The Heat-Labile Enterotoxin of *Escherichia coli* Binds to Polylactosaminoglycan-Containing Receptors in CaCo-2 Human Intestinal Epithelial Cells

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ABSTRACT: The *E. coli* type I heat-labile enterotoxin (LT-I) shares considerable functional, structural, and immunological homology with cholera toxin (CT). Although the ganglioside G_{M1} is the sole receptor for CT, LT-I also appears to utilize additional, unique receptors on intestinal cells not recognized by CT. We characterized this second class of LT-I receptors using the human intestinal epithelial cell line, CaCo-2. CaCo-2 cells bound 8-fold more LT-I than CT, and some of these additional LT-I receptors appeared to be functional, as CT-B only partially inhibited LT-I activity at concentrations that completely inhibited CT activity. Membranes from unlabeled or [^{3}H]galactose-labeled cells were incubated with toxin B subunits and extracted with Triton X-100, and the solubilized toxin B–receptor complexes were immunoabsorbed with anti-B bound to protein A–Sepharose. When organic extracts of the complexes were separated by thin-layer chromatography and overlayed with [^{125}I]toxin, both toxins were found to bind only G_{M1}. Separation of the complexes from [^{3}H]galactose-labeled membranes by sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed a series of galactoproteins specifically recognized by LT-I but not by CT. Similar proteins were detected on Western blots probed with [^{125}I]toxin. LT-I activity on intact cells and binding to membranes and the above galactoproteins were enhanced by neuraminidase treatment even in the presence of CT-B. β-1,4-Galactosidase and endo-β-1,4-galactosidase, but not β-1,3-galactosidase, significantly reduced LT-I binding. LT-I binding to fetuin and transferrin exhibited a similar glycosidase sensitivity. When analyzed by Western blotting with [^{125}I]toxin, LT-I cross-reacted with glycoproteins immunoprecipitated from membrane extracts with antibodies specific for Le^x and the I/i blood groups and for the lysosomal membrane glycoproteins LAMP 1 and 2. These results indicated that polylactosylaminylated membrane glycoproteins represented an alternate class of receptors for LT-I in CaCo-2 cells.

Enterotoxigenic *Escherichia coli* produces the heat-labile toxin (LT[1]), the causative agent in "traveler's diarrhea" [Sack, 1975; for reviews, see Eidels *et al.* (1983) and Spangler (1992)]. The 86 kDa heterohexamer is composed of an A subunit possessing latent ADP-ribosyltransferase activity and a pentameric B subunit responsible for receptor recognition and cellular attachment. In the intestinal epithelial cells of the host, the internalized A subunit undergoes selective proteolysis and reduction of the lone disulfide bond to generate the A_1 peptide. The latter mediates the irreversible activation of adenylyl cyclase through transfer of ADP-ribose from NAD to the α subunit of the stimulatory G protein (G_s). The resulting elevation of intracellular cyclic AMP levels is responsible for the extensive loss of water and electolytes into the intestinal lumen and the characteristic diarrhea associated with *E. coli* infection.

The type I heat-labile toxin of *E. coli* (LT-I) and cholera toxin (CT) produced by *Vibrio cholerae* are immunologically, structurally, and functionally similar (Honda *et al.*, 1981; Pickett *et al.*, 1986). Two antigenic variants of type I heat-labile toxin are produced by isolates of *E. coli* from humans and pigs (Honda *et al.*, 1981). Antisera raised against CT will cross-react and neutralize both forms of LT-I, which together with CT form serogroup I. LT-I and its more virulent relative CT are highly homologous, with almost 80% identity at both the nucleotide and amino acid levels (Mekalanos *et al.*, 1983; Yamamoto *et al.*, 1984). The crystal structure of LT-I at a resolution of 2.3 Å has been published (Sixma *et al.*, 1991).

The biologically active receptor for cholera toxin (CT) has been well established as the ganglioside G_{M1} [for reviews, see Fishman (1982, 1986, 1990)]. It has also been shown that CT will bind weakly to the galactoproteins of rat intestinal brush border membranes and gastric mucins (Morita *et al.*, 1980). It is unclear, however, whether this binding is specific or functional (Critchley *et al.*, 1981). As with CT, G_{M1} also serves as a functional receptor for LT-I when the ganglioside is incorporated into G_{M1}-deficient cells (Moss *et al.*, 1979, 1981; Fishman *et al.*, 1993). Unlike CT, LT-I exhibits a relatively high affinity for galactose polymers (Clements *et al.*, 1980) and, in several instances, has been shown to bind specifically to glycoproteins from the intestinal brush border membranes of several species (Holmgren *et al.*, 1982; Griffiths *et al.*, 1986; Zemelman *et al.*, 1989; Griffiths & Critchley, 1991). The apparent differences in toxin binding specificity for CT and LT-I suggest subtle variations in their B subunits.

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[1] Abbreviations: CT, cholera toxin; LT, *E. coli* heat-labile toxin; LT-I, heat-labile toxin type I; G_{M1}, II^{3}Neu5Ac-GgOse$_4$Cer; G_s, stimulatory G protein; IBMX, 3-isobutyl-1-methylxanthine; TBS, Tris-buffered saline; LAMP, lysosomal membrane glycoprotein; SSEA, stage-specific mouse embryonic antigen; Le^x, Galβ1–4[Fucα1–3]GlcNAcβ1–3Galβ1–4Glc; i blood group, Galβ1–4GlcNAcβ1–3Galβ1–4Glc; I blood group, Galβ1–4GlcNAcβ1–3[Galβ1–4GlcNAcβ1–6]Galβ1–4Glc; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

In addition to receptor specificity for both toxins, the question arises as to whether or not these additional receptors for LT-I are biologically active. This possibility is supported by the inability of the B subunit of CT (at concentrations capable of blocking the action of the holotoxin) to prevent LT-I activity in rabbit and rat intestinal cells (Holmgren *et al.*, 1982; Zemelman *et al.*, 1989).

In this study, we examine the differences in LT-I and CT receptors using the culture-adapted human intestinal epithelial cell line, CaCo-2. In culture, these cells behave as highly differentiated enterocytes (Hidalgo *et al.*, 1989). We found that, whereas both toxins utilized G_{M1} as a receptor, LT-I uniquely bound to the terminal sequence Gal(β1–4)GlcNAc-(β1–3)-R commonly associated with N-linked complex carbohydrate chains and the poly-*N*-lactosamine series (of the lacto-*N*-neotetraosyl type) of glycoproteins. We also present evidence that suggests that a subset of cell surface proteins expressing this determinant act as biologically functional receptors for LT-I.

EXPERIMENTAL PROCEDURES

Materials. CT, CT-B, and anti-CT-B antibodies were obtained from List Biological Laboratories (Campbell, CA). Human isolates of LT-I and and its B subunit were the generous gifts of Drs. R. Holmes, Uniformed Services University of the Health Sciences (Bethesda, MD) and J. D. Clements, Tulane Medical Center (New Orleans, LA), respectively. $Na^{125}I$ was obtained from DuPont-NEN, and D-[4,5-$^3H_2$]galactose was from American Radiolabeled Chemicals, Inc. Sigma was the source of 3-isobutyl-1-methylxanthine (IBMX). Poly-(isobutyl methacrylate) was obtained from Polysciences (Warrington, PA). All glycosidases were obtained from Boehringer Mannheim. Fetuin and human transferrin were obtained from Calbiochem. Antibodies to the lysosomal membrane glycoproteins 1 and 2 (LAMP 1 and 2) were the generous gift of Dr. Minoru Fukuda, La Jolla Cancer Research Foundation (La Jolla, CA). Stage-specific mouse embryonic antigen (SSEA-1) antisera were provided by Dr. Barbara B. Knowles of the Wistar Institute of Anatomy and Biology (Philadelphia, PA). Blood-group-specific I/i antisera were provided by the American Red Cross Reference Laboratories (Gaithersburg, MD).

Cells and Cell Culture. Human intestinal CaCo-2 and neurotumor SK-N-MC cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in Eagle's minimal essential medium supplemented with nonessential amino acids and sodium pyruvate (BioWhittaker, Walkersville, MD) and either 20% (CaCo-2) or 10% (SK-N-MC) NUSERUM IV (Collaborative Biomedical Products, Bedford, MA). For experiments on intact cells, cultures were grown to confluency in 24-well cluster plates; the medium was replaced with serum-free medium buffered with 25 mM HEPES containing 0.01% bovine serum albumin (medium A), and the cells were used as will be described. To label cells with [^{3}H]galactose, confluent cultures of CaCo-2 cells were washed in serum-free medium and incubated at 37 °C overnight in glucose-free medium containing 5% NUSERM and 100 μCi/mL [^{3}H]galactose. Crude membranes then were prepared from these cells as will be described.

Toxin Binding Assays. Intact cells were washed once and chilled at 4 °C for 15 min. Cells then were incubated with iodinated toxin (10^5 cpm/pmol) in 0.5 mL of medium A for 1 h at 4 °C, after which time they were washed three times with 0.5 mL of phosphate-buffered saline (pH 7.4), solubilized with 0.5 mL of 1 M NaOH, and counted. Assays to determine the ability of toxin B subunits to inhibit binding were conducted as described earlier, except that the cells were treated with the indicated concentration of B subunit for 30 min at 4 °C and the medium was aspirated prior to the addition of [^{125}I]-toxin. Nonspecific binding was routinely determined in the presence of 0.5–1.0 μM unlabeled B subunit. For toxin binding to crude membranes, membranes were prepared from confluent cultures of CaCo-2 cells, as described by Zhou and Fishman (1991), and suspended in 10 mM Tris-HCl (pH 7.7). Assays routinely contained 7–17 μg of membrane protein and increasing concentrations of [^{125}I]toxin (10^5 cpm/pmol) in a total volume of 0.5 mL of 0.15 M NaCl/1 mM EDTA/25 mM Tris-HCl (pH 7.4)/0.1% bovine serum albumin. Competition binding assays used 3.3×10^5 cpm of toxin (~110–132 fmol) per 0.5 mL of reaction mixture in the presence of increasing concentrations of toxin B subunit. Samples were then incubated for 1 h at 4 °C. Three 0.15 mL portions of each sample (~2–5 μg membrane protein) then were collected on Millititer GV 96-well microfiltration plates (Millipore) and washed 3 times with 0.2 mL of buffer, and the filters were counted.

Toxin-Stimulated Accumulation of Intracellular Cyclic AMP. Cultures were washed and chilled at 4 °C in medium A for 15 min prior to exposure to B subunit or holotoxin. Commercial preparations of CT-B were affinity-purified to remove any residual toxin activity with anti-CT-A_1 antiserum, as previously described (Orlandi & Fishman, 1993). Where indicated, the cells then were incubated with B subunit for 30 min at 4 °C, and the medium was aspirated and replaced with medium A containing either 0.03 nM CT or 0.3 nM LT-I for an additional 30 min at 4 °C. The medium then was removed, and the cells were incubated at 37 °C in medium A containing 1 mM IBMX for 2 h. The cells were extracted with 0.1 M HCl, and the extracts were assayed for cyclic AMP by radioimmune assay (Zaremba & Fishman, 1984).

Glycosidase Digestions. Confluent cells grown in 24-well clusters were washed once in serum-free medium and incubated in the same medium containing 10 munits/mL *Vibrio cholerae* neuraminidase at 37 °C for 60 min. Treated monolayers then were rinsed, exposed to either toxin B subunits and/or toxin as indicated, and subsequently assayed for cyclic AMP accumulation. Glycosidases were also used to study their effects on toxin binding to either crude membrane preparations or detergent extracts of crude membrane preparations separated by SDS–PAGE and electophoretically transferred onto nitrocellulose. In membrane binding studies, membranes were prepared as described here and suspended in the appropriate buffer at 1 mg/mL. Samples were treated overnight at 37 °C in 50 mM sodium acetate with 10 munits/mL either *V. cholerae* neuraminidase at pH 5.5, *Diplococcus pneumoniae* β-galactosidase at pH 6.0, or *Bacteroides fragilis* endo-β-galactosidase at pH 6.0. For bovine testes β-galactosidase, 100 mM sodium citrate (pH 4.5) was used. Following glycosidase treatment, membranes were pelleted (16000*g* for 10 min) and suspended in 10 mM Tris-HCl (pH 7.7) for binding assays. Glycosidase digestions of fetuin and transferrin were essentially the same with the exception of the sequential neuraminidase and bovine testes β-galactosidase treatments, which required a buffer exchange to 100 mM sodium citrate (pH 4.5) for the latter activity. Fetuin and transferrin were treated with endoglycosidase F/*N*-glycosidase in 20 mM sodium cacodylate (pH 6.0) containing 0.1% SDS and 1% Triton X-100. Glycosidase treatments of electroblots were performed in the same buffers as before and included 0.05% Tween 20. When nitrocellulose blots were treated sequen-

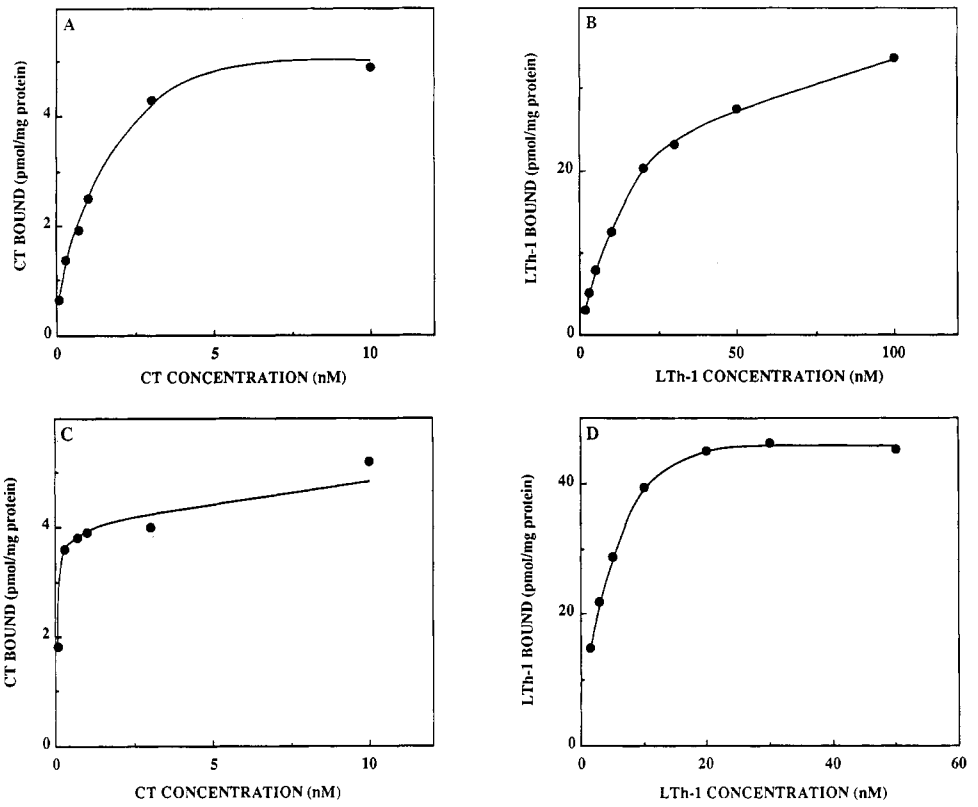

FIGURE 1: Concentration-dependent binding of ^{125}I-labeled CT and LT-I. [^{125}I]CT (A, C) and [^{125}I]LT-I (B, D) were incubated with either intact CaCo-2 cell monolayers (A, B) or crude membrane preparations (C, D), as described in the Experimental Procedures. Each point is the mean of triplicate determinations with standard deviations of <10%. Results also have been corrected for nonspecific binding in the presence of 1 μM CT-B or LT-B.

tially with neuraminidase and a subsequent glycosidase, strips were incubated for 60 min at 37 °C with neuraminidase, washed, and incubated overnight at 37 °C with the indicated glycosidase.

Detection of Toxin–Receptor Complexes. Toxin–receptor complexes were isolated and analyzed by established procedures (Critchley *et al.*, 1981; Spiegel *et al.*, 1985). Briefly, unlabeled or [^{3}H]galactose-labeled membrane preparations were incubated with either 30 nM CT-B or 100 nM LT-I-B for 1 h at 4 °C. Membranes were pelleted, suspended in TBS (50 mM Tris-HCl/150 mM NaCl (pH 8.0)) containing 1% Triton X-100, and extracted for 1 h at 4 °C. Any insoluble material was removed by centrifugation (16000*g*, for 10 min), and the soluble toxin B–receptor complexes were immunoabsorbed with anti-CT-B bound to protein A–Sepharose 4B CL (Pharmacia). Glycolipid analysis was performed by extracting the absorbed material with chloroform/methanol (1:1, v/v) and chromatographing the extracts on plastic-backed silica gel sheets in a solvent system of chloroform/methanol/water (60:35:8, v/v). To identify galactoproteins, [^{3}H]-galactose-labeled immunoabsorbed material was resolved by SDS–PAGE under reducing conditions using 5–20% acrylamide gradient gels and detected by fluorography. Extracts also were incubated with I/i blood-group-specific antiserum, anti-Le^x (SSEA) antiserum, and anti-sera to LAMP 1 and 2. Samples then were absorbed with the appropriate secondary antibody and protein A–Sepharose. Proteins were separated by SDS–PAGE, electroblotted onto nitrocellulose, and probed with [^{125}I]toxin.

Toxin Overlays. Thin-layer chromatograms of glycolipid extracts were analyzed for toxin binding components by a slight modification of the method of Magnani *et al.* (1980). Briefly, chromatograms were treated for 1 min in 0.1% (w/v) poly(isobutyl methacrylate), air-dried, and blocked for 1 h in TBS/0.3% Tween 20 (pH 7.4). The chromatograms were then overlayed with [^{125}I]toxin (10^6 cpm/mL, 0.3–0.4 nM) in TBS/0.05% Tween 20 (pH 7.4) overnight at 4 °C. After the toxin solutions were removed, the plates were washed extensively with TBS/0.3% Tween 20, air-dried, and subjected to autoradiography. Toxin overlays of electroblots were performed in essentially the same manner, with the exception of the poly(isobutyl methacrylate) treatment.

Other Methods. Toxins were iodinated by a chloromine-T procedure, as described previously (Griffiths *et al.*, 1986), and routinely had a specific activity of ~15–20 μCi/μg. Protein content was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard. SDS–PAGE was performed according to Laemmli (1970). The electrophoretic transfer of proteins separated by SDS–PAGE was performed as described elsewhere (Towbin *et al.*, 1979).

RESULTS

Differential Binding of CT and LT to Intact CaCo-2 Cells and Membranes. To determine whether the human intestinal epithelial cell line, CaCo-2, expressed additional receptors for LT-I other than G_{M1}, we first compared the binding of [^{125}I]CT and [^{125}I]LT-I to these cells (Figure 1). The binding of both iodotoxins to intact cell monolayers (Figure 1A,B) indicated that CT had an apparently higher affinity for the cells than LT-I (half-maximal binding occurred at ~0.5 and 10 nM, respectively), whereas LT-I maximally bound to ~8-fold more sites than CT (34 vs 4.3 pmol/mg of cell protein).[2]

[2] Because previous studies reported that toxin binding is complex and yields curvilinear Scatchard plots exhibiting positive cooperativity (Fishman *et al.*, 1978; Fishman & Atikkan, 1980; Griffiths *et al.*, 1986; Lencer *et al.*, 1987), we did not further analyze the binding data.

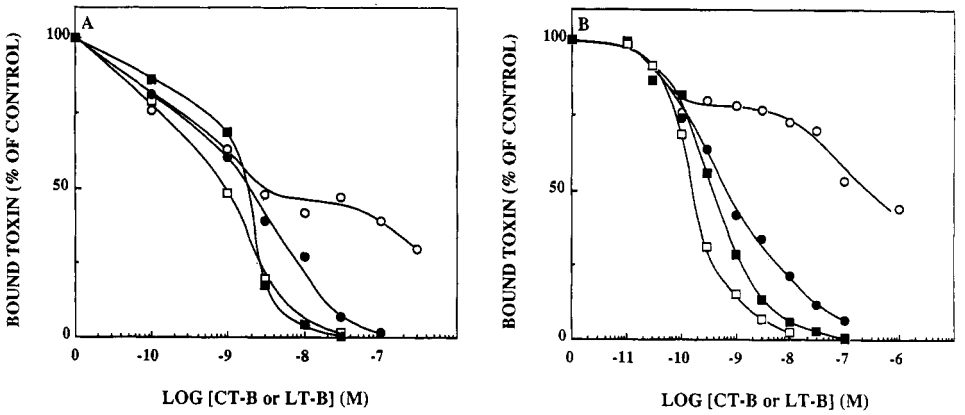

FIGURE 2: Concentration-dependent effects of toxin B subunits on the binding of ^{125}I-labeled CT or LT-I. Iodinated toxin (0.2 nM) was incubated with either intact CaCo-2 cell monolayers (A) or crude membrane preparations (B) pretreated with increasing concentrations of either CT-B or LT-B, as described in the Experimental Procedures: [^{125}I]CT + LT-B (■); [^{125}I]LT-I + LT-B (□); [^{125}I]CT + CT-B (●); [^{125}I]LT-I + CT-B (○). The results are expressed as a percent of control binding in the absence of B subunit. Each point is the mean of triplicate determinations with standard deviations of <10%.

Similar results were obtained when toxin binding to crude membrane preparations was determined (Figure 1C,D). The binding of [^{125}I]CT was inhibited in a similar concentration-dependent manner when intact cells or membranes were preincubated with either unlabeled CT-B or LT-B (Figure 2A,B, respectively). Complete inhibition of binding was observed at 10–30 nM B subunit. Binding of [^{125}I]LT-I was inhibited in a similar manner by its B subunit, but only partially by CT-B (50% of control binding at 300 nM CT-B). Further inhibition by higher concentrations of CT-B suggested that CT-B may weakly recognize the additional LT-I receptors.

LT-I Binding to Gangliosides and Galactoproteins. Crude CaCo-2 membranes were incubated with either toxin B subunit, washed, and extracted with nonionic detergent. The extracts then were immunoadsorbed with anti-CT-B antibodies bound to protein A–Sepharose. The immunoadsorbed samples were extracted with solvent and separated by thin-layer chromatography. The chromatograms were overlayed with ^{125}I-labeled toxin to detect any glycolipid(s) that may have preferentially bound LT-I but not CT. In each instance, however, the only glycolipid bound by either toxin was identified as ganglioside G_{M1} (Figure 3A,B). Additional experiments were carried out using membranes prepared from cells labeled with [^{3}H]galactose to examine specific toxin binding to glycoproteins. Whereas CT-B failed to recognize any galactoproteins when the immunoadsorbed samples were analyzed by SDS–PAGE, LT-I-B was found to specifically recognize a series of galactoproteins (Figure 3C). The most notable were several galactoproteins clustered between 92 and 120 kDa. Both toxin B subunits recognized material that migrated near the dye front and was presumably G_{M1} (Critchley *et al.*, 1981, 1982; Spiegel *et al.*, 1985).

Effects of Glycosidase Treatment on the Binding of LT-I to Membrane Glycoproteins. Enzymatic digestion of CaCo-2 membranes with *V. cholerae* neuraminidase, prior to exposure to the toxins, revealed contrasting effects on the binding of [^{125}I]CT and [^{125}I]LT-I (Table 1). As expected, the binding of CT was increased nearly 4-fold by the neuraminidase treatment due to the conversion of more complex gangliosides to G_{M1} (Miller-Podraza *et al.*, 1982). This is supported by the ability of CT-B to effectively inhibit CT binding even to neuraminidase-treated membranes. In comparison, treatment with neuraminidase increased LT-I binding >6-fold and appeared to expose new, previously cryptic sites that were largely insensitive to CT-B. We examined this effect further by using the methods to analyze immunoabsorbed toxin–receptor complexes described earlier. Toxin overlays of thin-layer chromatograms indicated that, while binding to G_{M1} increased for both LT-I and CT, neither toxin bound to any additional glycolipids (data not shown). Binding of the B subunit of LT-I to the family of galactoproteins in the 92–120 kDa molecular mass range was enhanced by prior neuraminidase treatment of the [^{3}H]galactose-labeled membranes (Figure 4C, lane 3). Whereas neuraminidase treatment enhanced toxin binding, *D. pneumoniae* β-1,4-galactosidase and *B. fragilis* endo-β-1,4-galactosidase treatment resulted in significant inhibition of LT-I binding to receptors other than G_{M1}, in contrast to the negligible effects on CT binding (Table 1).

The effect of glycosidases on the binding of LT-I was examined further with a modified Western blot technique. Membrane proteins were separated by SDS–PAGE and transferred to nitrocellulose; the latter was treated with a panel of glycosidases and overlayed with [^{125}I]toxin (Figure 4). The resulting protein profile recognized by [^{125}I]LT-I in the toxin overlays closely resembled those galactoproteins identified by immunoadsorption of the toxin B–receptor complexes (compare Figures 3 and 4). Similarly, the effects of the different glycosidases on the binding of LT-I to those proteins in the 92–120 kDa range and in membrane binding assays (Table 1) were quite similar. As observed earlier, [^{125}I]-LT-I binding to glycoproteins was enhanced significantly by neuraminidase treatment (Figure 4B, lane 1); however, this modification now made the receptors more susceptible to the action of *B. fragilis* endo-β-1,4-galactosidase, which abrogated LT-I binding (Figure 4B, lane 5). In addition, bovine testes β-1,3-galactosidase had no effect on LT-I binding (lane 4), in direct contrast to the ability of *D. pneumoniae* β-1,4-galactosidase to completely inhibit toxin binding (lane 3). Whereas LT-B (Figure 4A,B, lanes 6) completely blocked [^{125}I]LT-I binding, CT-B had no effect (lanes 2), and [^{125}I]-CT bound neither to untreated nor neuraminidase-treated membrane blots (lanes 7). Both the membrane binding studies and toxin overlay experiments were indicative that the minimal requirement for LT-I binding to receptors other than G_{M1} was the presence of terminal β-1,4-linked galactosyl residues. The inhibitory effects of endo-β-1,4-galactosidase treatment of membranes on the binding of LT-I further indicated that polylactosaminyl determinants with a terminal β-1,4-linked galactose were competent receptors for LT-I.

LT-I Binding to Complex and Polylactosaminyl-Containing Glycoproteins. To confirm that LT-I utilized glycoproteins

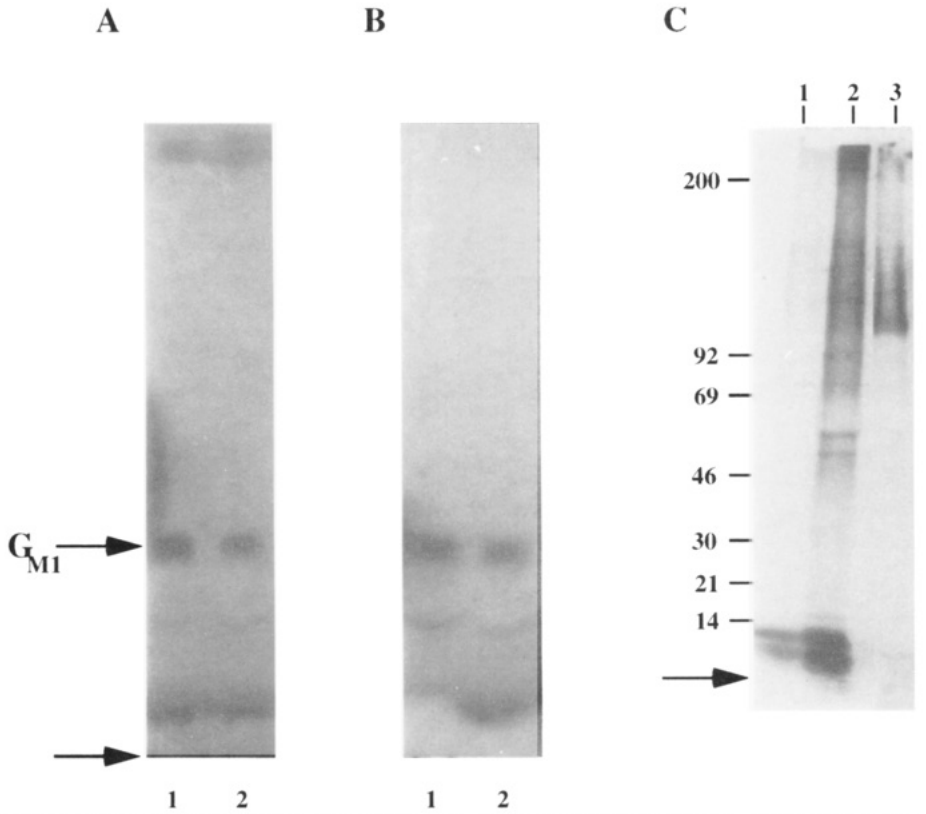

FIGURE 3: Identification of CT and LT-I binding components in CaCo-2 membranes. Unlabeled (A, B) and [^{3}H]galactose-labeled (C) CaCo-2 membranes were incubated with either CT-B or LT-B, washed, and extracted with nonionic detergent. The extracts then were immunoabsorbed with anti-CT-B antibodies and analyzed for glycolipids and glycoproteins, as described in the Experimental Procedures. Panels A and B: Immunoabsorbed material from membranes containing bound CT-B (lane 1) or LT-B (lane 2) was extracted with chloroform/methanol and separated by thin-layer chromatography, and the chromatograms were overlayed with either ^{125}I-labeled CT (A) or LT-I (B). The mobility of standard G_{M1} is shown on the left-hand side. The arrow at the bottom of the chromatograms represents the point of sample application. Panel C: Immunoabsorbed material from [^{3}H]galactose-labeled membranes containing bound CT-B (lane 1) or LT-B (lane 2) was separated by SDS–PAGE and detected by fluorography. Lane 3: Same as lane 2 except that the membranes were treated first with *V. cholerae* neuraminidase. The arrow represents the dye front.

Table 1: Effects of Glycosidases on the Binding of ^{125}I-Labeled LT-I and CT to CaCo-2 Membrane[a]

glycosidase treatment	binding (% of control) [^{125}I]CT	[^{125}I]LT-I
untreated membranes	100[b]	100[b]
+ 30 nM CT-B	4	56.3
V. cholerae neuraminidase	388	634
+ 30 nM CT-B	33	491
D. pneumoniae β-galactosidase	87.2	16.7
+ 30 nM CT-B		6.1
endo-β-galactosidase	119	37.9
+ 30 mM CT-B		0.5

[a] Membranes were prepared from CaCo-2 cells, and portions were treated with the indicated glycosidases. The control and treated membranes then were assayed for specific binding of either 1 nM ^{125}I-labeled CT or 30 nM ^{125}I-labeled LT-I in the absence or presence of 30 nM CT-B. See Experimental Procedures for further details. [b] Control membranes bound 16.3-fold more LT-I than CT at concentrations that reflected saturable binding (30 and 1.0 nM, respectively).

with terminal β-1,4-linked galactosyl residues as alternate receptors, the binding of the toxin to fetuin and human transferrin as model proteins was examined. The carbohydrate structures of these glycoproteins have been characterized extensively (Nilsson *et al.*, 1979; März *et al.*, 1982): both contain N-linked complex carbohydrate chains in which β-1,4-linked galactose is present as the penultimate residue. Using the Western blot technique, [^{125}I]LT-I failed to bind to either glycoprotein (Figure 5A,B, lanes 1). Treatment of the glycoproteins with *V. cholerae* (lanes 2) or *Arthrobacter ureafaciens* (lanes 3) neuraminidase, which exposes these galactose residues, was equally effective in generating LT-I binding sites on these glycoproteins. Toxin binding to these neuraminidase-generated sites was sensitive to the action of *D. pneumoniae* β-1,4-galactosidase and endoglycosidase F/*N*-glycosidase F. Sequential treatment of both fetuin and transferrin with *A. ureafaciens* neuraminidase and either of these glycosidases destroyed LT-I binding sites (Figure 5, lanes 4 and 6, respectively). In contrast, bovine testes β-1,3-galactosidase had no effect on these new sites (lanes 5). Removal of the O-linked oligosaccharides of asialofetuin also had no effect on LT-I binding (data not shown).

Antisera to the I/i and Le^x (SSEA-I) blood groups, as well as the lysosomal membrane glycoproteins LAMP 1 and 2, were employed to further analyze the carbohydrate determinants of the alternate LT-I receptors. The common characteristic of each of the anti-blood group antisera is their reactivity toward Galβ1–4GlcNAcβ1–3-containing determinants. While LAMP 1 and 2 antisera are directed against polypeptide rather than carbohydrate, these glycoproteins are heavily glycosylated with polylactosaminoglycans (Viitala *et al.*, 1988; Carlsson *et al.*, 1988). Crude membranes were prepared, extracted, and immunoabsorbed with the above-mentioned antisera. The immunoabsorbed proteins were then separated by SDS–PAGE, electroblotted onto nitrocellulose, and probed with [^{125}I]LT-I. Electroblots of extracts immunoabsorbed with a mixture of anti-LAMP 1 and 2 antisera showed weak toxin binding to a diffuse band in the 100–120 kDa range, whose intensity increased significantly with neuraminidase treatment of the blots (Figure 6A, lanes 1 and

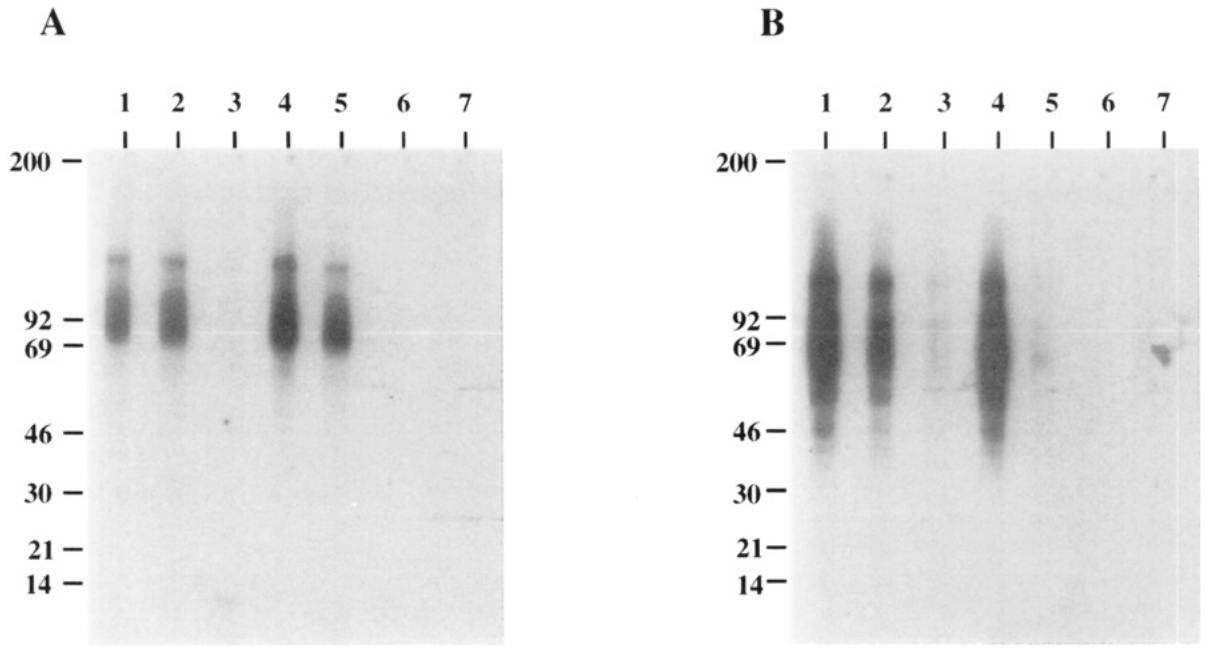

FIGURE 4: Binding of iodotoxins to electroblots of CaCo-2 membrane proteins. Membrane extracts were separated by SDS–PAGE and electroblotted onto nitrocellulose sheets, which were treated with toxin B subunits or glycosidases and overlayed with ^{125}I-labeled LT-I (lanes 1–6) or CT (lane 7) as described in the Experimental Procedures. Electroblots were either untreated (A) or treated with *V. cholerae* neuraminidase (B) prior to the indicated treatment: lanes 1 and 7, control; lane 2, 30 nM CT-B; lane 3, *D. pneumoniae* β-galactosidase; lane 4, bovine testes β-galactosidase; lane 5, *B. fragilis* β-galactosidase; lane 6, 1 μM LT-B.

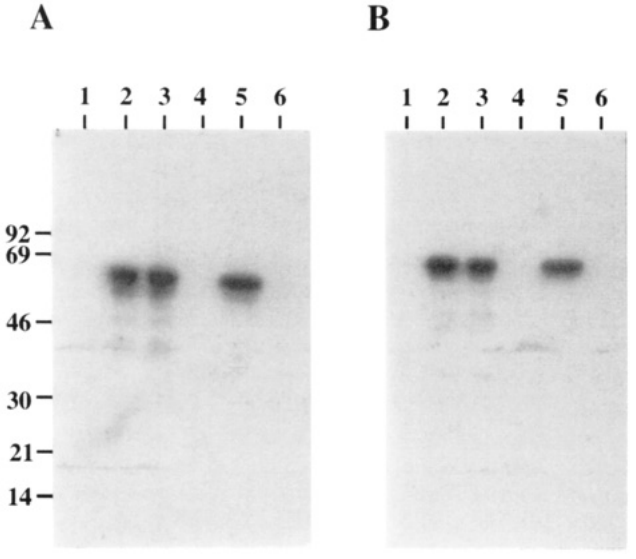

FIGURE 5: Binding of [^{125}I]LT-I to fetuin and human transferrin. Fetuin (A) and human transferrin (B) were treated with the indicated glycosidases, separated by SDS–PAGE, and electroblotted onto nitrocellulose sheets that were overlayed with [^{125}I]LT-I: lane 1, untreated; lane 2, *V. cholerae* neuraminidase; lanes 3–6, *A. ureafaciens* neuraminidase; lane 4, plus *D. pneumoniae* β-galactosidase; lane 5, plus bovine testes β-galactosidase; lane 6, plus endoglycosidase F/*N*-glycosidase. Relative molecular weight markers are shown on the left-hand side.

2). [^{125}I]LT-I recognized a less abundant glycoprotein, which migrated slightly faster and was more sharply defined in blots of membrane extracts immunoabsorbed with anti-I serum (Figure 6B, lane 1). More efficient binding to this band again was dependent on neuraminidase treatment (lane 2). In contrast, [^{125}I]LT-I bound predominantly to glycoproteins that migrated around 69 kDa on blots of membrane extracts immunoabsorbed with either anti-i or anti-Le^x antisera (Figure 6C,D, lanes 1). The binding of [^{125}I]LT-I to these glycoproteins was also enhanced by neuraminidase treatment (lanes 2). In addition, LT-I appeared to recognize, to varying degrees, glycoproteins that migrated around 50 kDa from membrane extracts immunoabsorbed with some of these antisera. We cannot rule out the possibility that these represent the degradation of the high molecular weight glycoproteins. Finally, in some of the blots, [^{125}I]LT-I bound to lower weight material migrating near the dye front. As the transfer solution contained methanol, it is unlikely that this material represents gangliosides that do not absorb to the nitrocellulose under these conditions (Critchley *et al.*, 1986; also compare Figure 3C, lane 1, with Figure 4A, lane 7).

To further identify the LAMP glycoproteins as receptors for LT-I, extracts of control and neuraminidase-treated membranes from cells labeled with [^{3}H]galactose were immunoabsorbed with either anti-LAMP 1 or 2 antiserum, separarated by SDS–PAGE, and detected by fluorography (Figure 7). In each case, the predominant labeled band migrated slightly above the 92 kDa standard, which agreed with the results of Youakim *et al.* (1989). They obtained remarkably similar glycoproteins when they immunoprecipitated membrane extracts from [^{3}H]glucosamine-labeled CaCo-2 cells with anti-LAMP 1 and 2 antisera. Whereas LAMP 1 appeared to migrate slightly faster than LAMP 2 (panel A, lanes 3 and 4), this difference seemed to disappear when the membranes were first treated with neuraminidase (panel B, lane 2 and 3). The latter also appeared to correspond to galactoproteins extracted from labeled membranes treated with LT-I-B and immunoadsorbed with anti-B (panel A, lane 2). As expected, more of these galactoproteins were recovered from neuraminidase-treated membranes (panel B, lane 1), whereas none were detected from membranes containing bound CT-B (panel A, lane 1). Taken together with the results shown in Figure 6A, the data were strongly consistent with LT-I binding to the LAMP glycoproteins.

Differences in CT and LT-I Stimulation of Cyclic AMP Accumulation in CaCo-2 Cells. We have shown previously that CaCo-2 cells are highly sensitive to CT, as measured by intracellular cAMP accumulation, with half-maximal stimulation occurring at 50 pM CT (Orlandi *et al.*, 1993). CaCo-2

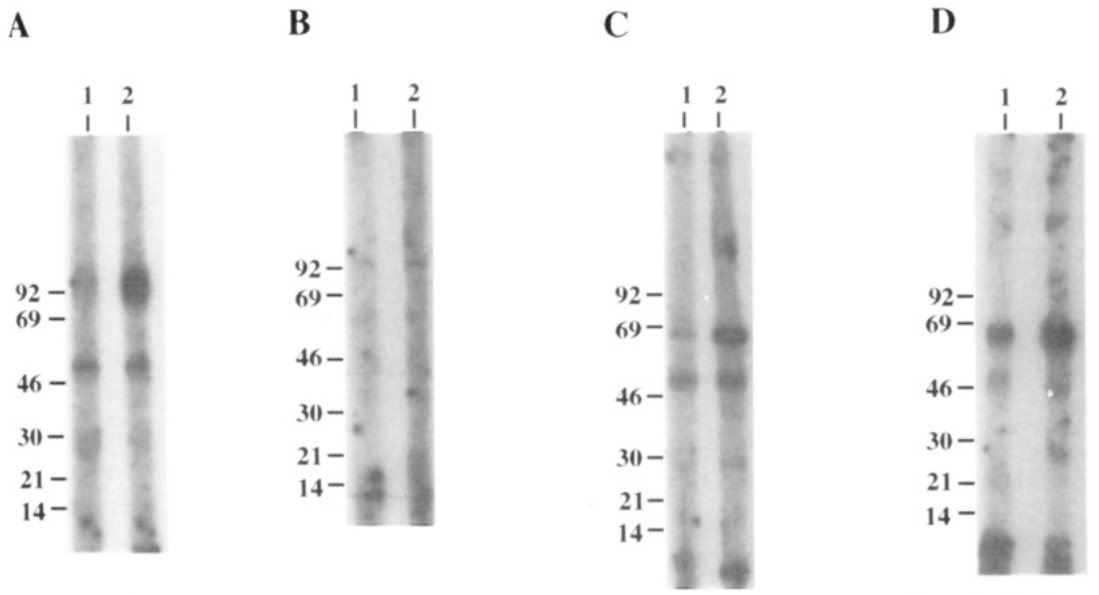

FIGURE 6: Binding of [^{125}I]LT-I to CaCo-2 membrane extracts immunoabsorbed with polylactosaminyl-specific antibodies. Detergent extracts of CaCo-2 membranes were incubated with the following antisera: (A) anti-LAMP 1 and 2 mixture; (B) anti-blood group I; (C) anti-blood group i; (D) anti-Le^x. The extracts then were immunoabsorbed with the appropriate secondary antibody bound to protein A–Sepharose. The immunoabsorbed samples were separated by SDS-PAGE and electroblotted onto nitrocellulose sheets that were probed with ^{125}I-labeled LT-I. Each nitrocellulose strip was either not treated (lane 1) or treated with neuraminidase (lane 2) prior to toxin binding.

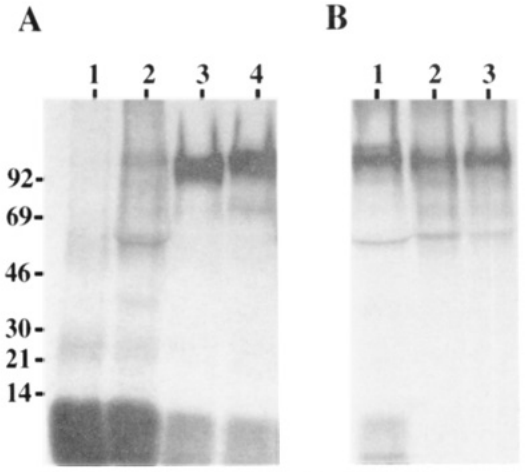

FIGURE 7: Comparison of CaCo-2 LAMP glycoproteins with galactoproteins that bind LT-I. Detergent extracts of control (A) and neuraminidase-treated (B) membranes from [^{3}H]galactose-labeled cells were immunoabsorbed as described, separated by SDS–PAGE, and detected by fluorography. Panel A: Extracts were absorbed with either CT-B (lane 1) or LT-I-B (lane 2) and then anti-CT-B antibodies or with anti-LAMP 1 (lane 3) or anti-LAMP 2 (lane 4) antibodies. Panel B: Extracts first were absorbed with CT-B and anti-B antibodies to remove excess G_{M1}. The extracts then were absorbed with LT-I-B and anti-B antibodies (lane 1) or with anti-LAMP 1 (lane 2) or anti-LAMP 2 (lane 3) antibodies. Equal amounts of ^{3}H were applied to each lane; both panels A and B were derived from the same gel, but were exposed for 96 and 24 h, respectively.

cells appeared to be less sensitive to LT-I as 300 pM was required to produce a half-maximal response (data not shown). This agreed with the differences in binding affinity (see Figure 1). Preincubation of intact cells with increasing concentrations of CT-B resulted in a corresponding decrease in the CT-stimulated accumulation of cyclic AMP (Figure 8A). LT-I-B had similar effects on CT activity (94% inhibition by 200 nM LT-I-B vs 92% inhibition by CT-B). In contrast, CT-B was relatively ineffective at inhibiting the activity of LT-I (Figure 8A). At concentrations of CT-B that inhibited CT responsiveness by >95%, LT-I stimulation of cyclic AMP

Table 2: Effects of *Vibrio cholerae* Neuraminidase on CT and LT-I Stimulation of Cyclic AMP Accumulation in Intact CaCo-2 Cells[a]

treatment	cyclic AMP (pmol/mg of protein)[b]	
	CT	LT-I
untreated cells	1630 ± 68 (100)[c]	2330 ± 44 (100)[c]
+ 300 nM CT-B	235 ± 31 (14.4)	1260 ± 92 (54.1)
V. cholerae neuraminidase	2850 ± 273 (174)	3630 ± 652 (156)
+ 300 nM CT-B	317 ± 24 (19.4)	1970 ± 131 (84.5)

[a] Cells grown in 24-well clusters were treated, where indicated, for 60 min at 37 °C with 10 munits/0.5 mL of *V. cholerae* neuraminidase. Cultures then were chilled at 4 °C, washed, incubated with CT-B and the respective toxin, and assayed for intracellular cyclic AMP, as described in the Experimental Procedure. [b] The amount of cyclic AMP in pmol/mg of protein is shown for LT-I and CT at concentrations of 0.30 and 0.03 nM, respectively, which reflect the concentrations necessary to achieve half-maximal levels of intracellular cyclic AMP. Basal levels were 40–50 pmol/mg of protein. [c] Values in parentheses represent the percentage of cyclic AMP detected compared to untreated cells.

accumulation was reduced by 50%. This effect was not seen with other human cell lines, such as SK-N-MC (Figure 8B) or HeLa (data not shown).

The differential effects of CT-B on the activity of CT and LT-I were enhanced further when intact CaCo-2 cells were pretreated with *V. cholerae* neuraminidase. It had previously been shown that exposure of intact cells to neuraminidase will result in the degradation of more complex gangliosides to G_{M1} and, hence increase the level of CT binding and activity (Miller-Podraza *et al.*, 1982; Fishman *et al.*, 1984). As expected, the activity of both CT and LT-I increased significantly when cells were treated with neuraminidase (Table 2). In contrast to the effects on CT activity (regarded as due solely to the increase in cell surface G_{M1}), neuraminidase treatment appeared to reveal new biologically active binding sites for LT-I that were only partially sensitive to the presence of CT-B. These results were consistent with our earlier studies on the effects of neuramindase treatment on the binding of LT-I to CaCo-2 membrane galactoproteins and suggested that neuraminidase treatment generated additional alternate receptors for LT-I that were biologically active.

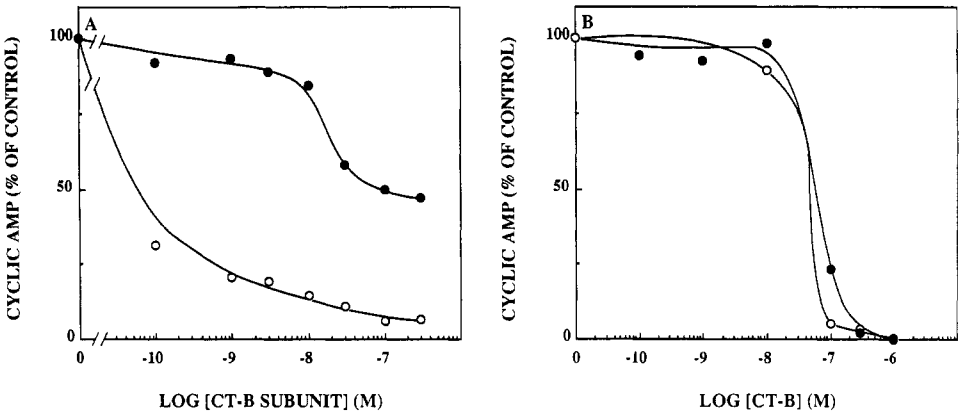

FIGURE 8: Effects of CT-B on toxin stimulation of intracellular cyclic AMP levels. Monolayers of intact CaCo-2 (A) or SK-N-MC (B) cells were pretreated with increasing concentrations of affinity-purified CT-B, exposed to either 0.03 nM CT (○) or 0.3 nM LT-I (●), and assayed for cyclic AMP as described in the Experimental Procedures. The concentrations of toxins used were those that elicited half-maximal levels of intracellular cyclic AMP. The results are expressed as a percent of control levels of cyclic AMP in the absence of B subunit (for CT and LT-I, 1880 and 2280 pmol/mg of protein respectively). Each point is the mean of triplicate determinations with standard deviations of <10%.

DISCUSSION

The heat-labile toxin of enterotoxogenic *E. coli* (LT-I) and CT both utilize the ganglioside G_{M1} as a cell surface receptor for binding to target cells. The intestinal epithelium of several species also expresses an alternate class of receptors that is recognized solely by LT-I (Holmgren *et al.*, 1982; Griffiths *et al.*, 1986; Zemelman *et al.*, 1989; Griffiths & Critchley, 1991). In this study, we used the culture-adapted human colonic carcinoma cell line CaCo-2 to characterize the carbohydrate determinant of these alternate receptors. As judged by toxin overlays of thin-layer chromatograms of lipid extracts, G_{M1} was the only CaCo-2-derived glycolipid recognized by LT-I and CT. Similar conclusions were reached in studies with intestinal epithelium from rabbits (Holmgren *et al.*, 1982; Griffiths *et al.*, 1986) and humans (Holmgen *et al.*, 1985).

We found that LT-I bound to additional sites on CaCo-2 cells and membranes and identified them as a series of galactoproteins not recognized by CT. Whereas a terminal β-1,3-linked galactose is found in G_{M1}, LT-I binding to these galactoproteins required the presence of terminal β-1,4-linked galactosyl residues. This distinction was borne out by the differential effects of *D. pneumoniae* and bovine testes β-galactosidases on LT-I binding. The former enzyme selectively hydrolyzes terminal β-1,4-linked galactosyl residues (Kojima *et al.*, 1987), whereas the latter recognizes terminal β-1,3-linked galactose as its preferred substrate (Distler & Jourdian, 1973). The binding of LT-I to asialofetuin and asialotransferrin further supported this conclusion. The N-linked complex carbohydrate structures of asialotransferrin (März *et al.*, 1982) and asialofetuin (Nilsson *et al.*, 1979) each terminate with Galβ1–4GlcNAc. As expected, binding to each was dependent on the exposure of terminal galactose residues by neuraminidase treatment and was abrogated either by β-1,4-galactosidase or endoglycosidase F treatment. Treatment of the asialoglycoproteins with the β-1,3-galactosidase had no effect on LT-I binding.

Binding studies and [^{125}I]LT-I overlays of CaCo-2 membrane proteins separated by SDS–PAGE and transferred onto nitrocellulose suggested that the alternate LT-I receptor(s) belonged to the poly-*N*-lactosamine series (of the lacto-*N*-neotetraosyl type) with the common structure

Galβ1–4GlcNAcβ1–3GalB1–4GlcNAc–R

This was substantiated by two independent methods: (1) the effect on LT-I binding to CaCo-2 membranes following digestion with glycosidases known to act on polylactosaminyl structures and (2) the ability of LT-I to bind to membrane glycoproteins with defined carbohydrate structures immunoprecipitated by specific antisera. Firstly, treatment with either *D. pneumoniae* β-galactosidase or *B. fragilis* endo-β-galactosidase selectively destroyed LT-I binding without a significant effect on CT binding. While the requirement for terminal β-1,4-linked galactose was firmly established by the effects of *D. pneumoniae* β-galactosidase, the effects of *B. fragilis* endo-β-galactosidase indicated that the required terminal galactose was a component of polylactosaminyl determinants. *B. fragilis* endo-β-galactosidase preferentially hydrolyzes internal β-1,4-galactosidic linkages of the lacto-*N*-neotetraosyl type (Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc/Glc), provided that the galactose residue is not branched (Scudder *et al.*, 1984).

Secondly, LT-I specifically bound to several CaCo-2 membrane glycoproteins immunoabsorbed with antibodies against blood group I, i, or Le^x antigens, as well as antisera against the lysosomal membrane glycoproteins, LAMP 1 and 2. The common feature that defines each antiserum is its ability to recognize structures containing the core carbohydrate sequence: Galβ1–4GlcNAcβ1–3. Interestingly, one of the determining factors in the level of LT-I binding to those immunoprecipitated glycoproteins was the level of terminal sialic acid. In each instance, the binding of LT-I was substantially increased following exposure to neuraminidase. These results correlated quite closely with those obtained in LT-I membrane binding studies in which binding to alternate receptors can be increased significantly by such treatment. The elevated LT-I binding observed following neuraminidase treatment also resulted in a concomitant increase in toxin activity when intact CaCo-2 cells were treated similarly. As the stimulation through these additional receptors was only partially blocked by excess CT-B, these observations suggested the presence of cryptic glycoproteins that, upon removal of terminal sialic acid residues, can become functional receptors for LT-I.

Of particular interest were the lysosomal membrane glycoproteins LAMP 1 and LAMP 2, which appeared to represent a major class of galactoproteins recognized by LT-I. These heavily polylactosaminylated glycoproteins are the major carriers of polylactosaminoglycans in a variety of cells including CaCo-2, and they display considerable heterogeneity

in the polylactosaminoglycans expressed (Viitala *et al.*, 1988; Carlsson *et al.*, 1988; Youakim *et al.*, 1989). While the majority of these glycoproteins reside in lysosomes, it has been shown that some LAMP 1 and LAMP 2 are expressed on the cell surface (Carlsson *et al.*, 1988; Harter & Mellman, 1992).

In addition to defining the oligosaccharide determinant of the alternate LT-I receptors, we also found that this class of receptors is biologically active. When CaCo-2 cells were exposed to concentrations of CT-B that completely inhibited the binding of CT, the binding of LT-I was only partially inhibited. Similarly, toxin activities under these same conditions also reflected the differential effects on toxin binding. The expression of this alternate class of receptors specific for LT-I appeared to be restricted to the cells of the intestinal epithelium. Thus, the binding and activity of LT-I to other human nonintestinal cell lines, such as SK-N-MC or HeLa cells (data not shown), were identical to those of CT and consistent with G_{M1} as the sole toxin receptor in these cells. In both instances, the B subunits of CT and LT-I were equally effective at inhibiting LT-I activity.

While G_{M1} is a ubiquitous glycolipid that is expressed in a wide variety of tissue types, the intestinal epithelium is also lined with a multitude of glycoproteins and high molecular weight mucins, which appear to provide additional sites for LT-I to bind. As was observed in several previous studies, the intestinal epithelial cells of most species (including humans) have additional receptors for LT-I; however, the proportion of these receptors varies greatly among those species examined (Griffiths & Critchley, 1991). Holmgren *et al.* (1982) reported that rabbit intestinal epithelial cells and brush border membranes bind up to 13-fold more LT-I than CT. Griffiths *et al.* (1986) obtained similar results, and in addition, they showed that LT-I binds to proteins with mobilities identical to those major brush border galactoproteins. The appearance of these alternate receptors for LT-I may be developmentally regulated, as their levels increased with the postnatal age of the rabbit (Griffiths & Critchley, 1991).

Similar, additional binding sites for LT-I are also present in rat intestinal brush border membranes (Zemelman *et al.*, 1989; Griffiths & Critchley, 1991). Differences were noted between strains, however, with Wistar rats having 20–30-fold more sites and Sprague–Dawley rats having only 1.3–2-fold more such sites. Most of the binding activity is associated with proteins of 130–140 kDa, with lesser binding to smaller proteins. That these proteins are galactoproteins is supported by the ability of ricin, a galactose-specific lectin, to inhibit LT-I binding to them. Furthermore, LT-I is able to bind to the sucrase–isomaltase complex purified from the rat brush border membranes (Griffiths & Critchley, 1991). As was observed in rabbits, expression of these galactoproteins appears to be age-related in rats; no LT-I binding proteins are detected in 2-week-old neonatal intestine (Zemelman *et al.*, 1989). Finally, in the mature rat intestine, more of these LT-I binding proteins are detected in the distal than the proximal gut.

Whereas we found that CaCo-2 cells bound 8-fold more LT-I than CT, human intestinal cells and brush borders prepared from surgically excised tissue bind only 50% more LT-I than CT (Holmgren *et al.*, 1985). The latter group, however, found considerable variation in the levels of these additional sites among tissue samples from different individuals. Other factors, such stage of development, degree of differentiation, region of the intestine, and extent of sialylation, may influence the expression of glycoproteins that bind LT-I. CaCo-2 cells are derived from a carcinoma and may represent a oncofetal cell type. CaCo-2 cells differentiate into enterocytes when maintained in culture for 2–3 weeks postconfluency (Quaroni *et al.*, 1979). As the cells differentiate, levels of α-2,6-sialyltransferase activity, which sialylates *N*-acetyllactosaminyl sequences, increase (Dall'Olio *et al.*, 1992). On the basis of the effects of neuraminidase, the alternate LT-I receptors were mostly sialylated in the CaCo-2 cells used in our experiments.

In conclusion, our results indicate that the oligosaccharide binding determinants on human intestinal epithelial galactoproteins recognized solely by LT-I are significantly different from G_{M1}–oligosaccharide recognized by both LT-I and CT, but can serve as alternate, biologically active receptors. Thus, while the two enterotoxins exhibit remarkable similarities in structure and mechanism of action, subtle structural differences in their B subunits must exist to account for the variation in binding specificities. Also, the utilization of a glycoprotein as a receptor, in contrast to the use of a ganglioside for toxin binding and entry into the cell, may contribute in part to the observed differences in CT and LT-I potency. The presence of glycoproteins as biologically active receptors for LT-I (primarily polylactosaminoglycans) raises several interesting questions concerning the mechanism of toxin internalization and activation. These include whether distinct pathways exist for toxin activation when bound to either ganglioside G_{M1} or glycoproteins. Neoglycoproteins containing G_{M1}–oligosaccharide, when generated on rat glioma C6 and HeLa cells, efficiently bind CT (Pacuszka & Fishman, 1991; 1992) as well as LT-I (Fishman *et al.*, 1993), but are nonfunctional in terms of a cyclic AMP response unless the cells are treated with chloroquine. Under the latter conditions, the toxins are entering the cell via the clathrin-coated pit/endocytic pathway, whereas it is believed that toxin bound to G_{M1} enters the cell through non-clathrin-coated invaginations known as caveolae (Tran *et al.*, 1987; Parton, 1994). Future studies will be required to determine the pathway by which LT-I bound to intestinal galactoproteins enters the cell and is activated.

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