Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 524–528, January 1992
Biochemistry

Alteration of the carbohydrate binding specificity of verotoxins from Galα1-4Gal to GalNAcβ1-3Galα1-4Gal and vice versa by site-directed mutagenesis of the binding subunit

(glycosphingolipid binding/cytotoxicity/protein tertiary structure)

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Communicated by R. John Collier, September 30, 1991 (received for review June 6, 1991)

ABSTRACT **Verotoxin 1 (VT-1) and Shiga-like toxin II (SLT-II) bind to the glycosphingolipid (GSL), globotriaosylceramide (Gb_3), whereas pig edema disease toxin (VTE) binds to globotetraosylceramide (Gb_4) and to a lesser degree Gb_3. Amino acids important in the GSL binding specificity of VT-1 and VTE have been identified by site-directed mutagenesis. One mutation, Asp-18 → Asn, in VT-1 resulted in binding to Gb_4 in addition to Gb_3 in a manner similar to VTE. Several mutations in VTE resulted in the complete loss of GSL binding; however, one mutation resulted in a change in the GSL binding specificity of the VTE B subunit. The double mutation Gln-64 → Glu and Lys-66 → Gln (designated GT3) caused a selective loss of Gb_4 binding, effectively changing the binding phenotype from VTE to VT-1. Both wild-type VTE and GT3 were purified to homogeneity and binding kinetics *in vitro* were determined with purified GSLs from human kidney. The cell cytotoxicity spectrum of the mutant toxin was also found to be altered in comparison with VTE. These changes were consistent with the GSL content of the target cells.**

The verotoxin (or Shiga-like toxin, SLT) family is a group of subunit toxins that have been associated with disease in humans and animals. *Escherichia coli* strains producing verotoxin 1 (VT-1), SLT-II, and VT-2 are associated with diarrhea, hemorrhagic colitis (1), and the hemolytic uremic syndrome in humans (2), while pig edema disease toxin (VTE or SLT-IIv) has been associated with edema disease of swine (3). Pathologically, these diseases are characterized by microvascular thrombosis in various organs. Similar lesions have been produced by parenteral injection of purified toxin in rabbits or crude toxin preparations in pigs (4, 5). The toxin family can be divided into two main groups, the first of which consists of Shiga toxin and VT-1. These are fully cross-neutralizable and differ only in a single conservative amino acid substitution in the A subunit (6, 7). The second group includes SLT-II, VT-2, and VTE which are partially cross-neutralizable but are not neutralized at all by antiserum raised against Shiga toxin (3). The amino acid sequence of the B subunit of VT-1 is identical to that of Shiga toxin and is 62% identical to that of SLT-II and VT-2 (8). VT-1, VT-2, and SLT-II have been shown to bind specifically to globotriaosylceramide (Gb_3; Galα1-4Galβ1-4GlcCer, in which Cer is ceramide) (9, 10). Although the B subunit of SLT-II is 84% identical to that of VTE (11, 12), the latter was found to bind primarily to globotetraosylceramide (Gb_4; GalNAcβ1-3Galα1-4Galβ1-4GlcCer) (13, 14) and to a lesser extent to Gb_3 (13, 14). Because of the homology between the B subunits, we were able to target a limited number of codons in the B cistrons of VTE and VT-1 for site-directed mutagenesis. The aim of these experiments was to investigate the role of the amino acid substitutions in determining carbohydrate binding specificity. This study describes the mutations performed and focuses on one VTE mutant in which the glycosphingolipid (GSL) binding specificity was changed from Gb_4 to Gb_3.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* JM101 F′ *traD36, lacZ*ΔM15, *proA*$^+$, *proB*$^+$, *lacI*^{q}/*lac, pro, supE, thi* (ref. 15, p. A.10) was used as the host for plasmid DNA preparation and as a source of periplasmic extracts for crude toxin preparations. The nonsuppressor *E. coli* strain W3350 (*sup*^{0}) (16) was used for expression of the truncation mutant GT19, which contains an amber termination codon.

The VTE operon was isolated on a 2.4-kilobase (kb) fragment by *Sal* I cleavage of plasmid pCG6 (12) and was cloned in the *Sal* I site of pTZ18-R. A plasmid carrying the insert with the A subunit cistron proximal to the *Eco*RI site of the polylinker of pTZ18-R was designated pGT100.

The VT-1 B subunit cistron was recovered from pJLB34 (17) as a 1.1-kb *Pst* I fragment and was cloned in the *Pst* I site of the multiple cloning site of pTZ18-R. A plasmid with the B coding sequence oriented such that the start codon was proximal to the T7 promoter was identified by restriction endonuclease analysis and was designated pJLB118.

Enzymes, Biochemicals, and Radionuclides. Restriction enzymes, DNA polymerase I (Klenow fragment), calf intestinal alkaline phosphatase, polynucleotide kinase, T4 DNA ligase, and dATP, dGTP, dCTP, and dTTP were purchased from Boehringer Mannheim. Oligonucleotides used in mutagenesis were purchased from Allelix, Mississauga, ON and The Hospital for Sick Children Biotechnology Center, Toronto.

DNA Manipulations. Plasmid DNA was prepared by the method of Birnboim and Doly (18). DNA fragments were purified from agarose by using the Gene Clean Kit (Bio 101, La Jolla, CA).

For mutagenesis, single-stranded DNA template containing uracil was prepared from *E. coli* RZ1032 HfrKL16 *dut-1, ung-1, thi-1, relA1, lysA, zbd-297*::Tn*10, supE44* (19) containing pGT100 or pJLB118. The organisms were grown in 2X YT broth (ref. 15, p. A.3) and were superinfected with the bacteriophage M13K07 (ref. 15, p. 4.48). Carbenicillin (Cb) was added at 150 μg/ml and kanamycin (Km) at 75 μg/ml as necessary. After overnight growth, phage were precipitated with polyethylene glycol and single-stranded DNA was pu-

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Abbreviations: VT-1, verotoxin 1; SLT-II, Shiga-like toxin II; VT-2, verotoxin 2; VTE, pig edema disease toxin; GSL, glycosphingolipid; Gb_3, globotriaosylceramide; Gb_4, globotetraosylceramide; LacCer, lactosylceramide.

rified as previously described (ref. 15, pp. 4.29). Mutagenesis was performed as described by Kunkel (19). Mutants were identified by DNA sequencing, which was performed by the dideoxy chain-termination method of Sanger, using the Sequenase 2 modified T7 polymerase (United States Biochemical) (20).

Maxicell Analysis and Immunoprecipitation of VTE and Mutant VTEs. Plasmid-encoded proteins were analyzed by using the maxicell method of Sancar *et al.* (21). *E. coli* DR1984 *recA1, uvrC34* (obtained from A. Bognar, University of Toronto) was transformed with plasmids containing wild-type and mutant VTE genes. Bacteria were grown and irradiated with UV, and plasmid-encoded proteins were labeled with [^{35}S]methionine (21).

Bacterial cells were lysed in RIPA lysis buffer (10 mM Tris·HCl, pH 8.0/100 mM NaCl/1 mM EDTA/1% Nonidet P-40/0.1% SDS) containing 1 mM phenylmethanesulfonylfluoride. Labeled proteins were immunoprecipitated with absorbed polyclonal porcine VTE antiserum (gift of Carlton Gyles, University of Guelph) and protein A-Sepharose CL-4B (Sigma) by using the procedure of Greer *et al.* (22). The labeled proteins were then separated by electrophoresis in a 16.5% polyacrylamide gel using the *N*-[tris(hydroxymethyl)methyl]glycine (Tricine)/SDS buffer system as previously described (17, 23). The position of A and B subunits of VTE on the gels was determined by coelectrophoresing either purified VTE holotoxin or VT-1 B subunit with the labeled sample.

Extraction of Toxin and GSL Binding Assay Using TLC. VT-1 B subunit, mutant VT-1 B subunit, VTE, and mutant VTE were extracted from the periplasmic space by using polymyxin B as previously described (17). GSLs Gb_3, Gb_4, and lactosylceramide (LacCer) were purified from human kidney as previously described (24) and were used for TLC overlay assays as described by Head *et al.* (25, 26). Polyclonal porcine VTE-specific antiserum was used to detect bound VTE and mutant VTEs, while monoclonal antibody 13C4 (27) was used to detect bound VT-1 B subunit and VT-1 B subunit mutants. The blots were visualized by using peroxidase-conjugated anti-mouse IgG (for monoclonal antibody 13C4) or anti-porcine IgG (both obtained from Sigma) followed by development with 4-chloro-1-naphthol peroxidase substrate (Bio-Rad).

Purification of VTE and Mutant Toxin GT3. VTE was purified from JM101 (pGT100) and mutant toxin was from JM101 (pGT3). For both toxins, the initial purification consisted of concentration followed by anion-exchange chromatography on Q Sepharose (Pharmacia) at pH 8.0 in 50 mM Tris·HCl. Both toxins appeared in the flow-through fractions. This was followed by cation-exchange chromatography on S Sepharose in 10 mM phosphate buffer, pH 6.8, eluting with a 0–0.5 M NaCl gradient. After this step, VTE was purified by chromatofocusing using Polybuffer Exchanger 118 (Pharmacia). GT3 was further purified by using an immunoaffinity column made with monoclonal antibody BC5:BB12 (28). Protein was determined by using the BCA (bicinchoninic acid) protein assay. Purified VTE and GT3 displayed A and A1 subunit bands and a single B subunit band on SDS/PAGE (data not shown).

VTE and GT3 were radiolabeled with Iodogen (Pierce) (29). Forty micrograms of toxin and 0.5 mCi (1 Ci = 37 GBq) of [^{125}I]iodide (New England Nuclear) was added to a tube coated with 40 μg of Iodogen. This was allowed to react for 1 min, and the unreacted iodide was removed by gel filtration (Sephadex G-25, Pharmacia).

GSL Binding Assay Using Microtiter Plates. To measure the affinities of VTE and GT3 for Gb_3 and Gb_4, a direct binding assay was performed in microtiter wells as previously described (26). Each well was coated with 100 ng of either Gb_3 or Gb_4 mixed with phosphatidylcholine (500 ng) and cholesterol (250 ng) per well. Binding of increasing levels of ^{125}I-labeled VTE or ^{125}I-labeled GT3 at 25°C was analyzed by the LIGAND program for Scatchard analysis (BioSoft, Milltown, NJ).

GSL Analysis. Vero, MRC-5, and HEp-2 cells were grown to confluency and trypsinized. Lipids were extracted and Gb_3 and Gb_4 were quantitated by HPLC after benzoylation as described (30).

Cytotoxicity. Cell lines (Vero, MRC-5, or HEp-2) were grown at 37°C to 80% confluency and pure VTE or GT3 was added in triplicate at concentrations from 10 μg to 100 fg per ml. After four days the cells were fixed, stained with Giemsa stain, and scanned at 470 nm as described (31) and compared with cells from control wells.

RESULTS

Mutagenesis of VT-1 and VTE B Subunits. The goal of the mutagenesis experiments was to exchange the GSL binding specificity of the VT-1 and VTE B subunits. Some of the amino acids conserved in SLT-II and VT-1 yet distinct in the VTE B subunit (Fig. 1) must be responsible for the different binding specificities of these toxins. We therefore targeted the corresponding codons for substitution, using oligonucleotide-directed site-specific mutagenesis. We initially selected amino acids in the VT-1 B subunit sequence for mutagenesis. Plasmid pJLB118, which contains the VT-1 B subunit, was used to change targeted codons to those found in the VTE B subunit sequence. Crude VT-1 B subunit, prepared as periplasmic extracts of *E. coli* containing the wild-type and mutant VT-1 B subunit plasmids, was then examined for binding to Gb_3 and Gb_4 in the TLC overlay assay. In the case of VT-1 B subunit, five mutations were made. All mutants containing the change Asp-18 → Asn (KR7, KR9, KR10) resulted in binding to Gb_4 as well as Gb_3, effectively mimicking the VTE phenotype (Table 1, Fig. 2 *Upper*).

Mutations were produced in the VTE B cistron sequence which would alter single or various combinations of codons to those found in the SLT-II B cistron. Mutations were confirmed by nucleotide sequence analysis of the B cistron. The amino acids targeted for mutation are shown in Fig. 1. Toxins from periplasmic extract were prepared and examined in the TLC binding assay. Six mutant toxins showed no alteration of GSL binding compared with wild-type VTE

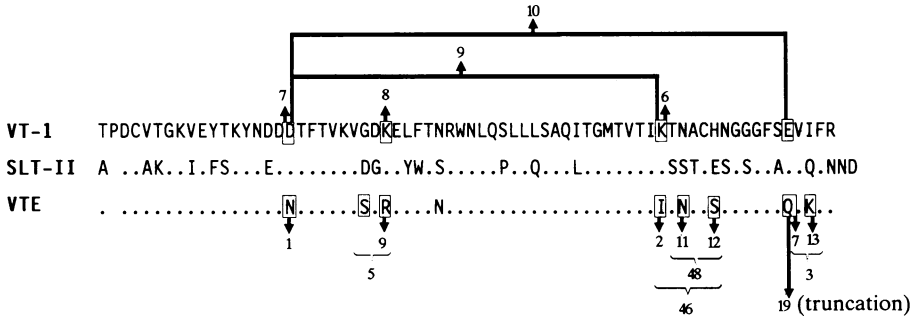

FIG. 1. The complete amino acid sequence of the VT-1 B subunit is shown. The amino acids that are different in SLT-II are shown below this sequence, and the amino acids that differ in VTE are shown below the SLT-II sequence. Amino acids selected for mutagenesis are boxed. Mutations are numbered above the VT-1B subunit sequence and below the VTE subunit sequence. GT19 changes a glutamine codon to an amber stop codon, truncating the B subunit at amino acid 63.

Table 1. VT-1 and VTE B subunit mutations: Binding phenotype on TLC

No binding change		Binding change		No binding	
		VT-1 B subunit mutations			
KR6	K53 → I	KR7	D18 → N		
		KR9	D18 → N + K53 → I		
KR8	K27 → R				
		KR10	D18 → N + E65 → Q		
		VTE B subunit mutations			
GT1	N17 → D	GT48	N54 → S + S57 → E	GT5	S24 → E + R26 → K
GT9	R26 → K				
GT11	N54 → S	GT3	Q64 → E + K66 → Q	GT46	I52 → K + N54 → S + S57 → E
GT12	S57 → E				
GT7	Q64 → E				
GT13	K66 → Q			GT2	I52 → K
				GT19	Q64 → amber; truncation at residue 63

Substitutions constructed in the VT-1 and VTE B subunits are grouped according to their binding characteristics in the TLC assay. The designations used in the text are also listed.

(Table 1). One other mutant, designated GT48, showed reduced binding to Gb_4 and no binding to Gb_3 (Fig. 2 *Lower*). For three other mutants, there was no detectable binding of toxin to Gb_3 or Gb_4 (GT2 and GT5 binding shown in Fig. 2 *Lower*; GT46 data not shown).

The most significant mutant was designated GT3 (Gln-64 → Glu and Lys-66 → Gln). It was found to have markedly reduced binding to Gb_4 while maintaining binding to Gb_3 (Fig. 2 *Lower*). It is significant that when the mutations Gln-64 → Glu and Lys-66 → Gln were performed separately (mutants GT7 and GT13, Table 1) there was no significant change in GSL binding (data not shown). Because this mutation in the carboxyl-terminal portion of the molecule had such a profound effect on binding specificity, we examined the effect of removing the five carboxyl-terminal amino acids. This was done by placing a TGA stop codon corresponding to amino acid 64 of the mature B subunit. The mutant plasmid, designated pGT19, and the wild-type plasmid were used to transform the nonsuppressor host *E. coli* W3350. We did not detect any GSL binding for this truncation mutant.

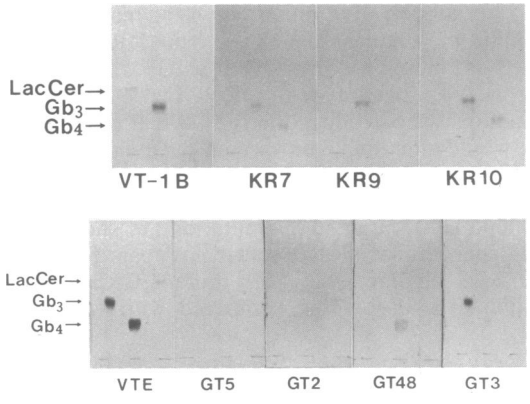

FIG. 2. TLC binding assays of VT-1 B subunit and VT-1 B subunit mutants (*Upper*) and VTE and VTE mutants (*Lower*) to Gb_3, Gb_4, and LacCer. Each blot contains three lanes. The VT-1 B subunit blots contain from left to right LacCer, Gb_3, and Gb_4, 2 μg each. The VTE blots contain from left to right Gb_3, Gb_4, and LacCer also at 2 μg each. The binding of toxin present in the LacCer lanes is due to the presence of galabiosylceramide. The mutations corresponding to the blots are noted below each chromatogram. The ligands are VT-1 B, KR7, KR9, and KR10 for VT-1 and VTE, GT5, GT2, GT48, and GT3 for VTE.

Maxicell Expression and Immunoprecipitation. GT2, GT46, and the truncation mutant GT19, all of which appeared not to bind to GSLs, were examined by immunoprecipitation with polyclonal VTE antiserum. The wild-type control yielded two polypeptides, 32 and 7 kDa, corresponding to the A and B subunits, respectively (data not shown). In contrast, the truncation mutant GT19 yielded markedly reduced amounts of 7-kDa B subunit. In addition to the 32-kDa A subunit band, multiple bands with lower molecular mass were also seen (data not shown). This was also seen with mutant GT46 (data not shown). In the case of mutant GT2, the same multiple bands below 32 kDa were observed; however, the amount of B subunit precipitated was similar to that of wild type (data not shown). Negative controls included immunoprecipitation with preimmune antiserum and immunoprecipitation of extracts of maxicells containing the vector pTZ18-R, which yielded no bands (data not shown).

Determination of Affinity Constants for VTE and GT3. The saturation binding curves for binding of purified VTE and GT3 to Gb_3- and Gb_4-coated microtiter plate wells are shown in Fig. 3. They confirm that compared with VTE, GT3 binding to Gb_4 is dramatically reduced. Scatchard analysis of the data showed that for Gb_4, VTE bound via a single site with $K_d = 1.48 \times 10^{-8}$ M and $B_{max} = 8.61 \times 10^{-9}$ M. GT3 fit a two-site model with a high-affinity site of $K_d = 1.44 \times 10^{-8}$ M ($B_{max} = 4.25 \times 10^{-10}$ M) and a low-affinity site of $K_{d2} = 1.00 \times 10^{-6}$ M ($B_{max} = 9.82 \times 10^{-9}$ M).

The Scatchard plots for Gb_3 were essentially opposite to those seen for Gb_4. VTE best fit a two-site model with dissociation constants of $K_{d1} = 3.11 \times 10^{-9}$ M and $K_{d2} = 1.36 \times 10^{-7}$ M. GT3, however, fit a single-site model with a dissociation constant of 1.82×10^{-8} M. The B_{max1} for VTE binding to Gb_3 was 6.92×10^{-10} M and B_{max2} was 5.21×10^{-9} M. The B_{max} for GT3 binding to Gb_3 was 2.39×10^{-9} M. Thus there was a significantly higher number of high-affinity Gb_3 binding sites for GT3.

Cell Cytotoxicity and GSL Content. The Gb_3 and Gb_4 contents of Vero, MRC-5, and HEp-2 cells are shown in Table 2. Comparative cytotoxicity assays were performed and compared with cell GSL content. Vero cells contain 1.6-fold more Gb_4 than Gb_3 and were approximately 100-fold more susceptible to VTE than GT3 (Fig. 4). MRC-5 cells contain 5.5-fold more Gb_3 than Gb_4. GT3 showed a greater cytotoxicity on these cells than did VTE. HEp-2 cells contained Gb_3 without detectable Gb_4. These cells were highly resistant to VTE but markedly sensitive to GT3 (Fig. 4).

DISCUSSION

In our studies of VT-1, changing Asp-18 to Asn (KR7) resulted in binding of the mutant to Gb_4 as well as Gb_3. This effectively changes the GSL binding phenotype to that of VTE and provides what is to our knowledge the first demonstration of a change in carbohydrate binding specificity mediated by a single amino acid substitution. Interestingly, the reciprocal change in VTE Asn-17 → Asp (GT1) did not decrease binding to Gb_4, indicating that additional long-range factors are involved in defining binding specificity. This region of the B subunit has been previously demonstrated to be important in receptor binding, since mutation of Asp-16 and Asp-17 to His abolished binding in a Gb_3 analogue ELISA (32). Jackson *et al.* (32) also changed Asp-18 to Asn in the VT-1 holotoxin (the same mutation as KR7 B subunit) but did not examine Gb_4 binding. The biological significance of this mutation has yet to be determined.

Substitutions of SLT-II residues for those of VTE were made with the aim of changing the binding specificity of VTE to that of SLT-II (from Gb_4 to Gb_3). No single mutation

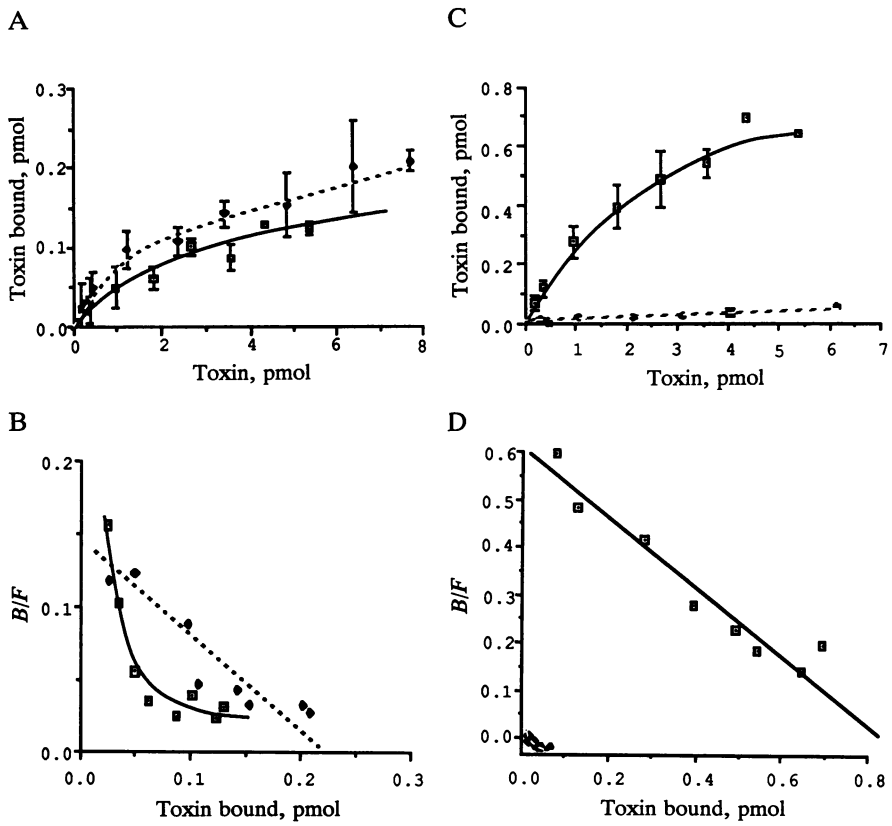

FIG. 3. Binding of ^{125}I-labeled VTE and ^{125}I-labeled GT3 to GSLs in the microtiter assay. (*A*) Curves for binding of radioiodinated VTE (solid line) and GT3 (broken line) to Gb_3. (*C*) Curves for binding of radioiodinated VTE (solid line) and GT3 (broken line) to Gb_4. (*B* and *D*) Scatchard plots for binding of wild-type (solid line) and mutant (broken line) toxin to Gb_3 and Gb_4, respectively. *B/F*, bound/free toxin. Error bars indicate ± one standard deviation.

altered binding specificity. One peptide with a double mutation (GT5), one with a triple mutation (GT46), and one with a single mutation (GT2) did not bind to Gb_3 or Gb_4. Immunoprecipitation of polypeptides produced in maxicells with VTE-specific polyclonal antiserum was examined for pGT2 and pGT46. In addition to the 32-kDa A subunit, both produced multiple bands that migrated faster. Since these species were never seen in the negative controls or the wild type, they likely represent proteolytic degradation products of the A subunit, which might result from lack of association with the B subunit due to marked conformational changes in the latter. Several mutants also showed markedly reduced amounts of B subunit (GT46 and GT19). Collectively, the results suggest that lack of binding in these mutants reflects major conformational changes rather than alteration of the amino acids directly involved in GSL binding.

Mutant GT3, which carries the double mutation Gln-64 → Glu and Lys-66 → Gln, is the most interesting, since it exhibited a marked and selective reduction in Gb_4 binding. VTE binding to Gb_4 gave a single high-affinity site. In contrast, analysis of the GT3 binding data showed biphasic kinetics. The minimal residual binding of GT3 to Gb_4 involved a 10-fold reduction in the number of high-affinity sites. The Gb_3 binding data for GT3 best fit a one-site model. In contrast, the VTE data fit a two-site model with a small number of high-affinity sites and a large number of lower-affinity sites.

Table 2. GSL contents of cell lines

Cell line	GSL, nmol per 10^6 cells	
	Gb_3	Gb_4
Vero	0.272	0.491
MRC-5	1.103	0.179
HEp-2	0.388	0.000

Whole cell lipids were purified and subjected to HPLC. Standards were used to identify the locations of Gb_3 and Gb_4.

It is entirely possible that the small number of high-affinity sites calculated for VTE binding to Gb_3 and for GT3 binding to Gb_4 represent trace contamination of the Gb_3 with Gb_4 and vice versa. Thus the GT3 mutation would essentially have resulted in a large reduction in Gb_4 binding affinity from $K_d = 1.48 \times 10^{-8}$ M to $K_d = 1.00 \times 10^{-6}$ M and an increase in Gb_3 binding affinity from $K_d = 1.36 \times 10^{-7}$ M to $K_d = 1.82 \times 10^{-8}$ M.

The results obtained with mutant GT3 permit some speculation regarding the nature of the GSL binding site on the VTE B subunit. Presumably, the terminal β1-3GalNAc residue of Gb_4 stabilizes the interaction of this GSL with VTE and this interaction is compromised in GT3. Thus, Gln-64 and Lys-66 may be important in forming the part of the binding pocket that interacts with this terminal residue of Gb_4. The crystal structure of the VT-1 B subunit has now been determined (P. Stein and R. J. Read, personal communication). There is significant homology between the VT-1 and VTE B subunits; therefore the structure is expected to be similar. The residues in the VT-1 B subunit that correspond to Gln-64 and Lys-66 are exposed on the surface and are located at the edge of a putative binding cleft. The carboxyl-terminal region also appears to have an important structural role (P. Stein and R. J. Read, personal communication). This may explain the low levels of immunoprecipitable B subunit found in the GT19 carboxyl-terminal truncation mutant.

To investigate the role of GSL binding in the mechanism of toxin action, VTE and GT3 cytotoxicity were compared in Vero, MRC-5, and HEp-2 cell lines. GT3 was less cytotoxic than VTE on Vero cells, an observation that correlated with the higher level of Gb_4 compared to Gb_3 in these cells (Table 2). The cytotoxicity of the two toxins on MRC-5 cells, which have higher levels of Gb_3 than Gb_4, was similar, representing a reversed trend compared to Vero cells. HEp-2 cells were found to contain no detectable Gb_4 and were essentially insensitive to VTE (Table 2) but were sensitive to GT3. The increased sensitivity of MRC-5 and HEp-2 cells to GT3 compared to Vero cells may be related to the increased

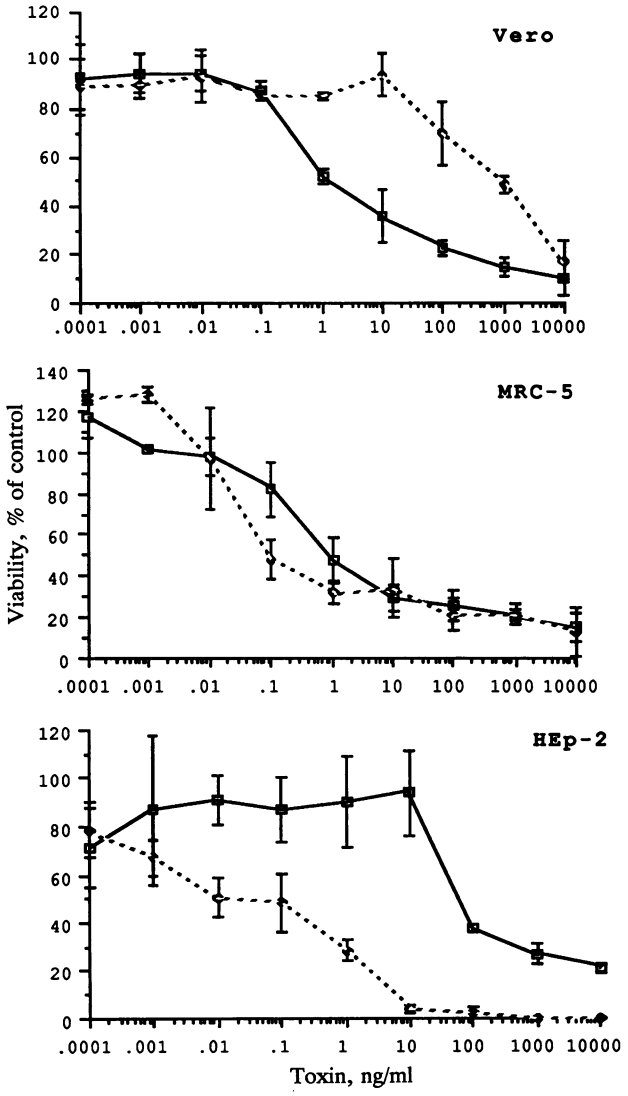

FIG. 4. Cytotoxicity of VTE and GT3 to Vero, MRC-5, and HEp-2 cells. VTE and GT3 were used in 10-fold dilutions ranging from 10 μg to 100 fg of toxin. Cells were exposed to toxin for 4 days and then fixed with methanol and stained with Giemsa stain. Cytotoxicity was measured as number of viable cells relative to control cells not exposed to any toxin. The solid line represents VTE and the broken line represents GT3. All assays were performed in triplicate and error bars indicate ± one standard deviation.

number of high-affinity interactions with Gb_3 for this toxin. Our previous studies have suggested that given equal receptor numbers, affinity of GSL binding is of prime importance in determining cytotoxicity (26). However, the relative importance of receptor affinity and number in determining toxin internalization into cells is not yet clear. The mutant toxins may prove useful tools to analyze this problem.

Our studies demonstrate a change in protein carbohydrate binding specificity following site-directed mutagenesis. This resulted in a corresponding change of the cytotoxic specificity on several cell lines, indicating that the binding changes detected *in vitro* may have biological significance. Preliminary experiments have now shown that the distribution of GT3 mutant toxin to various organs after intravenous injection in pigs differs significantly from that of wild-type VTE. This is accompanied by differences in the clinical characteristics of toxin-induced disease; however, the basic histologic lesion remains unchanged (B.B., G.J.T., and C. L. Gyles, unpublished results). Clearly this mutant will be useful in studying the pathogenesis of toxin-induced disease.

We gratefully acknowledge James Samuel for kindly providing us with purified VTE. This work was supported by Program Grant 11123 from the Medical Research Council of Canada. G.J.T. and K.R. are recipients of fellowships from the Ontario Ministry of Health.

1. Riley, L. W., Remis, R. S., Helgerson, S. D., McGee, H. B., Wells, J. G., Davis, B. R., Hebert, R. J., Olcott, E. S., Johnson, L. M., Hargett, N. T., Blake, P. A. & Cohen, M. L. (1983) *N. Engl. J. Med.* **308,** 681–685.
2. Karmali, M., Petric, M., Lim, C., Fleming, R., Arbus, G. & Lio, H. (1985) *J. Infect. Dis.* **151,** 775–782.
3. Marques, L. R. M., Peiris, J. S. M., Cryz, S. J. & O'Brien, A. (1987) *FEMS Microbiol. Lett.* **44,** 33–38.
4. Gannon, V., Gyles, C. & Wilcock, B. (1989) *Can. J. Vet. Res.* **53,** 306–312.
5. Pai, C. H., Kelly, J. K. & Meyers, G. L. (1986) *Infect. Immun.* **51,** 16–23.
6. DeGrandis, S., Ginsberg, J., Toone, M., Climie, S., Friesen, J. & Brunton, J. (1987) *J. Bacteriol.* **169,** 4313–4319.
7. Strockbine, N., Marques, L., Newland, J., Smith, H., Holmes, R. & O'Brien, A. (1986) *Infect. Immun.* **53,** 135–140.
8. Jackson, M., Neill, R., O'Brien, A., Holmes, R. & Newland, J. (1987) *FEMS Lett.* **44,** 109–114.
9. Lingwood, C. A., Law, H., Richardson, S., Petric, M., Brunton, J. L., DeGrandis, S. & Karmali, M. (1987) *J. Biol. Chem.* **262,** 8834–8839.
10. Waddell, T., Head, S., Petric, M., Cohen, A. & Lingwood, C. A. (1988) *Biochem. Biophys. Res. Commun.* **152,** 674–679.
11. Weinstein, D., Jackson, M., Samuel, J., Holmes, R. & O'Brien, A. (1988) *J. Bacteriol.* **170,** 4223–4230.
12. Gyles, C., DeGrandis, S., MacKenzie, C. & Brunton, J. (1988) *Microb. Pathog.* **5,** 419–426.
13. DeGrandis, S., Law, H., Brunton, J., Gyles, C. & Lingwood, C. (1989) *J. Biol. Chem.* **264,** 12520–12525.
14. Samuel, J. E., Perera, L. P., Ward, S., O'Brien, A. D., Ginsburg, V. & Krivan, H. C. (1990) *Infect. Immun.* **58,** 611–618.
15. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), 2nd Ed.
16. Campbell, A. (1961) *Virology* **14,** 22–32.
17. Ramotar, K., Boyd, B., Tyrrell, G. J., Gariepy, J., Lingwood, C. A. & Brunton, J. L. (1990) *Biochem. J.* **272,** 805–811.
18. Birnboim, H. & Doly, J. (1979) *Nucleic Acids Res.* **7,** 1513–1523.
19. Kunkel, T. A. (1987) in *Current Protocols in Molecular Biology*, eds. Ausubel, F., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (Wiley, New York), 8.1.1–8.1.6.
20. Sanger, F., Nicklen, S. & Coulson, A. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
21. Sancar, A., Wharton, R. P., Seltzer, S., Kacinski, B. M., Clark, N. D. & Rupp, W. D. (1981) *J. Mol. Biol.* **148,** 45–62.
22. Greer, P. A., Meckling-Hansen, K. & Pawson, T. (1988) *Mol. Cell. Biol.* **8,** 578–587.
23. Schagger, H. & Von Jagow, G. (1987) *Anal. Biochem.* **166,** 368–379.
24. Boyd, B. & Lingwood, C. A. (1989) *Nephron* **51,** 207–210.
25. Head, S., Ramotar, K. & Lingwood, C. A. (1990) *Infect. Immun.* **58,** 1532–1537.
26. Head, S. C., Karmali, M. A. & Lingwood, C. A. (1991) *J. Biol. Chem.* **266,** 3617–3621.
27. Strockbine, N., Marques, L., Holmes, R. & O'Brien, A. (1985) *Infect. Immun.* **50,** 695–700.
28. Downes, F. P., Barrett, T. J., Green, J. H., Aloisio, C. H., Spika, J. S., Strockbine, N. A. & Wachsmuth, I. K. (1988) *Infect. Immun.* **56,** 1926–1933.
29. Fraker, P. J. & Speck, J. C., Jr. (1978) *Biochem. Biophys. Res. Commun.* **80,** 849–857.
30. Strasberg, P., Grey, A., Warren, I. & Skomoroski, M.-A. (1989) *J. Lipid Res.* **30,** 121–127.
31. Gentry, M. K. & Dalrymple, J. L. (1980) *J. Clin. Microbiol.* **12,** 361–366.
32. Jackson, M. P., Wadolkowski, E. A., Weinstein, D. L., Holmes, R. K. & O'Brien, A. D. (1990) *J. Bacteriol.* **172,** 653–658.