Two Distinct Gb3/CD77 Signaling Pathways Leading to Apoptosis Are Triggered by Anti-Gb3/CD77 mAb and Verotoxin-1*

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Globotriosylceramide (Gb3), a neutral glycosphingolipid, is the B-cell differentiation antigen CD77 and acts as the receptor for most Shiga toxins, including verotoxin-1 (VT-1). We have shown that both anti-Gb3/CD77 mAb and VT-1 induce apoptosis in Burkitt’s lymphoma cells. We compared the apoptotic pathways induced by these two molecules by selecting cell lines sensitive to only one of these inducers or to both. In all these cell lines (including the apoptosis-resistant line), VT-1 was transported to the endoplasmic reticulum and inhibited protein synthesis similarly, suggesting that VT-1-induced apoptosis is dissociated from these processes. VT-1 triggered a caspase- and mitochondria-dependent pathway (rapid activation of caspases 8 and 3 associated with a loss of mitochondrial membrane potential (ΔΨm) and the release of cytochrome c from mitochondria). In contrast, the anti-Gb3/CD77 mAb-induced pathway was caspase-independent and only involved partial depolarization of mitochondria. Antioxidant compounds had only marginal effects on VT-1-induced apoptosis but strongly protected cells from anti-Gb3/CD77 mAb-induced apoptosis. VT-1 and anti-Gb3/CD77 mAb-treated cells displayed very different features on electron microscopy. These results clearly indicate that the binding of different ligands to Gb3/CD77 triggers completely different apoptotic pathways.

Globotriasoylceramide (Gb3: Galα1→4Galβ1→4Glcβ1→Cer), a neutral glycosphingolipid ( GSL), has been identified as three different entities according to the cell type on which it is found. On erythrocytes, it constitutes the rare P blood group antigen (1). On lymphocytes, it constitutes the CD77 differentiation antigen and its expression is limited to a subset of marginal center B cells (2, 3). On endothelial cells, it serves as the natural receptor for bacterial toxins of the Shiga family (Stx) (4). The function of Gb3/CD77 has been extensively investigated. We previously reported that the ligation of Gb3/CD77 by specific monoclonal antibodies (mAb) induces apoptosis in Burkitt’s lymphoma (BL) cells, the tumoral counterparts of Gb3/CD77(+) germinal center B lymphocytes. We have also demonstrated that this apoptosis involves a rapid, sustained increase in intracellular Ca2+ concentration, a rapid, transient increase in cAMP levels, protein kinase A activation, and, finally, ceramide accumulation (5, 6).

Because Gb3/CD77 is the Shiga toxin receptor, numerous studies have analyzed the role of these toxins and their receptor in pathogenesis (reviewed in Ref. 7). In humans, infection by Stx-producing organisms (some strains of Escherichia coli and Shigella dysenteriae type 1) causes gastrointestinal diseases and the more serious hemolytic uremic syndrome, both of which are responsible for large numbers of deaths (reviewed in Refs. 4 and 8). Stx (also called verotoxins (VT) because the E. coli toxins were originally described to be cytotoxic to Vero cells (8)) are bipartite molecules comprised of an A catalytic subunit and five B subunits that specifically bind Gb3/CD77. The A subunit has RNA N-glycosidase activity; it removes an adenosine base from 28 S RNA, thereby inhibiting protein synthesis and killing target cells (9). Several recent studies have demonstrated that these toxins also induce apoptosis in various cell types. These studies have led to an ongoing controversy about the relationship between apoptosis and cell death due to protein synthesis inhibition (10–13). Studies of Stx and Gb3/CD77 have also helped to elucidate intracellular transport events (14, 15). It has been shown that the targeting of the toxin to a specific intracellular transport pathway can be determined by the Gb3/CD77 isofrom expressed on the cell surface and by the presence or absence of Gb3/CD77 in the lipid raft microdomains of the membrane (16, 17).

In this study, we investigated the nature of the apoptotic signals mediated by Gb3/CD77. Recent reports have shed light on both toxin- and anti-Gb3/CD77 mAb-induced apoptotic pathways (6, 18, 19), but various questions remain unresolved: Is ligand binding to Gb3/CD77 sufficient to induce apoptosis? Do ligands trigger a single intracellular mechanism? What is the relationship between protein synthesis inhibition and apoptosis for toxins? We investigated these issues by comparing the apoptotic mechanisms induced by anti-Gb3/CD77 mAb and verotoxin-1 (VT-1) in various BL cells. Cellular toxicity was strictly dependent on the expression of Gb3/CD77 on the cell surface in both cases, but some cell lines strongly expressing Gb3/CD77 were killed by both inducers, whereas others were...
only killed by one. Our results indicate that these two ligands trigger different signaling pathways: VT-1-induced apoptosis involves caspase activation and mitochondrial depolarization, whereas oxidative stress seems to mediate anti-Gb3/CDD7 mAb-induced cell death. Finally, the ability of VT-1 to induce apoptosis was not correlated with the transport of this molecule to the endoplasmic reticulum (ER) or with the inhibition of protein synthesis.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—All cell lines used in this study were originally established from endemic or sporadic cases of Burkitt’s lymphoma. The Ramos BL cell line was obtained from ATCC (Rockville, MD), whereas the Namalwa and P3HR1 cell lines were kindly provided by Prof. G. Klein (Stockholm, Sweden) and the BL2 cells by Prof. G. Lenoir (Lyons, France). These cell lines were cultured in RPMI 1640 medium (Invitrogen, France) containing 2 mM L-glutamine, 1 mM sodium pyruvate, 20 mM glucose, 100 units/ml penicillin, and 100 µg/ml streptomycin, and supplemented with 5% heat-inactivated fetal calf serum. Gb3/CDD7 (–) Ramos cells were obtained after 10 days of culture in media containing 2 µM PPMP (1-phenyl-2-palmitylamino-3-morpholino-1-propanol HCl), a glucosylceramide synthase inhibitor causing reversible GSL degradation (25). Gb3/CDD7 (+) Ramos cells were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA), the anti-cytochrome c mAb (clone 7H8.2C12) was from BD Pharmingen (Palo Alto, CA), the anti-PARP mAb (Ab-2, clone c-2-10) and the anti-caspase-8 mAb (Ab-3, clone 1-3) were from Oncogene Research Products (Boston, MA). The anti-human IgM (F(ab’2) fragment) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA), the anti-cytochrome c mAb (clone 7H8.2C12) was from BD Pharmingen (Palo Alto, CA), the anti-PARP mAb (Ab-2, clone c-2-10) and the anti-caspase-8 mAb (Ab-3, clone 1-3) were from Oncogene Research Products (Boston, MA). The goat anti-mouse IgM (GAM), used to cross-link 1A4 mAb in apoptosis experiments, was obtained from Pierce (Pleasant Gap, PA). Recombinant Goat anti-mouse IgM (GAM), used to cross-link 1A4 mAb in apoptosis experiments, was obtained from Pierce (Pleasant Gap, PA). Recombinant goat anti-mouse IgG plus IgM, used for FACS analysis, was from Caltag Laboratory (Burlingame, CA).

Reagents—1A4 ascites (mouse monoclonal IgM anti-Gb3/CDD7) was provided by Dr. S. Hakomori (Seattle, WA) (21). IgM were purified with the ImmunoPure IgM purification kit (Pierce, Rockford, IL), according to the manufacturer’s instructions. Purified recombinant verotoxin 1 (VT-1) was kindly provided by Dr. C. Lingwood (Toronto, Canada). The goat anti-human IgM (F(ab’2) fragment) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA), the anti-cytochrome c mAb (clone 7H8.2C12) was from BD Pharmingen (Palo Alto, CA), the anti-PARP mAb (Ab-2, clone c-2-10) and the anti-caspase-8 mAb (Ab-3, clone 1-3) were from Oncogene Research Products (Boston, MA). The goat anti-mouse IgM (GAM), used to cross-link 1A4 mAb in apoptosis experiments, was obtained from Pierce (Pleasant Gap, PA). Recombinant Goat anti-mouse IgM (GAM), used to cross-link 1A4 mAb in apoptosis experiments, was obtained from Pierce (Pleasant Gap, PA).

**Western Blot Analysis**—Cells were lysed in SDS sample buffer (25 mM Tris HCl pH 6.8, 1% SDS, 5% glycerol, 5% β-mercaptoethanol) and were run on a 10% SDS–polyacrylamide gel and transferred to nitrocellulose. The membranes were blocked with 5% non-fat milk powder and incubated for 1 h in a wet chamber with the primary antibody (primary antibody was used at a 1:1000 dilution). The membranes were then washed once with PBS, incubated with HRP-conjugated goat anti-mouse IgG for 1 h and washed again. The membranes were then incubated with HRP-conjugated goat anti-mouse IgG for 1 h and washed again. The membranes were then incubated with HRP-conjugated goat anti-mouse IgG for 1 h and washed again. The membranes were then exposed to X-ray film for 1 min.

**Electron Microscopy**—Cells were fixed in 2% paraformaldehyde in PBS for 15 min, then post-fixed in 2% osmium tetroxide in the same buffer. They were dehydrated in a series of ethanol solutions and propylene oxide, before being embedded in Epon (R.L. Electron microscopy, Rueil-Malmaison, France). The fluorescent dye 3,3′-dihexyloxacarbocyanine iodide (DiOC6 (3)) was obtained from Molecular Probes (Eugene, OR). A stock solution (1 mM in Me2SO) was stored at room temperature. The antibody was dissolved in Z-DEET-FMK (fluoromethylketone) was purchased from Calbiochem (Darmstadt, Germany).

**RESULTS**

**Gb3/CDD7 (–) BL Cells Display Differential Sensitivity to Apoptosis Induced by Cross-linked Anti-Gb3/CDD7 mAb and VT-1**—We investigated the mechanism of Gb3/CDD7-mediated apoptosis by treating a series of Burkitt’s lymphoma cell lines with VT-1 or an anti-Gb3/CDD7 mAb (clone 1A4) cross-linked with a goat anti-mouse antibody (GAM). We selected cell lines that were spontaneously Gb3/CDD7 (+) (Ramos, P3HR1, BL2) or Gb3/CDD7 (–) (Namalwa), and we also generated Gb3/CDD7 (–) cells from cells that were originally positive and Gb3/CDD7 (+) cells from cells that were originally negative. Gb3/CDD7 (–) converted Ramos cells were established by treatment with POMP, a glycolipid biosynthesis inhibitor, and are referred to as Ramos PPPM. Gb3/CDD7 (–) converted Namalwa cells were obtained by stable transfection with the Gb3/CDD7 cDNA (Gb3/CDD7 (–) and (22) and are referred to as Nam/
Gb3(+). Cell surface expression of Gb3/CD77 was evaluated by FACS analysis, using an anti-Gb3/CD77 mAb (clone 1A4). Ramos, P3HR1, BL2, and Nam/Gb3(+) cells strongly expressed Gb3/CD77 (mean fluorescence index (MFI): 228, 214, 165, and 168, respectively) (Fig. 1). The Namalwa cells transfected with the insert-less vector (NampDR2) did not express Gb3/CD77 (MFI: 14), and treatment with PPMP greatly decreased expression of this antigen (MFI: 33) (Fig. 1).

We then assessed the sensitivity of these cell lines to VT-1 and an anti-Gb3/CD77 mAb (Table I). VT-1 and anti-Gb3/CD77 mAb had different effects: apoptosis levels were high in Ramos, BL2, and P3HR1 cells treated with anti-Gb3/CD77 mAb (74 ± 13%, 71 ± 11%, and 89 ± 5%, respectively), whereas Nam/Gb3(+) cells responded only weakly to this treatment (28 ± 4%). In contrast, VT-1 strongly induced apoptosis in Ramos, BL2, and Nam/Gb3(+) cells (98 ± 1%, 96 ± 4%, and 66 ± 7%, respectively), whereas it had almost no effect on P3HR1 cells (11 ± 3%). We investigated whether the resistance of P3HR1 to VT-1-induced apoptosis was dose-dependent by treating these cells with 500 ng/ml and 5 µg/ml VT-1. No apoptosis was observed at either concentration (data not shown). Gb3/CD77(−) NampDR2 and Ramos PPMP cells were not killed by anti-Gb3/CD77 mAb or VT-1 (Table I).

Two of the Gb3/CD77(+) BL cell lines tested (Ramos and BL2) were therefore sensitive to both anti-Gb3/CD77 mAb and VT-1; one cell line (P3HR1) was sensitive to anti-Gb3/CD77 mAb only, and one (Nam/Gb3(+)) was mostly sensitive to VT-1. Thus, the induction of apoptosis by anti-Gb3/CD77 mAb and VT-1 is strictly dependent on cell-surface Gb3/CD77 expression, but this expression is not sufficient to confer sensitivity to these apoptosis inducers.

**Retrograde Transport of the VT-B Subunit Is Similar in All Gb3/CD77(+) BL Cells**—To determine why P3HR1 cells do not undergo apoptosis when treated by VT-1, we investigated the pathway by which verotoxin is taken up by BL cell lines. The retrograde transport of VT to the ER has been shown to be necessary for maximal toxicity (25). We evaluated the uptake of VT into the ER, using a previously described modified VT-B subunit (B-glyc-KDEL) (23). B-glyc-KDEL carries an N-glycosylation site at the C terminus of the VT-B fragment, making it possible to detect its arrival in the ER by monitoring glycosylation by the ER-located oligosaccharyltransferase. After iodinated B-glyc-KDEL had been taken up (at 37 °C for 15 h), we lysed the cells (Ramos, P3HR1, Nam/Gb3(+), NampDR2, and HeLa (used as a positive control)) and analyzed the lysates by high resolution gradient gel (10–20%) electrophoresis and autoradiography. In addition to B-Glyc-KDEL, a band with a lower electrophoretic mobility was detected in HeLa, Ramos, P3HR1, and Nam/Gb3(+) extracts. No such band was detected in Gb3/CD77(−) NampDR2 extracts, in which B-Glyc-KDEL had not been internalized (Fig. 2). Treatment of the cells with tunicamycin, an inhibitor of N-glycosylation, totally abolished this band. Thus, this band corresponds to glycosylated B-Glyc-KDEL. This glycosylated product was quantified with a PhosphorImager: 21.8% of the total B-fragment was glycosylated in Ramos and Nam/Gb3(+) cells (98% and 96%, respectively), whereas it had almost no effect on P3HR1 cells (11 ± 3%).

**Table I**

| Cell line   | Apoptotic cells | GAM | Anti-Gb3 | Medium | VT-1 |
|-------------|-----------------|-----|----------|--------|------|
| CD77(+)     | Ramos           | 9 ± 2 | 74 ± 13 | 10 ± 3 | 98 ± 1 |
|             | BL2             | 9 ± 3 | 71 ± 11 | 9 ± 2  | 96 ± 4 |
|             | P3HR1           | 7 ± 2 | 89 ± 5  | 6 ± 1  | 11 ± 3 |
|             | Nam/Gb3(+)     | 6 ± 2 | 28 ± 4  | 7 ± 1  | 66 ± 7 |
| CD77(−)     | Ramos PPMP      | 6 ± 1 | 8 ± 1   | 6 ± 1  | 13 ± 5 |
|             | NampDR2         | 5 ± 1 | 6 ± 2   | 6 ± 2  | 5 ± 1  |

*Fig. 1. Cell surface expression of Gb3/CD77 antigen in various BL cell lines.* Cells were labeled with an anti-Gb3/CD77 monoclonal antibody (clone 1A4) and FITC-conjugated goat anti-mouse IgG plus IgM (shaded histograms) or with FITC-conjugated goat anti-mouse IgG plus IgM alone (open histograms). Cells were then analyzed with a FACS Calibur flow cytometer.
Gb3/CD77 Antigen Mediates Different Apoptotic Pathways

VT-1 and Cross-linked Anti-Gb3/CD77 mAb Induce a Loss of Mitochondrial Membrane Potential in Different Ways—Mitochondria are a key element in many apoptotic pathways (reviewed in Ref. 26). We therefore measured mitochondrial membrane potential (Δψ<sub>m</sub>) before and after treatment of the cells with VT-1 or anti-Gb3/CD77 mAb. We measured Δψ<sub>m</sub> by cytofluorimetry, using the carbocyanine dye DiOC<sub>6</sub>(3). In Ramos cells, VT-1 induced a loss of Δψ<sub>m</sub> after 4 h of treatment, which gradually increased until 16 h, when mitochondrial membrane depolarization was observed in all cells (Fig. 5A). In Nam/Gb3(<sup>+</sup>) cells, the loss of Δψ<sub>m</sub> was delayed, and only 18% of cells were DIOC<sub>6</sub>(3)<sup>low</sup> after 8 h of treatment. However, Δψ<sub>m</sub> loss then rapidly increased and 66% of cells were DIOC<sub>6</sub>(3)<sup>low</sup> after 24 h of treatment. No Δψ<sub>m</sub> loss was observed in P3HR1 and NampDR2 cells. We investigated whether caspase-8 activation occurred upstream or downstream from mitochondrial perturbation by studying the effects of a caspase-8-specific inhibitor (Z-IETD-fmk) on Δψ<sub>m</sub> loss. Preincubation of the cells with this tetrapeptide inhibited the loss of Δψ<sub>m</sub> induced by VT-1 (after 24 h, 69% of pre-treated Ramos cells were DIOC<sub>6</sub>(3)<sup>low</sup> versus 100% of cells that had not been pre-treated, and 48% of pre-treated Nam/Gb3(<sup>+</sup>) cells were DIOC<sub>6</sub>(3)<sup>low</sup> versus 66% of non-pre-treated cells) (Fig. 5A). Thus, mitochondrial depolarization appears to depend, at least partly, on caspase-8 activation.

We also treated the BL cell lines for the same length of time with anti-Gb3/CD77 mAb. Anti-Gb3/CD77 mAb induced a moderate, sustained loss of Δψ<sub>m</sub> in P3HR1 cells (~30% of the cells were DIOC<sub>6</sub>(3)<sup>low</sup> following 4–24 h of treatment) but had no effect on Δψ<sub>m</sub> in Ramos cells (although these cells are as sensitive as P3HR1 to anti-Gb3/CD77 mAb) or on Δψ<sub>m</sub> in Nam/Gb3(<sup>+</sup>) and NampDR2 cells (Fig. 5B).

VT-1 and Cross-linked Anti-Gb3/CD77 mAb Induce Cytochrome c Release from Mitochondria in Different Ways—During apoptosis, Δψ<sub>m</sub> disruption is generally accompanied by the release of cytochrome c from the mitochondria to the cytoplasm, where it forms complexes with Apaf-1, dATP, and caspase-9. This activates caspase-9, which in turn activates caspase-3. We investigated cytochrome c release following treatment with VT-1 and anti-Gb3/CD77 mAb by treating BL cell lines for various lengths of time (2, 4, 6, 8, 16, and 24 h) and preparing subcellular fractions as previously described (24). Western blot analysis of the cytosolic and mitochondrial fractions was carried out using an anti-cytochrome c mAb (Fig. 6). Cytochrome c levels in the mitochondrial fractions of Ramos cells treated with VT-1 decreased slightly after 4 h of treatment, were only just detectable after 6 h, and were completely undetectable after 8, 16, and 24 h. Conversely, cytochrome c was first detected in the cytosolic fractions of these cells after 2 h of treatment, and its levels gradually increased until 24 h. No changes in cytochrome c levels were observed in the mitochondrial or cytosolic fractions of cells treated with anti-Gb3/CD77 mAb, other than slight changes after 24 h of treatment.
P3HR1 cells treated with VT-1, no significant changes in cytochrome c levels were observed in the mitochondrial or cytosolic fractions. By contrast, cytochrome c became clearly detectable in the cytosolic fraction of these cells after 4 h of treatment with anti-Gb3/CD77 mAb. However, this release appeared to be moderate as no clear decrease in cytochrome c level was observed in the mitochondrial fraction. The cytochrome c levels in the mitochondrial fraction of NamGb3(+) cells treated with VT-1 clearly decreased 4 h after treatment and was accompanied by an increase in cytochrome c levels in the cytosolic fraction. Treatment of these cells with anti-Gb3/CD77 mAb did not change the cytochrome c levels. Thus, VT-1 induces the concomitant release of cytochrome c and loss of Δψm. Our results also show that in P3HR1, but not in Ramos cells, anti-Gb3/CD77 mAb induces a moderate depolarization of mitochondria, together with a release of cytochrome c without caspase activation.

**Cells Treated with VT-1 and Cross-linked Anti-Gb3/CD77 mAb Display Different Morphological Changes**—We used electron microscopy to investigate Ramos cells, which are sensitive

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**TABLE II**

Analysis of protein synthesis after treatment with VT-1

| Cell line          | [3H]Leucine uptake (% of control) | Cycloheximide |
|--------------------|-----------------------------------|---------------|
| Control VT-1       | 103,823 ± 8,161                   | 589 ± 105 (0.6%) | 479 ± 63 (0.5%) |
| Ramos              | 96,904 ± 9,840                    | 989 ± 242 (1%)  | 1,979 ± 275 (2%)  |
| P3HR1              | 82,794 ± 15,505                   | 7,039 ± 758 (8.5%) | 1,568 ± 206 (1.9%) |
| Nam/Gb3(+)         | 89,849 ± 11,863                   | 90,506 ± 9,796 (101%) | 1,357 ± 299 (1.5%) |
| Nam/GD2R2          | 785 (8.5%)                        | 1,568 (101%)    | 1,357 (1.5%)      |

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**FIG. 3.** VT-1, but not anti-Gb3/CD77 mAb, induces caspase-8 activation. Cells were treated with VT-1 or an anti-Gb3/CD77 mAb (clone 1A4 cross-linked with GAM) for various periods of time. Cell pellets were then solubilized, and equal amounts of proteins were subjected to electrophoresis on a 10% polyacrylamide-SDS gel. Proteins were then transferred to PVDF membranes, which were probed with an anti-caspase-8 mAb and a horseradish peroxidase-conjugated rabbit anti-mouse Ig antibody. Antibody complexes were detected by enhanced chemiluminescence.

**FIG. 4.** VT-1, but not anti-Gb3/CD77 mAb, induces cleavage of the PARP protein. Cells were treated with either VT-1 or an anti-Gb3/CD77 mAb (clone 1A4 cross-linked with GAM) for various periods of time. Cell pellets were then solubilized and equal amounts of protein were subjected to electrophoresis on a 10% polyacrylamide-SDS gel. Proteins were then transferred to PVDF membranes, which were probed with an anti-PARP (a target of activated caspase-3) mAb and a horseradish peroxidase-conjugated rabbit anti-mouse Ig antibody. Antibody complexes were detected by enhanced chemiluminescence.
large cytosolic vacuoles. Cells treated with anti-Gb3/CD77 mAb were also small but, unlike VT-1-treated cells, they had lost their rounded shapes, showed less compact chromatin condensation (with more complex, lumpier shapes), and the cytosolic vacuoles were smaller and more numerous. These features are reminiscent of those described by several authors for caspase-independent apoptosis (for review see Ref. 27).

The Antioxidants NAC and GSH Protect BL Cells Strongly from Anti-Gb3/CD77 mAb-induced Apoptosis but Barely from VT-1-induced Apoptosis—The intracellular regulation of redox processes seems to be involved in cell death induced by various stimuli. Several studies have demonstrated that reactive oxygen species (ROS) regulate many important cellular events, including apoptosis (reviewed in Refs. 28 and 29) and that various antioxidants block apoptosis (30). We therefore investigated the effects of two antioxidant agents, NAC and GSH-EE (a cell-permeable form of reduced glutathione), on the percentage of apoptotic cells induced by anti-Gb3/CD77 mAb and VT-1. Because NAC and GSH-EE were toxic to Ramos cells, we used BL2 cells, which are also sensitive to both anti-Gb3/CD77 mAb and VT-1 (see Table I). Pre-treatment of the cells for 2 h with 20 mM NAC partially or almost completely protected cells (BL2, Nam/Gb3(±), and P3HR1 cells, respectively) from the apoptosis induced by anti-Gb3/CD77 mAb, but had no effect (BL2 cells) or only a slight effect (Nam/Gb3(±) cells) on VT-1-induced apoptosis (Fig. 8). Preincubation of P3HR1 and Nam/Gb3(±) cells with 15 mM GSH EE gave similar results, with slightly stronger protection for VT-1-treated Nam/Gb3(±) cells. Thus, ROS are important elements of the apoptotic pathway induced by anti-Gb3/CD77 mAb, whereas they play little or no role in the pathway induced by VT-1.

FIG. 5. Loss of mitochondrial membrane potential ($\Delta \psi_m$) following treatment with VT-1 or anti-Gb3/CD77 mAb. A, cells were either directly treated with VT-1 or incubated with a caspase-8-specific inhibitor (C8i) before being treated with VT-1. B, cells were treated with an anti-Gb3/CD77 mAb (clone IA4 cross-linked with GAM). After various periods of time, cells were stained with DIOC6(3) and analyzed on a FACSCalibur flow cytometer. DIOC6(3) stain mitochondria in a potential-dependent fashion. Cells with a low $\Delta \psi_m$ are therefore poorly fluorescent and are called “DIOC6(3) low.”

to both VT-1- and anti-Gb3/CD77 mAb-induced apoptosis. The morphological features of cells treated with VT-1 (Fig. 7, B and E) and anti-Gb3/CD77 mAb (Fig. 7, C and F) were very different. Cells treated with VT-1 were smaller than control cells (Fig. 7, A and D). They also presented classic heavy condensation of chromatin into globular or crescent-shaped figures, and
VT-1 reaches the ER. The second of these two possibilities is the most likely as treatment of the cells with brefeldin A (a fungal metabolite that provokes disassembly of the Golgi apparatus) inhibits VT-induced apoptosis (13, 37). Our data therefore imply that, in resistant cells, apoptosis is inhibited after delivery of the toxin to the ER.

The efficient inhibition of protein synthesis by VT-1 in P3HR1 cells, in the absence of apoptosis, provides new insight into the relationship between these two processes. Although it remains possible that protein synthesis inhibition triggers apoptosis, our data strongly suggest that these two processes are independent. Furthermore, the rapid activation of caspases and mitochondria in sensitive cells are also consistent with this. Various studies have already addressed this issue and have generated conflicting conclusions: apoptosis induced by the B subunit alone has led some authors to conclude that protein synthesis inhibition and apoptosis are independent (5, 12, 13), whereas another study, in which the VT-1 B chain did not induce apoptosis (10), led to the opposite conclusion. Other studies with VT-1 and cycloheximide also came to conflicting conclusions (11, 38, 39). We are currently comparing the abilities of various protein synthesis inhibitors and VT-1 to induce apoptosis in a panel of Gb3/CD77(−)/H11001 BL cells.

The prior incubation of BL cells with antioxidant compounds strongly protected against anti-Gb3/CD77-induced apoptosis. Oxidative stress has been shown to mediate apoptosis in several independent experiments (reviewed in Ref. 29). In these experiments, oxidants induced apoptosis (40), ROS were generated during apoptosis (41), or antioxidants blocked apoptosis (29, 42). We show here that two antioxidants, NAC and GSH, strongly protected the cells against apoptosis induced by anti-Gb3/CD77 mAb. We also recently observed that preincubation with L-buthioninesulfoximine (a specific inhibitor of GSH synthesis) sensitizes Ramos and P3HR1 cells to low doses of anti-Gb3/CD77 mAb (5 and 15 μg/ml) (data not shown), thereby confirming that GSH is an important regulator of anti-Gb3/CD77-mediated apoptosis.

**Fig. 6.** Release of cytochrome c from mitochondria after treatment with VT-1 or anti-Gb3/CD77 mAb. Cells were treated with either VT-1 or an anti-Gb3/CD77 mAb (clone 1A4 cross-linked with GAM) for various periods of time and then fractionated into mitochondrial and cytosolic fractions as described under “Experimental Procedures.” Equal amounts of each fraction (20 μg of mitochondrial protein and 40 μg of cytosolic protein) were subjected to electrophoresis on a 15% polyacrylamide-SDS gel. Proteins were then transferred to PVDF membranes, which were probed with an anti-cytochrome c mAb and a horseradish peroxidase-conjugated rabbit anti-mouse Ig antibody. Antibody complexes were detected by enhanced chemiluminescence.

**Fig. 7.** Cells treated with VT-1 and anti-Gb3/CD77 mAb displayed different morphological changes. Ramos cells were cultured for 24 h in RPMI 1640 medium alone (A and D), 16 h in the presence of VT-1 (B and E), or 24 h in the presence of an anti-Gb3/CD77 mAb (clone 1A4 cross-linked with GAM) (C and F), and then fixed in isotonic 1.6% glutaraldehyde solution. Electron micrographs of thin, plastic-embedded sections were taken on a Philips EM 400 electron microscope. Bars, 2.7 μm (A–C) and 1 μm (D–F).
CD77-induced apoptosis and substantiating the role of redox reactions in this process.

ROS production and ceramide generation were recently shown to be closely linked: ceramide generation was shown to be a redox-sensitive process (through regulation of the neutral sphingomyelinase) and ROS, produced by mitochondria, were shown to be early mediators of ceramide-induced cell death (43, 44). It was also shown that the addition of ceramide to isolated mitochondria induces cytochrome c release and the loss of \( \Delta \psi_m \) (45). Because ceramide accumulates in BL cells exposed to an anti-Gb3/CD77 mAb (6), we are now conducting experiments to confirm the role of oxidative stress in anti-Gb3/CD77 mAb-induced apoptosis and to determine the contribution of ceramide to this signaling pathway. However, as some glycosphingolipids (GD3 and LacCer) also induce ROS formation, a decrease in \( \Delta \psi_m \), and the release of cytochrome c from mitochondria (46), we are currently testing the effect of Gb3/CD77 on isolated mitochondria.

In conclusion, we demonstrate here that two different apoptotic pathways are triggered depending on the nature of the ligand binding to the Gb3/CD77 glycosphingolipid receptor: a caspase- and mitochondria-dependent pathway or a ROS-dependent pathway.

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Fig. 8. Antioxidants protect BL cells strongly from anti-Gb3/CD77 mAb-induced apoptosis but only slightly from VT-1-induced apoptosis. Cells were incubated for 2 h with medium, N-acetyl-l-cysteine (NAC) or glutathione ethyl ester (GSH-EE, a cell-permeable form of reduced glutathione) and were then treated with VT-1 or an anti-Gb3/CD77 mAb (clone 1A4 cross-linked with GAM). After 24 h, cells were labeled with annexin V-FITC and propidium iodide and analyzed with a FACSCalibur flow cytometer to determine the percentage of apoptotic cells.

Table III

| Cell line  | Gb3/CD77 expression | Apoptosis sensitivity | Transport of VT-B fragment | Protein synthesis inhibition by VT-1 |
|------------|---------------------|-----------------------|---------------------------|-----------------------------------|
| Ramos      | +++                 | +++                   | ++\(^a\)                   | +++                                |
| P3HR1      | +++                 | +++                   | ++                        | +++                                |
| Nam/Gb3(+) | +                   | +                     | +                         | ++\(^a\)                           |
| NampDR2    | –                   | –                     | –                         | –                                  |
| Ramos PPMP | ND\(^b\)            | ND\(^b\)              | ND\(^b\)                  | ND\(^b\)                           |

\(^a\) \( + + \) indicates that the transport of the VT-B fragment to ER was slightly less efficient than in control HeLa cells.

\(^b\) ND, not determined.

Table IV

Characteristics of the apoptotic pathways induced by VT1 and anti-Gb3/CD77 mAb in BL cells

| VT-1 | Anti-Gb3/CD77 mAb |
|------|------------------|
| Caspase activation | ++ |
| Loss of \( \Delta \psi_m \) | ++ |
| Cytochrome c release | ++ |
| Chromatin condensation | ++ |
| Antioxidant protection | ++ |

\(^a\) Low or negative depending on the cell line.
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Two Distinct Gb3/CD77 Signaling Pathways Leading to Apoptosis Are Triggered by Anti-Gb3/CD77 mAb and Verotoxin-1

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