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Induction of verotoxin sensitivity in receptor-deficient cell lines using the receptor glycolipid globotriosylceramide

(liposome/exogenous glycolipid incorporation/receptor-mediated endocytosis)

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ABSTRACT **Verotoxin 1 is an *Escherichia coli*-derived subunit toxin that specifically binds to the glycolipid globotriosylceramide and is cytotoxic for cells that contain this plasma membrane glycolipid. Glycolipid incorporation experiments have now been performed using human lymphoid cells of the B lineage that lack this receptor, to conclusively demonstrate that globotriosylceramide alone is a functional receptor for this toxin. Globotriosylceramide incorporated into the membrane of toxin-resistant cells provides intracellular access to verotoxin by receptor-mediated endocytosis. Protein synthesis is then inhibited and globotriosylceramide-containing cells are killed.**

Verotoxin (VT) is an *Escherichia coli* elaborated subunit toxin (1, 2) highly homologous with Shiga toxin (from *Shigella dysenteriae* type 1) and, therefore, also referred to as Shiga-like toxin or SLT (2, 3). At least two variants of VT have been described, termed VT1 and VT2 (4) [or SLTI and SLTII (5)]. Research interest has focused on these toxins since they have been strongly implicated as the causative agent of the renal damage seen in the hemolytic uremic syndrome (HUS) (6, 7). Evidence for the presence of VT has been found in nearly 90% of patients with HUS in this hospital over the last 5 years.‡‡ HUS is usually preceded by hemorrhagic colitis (6) and similar gastrointestinal symptoms are obtained when purified VT is administered to rabbits (8).

Most cells are entirely resistant to VT cytopathology. HeLa (9), Vero (6), and Daudi human lymphoma cells (10) have, however, been shown to be highly susceptible. It is possible that the basis of such differential sensitivity resides at the level of the cell surface toxin receptor.

VT1 (11), VT2 (12), and Shiga toxin (9, 13) have been shown to bind specifically to the neutral glycolipid globotriosylceramide (galactose α1-4galactoseβ1-4glucosylceramide; Gb_3). We have proposed (10) that this binding mediates the entry of the toxin into susceptible cells, since VT is noncytotoxic for cells that do not contain Gb_3, and Daudi cells selected for resistance to VT1 show a selective and dramatic decrease in the cellular content of Gb_3.

We now show that incorporation of exogenous Gb_3 into Gb_3-deficient, toxin-resistant mutant Daudi cells and a wild-type human B-cell line that does not contain Gb_3, sensitizes the recipient cells to the cytopathology of VT1.

MATERIALS AND METHODS

Gb_3 and globotetraosylceramide (Gb_4) were purified from human renal tissue by a modification of the procedure as described by Strasberg *et al.* (14). The chloroform/methanol tissue extract was first applied on a Bio-Sil A (Bio-Rad) silica column in chloroform. The column was extensively washed with chloroform and neutral glycolipids were eluted with acetone/methanol, 9:1 (vol/vol). The neutral glycolipid fraction was then applied on a second Bio-Sil A column in chloroform/methanol, 98:2 (vol/vol). Glycolipids were then resolved with a linear solvent gradient comprising equal weights of chloroform/methanol, 15:1 (vol/vol), to chloroform/methanol, 4:1 (vol/vol). Digalactosyldiacylglycerol (DGDG) was purchased from Supelco (Ind), galactosylceramide (GC) was from Sigma (Ill), and phosphatidylethanolamine (PE) and phosphatidylserine (PS) were from Avanti. VT1 was purified as described (1). Rabbit anti-VT antibodies were kindly provided by S. Richardson (Department of Microbiology, Hospital for Sick Children). Monoclonal antibody against the B subunit of VT1 (13-C4) was a generous gift from A. O'Brien (Uniformed Services University of the Health Sciences, Bethesda, MD). Plastic-backed Polygram TLC sheets were purchased from Brinkman Instruments. [^{35}S]Methionine was from DuPont/New England Nuclear.

Cells. Human Daudi lymphoma cells, the VT-resistant VT20 mutant clone derived from these cells (10), and the human fetal B-cell line HFB18.5 (15) were cultured in suspension in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum. The HFB18.5 cell line was a cloned Epstein–Barr virus-transformed human fetal B lymphocyte line that was isolated (15) and kindly provided by H.-M. Dosch, Department of Immunology and Rheumatology, Hospital for Sick Children.

Preparation of Glycolipid-Containing Liposomes. Four hundred micrograms of Gb_4, Gb_3, GC, or DGDG was dried with 200 μg of PE and 200 μg of PS under a stream of nitrogen. Sterile isotonic phosphate-buffered saline (PBS, pH 7.4; 400 μl) was added to the lipid and the mixture was sonicated using a water bath sonicator for 30 min. Liposome preparations were used immediately.

Incorporation of Exogenous Glycolipid into Cells. VT20 or HFB18.5 cells (1.6×10^7 cells) in late logarithmic growth phase were washed twice with PBS to remove serum proteins and then suspended in serum-free RPMI 1640 medium at 4×10^6 cells per ml. The cells were incubated in the presence of liposomes (or PBS for controls) prepared as above with rotary shaking (100 rpm) at 37°C for 1 hr, washed twice (5 min, 800 $\times$ *g*), with PBS, and incubated for 18–24 hr at 37°C in the presence of RPMI 1640 medium containing 10% fetal calf serum prior to experimentation.

Toxin Binding and Internalization. Toxin binding and internalization was assayed by fluorescence-activated cell sort-

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Abbreviations: VT, verotoxin; HUS, hemolytic uremic syndrome; Gb_3, globotriosylceramide; Gb_4, globotetraosylceramide; DGDG, digalactosyldiacylglycerol; GC, galactosylceramide; PE, phosphatidylethanolamine; PS, phosphatidylserine; FACS, fluorescence-activated cell sorting.

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ing (FACS) (Coulter-Epics IV) as described (10). Approximately 1×10^6 cells were incubated at 4°C in the presence of 0.1 μg of VT1 per ml for 30 min and washed three times with RPMI by centrifugation (5 min, 800 × *g*). Monoclonal anti-VT1 was added and excess antibody was removed by washing after 30 min. A goat anti-mouse Fab immunoglobulin conjugate was added for a further 30 min at 4°C and, after washing, cell bound fluorescence was assayed by FACS. For assay of toxin internalization, cells were incubated at 37°C for 30 min prior to the addition of anti-VT1 antibody.

Cytotoxicity. Glycolipid or control cells were cultured in the presence or absence of VT1 added 24 hr after glycolipid incorporation. For VT20 cells, protein synthesis was determined by incorporation of the [^{35}S]methionine as described (10) after overnight culture. For HFB18.5 cells, cytotoxicity was measured by determination of viable cells by trypan blue exclusion 6 days after addition of toxin.

Glycolipid Extraction and Toxin Binding. After glycolipid incorporation and cell culture for 18 hr, treated and control cells were washed twice with PBS and glycolipids were extracted with 20 vol of chloroform/methanol, 2:1 (vol/vol), and partitioned against 4 vol of water.

For toxin binding, a sample of the lower phase was separated by TLC in chloroform/methanol/water, 65:25:4 (vol/vol). The plates were then blocked by incubation in 3.0% (wt/vol) gelatin for 2 hr at 37°C. After washing, the plates were incubated overnight with purified VT1 in 100 mM Tris·HCl (pH 7.4) at 4°C. The plates were washed three times in buffer and incubated overnight with 0.1% rabbit anti-VT1 (6) in 100 mM Tris·HCl (pH 7.4) at 4°C. The plates were washed again and incubated in the presence of goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Bio-Rad) for 2 hr at 4°C. After washing, bound antibody was visualized with the peroxidase substrate 4-chloro-1-naphthol as described (8).

RESULTS

Incorporation of Gb_3. Incorporation of Gb_3 in PE/PS liposomes into cells is shown in Fig. 1. A representative experiment with HFB18.5 cells is shown. Incorporated glycolipids were detected in the extract of reconstituted cells with orcinol spray for carbohydrate and by VT1 binding (Fig. 1 *A* and *B*, respectively). The extract of cells supplemented with other glycolipids did not bind VT. In subsequent experiments, cells were treated with liposomes at 37°C for 1 hr

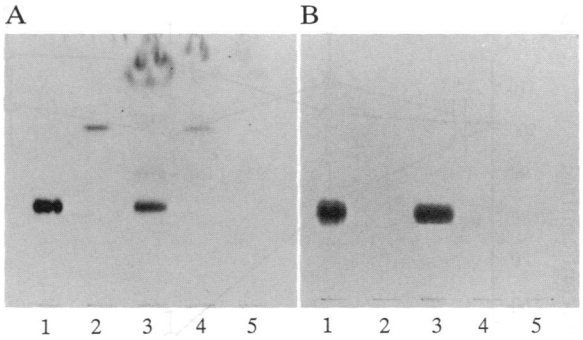

FIG. 1. Incorporation of liposomal glycolipid into cells. HFB18.5 cells were treated with PE/PS vesicles containing either Gb_3 or GC. After extensive washing and 18 hr in culture, the cells were harvested and glycolipids were extracted and tested for VT binding. (*A*) Gb_3 visualized by orcinol spray for carbohydrate. (*B*) VT binding. Lanes: 1, Gb_3 standard; 2, GC standard; 3, HFB18.5 cells treated with Gb_3 liposomes; 4, HFB18.5 cells treated with GC liposomes; 5, HFB18.5 cells treated with PBS.

when a total of 2.0% of the exogenous Gb_3 became cell associated, giving a concentration of 0.5 μg of exogenous glycolipid per 10^6 cells.

Binding and Internalization of VT1. Toxin binding and subsequent internalization were monitored by FACS. Wild-type Daudi cells efficiently bound VT at 4°C that was internalized on warming the cells to 37°C (Fig. 2*A*), as monitored by the dramatic shift in the intensity of fluorescence at this temperature. Some low-intensity residual surface bound toxin was observed at 37°C. The VT20 toxin-resistant receptor-deficient mutant cell line, however, did not bind the toxin at either temperature (Fig. 2*B*). When these cells were reconstituted with Gb_3, significant reproducible cell surface binding of the toxin was observed (Fig. 2*C*). Toxin binding to reconstituted cells was not as effective as to wild-type cells (Fig. 2*A*, cf. Fig. 2*C*) but the profiles after internalization are identical. Reconstitution of the mutant cells with DGDG, a glycerolipid analogue of Gb_3, which does not bind VT1 *in vitro* (11), resulted in no alteration in VT binding to the mutant VT20 cells (Fig. 2*D*).

Cytotoxicity. Protein synthesis in VT20 cells was found to be highly resistant to inhibition in the presence of VT1. VT20 mutant cells reconstituted with Gb_3 were now found to be sensitive to the cytopathology of VT, and protein synthesis was inhibited by 50% with a VT dose between 10 and 100

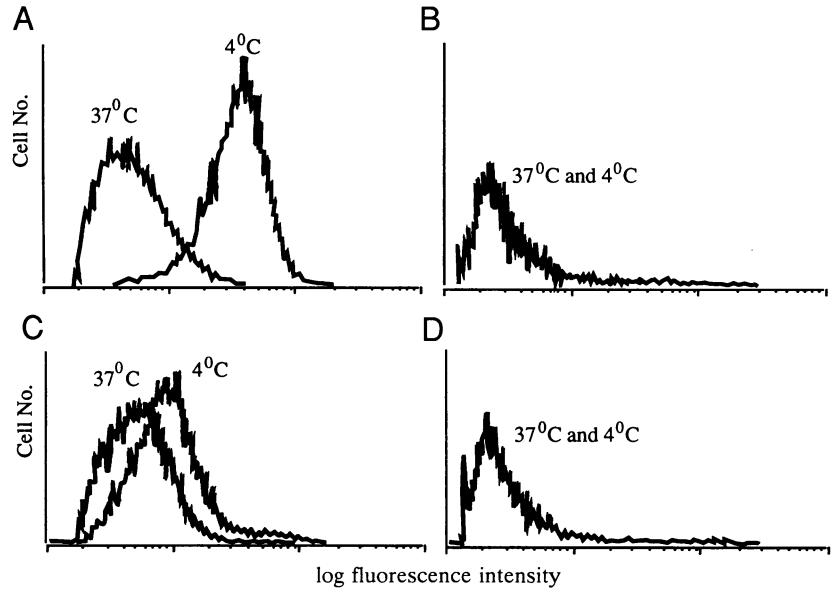

FIG. 2. Binding and internalization of VT by Daudi, VT20, and glycolipid-reconstituted VT20 cells. Cells were analyzed by FACS after toxin binding at 4°C. Loss of bound toxin due to internalization was observed after subsequent incubation at 37°C. (*A*) Wild-type Daudi cells. (*B*) VT20 cells. (*C*) Gb_3-treated VT20 cells. (*D*) DGDG-treated VT20 cells.

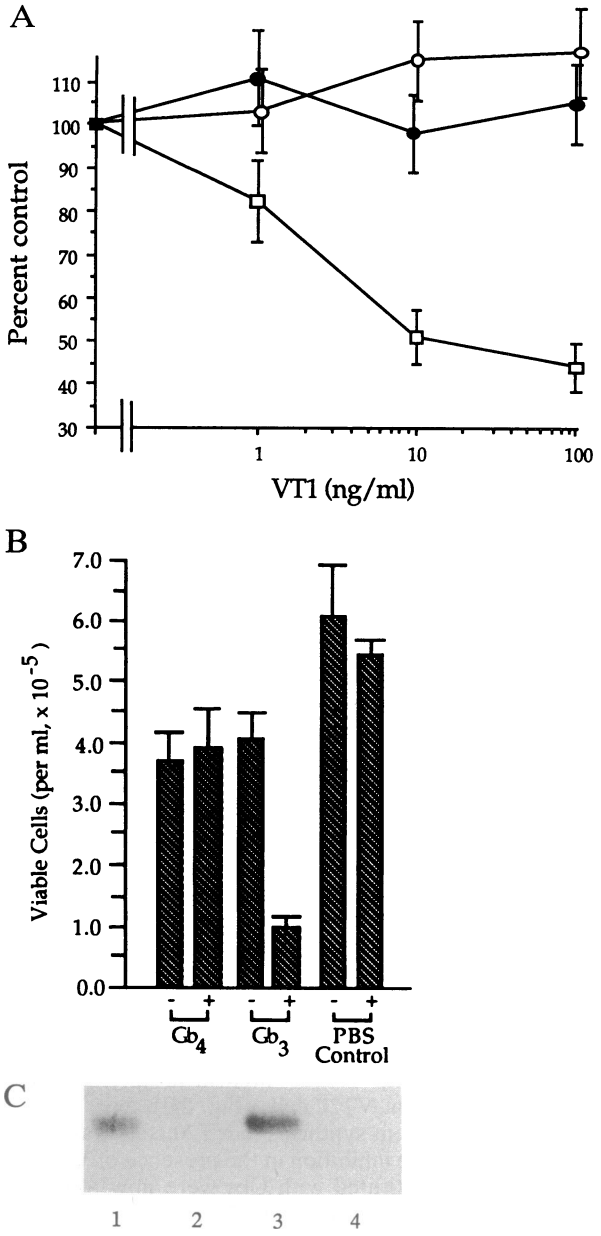

FIG. 3. Assay of VT cytotoxicity on reconstituted cells. (*A*) Glycolipid-reconstituted VT20 cells were incubated in the presence of VT for 48 hr and pulse-labeled for 4 hr with [^{35}S]methionine to measure protein synthesis. The average of duplicate determinations is shown. ●, VT20; □, Gb_3-reconstituted VT20; ○, DGDG-reconstituted VT20. (*B*) HFB18.5 and glycolipid-supplemented HFB18.5 cells, as indicated, were cultured with (+) or without (−) VT1 and the viable cell density was measured. Average of triplicate determinations and standard error is shown. (*C*) VT binding by TLC overlay. Lanes: 1, 0.2 nmol of Gb_3 standard; 2, Gb_4-reconstituted cells; 3, Gb_3-reconstituted cells; 4, PBS control-treated cells.

ng/ml (Fig. 3*A*). Cells supplemented with DGDG remained insensitive to VT1. The growth of HFB18.5 cells supplemented with Gb_3 was prevented in the presence of VT1 at 1.0 μg/ml (Fig. 3*B*). The growth of PBS control or HFB18.5 cells incorporated with Gb_4, which does not bind VT1 (11), was unaffected by VT1 at 1.0 μg/ml. VT was shown to bind only to the glycolipid extract of Gb_3-treated HFB18.5 cells.

DISCUSSION

VT shows a unique cell selectivity *in vitro* (16). Green monkey kidney (Vero) cells have been routinely used to assay for this toxin in experimental (11) and clinical (6) investigations. HeLa cells (9) and some cells of the B lineage are also sensitive (17). We have shown Gb_3 is the only VT1-binding glycolipid in Vero cells (12) and that Gb_3 is present in all sensitive and absent in all insensitive cell lines studied thus far (17), although the degree of sensitivity is not proportional to the Gb_3 content. The strongest evidence for a functional role for Gb_3 in VT cytopathology has been, however, the specific deletion of this glycolipid in mutant Daudi cells selected for resistance to VT1 (10).

The nature of the target cells *in vivo* has been a question of some speculation. The histopathology of the lesions observed in HUS is consistent with a primary effect on the endothelial cells of the renal vasculature (18, 19). Indeed, human endothelial cells are sensitive to this toxin in culture (20) and Gb_3 is indeed expressed on these cells (T. Obrig, A. Branca, C.A.L., P. Del Vecchio & T. Moran, unpublished work). We also found that Gb_3 is the major neutral glycolipid of the human kidney, particularly the cortex (22), which is the major site of renal lesions in HUS (23). Renal pathology is not observed in animals infected with VT-producing *E. coli* (24) and significant levels of Gb_3 have not been found in animal kidneys (22). Certain cells of the human B lineage also contain Gb_3 and are sensitive to VT1 (17) whereas T cells do not contain this receptor and are resistant.

These studies provide the rationale for our present attempts to induce VT sensitivity by using Gb_3.

VT20 cells were chosen since these cells had been specifically selected for resistance to VT1 from a highly sensitive parental cell line and showed a lack of VT1 surface receptors and a specific decrease in the level of Gb_3 (10). If this decrease were the primary mutation, it follows that the machinery for protein synthesis would be sensitive to VT1 inhibition should the receptor defect be experimentally corrected. HFB18.5 cells are also of the human B-cell lineage and were chosen to demonstrate the generality of Gb_3 induction of VT1 sensitivity. HFB18.5 cells contain no Gb_3 detectable by orcinol spray or by the more sensitive VT1 TLC overlay procedure (Fig. 1) [traces of Gb_3 are detectable in VT20 cells by this procedure (10)]. HFB18.5 cells are resistant to very high doses of VT1. If availability of membrane glycolipid receptor is the only factor that restricts sensitivity to this toxin, incorporation of Gb_3 into HFB18.5 cells should also result in the induction of sensitivity in these cells.

Initial attempts to alter the VT1-resistant VT20 phenotype by using purified Gb_3 alone were unsuccessful. Gb_3 liposomes containing PE and PS were chosen as a more effective vehicle for the reconstitution of the VT20 cells due to the "fusigenic" potential of these phospholipids that results from their tendency to form the HII hexagonal phase in membranes, particularly in the presence of calcium ions (25). By using this system, we were able to incorporate up to 0.5 μg of Gb_3 per 10^6 cells without adverse effect on the cell viability. This represents a level 5-fold higher than the original wild-type Daudi cells (17). However, at least 50% of this Gb_3 will be exposed to the cytosolic compartment of the cell. Indeed this value will be higher since our liposomes contain a multilammellar fraction. Although such "mislocated" Gb_3 does not cause a major perturbation of cell physiology, it might interfere with the intracellular routing of internalized toxin.

Our results show that the Gb_3-reconstituted VT20 cells, although clearly able to bind the toxin (Fig. 2), do not bind toxin as effectively as wild-type cells, but internalization of bound toxin is as in wild-type cells. This suggests that the process of receptor-mediated endocytosis is maintained in the reconstituted membrane. It is probable that the long period of culture after the addition of Gb_3 liposomes has allowed the functional integration of the exogenous glycolipid. Indeed attempts to demonstrate internalization of

bound VT 2 hr after glycolipid supplementation of VT20 cells were largely unsuccessful (data not shown).

Since the toxin could be effectively internalized in the reconstituted cells, it was expected that the Gb_3-reconstituted VT20 cells would now be susceptible to VT1 cytotoxicity. This was found to be the case. Wild-type Daudi cells are killed at toxin concentrations as low as 0.01 ng/ml (10). VT20 mutants are resistant to toxin concentrations up to 1.0 μg/ml (10). Although protein synthesis in DGDG-reconstituted VT20 cells was unaffected for cells cultured in the presence of VT1, Gb_3-reconstituted cells were markedly inhibited (Fig. 3*A*). A 50% reduction in protein synthesis was observed with VT1 at 100 ng/ml. The demonstration that Gb_3-supplemented HFB18.5 cells become sensitive to VT1 at 1.0 μg/ml (Fig. 3*B*) strongly indicates that the availability of cell surface Gb_3 alone regulates sensitivity to this toxin. Glycolipid-treated cells were found to grow slightly less well than the controls. Nevertheless, in both cases our results conclusively demonstrate that the glycolipid Gb_3 alone can function as the cell surface receptor to mediate the cytopathology of this toxin. Incorporation of Gb_4 into HFB18.5 cells has no effect on sensitivity to VT1, verifying our results (11, 12) that VT1 does not bind to this glycolipid. Thus in this case, the results of TLC overlay binding assays genuinely reflect the ability of glycolipids to function as receptors within the plasma membrane.

Evidence for the ability of plasma membrane glycolipids to function as cell membrane receptors for biological ligands is limited. The best studied example is that of the interaction of cholera toxin with membrane GM1 ganglioside in sensitive cells (26). Although such binding also cannot be considered as physiological, it is proposed that the toxin mimics some endogenous signaling pathway. The toxin-binding results in the ADP-ribosylation of adenylate cyclase and the subsequent elevation of intracellular cAMP levels (27). For VT, however, cAMP has not been shown to be involved in the mechanism of cytotoxicity. Cytotoxicity is by a direct enzymatic effect of the A subunit upon the 28S RNA of the 60S ribosome (28).

Cells unresponsive to cholera toxin can be rendered sensitive by incorporation of exogenous GM_1 into the cell membrane (29). GM_1, however, is water soluble and reconstitution is more easily accomplished than with the VT1 receptor. As for VT1 (9), it is the cholera toxin B subunit, which contains the receptor binding site (30). In cholera toxin, five B subunits are involved in receptor binding (30). It is likely that a similar model would apply to VT1. Other examples of specific protein glycolipid interactions include several proteins that show affinity for sulfatide (31) and our own work on sulfogalactolipid binding proteins in the testis (32).

The ability to introduce significant long-lived levels of neutral glycolipids into cultured cells so that function is maintained provides a useful adjunct for the study of the function of these membrane components in cell physiology.

Our present results demonstrate that Gb_3 is a functional receptor for VT1 and suggest that the mechanism of internalization of bound toxin is due to aggregation of receptor-bound toxin, probably a result of crosslinking due to the multivalency of the toxin, followed by coalescence at one site on the cell surface and subsequent internalization, perhaps by a "lipid flow" process (33). This would be in part consistent with the work of Sandvig *et al.* (21) that demonstrated internalization of Shiga toxin by way of coated pits.

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