

PATHOGENESIS OF SHIGELLA DIARRHEA

XI. Isolation of a Shigella Toxin-Binding Glycolipid from Rabbit Jejunum and HeLa Cells and Its Identification as Globotriaosylceramide

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Shigella toxin is a multimeric protein consisting of one A subunit (M_r , 32,000) and five B subunits (M_r , 6,500) (1). It is produced by all species of the genus and is believed to play an important role in pathogenesis of clinical shigellosis (2). Shigella toxin is particularly interesting because several different biological effects are mediated by the same highly purified molecule (1, 3). For example, it causes fluid secretion when placed in the lumen of rabbit small intestine (enterotoxicity) (2), but results in delayed limb paralysis and then death if administered parenterally in the same species (neurotoxicity) (4). When directly injected into the vagus nerve trunk of rats, shigella toxin travels in retrograde fashion via the axon to the neuronal cell body, causing dissolution of Nissl substance and death of the neuron (neuronotoxicity) (5). It is also lethal to certain cell culture lines (cytotoxicity) due to inhibition of protein synthesis (6, 7). The latter effect has also been shown in cell-free protein synthesis systems (8). The mechanism appears to be an irreversible and probably catalytic inactivation of the 60 S ribosomal subunit by the toxin A subunit (9).

It is not known how shigella toxin produces these various manifestations. It is possible that there are distinctive binding sites on different tissues for the toxin B subunit that result in diverse effects and that the B subunit has multiple binding domains that recognize these distinctive receptors. It is also conceivable that direct binding of the A subunit could cause some of the observed effects. In HeLa cells, the cytotoxic effect of shigella toxin appears to be related to binding of the B subunit to an *N*-linked glycoprotein on the cell surface that contains oligomeric $\beta 1 \rightarrow 4$ linked *N*-acetyl-D-glucosamine (GlcNAc)¹. The evidence for

This work was supported in part by research grants AI-16242, AI-20325, CA-2006, and Center Grant 1P3D-AM-39428 (Gastroenterology Research on Absorptive and Secretory Processes) from the National Institutes of Health, and a grant in Geographic Medicine from the Rockefeller Foundation. H. Clausen is supported by Ingeborg og Leo Dannin Fonden and Vera og Carl Johan Michaelsons Legat, Denmark.

¹Abbreviations used in this paper: GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine; HPTLC, high-performance thin-layer chromatography; LP, lower phase; MVM, microvillus membranes; Rab, rabbit polyclonal antitoxin; TC_{50} , 50% tissue culture lethal dose; UN, upper neutral.

this statement includes: (a) the loss of sensitivity to toxin when HeLa cells are treated with tunicamycin (10), an inhibitor of *N*-linked glycoprotein synthesis; (b) competitive inhibition of the cytotoxic response of HeLa cells when incubated with GlcNAc-specific lectins (10, 11) or oligomeric β 1 \rightarrow 4-linked GlcNAc (11); (c) inhibition of toxin binding and bioactivity in HeLa cells by B subunit-specific antibodies but not by A subunit-specific antibodies (1); and (d) the demonstration that renatured, purified B subunits bind to the HeLa cell surface and compete for binding of the holotoxin, whereas purified A subunit neither binds nor inhibits binding of the intact toxin (Donohue-Rolfe, A., and G. T. Keusch, manuscript in preparation).

Overnight incubation of cells in the presence of tunicamycin, which completely inhibits the cytotoxic response to 100 50% tissue culture lethal doses (TC₅₀) of toxin, reduces toxin binding by only 55–60% (10). We have therefore suggested that a second toxin-binding site is present on HeLa cells and that this site is not involved in the cytotoxic response (10). This is the probable explanation for our previous observations (2) and those of Eiklid and Olsnes (7) that the response of HeLa cell lines of widely differing sensitivity to toxin fails to correlate with the number of receptors present. Preliminary experiments have indicated that this second HeLa cell receptor is a glycolipid, similar to a chloroform/methanol soluble toxin-binding site on rabbit jejunal brush border membranes (2, 12). The present study was undertaken to isolate and identify this glycolipid from both HeLa cells and rabbit intestinal epithelial cells.

Materials and Methods

Materials. Casamino acids were obtained from Difco Laboratories Inc., Detroit, MI. Blue Sepharose CL-6B, polybuffer exchanger 94 and polybuffer 96, and DEAE-Sephadex were purchased from Pharmacia Fine Chemicals, Piscataway, NJ. BioGel P-60 was obtained from Bio-Rad Laboratories, Richmond, CA. Chloramine T, Na metabisulfite, rabbit hemoglobin, and BSA were from Sigma Chemical Co., St. Louis, MO. Na¹²⁵I (17.4 Ci/mg) and Bolton Hunter reagent were purchased from New England Nuclear, Boston, MA. All tissue culture media were from Gibco Laboratories, Grand Island, NY. High-performance thin-layer chromatography (HPTLC) plates were purchased from either Merck, Darmstadt, West Germany, or J.T. Baker Chemical Co., Phillipsburg, NJ. Primuline was obtained from Aldrich Chemical Co., Milwaukee, WI. Soluble Gal α 1 \rightarrow 4Gal was purchased from Socker-Bolaget Fine Chemicals, Arlov, Sweden.

Toxin Production. *Shigella* toxin was produced and purified in three steps from *Shigella dysenteriae* 1, strain 60 R, as previously described (1). The pure toxin was lyophilized in ammonium bicarbonate and stored at -70°C . The A and B subunits were dissociated in 5% formic acid and isolated by chromatography on BioGel P-60, as previously reported (1). Toxin or isolated A or B subunits were labeled with ¹²⁵I by a modification of the chloramine T procedure (1).

Antibodies. Rabbit polyclonal (Rab) and mouse monoclonal A and B subunit-specific antibodies (designated 5B2 and 4D3, respectively) have been described before (1). Rat IgM mAb to Gb₃ (globotriaosylceramide)² and an IgG₃ mouse mAb raised against III³ FucnLc₄, designated 38.13 and FH3, respectively, were prepared and characterized as previously reported (13, 14). For some experiments, these antibodies were iodinated by the Bolton Hunter method (15).

Growth of HeLa Cells and Assay of Shigella Cytotoxicity. HeLa cells (CCL-2; American Type Culture Collection, Rockville, MD) were grown at 37°C in McCoy's 5a (modified)

² Glycolipids are abbreviated according to the recommendations of the Nomenclature Committee of the International Union of Pure and Applied Chemistry.

medium containing 10% FCS (16). 50 ml of packed HeLa cells were obtained for lipid studies after passaging cells to multiple 150-mm Petri dishes. Cells were harvested by scraping with a rubber policeman, centrifuged at low speed, and the pellet was frozen until used. Toxin was assayed with a previously described cytolethal assay using HeLa cells in monolayer culture in microtiter plates (16).

Extraction and Purification of Lipids. The total lipid was extracted from packed HeLa cells or rabbit jejunal mucosal scrapings in isopropanol/hexane/water (55:25:20) and dried in a rotary evaporator (17). A Folch partition was performed in chloroform/methanol (2:1) (18). The upper phase glycolipids were then fractionated by DEAE-Sephadex chromatography to obtain the upper neutral (UN) glycolipids, while leaving gangliosides still bound to the column (19). The lower phase (LP) glycolipids from the Folch partition were acetylated, purified on a Florisil column, and deacetylated (20). Authentic Gb₃ standard was purified from human erythrocyte membranes by HPLC, as previously described (21). IsoGb₃ was prepared from rat globoisotetraosylceramide by enzymatic hydrolysis with β -galactosidase (22).

High-Performance Thin-Layer Chromatography. Gb₃ was purified from LP glycolipids from HeLa cells and rabbit jejunal mucosa by preparative HPTLC, as previously described, using the solvent system chloroform/methanol/water (60:35:7) and detected by staining with primuline (13). The upper (U) and lower (L) bands of the Gb₃ doublet were separated, isolated for use in some studies, and designated Gb₃-U and Gb₃-L, respectively. The specificity of the toxin was tested by direct binding studies to glycolipids separated on HPTLC SI plates (J. T. Baker Chemical Co.) in chloroform/methanol/water (50:40:10) containing 0.02% CaCl₂ (final concentration). Dried plates were soaked for 1 min in 0.5% polyisobutylmethacrylate in acetone, dried, sprayed with 5% BSA in PBS, and then soaked in the same solution for 2 h (13). Finally, the plates were incubated at 4°C for 4 h in PBS/0.1% BSA containing 2×10^5 cpm/ml of ¹²⁵I-toxin (sp act $\sim 5 \times 10^4$ cpm/ng), washed thoroughly, dried, and autoradiograms were prepared (XAR-5 film, Eastman Kodak Co., Rochester, NY). To visualize the separated glycolipids, plates were then sprayed with 0.5% orcinol in 10% aqueous H₂SO₄ and heated to 100°C.

Solid Phase Binding Studies. Five parts of lecithin and three parts of cholesterol were mixed with one part (w/w) of purified glycolipid in absolute ethanol to achieve a concentration of 4 mg glycolipid per ml. Glycolipids used included LacCer, iGb₃, Gb₃, Gb₄, nLc₆, and Gg₄. 50 μ l of this mixture was added to the first well and serial twofold dilutions were made with absolute ethanol in 96-well, soft flat-bottomed microtiter plates. The plates were dried at 37°C for 1 h and then incubated with PBS/5% BSA for 2 h to block nonspecific binding sites. Blocking solution was then replaced with either iodinated toxin or purified A or B subunits in 30 ml of PBS/0.1% BSA for 4 h at 4°C. Wells were washed five times with PBS, cut with a hot wire, and radioactivity was counted in a gamma spectrometer.

In some experiments, liposomes were prepared by sonication of the same lecithin/cholesterol/glycolipid mixtures in PBS. HeLa cells were grown to near confluency in quadruplicate tissue culture microtiter plates and then incubated with 5% BSA in PBS for 1 h. Plates were chilled to 4°C and serial dilutions of the liposomes were added so that the first well contained 500 ng of glycolipid. Control plates were identically prepared except for the omission of liposomes. ¹²⁵I-toxin (10⁵ cpm) was then added. After 2 h of incubation with shaking at 4°C, all plates were washed five times in PBS. One set of plates was used to measure bound radioactivity in a gamma scintillation counter. The second set of plates was inoculated with 200 μ l of fresh McCoy's medium per well, incubated at 37°C overnight, and cytotoxicity was measured as described above.

Results

HPTLC of HeLa Cell and Rabbit Jejunal Lipids and Toxin Binding. Fig. 1A shows the orcinol staining pattern of isolated UN and LP lipids obtained from HeLa cells and rabbit jejunum, and standards separated by HPTLC. The autoradiogram obtained after incubation of this plate with iodinated toxin is

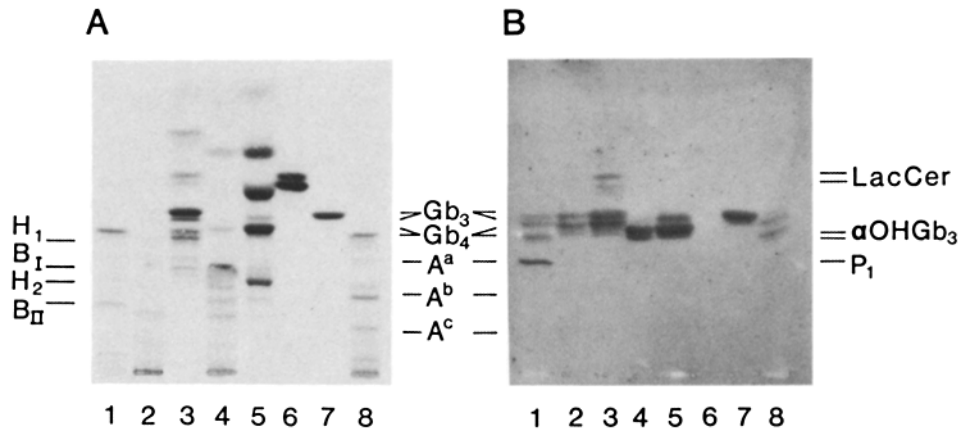


FIGURE 1. HPTLC separation of glycolipid preparations. Lane 1, UN glycolipid fraction of group B human erythrocytes; lane 2, UN glycolipid fraction of HeLa cells; lane 3, LP fraction of HeLa cells; lane 4, UN glycolipid fraction of rabbit jejunum; lane 5, LP fraction of rabbit jejunum; lane 6, LacCer standard; lane 7, Gb₃ standard; lane 8, UN glycolipid fraction of group A human erythrocytes. The plate was developed in chloroform/methanol/water (50:40:10). (A) HPTLC plate stained by orcinol/H₂SO₄ reaction. (B) autoradiogram after incubating plate with ¹²⁵I-shigella toxin. *Markers*, the known mobility of various glycolipids.

shown in Fig. 1B. Toxin bound strongly to authentic Gb₃ and to Gb₃ present in UN and LP glycolipid preparations from both HeLa cells and rabbit jejunum, and from human type A and type B erythrocytes. Toxin also bound to a doublet band migrating in the position where Gb₄ is found. In addition, a labeled band was detected in type B erythrocyte glycolipids, migrating between B₁ and H₁ in the expected position of P₁ antigen. Overnight incubation of cells in the presence of 0.05 μg/ml of tunicamycin before extraction of the glycolipid fraction failed to diminish toxin binding to HeLa Gb₃ on HPTLC plates (data not shown).

Solid-Phase Binding Studies. Fig. 2 shows the binding of ¹²⁵I-toxin to glycolipid-coated microtiter plates. Toxin bound to purified erythrocyte Gb₃ and to the isolated upper and lower bands of the Gb₃ doublet (Gb₃-U and Gb₃-L, respectively) in both HeLa cell and rabbit jejunal LP glycolipids. The extent of binding to Gb₃-U and Gb₃-L lipids from each tissue was similar, and was ~50-fold greater to Gb₃ than to LacCer, as estimated from the titration curve shown in Fig. 2. There was no detectable binding to iGb₃, Gb₄, nLc₄, or nLc₆.

Consistent results were observed when glycolipid-containing liposomes were used to compete for toxin binding to HeLa cell monolayers (Fig. 3). Thus, purified Gb₃ was a highly effective inhibitor, whether obtained from erythrocyte membranes or from HeLa cells or rabbit jejunum. Isolated Gb₃-U and Gb₃-L from HeLa and rabbit LP glycolipid extracts were similar in ability to inhibit binding of toxin to HeLa cell monolayers. Liposomes containing LacCer, nLc₆, or IV²FucnLc₄ were inactive in these experiments. In addition, neither soluble chitotriose (1 mg/ml) nor soluble Galα1→4Gal (1 mg/ml), the terminal disaccharide of Gb₃, inhibited toxin binding (data not shown).

The effect of anti-glycolipid and anti-toxin antibodies on toxin binding to Gb₃ from the different tissues was then studied (Fig. 4). In contrast to antibody FH3, which was raised against an unrelated glycolipid antigen and had no discernible

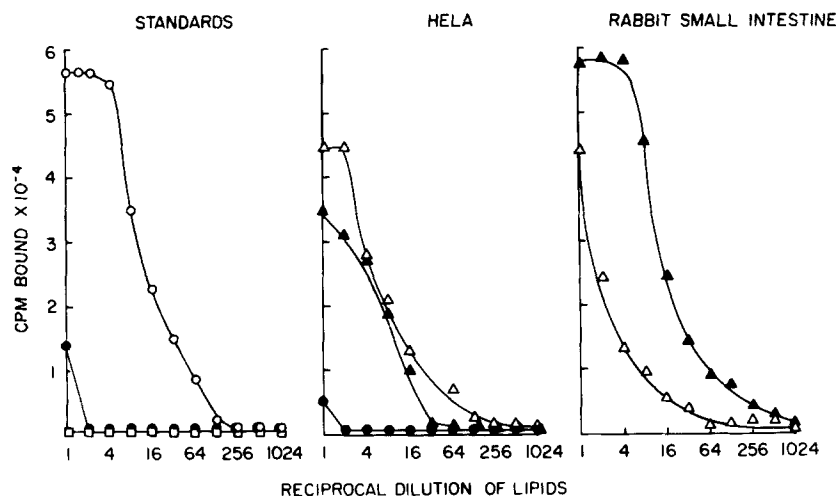


FIGURE 2. Solid-phase binding of ¹²⁵I-shigella toxin to glycolipids. Lecithin/cholesterol/glycolipid mixtures (5:3:1) were prepared and serial dilutions were coated on microtiter plates so that the first well contained 200 ng of glycolipid. Wells were blocked with PBS/5% BSA and 1 ng of iodinated toxin was added for 4 h at 4°C. Plates were washed thoroughly and radioactivity was determined. ○, Gb₃ standard; ●, LacCer; △, Gb₃-U; ▲, Gb₃-L; □, mixture of iGb₃, Gb₄, nLc₄, and nLc₆.

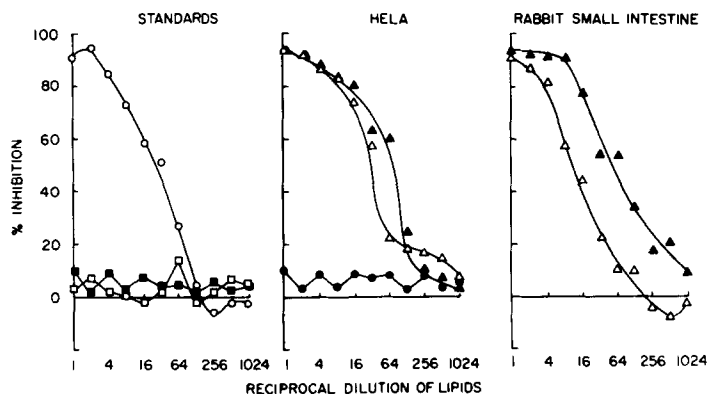


FIGURE 3. Liposomes were prepared by sonicating a 5:3:1 mixture in PBS of lecithin/cholesterol/glycolipid. Beginning with a concentration of 500 ng of glycolipid, serial dilutions of liposomes were added to HeLa cell monolayers in microtiter plates that had been previously incubated with PBS/5% BSA for 1 h at 4°C. 1 ng of ¹²⁵I-toxin was added to each well and incubated at 4°C for 2 more h. Plates were thoroughly washed and radioactivity was determined. Inhibition of binding in the presence of the different glycoprotein-containing liposomes was calculated by comparison with identically treated control wells containing no liposomes. Duplicate plates were used for the cytotoxicity data shown in Table I. ○, Gb₃ standard; ●, LacCer; △, Gb₃-U; ▲, Gb₃-L; □, nLc₆; ■, 1V²FucnLc₄.

effect (●), antibody 38.13 directed to Gb₃ resulted in a small but definite decrease in toxin binding, best seen with rabbit Gb₃-U and HeLa Gb₃-L (○). In contrast, both Rab, rabbit polyclonal antitoxin (△), and 4D3, mouse monoclonal B subunit-specific antibody (▲), virtually eliminated toxin binding at a titer of 1:1,000. It should be noted that the initial concentration of these two antibodies was 10-fold less than either FH3 or 38.13. The weak inhibitory effect of 38.13 antibody

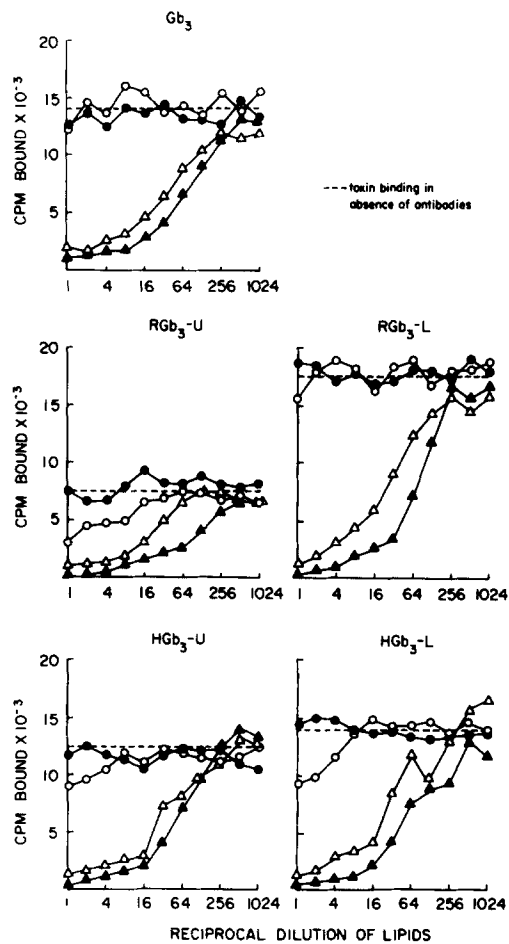


FIGURE 4. Inhibition of toxin binding to glycolipid-coated microtiter plates in the presence of antibody. Plates were coated with a mixture of lecithin/cholesterol/glycolipid (5:3:1) so that each well contained 100 ng of glycolipid, and then were blocked with PBS/5% BSA. Serial dilutions of antibodies were added to the wells so that the initial dilution of 38.13 and FH3 was 1:100 and the dilution of Rab and 4D3 was 1:1,000 in the first well. Plates were incubated with ^{125}I -toxin and binding determined as described. \circ , plates treated with antibody 38.13 (anti- Gb_3); \bullet , plates treated with antibody FH3 (anti-III 5 FucnLc $_4$); Δ , plates treated with antibody Rab; and \blacktriangle , plates treated with 4D3 (mouse monoclonal anti-B subunit).

is probably related to its inability to bind strongly to Gb_3 -coated plates, since only 0.5% of the input counts of ^{125}I -labeled antibody were observed to bind (data not shown).

Binding of Isolated A and B Subunits to Gb_3 . Fig. 5 shows the binding of toxin subunits to glycoprotein-coated microtiter plates. ^{125}I -B subunit bound to Gb_3 (Δ) but not to LacCer (\blacktriangle), whereas the A subunit failed to bind to either glycolipid (\circ \bullet). Fig. 6 shows competitive inhibition of binding of intact ^{125}I -toxin to Gb_3 by unlabeled shigella toxin or isolated A and B subunits. Both intact toxin (\square) and B subunit (Δ) were good competitive inhibitors, whereas A subunit (\circ) or cholera toxin (\blacksquare) were not.

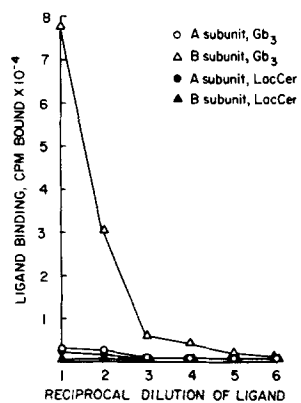


FIGURE 5. Binding of ¹²⁵I-toxin subunits to microtiter plates coated with either Gb₃ (open symbols) or LacCer (closed symbols). Incubation was for 4 h at 37°C.

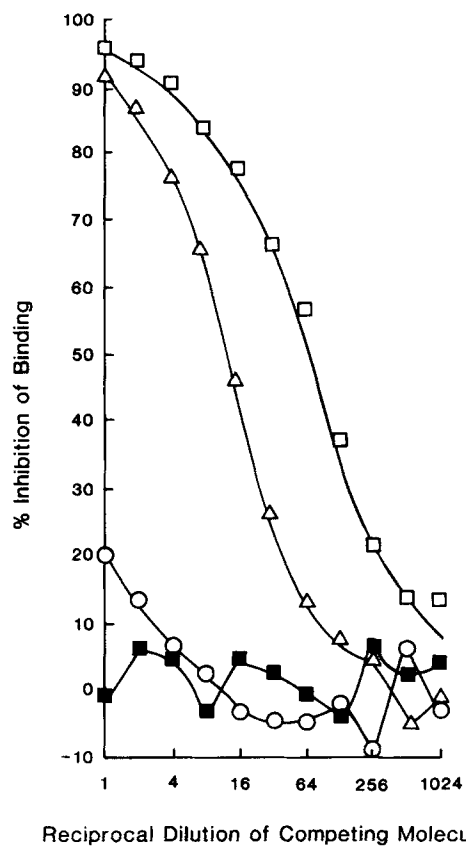


FIGURE 6. Inhibition of binding of ¹²⁵I-shigella toxin to Gb₃-coated microtiter plates by unlabeled shigella toxin (□) or isolated A or B subunits (○ and △, respectively) and cholera toxin (■). The initial concentration of the competing molecule represents a 100-fold molar excess in relation to iodinated holotoxin.

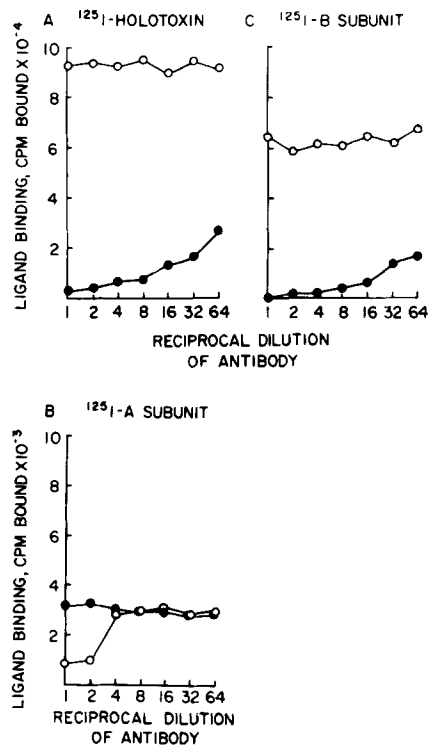


FIGURE 7. Effect of subunit-specific antibodies 4D3 (B subunit-specific, ●) and 5B2 (A subunit-specific, ○) on binding of ^{125}I -labeled holotoxin (A), A subunit (B), or B subunit (C) to Gb_3 -coated microtiter plates. Beginning with a dilution of antibody of 1:1,000 in the first well, serial dilutions were made and 5×10^5 cpm of toxin or subunit were added. Incubation proceeded for 4 h at 4°C .

Additional experiments were then performed to determine the effect of subunit-specific antibodies on the binding of intact toxin or isolated A and B subunits to Gb_3 -coated microtiter plates (Fig. 7). The binding of holotoxin and B subunit (Fig. 7, A and C, respectively) was inhibited by the B subunit-specific antibody, 4D3 (●), but not by the A subunit-specific antibody, 5B2 (○). In contrast, the limited binding of the A subunit (panel B) was inhibited by the A-specific antibody but not by the B-specific antibody (note the 10-fold decrease in scale of the ordinate). These data show that the binding of the A subunit to Gb_3 was not due to contamination of the A subunit preparation by the B subunit.

Competitive Inhibition of the Cytotoxic Response of HeLa Cells to Shigella Toxin by Glycolipid-Containing Liposomes and Soluble Oligosaccharides. In parallel with the competitive binding studies shown in Fig. 3, the cytotoxic response of HeLa cells was determined in the presence of serial dilutions of glycolipid-containing liposomes (Table I). Neither Gb_3 (whether obtained from erythrocytes, HeLa cells, or rabbit jejunum) nor non-toxin-binding glycolipids inhibited cytotoxicity, although all Gb_3 liposomes inhibited binding of toxin to HeLa cells by $>90\%$ (Fig. 3). The soluble terminal $\text{Gal}\alpha 1 \rightarrow 4\text{Gal}$ disaccharide of Gb_3 also failed to affect toxin activity in HeLa monolayers, whereas chitotriose had a marked inhibitory effect (Table II).

TABLE I
Effect of Glycolipids Incorporated into Liposomes on the Cytotoxic Response of HeLa Cells to Shigella Toxin

Glycolipid present in liposomes	Percent HeLa cell mortality	
	Control (no toxin)	Toxin
None	0.0	46.0
nLc ₆	0.3	46.4
IV ² FucnLc ₄	0.8	46.4
HeLa LacCer	0.3	46.4
HeLa Gb ₃ -U	0.1	47.1
HeLa Gb ₃ -L	0.0	46.4
Rabbit Gb ₃ -U	0.1	46.8
Rabbit Gb ₃ -L	0.8	47.1
Erythrocyte Gb ₃	0.1	45.9

TABLE II
Effect of Oligosaccharide Hapten Inhibitors on the Cytotoxic Response of HeLa Cells to Shigella Toxin

Oligosaccharide added <i>mg/ml</i>	Percent HeLa cell mortality	
	Toxin + Chitotriose	Toxin + Gal-Gal
0.0	57.3	58.2
1.0	27.0	58.4
0.5	30.5	57.0
0.25	33.6	57.0
0.125	35.9	57.5

Discussion

In this paper we have shown that shigella toxin can specifically bind to a glycolipid present in both rabbit jejunal mucosa and HeLa cells. This glycolipid was identified as Gb₃ (globotriaosylceramide) by comparison of its mobility on HPTLC with that of authentic Gb₃ isolated from human type B erythrocyte membranes. Rabbit jejunal or HeLa cell Gb₃ and purified Gb₃ derived from human type B erythrocytes all showed similar toxin binding capacity. Moreover, when these sources of Gb₃ were incorporated into liposomes they possessed similar ability to competitively inhibit the binding of toxin to HeLa monolayers. Toxin also bound to a second band in the glycolipid extracts of erythrocyte membranes in the position at which Gb₄ and α OH-Gb₃ (Gb₃ containing α -hydroxylated fatty acid and phytosphingosine) are known to comigrate. Since toxin was unable to bind to purified Gb₄ in solid phase binding studies it is likely that the minor band detected is α OH-Gb₃. A third toxin-binding band was identified in type B human erythrocyte extracts, which is probably the proposed P₁ erythrocyte antigen structure, IV⁴GalnLc₄ (23). This has a Gal α 1 \rightarrow 4 linked to nLc₄ so that it possesses the same terminal Gal α 1 \rightarrow 4Gal disaccharide structure as Gb₃, but linked β 1 \rightarrow 4 to GlcNac instead of Glc. As this glycolipid was not available for further study, experiments to confirm this interpretation were not possible. The important observation, however, is the identification of the Gb₃

glycolipid-binding site for shigella toxin in rabbit jejunum and HeLa cells. This is consistent with preliminary studies of other investigators, who have reported that the specific disaccharide, Gal α 1 \rightarrow 4Gal coupled to BSA, inhibits the binding of shigella toxin to HeLa cells (24).

Our data show the fine specificity of the binding for Gb₃, since neither iGb₃ (which is identical to Gb₃ except that the Gal-Gal disaccharide is linked α 1 \rightarrow 3 instead of α 1 \rightarrow 4) nor LacCer (which differs from Gb₃ in the deletion of the terminal galactose residue) or Gb₄ (which has a terminal GalNAc linked β 1 \rightarrow 3 to Gb₃) were able to bind shigella toxin. Since soluble Gal α 1 \rightarrow 4Gal failed to competitively inhibit binding of toxin to HeLa cells, our evidence indicates that receptor activity for shigella toxin requires terminal Gal α 1 \rightarrow 4Gal linked to another residue (either Glc or GlcNAc from the studies described here). It is also clear that the binding of toxin to Gb₃ is via the toxin B subunit. Purified B subunit bound to Gb₃ but not to LacCer, whereas purified A subunit bound to neither glycolipid. This is consistent with our observation that only the holotoxin or the B subunit, but not the A subunit or cholera toxin, inhibited binding of intact ¹²⁵I-holotoxin to Gb₃-coated microtiter plates. A third piece of evidence is that binding of toxin or B subunit to Gb₃ was completely inhibited by a B subunit-specific mAb, whereas an A subunit-specific mAb had no effect.

The Gb₃ toxin-binding component was detected in HeLa cells. Moreover, the binding of shigella toxin to these cells was competitively reduced by >90% in the presence of Gb₃ incorporated into liposomes. Nevertheless, we found no evidence that binding to Gb₃ was responsible for the HeLa cell cytotoxicity of shigella toxin, since the addition of Gb₃-containing liposomes failed to diminish the cytotoxic response of HeLa monolayers to toxin. This is not entirely surprising because we have previously presented evidence that cytotoxicity is mediated by a tunicamycin-sensitive glycoprotein containing GlcNAc linked β 1 \rightarrow 4 to GlcNAc (10). The present studies provide additional data to support this, as shown by the competitive inhibition of toxicity by trimeric β 1 \rightarrow 4-linked GlcNAc (chitotriose). We have also found that overnight incubation of HeLa cells in the presence of a sufficient concentration of tunicamycin to completely abrogate the response to 100 TC₅₀ doses of toxin, reduces specific binding of toxin by only 55–60% (10), and does not appear to diminish the amount of the extractable glycolipid toxin-binding component of HeLa cells (present study). This residual binding can now be explained, at least in part, by the tunicamycin-resistant Gb₃ receptor. That this is distinct from a glycoprotein cytotoxic receptor is also shown by the observation that chitotriose inhibited the toxicity of shigella toxin for HeLa cells but not its binding to Gb₃-coated microtiter plates. It is most interesting to note that while toxin binding to both receptors appears to be via the same toxin B subunit, as indicated by studies with purified subunits and subunit-specific mAbs, the glycoprotein and glycolipid binding sites exhibit distinctive sugar specificities (GlcNAc β 1 \rightarrow 4GlcNAc- and Gal α 1 \rightarrow 4Gal β 1 \rightarrow 4 Glc or GlcNAc, respectively). We do not at present know how many different functional domains are present on the M_r 6,500 B subunit peptide, nor whether the B subunit-specific monoclonal, 4D3, recognizes more than one domain. We believe a more likely hypothesis is that 4D3 directly binds to one binding domain and sterically inhibits, but does not bind to, a second domain. Thus we propose a

novel mechanism to explain the diverse effects of toxin that involves two distinct sugar-specific binding sites on the same small peptide that mediate the different biological activities. More data are certainly needed to reach a firm conclusion about this.

Rabbit jejunum responds *in vivo* to binding of toxin to the brush border surface by net secretion of isotonic fluid, but in contrast to the HeLa cell does not exhibit any histologically demonstrable cytotoxic response (25). Preliminary studies with purified rabbit jejunal microvillus membranes (MVM) have shown a single class of binding site for toxin (26, 27), and the present study indicates that this is Gb₃. Since Gb₃ apparently does not mediate toxin effects in HeLa cells, the obvious question is whether or not binding to gut Gb₃ initiates the fluid secretory response of rabbit small bowel. We cannot completely answer this question now, but other preliminary observations from our laboratory are consistent with such a role. We have reported that the appearance of Gb₃ in rabbit jejunum is developmentally regulated in the New Zealand white rabbit (12). Thus, animals younger than 16 d (and responsive to cholera toxin) do not secrete fluid when shigella toxin is placed in ligated small bowel loops (27). However, a consistently positive fluid response occurs in animals older than 18 d when Gb₃ becomes detectable in jejunal MVM, and binding of ¹²⁵I-toxin to MVM is also demonstrable. While these results do not prove the physiological relevance of the Gb₃ receptor, the data certainly suggest that binding to Gb₃ in rabbit gut is coupled to a fluid secretory process. We presume that we cannot show any effect of toxin binding to HeLa cell Gb₃ because HeLa cells are not secretory cells.

Gb₃ is also the determinant of the P^k blood group antigen (28). The Gal α 1 \rightarrow 4Gal disaccharide of P^k, whether terminal as in Gb₃ or internal as in Gb₄, is recognized by the Gal-Gal pilus of uropathogenic *Escherichia coli* (29–31). This structure, also termed P pilus (after the P blood group system) or PAP pilus (for pyelonephritis-associated pilus), has been shown to be a virulence determinant of pili-positive *E. coli* strains, since it allows recognition and binding to renal epithelial membrane glycosphingolipids containing Gal α 1 \rightarrow 4Gal (32, 33). Progress towards developing a vaccine for these strains of *E. coli* based on immunization with Gal-Gal pili or synthetic Gal-Gal has been reported (33, 34). The common Gb₃ receptor for shigella toxin and Gal-Gal positive uropathogenic *E. coli* suggests homology between domains of the shigella toxin B subunit and the Gal-Gal pilus. It is therefore possible that antitoxin antibodies might be generated by immunization with a Gal-Gal pilus vaccine. This might prove to be of practical use if immunization with receptor analogues fails to significantly block toxin activity, as already observed with the anti-Gb₃ mAb used in these studies. It would be most interesting if a Gal-Gal pilus vaccine generated both antipilus and antitoxin antibodies to induce simultaneous resistance to pyelonephritis and shigellosis.

Summary

A glycolipid that specifically binds shigella toxin was isolated from both HeLa cells and rabbit jejunal mucosa and identified as globotriaosylceramide (Gb₃) by its identical mobility on HPTLC to authentic erythrocyte Gb₃. Toxin also bound

to a band tentatively identified as α -hydroxylated Gb₃. In addition, toxin bound to P₁ antigen present in group B human erythrocyte glycolipid extracts. The common feature of the three binding glycolipids is a terminal Gal α 1 \rightarrow 4Gal disaccharide linked β 1 \rightarrow 4 to either Glc or GlcNAc. Globoisotriaosylceramide, which differs from Gb₃ only in possessing a Gal α 1 \rightarrow 3Gal terminal disaccharide, and LacCer, which lacks the terminal Gal residue of Gb₃, were incapable of binding the toxin. Binding was shown to be mediated by the B subunit by the use of isolated toxin A and B subunits and monoclonal subunit-specific antibodies. Gb₃-containing liposomes competitively inhibited the binding of toxin to HeLa cell monolayers but did not inhibit toxin-induced cytotoxicity.

These studies show an identical carbohydrate-specific glycolipid receptor for shigella toxin in gut and in HeLa cells. The toxin B subunit that mediates this binding has also been shown to recognize a glycoprotein receptor with different sugar specificity. Thus, we have demonstrated that the same small (M_r 6,500) B subunit polypeptide has two distinctive carbohydrate-specific binding sites. The Gal α 1 \rightarrow 4Gal disaccharide of the glycolipid toxin receptor is also recognized by the Gal-Gal pilus of uropathogenic *E. coli*. This suggests the possibility that the pilus and toxin B subunit contain homologous sequences. If this is true, it may be possible to use the purified Gal-Gal pilus to produce toxin-neutralizing antibodies.

Received for publication 3 February 1986.

References

1. Donohue-Rolfe, A., G. T. Keusch, C. Edson, D. Thorley-Lawson, and M. Jacewicz. 1982. Pathogenesis of shigella diarrhea. IX. Simplified high yield purification of shigella toxin and characterization of subunit composition and function by the use of subunit-specific monoclonal and polyclonal antibodies. *J. Exp. Med.* 160:1767-1781.
2. Keusch, G. T., A. Donohue-Rolfe, and M. Jacewicz. 1985. Shigella toxin and the pathogenesis of shigellosis. *Ciba Found. Symp.* 112:193-214.
3. Eiklid, K., and S. Olsnes. 1983. Animal toxicity of *Shigella dysenteriae* cytotoxin: evidence that the neurotoxic, enterotoxic and cytotoxic activities are due to one toxin. *J. Immunol.* 130:380-384.
4. Keusch, G. T., A. Donohue-Rolfe, and M. Jacewicz. 1982. Shigella toxin(s): description and role in diarrhea and dysentery. *Pharmacol. & Ther.* 15:403-438.
5. Wiley, R. G., A. Donohue-Rolfe, and G. T. Keusch. 1985. Axonally transported shigella cytotoxin is neuronotoxic. *J. Neuropathol. & Exp. Neurol.* 44:496-506.
6. Keusch, G. T. 1973. Pathogenesis of shigella diarrhea. III. Effects of shigella enterotoxin in cell culture. *Trans. N.Y. Acad. Sci.* 35:51-58.
7. Eiklid, K., and S. Olsnes. 1980. Interaction of *Shigella shigae* cytotoxin with receptors on sensitive and insensitive cells. *J. Receptor. Res.* 1:199-213.
8. Olsnes, S., and K. Eiklid. 1980. Isolation and characterization of *Shigella shigae* cytotoxin. *J. Biol. Chem.* 256:284-289.
9. Reisbig, R., S. Olsnes, and K. Eiklid. 1981. The cytotoxic activity of shigella toxin: evidence for catalytic inactivation of the 60S ribosomal subunit. *J. Biol. Chem.* 256:8739-8744.
10. Keusch, G. T., M. Jacewicz, and A. Donohue-Rolfe. 1986. Pathogenesis of shigella diarrhea. Evidence for an N-linked glycoprotein receptor and receptor modulation by β -galactosidase. *J. Infect. Dis.* 153:238-248.

11. Keusch, G. T., and M. Jacewicz. 1977. Pathogenesis of shigella diarrhea. VII. Evidence for a cell membrane toxin receptor involving β 1 \rightarrow 4-linked *N*-acetyl-D-glucosamine oligomers. *J. Exp. Med.* 146:535-546.
12. Mobassaleh, M., A. Donohue-Rolfe, R. K. Montgomery, G. T. Keusch, and R. J. Grand. 1985. Postnatal development of shigella toxin binding sites on rabbit intestinal microvillus membranes. *Abstracts of the Twenty-fifth Interscience Conference on Antimicrobial Agents and Chemotherapy.* 149.
13. Nudelman, E., R. Kannagi, S. Hakomori, M. Parsons, M. Lipinski, J. Wiels, M. Fellous, and T. Tursz. 1983. A glycolipid antigen associated with Burkitt lymphoma defined by a monoclonal antibody. *Science* (Wash. DC). 220:509-511.
14. Fukushi, Y., S. Hakomori, E. Nudelman, and N. Cochran. 1984. Novel fucolipids accumulating in human adenocarcinoma. II. Selective isolation of hybridoma antibodies that differentially recognize mono-, di-, and trifucosylated type 2 chain. *J. Biol. Chem.* 259:4681-4685.
15. Bolton, A. E., and W. M. Hunter. 1973. The labelling of proteins to high specific radioactivities by conjugation to a ¹²⁵I-containing acylating agent. *Biochem. J.* 133:529-539.
16. Keusch, G. T., M. Jacewicz, and S. Z. Hirschman. 1972. Quantitative microassay in cell culture for enterotoxin of *Shigella dysenteriae* 1. *J. Infect. Dis.* 125:539-541.
17. Kannagi, R., M. N. Fukuda, and S. Hakomori. 1982. A new glycolipid antigen isolated from human erythrocyte membranes reacting with antibodies directed to globo-*N*-tetraosylceramide (globoside). *J. Biol. Chem.* 257:4438-4442.
18. Folch, J., S. Arsove, and J. A. Meath. 1972. Isolation of brain strandin. A new type of large molecule tissue component. *J. Biol. Chem.* 191:819-831.
19. Yu, R. K., and R. W. Ledeen. 1972. Gangliosides of human, bovine, and rabbit plasma. *J. Lipid Res.* 13:680-686.
20. Saito, T., and S. Hakomori. 1971. Quantitative isolation of total glycosphingolipids from animal cells. *J. Lipid Res.* 12:257-259.
21. Watanabe, K., and Y. Arao. 1981. A new solvent system for the separation of neutral glycosphingolipids. *J. Lipid Res.* 22:1020-1024.
22. Siddiqui, B., J. Kawanami, Y. -T. Li, and S. Hakomori. 1972. Structures of ceramide tetrasaccharides from various sources: uniqueness of rat kidney ceramide tetrasaccharide. *J. Lipid Res.* 13:657-662.
23. Naiki, M., J. Fong, R. Ledeen, and D. M. Marcus. 1975. Structure of the human erythrocyte blood group P₁ glycosphingolipid. *Biochemistry.* 14:4831-4836.
24. Brown, J. E., G. R. Fanning, and A. A. Lindberg. 1985. Identification of a glycolipid receptor for Shiga toxin from *Shigella dysenteriae* 1. *Abstr. Annu. Meet. Am. Soc. Microbiol.* 36.
25. Steinberg, S. E., J. G. Banwell, J. H. Yardley, G. T. Keusch, and T. R. Hendrix. 1975. Comparison of secretory and histologic effects of shigella and cholera enterotoxins in rabbit jejunum. *Gastroenterology.* 68:309-317.
26. Fuchs, G., A. Donohue-Rolfe, R. K. Montgomery, G. T. Keusch, and R. J. Grand. 1984. Evidence of a receptor for shigella toxin on rabbit intestinal microvillus membranes. *Gastroenterology.* 86:1083. (Abstr.)
27. Mobassaleh, M., A. Donohue-Rolfe, R. K. Montgomery, R. J. Grand, and G. T. Keusch. 1986. Functional characterization of age dependent binding of shigella toxin to rabbit intestinal microvillus membranes. *Gastroenterology.* In press. (Abstr.)
28. Marcus, D. M., S. K. Kundu, and A. Suzuki. 1981. The P blood group system: recent progress in immunochemistry and genetics. *Semin. Hematol.* 18:63-71.
29. Vaisanen, V., J. Elo, L. Tallgren, A. Siitonen, P. Makela, C. Svanborg-Eden, G. Kallenius, S. B. Svenson, H. Hultberg, and T. Korhonen. 1981. Mannose-resistant

- hemagglutination and P antigen recognition characteristic of *E. coli* causing primary pyelonephritis. *Lancet*. 2:1366-1369.
30. Kallenius, G., R. Mollby, S. B. Svenson, J. Winberg, A. Windblod, S. Svenson, and B. Cedergren. 1980. The P^k antigen as receptor for the hemagglutinin of pyelonephritic *Escherichia coli*. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 7:297-302.
 31. Bock, K., M. E. Breimer, A. Brignole, G. C. Hansson, K-A. Karlsson, G. Larson, H. Leffler, B. E. Samuelsson, N. Stromberg, and C. Svanborg Eden. 1985. Specificity of binding of a strain of uropathogenic *Escherichia coli* to Gal α 1 \rightarrow 4Gal-containing glycosphingolipids. *J. Biol. Chem.* 260:8545-8551.
 32. Leffler, H., and C. Svanborg-Eden. 1981. Glycolipid receptors for uropathogenic *Escherichia coli* on human erythrocytes and uro epithelial cells. *Infect. Immun.* 34:920-929.
 33. O'Hanley, P., D. Lark, S. Falkow, and G. Schoolnik. 1985. Molecular basis of *Escherichia coli* colonization of the upper urinary tract in BALB/c mice. *J. Clin. Invest.* 75:347-360.
 34. Schmidt, M. A., P. O'Hanley, and G. Schoolnik. 1985. Gal-Gal pyelonephritis *Escherichia coli* pili linear immunogenic and antigenic epitopes. *J. Exp. Med.* 161:705-717.