Modification of the Glycolipid-Binding Specificity of Vero Cytotoxin by Polymyxin B and Other Cyclic Amphipathic Peptides

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Polymyxin B, an amphipathic cyclic decapeptide produced by Bacillus polymyxa, is routinely used in the extraction of the components from the periplasmic space of gram-negative bacteria. Vero cytotoxin 1 (VT1) is an Escherichia coli-elaborated subunit toxin which binds to the glycolipid globotriosyleceramide (Galα1-4Gal β1-4Glc-ceramide [Gb3]) and has been strongly implicated in the etiology of the hemolytic uremic syndrome and hemorrhagic colitis. We now show by in vitro glycolipid-binding assays that in the presence of low concentrations of polymyxin B, globotetraoyleceramide (GalαNacβ1-3Galα1-4Galβ1-4Glc-ceramide [Gb4]) is also recognized by both the VT1 B (binding) subunit and holotoxin. Melittin, a 26-amino-acid cyclic peptide of similar amphipathic nature, produced the same effect, whereas a hydrophobic blocking agent did not. Triton X-100 did not increase binding of VT1 to Gb3 but prevented glycolipid binding in toto at concentrations above 0.5%. Caution is therefore advised in the analysis of VT1 glycolipid binding in the presence of amphipathic peptides.

Vero cytotoxin (VT) (or Shiga-like toxin)-producing Escherichia coli have been strongly implicated in the etiology of hemorrhagic colitis (25) and the hemolytic uremic syndrome (16). These toxins are subunit proteins comprising an A subunit which inactivates the 28S rRNA of susceptible cells and a pentamer of smaller B subunits which bind to a cell surface receptor to facilitate entry of the cytotoxic subunit into cells. The B subunit recognizes the glycolipid globotetraoyleceramide (Gb4), and binding is specific for glycosphingolipids containing a terminal Galα1-4Gal residue (20). Several variants of these toxins have been described, most notably VT1 (18), VT2 (17, 26), and Shiga-like toxin II (27) (VT2 and Shiga-like toxin II are not equivalent [10]). All these toxins, including Shiga toxin itself (12), share the same glycolipid-binding specificity (7). Recently a further variant, isolated from pigs with edema disease (edema disease toxin VTE or Shiga-like toxin IV), was described which although highly homologous with other members of the VT family, particularly VT2, bound to an additional glycolipid, globotetraoyleceramide (Gb5), the next homolog in the globo series of neutral glycosphingolipids, containing a terminal β1-3-linked N-acetylgalactosamine residue (7). These toxins have been cloned (11, 23, 28) and sequenced (6, 13), and studies are currently in progress to determine the amino acids involved in the glycolipid-binding site.

Polymyxin B is an antibiotic routinely used in extraction of these toxins from the intact bacterium. The peptide (Mx, 1,202.5) selectively permeabilizes the outer membrane of E. coli, releasing the contents of the periplasmic space (4), thereby increasing the specific activity of toxin extracts (15).

Caution is now advised in the analysis of glycolipid binding in the presence of this peptide, since we present data to show that the glycolipid-binding specificity of VT1 is specifically modified in the presence of this antibiotic. A possible mechanism for this alteration is suggested.

MATERIALS AND METHODS

Ceramide dihexoside (CDH), Gb3, and Gb4 were separated from human kidney (3), digalactadiacylgycerol (DGDG) was from Supelco, phosphatidylcholine and cholesterol were from Sigma Chemical Co., GM3 and GM2 gangliosides were generous gifts from J. Callahan, Department of Neurosciences, The Hospital for Sick Children, asialo GM1 was prepared by hydrolysis of 2 to 5 mg of GM1 per ml of 1 M acetic acid for 1 h at 80°C. After neutralization, the products were partitioned (chloroform-methanol-H2O, 2:1:0.6 [vol/vol/vol]), and the lower phase dried (9). The CDH fraction was treated with α-galactosidase (Sigma) as described previously (2).

Polymyxin B sulfate (Aerosporin) was from Burroughs Wellcome Co., melitin, toluidinylnaphthalene-6-sulfonate (TNS), and Triton X-100 were from Sigma, goat anti-rabbit immunoperoxidase conjugate was from Bio-Rad Laboratories, ultrapure gelatin was from Fisher Scientific Co., bovine serum albumin was from ICN, and flexible microtiter trays were from Dynatech Laboratories, Inc. Polygram SIL G plastic backed thin-layer chromatography (TLC) plates (Brinkmann Instruments, Inc.) were used for glycolipid separation and the overlay procedure. VT1 was radiolabeled by using the 125I Bolton-Hunter kit (ICN).

Bacterial strains and products. Recombinant E. coli (pJLB28) (11) was used as a source of VT1. High yields of the toxin (10 to 15 mg/liter of broth culture) were purified by a modification of our previously developed method involving polymyxin extraction, ultrafiltration, hydroxylapatite chromatography, chromatofocusing, and Cibachron blue chromatography (24).

Recombinant E. coli(pJLB120) was used as a source of the

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VT1 B subunit (K. Ramotar et al., Biochem. J., in press). The expression product was extracted from the bacteria by three techniques.

(i) Polymyxin B. The standard polymyxin procedure of Karmali et al. (15) was used. Overnight 2-ml broth cultures were harvested, the pellets were suspended in polymyxin B (2 ml of 1.7 mM polymyxin B or 0.3 ml of 0.12 mM polymyxin B), incubated at 37°C for 1 h, and pelleted, and the supernatants were diluted to 10 ml to yield final polymyxin B concentrations of 0.33 mM (high) or 3.7 μM (low) (assuming 100% recovery in the supernatant).

(ii) Osmotic shock. Overnight 2-ml broth cultures were harvested, pellets were suspended in 2 ml of 30 mM Tris-20% sucrose-1 mM EDTA (pH 8.0) (TSE buffer), incubated at 30°C for 10 min, and reharvested. The pellets were resuspended in 1 ml of TSE, centrifuged, vigorously suspended in 1 ml of ice-cold water, stirred for 10 min, and pelleted, and the supernatants were collected (22).

(iii) Spheroplast preparation. Overnight 2-ml broth cultures were harvested, the pellets were suspended in 2 ml of TSE buffer, stirred for 10 min at 4°C, incubated with 5 mg of lysozyme per ml for 30 min at 37°C, and pelleted, and the supernatants were collected (1).

Glycolipid binding. The binding of VT1 holotoxin and the VT1 B subunit was assayed by a TLC overlay procedure previously described (20). Purified glycolipids (2 μg) were separated by TLC in chloroform-methanol-H₂O (65:25:4 [vol/vol/vol]) after separation, the plates were blocked in 3% gelatin at 37°C overnight, washed with 50 mM Tris-saline (pH 7.4) (TBS), and incubated for 1 h with VT1 holotoxin (1 μg per plate) or the VT1 B subunit (approximately 50 μg per plate) with or without modifying agents in 50 mM TBS (pH 7.4). Holotoxin or B-subunit binding was visualized with specific rabbit anti-VT1 polyclonal serum (neutralization titer, 1:024; diluted 1:100) followed by goat anti-rabbit immunoperoxidase conjugate (diluted 1:2,000), with 4-chloro-1-naphthol as the substrate (20). Toxin binding was determined.

![FIG. 1. Effect of polymyxin B on binding of VT1 B subunit to glycosphingolipids. Glycolipid standards (2 μg per lane) were separated by TLC (chloroform-methanol-water [65:25:4, vol/vol/vol]) and visualized by B-subunit (approximately 50 μg per plate) binding, using anti-VT1 and an immunoperoxidase conjugate as described in Materials and Methods. (a) B subunit extracted with 0.0037 mM polymyxin B; (b) B subunit extracted with 0.33 mM polymyxin B; (c) purified VT1 (1 μg per plate) standard (no polymyxin B); (d) B subunit released by osmotic shock; (e) B subunit released by spheroplast preparation. Left lane, CDH; center lane, Gb₄; right lane, Gb₅.](http://iai.asm.org/)

![FIG. 2. Purified VT1 binding to glycolipids (with or without polymyxin B). Purified glycolipids (2 μg per plate) were separated by TLC and visualized by VT1 overlay (1 μg per plate) in the presence of polymyxin B at 0 mM (a), 0.0033 mM (b), 0.017 mM (c), 0.083 mM (d), or 0.33 mM (e). Lanes (from left): DGDG, CDH, Gb₄, and Gb₅. (f) VT1 binding in the presence of 0.005 mM polymyxin B to CDH (left lane) and α-galactosidase-digested CDH (right lane). Anti-VT1 and immunoperoxidase conjugate were used as described in Materials and Methods.](http://iai.asm.org/)

![FIG. 3. Quantitation of increased Gb₄ binding of VT1 in the presence of polymyxin B. Gb₄ or DGDG (100 ng per well) was mixed with phosphatidylycholine and cholesterol (1:5:2:5) in microtiter wells as described in Materials and Methods. Serial twofold dilutions of [125I]VT1 (3.96 x 10⁶ cpm/μg) were added to the wells in the presence (0.25 mM) or absence of polymyxin B. Symbols: ■, Gb₄ (no polymyxin B); □, Gb₄ plus polymyxin B; ○, DGDG (no polymyxin B); X, DGDG plus polymyxin B. Averages of duplicates are shown.](http://iai.asm.org/)
by comparison with a similar plate visualized for carbohydrate with orcinol spray. Control TLC plates without toxin were also sprayed with orcinol after the procedure to monitor any possible glycolipid loss during washing.

The effect of polymyxin B on binding of $^{125}$VT1 to Gb$_4$ in a lipid mixture was quantitated in microtiter wells. Gb$_3$ (or DGDG as a control) was mixed with phosphatidylcholine and cholesterol (to facilitate immobilization) at a ratio of 1:5:2.5 in methanol; 100 µl (100 ng of glycolipid) was added to the wells, allowed to dry overnight, and blocked with 3% bovine serum albumin in TBS for 2 h. The wells were then washed three times with 50 mM TBS (pH 7.4). Dilutions of $^{125}$VT1 (specific activity, 3.96 × 10$^4$ cpm/µg of protein) were made with and without polymyxin B (0.25 mM), added to the wells, and incubated for 2 h. The wells were thoroughly washed with 50 mM TBS (pH 7.4) and excised, and the radioactivity counted in a Beckman 5500 Gamma Counter. The entire procedure was performed at room temperature.

**RESULTS**

Figure 1 shows the analysis of glycolipid-binding specificity by TLC overlay of the expression products of the recombinant B-subunit gene. *E. coli*(pJLB120) was extracted with low or high polymyxin B concentrations, by osmotic shock, or with lysozyme. Binding of the B subunit to CDH, Gb$_3$, and Gb$_4$ was compared. In the absence of polymyxin B, binding was restricted to Gb$_3$ (Fig. 1c to e). However, the presence of polymyxin B at a concentration as low as 3.7 µM resulted in significant additional binding to Gb$_4$ and CDH (Fig. 1a and b).

Glycolipid-binding specificity determined by TLC overlay of purified recombinant VT1 holotoxin is shown in Fig. 2. Increasing concentrations of polymyxin B were added to the overlays during the VT incubation. As indicated above, binding to CDH and Gb$_4$ increased in the presence of polymyxin B. However, no binding to DGDG was observed at any polymyxin B concentration. VT1 binding in the presence of polymyxin B was eliminated for CDH samples treated with α-galactosidase (Fig. 2d). A linear increase in the binding of VT1 to Gb$_4$ in microtiter plates was observed in the presence of polymyxin B. Binding of VT1 to DGDG was unaffected (Fig. 3). An approximate fourfold increase in binding was observed over a VT concentration of 1 to 10 µg per well.

Polymyxin B contains significant hydrophobic character.
(4). We therefore investigated the effect of a known hydrophobic blocking agent, as well as another amphipathic cyclic peptide, melittin, known to interact with glycolipids (8), on the glycolipid-binding specificity of VT1. Melittin caused the same change in binding specificity of VT1 such that Gb$_4$ was also recognized (Fig. 4, row b). The hydrophobic blocking agent TNS (soluble only up to a concentration of 0.12 mM) did not alter binding specificity (Fig. 4, row c). Triton X-100 also did not alter binding specificity but inhibited all binding at a concentration of 0.5% (Fig. 4, row d).

In order to determine the specificity of polymyxin-mediated VT1 binding, we examined VT1 binding to other glycosphingolipids in the presence of polymyxin B (Fig. 5). The increased binding was found to be specific for Gb$_4$, Gangliosides, and more notably asialo GM1, another ceramide tetrahexoside, were not bound.

**DISCUSSION**

Carbohydrate recognition is a common motif for microorganisms and their toxic products, and many of the receptors are glycolipids (14). VT1 holotoxin (20) and, as we now show, the VT1 B subunit specifically recognize the glycolipid Gb$_3$, and there is substantial evidence that this glycolipid acts as the functional receptor for VT1 (5, 21). Galabiosylceramide (5) and P$_1$ glycolipid (19) (both containing terminal Galal-4Gal) have also been shown to bind VT. Our study clearly shows that in the presence of polymyxin B or melittin (small amphipathic peptides), the toxin and the B subunit are also capable of recognizing Gb$_4$. The observed increase in binding to CDH in the presence of polymyxin B was due to galabiosylceramide in this preparation, since VT binding could be abolished by pretreatment with α-galactosidase (the gross glycolipid content, as detected by orcinol spray, was unaffected by treatment with this enzyme [results not shown]).

Although melittin has been reported to interact with glycosphingolipids as a function of carbohydrate chain length (8), the change in VT1 binding specificity was not directly related to carbohydrate chain length, since asialo GM1, a similar ceramide tetrahexoside in which the terminal sugars are essentially reversed, was not recognized (Fig. 5). Moreover, the change in binding was not a result of nonspecific toxin-peptide-glycolipid interaction since (i) the binding specificity did not change to include unrelated glycolipids such as DGDG, GM1, asialo GM1, or GM2; and (ii) the toxin did not bind directly to melittin or polymyxin B spotted on nitrocellulose paper (results not shown).

Our finding that the effect of polymyxin B on VT binding was restricted to glycosphingolipids containing the Gal α1-4Gal structure may suggest a mechanism of action. Gb$_4$ differs from Gb$_3$ by an additional terminal β1-3-linked N-acetylgalactosamine residue but is not recognized by VT1 (20). This has been interpreted to suggest that the 3' position of the terminal α-Gal of Gb$_3$ must be free for VT binding. Alternatively, the terminal GalNAc residue of Gb$_4$ may contain a structural feature which prevents binding, which, when masked by polymyxin B or melittin, is no longer inhibitory. Such masking might result from a change in the glycolipid conformation in the presence of polymyxin B, allowing VT1 to bind to an internal α-Gal residue. However, the glycolipid-binding site nevertheless has limited tolerance since no binding to Forssman glycolipid (GalNAca1-3GalNAcβ1-3Galal-4Galβ1-4Glcβ1-ceramide) was observed in the presence of polymyxin B (data not shown), despite the presence of an internal Galal-4Gal. Binding of VT to galabiosylceramide might be limited by the close apposition of the ceramide moiety. Such hydrophobic inhibition might well be reduced, and the Galal-4Gal be more available, in the presence of an amphipathic molecule such as polymyxin B. It is interesting that no binding to DGDG was observed even in the presence of polymyxin B despite the presence of the required carbohydrate sequence.

**FIG. 5.** Specificity of effect of polymyxin B on VT1 binding to glycosphingolipids. Purified glycolipids (2 μg per lane) were separated by TLC and visualized by VT1 binding (1 μg per plate) in the presence of polymyxin B at 0 mM (a), 0.005 mM (b), 0.05 mM (c), 0.1 mM (d), or 0.5 mM (e). Lanes (from left): Gb$_3$, Gb$_4$, GM$_1$, asialo GM$_1$, and GM$_2$. 

[Image of Figure 5]
We were unable to show any direct effect of polymyxin B on the oligomerization of the B subunit. High pressure liquid chromatography analysis of the B subunit associates as a pentamer both in the absence (Ramotar et al., in press) and presence (data not shown) of polymyxin B. Polymyxin B does, however, interact directly with the glycolipids, since pretreatment of glycolipid-containing TLC plates with polymyxin B and washing prior to VT1 incubation resulted in the same shift in glycolipid-binding specificity, that is, to include recognition of Gb4 and galabiosylceramide. This confirms that the effect is via an interaction of the amphipathic peptide with the glycolipid. Although an exact mechanism was not established, we suggest that the polymyxin B (or melittin) can interact with Gb4 and alter its structural conformation such as to optimize the presentation of the Galα1-4Gal residue for VT1 binding. Such an effect may result from a combination of hydrophobic interaction with the ceramide moiety and hydrophilic interaction with the carbohydrate. It is nevertheless surprising that neither polymyxin B (Mr, 1,202.5) nor melittin (Mr, 2,847.83) sterically prevent glycolipid binding. The hydrophobic blocking agent TNS was not seen to change binding specificity up to concentrations of 0.12 mM, suggesting that the amphipathic nature of polymyxin B is essential for this effect. Triton X-100 inhibited all VT1 binding at 0.5%, suggesting that hydrophobic interactions are nevertheless important in toxin receptor binding.

VTE (pig edema toxin) has been shown to bind primarily to Gb4 (7). Studies are currently under way to determine which residues are responsible for this specificity. It is interesting that the addition of polymyxin B can change the binding specificity of VT1 to a specificity similar to that seen for VTE. The VT family thus provides a unique opportunity to study the molecular basis of such specific glycolipid recognition.

The data presented here demonstrate that low concentrations of certain amphipathic agents significantly alter the glycolipid-binding specificity of the bacterial toxin VT1. Thus, caution is advised when preparing toxin extracts for use in binding studies. The use of purified toxin or an alternate extraction procedure (lysozyme or osmotic shock) would eliminate spurious results.

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