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Pathogenesis of *Shigella* Diarrhea: XVII. A Mammalian Cell Membrane Glycolipid, Gb3, Is Required but Not Sufficient to Confer Sensitivity to Shiga Toxin

Mary S. Jacewicz, Munir Mobassaleh, Sonja K. Gross, K. A. Balasubramanian, Peter F. Daniel, Srinivasa Raghavan, Robert H. McCluer, and Gerald T. Keusch

Shiga toxin recognizes a galactose-α1→4-galactose terminal glycolipid, globotriaosylceramide (Gb3), in sensitive mammalian cells and is translocated by endocytosis to the cytoplasm, where it blocks protein synthesis. To determine if Gb3 is both required and sufficient for toxicity, Gb3 content in cells was altered by blocking key biosynthetic or degradative path enzymes with specific inhibitors. The resulting decrease or increase in cellular Gb3 was associated with a decrease or increase in binding of and response to Shiga toxin. Toxin-resistant Gb3-deficient variants of sensitive cells fused with liposomes containing Gb3 but not globotetraosylceramide (Gb4) became susceptible, whereas fusion of Gb3 liposomes to naturally resistant Gb3-deficient CHO cells increased toxin binding but not cytotoxicity. These data demonstrate that Gb3 is required, but not sufficient, for the action of Shiga toxin and suggest the existence of a toxin translocation mechanism linked to surface glycolipids that is not expressed in CHO cells.

The Shiga family of toxins is linked to the pathogenesis of hemolytic uremic syndrome, which complicates diarrheal disease due to cytotoxin-producing *Shigella dysenteriae* type 1 or certain serotypes of *Escherichia coli* associated with hemorrhagic colitis [1]. These toxins are heterodimeric proteins that comprise an enzymatically active A subunit that irreversibly inhibits protein synthesis by mammalian ribosomes [2] and multiple B subunits that recognize neutral glycolipids on the target cell surface. Shiga toxin–binding glycolipids all contain a terminal Gal-α1→4-Gal disaccharide, such as globotriaosylceramide (Gb3 or Gal-α1→4-Gal-β1→4-Glc-β1- ceramide) [3–5]. Because their biochemical target is the ribosome, Shiga family toxins must be internalized by cells to exert toxicity. Internalization proceeds by receptor-mediated endocytosis [6] from clathrin-coated regions of the plasma membrane [7].

The evidence that Gal-α1→4-Gal terminal glycolipids serve as the receptors for internalization of Shiga toxin consists of three observations: holotoxin or purified toxin B subunit binds specifically to purified Gb3 in vitro and to Gb3 extracted from toxin-sensitive cells and tissues [3–5]; cytotoxicity in tissue culture lines and cloned HeLa cells correlates with the cell content of Gb3 [8]; and enterotoxicity (intestinal fluid secretion) in rabbit small bowel loops correlates with the appearance of Gb3 in the microvillus membrane at day 16 of postnatal life [9, 10]. In piglets, tissue localization of intravenously injected *E. coli* Shiga-like toxin (SLT-I), which has a B subunit identical to that of Shiga toxin, is proportional to tissue content of Gb3 [11]. However, lesions in this species occur primarily in brain and not necessarily in tissues to which toxin binds at higher levels, suggesting that some but not all Gb3 binding sites are functional receptors. The only direct evidence that Gb3 is a required receptor is the report that Shiga toxin–resistant Daudi cells become sensitive after incubation with Gb3-containing liposomes [12].

The experiments presented here were designed to more fully investigate the role of and requirement for Gb3 in the toxic effects of Shiga toxin by examining the consequence of altering, by various means, the Gb3 content in sensitive and resistant cells.

### Materials and Methods

**Toxin purification.** Toxin was purified from sonic lysates of *S. dysenteriae* 1 60R by affinity chromatography on the P1–blood group–reactive glycoprotein of hydatid cysts as previously described by our laboratory [13]. Purified toxin was lyophilized and stored dry at −70°C until used. Shiga toxin was iodinated by a modification of the chloramine T procedure as previously reported [14].

**Selection and maintenance of cell lines.** HeLa CCL2 and 229 cells, Vero cells, and CHO K1 cells were used (American Type...
Culture Collection, Rockville, MD). HeLa and Vero cells were maintained in McCoy's 5A modified medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies GIBCO BRL, Gaithersburg, MD). CHO cells were grown in F12 nutrient mixture containing proline with FBS and antibiotics (Life Technologies Gibco BRL). To select toxin-resistant lines, parent strain HeLa cells in logarithmic phase were exposed overnight at 37°C to 0.1 μg/mL Shiga toxin in medium, washed, and then allowed to grow to confluence in fresh medium. The surviving cells, designated T1, were sequentially passaged four more times (T2–T5) in increasing concentrations of toxin, up to 10 μg/mL for 7 days. T5 cells were resistant to overnight incubation with 10 μg/mL toxin.

Cytotoxicity and amino acid uptake assays. Cytotoxicity was assessed either by assaying overnight detachment of cells from the monolayer as previously reported [15] or by measuring incorporation of [3H]leucine into protein by cell monolayers in 96-well microtiter plates (Nunc, Roskilde, Denmark). In the latter assay, cells were incubated for 3 h with Shiga toxin, washed with leucine-free medium, incubated for 30 min with 1 μCi/0.1 mL [3H]leucine/well (Du Pont NEN Research Products, Boston), washed, and solubilized with 100 μL of 0.1 M KOH/well. Protein was precipitated with 150 μL of 10% trichloroacetic acid and harvested under suction onto 25-mm glass fiber filters (Whatman, Maidstone, UK) and air dried. Radioactivity was determined, and cytotoxicity was calculated as percentage inhibition of [3H]leucine incorporation compared with cells not exposed to Shiga toxin.

Amino acid uptake was measured by adding 1 μCi/0.1 mL [α-3H]l-aminoisobutyric acid; (Du Pont NEN Research Products) in place of [3H]leucine. After 30 min, cells were washed and solubilized with 0.1 M KOH as above, and radioactivity was determined.

Toxin binding. 125I-labeled Shiga toxin was serially diluted in medium at 4°C, and 30 μL of each dilution was applied in triplicate to cells grown in 96-well microtiter plates chilled to 4°C. After 1 h at 4°C, monolayers were washed three times with medium and twice with PBS and then solubilized in 100 μL of 0.1 M KOH; radioactivity was determined. Capacity and affinity of binding were calculated by Scatchard analysis [8].

Glycolipid content of cells. To determine glycolipid content, monolayers were washed in PBS, scraped, and extracted in 2 mL of methanol at 55°C for 15 min. A Folch partition was established by addition of 4 mL of chloroform and 1.5 mL of H2O [16]. Lower-phase lipids were applied to a Unisil column (Clarkson Chemical, Williamsport, PA) and the neutral glycolipids, eluted with acetone:methanol (9:1, vol:vol), were hydrolyzed with methanolic NaOH, subjected to a Folch partition, and benzoylated with 10% benzoyl chloride in pyridine [17]. Samples were dissolved in carbon tetrachloride and injected onto a pellicular Zipax column (Du Pont, Wilmington, DE) for separation by high-performance liquid chromatography (HPLC; Waters, Milford, CT) with a linear 2%–42% gradient of 46% dioxane in hexane diluted into hexane at a flow rate of 2 mL/min over 13 min [10]. All HPLC grade solvents were from Fisher Scientific (Pittsburgh). Eluted peaks were detected by absorption at 230 nm. Protein content of the scrapings was determined with a protein assay reagent (BioRad Laboratories, Richmond, CA).

Surface labeling of HeLa cells. Exposed galactose residues were labeled by oxidation with galactose oxidase followed by reduction with tritiated sodium borohydride [3H]NaBH4 (Du Pont NEN Research Products) [18]. Monolayers (5 × 104 cells) were grown in 150-mm diameter tissue culture plates and preincubated by incubation with NaBH4 (1.75 mg in 5 mL of PBS) for 5 min at 25°C, washed twice with serum-free medium, and harvested by scraping and centrifugation at 1000 rpm for 5 min. Cells were incubated with 10 units of galactose oxidase in 100 μL of serum-free medium for 3 h at 37°C, washed twice with 10 mL of 0.15 M PBS, pH 7.2, and centrifuged as above; 1 μCi of [3H]NaBH4 in 50 μL of PBS was added to each pellet for 30 min at 25°C with shaking. After five washes with 10 mL of PBS, neutral glycolipids were isolated and separated by quantitative HPLC over 40 min at a flow rate of 1 mL/min, as described above. Glycolipid content was measured by integrating the area under the curve. Radioactivity associated with each peak was measured simultaneously using a flow-through scintillation counter attached to the HPLC column system. Specific activity of each peak was calculated.

Preparation of Gb3-containing liposomes and fusion conditions. A mixture of 500 μg of Gb3, 500 μg of phosphatidylethanolamine, 500 μg of phosphatidylserine, and 75 μg of palmitic acid (all from Matreya, Pleasant Gap, PA) in CHCl3 was dried under nitrogen, resuspended in 1 mL of PBS, and sonicated to clarity (450 Sonifier [power setting 3, 50% cycle, 5 min]; Branson Ultrasonics, Danbury, CT). For fusion experiments, CHO or HeLa T5 cells were trypsinized, washed, and resuspended in medium at 2 × 105 cells/mL. Cells (100 μL) were mixed with 100 μL of PBS or the liposome preparation and rotated at 37°C for 2 h, when 500 μL of 50% polyethylene glycol (PEG; Life Technologies GIBCO BRL) was added. After a further 10 min of rotation, cells were washed twice with 10 mL of medium and resuspended in medium at 5 × 105 cells/mL. Cell suspension (100 μL) was dispensed into 96-well microtiter plates and incubated overnight at 37°C. Binding of 125I-labeled Shiga toxin was measured and, in companion cells, cytotoxicity was assayed by the [3H]leucine incorporation method.

To optimize fusion conditions and to assess fusion in these experiments, liposomes were prepared in the presence of 100 mM 5(6)-carboxyfluorescein (Sigma, St. Louis) [19]. Free dye was removed by column chromatography (Sephadex G-25; Pharmacia LKB Biotechnology, Piscataway, NJ) and liposomal fusion was monitored by fluorescence microscopy. 5(6)-Carboxyfluorescein is self-quenched at high concentration, as in liposomes, but fluoresces when diluted into the cytoplasm after fusion.

Effects of 1,5-dideoxy-1,5-imino-D-galactitol (DIG). HeLa T5 cells were incubated for 6 days with daily changes of medium containing 10 μM DIG, a competitive inhibitor of lysosomal α-galactosidase (provided by B. Ganem, Cornell University, Ithaca, NY) [20, 21]. On day 6, treated cells were dispensed into 96-well microtiter plates and incubated overnight in medium with 10 μM DIG. The following day, leucine incorporation, amino acid uptake, and glycolipid content were measured as described above.

Effects of 1,3-threo-1-phenyl-2-decanoylimino-3-morpholino-1-propanol HCl (PDMP). Two Shiga toxin–sensitive cell lines,
HeLa 229 and Vero were grown for 7 days with daily changes of medium containing 40 μM PDMP, an inhibitor of UDP-glucose:ceramide glucosyltransferase (Matreya) [22]. Leucine incorporation and glycolipid content were determined as described above.

α-galactosidase determination. α-galactosidase activity was measured by a modification of the method of Kano and Yama-kawa [23]. Cells were washed twice with PBS, harvested by scraping, sonicated in water, and incubated in the presence of 5 mM 4-methylumbelliferyl-α-D-galactopyranoside (Sigma) in 0.1 M citrate-phosphate buffer, pH 4.0, for 30 min at 37°C with shaking. The reaction was stopped with 2 mL of 0.25 M glycine-KOH, pH 10.3, and relative intensity of emission was measured in a ratio photometer with a Xenon lamp power supply (American Instrument, Silver Spring, MD), stimulating at 366 nm and measuring emission at 446 nm. Release of 4-methylumbelliferyl-beta-D-galactoside was estimated from the relative intensity of standards. Protein content was measured by the Lowry method, and specific enzyme activity was expressed as nanomoles of 4-methylumbelliferone/minute/milligram of protein.

Galactosyltransferase assay. UDP-galactose:lactosylceramide galactosyltransferase (galactosyltransferase) was determined by a new immunostaining technique that detects the synthesis of Gb3 [24]. Cell monolayers were scraped, homogenized, and incubated for 1 h at 37°C with the following reaction mixture: 200 μg of homogenate protein, 0.25 mg of sodium cholate, 50 mM 2-[N-morpholino]ethanesulfonic acid buffer (pH 6.5), 10 mM MnCl2, 0.1 mM 5′-adenylimidodiphosphate, 0.5 mM UDP-galactose (all from Sigma), with or without 0.05 μmol of lactosylceramide acceptor (Matreya). The reaction was stopped by addition of 5 mL of Folch theoretical upper-phase (CHCl3:methanol:water, 3:48:47, vol/vol) containing 0.1 M KCl, and glycolipids were extracted and separated by high-performance thin-layer chromatography (HPTLC-SI plates; J. T. Baker Chemical, Phillipsburg, NJ) in CHCl3:methanol:water, 3:48:47, vol/vol) containing 0.1 M KCl, and glycolipids were extracted and separated by high-performance thin-layer chromatography (HPTLC-SI plates; J. T. Baker Chemical, Phillipsburg, NJ) in CHCl3:methanol:water, 3:48:47, vol/vol) containing 0.1 M KCl, and glycolipids were extracted and separated by high-performance thin-layer chromatography (HPTLC-SI plates; J. T. Baker Chemical, Phillipsburg, NJ) in CHCl3:methanol:water, 3:48:47, vol/vol) containing 0.1 M KCl, and glycolipids were extracted and separated by high-performance thin-layer chromatography (HPTLC-SI plates; J. T. Baker Chemical, Phillipsburg, NJ) in CHCl3:methanol:water, 3:48:47, vol/vol) containing 0.1 M KCl, and glycolipids were extracted and separated by high-performance thin-layer chromatography (HPTLC-SI plates; J. T. Baker Chemical, Phillipsburg, NJ) in CHCl3:methanol:water, 3:48:47, vol/vol). After blocking with 5% skim milk (wt/vol), Gb3 was detected by binding of Shiga toxin, followed by high-titer polyclonal rabbit anti-Shiga toxin and commercial goat anti-rabbit immunoglobulin alkaline phosphatase conjugate and color reagents (Promega, Madison, WI) and quantified by laser densitometry (Ultrascan XL; Pharmacia LKB Biotechnology). Total Gb3 in the absence of added lactosylceramide was subtracted from the value in the presence of acceptor; galactosyltransferase activity was calculated as follows: nanomoles of Gb3 formed/milligram of homogenate protein/hour.

Results

Toxin-Resistant HeLa Cell Lines

Increasingly resistant HeLa CCL2 cells were obtained by serial passage in increasing concentrations of Shiga toxin (table 1). T4 and T5 cells were so resistant that the TCLD50 end point could not be calculated. Comparable results were obtained by the two cytotoxity assays. Toxin resistance was not due to a change in amino acid uptake in T1–T5 cells, as assessed by the uptake of [α-3H]aminoisobutyric acid (table 2). Cytotoxicity was directly related to binding of 125I-labeled Shiga toxin. Binding capacity of the lines for Shiga toxin, derived by Scatchard analysis of the equilibrium binding data, decreased with selection for toxin resistance (table 1), with no change in binding affinity (1.12 ± 0.08 × 107 M⁻¹). The magnitude of the change in sensitivity to Shiga toxin was proportionately greater than the decrease in the number of binding sites.

Total Gb3 and Gb4 content in these cells was calculated from the HPLC elution profile of extracted neutral glycolipids (data not shown). Gb3 and Gb4 content progressively decreased as cells were selected for increasing resistance to Shiga toxin (table 1). Only Gb3 and Gb4, and not the monoo- or diglycosylceramides, could be surface-labeled by galac-tose oxidase-[3H]NaBH₄, regardless of the sensitivity of the cells for Shiga toxin (data not shown). Specific activity of labeled Gb3 and Gb4 was similar in all of the lines, independent of Gb3 or Gb4 content. Specific activity of Gb4 (11.76 ± 20.1 cpm/μmol) was higher than Gb3 (44.6 ± 5.8 cpm/μmol), but because Gb4 has two sites available for labeling and Gb3 has only one, this cannot be taken as evidence that Gb4 is more surface-exposed and more readily labeled than Gb3.

To determine the basis for these differences in glycolipid content, we assayed the galactosyltransferase and α-galacto-sidase enzyme activities involved in biosynthesis and degra-dation of Gb3. Galactosyltransferase directly correlated with both Gb3 content and sensitivity to Shiga toxin (figure 1). No difference was found in cellular α-galactosidase activity between parent CCL2 cells and resistant lines derived from them (data not shown). Galactosyltransferase activity was also greater in the more sensitive ATCC HeLa 229 line (1.332 vs. 0.895 nmol of Gb3/mg cell protein/h) compared with ATCC CCL2 cells, whereas the reverse was true of α-galactosidase activity (1.43 ± 0.13 vs. 4.48 ± 0.08 nmol/min/ mg; P < .001).

Effects of Increasing Cellular Gb3 Content

Treatment of cells with DIG. Incubation of toxin-resis-tant T5 cells for 7 days in the presence of DIG changed the cellular phenotype to sensitive (figure 2A), comparable to T1 cells. This was associated with a >2-fold increase in Gb3 content (data not shown) and specific binding of 125I-labeled Shiga toxin (figure 2B). Although growth of the monolayer was not affected by DIG, as assessed by direct microscopy, uptake of AIB diminished by 70.2% and incorporation of leucine into protein in the presence of Shiga toxin decreased by 82.5% compared with control cells incubated without DIG. In contrast, DIG treatment of CHO cells did not alter Gb3 content or sensitivity to Shiga toxin (data not shown).

Treatment of cells with Gb3-containing liposomes. To insert Gb3 by liposomal fusion, we first optimized conditions for fusion, which required addition of palmitate to the lipid membrane and PEG to the incubation medium (data
Cellular Gb3 and Shiga Toxin Action

Table 1. Sensitivity and neutral glycolipid content of selected HeLa cell lines to Shiga toxin.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>TC₅₀ (µg/mL)</th>
<th>Binding capacity (molecules bound/cell)</th>
<th>Glycolipid content (pmol/mg cell protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS</td>
<td>6.5 x 10⁻²</td>
<td>7.2 ± 1.1 x 10⁶</td>
<td>420.0</td>
</tr>
<tr>
<td>T1</td>
<td>2.4 x 10⁻²</td>
<td>4.9 ± 0.6 x 10⁶</td>
<td>458.7</td>
</tr>
<tr>
<td>T2</td>
<td>1.3 x 10⁻⁴</td>
<td>3.7 ± 0.5 x 10⁶</td>
<td>415.2</td>
</tr>
<tr>
<td>T3</td>
<td>~10³</td>
<td>5.6 ± 0.8 x 10⁵</td>
<td>625.3</td>
</tr>
<tr>
<td>T4</td>
<td>NC</td>
<td>3.0 ± 0.4 x 10⁴</td>
<td>464.2</td>
</tr>
<tr>
<td>T5</td>
<td>NC</td>
<td>1.2 ± 0.3 x 10⁴</td>
<td>318.2</td>
</tr>
</tbody>
</table>

NOTE. NC = TC₅₀ cannot be calculated; cell detachment at 10 µg/mL Shiga toxin was 4.7% for T4 cells and 0 for T5 cells. GluCer = glucosylceramide; LacCer = lactosylceramide.

* Extrapolated value.

not shown). These conditions were used for all experiments. Binding of [¹²⁵I]-labeled Shiga toxin to toxin-resistant T5 and CHO cells preincubated with liposomes loaded with Gb3 or Gb4 is shown in figure 3A and B, respectively. No effect of insertion of lactosylceramide was noted (data not shown). Binding of Shiga toxin increased when either cell type was incubated with Gb3-containing liposomes but not with Gb4-containing liposomes, except at the highest concentrations of labeled toxin in T5 cells. T5 cells fused with Gb3- but not Gb4-loaded liposomes also became markedly more sensitive to toxin (figure 4A). In contrast, CHO cells remained resistant to Shiga toxin after fusion with either Gb3- or Gb4-containing liposomes, except at the highest concentration of toxin in cells fused with Gb3-containing liposomes (figure 4B).

To determine if the failure to affect CHO cell susceptibility to Shiga toxin when Gb3 was fused into the cell membrane was due to an intrinsic resistance of CHO cell liposomes to the enzymatic action of the Shiga toxin A subunit, we examined the toxicity of the plant lectin ricin, which inhibits protein synthesis by the identical enzymatic activity as Shiga toxin but enters cells after binding to different galactose terminal receptors [25]. Shiga toxin-resistant CHO cells were still susceptible to ricin, with a sharp dose-response curve and a TC₅₀ of ~0.5 µg/mL (data not shown). In Shiga toxin-sensitive HeLa cells, Shiga toxin was more active than ricin by approximately 2 orders of magnitude on a weight basis.

Effects of Inhibiting Biosynthesis of Gb3

Two Shiga toxin-sensitive cell lines, HeLa 229 and Vero, were incubated with PDMP to block the initial step in the Gb3 synthetic pathway. Compared with controls, these cells grew more slowly in the presence of PDMP and required daily medium changes to maintain viability; otherwise they did not differ from control cells. All neutral glycolipids were reduced in cells exposed to PDMP, with a >10-fold decrease...
in Gb3 content in both HeLa (1329 ± 216 vs. 125 ± 16 pmol/mg cell protein) and Vero (1137 ± 118 vs. 107 ± 35 pmol/mg cell protein) cells. At comparable cell density, both cell types incorporated similar amounts of labeled leucine into protein in either the presence or absence of PDMP and both showed high-level toxin resistance after growth in the presence of PDMP (figure 5A and B, respectively). To determine if this resistance was due solely to the reduced Gb3 content, we attempted to add back Gb3 by liposomal fusion to PDMP-treated Vero cells. In the absence of PDMP, liposomal fusion had no effect on cytotoxicity, whereas insertion of Gb3, but not Gb4, reversed the effect of the inhibitor (figure 6A and B).

Discussion

These studies demonstrate that Gb3, a neutral glycolipid expressed at the cell surface, is necessary but not sufficient to confer sensitivity of mammalian cells to Shiga toxin. The data confirm and extend prior observations correlating Gb3 content and sensitivity to Shiga toxin using resistant HeLa cell lines selected by passage of sensitive cells in increasing concentrations of toxin. However, we observed a discordance between the percentage change in Gb3 content and cytotoxicity. For example, although the maximum observed difference between the parent and resistant lines in toxin-binding capacity and sensitivity to toxin was 600-fold and at least 10 orders of magnitude, respectively, sensitive parent HeLa cells contained only five times as much Gb3 as the highly resistant T5 cells. We surface-labeled exposed glyco-

Figure 2. Treatment of HeLa T5 cells with 1,5-dideoxy-1,5-imino-D-galactitol. Effect on sensitivity of cells to Shiga toxin (ShT), measured by inhibition of [3H]leucine incorporation into protein (A) and on binding of 125I-labeled Shiga toxin (B).

Figure 3. Binding of 125I-labeled Shiga toxin to toxin-resistant HeLa T5 (A) and CHO cells (B) and to cells fused with Gb3- and Gb4-loaded liposomes.
Gb3 inhibition

\[
\% \text{ inhibition}
\]

...where Shiga toxin uptake occurs \[7\]. In this way, small changes in the total receptor content might mediate the greater observed changes in the biologic effects of the toxin. Alternatively, if there is a separate translocation mechanism with which Gb3-bound toxin must interact, increased membrane Gb3 content could also enhance the chance of this occurring, again resulting in a sharp increase in toxicity as receptor content slowly increased. Thus, when the content of Gb3 is low and dispersed, much of the surface Gb3 may be unable to function as a receptor.

The differences in Gb3 content of the HeLa and Vero cells can now be attributed primarily to the endogenous activity of the Gb3 biosynthetic galactosyltransferase enzyme. Not only did HeLa cells selected for resistance show progressively less...
Gb3 transferase activity, but the more sensitive HeLa 229 cells also contained significantly higher levels of activity than did HeLa CCL2 cells. In addition to this greater synthetic enzyme activity, HeLa 229 cells expressed less Gb3-degradative α-galactosidase activity than did HeLa CCL2 cells.

We also used two strategies to directly increase cellular Gb3 content. We incubated resistant cells with DIG, a competitive inhibitor of lysosomal α-galactosidase A [20, 21], to reduce the degradation rate of Gb3. As expected, this led to an increase in cellular content of Gb3 and Shiga toxin binding and a highly significant increase in sensitivity to toxin. We next inserted Gb3 into resistant target cells using liposome fusion techniques. Waddell et al. [12] had previously reported that toxin-resistant mutant human Daudi lymphoma cells became sensitive when incubated with Gb3 liposomes, although liposome fusion itself was not assessed. However, we were unable to confirm these results in HeLa cells, using either micellar Gb3 or liposomes prepared as described by Waddell et al. We therefore optimized the conditions with palmitate and polyethylene glycol and monitored fusion by fluorescence microscopy using carboxyfluorescein incorporated into the liposomes, a dye that fluoresces only when diluted into the cytoplasm after fusion [19]. Under these conditions, insertion of Gb3 but not Gb4 in liposomes resulted in an increase in both Shiga toxin binding and toxicity in HeLa T5 cells.

We also used PDMP, a specific competitive inhibitor of UDP-glucose:ceramide glucosyltransferase [22], the first enzyme in the glycosylation pathway of globo-series glycosphingolipids such as Gb3 and Gb4, to reduce Gb3 synthesis. PDMP treatment of BALB/c 3T3 cells alters cell morphology, increases lactate production, and enhances sugar uptake [28]. In Lewis murine lung carcinoma cells, PDMP reversibly impairs laminin-mediated attachment and migration [29]; in B16 melanoma cells, expression of cell surface GM3 is reduced and the ability to bind adhesive proteins is lost [30]. In this study, incubation of toxin-sensitive Vero or HeLa 229 cells with PDMP reduced the content of Gb3 and its glycolipid precursors and resulted in high-level toxin resistance. However, using the optimized liposomal delivery system to add back Gb3 we could restore toxin binding and sensitivity, which demonstrates the essential role of Gb3 in the cellular response to Shiga toxin. Moreover, the ability to confer sensitivity to T5 cells or PDMP-treated Vero cells by fusion with Gb3 liposomes but not other glycolipids, such as lactosylceramide or Gb4, makes it unlikely that the resistance of these cells to the toxin is due to any mechanism other than that of receptor deficiency.

Distinctive results were obtained with CHO-K1 cells, which normally lack Gb3 and do not increase Gb3 content in the presence of DIG and thus can be considered to be intrinsically resistant to Shiga toxin. When liposomal Gb3 was introduced into these cells by the identical fusion protocol used for HeLa and Vero cells, there was no change in the sensitivity of CHO cells, even though increased Shiga toxin binding was readily demonstrated. This raises the possibility that CHO cells may lack either the mechanism for translocation of Gb3-bound toxin from the cell surface to the cytoplasm or a second protein or glycoprotein receptor needed to interact with the uptake mechanism and/or direct the complex to the trans-Golgi region of the cell [31] and beyond to the endoplasmic reticulum [32], where Shiga toxin exerts its biochemical action. The minimal toxicity observed in Gb3-deficient cells incubated with very high concentrations of Shiga toxin is probably due to non-receptor-based toxin uptake, as reported for receptor-negative diphtheria toxin-resistant mouse L cells [33].

It is also remotely possible that CHO cell ribosomes are
not susceptible to the action of Shiga toxin. Although we have not directly tested the effect of Shiga toxin on isolated CHO cell ribosomes, these cells are sensitive to ricin, which has enzymatic specificity identical to Shiga toxin but a different route of uptake [25]. Our working hypothesis is that CHO cells lack a necessary postbinding translocation mechanism for Gb3-bound toxin. This hypothesis explains the results of Boyd et al. [11], who found that some piglet tissues able to bind toxin in vivo are not damaged. The CHO cell with exogenously added Gb3 may provide the test system we need to investigate the nature of this mechanism.

Acknowledgments

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References


