Importance of Glycolipid Synthesis for Butyric Acid-induced Sensitization to Shiga Toxin and Intracellular Sorting of Toxin in A431 Cells

Kirsten Sandvig,^{*†} Øystein Garred,^{*} Ardy van Helvoort,[‡] Gerrit van Meer,[‡] and Bo van Deurs[§]

^{*}Institute for Cancer Research at The Norwegian Radium Hospital, Montebello, 0310 Oslo, Norway;

[†]University of Utrecht, Department of Cell Biology, Medical School AZU H02.314, 3584 CX Utrecht, The Netherlands; and [§]Structural Cell Biology Unit, Department of Anatomy, The Panum Institute, University of Copenhagen, DK-2200 Copenhagen N, Denmark

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The human epidermoid carcinoma cell line A431 becomes highly sensitive to Shiga toxin upon treatment with butyric acid. This strong sensitization (>1000-fold) is accompanied by an increase in the fraction of cell-associated toxin transported to the Golgi apparatus and to the endoplasmic reticulum (ER). Furthermore, our previous work showed that the length of the fatty acyl chain of Gb3, the Shiga toxin receptor, also was changed (longer fatty acids). We have now investigated the importance of this change by testing whether glycolipid synthesis is required for the changed intracellular sorting and the toxin sensitivity. We demonstrate here that inhibition of glycosphingolipid synthesis by inhibition of *N*-acyltransferase with fumonisin B₁, by inhibition of glucosylceramide synthetase by PDMP or PPMP, or by inhibition of serine palmitoyl transferase by β -fluoroalanine, inhibited the butyric acid-induced change in sensitivity and the increase in the fraction of cell-associated Shiga toxin transported to the Golgi apparatus and the ER. The block in butyric acid-induced sensitization caused by β -fluoroalanine could be abolished by simultaneous addition of sphinganine or sphingosine. Thus, the data suggest that the fatty acyl chain length of glycosphingolipids is important for intracellular sorting and translocation of Shiga toxin to the cytosol.

INTRODUCTION

Shiga toxin, a glycolipid-binding toxin, is of interest not only in connection with disease, but the toxin has proven valuable as a tool to study intracellular traffic. Shiga toxin was the first molecule shown to be transported all the way from the cell surface, through the Golgi apparatus and to the endoplasmic reticulum (ER) and the nuclear membrane (Sandvig *et al.*, 1992b, 1994). This toxin, which is produced by *Shigella dysenteriae*, belongs to a group of toxins consisting of two moieties, one that binds to cell surface receptors and another that enters the cytosol and inhibits protein synthesis enzymatically (for review, Olsnes and Sand-

vig, 1988; O'Brien *et al.*, 1992). The binding moiety in Shiga toxin consists of five small B subunits that bind specifically to the glycosphingolipid receptor Gb3 (Jacewicz *et al.*, 1986, 1994; Cohen *et al.*, 1987; Lindberg *et al.*, 1987). After binding, the toxin is endocytosed from clathrin-coated pits (Sandvig *et al.*, 1989, 1991b), and in a number of cells a fraction of the toxin is transported to the trans-Golgi network (TGN) (Prydz *et al.*, 1992; Sandvig *et al.*, 1992a,b; Garred *et al.*, 1995a). There are different lines of evidence suggesting that transport to the Golgi apparatus is required for intoxication; for instance, the drug brefeldin A, which disrupts the Golgi cisterns (Lippincott-Schwartz *et al.*, 1989), protects against Shiga toxin as well as several other protein toxins (Sandvig *et al.*, 1991a; Yoshida *et al.*, 1991). Furthermore, overexpression of GTPase mu-

[†] Corresponding author.

tants of Rab1, Sar1, and Arf1 in HeLa cells protects against Shiga-like toxin 1, suggesting that transport in the ER/Golgi area is important for intoxication (Simpson *et al.*, 1995). It has been speculated that Shiga toxin and protein toxins such as ricin, pseudomonas exotoxin A, and modeccin may be translocated to the cytosol from the ER after retrograde transport from the TGN (Pelham *et al.*, 1992; Sandvig *et al.*, 1992b). However, it was only recently shown that such retrograde toxin transport can take place (Sandvig *et al.*, 1992b, 1994; Khine and Lingwood, 1994). Retrograde transport of Shiga toxin was first demonstrated in butyric acid-treated A431 cells, which concomitantly with the retrograde transport became very sensitive to the toxin, supporting the idea that retrograde transport is important for intoxication (Sandvig *et al.*, 1992b). Retrograde transport to the ER in A431 cells can also be induced by treatment with 8-Br-cAMP or agents that increase the intracellular level of cAMP (Sandvig *et al.*, 1994). Recently, such retrograde toxin transport was demonstrated in the toxin-sensitive cell line T47D even without any pretreatment (Garred *et al.*, 1995a). Thus, retrograde transport to the ER could be a process that is under regulation by physiologically important molecules such as hormones or growth factors. In A431 cells, butyric acid not only induced increased toxin transport to the Golgi apparatus, retrograde transport to the ER, and a >1000-fold sensitization to Shiga toxin, but there also was a change in the fatty acid composition of the receptor (Sandvig *et al.*, 1994). After butyric acid treatment, the receptors contained longer fatty acids than in the untreated cells. There was also a small (two- to threefold) increase in toxin binding to the cells. In this paper, we have addressed the question of whether the changed lipid composition is important for the induced toxin sensitivity and the changed routing in the butyric acid-treated cells. We have inhibited different steps of glycosphingolipid synthesis (Figure 1), and the results shown below demonstrate that when glycosphingolipid synthesis is inhibited during incubation with butyric acid, the strong sensitization to Shiga toxin, the increased Shiga toxin binding, the increased fraction of toxin found in the Golgi apparatus, and the appearance of Shiga toxin in the ER (visualized by using Shiga-horseradish peroxidase [HRP]) were abolished. The data suggest that glycolipid composition is important for the fraction of toxin transported to the Golgi apparatus and the ER as well as for the sensitivity to Shiga toxin.

MATERIALS AND METHODS

Materials

Fumonisin B₁, HRP type VI, pronase, diaminobenzidine, SPDP (3-[2-pyridyldithiol]-propionic acid *N*-hydroxysuccinimide ester), sphingosine, sphinganine, HEPES, and Tris were obtained from Sigma

(St. Louis, MO). DL-PDMP (DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol) and DL-PPMP (DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol), and L-cycloserine were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Nycodenz was obtained from NYCOMED (Oslo, Norway); [³H]leucine and Na¹²⁵I were from the Radiochemical Center (Amersham, UK). L-[³H(G)]serine (802.9 GBq/mmol) was from New England Nuclear Products (Boston, MA). β-Fluoroalanine was a gift from Merck Research Laboratories (Rahway, NJ); Furin was a gift from Dr. G. Thomas, Vollum Institute, Oregon Health Sciences University (Portland, OR); Shiga toxin was provided by Drs. J.V. Kozlov (Academy of Sciences of Russia, Moscow, Russia) and J.E. Brown (USAMRIID, Fort Detrick, MD), and Shiga toxin subunit B was a generous gift from Dr. J.E. Brown. Conjugates of Shiga toxin and Shiga toxin B subunit with HRP were prepared by the SPDP method as described previously (van Deurs *et al.*, 1986). Iodination of toxins and transferrin was performed by the iodogen method (Fraker and Speck, 1978).

Cells

A431 cells were from the American Tissue Type Collection (Rockville, MD). A431 cells were grown in Costar 3000 flasks (Costar, Badhoevedorp, The Netherlands). The medium used was DMEM (3.7 g/l sodium bicarbonate) (Flow Laboratories, Irvine, Scotland) containing 10% fetal calf serum (Life Technologies, Paisley, Scotland) and 2 mM L-glutamine (Life Technologies).

Measurement of Cytotoxic Effect

The cells were incubated with toxins as described in the figure legends, and at the end of the incubation, protein synthesis was measured in the following way: The medium was removed, and the cells were incubated in a leucine-free HEPES-containing medium (MEM with 20 mM HEPES and without bicarbonate) supplemented with 1 μCi of [³H]leucine per milliliter for 10 min at 37°C. Then the medium was removed, the cells were washed twice with 5% (wt/vol) trichloroacetic acid and solubilized in KOH (0.1 M) for a minimum of 30 min at room temperature, and the radioactivity was measured. The differences between duplicates in such experiments were <10% of the average value, and representative experiments are shown in the figures.

Endocytosis of ¹²⁵I-Transferrin and ¹²⁵I-Ricin

Internalization of transferrin was measured as described by Ciechanover *et al.* (Ciechanover *et al.*, 1983), whereas endocytosis of ¹²⁵I-labeled ricin was measured as the amount of toxin that could not be removed with lactose (Sandvig and Olsnes, 1979).

Subcellular Fractionation of A431 Cells Incubated with ¹²⁵I-Shiga Toxin

¹²⁵I-Shiga toxin (~100 ng/ml) was added to A431 cells seeded at a density of 1.3×10^4 cells/cm² in 75-cm² Costar flasks and preincubated for 22 h with and without 1.5 mM butyric acid or butyric acid and fumonisin B₁ (10 μM). The toxin was continuously present during the incubation (2 h at 37°C), and HRP (0.5 mg/ml) was also added to label endosomal/lysosomal compartments. The fractionation was performed essentially as described by Sandberg *et al.* (1980), but with some modifications (Sandvig *et al.*, 1991a).

Enzyme Analysis

HRP was measured according to Steinman *et al.* (1976); UDP-galactose:glycoprotein galactosyl transferase according to Brändli *et al.* (1988); and β-N-acetyl-glucosaminidase according to Beaufay *et al.* (1974).

Lipid Analysis

A431 cells (6.5×10^5 cells/dish with a diameter of 10 cm) were incubated with and without fumonisin B₁ (10 μ M) or PDMP (1 μ M) and with and without butyric acid (2 mM) for 30 min at 37°C. Then L-[³H(G)]serine (5 ml with 2 μ Ci/ml medium) was added to each dish, and the cells were incubated for 20 h in a CO₂ incubator. The cells were then trypsinized, pelleted, and lyophilized. Lipids were extracted from the lyophilized cells according to Bligh and Dyer (Bligh and Dyer, 1959) and separated on borate-impregnated TLC plates in two dimensions: I, chloroform/methanol/25% NH₄OH/water (65:35:4:4; v/v) and II, chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5; v/v). Radiolabeled spots were visualized by fluorography, identified, and quantified as before (van der Bijl *et al.*, 1995).

Processing for Electron Microscopy

A431 cells grown as monolayers in T-25 flasks were treated, as described in the figure legends, and fixed with 2% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.2, for 60 min at room temperature. The cells were then carefully washed with PBS and incubated with diaminobenzidine and H₂O₂ as described previously (van Deurs *et al.*, 1993). The cells were postfixed with OsO₄, treated with 1% uranyl acetate in distilled water, embedded in Epon, cut at 50 nm, and examined in a JEOL 100 CX electron microscope without further contrasting as described previously (van Deurs *et al.*, 1993).

RESULTS

Fumonisin B₁ Inhibits Butyric Acid-induced Sensitization of A431 to Shiga Toxin

To test whether glycolipid synthesis is essential for butyric acid-induced sensitization of A431 cells to Shiga toxin, we first incubated the A431 cells with and without butyric acid and fumonisin B₁, an inhibitor of N-acyltransferase (Figure 1), for 22 h before toxin sensitivity was tested. As shown in Figure 2A, fumonisin B₁ completely inhibited the sensitization obtained with butyric acid alone. Lipid analysis of cells incubated with and without fumonisin B₁ and [³H]serine to label lipids demonstrated that fumonisin B₁ as expected decreased the synthesis of glucosylceramide in A431 cells by >90% (our unpublished results). Also, as shown in Figure 2B, fumonisin B₁ did not seem to have any toxic effect on the cells, and the protein synthesis was not reduced by the presence of this compound during the 26 h of this experiment. Butyric acid alone reduced the protein synthesis somewhat, but addition of fumonisin B₁ in addition to butyric acid gave no further reduction. Fumonisin B₁ does not in itself block the intoxication with Shiga toxin. When the compound was added 30 min before Shiga toxin, the cells that had been sensitized with butyric acid were still sensitive. Thus, ongoing lipid synthesis does not in itself seem to be essential for intoxication. This is supported by the finding that T47D cells, which are very sensitive to Shiga toxin, were equally sensitive after 24 h in the presence of fumonisin B₁ (our unpublished results).

The finding that A431 cells remain resistant to Shiga toxin when fumonisin B₁ is added in addition to butyric acid is not due to reduced endocytosis in these cells. Thus, both ricin endocytosis, which occurs both by clathrin-dependent and -independent mechanisms (Sandvig and van Deurs, 1994a), and transferrin uptake (Ciechanover *et al.*, 1983), which like Shiga toxin uptake occurs from clathrin-coated pits, are not reduced by fumonisin B₁ treatment (our unpublished results). Furthermore, both electron microscopy (EM) and cell-fractionation experiments confirmed that Shiga toxin is endocytosed in fumonisin B₁-treated cells (see below). Also, ricin is still able to intoxicate these cells (our unpublished results).

Proteolytic processing of Shiga toxin A-fragment appears to be important for rapid intoxication of cells (Garred *et al.*, 1995a,b). However, control experiments revealed that neither butyric acid treatment nor fumonisin B₁ treatment or the combination of the two af-

SPHINGOLIPID BIOSYNTHESIS

Figure 1. Sites of action of inhibitors of sphingolipid synthesis. Serine palmitoyl transferase is inhibited by β-fluoroalanine (Radin and Inokuchi, 1991; Merrill *et al.*, 1993; Schroeder *et al.*, 1994), N-acyltransferase is inhibited by fumonisin B₁ (Merrill *et al.*, 1993; Schroeder *et al.*, 1994), and glucosylceramide synthetase is inhibited by PDMP and PPMP (Radin and Inokuchi, 1991; Abe *et al.*, 1992; Barbour *et al.*, 1992).

Figure 2. Fumonisin B₁ inhibits butyric acid-induced sensitization to Shiga toxin in A431 cells. A431 cells growing in 24-well disposable trays were incubated for 22 h with and without butyric acid (1.5 mM) and fumonisin B₁ (10 μ M), or fumonisin B₁ (10 μ M) alone. Then increasing concentrations of Shiga toxin were added, and protein synthesis was measured as described in MATERIALS AND METHODS 4 h later. (A) Toxin-induced inhibition of protein synthesis. (B) Incorporation of [³H]leucine in cells pretreated under different conditions for 22 h. The error bars show deviations between duplicates.

affected cellular processing of the A-fragment (our unpublished observations). Also, the results with butyric acid sensitization and fumonisin B₁ were similar when Shiga toxin pretreated with a soluble form of furin (Garred *et al.*, 1995b) was used, supporting the idea

that the effects obtained are independent of toxin processing.

The effect of fumonisin B₁ on Shiga toxin sensitivity was measured after 1 d instead of 2 d with butyric acid (maximal sensitization; Sandvig *et al.*, 1992b, 1994) in the experiment shown in Figure 2, because after longer periods of time not only will the appearance of new receptors be inhibited by fumonisin B₁, but at the same time the number of the original receptors will be reduced by turnover (see below).

Fumonisin B₁ Inhibits the Butyric Acid-induced Increase in Shiga Toxin Binding to A431 Cells

Because fumonisin B₁ inhibits sphingosine and sphinganine *N*-acyltransferase, addition of this compound together with butyric acid might reduce the butyric acid-induced increase in Shiga toxin binding. As shown in Figure 3, that was indeed the case. Butyric acid induced a time-dependent increase in Shiga toxin binding, an increase that was essentially abolished by fumonisin B₁ (Figure 3, A and B). It is important to note that after 1 d, the binding of Shiga toxin to cells treated with fumonisin B₁ and butyric acid was not lower than binding to control cells. Thus, the lack of sensitivity to Shiga toxin shown in Figure 2 is not due to lack of receptors. As also shown in Figure 3, fumonisin B₁ alone gives a slight reduction in the binding of Shiga toxin compared with control cells even after 1 d, and the binding to fumonisin B₁-treated cells is clearly lower than to cells treated with the combination of butyric acid and fumonisin B₁. After a 44 h incubation in the presence of fumonisin B₁ and butyric acid, the binding of Shiga toxin is strongly reduced compared with control cells, and the binding is almost as low as in cells treated with fumonisin B₁ alone. Control cells will at the end of the experiment bind more toxin than at the beginning of the experiment because of cell growth. In agreement with the lack of effect of fumonisin B₁ on protein synthesis after 22 h (Figure 2B), there was no difference in the amount of cells after this time (Figure 3C). Treatment with butyric acid or the combination of butyric acid and fumonisin B₁ slightly reduced the amount of cells even after this time of incubation. After 44–46 h with fumonisin B₁, there was in some of the experiments a slight inhibition of cell growth, whereas in others there was no reduction (Figure 3C).

Inhibitors of Glucosylceramide Synthetase, PDMP and PPMP, Prevent the Butyric Acid-induced Sensitization to Shiga Toxin and the Increased Toxin Binding

Treatment of cells with fumonisin B₁ not only affects formation of glycosphingolipids, but also the formation of sphingomyelin. Thus, if butyric acid treatment also changes the fatty acid composition of sphingomy-

Figure 3. Fumonisin B₁ inhibits butyric acid-induced increase in Shiga toxin binding to A431 cells. A431 cells (2.5×10^4 cells/well) growing in 24-well disposable trays were incubated for the indicated periods of time with and without 1.5 mM butyric acid (b.a.) and with and without 10 μM fumonisin B₁ (fum.). (A) The ability of the cells to bind ^{125}I -Shiga toxin after various times is shown. ^{125}I -Shiga toxin was added, and the amount of bound toxin was measured after a 20-min incubation at room temperature by washing the cells three times in cold PBS, dissolving the cells in KOH (0.1 M), and counting the radioactivity. The counts were corrected for the difference in the numbers of cells by measuring optical density of the cells as described in C. (B) Shown are the average and SD from several experiments ($n = 4-6$), such as the one in A. Lanes 1-4, incubation times 20-22 h; lanes 5-8, incubation times 26-28 h; lanes 9-12, incubation times 44-46 h. (C) The numbers of cells after 20-22 h (lanes 1-4) and 44-46 h (lanes 5-8) were quantified by dissolving them in 1 ml of KOH (0.1 M) and measuring the optical density (280 nm; $n = 6-12$).

elin, this change might also be inhibited by fumonisin B₁. To investigate whether the butyric acid-induced sensitization to Shiga toxin was dependent on changes in glycosphingolipid composition and not a possible change in sphingomyelin, we incubated A431 cells with butyric acid and with and without two inhibitors of glucosylceramide synthetase, PDMP and PPMP (Inokuchi and Radin, 1987; Radin and Inokuchi, 1991; Abe *et al.*, 1992; Barbour *et al.*, 1992; Abe *et al.*, 1995). Both of these compounds are analogues of ceramide and glucosylceramide, but with different acyl chain length and therefore different abilities to penetrate the cell membrane (Inokuchi and Radin, 1987; Radin and Inokuchi, 1991; Abe *et al.*, 1992, 1995; Barbour *et al.*, 1992). The presence of these inhibitors in low concentrations should not affect the sphingomyelin synthesis, but because they inhibit formation of glucosylceramide, they will inhibit the butyric acid-induced change in Gb3, the Shiga toxin receptor. Lipid analysis after labeling with [^3H]serine (see MATERIALS AND METHODS) revealed that PDMP (1 μM) only had a slight effect on the amount of labeled sphingomyelin after a 20 h incubation, whereas as expected there was >80% reduction in the amount of labeled glucosylceramide in cells incubated with PDMP (Figure 4). As demonstrated in Figure 5, both PDMP and PPMP inhibited the butyric acid-induced increase in Shiga toxin binding, and they also counteracted the sensitization to Shiga toxin, suggesting that a change in

glycosphingolipid composition and not in sphingomyelin composition is important for the intoxication. Control experiments showed that butyric acid-treated cells were not protected against Shiga toxin when PDMP or PPMP was added 30 min before the toxin (our unpublished results). Furthermore, a 24 h preincubation of A431 cells with these compounds did not protect the cells against ricin, another protein toxin believed to undergo retrograde transport through the Golgi apparatus before translocation to the cytosol (our unpublished results).

The Serine Palmitoyl Transferase Inhibitor β -Fluoroalanine Counteracts the Butyric Acid-induced Sensitization to Shiga Toxin and the Increased Toxin Binding

When cells are incubated with fumonisin B₁, accumulation of intermediates in the sphingolipid biosynthesis pathway has been reported to occur (Radin and Inokuchi, 1991). Some of these intermediates, like sphingosine, may in themselves affect cells, and could in theory also affect the sensitivity to Shiga toxin. However, as shown in Figure 6, even when the first step in the glycosphingolipid synthesis pathway, the formation of 3-ketosphinganine from palmitoyl-CoA and serine, was inhibited by addition of β -fluoroalanine (Merrill *et al.*, 1993; Schroeder *et al.*, 1994), the butyric acid-induced sensitization to Shiga toxin as

Figure 4. Lipid chromatogram of [^3H]serine-labeled cells. A431 cells were preincubated in the absence of PDMP (A) or in the presence of $1\ \mu\text{M}$ PDMP (B) for 30 min at 37°C . Then [^3H]serine was added ($2\ \mu\text{Ci}/\text{ml}$ medium), and the incubation was continued for 20 h in the absence and presence of PDMP. Lipids were extracted and separated by two-dimensional TLC as described in MATERIALS AND METHODS but without using borate. GlcCer, glucosylceramide; o, origin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin. Bar, 2 cm.

well as the butyric acid-induced change in toxin binding was counteracted. As also shown in Figure 6, addition of β -fluoroalanine alone (without butyric acid present) reduced the binding of Shiga toxin more than simultaneous addition of butyric acid and β -fluoroalanine. This is similar as when fumonisin B_1 , PDMP, or PPMP was added in the absence of butyric acid (Figures 3, A and B, and 5C). Control experiments showed that the concentration of β -fluoroalanine used did not affect cell growth during

the time of the experiment, there was no effect on protein synthesis or on the optical density (280 nm) of the dissolved A431 cells (Hansen *et al.*, 1992) at the end of the incubation (our unpublished observations). Also, when β -fluoroalanine was added to the cells 30 min before addition of toxin, it did not protect (our unpublished results). Because β -fluoroalanine inhibits formation of sphinganine (see Figure 1), addition of this compound should overcome the block in sensitization caused by β -fluoroalanine. As shown in Figure 6C, this was indeed the case. Similarly, addition of sphingosine could overcome the block (Figure 6D). In the experiment shown in Figure 6, a lower concentration of sphinganine ($2.5\ \mu\text{M}$) than sphingosine ($10\ \mu\text{M}$) was used because sphinganine at higher concentrations reduced the protein synthesis. Furthermore, sphingosine is somewhat less efficient than sphinganine in overcoming the block caused by β -fluoroalanine because $2.5\ \mu\text{M}$ sphingosine only partly sensitized the cells in the presence of β -fluoroalanine and butyric acid (our unpublished observations). Also, another inhibitor of serine palmitoyl transferase, L-cycloserine (Sundaram and Lev, 1984), blocked the sensitizing effect of butyric acid (our unpublished observations). Thus, the data suggest that the increase in fatty acid chain length seen after addition of butyric acid to A431 cells is important for the induced sensitization to Shiga toxin, and the data indicate that lipid synthesis is required for the change in Shiga toxin binding.

Fumonisin B_1 Inhibits the Butyric Acid-induced Increase in Toxin Transport to the Golgi Apparatus

We have found previously that butyric acid treatment of A431 cells increases the transport of Shiga toxin to the Golgi apparatus and the ER (Sandvig *et al.*, 1992a,b, 1994). To test whether the change in lipid composition of butyric acid-treated cells (Sandvig *et al.*, 1994) is important for the increased transport to the TGN, A431 cells were incubated with and without butyric acid and fumonisin B_1 for 23 h. Then ^{125}I -Shiga toxin was added to the cells, and after 2 h of further incubation the cells were subjected to subcellular fractionation. As shown in Figure 7, fumonisin B_1 reduced the butyric acid-induced transport of Shiga toxin to the Golgi apparatus, suggesting that the lipid composition is important for sorting of Shiga toxin to the Golgi apparatus. Control experiments showed that the localization and the amount of the enzyme galactosyltransferase, a Golgi marker, was not changed by the fumonisin treatment. Furthermore, no morphological changes of the Golgi apparatus could be observed by EM in fumonisin-treated cells (our unpublished observations).

Figure 5. Ability of PDMP and PPMP to counteract the butyric acid-induced sensitization to Shiga toxin (A and B) and the increased binding of toxin observed after butyric acid treatment (C). A431 cells growing in 24-well disposable trays were incubated for 24 h in the absence and presence of butyric acid (2 mM) and the indicated concentrations of PDMP (A) and PPMP (B). Then the medium was removed, and HEPES-containing medium with increasing concentrations of Shiga toxin was added. After 3 h incubation with toxin, the protein synthesis was measured as described in MATERIALS AND METHODS. (C) The cells were incubated for 24 h, as in A and B. Then the medium was removed, and ice-cold HEPES-containing medium was added. 125 I-Shiga toxin (0.3 ml/well; 0.11 μ g/ml; specific activity 32,000 cpm/ng) was added to the cells, and after 1 h incubation at 0°C the cells were washed three times with ice-cold PBS. The cells were dissolved in KOH (0.1 M), and the radioactivity was measured. The optical density at 280 nm of cells grown in parallel was measured to quantitate the possible reduction in the number of cells after incubation with the various additions. Only butyric acid reduced this value (to 80% of the control), and the binding data were corrected for this difference. The error bars show deviations between duplicates.

Fumonisin B₁ Inhibits Toxin-induced Apoptosis and the Visualization of Shiga-HRP in the ER of Butyric Acid-treated Cells

Cells that are intoxicated with Shiga toxin or other protein toxins seem to undergo an apoptosis-like process (Creppy *et al.*, 1981; Griffiths *et al.*, 1987; Chang *et al.*, 1989; Waring, 1990; Sandvig and van Deurs, 1992; Inward *et al.*, 1995). The same is the case with A431 cells that have been sensitized to Shiga toxin with butyric acid (Figure 8, A and B). Even after a 22 h incubation with butyric acid (maximal sensitization after 48 h), the sensitivity of the cells to Shiga toxin is sufficient to see toxin-induced formation of apoptotic bodies and condensation of chromatin (Figure 8, A and B). These morphological changes are not observed when sensitization to Shiga toxin by butyric acid is inhibited by simultaneous addition of fumonisin B₁ (Figure 8E), supporting the idea that toxin-induced inhibition of protein synthesis is required for the toxin-induced changes in morphology.

When A431 cells are incubated with butyric acid for 48 h to induce maximal sensitivity to Shiga toxin (Sandvig *et al.*, 1992b, 1994), strong staining of the ER

can be seen after incubation with a Shiga-HRP conjugate. However, in the present study the cells were incubated with butyric acid for only 22 h to prevent disappearance of Shiga toxin receptors in those experiments where fumonisin B₁ also was added. As shown in Figure 8, although Shiga-HRP can easily be visualized in endocytic structures, only a weak staining of the ER can be seen (Figure 8, C and D). As expected, when fumonisin B₁ was added in addition to butyric acid, Shiga-HRP still stained the endocytic structures, but no staining of the ER could be observed (Figure 8E).

Shiga Toxin Binds Equally Well at Low pH to Receptors on Control A431 Cells as to Receptors on Butyric Acid-treated Cells

The binding of Verotoxin 1 (VT1), which is almost identical to Shiga toxin, to glycosphingolipid receptors is dependent on the fatty acid content of the receptor (Pellizzari *et al.*, 1992; Maloney and Lingwood, 1993; Kiarash *et al.*, 1994); long fatty acids increased the VT1 binding apparently by increasing the binding capacity

Figure 6. Effect of β -fluoroalanine on butyric acid-induced sensitization of A431 cells to Shiga toxin and the concomitant increase in Shiga toxin binding. (A) A431 cells growing in 24-well disposable trays were incubated for 24 h with and without 2 mM butyric acid (b.a.) and with and without 10 or 20 μM β -fluoroalanine ($\beta\text{-F-Ala}$), as indicated. The medium was then removed, and the cells were incubated in a HEPES-containing medium with increasing concentrations of Shiga toxin for 3 h before protein synthesis was measured as described in MATERIALS AND METHODS. (B) After the 24 h incubation with and without butyric acid and $\beta\text{-F-Ala}$, the cells were incubated on ice in HEPES for 1 h in the presence of ^{125}I -Shiga toxin (0.3 ml/well; 0.11 μg /ml; specific activity 32,000 cpm/ng). The cells were then washed three times in ice-cold PBS, dissolved in KOH (0.1 M), and the radioactivity was measured. Cells incubated in the same way for the first 24 h were washed in PBS and dissolved in KOH, and the optical density at 280 nm was measured. This value indicates whether cell growth was inhibited, and the binding data were corrected for the reduced number of cells after butyric acid treatment (80% of the control). The error bars show deviations between duplicates. (C) The cells were treated as in A, but sphinganine (2.5 μM) was in some cases added together with butyric acid as indicated in the figure. The concentration of β -fluoroalanine used was 20 μM . (D) The cells were also treated as in A, but sphingosine (10 μM) was added together with butyric acid (b.a.) when indicated.

(Kiarash *et al.*, 1994). Because Shiga toxin receptors in butyric acid-treated cells have longer fatty acids than

in control cells, the possibility existed that the low efficiency of Shiga toxin transport to the Golgi appa-

Figure 7. Effect of fumonisin B₁ on the butyric acid-induced increase in Shiga toxin transport to the Golgi apparatus. A431 cells were incubated in the presence or absence of 1.5 mM butyric acid or a combination of 1.5 mM butyric acid and 10 μ M fumonisin B₁ for 23 h in 10% fetal calf serum DMEM medium. The cells were then washed and incubated in HEPES containing medium with ¹²⁵I-Shiga toxin (36,000 cpm/ng; 10 ng/ml) and 0.5 mg/ml HRP for 2 h at 37 or 17°C in the presence or absence of fumonisin B₁. The cells were then washed and homogenized, and the PNS was subjected to a discontinuous sucrose gradient as described in MATERIALS AND METHODS. The bars indicate deviations between duplicates.

ratus in control A431 cells could be because of a different pH dependency of the binding of toxin to the receptor. If the toxin dissociated at low pH in endosomes, the toxin might end up in lysosomes. In contrast, if the toxin did not dissociate at low pH in the butyric acid-treated cells with longer fatty acids in the receptor, that could explain the increased transport to the Golgi apparatus. However, as shown in Figure 9, the binding of Shiga toxin to its receptor is very stable over a wide pH range both in control A431 cells and in butyric acid-treated cells. In Figure 9A, the toxin was first bound to the receptors at neutral pH in the cold. Then the cells were exposed to medium with different pH values at 37°C. As shown, no dissociation of toxin was observed at the pH values found in endosomes. The results shown in Figure 9B demonstrate that Shiga toxin binds equally well over a large pH interval even when added to cells directly at the indicated pH value.

DISCUSSION

The results described here indicate that glycosphingolipid composition is essential both for intoxication

of A431 cells with Shiga toxin and for intracellular sorting of the toxin. We have shown previously that butyric acid-induced sensitization of A431 cells to Shiga toxin was associated with a shift in the fatty acyl chain length of Gb3, the Shiga toxin receptor (Sandvig *et al.*, 1994). Concomitant with the sensitization was an increased binding of toxin to the cells as well as an increase in the fraction of toxin transported to the Golgi apparatus (Sandvig *et al.*, 1994). Also, in butyric acid-treated A431 cells, a Shiga-HRP conjugate could be observed in the ER. However, whether the shift in lipid composition was required for the changed sorting and sensitivity has not previously been investigated, and no evidence has been published so far indicating that glycosphingolipid composition may influence transport of any ligand to the Golgi apparatus. We show here that when we interfere with glycosphingolipid synthesis during the incubation with butyric acid, the strong sensitization to Shiga toxin does not occur, there is no increase in toxin binding, and importantly, there is no increase in the fraction of toxin transported to the Golgi apparatus. Furthermore, Shiga-HRP cannot be seen in the ER of butyric acid-treated cells when glycosphingolipid synthesis is inhibited with fumonisin B₁. For reasons described below, we inhibited glycosphingolipid biosynthesis at different steps. In all cases, the butyric acid-induced changes were inhibited. It is important to note that the cells were still able to bind Shiga toxin after 20–26 h with butyric acid and the inhibitors of glycosphingolipid synthesis; only after prolonged incubations did the inhibitors reduce the toxin binding to the cells. Thus, lack of sensitization was clearly not because of lack of toxin binding under the conditions used. The decreased binding of Shiga toxin in the presence of inhibitors of glycolipid synthesis is probably a result of endocytosis and degradation of Gb3 molecules. The finding that there was a more rapid decrease in Shiga toxin binding when the cells were incubated only in the presence of fumonisin B₁, PDMP, PPMP, or β -fluoroalanine than when butyric acid was present as well, might be due to a butyric acid-induced synthesis of glycolipid receptors containing already-preformed (dihydro)ceramide, glucosylceramide, or lactosylceramide as precursors.

Fumonisin B₁, a specific inhibitor of *N*-acyltransferase (Merrill *et al.*, 1993; Schroeder *et al.*, 1994), will inhibit not only glycosphingolipid synthesis but also sphingomyelin synthesis, leaving the possibility that butyric acid induced sensitization by a change in the composition of sphingomyelin rather than in glycolipid composition. To exclude this, we used PDMP and PPMP, two different drugs that specifically inhibit glucosylceramide synthetase and not sphingomyelin synthesis at low concentrations (Radin and Inokuchi,

Figure 8. Electron micrographs of A431 cells incubated with 2 mM butyric acid for 22 h and then with a Shiga-HRP conjugate for 1 h 20 min before fixation and processing for EM. (A and B) Shown are characteristic features of apoptosis induced by the toxin: formation of apoptotic bodies (AB) budding from the cell body and chromatin condensation (CC). Shiga-HRP is seen in endocytic structures (En), whereas endoplasmic reticulum (ER) in general remains unlabeled. However, a faint Shiga-HRP labeling of ER cisterns was sometimes noticed, as shown in C. A portion of C has been enlarged and printed with higher contrast in D to better reveal the HRP labeling (arrowheads), in particular one ER cistern. (E) Shown is a portion of an A431 cell from an experiment in which the cells, simultaneously with the butyric acid incubation and throughout the experiment, were also incubated with 10 μ M fumonisin B₁. This clearly counteracts the butyric acid-induced effect of Shiga toxin: no labeling of ER cisterns was found, and no apoptosis was induced; the cell surface looks normal with scattered microvilli (asterisks). En, labeled endocytic structures. Bars, 0.5 μ m.

1991; Abe *et al.*, 1992; Barbour *et al.*, 1992). Prolonged incubations with PDMP (7 d) have been reported to result in a strong reduction in the content of Gb3 in

cells (Jacewicz *et al.*, 1994), and these cells will therefore become resistant to Shiga toxin because of lack of toxin binding (Jacewicz *et al.*, 1994). However, in the

Figure 9. Effect of pH on the binding of Shiga toxin to control A431 cells and to butyric acid-treated A431 cells. A431 cells were incubated with and without butyric acid (1.5 mM) for 48 h. (A) Cells were incubated with ^{125}I -Shiga toxin at neutral pH for 30 min at 0°C. Then the cells were washed in ice-cold medium and exposed to medium at 37°C with the indicated pH values for 15 min. The medium was then removed, and the cell-associated radioactivity was measured. (B) ^{125}I -Shiga toxin was added to the cells at 0°C at the indicated pH value; after 1 h incubation, the cells were washed, and the cell-associated radioactivity was measured.

present study PDMP was used only to inhibit changes in glycolipid composition and for a short enough time not to remove the toxin binding.

Incubation with fumonisin B₁, PDMP, and PPMP may lead to an accumulation of intermediates, which by themselves might affect the cells (Radin and Inokuchi, 1991). Therefore, even the first step in the sphingolipid synthesis, the enzyme serine palmitoyl transferase, was inhibited by using β -fluoroalanine (Merrill *et al.*, 1993; Schroeder *et al.*, 1994) or cycloserine (Sundaram and Lev, 1984). The similar effect of the various inhibitors excludes the possibility that the change in the fatty acyl chain length in the presence of butyrate is due to transacylation of the pre-existing Gb3. Thus, the data strongly suggest that the change in lipid composition observed upon butyric acid incubation is essential for the sensitization to Shiga toxin and also for the changed intracellular sorting of the toxin. Whether lipid composition and differences in sorting also can explain that a number of cell lines have receptors for Shiga toxin but are completely resistant to the toxin (Eiklid and Olsnes, 1980) is not yet known. So far, there is not much information about the role of lipid structure for sorting processes. However, it has been shown that ceramide structure can affect glycolipid sorting in the exocytotic pathway in polarized epithelial cells (van't Hof *et al.*, 1992).

The binding of the receptor-binding subunit of a Shiga-like toxin (VT1) to the carbohydrate part of the glycolipid receptor is strongly pH-dependent (St. Hilaire *et al.*, 1994); it was impaired at pH 6 and almost abolished below pH 5. However, the lipid part of the receptor also is important for binding of the toxin (Kiarash *et al.*, 1994), and, as shown here, the binding of Shiga toxin to the receptors is stable over a large pH interval both before and after butyric acid treatment of A431 cells. Thus, the increased transport to the Golgi

apparatus after butyric acid treatment is not due to a decreased dissociation of the toxin from the receptor at the low endosomal pH, but probably is a result of a different sorting of the toxin-receptor complex. How the fatty acyl chain length might affect intracellular transport and sensitivity to Shiga toxin is not clear, but an increased chain length may affect the positioning of the lipid in the membrane and the presentation of the sugars at the cell surface (Kannagi *et al.*, 1983; Crook *et al.*, 1986), and could thereby change the interaction of the carbohydrates with other structures in the lumen of endosomes or in the Golgi cisterns. Furthermore, because even the small binding subunit of Shiga toxin can be transported in a retrograde manner in the absence of the A fragment (Khine and Lingwood, 1994; Sandvig *et al.*, 1994), it might be the interaction of the B fragment with neighboring molecules that is changed. Clearly, binding of Shiga toxin to cell-surface receptors affects the distribution of the receptors: upon toxin binding they aggregate in clathrin-coated pits (Sandvig *et al.*, 1989). Similarly, toxin transport to the Golgi apparatus and retrogradely to the ER may involve interaction of the B-fragment with other molecules. Recently, sorting based on the different distribution of lipids in organelles and the properties of these lipids have been suggested to occur (for review, Bretscher and Munro, 1993; Munro, 1995). Whether this could be important for the change in trafficking of glycolipids with different tail lengths is, of course, still an open question.

So far, there is limited information about retrograde transport of lipids and lipid-binding ligands, and the regulation of such transport. Based on quantitative measurements of bulk flow from the ER, retrograde transport of lipids has been suggested to occur (Wieland *et al.*, 1987), and retrograde transport of fluorescent lipids from the Golgi apparatus to the ER has

been reported (Hoffmann and Pagano, 1993). Thus, Gb3 may (depending perhaps on the fatty acid tail length) be transported in a retrograde manner even without bound toxin. As shown here, retrograde transport of Shiga toxin cannot be observed when the lipid changes are inhibited with fumonisin B₁, suggesting that the retrograde transport of Shiga toxin from the Golgi apparatus to the ER may depend on the fatty acid tail of Gb3.

The detailed mechanisms involved in regulation of ER to Golgi and intra-Golgi transport are being studied in several laboratories. Recently, rab6 was shown to play a role for intra-Golgi transport, and it was suggested that this protein might increase the rate of retrograde transport as its overexpression reduced the outward transport of two different proteins (Martinez *et al.*, 1994). Also, COP (coatamer proteins)-coated vesicles may be involved in retrograde transport because COPI binds to the cytoplasmic dilysine motive, which seems to be required for retrograde transport of transmembrane ER proteins (Letourneur *et al.*, 1994). It was shown several years ago that proteins with the carboxyl-terminal sequence KDEL are retained in the ER by KDEL receptors and that these receptors can retrieve proteins also from a post-ER compartment (Lewis and Pelham, 1992). Recently it was shown that although KDEL receptors are concentrated in the intermediate compartment between ER and Golgi, a small amount of these receptors could be detected throughout the Golgi apparatus and even in the TGN (Griffiths *et al.*, 1994; Miesenböck and Rothman, 1995). Thus, toxins having a KDEL sequence (Seetharam *et al.*, 1991) could be transported retrogradely after binding to these receptors in the TGN, a location reached by several protein toxins (Joseph *et al.*, 1978; Sandvig and van Deurs, 1994b). Even toxins without such a sequence (Pelham *et al.*, 1992), i.e., toxins such as Shiga toxin, ricin, and modeccin, may somehow interact with this system. Not only toxins, but also other types of molecules seem to be transported in a retrograde manner from the TGN. The presence of MG160, a sialoglycoprotein, in medial Golgi is consistent with the hypothesis that either this protein is sialylated in TGN and then transported in a retrograde manner, or the sialyltransferase undergoes retrograde transport (Johnston *et al.*, 1994). Also, after long incubation (7.5 h), WGA-HRP labels several Golgi cisterns, suggesting a slow retrograde transport (Johnston *et al.*, 1994). Clearly, more work is required to clarify how Shiga toxin transport and the intoxication of cells with this molecule is regulated. It is not known whether glycolipid composition is important for the membrane translocation of toxin into the cytosol. However, the present study strongly suggests that glycolipid composition is important for sorting of Shiga toxin to the Golgi apparatus and the ER.

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