Ability of Blood Group A–Active Glycosphingolipids to Act as Escherichia coli Heat-Labile Enterotoxin Receptors in HT-29 Cells

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We examined the ability of blood group A–active glycoconjugates to act as receptors for Escherichia coli heat-labile type I enterotoxin (LT-I) in HT-29 cells. These cells contained ~4 times more specific binding sites for LT-I than for cholera toxin (CT). Binding of LT-I could not be blocked by the B subunit of CT (CT-B), indicating the existence of LT-I receptors in addition to the glycosphingolipid GM1. LT-I was able to increase levels of cyclic adenosine monophosphate (AMP), even in the presence of CT-B. *Helix pomatia* and anti–blood group A antibody caused a dose-dependent inhibition of binding of LT-I to cells and production of cyclic AMP. LT-I recognized several complex blood group A–active glycosphingolipids from cells, and this interaction was also interfered with by *H. pomatia*. Treatment of cells with D,L-threo-1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol diminished surface expression of blood group A–active glycosphingolipids and binding of LT-I to non-GM1 receptors. These observations suggest that blood group A–active glycosphingolipids can function as alternative receptors for LT-I in HT-29 cells.

*Escherichia coli* heat-labile type I enterotoxin (LT-I) and cholera toxin (CT) are the bacterial virulence factors responsible for traveler’s diarrhea and cholera, respectively. These enterotoxins are the most closely related members of the CT family, sharing structural, immunological, and functional properties [1]. LT-I and CT are oligomeric proteins, composed of a single A subunit, that have latent ADP-ribosyltransferase activity and 5 identical B chains assembled into a pentameric ring (LT-B and CT-B). This nontoxic pentamer B subunit mediates binding of toxins to receptors on intestinal epithelial cells. Recent reports indicate that membrane-bound holotoxins undergo internalization and transport into the cell via membrane traffic [2, 3]. Intracellular proteolytic processing renders A1-peptide from the A subunit, which catalyzes ADP-ribosylation of the α-subunit of the heterotrimeric stimulatory G protein, thereby irreversibly activating adenylate cyclase. Permanent activation of adenylate cyclase increases levels of intracellular cyclic AMP, leading to net fluid secretion [4, 5].

In several systems, *E. coli* LT-I and CT bind with high affinity to GM1 ganglioside (Galβ3GalNAcβ4-(NeuAcα3)Galβ4Glcβ1Cer) [1], which functions as the natural receptor for these toxins. In addition, LT-B and, to a much lesser degree, CT-B behave as bacterial lectins that also recognize non-GM1 carbohydrate structures in glycolipids and glycoproteins from intestinal mucosal cells of several animal species [6–10]. Most of these glycoconjugates remain as LT-I putative receptors. In this regard, we have previously described an association between the ability of glycosphingolipids, glycoproteins, and mucin-derived glycopeptides obtained from pig and rabbit gastrointestinal tract tissue to interact with...
CT and LT-I and the type of ABH blood group determinant carried by these glycoconjugates [6, 7, 11, 12]. Recently, we have also shown that LT-I is able to interact preferentially with several blood group A− and blood group B−active glycolipids and glycoproteins obtained from rabbit intestinal brush border membranes and, to a lesser extent, with those glycoconjugates isolated from animals lacking these antigens (H rabbits). Conversely, CT did not interact with either blood group−active glycolipids or glycoproteins [13]. Furthermore, LT-I recognized ABH glycoconjugates among the abundant non-GM1 receptor population on rabbit intestinal brush border membranes and caused activation of adenylate cyclase in vivo, suggesting that ABH glycolipids and glycoproteins are LT-I functional receptors in rabbit intestinal ileal loops [14].

Because the ABH blood group polymorphism in rabbits is carried by membrane glycolipids and glycoproteins and because 2 blood group activities can be found in the same rabbit, the present study was designed to examine the interaction of LT-I with cells with membrane glycosphingolipids bearing a single type of ABH blood group activity. For this purpose, we characterized binding of LT-I to HT-29 colon carcinoma cells and examined the ability of cellular blood group A−active glycoconjugates to interact with this toxin. In addition, we attempted to assess the contribution of blood group A−active glycosphingolipids in the toxin-induced signal transduction mechanism that leads to production of intracellular cyclic AMP.

MATERIALS AND METHODS

Materials. Recombinant human LT-I [15] was isolated as described elsewhere [14]. CT (Sigma) and LT-I were radioiodinated as described elsewhere [12] (specific activity, 0.5−5.0 μCi/μg; 0.33−1.0 mmol iodide/mmol toxin). CT-B was purchased from List Laboratories, and LT-B was provided by J. D. Clements (Tulane University Health Science Center, New Orleans, LA). Polyclonal rabbit anti-CT and anti−LT-I antibodies were prepared as described elsewhere [14]. Monoclonal antibodies (MAbs) against blood group A and blood group B were purchased from Immucor. MAb against Lewis (b) antigen (Le[b]) was purchased from Organon Teknika.

Cell culture. Human adenocarcinoma HT-29 cells (American Type Culture Collection) and National Institutes of Health (NIH) 3T3 cells (provided by H. F. J. Maccioni, Universidad Nacional de Córdoba, Córdoba, Argentina) were grown in Dulbecco’s MEM (DMEM; PAA Laboratory) containing 25 mmol/L 3-isobutyl-1-methylxanthine (Sigma) plus CT or LT-I, and cells were further incubated for 60 min at 37°C. Cyclic AMP was extracted with 0.1 mol/L HCl, and the amount was determined by use of radioimmunoassay (Immunotech).

ABH phenotyping of cellular glycoconjugates and toxin-binding assays. Metabolic-labeled or unlabeled cells were harvested and washed with DPBS. Lipids from cells were successively extracted by use of sonication with 1.2 mL of chloroform:methanol:water (4:8:3, by vol) and chloroform:methanol (1:1, by vol) for 30 min. Lipid extracts were pooled and desalted by passing through a C18 column (Waters), as described elsewhere [7], applied to high-performance thin-layer chromatography (HPTLC) silica gel plates (Merk), and chromatographed with chloroform:methanol:
0.2% CaCl₂ in water (60:35:8, by vol) used as a solvent system. Radioactive fucolipids were visualized by exposing the plate to a PhosphorImager screen (Fuji Film) for 2 days.

Glycolipids that bind either the toxins or the anti–blood group MAbs were immunodetected, essentially as described elsewhere [13]. The effect of H. pomatia lectin on the interaction of LT-I with glycolipids was assayed by incubating the plate with a mixture of 300 nmol/L H. pomatia lectin and 5.0 nmol/L LT-I for 60 min at room temperature. In some experiments, the HPTLC plate was preincubated with H. pomatia for 30 min at room temperature, and then toxin was added. Both protocols yielded the same results.

Immunodetection assays were also used to monitor ABH blood group activity and toxin interaction of cellular glycoproteins from membrane preparations. Cells grown to confluence were harvested in DPBS containing protease inhibitors (1.0 μg/mL antipain, 17.5 μg/mL benzamidine, 1.0 mmol/L phenylmethylsulfonylfluoride, 1.0 μg/mL pepstatin, and 1.0 μg/mL leupeptin). Cell suspension was mechanically disrupted at 9500 rpm (Ultra-turrax T25; Janke & Kunkel), sonicated, and centrifuged at 100,000 g for 30 min. Total cell membranes (70 μg) were separated by use of SDS-PAGE in 7.5% gels, as described by Laemmli [18], followed by electrotransfer to nitrocellulose membranes [19]. To detect blood group–active glycoproteins, the membrane was blocked with 5% nonfat dried milk in PBS (pH 7.4) containing 0.1% Tween 20 (PBS-T), followed by incubation with anti-A and anti-B MAbs (1:500 dilution) or horseradish peroxidase (HRP)–conjugated UEA lectin (42 μg/mL) in 0.5% nonfat dried milk in PBS-T. The bound primary antibody was detected with a secondary antibody coupled to peroxidase (1:1000 dilution). All incubations were done for 1 h at room temperature. Blots were visualized by use of an enhanced chemiluminescence immunodetection system (ECL; Amersham Pharmacia Biotech). To assay toxin binding, the nitrocellulose membrane was blocked with 5% nonfat dried milk in PBS-T for 1 h at room temperature and incubated with 1.0–3.0 nmol/L CT or LT-I in PBS-T containing 0.5% nonfat dried milk for 18 h at 4°C. Toxin binding was detected by successive incubation with rabbit polyclonal anti-CT or anti-LT antibodies (diluted 1:4000) and HRP-conjugated protein A (1:30,000) for 1 h at room temperature. Bound HRP-conjugated protein A was revealed by use of the ECL system.

Flow cytometry. Blood group A antigen present on the cell surface was detected by use of flow cytometry. Cells grown in the presence of PPMP or vehicle (control) were detached with 0.25% trypsin in DPBS, washed, and counted. Cells (1 × 10⁶) were suspended in 1.0 mL of Hank’s balanced salt solution containing 1% BSA (HBSS-BSA) plus 5.0 μg of mouse anti-A MAb and were incubated for 30 min on ice. Then cells were washed, suspended in 1.0 mL of HBSS-BSA containing 0.5 μg of phycerothrin-conjugated secondary antibody (Caltag Laboratories), and incubated for 30 min on ice. After washing with HBSS-BSA and DPBS, cells were suspended in 1.0 mL of isotonic solution for flow cytometric analysis. Cells (1 × 10⁶) treated with PPMP or vehicle and incubated with the same amount of secondary antibody were used as controls. Flow cytometric analysis was performed by use of a flow cytometer (Ortho). Approximately 30,000 cells were analyzed for each sample. Collected data were processed by use of WinMDI software (version 2.7; available at: http://facs.scripps.edu/software.html).

Other methods. IgM from anti-A MAb was concentrated by use of a Kaptiv-M column (Tecnogen). Protein concentrations were determined according to the method of Lowry et al. [20] using BSA as standard. The Student’s t test was used to evaluate statistical differences between independent means. Calculation of parameters related to curves was done using GraphPad Prism (version 3.0; GraphPad Software).

RESULTS

Toxin-binding assays. Binding of [125I]-LT-I and [125I]-CT to human intestinal HT-29 cells was performed to investigate the presence of LT-I receptors not shared with CT. Figure 1A shows a clear difference in the saturation curves obtained for the [125I]-labeled toxins at steady state. CT showed higher affinity for cells than did LT-I, because half saturation of [125I]-CT occurred at a concentration of ∼0.3 nmol/L, whereas half saturation of [125I]-LT-I occurred at a concentration of ∼6.6 nmol/L. At a toxin concentration of 10 nmol/L, the specific binding of [125I]-LT-I was four times higher than the specific binding of [125I]-CT to cells (2691 vs. 646 fmol/mg of protein). It is well known that the pentameric CT-B retains its ability to bind to ganglioside GM1 but is devoid of the holotoxin toxicity [21]. When cells were preincubated with 250 nmol/L CT-B, binding of [125I]-LT-I did not change (LT-I concentration range, 0.1–10 nmol/L), but preincubation with 250 nmol/L LT-B completely blocked binding of LT-I to cells (figure 1A). On the other hand, similar inhibition of binding of [125I]-CT to cells was observed with unlabeled CT-B or LT-B (figure 1B), with near complete inhibition reached at 50 nmol/L both B subunits. These results clearly indicate that, in HT-29 cell membranes, LT-I and CT have a common receptor, probably GM1 ganglioside, but that LT-I also has additional receptors not shared with CT. When these binding experiments were performed using NIH 3T3 cells, binding of [125I]-LT-I was completely blocked by CT-B, and binding of [125I]-CT was abolished by LT-B (figure 1C), strongly suggesting that both toxins share a single population of receptors in these cells. This receptor was tentatively identified as GM1 by use of a toxin-overlay technique performed on HPTLC-separated lipid extracts from NIH 3T3 cells, since CT and LT-I both recognized only a doublet that migrated in a pattern similar to that of the GM1 standard (data not shown).
To investigate whether blood group A–active glycoconjugates are acting as LT-I receptors, cells were preincubated with 2 different blood group A ligands. H. pomatia lectin and anti-A MAb both were effectively able to block binding of [125I]-LT-I to cells in a dose-dependent way but lacked this effect on binding of CT (figure 2). These results strongly suggest the involvement of blood group A epitope in binding of LT-I to cells.

**LT-I–induced production of cyclic AMP.** To investigate whether binding of LT-I to additional receptors in HT-29 cells can trigger the adenylate cyclase–dependent second messenger pathway, we measured the cyclic AMP content of cells. As expected, CT-B and LT-B both abolished the production of cyclic AMP elicited by CT in a dose-dependent way. Complete blocking of CT-induced production of cyclic AMP was reached at a concentration of 50 nmol/L, with the B subunit of either toxin (figure 3A). On the other hand, at concentrations >1.0 nmol/L, LT-I elicited more production of cyclic AMP than did CT, and this response was only partially inhibited by preincubation of cells with a 250 nmol/L excess of CT-B. Preincubation of cells with LT-B prevented LT-I toxic activity, and levels of cyclic AMP were similar to those observed in nonstimulated cells (figure 3B). As a result, the LT-I response measured in the presence of CT-B could be attributed to production of cyclic AMP induced by LT-I acting on non-GM1 receptors in HT-29 cells. The blocking effect of CT-B on LT-I responses was greater at lower concentrations of holotoxin, in a range that is expected to make a significant contribution to toxin activity because of the GM1 receptor.

H. pomatia lectin and anti-A MAb, which were able to interfere with binding of LT-I to cells, also blocked LT-I–induced
production of cyclic AMP, depending on the concentration of the blood group A ligand, without affecting CT-stimulated responses (figure 4). However, similar percentages of inhibition on LT-I–induced production of cyclic AMP were also observed when cells were preincubated with CT-B and \textit{H. pomatia} lectin or CT-B and anti-MAb (data not shown).

**ABH phenotyping and interaction of glycoconjugates with LT-I.** ABH blood group activity was monitored in membrane-associated glycoproteins from HT-29 cells. Cell membrane preparations separated by SDS-PAGE and electroblotted to nitrocellulose membranes failed to bind either anti-A or anti-B MAbs or \textit{Ulex europaeus} I lectin. Neither LT-I nor CT interacted with any glycoprotein component of HT-29 cell membranes (data not shown).

Total lipid extracts from HT-29 cells separated by HPTLC contained several blood group A–active glycosphingolipids and few compounds carrying the difucosylated Le(b) antigen, as detected by the corresponding antibody reflecting the blood group and secretor status of the cell human donor. \textit{H. pomatia} lectin showed a pattern of blood group A–active glycolipids similar to that detected by anti-A MAb (figure 5A). In addition, we assayed the interaction of LT-I and CT with lipid extracts by the thin-layer chromatography–overlay technique. As expected, both toxins recognized GM1 ganglioside from cell lipid extracts (figure 5B). Figure 5B also shows that the blood group A–active compounds that migrated less than GM1 can also be labeled by LT-I, but not by CT, and that \textit{H. pomatia} lectin could effectively block binding of LT-I to glycosphingolipids other than GM1. These results support the idea that glycosphingolipids carrying the blood group A epitope are able to bind LT-I in the cell membrane. Apparently, LT-I did not interact with Le(b)-active glycosphingolipids. The glycosphingolipid that moved in the HPTLC near the solvent front (figure 5B) that was recognized by LT-I probably corresponds to galactosylceramide. This compound was seen as a doublet and exhibited the same pattern of migration as did galactosylceramide from bovine brain on HPTLC developed using chloroform.

**Figure 2.** Concentration-dependent effect of blood group A ligands on [\textsuperscript{125}I]–\textit{Escherichia coli} heat-labile type I enterotoxin (LT-I) binding to HT-29 cells. Cells were preincubated for 30 min at 4°C with increasing concentrations of \textit{Helix pomatia} lectin (HP) or anti-A monoclonal antibody and then were further incubated with 5.0 nmol/L \([\textsuperscript{125}I]\)-LT-I or \([\textsuperscript{125}I]\)-cholera toxin (CT) for 60 min at 4°C. The bound labeled toxin was determined as described in Materials and Methods. Each point represents the mean ± SD of 3 experiments.

**Figure 3.** A. Competitive inhibition of CT-B (B subunit of cholera toxin [CT]) and LT-B (B subunit of \textit{Escherichia coli} heat-labile type I enterotoxin [LT-I]) on CT-induced production of cyclic AMP. Increasing CT-B or LT-B concentrations were preincubated for 30 min at 4°C with confluent HT-29 cells, and then cells were further incubated in fresh medium with 5.0 nmol/L CT for 60 min at 37°C. Cyclic AMP was measured as described in Materials and Methods. B. LT-I–stimulated production of intracellular cyclic AMP and effect of preincubation with CT-B or LT-B. Cells grown to confluence were preincubated in the presence or absence of 250 nmol/L CT-B or LT-B and then incubated with increasing concentrations of LT-I. Levels of cyclic AMP were determined as described in Materials and Methods. In nonstimulated cells, cyclic AMP content was 25 ± 8 pmol/mg of protein. Production of cyclic AMP induced by increasing concentrations of CT was also measured. In panel B, each point represents the mean ± SD of 2 independent experiments.
Figure 4. Effect of blood group A ligands on toxin-induced production of intracellular cyclic AMP. Confluent HT-29 cells were preincubated at 4°C with increasing concentrations of *Helix pomatia* lectin (HP) or anti-A monoclonal antibody (MAb), and then cells were further incubated at 37°C in fresh medium containing 5.0 nmol/L *Escherichia coli* heat-labile type I enterotoxin (LT-I). Each point is the mean ± SD of duplicated determinations of 2 experiments. The effect of preincubation with HP or anti-A MAb on cholera toxin (CT)–induced production of cyclic AMP in cells was also measured in similar conditions. Intracellular cyclic AMP was assayed as described in Materials and Methods.

Figure 5. A, Blood group antigenic activity of HT-29 glycosphingolipids. High-performance thin-layer chromatography (HPTLC) plates were overlaid with anti-A or anti-Lewis (b) antigen (Le[b]) monoclonal antibodies, followed by incubation with horseradish peroxidase (HRP)–conjugated secondary antibodies. The right plate was incubated with 5.0 nmol/L HRP-conjugated *Helix pomatia* lectin (HP). B, Toxin-binding properties of HT-29 glycosphingolipids and effect of HP on *Escherichia coli* heat-labile type I enterotoxin (LT-I)–glycosphingolipid interaction. HPTLC plates were overlaid with 5.0 nmol/L toxin (cholera toxin [CT] or LT-I) or 500 nmol/L HP plus 5.0 nmol/L LT-I. Toxin binding was detected with rabbit polyclonal antitoxin antibodies, followed by incubation with HRP-conjugated protein A. The band corresponding to GM1 is indicated. Lipid extracts were separated using chloroform:methanol:water (60:25:4, by vol) as a solvent system and revealed by orcinol-sulfuric acid reagent [22]. Galactosylceramide standard (2.0 nmol) also bound LT-I (data not shown).

Effect of PPMP on additional LT-I receptors. PPMP is a potent inhibitor of ceramide glucosyltransferase (EC 2.4.1.80), the enzyme that catalyzes the first glycosylation step in the synthesis of glucosylceramide-based glycosphingolipids in cells [16]. To examine the effect of lowered cellular glycosphingolipid expression on the amount of non-GM1 LT-I receptors, HT-29 cells were treated with PPMP. Cells cultured for 7 days in the continuous presence of 5.0 μmol/L PPMP did not decrease cell viability or change cell shape, as seen by use of phase-contrast microscopy (data not shown).

Fucose is a typical sugar that constitutes glycosphingolipids that carry ABH and/or Lewis blood group activity [23]. Incorporation of [3H]-fucose into total lipid extracts from cells was reduced to 40% and 17% of controls at 24 and 48 h, respectively. HPTLC analysis of [3H]-labeled fucolipids after 48 h of treatment with PPMP showed a clear reduction on de novo biosynthesis of fucolipids (figure 6A). To assess the effect of PPMP on expression of blood group A epitope on the cell surface, we performed flow cytometric analysis. Histogram profiles showed a strong decrease in blood group A fluorescent signals in PPMP-treated cells, compared with those in control cells, as indicated by the peak mean channel (figure 6B). The percentage of cells with fluorescence intensity above the negative control (the percentage of positive cells) was 95% and 64% in control and PPMP-treated cells, respectively. Binding of [125I]-LT-I to cells treated for 7 days in culture with 5.0 μmol/L PPMP was determined, to measure specific binding and non-GM1 receptors in the presence of CT-B. Figure 6C shows that treatment with PPMP reduced specific binding and the amount of additional LT-I receptors to 40%–60% of control cells (toxin concentration range, 1.0–10 nmol/L). These results clearly indicate that diminished glycosphingolipid expression on cell membrane reduced the amount of LT-I alternative receptor sites.

DISCUSSION

Blood group–related carbohydrate structures present on the mucosal surfaces of the gastrointestinal tract may act as either receptors for microorganisms or bacterial virulence factors. Several epidemiological studies (reviewed in [14]) have described a relationship between ABH blood group system and diarrheal diseases caused by CT and *E. coli* LT-I.

In the present report, we have demonstrated that LT-I is capable of binding to several complex blood group A–active
Figure 6. Effect of D,L-threo-1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (PPMP) on fucolipids expression and additional Escherichia coli heat-labile type I enterotoxin (LT-I) receptor population in HT-29 cells. A. Cells were labeled with \([3H]\)-fucose (5.0 \(\mu\)Ci/mL) during the last 24 h of a 48-h culture in control (vehicle) or in the presence of 5.0 \(\mu\)mol/L PPMP. Each lane contain lipid extract corresponding to 250 \(\mu\)g of protein separated by high-performance thin-layer chromatography using chloroform:methanol:0.25% CaCl\(_2\) in water (60:35:8, by vol) as a solvent system. B, Histogram profiles of A blood group antigen of HT-29 cells after treatment in culture for 7 days with vehicle (filled histograms) or with 5.0 \(\mu\)mol/L PPMP (unfilled histograms) are shown in the lower panel. Histogram profiles of cells incubated only with secondary antibody (controls) are shown in the upper panel. The logs of fluorescence intensities are plotted against cell nos. C, Specific binding of \([^{125}\text{I}]\)-LT-I to PPMP-treated HT-29 cells and LT-I additional receptors measured in the presence of 250 nmol/L CT-B (B subunit of cholera toxin). Control and PPMP-treated cells grown for 7 days were preincubated for 30 min at 4°C with CT-B and then were further incubated with increasing concentrations of \([^{125}\text{I}]\)-LT-I. Specific binding of \([^{125}\text{I}]\)-LT-I to PPMP-treated cells was determined as indicated in figure 1. OR-FL, orange fluorescence intensity.

glycosphingolipids from HT-29 human colon adenocarcinoma cells. In addition, we have provided some experimental evidence that allows us to speculate about the biological role of these blood group A–active glycosphingolipids as an alternative class of LT-I receptors on HT-29 cell membrane.

It is well known that HT-29 cells express blood group A antigenic activity on their plasma membranes [24]. Our present results indicate that the cell surface–associated signal for this blood group activity detected by flow cytometry is due to the presence of blood group A–active glycosphingolipids. Lipid extracts from cells contained various glycosphingolipids carrying blood group A antigenic activity, which were separated by HPTLC and immunodetected. Although we have failed to detect blood group A–active glycoproteins in cell membrane preparations, a minor contribution of these glycoconjugates should not be neglected, since blood group A epitopes are carried by an adhesin molecule from HT-29 cells [25]. Other investigators have reported impaired biosynthesis of glycoproteins in HT-29 cells, which accounts for the lack of expression of intestinal hydrolases in the undifferentiated stage of these cells [26].

Results of toxin-binding experiments in the present study clearly show that LT-B and CT-B are able to completely block both toxins in cellular lipid extracts. In contrast to CT, LT-I possess \(\sim\)4 times more specific binding sites not shared with CT in HT-29 cells. In addition, these non-GM1 LT-I receptors are capable of triggering a physiological response. It is well known that CT and LT-I elicit a second messenger pathway by activating adenylate cyclase and a subsequent increase in intracellular concentrations of cyclic AMP [4, 5]. This mechanism is also activated by LT-I when this toxin binds to additional receptors, since \(\sim\)50% of LT-I induced production of cyclic AMP in HT-29 cells could be measured in the presence of an excess of CT-B. However, we have observed that CT-B lacked the ability to interfere with binding of LT-I to cells, which may reflect the fact that a relatively small population of GM1 is responsible for approximately one-half of the LT-I functional response. Both anti-A MAb and \(H.\) pomatia lectin caused a dose-dependent inhibition of binding of LT-I and production of cyclic AMP in HT-29 cells. These results indicate that glycoconjugates bearing blood group A determinants participate as alternate LT-I receptors. In addition to observing the different effectiveness exhibited by both blood group A ligands, we have also observed that anti-A MAb has a greater effect on LT-I activity than on toxin binding, whereas \(H.\) pomatia seems to have much greater effect on binding of LT-I than on toxin activity. We do not have a clear explanation for these differential effects, but, apparently, both blood group A ligands do not affect a complex phenomenon such as receptor-mediated signal transduction cascade, per se, since they did not modify binding of CT or activity on cells. The specific prevention of binding...
of LT-I to cells and the subsequent production of cyclic AMP by ligands that recognize blood group A epitope strongly suggest that at least the blood group A determinant GalNac\(\alpha\)1,3\[L-Fuc\(\beta\)1,2\]Gal\(\beta\)1 is recognized by LT-I in membrane glycoconjugates. However, LT-I also recognized galactose from galactosylceramide, but the role of this glycosphingolipid as functional receptor to LT-I in HT-29 cells was not further investigated in the present study.

When glycoconjugates were assayed for in vitro binding of LT-I, only glycosphingolipids from total lipid extracts were able to interact with this toxin. In addition to GM1, LT-I recognized complex blood group A–active glycosphingolipids in toxin-overlap assays, an interaction that was also interfered with by \(H.\) \(pomatia\). These blood group A–active glycosphingolipids probably act as additional LT-I receptors in HT-29 cell membranes. However, it is apparent that short-chain blood group A glycosphingolipids and those bearing Le(b) blood group activity bound very poorly to this toxin. We speculate that glycosphingolipids carrying either blood group A and Le(b) blood group or Le(b) epitope alone may not be recognized by LT-I, since this toxin exhibited low binding activity to a human intestinal ALe(b) glycosphingolipid standard (E.M.G. and C.G.M., unpublished data). The observations of the present study suggest that LT-I seems to have an extended binding site, since LT-I does not tolerate the internal \(\alpha\)-(1,4)-L-fucosyl group from the Le(b) structure.

In an attempt to affect blood group A glycosphingolipid expression in cells and then measure the effect on the LT-I non-GM1 receptor population, we used PPMP. Cells treated with PPMP strongly reduced cell-surface expression of blood group A–active glycosphingolipids and concomitantly reduced to \(\sim\)50% the number of additional binding of LT-I to HT-29 cells, compared with control cells. Although the use of PPMP had a limited utility in the present study, since this compound will inhibit synthesis of all GlcCer and more-complex glycolipids [16], this approach supports the idea that the nature of the additional LT-I receptors in the cells are glycosphingolipid in nature.

In conclusion, the present study has indicated that HT-29 cells contain a significant amount of additional LT-I–functional receptors. The more-complex blood group A–active glycosphingolipids may act as some of the non-GM1 receptor sites recognized by LT-I in these cells. In addition, HT-29 cells appear to be a simplified but advantageous system over the in vivo rabbit intestine for study of the biological role of glycoconjugates carrying a single ABH blood group activity. In our previous report [14], the production of fluid was measured as an indication of an overall response to LT-I that was attributed to ABH-active glycoconjugate receptors in the rabbit intestine. However, in that study, we were unable to discern whether these additional receptors to LT-I were glycolipids or glycoproteins in nature. Several investigators have reported the existence of galactoproteins as functional receptors to LT-I in rabbit [27] and rat [10] intestines. More recently, a membrane glycoprotein containing neolactosaminoglycans was identified as a type of additional LT-I receptor in the CaCo-2 cell line [28]. However, Teneberg et al. [9] have postulated that neolactotetraosylceramide recognized by LT-I from \(E.\) \(coli\) porcine strains may be the sole LT-I receptor in infant rabbit intestine.

The present study has provided the first evidence that non-GM1 glycosphingolipids can function as alternative receptors to LT-I in cells. The internal structure of blood group A–active glycosphingolipids should be determined, to define completely the carbohydrate requirements for an efficient interaction with LT-I.

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