**Escherichia coli** verotoxin (also known as Shiga-like toxin) has been implicated in the aetiology of the hemolytic uremic syndrome and hemorrhagic colitis. The glycolipid binding specificity of verotoxin purified from *E. coli* H30 and verotoxin cloned from bacteriophage H19B has been examined. Verotoxin from both sources binds specifically to globotriosyl ceramide containing the carbohydrate sequence α1-4galactoseβ1-4glucose-ceramide. Removal of the terminal galactose or substitution with N-acetylglactosamine in β1-3 linkage deletes toxin binding activity. A ceramide trihexoside species, consistent with a globotriosyl ceramide structure was shown to be the major verotoxin-binding glycolipid of cultured vero cells which are routinely used to measure the cytotoxicity of toxin samples.

Some strains of *Escherichia coli* elaborate cytotoxins that are active on cultured vero cells (1). At least two of the verocytotoxins, VT1 and VT2 (2), are known to be produced by *E. coli* strains that are closely associated with a nonspecific diarrheal illness, as well as two distinct complications, the hemolytic uremic syndrome (HUS) (3) and hemorrhagic colitis (4). VT1 is closely related, both antigenically and biologically, to Shiga toxin produced by *Shigella dysenteriae* type 1 (5) and is thus referred to as Shiga-like toxin.

In *E. coli*, verotoxins are encoded for by one or more bacteriophages (6), and, furthermore, individual strains may produce either one or both VT1 and VT2 (2, 6). The nature of the genes coding for Shiga toxin production in *S. dysenteriae* type 1 remains to be established. VT1 and Shiga toxin are cytopathic to vero and HeLa cells, lethal to rabbits and mice, and induce fluid secretion in rabbit ileal loops (5). Both are subunit toxins with one "A" subunit having a molecular mass of approximately 31,000 daltons and several "B" subunits each with a molecular mass of about 5,500 daltons (5). The A subunit possesses the biological activity of the toxin which is involved in inhibiting protein synthesis (7), whereas the B subunits are presumed to mediate specific binding and recep-
tor-mediated uptake of the toxin. The subunits of VT1 have been cloned and expressed in *E. coli* (8–10).

Shiga dysentery (associated with infection by *S. dysenteriae* type 1) is the worst form of bacillary dysentery (11), often associated with a high morbidity and mortality. However, the precise role of Shiga toxin in this condition has yet to be established. Keusch and colleagues (12–15) have proposed that Shiga toxin is involved in the genesis of the early watery diarrheal phase of the illness. Their *in vitro* investigations have suggested Shiga toxin binds in *vitro* to glycoproteins containing terminal β1-4-linked N-acetyl-D-glucosamine (12) and have proposed that this may be the functional receptor for this toxin. However, Lindberg and his colleagues (15–17), using a 125I-Shiga toxin binding assay, were unable to confirm this but found binding of the toxin to glycolipids containing a terminal disaccharide Galα1-4Gal. This included a galabiosyl ceramide (Galα1-4Gal-ceramide), globotriosyl ceramide, and to a reduced extent, globotetrasyl ceramide. Recently, Jacewicz et al. (16) have confirmed this finding and have suggested that the effect of the toxin on fluid secretion in the intestine may be mediated via such an interaction. They have maintained, however, that a glycoprotein species serves as the Shiga toxin receptor mediating the cytotoxic effect on HeLa cells.

In this report, we describe studies with a simple binding assay using anti-verotoxin antibody to determine the glycolipid binding specificity of the natural and recombinant *E. coli* verotoxin.

**EXPERIMENTAL PROCEDURES**

**Materials**

The glycolipids, galactosyl ceramide (kerosin) 3'-sulfogalactosyl ceramide, and DGDG, were from Supelco. LacCer, 3'-sulfogalactosylglycerolipid, and galactoglycerolipid were prepared as previously described (17). Galα1-4Galβ1-4Glc-cer, Galβ1-4Glc-cer, globotetrasyl ceramide, Galα1-4Galβ1-4Glc-cer, and Galβ1-4Glc-cer were generous gifts from Dr. P. Strasberg, Division of Neurosciences and Dr. J. Clarke, Genetic Metabolic Program, The Hospital for Sick Children. All glycolipids gave a single spot on tlc (doublet for LacCer, Glα1-4Glc-cer, and Glβ1-4Glc-cer) when visualized with orcinol spray. [14C]Serine and [3H]Glucosamine were from NEN. Choloro-1-naphthol was from Sigma, and goat anti-rabbit immunoglobulins conjugated to horseradish peroxidase were from Miles. Plastic-backed Polygram SIL G tlc plates were purchased from Brinkmann Instruments (Ontario). Monoclonal antibody (Mab 13C4) against the B subunit of verotoxin (18) was generously supplied by Dr. A. O'Brien (Uniformed Services University, Bethesda, MD). Anti-Tja serum, protein A were purchased from Du Pont-New England Nuclear. Chloro-1-naphthol was from Sigma, and goat anti-rabbit immunoglobulins conjugated to horseradish peroxidase were from Miles. Plastic-backed Polygram SIL G tlc plates were purchased from Brinkmann Instruments (Ontario). Monoclonal antibody (Mab 13C4) against the B subunit of verotoxin (18) was generously supplied by Dr. A. O'Brien (Uniformed Services University, Bethesda, MD). Anti-Tja serum, protein A were purchased from Du Pont-New England Nuclear.
used by O'Brien and La Veck (5) for purifying Shiga-like toxin (verotoxin) from the same reference strain and by Brown et al. (19) for purifying Shiga toxin. Verotoxin containing a 31-kDa A subunit together with a B subunit of approximately 5 kDa (20) was used in the binding studies at a titer of $10^4$ as measured in the vero cell cytotoxicity assay (3).

Production of Antitoxin—Adult male rabbits weighing approximately 2 kg were given an intravenous dose corresponding to 20 ng/kg body weight of purified verotoxin. This dose was equivalent to one-tenth of the 50% lethal dose (LD$_{50}$). The primary immunization was followed by 6 booster doses (at concentrations of 10–100 LD$_{50}$) given at 2-week intervals. The rabbits were bled prior to each immunizing dose, and the verotoxin-neutralizing antibody titer determined as described previously (3). The verotoxin-neutralizing antibody titer of the serum collected in the final bleed was 8,192 while that of the preimmune serum was $<2$.

Cloning of Toxin—A 1.7-kilobase fragment of the genome of a toxin-converting bacteriophage H19B was cloned into pUC18 (10). E. coli TB1 which is nontoxigenic (10) was transformed with the recombinant plasmid resulting in production of high levels of verotoxin activity. The E. coli culture supernatant had a cytotoxin titer of $3 \times 10^9$ and was used as a source of cloned verotoxin (pJLB28).

Glycolipid Binding—Binding of verotoxin to purified glycolipids immobilized on tlc plates was performed essentially as previously described (21). Purified glycolipids (5 μg) were dot blotted on tlc plates. The tlc plate was then incubated in 10 mM Tris-saline, pH 7.4, containing 10% fetal bovine serum overnight at 4 °C. The tlc blots were washed with Tris-saline and incubated overnight with verotoxin preparation (3). The blots were washed 5 times with Tris-saline and incubated overnight at 4 °C with 1% polyclonal rabbit antitoxin which had been previously treated with 0.8% formaldehyde (21) (monoclonal antibodies were used at 1:10 dilution of hybridoma culture supernatant). The blots were washed 5 times with Tris-saline and further treated with peroxidase-conjugated goat anti-rabbit Ig for 2 h at room temperature (goat anti-mouse Ig peroxidase conjugate was used in the case of monoclonal antiveroantitoxin antibodies). Glycolipid-bound toxins was visualized after washing 5 times with Trissaline by treatment of the blots with 4-chloro-1-naphthol peroxidase substrate (21). The reaction was terminated by extensive washing with water. For quantitative analyses bound anti-verotoxin was detected by incubating with $^{125}$I-labeled Protein A (0.75 μCi/ml in phosphate-buffered saline) for 2 h at room temperature. The blots were washed extensively with saline, and bound Protein A was visualized by autoradiography. Glycolipid dots were cut out and counted in a γ counter. Some batches of fetal bovine serum were found to give high background staining and eliminate specific verotoxin binding to glycolipids, possibly due to the presence of serum glycolipids. In our later experiments 0.6% gelatin at room temperature was used to block nonspecific binding sites prior to verotoxin binding.

Radio labeling and Extraction of Vero Cell Glycolipids—Vero cells (No. 76, American Tissue Culture Collection, derived from the kidney of the green monkey) cultured as previously described (3) were grown for 72 h in the presence of [3H]serine. Cells were removed with 1% trypsin. The cell pellet containing approximately $10^9$ cells was mixed with an equal number of unlabeled cells, washed 3 times with phosphate-buffered saline, extracted with 20 volumes of chloroform/methanol, 2:1 (v/v), and filtered through glass wool. The residue was partitioned against an equal volume of water. The lower phase was saponified by partitioning against an equal volume of 1 N NaOH, and the lower phase was flash evaporated and lipids separated by tlc (chloroform/methanol/water, 65:25:4, by volume) without further purification. After toxin binding and immunostaining, radio-labeled lipids were detected by autoradiography. Nonlabeled standards were visualized using orcinol spray. For anti-Tjα binding, plates were treated with rabbit antihuman immunoglobulin prior to visualization with the peroxidase conjugate as above.

Digestion of Glycolipids with α-Galactosidase—Purified GbOse$_{4}$cer and crude vero cell glycolipid extract were treated with α-galactosidase from coffee beans essentially as described by Bailly et al. (22). 5 nmol of GbOse$_{4}$cer and 500 μg of sodium taurocholate in chloroform/methanol were dried together and incubated in 50 mM citrate buffer, pH 4.5, containing 2 mM EDTA, 1% bovine serum albumin (w/v), and 1.0 unit of α-galactosidase at 37 °C for 24 h. Control samples were incubated in the absence of enzyme. Under these conditions greater than 90% of the GbOse$_{4}$cer standard was digested as judged by orcinol spray after tlc separation. The digested glycolipids were

FIG. 1. Specific binding of verotoxin to GbOse$_{4}$cer. Purified glycolipids (5 nmol, panel a; 2.5 nmol, panel b) were separated by tlc (chloroform/methanol/water, 60:25:4, v/v) and visualized by orcinol spray (a) or verotoxin binding (b) using anti-verotoxin and an immunoperoxidase conjugate as described under “Methods.” Lane 1, DGDD; Lane 2, lactosyl ceramide; Lane 3, GbOse$_{4}$cer; Lane 4, GbOse$_{4}$cer.
analyzed for verotoxin binding using 125I-Protein A as previously described.

RESULTS

Purified Verotoxin Binding to Glycolipid Standards—Purified verotoxin was found to bind specifically to globotriosyl ceramide (GbOse3cer) (Fig. 1). No binding to lactosyl ceramide in which the terminal α-galactose residue is missing or globotetraosyl ceramide in which the terminal galactose is substituted in the 3 position with N-acetylgalactosamine was observed. Digalactosyl diglyceride, containing the same terminal carbohydrate sequence as GbOse3cer but linked to a glycerol lipid moiety, rather than sphingosine, was also unreactive with the toxin. Binding curves were generated using 125I-Protein A (Fig. 2). No binding above background was observed for LacCer, GbOse4cer, or DGDG at any concentration tested (0.01–50 nmol). However, binding to 0.1 nmol of GbOse3cer could be detected. Similar results were obtained when toxin binding was detected using a polyclonal toxin-neutralizing antibody or a monoclonal antibody raised against the B subunit of verotoxin (18) (Fig. 3). These results suggest that both the lipid moiety and the terminal glycose moiety are involved in verotoxin-glycolipid binding.

Recombinant Verotoxin-Glycolipid Binding—A similar glycolipid binding specificity was found for the cloned toxin pJLB28 expressing both the A and B subunits (Fig. 4). Specific binding to GbOse3cer was observed. As with the purified toxin, recombinant verotoxin binding was greatly reduced for lactosyl ceramide and globotetraosyl ceramide, and substitution of glycerol for the sphingosine base resulted in the loss

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**Fig. 2.** Quantitation of glycolipid binding. Increasing concentrations of glycolipid contained within 5 μl were dotted on tlc sheets. Toxin which bound was detected using 125I-labeled Protein A. Background values for binding without toxin have been subtracted. Average of duplicates is shown. ○, GbOse4cer; □, DGDG; ■, GbOse3cer; ●, LacCer.

**Fig. 3.** Binding of purified verotoxin to glycolipids. Binding of verotoxin to immobilized glycolipids was visualized immunologically as described under "Methods." a, control for background binding of polyclonal antitoxin in the absence of verotoxin. b, glycolipid dot blot incubated with verotoxin visualized with polyclonal antitoxin. c, glycolipid dot blot incubated with verotoxin visualized with monoclonal antitoxin. Background binding was as in a. Glycolipids: 1, 3′-sulfogalactosyl ceramide; 2, galactosyl ceramide; 3, GbOse4cer; 4, GM2; 5, DGDG; 6, 3′-sulfogalactosylglycerolipid; 7, GbOse3cer; 8, LacCer.
FIG. 4. Binding of cloned verotoxin to glycolipids. Culture supernatants of E. coli transformed with: A, pUC18 (containing no insert); B, pJLB28 (expressing A and B toxin subunits); C, purified verotoxin as used in Fig. 1 were assayed for glycolipid binding as in Fig. 3b. Glycolipids: 1, 3'-sulfogalactosyl ceramide; 2, galactosyl ceramide; 3, GbOse4cer; 4, LacCer; 5, GM3; 6, DGDG; 7, galactoglycerolipid; 8, GbOse3cer.

of binding. No background staining for the plasmid vector pUC18 carrying no insert was observed.

Verotoxin-Vero Cell Glycolipid Binding—Analysis of toxin binding to vero cell glycolipids shows that the natural toxin binds strongly to a species which migrates in the ceramide trihexoside region on tlc (Fig. 5). Metabolic labeling of glycolipids showed that this species was a relatively minor fraction of the vero cell glycolipid content (Fig. 5).

In order to further corroborate that this verotoxin binding glycolipid was GbOse3cer, we made use of the fact that naturally occurring antibodies specific for globotriosyl ceramide and globotetraosyl ceramide (anti-Tja antibodies) are found in the serum of individuals of p blood group status (23). Binding of such antibodies to the verotoxin binding trihexoside extracted from vero cells would provide further indirect confirmatory evidence of its chemical structure. The verotoxin binding trihexoside was eluted and was found to react with anti-Tja blood group antisera as detected by immunostaining after separation by two-dimensional tlc (Fig. 6).

α-Galactosidase Digestion—The specificity of verotoxin glycolipid binding was further confirmed by digestion of GbOse3cer and vero cell glycolipids with α-galactosidase prior to tlc and assay of verotoxin binding. The results (Fig. 7) show that removal of the terminal α-galactose residue deletes verotoxin binding both to GbOse3cer (now LacCer) and the vero cell glycolipids. The low level of residual verotoxin binding detected at higher glycolipid concentrations can be accounted for by residual undigested GbOse3cer.

DISCUSSION

Previous studies have strongly implicated verotoxin in the aetiology of the HUS and hemorrhagic colitis (3). We are, therefore, interested to determine the mechanism of verotoxin cytotoxicity and the nature of the component(s) on the cell surface which facilitate binding and subsequent entry into the cells.

Earlier work had shown that certain uropathogenic bacteria isolated from a patient with acute pyelonephritis were able to bind any glycolipid containing the structure galactose α1-4 galactose (24). Although binding of the bacterium to the epithelial cell surface is not considered to be mediated via the toxin, which is in fact concentrated in the periplasmic space between the inner and outer membrane of the bacterium, it has also been reported that the toxin purified from S. dysenteriae type 1 also binds to glycosphingolipids containing the Galα1-4Gal sequence in a terminal position (13, 14, 16) although residual binding to globotetraosyl ceramide was observed (13). These glycolipids form part of the P blood system (23). Galα1-4Gal-ceramide, Pk antigen (GbOse3cer), and the PI glycolipid (Galα1-4Galβ1-4GlcNACβ1-3Galβ1-4Glc-ceramide) were all found to bind the purified Shiga toxin. The cellular cytotoxicity could also be blocked by pretreating the Shiga toxin with Galα1-4Gal conjugated to bovine serum albumin (13, 15).

In light of these results, we have examined the glycolipid binding specificity of purified verotoxin and of the toxin cloned from a bacteriophage DNA sequence containing the structural genes for the A and B verotoxin subunits. Our results have shown that both toxins also bind to glycosphingolipids containing a Galα1-4Gal sequence at the nonreducing terminal. Thus, the toxin is strongly bound to GbOse3cer, but substitution of the terminal αGal with GalNAc substituted in β1-3 linkage completely removes binding of the natural toxin (Figs. 1–3) and vastly reduced the binding of the cloned
Glycolipid Binding of E. coli Produced Verotoxin in Vitro

FIG. 5. Verotoxin binding to vero cell glycolipids. Neutral glycolipids of vero cells were metabolically labeled, extracted, and separated by tlc as described under "Methods." Verotoxin binding to the separated glycolipids was determined as for Fig. 1. A, verotoxin binding; B, autoradiogram of labeled glycolipids: 1, galactosyl ceramide standard; 2, LacCer standard; 3, GbOse3cer standard; 4, GbOse3cer standard. Arrow in A indicates major toxin-binding species. Species (Fig. 4). Enzymatic cleavage of the terminal α-galactose residue results in the loss of verotoxin binding (Fig. 7). However, the sphingosine base of the glycolipid is also involved in the binding since DGDD containing the same terminal Galα1-4Gal sequence as GbOse3cer, but linked to a glycerol moiety, showed no verotoxin binding activity (Figs. 2 and 3). It has been previously suggested that the carbohydrate moiety of glycerol-based glycolipids may orient differently as opposed to sphingolipids with respect to the lipid moiety (25). It is perhaps of relevance to note that similar digalactosyl diglycerides are found in several bacteria themselves (26).

In order to study whether binding to GbOse3cer might be involved in the natural mechanism of verotoxin cytotoxicity, we analyzed binding to glycolipids isolated from vero cells which are used in the cytotoxicity assay (3). The toxin was found to bind very well to a glycolipid migrating in the ceramide trihexoside region after tlc separation (Fig. 5). This "receptor" glycolipid is a relatively minor component of the veroceil neutral glycosphingolipid complement (Fig. 5B), but this component is the major verotoxin binding site (Fig. 5A), suggesting that binding to this glycolipid on the cell surface may be involved in the physiological mechanism of verotoxin cytotoxicity. In order to check that the toxin-binding glycolipid contained the same carbohydrate sequence as the GbOse3cer standard, we made use of the fact that GbOse3cer forms part of the P blood group system (24). Anti-Tja blood group sera (from individuals lacking all P blood group antigens) contain antibodies reactive with the carbohydrate moieties of P1, P (globotetraosyl ceramide), and P, antigens (23). Both the verotoxin binding trihexoside glycolipid and GbOse3cer standard (not shown) were found to be immunoreactive with anti-Tja serum (Fig. 4) indicating that both contain the carbohydrate sequence of P1 (GbOse3cer). This reactivity was removed by preabsorbing the anti-Tja serum with P1 red blood cells but not with P red blood cells (lacking GbOse3cer, GbOse3cer) (not shown). The nature of the verotoxin binding glycolipid from vero cells was further confirmed by the finding that treatment with α-galactosidase removed verotoxin binding both for cell-extracted glycolipids and GbOse3cer standard (Fig. 7). Furthermore, this glycolipid (GbOse3cer) has been chemically identified in vero cells (27).

These results are of particular interest considering one of the more severe symptoms of verotoxin cytotoxicity in certain
the interpatient variation observed for this symptom might be related to the P blood group status of an individual and/or the serum levels of anti-blood group antibodies. (Anti-GbOse₃cer antibodies have also been described in a fraction of normal human sera, apparently unrelated to blood group status (29).)

The similarity of verotoxin and Shiga toxin glycolipid binding specificity argues in favor of the concept that these toxins share common (or very similar) binding subunits (B subunit), proposed to explain immunological cross-reactivity in cytotoxicity neutralization (18). The binding characteristics are distinct, however, from those of cholera toxin, the other bacterial toxin for which a specific glycolipid receptor has been defined (1, 29, 30). In this case, the B subunit specifically binds to G₀, ganglioside on the cell surface (31). It is now generally accepted that G₀ is the only functional receptor and that membrane glycoproteins are not involved (32). The question of involvement of membrane glycoproteins in the binding of Shiga toxin has also been raised. The experiments of Keusch et al. (12) suggest that N-linked glycoproteins containing terminal 2-N-acetylglucosamine residues function as the receptor for this toxin. Keusch has recently confirmed that Shiga toxin binds specifically to GbOse₃cer but maintains specificity for GbOse₃cer which contains a Gal₁-4Gal structure at the nonreducing terminus. The similar binding specificity of the cloned verotoxin offers the opportunity to determine the structural requirements for glycolipid binding, for modulation of cytotoxic activity, and eventual immunotherapy.

In conclusion, verotoxin shows a glycolipid binding specificity for GbOse₃cer which contains a Gal₀₁-4Gal structure at the nonreducing terminus. The similar binding specificity of the cloned verotoxin offers the opportunity to determine the structural requirements for glycolipid binding, for modulation of cytotoxic activity, and eventual immunotherapy.

Note Added in Proof—The binding of purified Shiga toxin to glycolipids containing a terminal Gal₀₁-4Gal sequence has been recently confirmed, although some binding to glycolipids with an internal galabiose sequence was also observed (Lindberg, A. A., Brown, J. E., Strömberg, N., Westling-Ryd, M., Schultz, J. E., and Karlsson, K.-A. (1987) J. Biol. Chem. 262, 1779–1785).

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