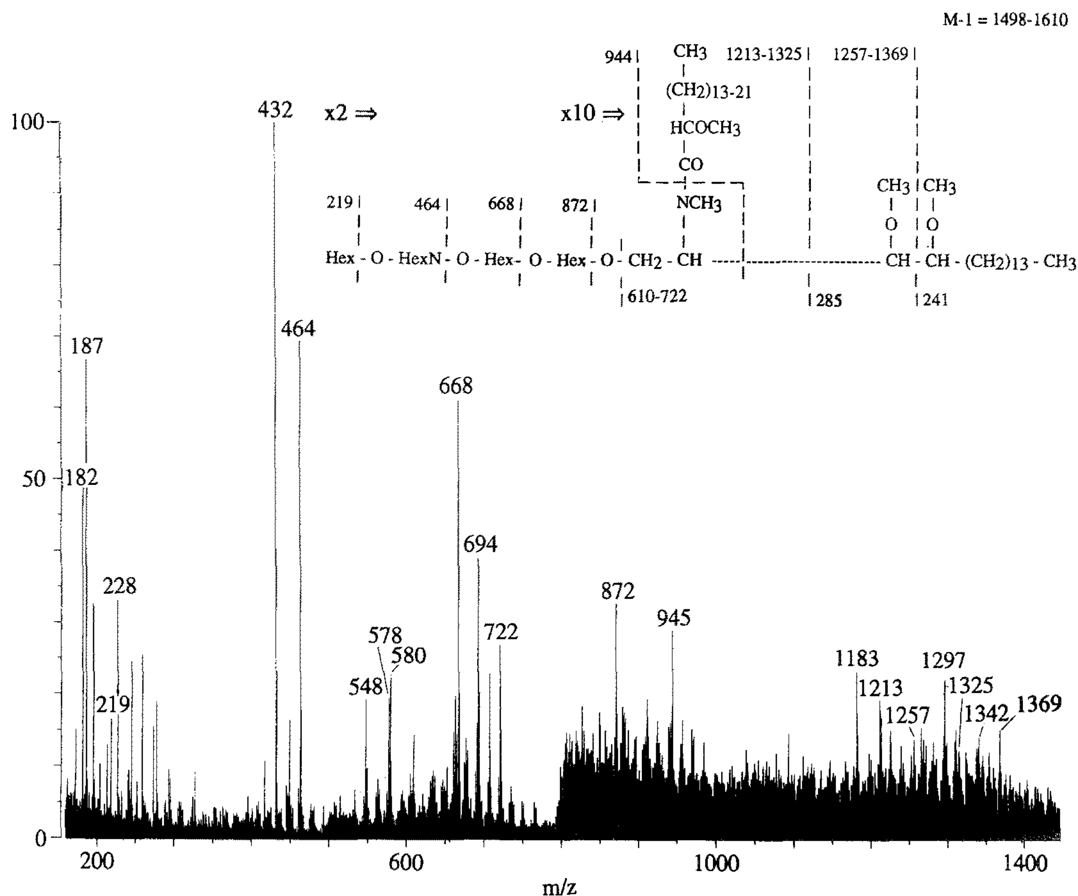

Figure 4. Average mass spectrum (scans 35–40) of the permethylated tetraglycosylceramide isolated from 5-day-old rabbit small intestine. Above the spectrum is shown a simplified formula for interpretation, representing the species with phytosphingosine and hydroxy 24:0 fatty acid. Mass spectra were obtained with a JEOL SX-102A mass spectrometer, using the in beam technique. Analytical conditions: sample amount 1 μg, electron energy 70 eV, trap current 300 μA and acceleration voltage 10 kV. Starting at 150°C, the temperature was raised by 10°C per min. The spectrum was recorded at 280°C.

(4-substituted glucose) and 3,6-dimethyl-2-*N*-methyl-acetamido-glucitol (4-substituted *N*-acetylglucosamine). When the permethylated and reduced glycosphingolipid was degraded (not shown), the peaks for 3-substituted galactose and 4-substituted *N*-acetylglucosamine disappeared, and a peak with long retention time (corresponding to 4*N*-acetylglucosamine-3galactose-1) appeared.

In summary, mass spectrometry and degradation studies demonstrated a galactose1-4*N*-acetylglucosamine1-3galactose1-4glucose1 carbohydrate sequence, linked to ceramide with sphingosine and phytosphingosine combined with hydroxy and non-hydroxy 16:0–24:0 fatty acids.

By ^{1}H-NMR spectroscopy (not shown) the anomeric configurations of the glucose, the two galactoses, and the *N*-acetylglucosamine were assigned as β. The four anomeric signals were both shift- and coupling-wise in agreement with data from spectra of reference lactoneotetraosylceramide from human erythrocytes published elsewhere [31].

Thus, the LT-binding glycosphingolipid isolated from infant rabbit small intestine was identified as Galβ4GlcNAc-β3Galβ4Glcβ1Cer (lactoneotetraosylceramide). A binding of the heat-labile *E. coli* enterotoxin causing diarrhoea in humans, to GM1, GD1b and gangliotetraosylceramide has earlier been reported by Fukuta *et al.* [32]. However, the interaction of LT with lactoneotetraosylceramide is a novel finding.

This binding of LT to lactoneotetraosylceramide is in accordance with the crystal structure of porcine LT complexed with lactose [27], where lactose is bound despite the absence of sialic acid, and all free hydroxyl groups of the galactose are engaged in hydrogen bond interactions with amino acids in the binding site. As shown in Fig. 6, docking of the oligosaccharide part of lactoneotetraosylceramide to LT, after a heating-cooling cycle as described in Materials and methods, results in a structure were the terminal 4-linked β-galactose occupies essentially the same position as the corresponding residue in the lactose complex, thus retaining all the hydrogen bond interactions described earlier [27]. Additionally, other favourable interactions occur; hydrogen bond formation between the hydroxymethyl and the *N*-acetylglucosamine and the Arg-13 side chain, as well as van der Waals interactions between

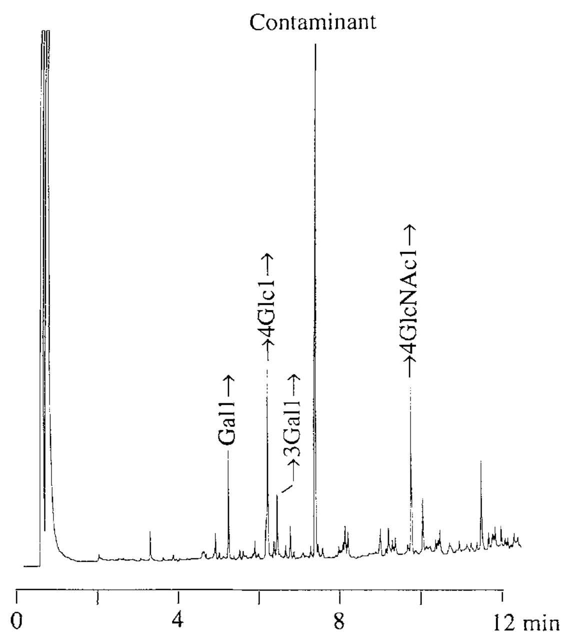

Figure 5. Gas chromatogram of partially methylated alditol acetates of the degraded permethylated tetraglycosylceramide from rabbit small intestine. The products were analysed on a Carlo-Erba 4169 gas chromatograph. Poly(arylene-methylphenyl)-siloxane (film thickness 0.2 μm) was used as stationary phase. After split injection at 150°C the oven temperature was raised by 8°C per min to 380°C.

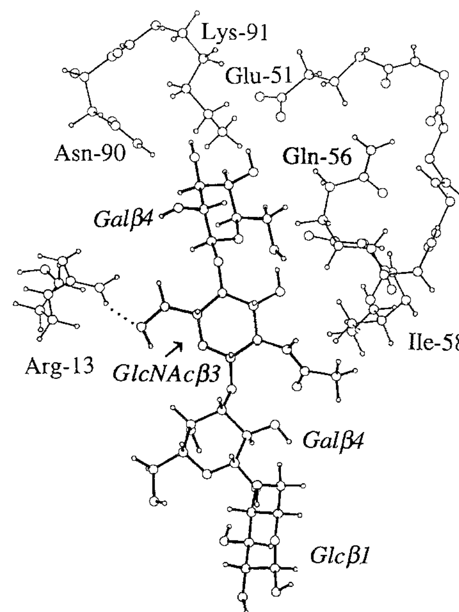

Figure 6. View of the lactoneotetraosyl-LT complex showing several of the more important interactions in the active site region. The tetrasaccharide is drawn with bold bonds. The structure is the result of the energy minimization – molecular dynamics – energy minimization cycle described in Materials and methods. The glycosidic torsion angles (Φ, Ψ) after this cycle were found to be (49/5), (23/−13) and (45/8) for Galβ4GlcNAc, GlcNAcβ3Gal and Galβ4Glc, respectively, compared with (61/−9), (52/−33) and (59/−4) from the minimum energy structure of the tetrasaccharide alone, yielding very similar overall structures in the two cases. The dotted line shows the hydrogen bond between Arg-13 of LT and the hydroxymethyl group of the *N*-acetylglucosamine.

the acetamido group and a shallow groove formed by the backbone stretch provided by residues 54–58 and the side chain of Ile-58 in the protein structure. In CT, however, Arg-13 is replaced by the considerably shorter His-13 [33], which would prevent the formation of a hydrogen bond between this residue and the hydroxymethyl group of the *N*-acetylglucosamine, which presumably accounts for the absence of a detectable binding to lactoneotetraosylceramide in this case.

The discrepancies between the binding of CT and LT in rabbit small intestine [10] may thus be due to an additional interaction of LT with lactoneotetraosylceramide. In addition, polyglycosylceramides with repetitive Galβ4GlcNAcβ-sequences are found in rabbit small intestine (Miller-Podraza H, submitted), and these may also be receptor-active.

The Galβ4GlcNAcβ-sequence is also a common constituent of the carbohydrate chains of glycoproteins, although it is in most cases substituted with fucose and/or sialic acid [34]. However, from the crystal structure of LT complexed with lactose [27] it was concluded that the initial binding of CT or LT occurs with the enzymatic A1 fragment directed away from the membrane, while the A2 fragment continues through the central pore created by the five B-subunits, and is able to interact with the membrane. This arrangement suggests that glycoproteins are less likely as functional receptors for LT. At least for CT, the membrane proximity afforded by binding to the GM1 ganglioside is a requirement of the biological effect, since no increased production of cAMP or activation of adenylate cyclase was obtained when the GM1 oligosaccharide was coupled to cell membrane proteins of toxin-insensitive rat glioma cells, although the neo-glycoproteins were bound by the toxin [35].

The dominating non-acid glycosphingolipids in the enterocytes of human small intestine are monohexosylceramide and glycosphingolipids with 5–7 carbohydrate residues [36]. However, by chromatogram binding experiments, using the Galβ4GlcNAcβ- and Fucα2Galβ4GlcNAcβ-binding lectin from *Erythrina cristagalli* [37], and glycosphingolipids isolated from mucosal scrapings of human duodenum and jejunum, a band with mobility in the region of lactoneotetraosylceramide was detected in four of five specimens (Fig. 7B). The fraction devoid of binding in the tetraglycosylceramide region contained a more slow-moving glycosphingolipid with binding activity (Fig. 7B, lane 4). In addition, slow-moving glycosphingolipids with lectin-binding activity were also seen in two other specimens. None of these lectin-binding glycosphingolipids were bound by monoclonal antibodies directed against the blood group

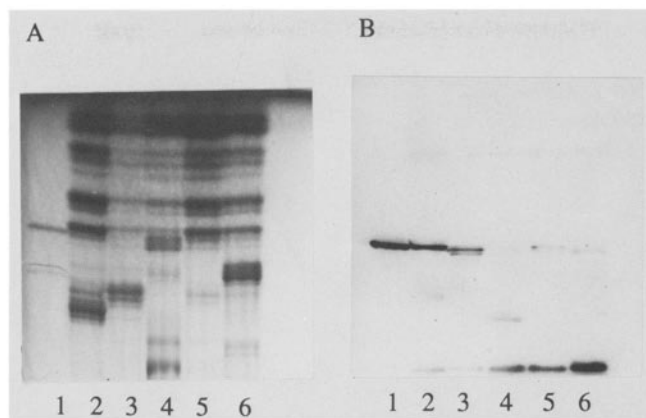

Figure 7. Thin-layer chromatogram with separated glycosphingolipids detected with anisaldehyde (A) and autoradiogram obtained by binding of ^{125}I-labelled *Erythrina cristagalli* lectin (B). The assay was done as described in the Materials and methods section, and autoradiography was for 12 h. Lane 1 contained reference lactoneotetraosylceramide from human erythrocytes, 4 µg, and lanes 2–6 were non-acid glycosphingolipids isolated from mucosal scrapings of human small intestine of five different individuals, 80 µg per lane.

H type 2 determinant (not shown). Thus, the pLT-binding Galβ4GlcNAcβ-sequence is most probably present in glycosphingolipids of the epithelial cells of human small intestine, and may, in addition to GM1 ganglioside [11], mediate the induction of diarrhoea. However, in some hLTs the suggested crucial amino acid at position 13 of the B-subunit is Arg, as in CT. Further investigations will thus be required to determine if this prevents the binding of hLT to lactoneotetraosylceramide.

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