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Globotriaosylceramide, Gb3, Is an Alternative Functional Receptor for Shiga-like Toxin 2e

GERALD T. KEUSCH,[1]* MARY JACEWICZ,[1] DAVID W. K. ACHESON,[1] ARTHUR DONOHUE-ROLFE,[1] ANNE V. KANE,[2] AND ROBERT H. MCCLUER[3]

The Division of Geographic Medicine and Infectious Diseases[1] and the Gastroenterology Research Center,[2] New England Medical Center, Boston, and the Eunice K. Shriver Center, Waltham,[3] Massachusetts

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We reexamined the binding specificity of the Shiga-like toxin variant associated with porcine edema disease, SLT2e, which is reported to be more cytotoxic for Vero cells than for HeLa cells, by using receptor-deficient cells and a liposomal insertion system for purified glycolipids. We found that SLT2e preferentially uses globotetraosylceramide as a receptor but can also cause cytotoxicity by using globotriaosylceramide, the SLT2 receptor. We conclude that the differential cytotoxicity of SLT2e on HeLa and Vero cells is a function of both the receptor preference of the toxin and the specific glycolipid content of the target cells being used.

Shiga toxin (STX), produced by *Shigella dysenteriae* type 1, is the prototype of a family of heterodimeric microbial toxins of similar structure, including the Shiga-like toxins (SLTs) of *Escherichia coli* associated with hemorrhagic colitis in humans and edema disease in pigs (1). STX and SLT1 are virtually identical, with just a single-amino-acid difference in their enzymatically active A subunits. They are only 55% homologous with SLT2 (8), with which they differ immunologically (11), but STX, SLT1, and SLT2 all bind to the same glycolipid receptor, globotriaosylceramide (Gb3) (1, 8). Variant toxins immunologically related to SLT2 have been isolated from animals (SLT2e) that bind to globotetraosylceramide (Gb4), a neutral glycolipid in which the trisaccharide of Gb3 is subterminal to an *N*-acetyl-D-galactosamine residue (3). This difference in binding is reported to explain the preferential cytotoxicity of the SLT2e for Vero cells over HeLa cells (7, 9); However, we have not been able to confirm these findings in our laboratory (2). In this study, we reexamined the specificity of SLT2e binding by using glycolipid-deficient cells containing purified glycolipids inserted by an optimized liposomal fusion method recently employed by us to document the specificity of STX and SLT1 for Gb3 (6).

SLT2e was purified in large scale from *E. coli* HB101 (pDLW5) containing the intact structural gene for SLT2e (provided by Alison O'Brien, Uniformed Services University of the Health Sciences, Bethesda, Md.) as previously described (2). Purified toxin was lyophilized and stored dry at −70°C until used. Toxin was iodinated by the chloramine T procedure as reported previously (4).

Cell lines, including STX- and SLT1-resistant HeLa lines, and media were identical to those used in our previous study (6). Toxin-resistant glycolipid-deficient HeLa T5 cells selected by passage in high-dose STX and HeLa and Vero cells made resistant by growth in the presence of 40 μM *d,1*-threo-1-phenyl-2-decanoylimino-3-morpholino-1-propanol HCl (PDMP) (Matreya, Inc., Pleasant Gap, Pa.), an inhibitor of UDP-glucose: ceramide glucosyltransferase (the first enzyme involved in the synthesis of neutral glycolipids [5]), were incubated with purified glycolipids in liposomes under optimal conditions for insertion into the cell membrane exactly as described in our prior study (6). Cytotoxicity of SLT2e, measured as [^{3}H]leucine incorporation into protein, binding of ^{125}I-labeled toxin, and glycolipid content were also determined as reported previously (6).

CHO cells contained little detectable Gb3 or Gb4, whereas the Vero and HeLa cells from our laboratory contained large amounts of both Gb3 and Gb4 (Table 1). ^{125}I-SLT2e bound poorly to glycolipid-deficient T5 and CHO cells, but binding increased significantly after fusion with Gb3 liposomes and increased to an even greater extent when Gb4 was inserted into the cells (Fig. 1). Cytotoxicity also increased significantly in T5 cells treated with Gb4 liposomes and, to our surprise, in cells treated with Gb3 liposomes (Fig. 2A). In contrast, no increase in cytotoxicity was detected in either Gb3- or Gb4-liposome-treated CHO cells (Fig. 3B), although toxin binding increased. These data suggest that CHO cells may be unable to translocate SLT2e bound to liposomally inserted Gb3 or Gb4 to the toxin's ribosomal target (1, 8).

PDMP-treated HeLa or Vero cells were glycolipid deficient (Table 2) and failed to bind SLT2e unless fused with Gb3- or Gb4-containing liposomes (Fig. 3A). PDMP-treated Vero cells also became sensitive to SLT2e after insertion of either Gb3 or, to a greater extent, Gb4 (Fig. 3B). To be certain that these results were not due to unusual experimental conditions, we compared the cytotoxicity due to SLT2e with that of SLT1, known to bind only Gb3 (6), by using PDMP-treated Vero cells fused with various ratios of Gb3- and Gb4-containing lipo-

TABLE 1. SLT2e cytotoxicity and neutral glycolipid content of cultured cell lines

Cell line	IC_{50} (μg/ml)[a]	Glycolipid content (pmol/mg of cell protein)[b]			
		GluCer	LacCer	Gb3	Gb4
HeLa 229	2×10^{-4}	432.7	76.5	1,136.8	552.8
HeLa CCL2	8×10^{0}	420.0	68.9	497.8	218.6
T5	NC[c]	318.2	101.3	90.2	19.5
Vero	3×10^{-5}	992.8	88.1	1,329.3	1,296.3
CHO-K1	NC[c]	1,516.0	1,288.6	ND[d]	24.8

[a] IC_{50}, 50% inhibitory dose of SLT2e (tissue culture).
[b] GluCer, glucocerebroside; LacCer, lactocerebroside.
[c] NC, IC_{50} value cannot be calculated.
[d] ND, none detected.

* Corresponding author. Phone: (617) 636-7001. Fax: (617) 636-5292.

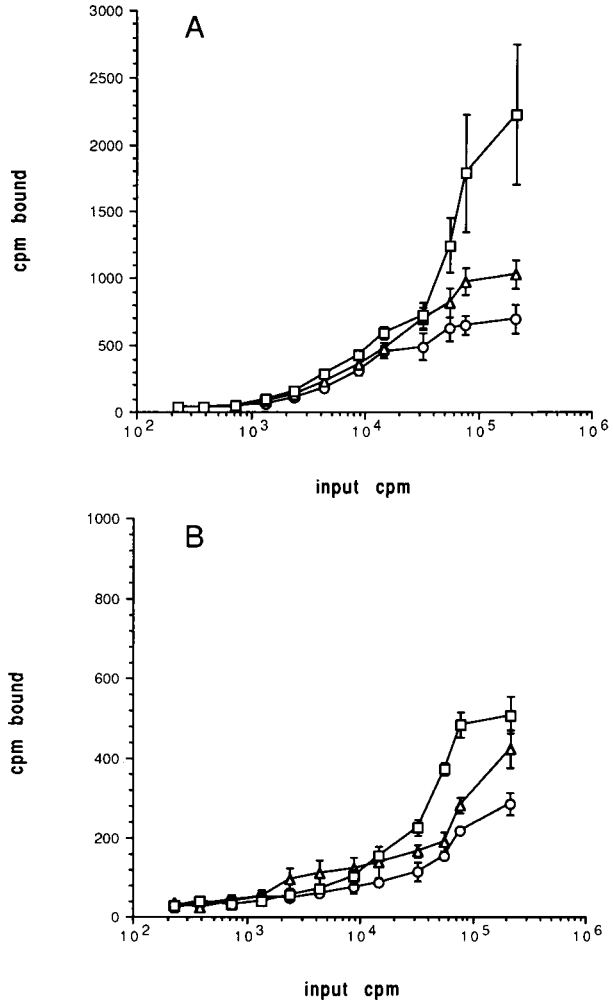

FIG. 1. Binding of ^{125}I-SLT2e to the STX-resistant T5 line of HeLa 229 cells (A) and CHO-K1 cells (B), shown by open circles. Binding of toxin to these cells after fusion with Gb3 liposomes is shown by open triangles, and binding to cells after fusion with Gb4 liposomes is shown by open squares. Conditions are as stated in Materials and Methods. Note the difference in the vertical scale between panels A and B. Error bars indicates 1 standard error.

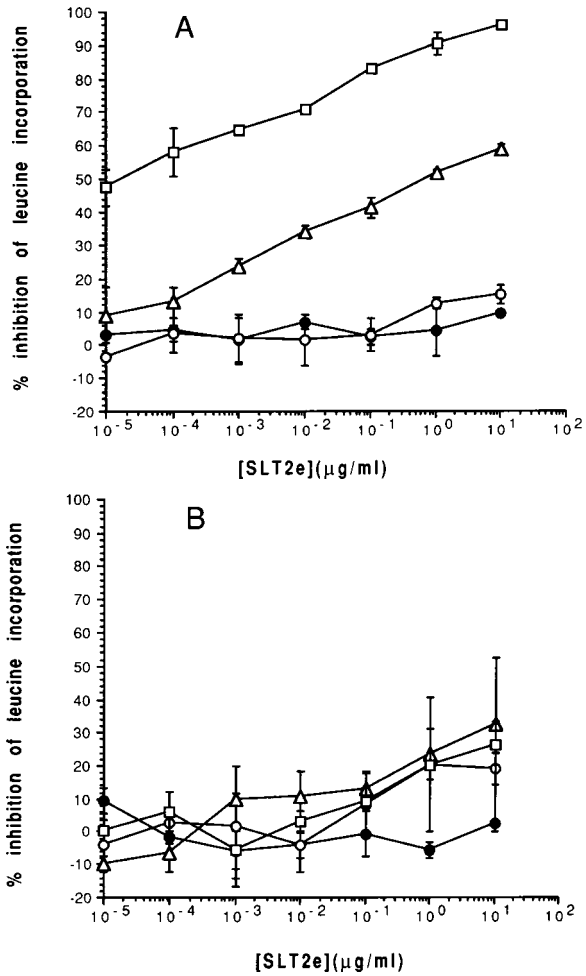

FIG. 2. Effect of SLT2e on leucine incorporation by HeLa T5 (A) and CHO-K1 (B) cells before and after preincubation with either Gb3-containing (open triangles) or Gb4-containing (open squares) liposomes. Two controls are shown in each panel, including cells grown in medium alone (solid circles) and cells treated with polyethylene glycol alone (open circles), exactly as described in the fusion protocol. Error bars indicate 1 standard error.

somes. Cytotoxicity due to SLT1 steadily diminished in direct proportion to the amount of Gb3-containing liposomes used, independent of the reciprocal increase in Gb4-containing liposomes (Fig. 4A). Moreover, insertion of Gb4 alone did not mediate cytotoxicity. In contrast, Gb3 could substitute for Gb4 in mediating the toxic effects of SLT2e (Fig. 4B), albeit not as efficiently as Gb4. High-performance liquid chromatography analysis of specific glycolipid content after insertion of Gb3 demonstrated that the results were not due to conversion of Gb3 to Gb4 during the course of the experiment (data not shown).

Thus, the present study shows that, in the absence of Gb4, SLT2e can use Gb3 as a functional receptor. Although we affinity purified the toxin for this study on immobilized hydatid cyst glycoproteins, which contain the P1 blood group-reactive analog of Gb3 (2), we do not believe that we have selected a population of SLT2e toxin molecules with Gb3 binding capacity. First, hydatid cyst fluid contains a mixture of glycoproteins and may well contain Gb4-like structures. Second, the SLT2e toxin used in these studies retained preferential binding to Gb4, rather than the reverse, and indeed bound to Gb4- but not to Gb3-coated microtiter plates (2).

In addition to the less-stringent receptor preference of SLT2e, our results demonstrate that the distribution of Gb3 and Gb4 on target cells is a determinant of the cytotoxic response to SLT2e. Although the content of Gb4 varied among the cell types in the same rank order as their sensitivity to SLT2e, if Gb4 is the sole receptor for this toxin our Vero cells should be much more sensitive to SLT2e than our HeLa 229 cells, because the former have more than twice the amount of Gb4 per milligram of cell protein. We did not observe this, however. The experiments in which different ratios of Gb3 and Gb4 were used to fuse the receptor glycolipid in the cell membrane are particularly compelling in supporting the role of Gb3 in mediating toxicity, albeit Gb4 appears to be more efficient than Gb3. Although we did not examine the impact of the carbon chain length of the ceramide moiety of the receptor glycolipid, which has been reported to influence the affinity but not the capacity of binding of SLT1 to target cells in solid-phase binding assays (10), the findings in this study demonstrate functional receptor activity of Gb3 for SLT2e within the natural environment of the cell membrane.

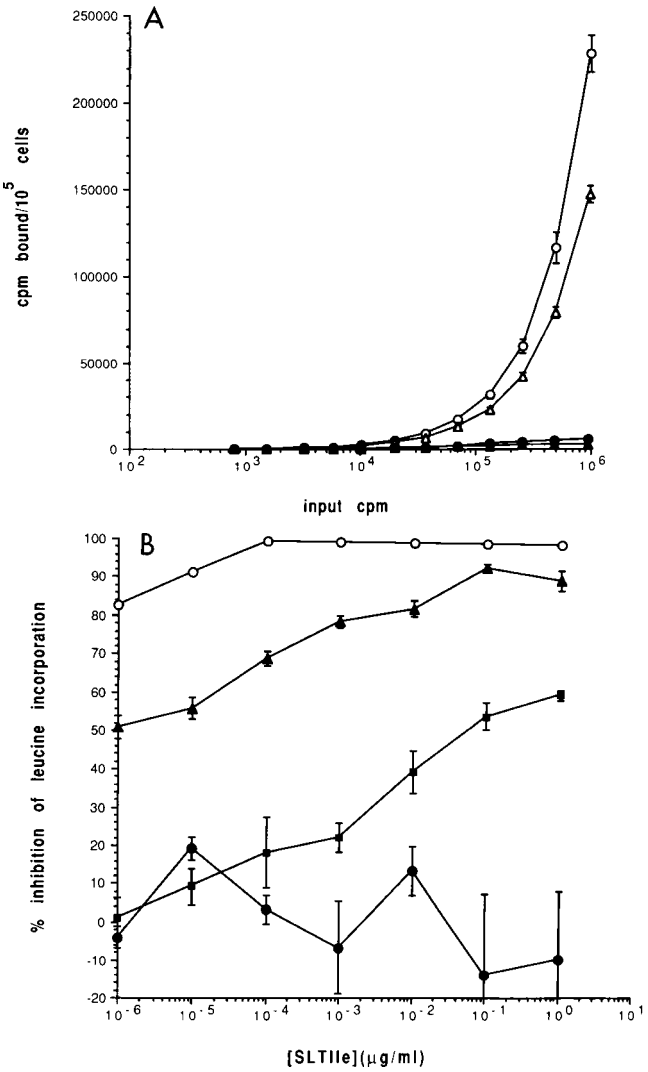

FIG. 3. (A) Binding of ^{125}I-SLT2e to sensitive HeLa 229 and Vero cells before (open circles and triangles, respectively) or after (solid circles and triangles, respectively) preincubation for 6 days with an inhibitor of glycolipid synthesis, PDMP. Conditions are as described in Materials and Methods. (B) Effect of SLT2e on leucine incorporation by Vero cells after preincubation for 6 days with PDMP to inhibit neutral glycolipid synthesis (solid circles) and after fusion of Gb3-containing (solid squares) or Gb4-containing (solid triangles) liposomes with PDMP-treated cells. The Vero cell controls (no PDMP exposure) are shown by open circles. Error bars indicate 1 standard error.

Thus, our data show that differential cytotoxicity for HeLa and Vero cells is not a good criterion for screening SLT2e and other variant toxins that may exhibit selective toxicity for Vero cells unless the glycolipid content of the cells used is known and there are large differences in the content of both Gb3 and Gb4.

TABLE 2. Effect of PDMP on glycolipid content in Vero and HeLa 229 cells

Cells	Glycolipid content (pmol/mg of cell protein)	
	Gb3	Gb4
HeLa 229	1,136.8 ± 118.4	552.8 ± 12.9
HeLa 229 + PDMP	107.0 ± 35.2	19.3 ± 7.6
Vero	1,329.3 ± 216.1	1,296.2 ± 305.1
Vero + PDMP	24.8 ± 15.7	287.1 ± 43.5

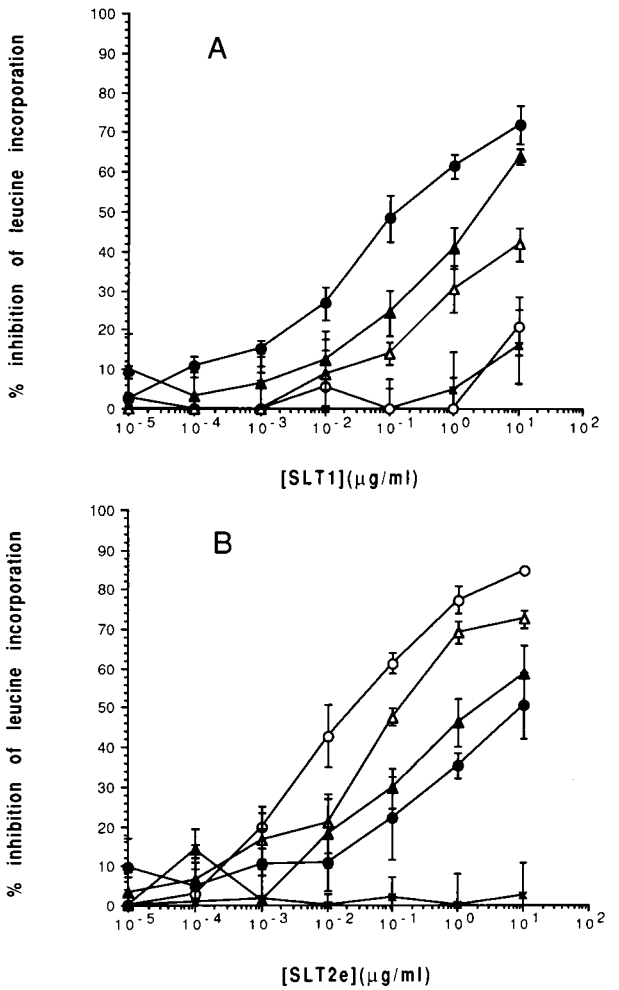

FIG. 4. Effect of fusion of different ratios of Gb3 and Gb4-containing liposomes on toxicity due to SLT1 (A) or SLT2e (B). The symbols indicate the following additions: x, none; open circles, all Gb4; open triangles, 2:3 Gb3/Gb4 ratio; solid triangles, 3:2 Gb3/Gb4 ratio; solid circles, all Gb3. Error bars indicate 1 standard error.

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