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Unexpected Carbohydrate Cross-binding by *Escherichia coli* Heat-labile Enterotoxin. Recognition of Human and Rabbit Target Cell Glycoconjugates in Comparison with Cholera Toxin

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Abstract—The bacterial protein enterotoxins, cholera toxin (CT) of *Vibrio cholerae* and heat-labile toxin (LT) of *Escherichia coli*, induce diarrhea by enhancing the secretory activity of the small intestine of man and rabbit (animal model). This physiological effect is mediated by toxin binding to a glycolipid receptor, the ganglioside GM1, Galβ3GalNAcβ4(NeuAcα3)Galβ4Glcβ1Cer. However, LT, but not CT, was recently shown by us to bind also to paragloboside, Galβ4GlcNAcβ3Galβ4Glcβ1Cer, identified in the target cells. By molecular modeling of this tetrasaccharide in the known binding site of LT, the saccharide–peptide interaction was shown to be limited to the terminal disaccharide (*N*-acetyllactosamine). This sequence is expressed in many glycoconjugates, and we have therefore assayed glycolipids and glycoproteins prepared from the target tissues. In addition to paragloboside, receptor activity for LT was detected in glycoproteins of human origin and in polyglycosylceramides of rabbit. However, CT bound only to GM1. Two variants of LT with slightly different sequences, human (hLT) and porcine (pLT), were identical in their binding to target glycoproteins and polyglycosylceramides, but different regarding paragloboside, which was positive for pLT but negative for hLT. This difference is discussed on basis of modeling, taking in view the difference at position 13, with Arg in pLT and His in hLT. Although *N*-acetyllactosamine is differently recognized in form of paragloboside by the two toxin variants, we speculate that this sequence in human glycoproteins and rabbit polyglycosylceramides is the basis for the common binding. Much work remains, however, to clear up this unexpected sophistication in target recognition. Copyright © 1996 Elsevier Science Ltd

Introduction

Microbes and microbial toxins causing various diseases of animals and man require attachment sites for the association to specific target cells and to avoid elution by secretions at mucous membranes. Such sites are mainly carbohydrate epitopes, probably due to their abundance at host cell surfaces and their often tissue-specific appearance. Recent advances in assaying and identification of receptor sequences in combination with access to a growing number of crystal structures of microbial receptor-binding proteins, mainly viral hemagglutinins and bacterial toxins, also in complex with saccharide, have allowed interesting conclusions in several cases concerning infection mechanisms (recent review, see ref. 1), of potential interest for therapy.

Bacterial toxins are mostly medium-sized secreted proteins accessible for preparation and detailed analysis of binding specificity. Bacteria causing diarrhea are common globally, including *Vibrio cholerae* secreting cholera toxin (CT), and *Escherichia coli* secreting the heat-labile toxin (LT).[2] These toxins are composed of one biologically active A subunit of about 27 kDa, and five receptor-binding B subunits of about 12 kDa each. The B subunits show great sequence homology (about 80%) and the two toxins probably induce the diarrhea by identical mechanisms in the epithelium of the small intestine. Both toxins have long been known to bind with high affinity to a glycolipid, the ganglioside GM1, Galβ3GalNAcβ4(NeuAcα3)-Galβ4Glcβ1Cer. The epitope is apparently selectively expressed in glycolipid form, since attempts to bind CT to non-glycolipids have been unsuccessful. It has also been shown by synthetic neoglycoconjugates, added to toxin-insensitive cell cultures, that this receptor saccharide epitope has to be located close to the membrane to mediate the toxin effect, probably for penetration of the toxin A subunit into the cell.[3] However, it was earlier shown for LT, but not for CT, that the small intestine of the rabbit (animal model) carried additional sites of attachment, assumed to be glycoprotein, resulting in binding of 10 times more toxin than expected from the amount of GM1 present.[4]

Recently, we reported the isolation and identification of a glycolipid of rabbit small intestine, which was receptor-active for LT but not for CT, namely paragloboside or neolactotetraosylceramide, Galβ4GlcNAc-β3Galβ4Glcβ1Cer.[5] This finding was surprising, since this compound lacked sialic acid, which earlier was shown to be essential for the binding by CT, and also had a different core structure compared to GM1. Modeling of the tetrasaccharide in the binding site of the B subunit of LT of porcine origin (pLT) was highly suggestive of a critical hydrogen bond between the

6-hydroxymethyl group of GlcNAc and the side chain of Arg-13. This amino acid makes up the major difference in the site when compared to CT, which at this position has a His, that is incapable of producing a corresponding interaction.

Modeling further revealed that only the terminal disaccharide was in direct contact with the toxin peptide. Galβ4GlcNAc, or *N*-acetyllactosamine, is a common sequence of many glycoproteins, and also of polyglycosylceramides (PGCs), complex glycolipids with possibly up to 50–60 sugar residues.[6] The disaccharide may, however, be substituted with sialic acid or blood group determinants. We therefore became interested in testing whether glycoproteins and PGCs of target cells (human and rabbit) also were binding-active with respect to LT. The results show that glycoproteins of human origin, but not of rabbit are binding-active, and that PGCs of rabbit, a glycoconjugate not yet detected in human small intestine, are receptor-active, but only in adult rabbits.

Results

Glycolipids and glycoproteins were prepared from the whole small intestine of 5-day, 10-day and 8-week old rabbits as well as from the small intestinal mucosa of five human individuals. Glycolipids were assayed for binding after separation on TLC plates, and glycoproteins on blots after electrophoresis, by overlay of ^{125}I-labeled CT, LT of human (hLT) and porcine (pLT) origin, and two plant lectins, one from *Erythrina cristagalli*, and the other from *Arachis hypogaea*.

In the earlier paper[5] pLT was compared with CT in their binding to a number of reference glycolipids, including gangliotetraosylceramide (Galβ3GalNAcβ4Galβ4Glcβ1Cer, asialo-GM1) and paragloboside, both of which are positive binders for pLT but negative for CT. Paragloboside, but not gangliotetraosylceramide, was isolated from rabbit small intestine and structurally characterized, and it was also detected in epithelial cells of human small intestine. In the present work, these two glycolipids were added as references in the comparison of the two toxins, both on TLC plates and in microtiter wells, the latter assay being more quantitative than the former. Furthermore, the two tetrasaccharides were modeled in the binding site of the two different toxin B subunits with the ambition to explain differences found in the binding selectivities.

Binding of the toxins to rabbit intestine glycoconjugates

The result from overlay of toxins and lectins on TLC plates with various rabbit glycolipids was very rewarding (Fig. 1). To make the complex PGC preparation move on silica gel plates one has to use a solvent for development that places the simpler glycolipids in the front of the interval. We used crude PGC fractions which may contain small amounts of short glycolipids (expected to travel in the front) and glycoproteins (expected to stay at the application line). However, the mobility of the active material is in accordance with earlier results for PGCs.[6] As shown by the autoradiogram of Figure 1(D), CT was bound only by the GM1 ganglioside. There was no binding to the two reference 4-sugar glycolipids or to PGCs. Figure 1(E) shows that hLT bound to PGCs of 8-week-old rabbits but not to those of 5- or 10-day-old rabbits or to the two 4-sugar glycolipids. Figure 1(F) shows that pLT similarly bound only to PGCs of 8-week-old rabbits, and also to the two 4-sugar glycolipids. Figure 1(B) shows the binding of the lectin of *Arachis hypogaea* (peanut lectin, PNA), known to recognize Galβ3GalNAc, showing gangliotetraosylceramide (lane 6) to be the only receptor-active substance. In Figure 1(C) the binding of the lectin from *Erythrina cristagalli* (ECL), known to bind Galβ4GlcNAc, is shown only for paragloboside.[7] Interestingly, the two lectins did not bind to PGCs (lane 4), in contrast to the toxins. Therefore, either the epitope on PGCs is not Galβ4GlcNAc (or Galβ3GalNAc) but a separate sequence not being recognized by the plant lectin, or it is Galβ4GlcNAc (or Galβ3GalNAc), but with a separate subepitope for the toxins, which is not accessible for binding due to the character of neighboring sugar groups. Alternatively, the epitopes are identical, but the proteins, due to different structures, may dock differently depending on neighboring sugars. That the binding sites of the lectin and the two LT variants differ, however, is indicated by binding to the two 4-sugar glycolipids: pLT bound both glycolipids, the lectins one each of them and hLT neither. This is further substantiated by the binding curves of Figure 2, showing that of the three toxins only pLT bound the 4-sugar glycolipids, which is summarized in Table 1.

Figure 3, lane 3, documents that glycoproteins of rabbit intestine were not receptor-active, neither before nor after removal of sialic acid. The binding spot in the low molecular mass region was probably due to receptor-active glycolipids.[8]

Binding of the toxins to human intestine glycoconjugates

Concerning glycoproteins prepared from the five individuals, the blots after electrophoresis (Fig. 3, lanes 4–8) document that neither the plant lectin ECL (blot A) nor hLT (blot B) bound to intact glycoproteins. After removal of sialic acid, the plant lectin bound mainly to one lane (blot C), but hLT bound to one major band with a relative molecular mass of about 41 kDa from all 5 individuals (blot D). As noted above, the binding-active spot in the low molecular mass region may be due to glycolipids.[8] This band was apparently only a minor component of the total fraction, as indicated by Coomassie staining (E). pLT bound in a similar way (not shown), whereas CT did not bind glycoprotein (not shown). The results are summarized in Table 1.

The more complex glycolipids, PGCs, found in the rabbit were not detected in human small intestine.

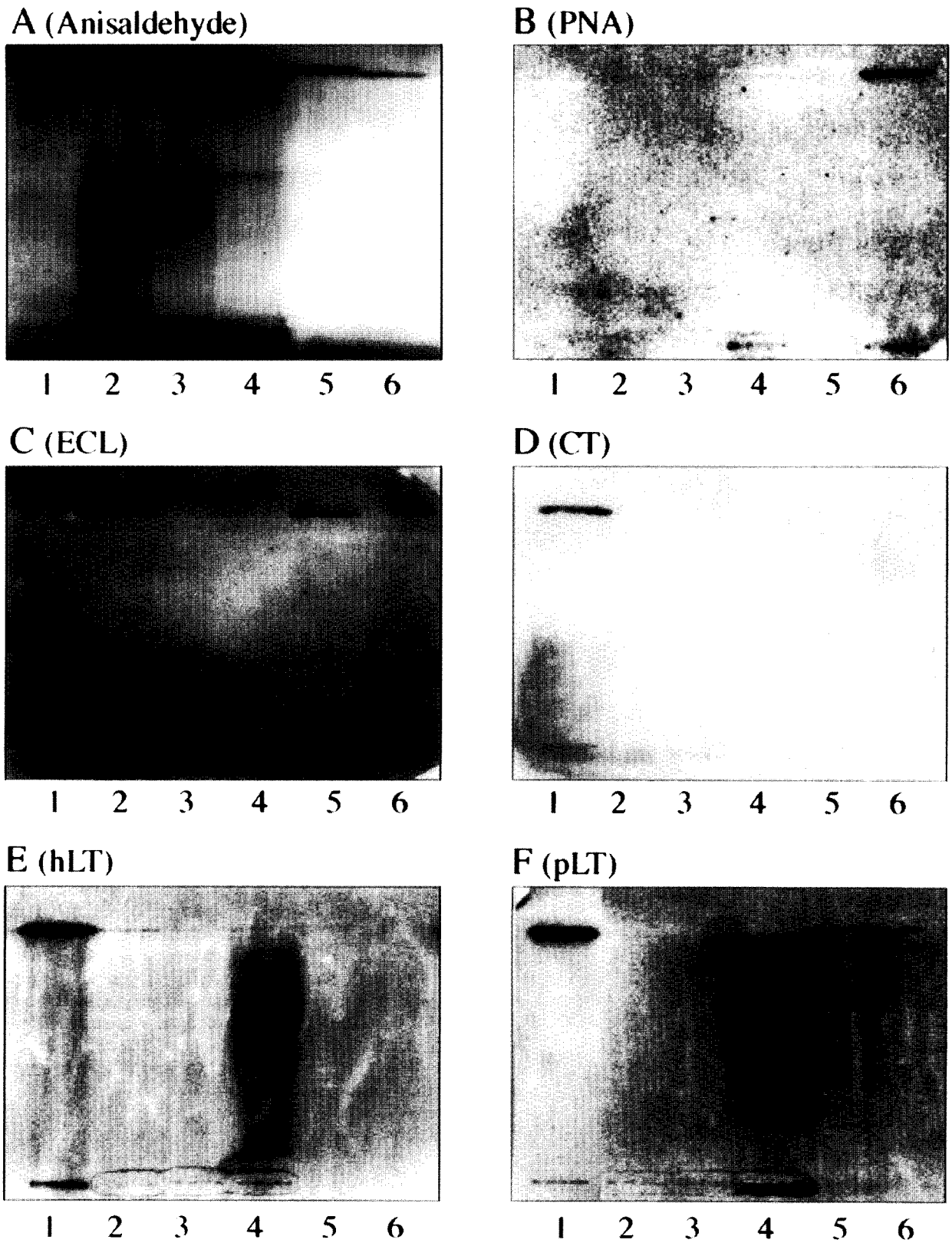

Figure 1. Results from binding of ^{125}I-labeled lectins of *Arachis hypogaea* (PNA) and *Erythrina cristagalli* (ECL), and bacterial toxins (CT, hLT and pLT) to TLC plates with separated rabbit and reference glycolipids as detected by autoradiography (plate B: PNA; plate C: ECL; plate D: CT; plate E: hLT; plate F: pLT). For comparison, a chromatogram detected with the anisaldehyde spray reagent is shown (plate A). Lane 1: GM1 ganglioside, 0.5 μg; lane 2: PGC of 5-day-old rabbit, 40 μg; lane 3: PGC of 10-day-old rabbit, 40 μg; lane 4: PGC of 8-week-old rabbit, 40 μg; lane 5: paragloboside, 2 μg; lane 6: gangliotetraosylceramide, 2 μg. The solvent was *n*-propanol–0.25% KCl in water–methanol–chloroform (7:5:1:0.5, by vol).

(However, we cannot exclude the presence of very low levels because of limited amounts of source material.) Results from overlay on traditional glycolipids separated on TLC plates are shown in Figure 4. Both the plant lectin ECL (plate B) and pLT (plate C) bound to a 4-sugar glycolipid, which most likely is paragloboside (Table 1) and which was present in all five individuals analysed. pLT (plate C) and CT (plate D) bound to a glycolipid in the mobility interval of GM1.

Molecular modeling

In order to reveal characteristics in the binding sites of the three toxins that may explain the different selectivities found (Table 1), the interaction was simulated by molecular modeling. In Figure 5, some of the important amino acids in the binding pocket of pLT

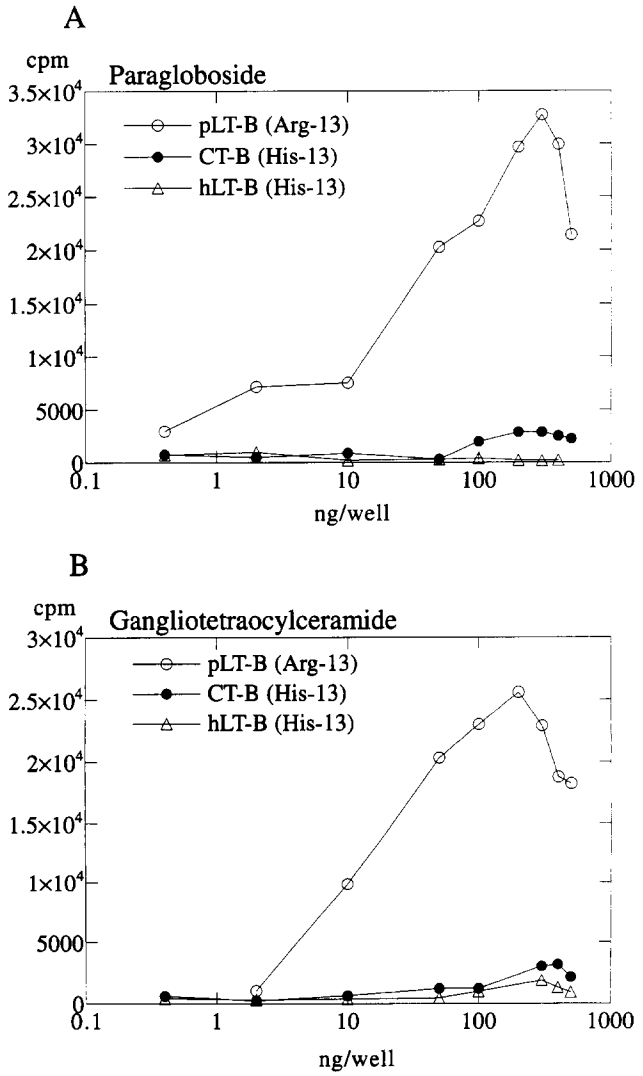

Figure 2. Curves from binding of ^{125}I-labeled pLT, CTB and hLT to dilutions of paragloboside (A) and gangliotetraosylceramide (B) coated in microtiter wells. Data are expressed as mean values of triplicate determinations.

are shown interacting with the terminal disaccharide part (the lactose segment has been removed for clarity only) of the two receptor-active 4-sugar glycolipids. The structures were obtained from molecular dynamics simulations as described in the Experimental section. Crystal complexes have been reported of B subunits of pLT with lactose[9] and of CT with GM1 pentasaccharide.[10]

The major important difference in the binding site between CT and pLT is at position 13, which is His in CT and Arg in pLT. Arg-13, but not His-13, may facilitate hydrogen bond interactions with the glycolipids, which most likely explains the binding of pLT and non-binding of CT by the two 4-sugar glycolipids lacking sialic acid. However, as shown in Figure 5, this possibility differs in the two cases. For Galβ3GalNAcβ, which is part of the GM1 sequence being bound by CT but where NeuAc is essential, likely hydrogen bond interactions are found between the guanidino moiety of Arg-13 and the glycosidic oxygen for the GalNAcβ4Galβ linkage and/or the carbonyl oxygen of the GalNAcβ acetamido group, whereas hydrogen bonds to the 2-OH of Galβ4 were rather infrequent. Alternative interactions, using a different starting conformation of the Arg-13 side chain in a separate dynamics run, are found between the guanidino moiety and the Galβ4 2-OH and 3-OH as well as the backbone carbonyl oxygen of Glu-18, thus lodging the Arg-13 side chain behind Galβ4 in the view shown in Figure 5(B). It is not possible to discriminate between these two possibilities at present. These results are in some details in agreement with the proposal of Merritt et al.[10] who postulated interactions with the carbonyl oxygen of the GalNAcβ4 acetamido group, the Galβ4 2-OH and/or the Glc 6-OH. However, the Φ dihedral angle of the Galβ4Glc linkage in the CT-GM1 pentasaccharide crystal structure[10] is most likely distorted due to the absence of a ceramide moiety, thus rendering any interactions with the Glc 6-OH unlikely.

For Galβ4GlcNAcβ of paragloboside, in which the amino sugar is rotated approximately 180 degrees compared to GalNAc, the hydrogen bond is instead to the 6-hydroxymethyl group of GlcNAcβ4. Further stabilizing interactions between the GlcNAc acetamido group and the peptide backbone were also found. hLT does not bind to the two 4-sugar glycolipids since it has a His at position 13 as found for CT. However, also other amino acid substitutions may produce a similar binding affinity, since a designed CT double mutant, in which His-94 and Ala-95, the latter being located just outside the strict binding site, were changed to Asn-94 and Ser-95 as in LT, did also bind these glycolipids (Table 1). This will be reported in more detail elsewhere.

Although hLT and pLT differ in binding to the two 4-sugar glycolipids, as explained above, they both recognize PGCs of the rabbit (Fig. 1) and desialylated glycoprotein of the human (only hLT is shown in Fig. 3). This is discussed below.

Table 1. Results from binding of ^{125}I-labeled toxins and *Erythrina* lectin (ECL) to reference and target cell glycoconjugates

Glycoconjugate	CT His-13	hLT His-13	pLT Arg-13	CT Asn-94 Ser-95	ECL
Galβ3GalNAcβ4(NeuAcα3)Galβ4GlcβCer Ganglioside GM1	+	+	+	+	–
Galβ3GalNAcβ4Galβ4GlcβCer Gangliotetraosylceramide	–	–	+	+	–
Galβ4GlcNAcβ3Galβ4GlcβCer Paragloboside or Neolactotetraosylceramide	–	–	+	+	+
Galβ4GlcNAcβ6(Galβ4GlcNAcβ3)Galβ4GlcβCer	–	–	+	+	+
Galβ4GlcNAcβ6(NeuAcα6Galβ4GlcNAcβ3)Galβ4GlcβCer	–	–	–	NA	–
Polyglycosylceramides, PGCs 5- and 10-day-old rabbit intestine	–	–	–	NA	–
Polyglycosylceramides, PGCs 8-week-old rabbit intestine	–	+	+	NA	–
Glycoproteins Human small intestinal epithelium	–	–	–	NA	–
Desialylated glycoproteins Human small intestinal epithelium	–	+	+	NA	–

NA: not analysed.

Discussion

The two enterotoxins, CT and LT, are mediators of diarrhea in globally important bacterial infections. They both bind with high affinity the GM1 ganglioside (Table 1), which is essential for secretion to occur from the small intestinal epithelium into the lumen. The GM1 epitope has not been detected in glycoproteins and may thus be a strictly membrane-bound receptor not appearing in (glycoprotein) secretions, which may compete with a membrane attachment. Furthermore, it is essential that this epitope is close to the bilayer,[1,3] otherwise the active A subunit cannot penetrate into the cell.

The recent finding[5] of paragloboside (Table 1) as a cross-binding glycolipid for pLT in rabbit and human small intestine was surprising in view of the lack in this sequence of NeuAc, and this compound has, furthermore, a core saccharide different from that of GM1. A cross-binding by LT to unidentified material in rabbit small intestine was found many years ago,[4] proposed to be glycoprotein, and which bound about 10 times more LT than the GM1 present. According to the results of the present paper, this receptor-positive material is most probably PGCs and not glycoprotein.

Molecular modeling revealed that only the terminal Galβ4GlcNAcβ, *N*-acetyllactosamine, was in direct contact with the binding site of the toxin B subunit.[5] This opened the question for the present work about possible receptor activity also in other glycoconjugates known to contain this disaccharide. Numerous glycoproteins carry in their *N*-linked saccharides *N*-acetyllactosamine, which may be blocked by sialic acid, blood group or similar determinants.[11] Polyglycosylceramides are very complex, branched-chain glycolipids based on *N*-acetyllactosamine.[6] We therefore investigated glycoproteins and PGCs prepared from target tissues, epithelial cells of human small intestine and rabbit small intestine (used as animal model), for receptor activity. We also used two LT variants, hLT produced by *E. coli* isolated from human infection, and pLT produced by bacteria originally isolated from pig infection.

The results were somewhat complicated (Table 1). Clearly, CT was binding only to the GM1 ganglioside. pLT bound not only to GM1 with an affinity similar to CT, but also to paragloboside and gangliotetraosylceramide,[5] with about 200 and 100 times lower affinity, respectively, as compared to GM1. However, hLT did not bind these two glycolipids (Figs 1 and 2, and Table 1).

Rabbit PGCs were active for both hLT and pLT (Fig. 1 and Table 1), but only for the 8-week-old and not for 5- or 10-day-old rabbits. However, as shown before,[5] paragloboside was active for pLT also in infant rabbit. This developmental change may be caused by a decrease in sialylation or blood group determinants on *N*-acetyllactosamine thus exposing an unprotected disaccharide, or change in branching. Glycoproteins were not receptor-active in the rabbit (Fig. 3, lane 3), neither before nor after desialylation.

In the human intestine, a 4-sugar glycolipid, probably paragloboside, was found receptor-active for pLT (Fig. 4) but not for hLT (not shown). PGCs were not found in human small intestine, but human glycoprotein was receptor-active for both hLT and pLT, but only after desialylation (Fig. 3 and Table 1). There was one major positive band found for all five individuals analysed.

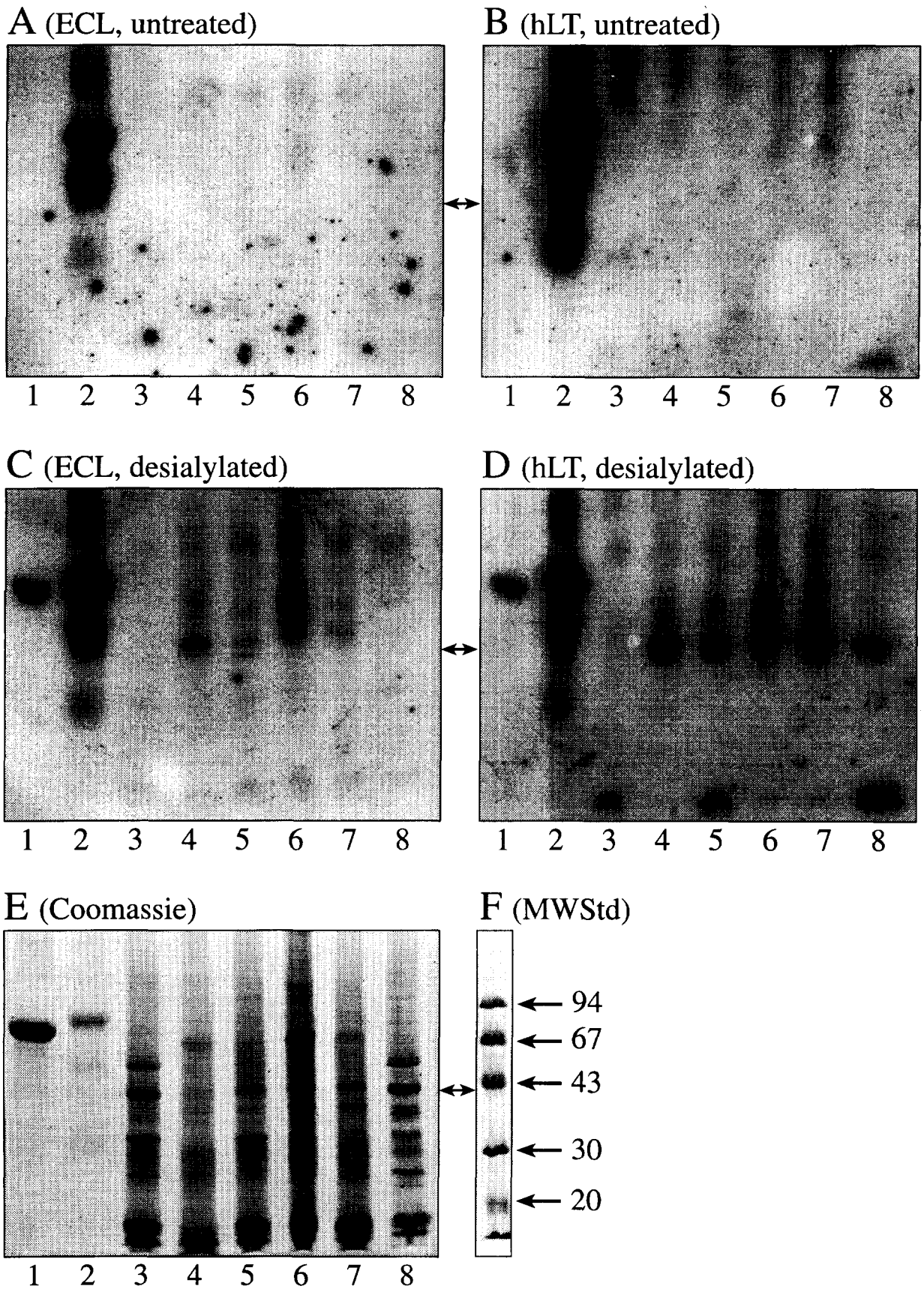

Figure 3. Results from binding of ^{125}I-labeled *Erythrina cristagalli* lectin and hLT to blots after electrophoresis of human and rabbit small intestine glycoproteins and to neoglycoproteins, as detected by autoradiography (A–D). (E) is the gel stained with Coomassie Blue and (F) is molecular mass markers (kDa). The double-arrows mark the position of the 41 kDa protein. Lane 1: NeuAcα3Galβ4GlcNAcβ-DCP-BSA, 1.5 μg; lane 2: Galβ4GlcNAcβ-CETE-BSA, 0.5 μg; lane 3: 8-week-old rabbit, 4 μg total protein; lanes 4–8: epithelium glycoproteins of five human individuals, 3.5 μg total protein each. Blot A: lectin and non-treated samples; blot B: hLT and non-treated samples; blot C: lectin and desialylated samples; blot D: hLT and desialylated samples.

The absence of binding to other glycoprotein bands, which very likely contain *N*-acetyllactosamine, may be due to the fact that a branch is blocked by a neighboring branch or by a blood group or similar determinant not being sensitive to the mild acid treatment.

Thus, both LT variants bound to 8-week-old rabbit PGCs, and to human glycoprotein after desialylation, in spite of the fact that they differed in activity for the two 4-sugar glycolipids (Table 1). The binding by pLT, but not by hLT, of Galβ3GalNAcβ and Galβ4GlcNAcβ

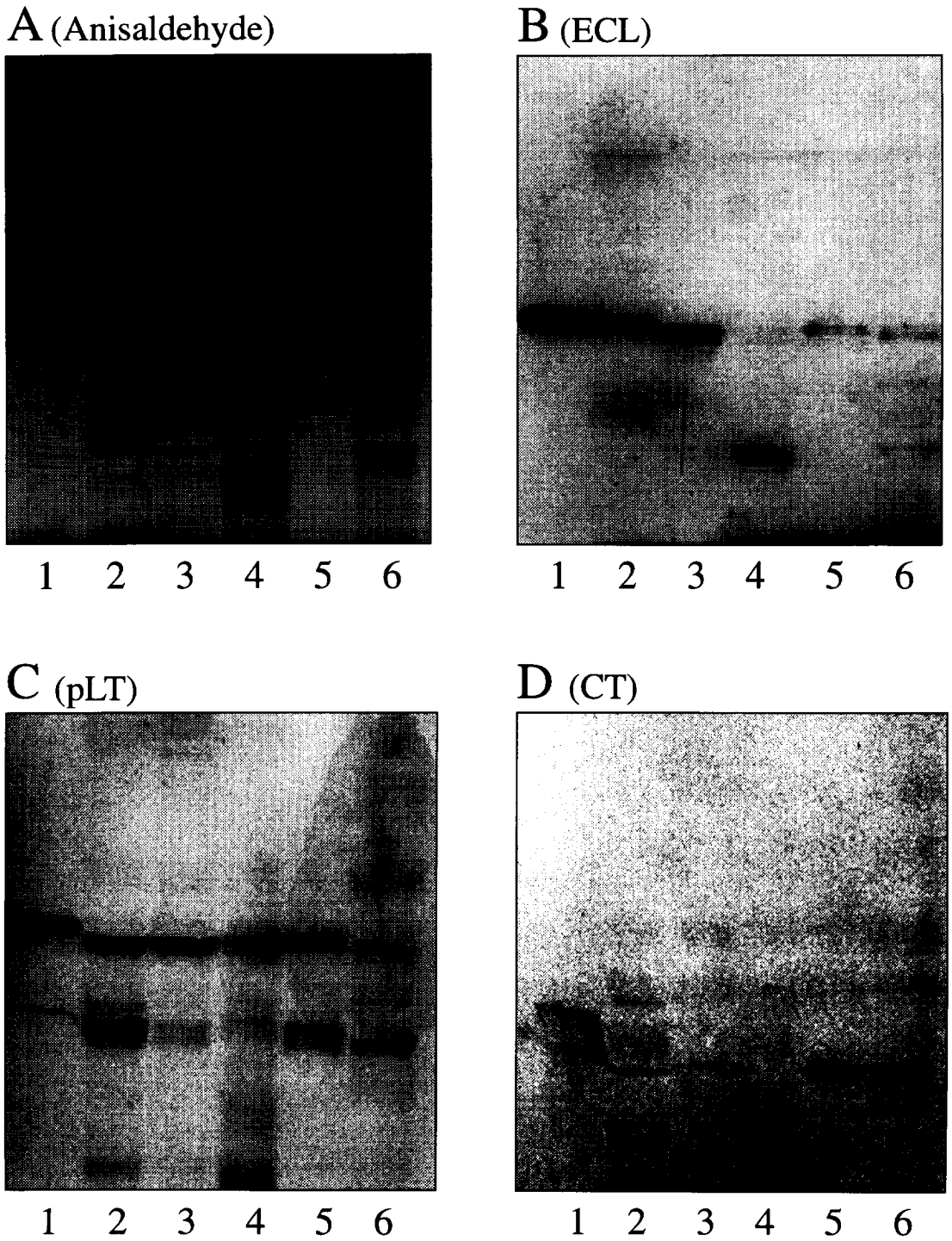

Figure 4. Results from binding of ^{125}I-labeled *Erythrina cristagalli* lectin, pLT and CT to non-acid glycolipids prepared from small intestinal epithelium of five human individuals, as detected by autoradiography (B–D). For comparison, a chromatogram after detection with the anisaldehyde spray reagent is shown (A). Lane 1: a mixture of reference paragloboside, 4 μg (upper), and GM1, 1 μg; lanes 2–6: glycolipids of five individuals, about 80 μg each. Plate B: lectin; plate C: pLT; plate D: CT. The band being positive in lanes 2–6 of D is probably GM1, this acid glycolipid being difficult to remove completely from the non-acid fraction. The upper binding-active band of lanes 2–6 of C is probably paragloboside while the lower binding-active double-band corresponds to GM1 and probably a non-acid glycolipid with six sugars. The solvent used was chloroform–methanol–water (60:35:8 by vol).

was explained (Fig. 5) by hydrogen bonding of Arg-13 and a glycosidic oxygen or a hydroxymethyl group, not possible for His-13. However, also a laboratory double mutant of CT (Table 1) with His-13 but with His-94-Asn, and Ala-95-Ser, as in LT, was binding to the two 4-sugar glycolipids. Therefore, other substitutions than His-13 to Arg-13 may induce this cross-binding. We are at present investigating this in greater detail.

The epitope on PGCs and glycoprotein being recognized by both hLT and pLT is most likely

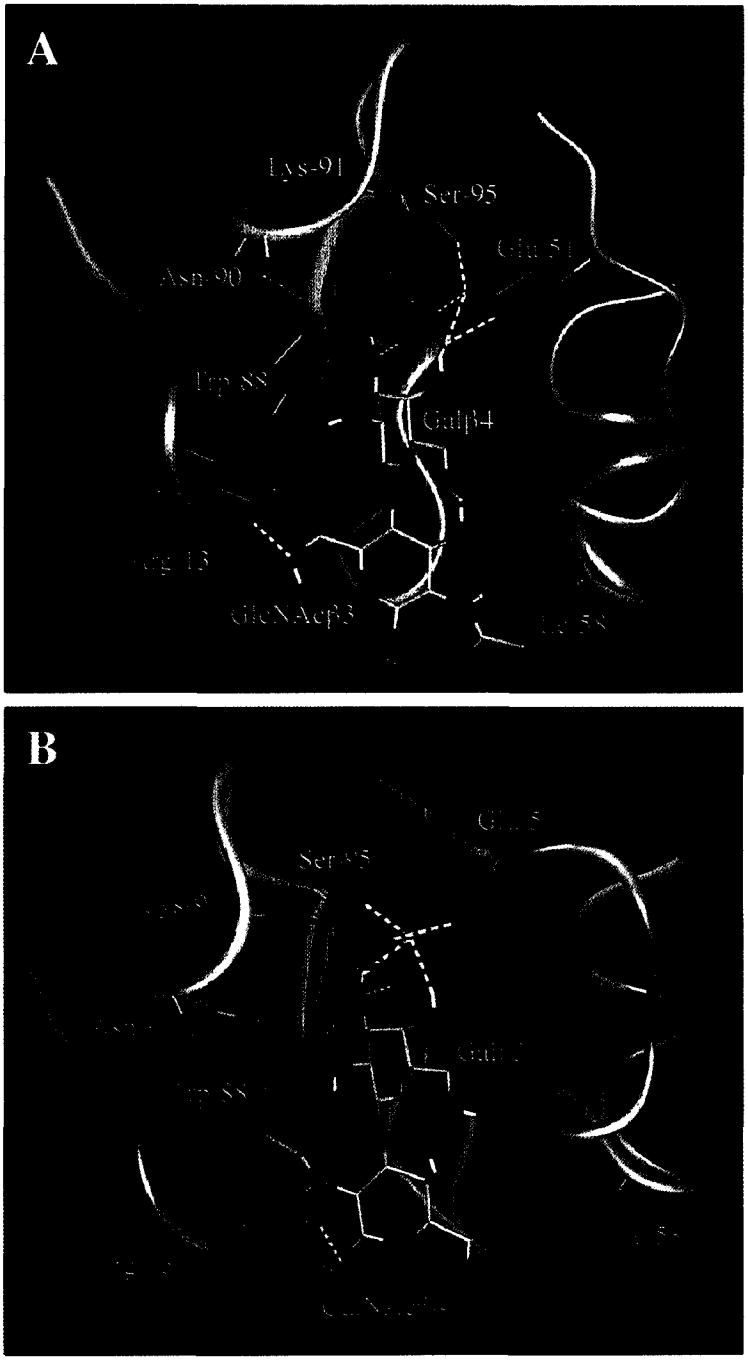

Figure 5. Models of paragloboside (A) and gangliotetraosylceramide (B) in the binding site of pLT (for clarity only the terminal disaccharides and selected amino acids are shown). Hydrogen bonds (dotted) are indicated for Gal interactions, and between Arg-13 and the hydroxymethyl group of GlcNAc and glycosidic oxygen and the acetamido carbonyl oxygen of GalNAc, respectively, proposed to be the basis for binding of these two glycolipids by pLT but not by hLT or CT having His at position 13 (Table 1). However, in the case of gangliotetraosylceramide alternative interactions between the side chain of Arg-13 (using a different starting conformation of the Arg-13 side chain in the dynamics run) and the Galβ4 2-OH and 3-OH as well as to the backbone carbonyl oxygen of Glu-18 were found, lodging the guanidino moiety behind the Galβ4 residue. The relative importance of these two configurations has not been established. Note also that GlcNAc is rotated about 180° compared to GalNAc. *Indicates the location of the glycosidic oxygens to which the remaining lactosylceramide part is attached. The two complexes are slightly rotated relative each other.

Galβ4GlcNAcβ, although this has to be carefully confirmed by chemical analysis. Galβ3GalNAcβ has not yet been safely documented in glycoproteins. However, the fact that hLT is not binding to paragloboside although it recognizes both PGCs and glycoprotein may be an argument against Galβ4GlcNAcβ as the determinant. Hypothetically, however, the physiological receptor for hLT on glycoprotein of human epithelium may be based on Galβ4GlcNAcβ but with an unidentified neighboring group essential for the apparently strong binding by both hLT and pLT (shown in Fig. 3 for hLT). The epitope is, however blocked by a sialic acid. Figure 3 also documents that *N*-acetyllactosamine coupled with a spacer to BSA is positive and that the corresponding sialylated neoglycoprotein is positive first after desialylation. A recent paper made it likely that the terminal Gal of a poly-*N*-acetyllactosamine series of glycoprotein in a human intestinal epithelial cell line, CaCo-2, was essential for LT binding, and this binding was enhanced after neuraminidase treatment.[12]

The lectin of *Erythrina cristagalli* has specificity for Galβ4GlcNAcβ but is binding about equally well to Fucα2Galβ4GlcNAcβ,[7] a sequence not recognized by the LT variants. The lectin did not bind significantly to the same glycoprotein band (Fig. 3) or to PGCs (Fig. 1) as hLT or pLT did, supporting that the binding subepitopes are non-identical.

It will thus be of interest to find out the nature of the epitope(s) on the glycoprotein and PGCs. *N*-Acetyllactosamine may be linked in various positions (2, 3, 4 or 6), to various sugars (Gal, GalNAc, Man), and with a variety of closely located branches.[11] Also, it may be substituted in different ways. Since hLT is negative for Galβ4GlcNAcβ3Gal in paragloboside it is not likely that the *N*-acetyllactosamine epitope is in a straight-chain poly-*N*-acetyllactosamine type of glycoprotein, which would render it negative also for hLT. May be a more mobile 6-linked *N*-acetyllactosamine without crowding neighboring groups is the essential requirement. A 6-link may be either to Gal, GalNAc or Man,[11] but in several cases there are close obligate branches which may prevent access for the toxin. For example, it was shown by modeling that an *N*-acetyllactosamine in 6-linkage to Gal is sterically hindered for binding by the plant lectin if the neighbor is a 3-linked *N*-acetyllactosamine on the same Gal, see the sequence in Table 1.[7] Thus, practically only a single glycoprotein may remain (Fig. 3D) with receptor activity among several carrying *N*-acetyllactosamine.

CT, being produced by *Vibrio cholerae*, is recognizing only GM1, while LT, being produced by *E. coli*, apparently has conserved a cross-binding with lower affinity to glycoprotein (or PGCs) in addition to the GM1 specificity. The reason for this difference between the two bacteria, which both colonize the same target, the small intestine, and apparently use the same mechanism mediated by the toxins, may at present only be speculated upon. Attachment to the membrane-close GM1 is apparently a prerequisite for the penetration of the active A subunit into the cell to produce the toxin effect. However, the dynamics of the intestine with motility, secretions, and food products in the lumen, may be critical for the toxin secreted by the bacterial cell, in order to reach the membrane epitope. Also considered essential is the bacterial cell adhesion to host receptors.[1] Hypothetically, the two bacteria may use different adhesion sites, where *Vibrio cholerae* adheres more intimately, thus allowing cholera toxin to be more directly exposed for GM1. *E. coli*, on the other hand, if adhering more distantly or further out from the membrane bilayer, may require a two-step attachment for its toxin, a first lower-affinity binding to glycoprotein epitopes followed by a final high-affinity binding to GM1. For this to take place, the bacterium has to cleave sialic acids to expose glycoprotein binding sites (Fig. 3), which may be possible through a hitherto unidentified neuraminidase secreted by the bacterium.

Conclusions

The situation concerning bacterial diarrhea-inducing toxins is more complex than first thought of. Cholera toxin and LTs are globally of major importance, show great sequence homology and are thought to use the same secretory mechanism in identical target cells mediated by high-affinity binding to GM1. However, LTs but not CT show an apparently conserved cross-binding to desialylated glycoproteins of human small intestinal epithelium (identical for five different individuals). The extra binding epitope is most probably Galβ4GlcNAcβ with critical neighboring groups and is explained by distinct point mutations in or close to the binding site of the LT B subunit. For pLT, Arg-13 (His-13 of CT) is at a favorable hydrogen bond distance of the hydroxymethyl group of GlcNAc. However, hLT with a His-13, is not binding to the reference paragloboside, but does bind to the desialylated glycoprotein similarly to pLT. More work is needed to precisely identify the binding epitope on the glycoprotein. The cross-binding of LTs may be an essential first-step attachment to more membrane-distant sites before the final docking to GM1 at the bilayer for toxin penetration takes place.

Experimental

B subunits of CT were from List Biological Laboratories, Inc., Campbell, CA, U.S.A. LTs were prepared as described,[13] the lectin of *Arachis hypogaea* (peanut lectin) was from Sigma, and the lectin of *Erythrina cristagalli* was from Vector Laboratories, Inc., Burlingame, CA, U.S.A. The CT mutant with Asn-94 and Ser-95, as in LT, was provided by Jan Holmgren. The proteins were labeled with ^{125}I and used as described in detail elsewhere.[5,7] Conditions for overlay of labeled toxins and lectin on TLC plates have been described,[5,7] as well as binding to blots after electrophoresis of glycoproteins.[8,14] Desialylation of glycoproteins on the blot was done as follows. After the blotting procedure, the nitrocellulose membrane was washed twice in 0.1

M sodium acetate buffer, pH 4.8, and incubated in 0.2 M glycine chloride buffer, pH 1.9 for 60 min at 95 °C. The membranes were washed twice in blocking solution[8] prior to blotting and binding experiments. Concerning neoglycoproteins used for Figure 3, Galβ4GlcNAcβ-CETE-BSA was purchased from BioCarb Ltd, Lund, Sweden (present supplier Accurate Chem. & Scien. Co, U.S.A.), and NeuAcα3Galβ4GlcNAcβ-DCP-BSA from Dextra Laboratories, Reading, U.K. The spacers to BSA (bovine serum albumin) were CETE: 2-(2-carbomethoxyethylthio)ethyl, and DCP: 1,5-dicarboxypentyl. Molecular modeling was conducted on a Silicon Graphics Indigo2 Extreme workstation using Quanta4.1/CHARMm23 software (Molecular Simulations, Inc.). Molecular dynamics simulations (100 ps) were performed using a water shell ($e=1$) or in vacuum ($e=4r$ or $8r$).

The methods used for the isolation of reference glycolipids listed in Table 1 were reported in previous papers.[5,7] Rabbit intestine was obtained fresh immediately after killing the animals. Mucosa scrapings of five human individuals were obtained at autopsy with due permission. Total non-acid glycolipids of rabbit and human were prepared according to a published procedure.[16] Polyglycosylceramides were prepared from the residues after traditional glycolipid extraction, using an acetylation-deacetylation procedure for solubilization.[6] Proteins were extracted by SDS from the residues after traditional glycolipid extraction.[8]

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