Retrograde Transport from the Golgi Complex to the ER of Both Shiga Toxin and the Nontoxic Shiga B-fragment Is Regulated by Butyric Acid and cAMP

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Abstract. Endocytosed Shiga toxin is transported from the Golgi complex to the endoplasmic reticulum in butyric acid-treated A431 cells. We here examine the extent of this retrograde transport and its regulation. The short B fragment of Shiga toxin is sufficient for transport to the ER. The B fragment of cholera toxin, which also binds to glycolipids, is transported to all the Golgi cisterns, but cannot be localized in the ER even after butyric acid treatment. Under all conditions the toxic protein ricin was found predominantly in the *trans*-Golgi network. There is no transport of endocytosed fluid to the Golgi apparatus or to the ER even after butyric acid treatment and in the presence of Shiga toxin, indicating that transport to the ER, through the *trans*-Golgi network and the cisterns of the Golgi apparatus, involves several sorting stations. Since Shiga toxin receptors (Gb3) in butyric acid-treated A431 cells seem to have a ceramide moiety with longer fatty acids than in untreated cells, the possibility exists that fatty acid composition of the receptor is important for sorting to the ER. Both retrograde transport and intoxication with Shiga toxin can also be induced by cAMP, supporting the idea that retrograde transport from the Golgi to the ER is required for intoxication. The data suggest that transport to the ER in cells in situ may depend on fatty acid composition and is regulated by physiological signals.

A number of protein toxins, like Shiga toxin, abrin, ricin, modeccin, pseudomonas toxin, and cholera toxin, all consist of a moiety that binds the toxins to cell surface receptors, and an enzymatically active moiety that acts on cytoplasmic targets (for review, see 42, 46, 80, 83). Several of the protein toxins are transported to the *trans*-Golgi network (TGN)[1] in the cells after endocytic uptake from the cell surface, and different lines of evidence suggest that this transport is necessary for translocation of the enzymatically active part of these toxins to the cytosol (22, 23, 51, 64, 65, 78, 79, 84). Studies of brefeldin A (BFA)-treated cells suggest that there is a correlation between an intact Golgi apparatus and intoxication (64). Since BFA does not seem to disrupt the TGN in cells (7, 64), one possible explanation for the protection afforded by BFA could be that these toxins normally are transported retrogradely through the Golgi apparatus and to the ER before translocation to the cytosol. The mechanism responsible for such a retrograde transport is not known. It has been demonstrated that some *trans*-membrane proteins, the KDEL receptors, can retain newly synthesized proteins with the sequence KDEL in the ER, and they can also bring such proteins from the Golgi apparatus to the ER in a retrograde manner (31). However, several of the protein toxins, including Shiga toxin, do not contain the KDEL signal (27, 51, 67, 70). It is of course possible that the toxins bind to other molecules which are transported to the ER. Recent findings supporting the idea that the toxins are transported to the ER before translocation is that addition of KDEL to ricin increases the toxic effect on cells (82), and removal of a KDEL-like signal on pseudomonas exotoxin A decreases the effect of this toxin (6, 66). Furthermore, the NH_2-terminal region of pseudomonas toxin may promote its own export from microsomes (74). However, not until recently was an endocytosed ligand seen both in the Golgi apparatus and in the ER. This was observed for the first time in butyric acid-treated A431 cells (60). After such treatment

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1. *Abbreviations used in this paper*: 8-Br-cAMP, 8-Bromoadenosine-3′,5′-cyclic monophosphate; BFA, brefeldin A; FAB MS, fast atom bombardment mass spectrometry; HPTLC, high performance thin layer chromatography; TGN, trans-Golgi network.

a Shiga–HRP conjugate was observed not only in the TGN, but also in the various Golgi cisterns, in the ER and in the nuclear envelope. At the same time the cells became extremely sensitive to this toxin, supporting the notion that retrograde transport is required for intoxication.

In the present article we compare the effect of butyric acid on intracellular transport of Shiga toxin with the effect on transport of other toxins (cholera toxin, ricin, and modeccin) and a fluid phase marker (HRP). We have tested to which extent the butyric acid treatment affects retrograde transport from the Golgi complex to the ER also of the nontoxic Shiga toxin B subunit. Furthermore, with the idea that the composition of glycolipids could be important for sorting (81), we have analyzed the composition of the Shiga toxin receptor in normal A431 cells as well as in cells treated with butyric acid. Moreover, we have studied whether the A431 cells can be sensitized to Shiga toxin by other types of treatment than exposure to butyric acid, and whether this sensitization is accompanied by retrograde transport. As shown here, cAMP can mediate similar changes as butyric acid on sensitivity and retrograde transport of Shiga toxin, thus strengthening the notion that these processes are linked, and suggesting that retrograde transport is a process that can be regulated also by signals imposed on the cells in situ.

Materials and Methods

Reagents

Type VIA HRP, diaminobenzidine, SPDP (3-[2 pyridyldithio]-propionic acid *N*-hydroxysuccinimideester), HRP-labeled cholera toxin B subunit, Hepes, and Tris were obtained from Sigma Chemical Co. (St. Louis, MO). We have also used HRP (sp act 938 U/mg) from Serva (Heidelberg, Germany). Nycodenz was obtained from Nycomed Pharma (Oslo, Norway) UDP[^{3}H]galactose, [^{3}H]leucine, Na^{125}I, the kit to measure cAMP, and goat anti-mouse IgG conjugated to 10 nm gold were from the Radiochemical Centre (Amersham, UK). Texas red-conjugated goat anti-mouse IgG was obtained from Southern Biotechnology Associates (030-07; Birmingham, AL). High performance thin layer chromatography (HPTLC) plates coated with Silica Gel 60 either on glass plates or on aluminum sheets were purchased from Merck (Darmstadt, Germany). Standard mixtures of pure neutral glycolipids containing Galβ1-1Cer(CMH), Galβ1-4Glcβ1-1Cer-(CDH), Galα1-4Galβ1-4Glcβ1-1Cer (CTH, Gb3), GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer(Gb4), GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer (Forssman pentasaccharide) were from Accurate Chem. & Science Corp. (Westbury, NY). Monoclonal antibody raised against globotriose was from MonoCarb AB (Lund, Sweden), and alkaline phosphatase anti-mouse Ig was obtained from Sigma Chemical Co.

Polyisobutylmethacrylate was obtained from PlexiGum, Polysciences, Inc. (Warrington, PA). All organic solvents used were of pro analysis from Merck. For the autoradiogram, X-OMAT x-ray films from Eastman Kodak Co. (Rochester, NY) were used. Silica gel and C 18 cartridges were obtained from Sep-Pak (Millipore, Waters Associates, Milford, MA). Ricin (44) and modeccin (45) were purified as described earlier. Gelonin was a kind gift from Dr. F. Stirpe (Università di Bologna, Bologna, Italy). Shiga toxin and the mutant Shiga-His (a mutant in which the two Arg between A_1 and A_2 were substituted with His) were provided by Dr. J. V. Kozlov (W. A. Engelhardt Institute for Molecular Biology, Academy of Sciences of Russia, Moscow, Russia). Shiga toxin subunit B was a generous gift from Dr. J. E. Brown (USAMRIID, Frederick, MD). Conjugates of Shiga toxin, Shiga-His, Shiga toxin B subunit or ricin and HRP were prepared by the SPDP-method as previously described (79). The anti-ER (anti-IP90) antibody AF8 (20) was kindly provided by Dr. M. B. Brenner (University of Pittsburgh, PA).

Cells

A431 (human epidermoid carcinoma) cells were from the American Tissue Type Collection (Rockville, MD). The medium used was Dulbecco's modification of Eagle's medium (3.7 g/l sodium bicarbonate) (Flow Laboratories, Irvine, Scotland) containing 5% fetal calf serum (Gibco, Ltd., Paisly, Scotland) and 2 mM L-glutamine (Gibco, Ltd.).

Measurement of Cytotoxic Effect

After incubation of cells with toxin as described in legends to figures, the medium was removed, and the cells were incubated in the same medium (no unlabeled leucine) for 10 min at 37°C with 1 μCi of [^{3}H]leucine per ml. Then the solution was removed, the cells were washed twice with 5% (wt/vol) trichloroacetic acid and solubilized in KOH (0.1 M). Finally, the acid-precipitable radioactivity was measured. The experiments were carried out in duplicate. The difference between duplicates was less than 10% of the average value.

Measurement of Receptor-mediated Binding and Endocytosis of ^{125}I-Ricin and ^{125}I-Modeccin

Binding and endocytosis of ^{125}I-labeled ricin and ^{125}I-modeccin were measured as previously described (61).

Measurement of cAMP

The content of cAMP in cells was measured by a cyclic AMP [^{3}H] assay system from Amersham Corp. In principle, 2×10^6 cells growing in Petri dishes (5-cm diam) were washed twice in PBS and then dissolved in 750 μl of ice-cold HCl (10 mM) in ethanol (96%). After 5-min incubation at 0°C, the cells were removed with a rubber policeman and the homogenate was centrifuged for 10 min in an Eppendorph centrifuge. The supernatant was freeze-dried and the pellet was dissolved in 2 ml KOH (0.2 M). The OD (280 nm) of this solution was used as a measurement of the amount of cells used. The freeze-dried supernatant was dissolved in 250 μl Na-acetate (0.5 M, pH 6.2). This solution was then used in the Amersham cAMP kit to measure the concentration of cAMP in the cells.

Subcellular Fractionation of Polarized Cells Incubated with Shiga Toxin

^{125}I-Shiga toxin (1 μg/ml) was administered to A431 cells incubated with and without 2 mM butyric acid. The toxin was continuously present during the incubation, and HRP was usually also added to label endosomal/lysosomal compartments. The fractionation was carried out essentially as described by Sandberg et al. (58) who separated rat liver Golgi fractions from the ER. The modification of this method has previously been described (64).

Enzyme Analysis

HRP was measured according to Steinman et al. (68), UDP-galactose:glycoprotein galactosyl transferase according to Brändli et al. (4), and β-*N*-acetyl-glucosaminidase according to Beaufay et al. (2).

Purification of Glycolipids

Total glycolipids from A431 cells, grown with and without 2 mM butyric acid (~10^9 cells, i.e., 100 Costar 3000 flasks [225 cm^2]) were extracted according to methods earlier described (57, 75). After harvest, the cells were pooled and washed in PBS, suspended in water and lyophilized. A crude glycolipid extract was achieved by extracting the dry cell material once with 50 ml of chloroform/methanol/water, 20:10:1 by volume overnight and then with chloroform/methanol/water, 10:20:1 and 10:10:1 by volume. Cell debris was removed by filtration through Whatman filter papers. The extracts were pooled and subjected to a mild alkaline hydrolysis with 0.2 M KOH in methanol for 2 h, thereafter neutralized with glacial acetic acid. Salts were removed through dialysis against ice-cold water using a membrane with mol wt cut off 500 (Spectrapor; Spectrum Medical Industries Inc., Houston, TX), and the material was lyophilized.

Separation of gangliosides (acidic glycolipids) from neutral glycolipids was performed as earlier described (72). The samples were dissolved in 90% methanol and 10% 5 mM phosphate buffer and passed over a SepPak C18 (Millipore, Waters Associates) cartridge, equilibrated with methanol (20 ml) followed by the methanol–phosphate buffer mixture (10 ml). Acidic gangliosides were washed out by adding another 10 ml of the 90% methanol. Neutral glycolipids were eluted with 10 ml of pure methanol. The different fractions were concentrated and the neutral glycolipids were additionally passed over a Sep-Pak silica cartridge dissolved in chloroform/methanol (1:1

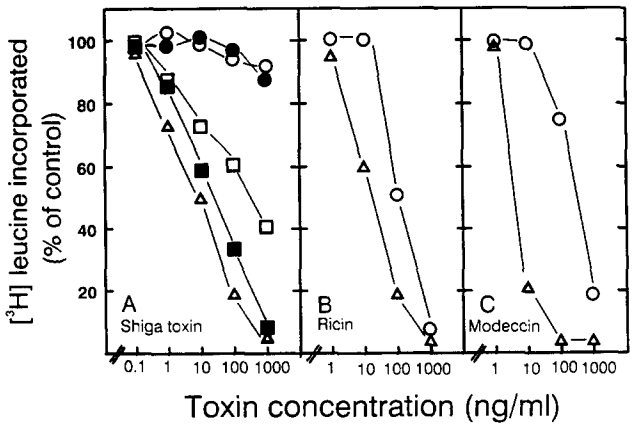

Figure 1. Butyric acid-induced sensitization of A431 cells to toxins. A431 cells growing in 24-well disposable trays were incubated with and without 2 mM butyric acid for the indicated periods of time. Then the cells were transferred to a Hepes-containing medium without serum, increasing concentrations of toxins were added, and the cells were incubated for 3 h more before protein synthesis was measured as described in Materials and Methods. (*A*) Shiga toxin; (*B*) ricin; (*C*) modeccin; (○), no butyric acid; (●) butyric acid for 3 h; (□) butyric acid for 17 h; (■), butyric acid for 24 h; (△), butyric acid for 48 h.

vol/vol). Further separation of the individual glycolipids was achieved by using HPTLC (57).

HPTLC Overlay Assay

Shiga toxin binding to glycolipids on HPTLC plates was performed according to methods described earlier (18, 33). After separation, the air-dried plate was immersed in 0.05% polyisobutylmethacrylate in hexane until translucency and then thoroughly air-dried. This was repeated three times. Unspecific binding sites were blocked by incubating the plate with 1% BSA in PBS for 15 min. The plate was then soaked for 1 h in a solution containing ^{125}I-labeled Shiga toxin in PBS with 0.05% Tween 20. After washing the plate five times in PBS with 0.05% Tween 20, it was exposed to a X-OMAT x-ray film (Eastman Kodak Co.). Toxin-binding glycolipids appeared as black bands on the film. A standard of known neutral glycolipids including the toxin receptor (Gb3) was used as a control.

Purification of Toxin Binding Glycolipids

Preparative HPTLC was used as the final step in purifying toxin-binding glycolipids, which had been identified with the HPTLC overlay described. The plates were run on Silica Gel 60 coated on alumina sheets. Gel containing the proper bands could then easily be cut out with a scissor and the pieces were placed in 5 ml methanol overnight, to extract the glycolipids. A final run on a C18 cartridge removed gel remnants.

Sugar Analysis

Purified toxin-binding glycolipids (~1–5 μg, as compared to known Gb3 standard on HPTLC) were hydrolyzed with 0.5 ml of 4 M trifluoroacetic acid at 98°C for 16 h. The samples were dried and dissolved in 0.5 ml methanol and dried again. This procedure was repeated three times. Finally the samples were dissolved in 100 μl water, 2 × 25 μl were run on a Dionex Bio LC-system (Sunnyvale, CA), and the Millipore calculation program Maxima, was used. Standard Gb3 and a blank were run at the same time.

Fast Atom Bombardment Mass Spectrometry

Purified glycolipids, both native and permethylated derivatives, were analyzed by fast atom bombardment mass spectrometry (FAB MS). Approximately 5 μg of glycolipids, as estimated from the sugar analysis, were permethylated. Methylation was performed with 4 M butyllithium in dimethylsulfoxide with a procedure adjusted for microgram quantities. The sample was dissolved in 200 μl DMSO before being sealed and thereafter flushed with nitrogen, according to standard methylation procedure. Butyllithium (100 μl) was added into the sealed tube, and the content was gently mixed. The vial was left at room temperature for 16 h. After chilling, 200 μl of cold methyliodide was added to the sealed tube, the content was gently mixed and left at room temperature for 30 min before the methyliodide was evaporated by flushing with nitrogen. The methylated glycolipids were finally purified on a Sep-Pak C18 cartridge (50). Glycolipids originating from the A431 cell line without butyric acid treatment were analyzed as permethylated derivatives only. FAB MS spectra were recorded on a NERMAG R10/10L quadropoule instrument and on a JEOL SX 102 instrument (JEOL U.S.A. Inc., Peabody, MA). Ions were produced from a matrix of triethanolamine for native glycolipids and from a matrix of thioglycerol or *m*-nitrobenzyl alcohol for permethylated material.

Processing for Electron Microscopy

A431 cells grown as monolayers in T-25 flasks were treated as described in the text, 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-H_2O_2 as previously described (79). The cells were postfixed with OsO_4, 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 previously described (79).

Results

Butyric Acid Induces Sensitization of A431 Cells to Shiga Toxin and Other Protein Toxins

Untreated A431 cells are resistant to Shiga toxin in spite of the fact that the cells are able to bind and endocytose the toxin. Endocytosis of Shiga toxin in the untreated cells has been shown to occur by EM (60) and cell fractionation as well as by quantification of cell surface bound Shiga toxin (data not shown). This raises the intriguing question why the cells are resistant to Shiga toxin. When A431 cells are incubated with butyric acid for increasing periods of time, they gradually become sensitive to Shiga toxin (Fig. 1). The cells were still resistant after a 3-h incubation with butyric acid, they obtained some sensitivity after a 17-h incubation, and maximal sensitivity was obtained after 24–48 h. Butyric acid had to be present in the medium during the whole incubation in order to give maximal sensitivity. When butyric acid was removed after 10 h, the cells were completely resistant to toxin added after 48 h. Also, when butyric acid was removed after 48 h, the cells again became resistant to Shiga toxin (data not shown).

The sensitization to Shiga toxin is dependent on protein synthesis. When cycloheximide was added at the same time as butyric acid, the cells remained resistant to Shiga toxin (data not shown), demonstrating that it is not the presence of butyric acid as such that makes the cells sensitive. Cycloheximide reversibly inhibits protein synthesis, and the effect of Shiga toxin could therefore be measured upon removal of cycloheximide. Similarly, as found for Shiga toxin, although not to the same extent, the A431 cells were sensitized by butyric acid also to ricin and modeccin (Fig. 1). The binding and endocytosis of these toxins were unaffected by the butyric acid treatment (data not shown). In A431 cells (data not shown) and in some other cell types (41, 48), BFA completely inhibits cholera toxin-induced increase in cAMP, suggesting that Golgi is involved also in the entry of this toxin. However, the ability of cholera toxin to raise the level of cAMP was not increased in the butyric acid-treated cells.

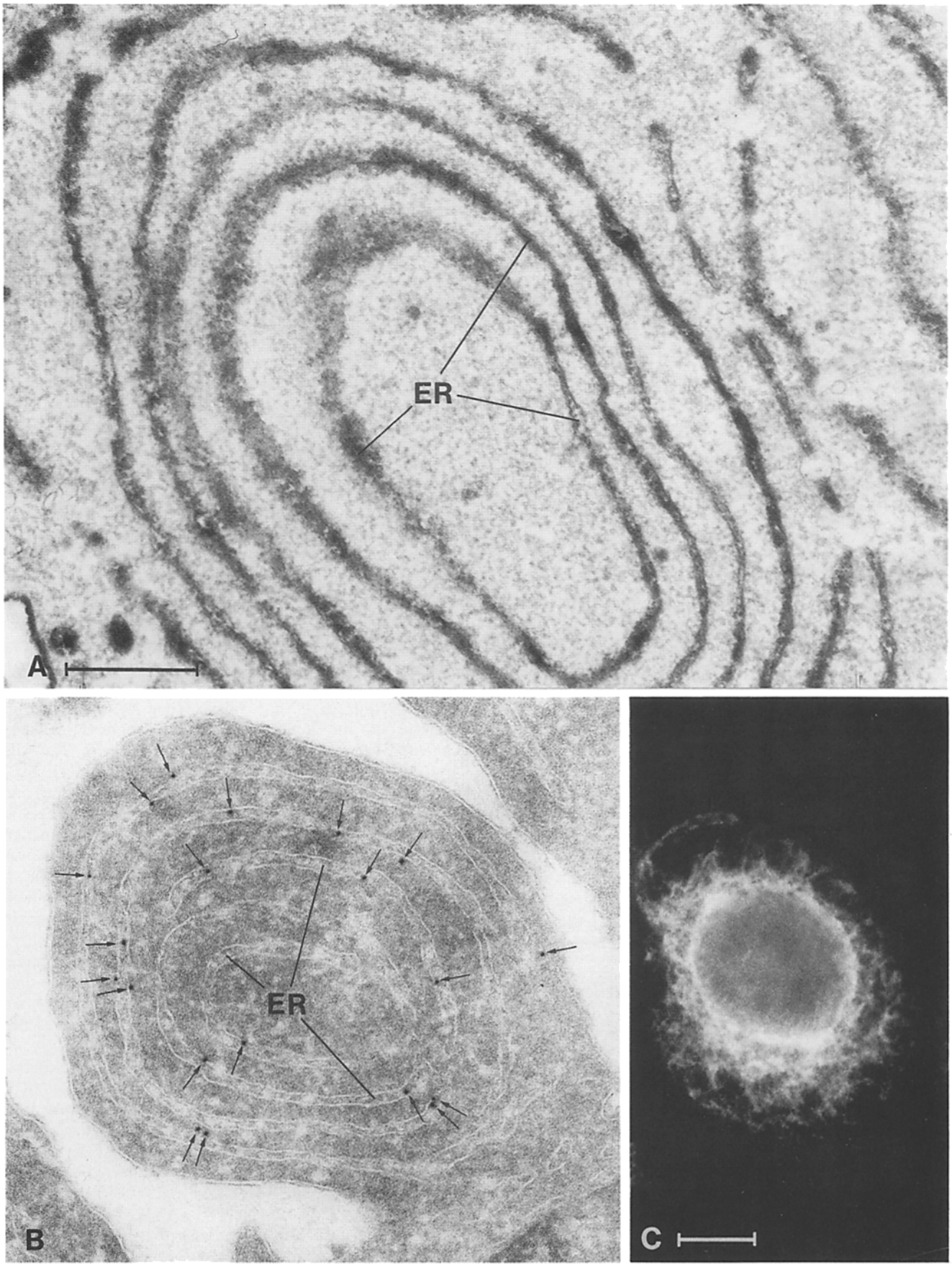

Figure 2. Retrograde transport of Shiga B fragment to the endoplasmic reticulum. A431 cells were incubated with 2 mM butyric acid for 48 h at 37°C and then with Shiga B-HRP for 1 h at 37°C. The cells were then processed for diaminobenzidine cytochemistry to detect the toxin conjugate in the ER, which sometimes forms characteristic whorls (*A*). Alternatively, cryosections of cells were incubated with the anti-IP90 monoclonal antibody AF8 followed by goat anti-mouse IgG conjugated to 10 nm gold (*B*, arrows) to detect ER cisterns (*B*). *C* is a corresponding immunofluorescence picture to show AF8-labeling of the ER and nuclear envelope. AF8 was detected with Texas red-conjugated goat anti-mouse IgG. Bars; (*A* and *B*) 0.5 μm, (*C*) 5 μm.

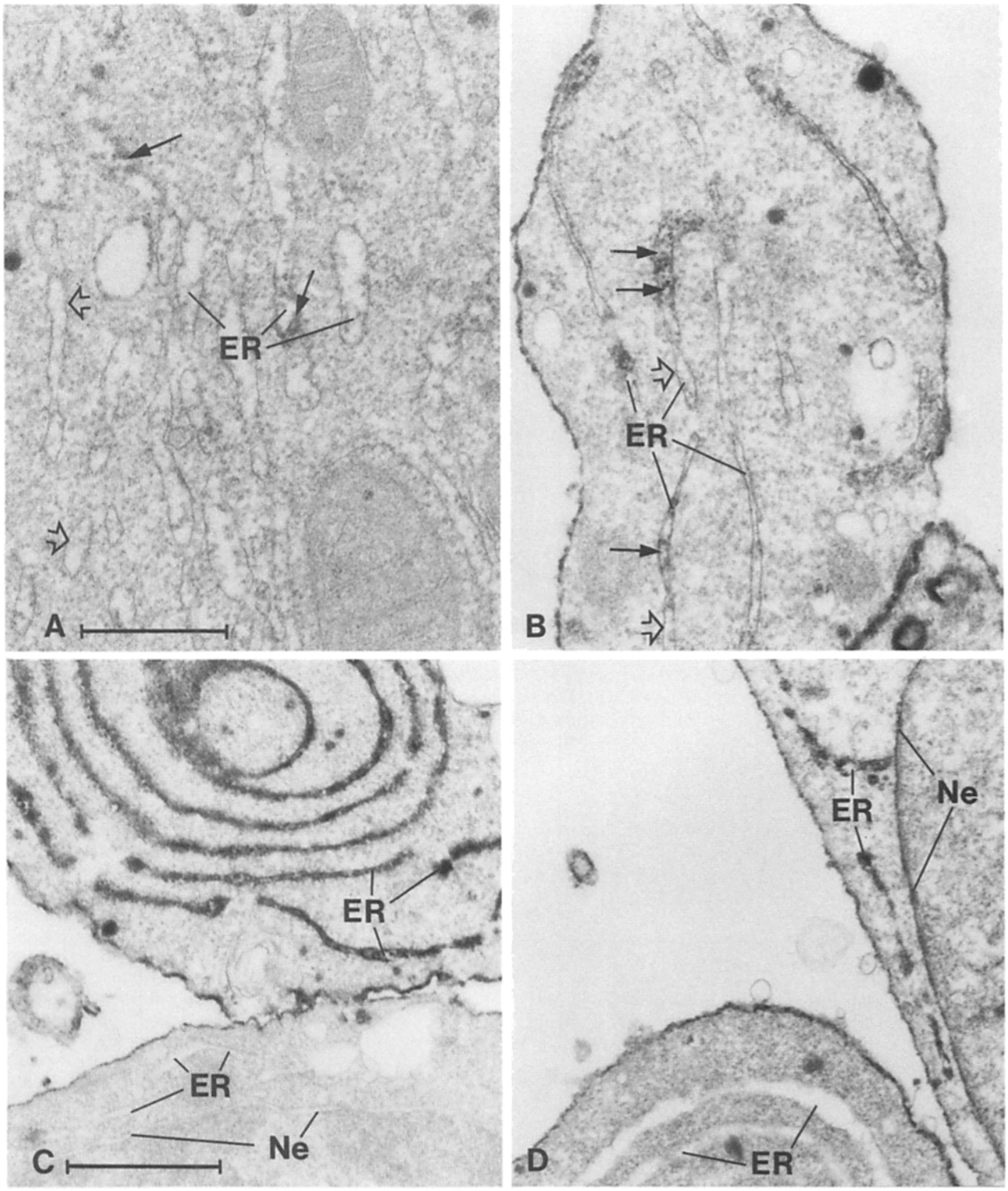

Figure 3. The ER labeling with Shiga–HRP varies considerably. A431 cells were incubated with butyric acid as in Fig. 2 and then they were incubated with Shiga–HRP (holotoxin) (*A*) or Shiga B–HRP (*B–D*) for 1 h. The toxin labeling (diaminobenzidine reaction product) varies considerably from ER cistern to cistern or even within the same ER cisterns (*A* and *B*) as well as from cell to cell (*C* and *D*). In *A* and *B*, small arrows indicate distinctly labeled portions of ER cisterns whereas open arrows indicate portions with very little or apparently no Shiga labeling. In *C* and *D* is shown how the ER and nuclear envelope (*Ne*) can be heavily labeled in some cells while these structures may appear unlabeled even in neighboring cells. Bars; (*A* and *B*) 0.5 μm; (*C* and *D*) 1 μm.

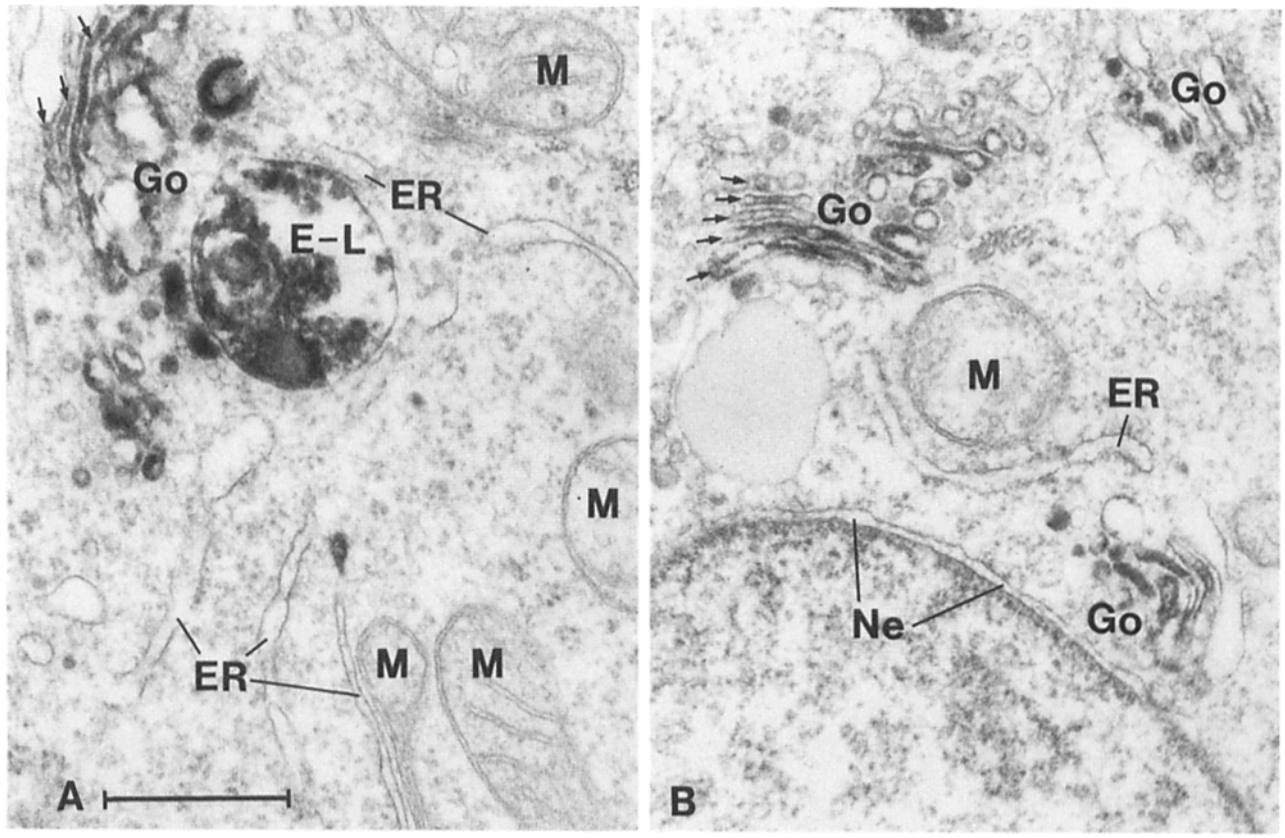

Figure 4. Endocytosed cholera-B-HRP is found throughout the Golgi apparatus but cannot be detected in the ER even after butyric acid treatment. A431 cells without (*A*) or with (*B*) a 48-h preincubation with butyric acid were incubated for 1 h at 37°C with a conjugate of HRP and cholera toxin B fragment. Even though reaction product is distinct in endosomes/lysosomes (*E–L*) and in the Golgi complexes (*Go*), where it often labels all cisterns from one side to the other (*small arrows*), it was never detected in the ER nor in the nuclear envelope (*Ne*). *M*, mitochondria. Bar, 0.5 μm.

Butyric acid treatment of A431 cells likewise had no sensitizing effect to gelonin (an A-chain–like toxin which does not bind to cells) (69) or to diphtheria toxin which is believed to enter the cytosol from early endosomes (13, 62).

Butyric Acid Induces Retrograde Transport of Shiga Toxin and Shiga B Fragment to the ER

Previous studies have suggested that Shiga toxin has to be transported to the Golgi apparatus before being translocated to the cytosol (63–65). Also in butyric acid-treated cells this seemed to be the case. When transport through the Golgi apparatus was interrupted by addition of the drug BFA, there was no effect of Shiga toxin in the butyric acid-treated cells (59). Interestingly, Shiga–HRP was never observed in the ER after BFA treatment (not shown). Also, Shiga toxin did not inhibit the protein synthesis in butyric acid-treated cells when added to cells at 18°C, a temperature at which transport to the Golgi apparatus is inhibited (76). Furthermore, the time between addition of toxin and the inhibition of protein synthesis was ~30 min, a lag time previously found in cell lines that are normally sensitive. The results thus suggest that the toxin follows the same pathway in the butyric acid-treated cells as in cell lines that are normally sensitive to the toxin.

To investigate whether retrograde transport to the ER somehow could be dependent on the A fragment of Shiga

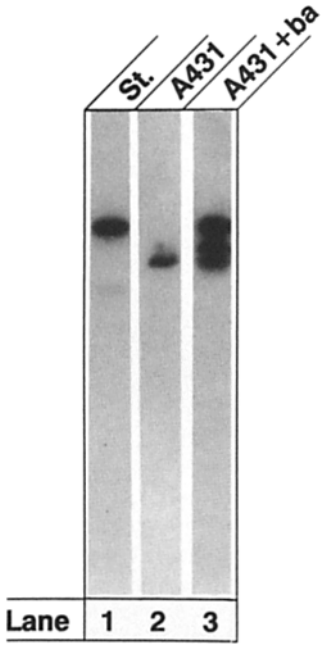

Figure 5. Autoradiogram showing binding of ^{125}I-labeled Shiga toxin to glycolipids. Neutral glycolipids were separated by HPTLC in the solvent system chloroform/methanol/water 60:35:8 (vol/vol/vol). The chromatogram was treated with 0.05% polyisobutylmethacrylate in hexane 3 × 1 min, followed by incubation in PBS supplemented with 1% BSA and 0.05% Tween 20 for 15 min. The plate was then incubated with 100 ml 20 μg/ml ^{125}I-labeled Shiga toxin (280 cpm/ng) for 1 h, washed 5 times with 0.05% Tween 20 in PBS, air-dried, and exposed to Kodak X-OMAT x-ray film for 24 h. The procedure was performed at room temperature. Standard mixture of neutral glycolipids containing monohexoside (*CMH*), galabiosylceramide (*CDH*), globotriaosylceramide (*CTH*), Globoside (*Gb4*) and Forssman pentasaccharide (lane *1*), neutral glycolipids from A431 cells (lane *2*) and from butyric acid-treated A431 cells (lane *3*).

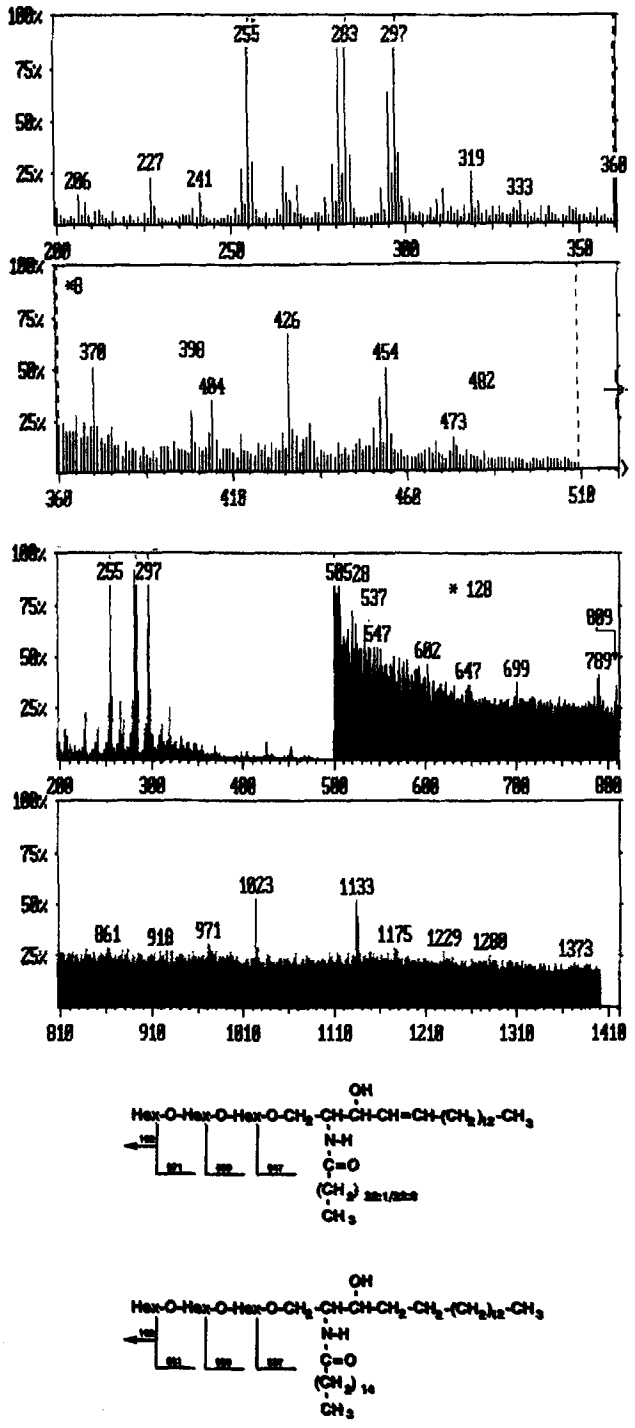

Figure 6. Negative ion FAB MS spectrum of Shiga toxin-binding glycolipids from butyric acid-treated A431 cells, native material. Molecular ions at m/z 1023 and 1133/1135 are observed. The fragment ions m/z 861, 699, 537 and m/z 971, 809, 647 confirmed that the major glycolipids contained three hexoses and a ceramide group. Fragment ions at m/z 398, 426, 452/454, and 482 are proposed to be derived from hex3-ceramides with short fatty acids (C6-C12).

toxin, we next tested whether the small B fragment of Shiga toxin alone also could be transported via the Golgi complex to the ER by using a Shiga B-HRP conjugate. As shown in Fig. 2, this was clearly the case. Since we were unable to detect Shiga toxin in the ER by immunogold labeling of ultracryosections, we documented that cisterns similar to the Shiga B-HRP-labeled cisternal structures were actually ER cisterns by immunogold labeling with the anti-ER antibody AF8 on ultracryosections (Fig. 2). While the labeling of ER cisterns with Shiga-HRP or Shiga-B-HRP was sometimes very heavy, a lower degree of labeling was often seen. Hence, in most cells only a few ER cisterns, or portions of cisterns, were distinctly labeled, the others appeared unlabeled. Also, apparently unlabeled ER cisterns could be observed in cells next to other cells with heavily labeled ER and nuclear envelope (Fig. 3).

The efficient retrograde transport observed for Shiga toxin in butyric acid-treated cells did not seem to be general for all ligands transported to the Golgi apparatus. Thus, we were not able to visualize retrograde transport of ricin-HRP, which could only or predominantly be observed in the TGN (76-79). Cholera B-HRP, a lipid-binding ligand with specificity for GM1 (15), showed a different labeling pattern. Both with and without butyric acid treatment, this ligand gave a very pronounced staining of the Golgi complexes, and the label was often present on both sides (*cis-trans*) of the stacks. However, no labeling of the endoplasmic reticulum or the nuclear envelope was seen (Fig. 4).

Intracellular Transport of the Fluid-phase Marker HRP in Butyric Acid-treated Cells

We were also interested in testing whether the fluid phase marker HRP was transported to the Golgi apparatus and further backwards to the ER in butyric acid-treated cells. As earlier observed in other cell types (for review, see 77), we could not see transport of HRP to the Golgi apparatus in A431 cells even after butyric acid treatment. Furthermore, no HRP was observed in the endoplasmic reticulum (not shown). The possibility existed that addition of Shiga toxin could lead to a rerouting of fluid in the butyric acid-treated A431 cells. However, even when unlabeled Shiga toxin was added together with HRP, this compound could not be observed in the Golgi apparatus or the endoplasmic reticulum.

Purification and Characterization of Shiga Toxin Receptors

Since butyric acid-induced retrograde transport could be demonstrated only for Shiga toxin which binds to neutral glycosphingolipids with the sequence galα1-4galβ (9, 21, 32), we decided to analyze whether butyric acid treatment caused any changes in the composition of the Shiga toxin receptors. For this purpose, glycosphingolipids from a large number of cells (10^9 cells) cultured with and without butyric acid were prepared and separated with HPTLC. Toxin-binding glycolipids were identified by overlaying the chromatogram with ^{125}I-labeled Shiga toxin. As shown in Fig. 5, both untreated and butyric acid-treated A431 cells contain neutral glycolipids that bind Shiga toxin in a HPTLC overlay system. The butyric acid-treated A431 cells contain three closely migrating bands with affinity for Shiga toxin. The upper band is close to the position for the standard Gb3, which is the main toxin-binding glycolipid in the standard mixture of glycolipids. On the other hand, untreated A431 cells contain one main glycolipid with affinity for the toxin, and a hardly discernible band corresponding to the middle

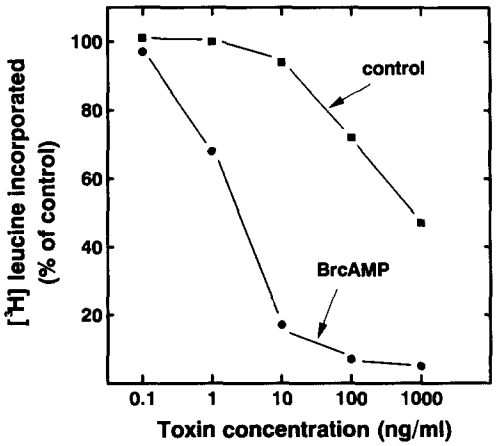

Figure 7. Sensitization of A431 cells to Shiga toxin by 8-Br-cAMP treatment. A431 cells were incubated with and without 8-Br-cAMP (2 mM) for 48 h. Then increasing concentrations of Shiga toxin were added, and the protein synthesis was measured 3 h later as described in Materials and Methods.

band found in butyric acid-treated cells. The main band in the untreated A431 cells has a low migration rate, suggesting a content of short fatty acids (see below). In the standard mixture of glycolipids, also Gb4, which has an internal galα1-4galβ, sequence shows a very weak affinity for ^{125}I-labeled Shiga toxin. When the HPTLC was overlaid with monoclonal antibodies recognizing galα1-4galβ1-4glc, only the two upper bands in the butyric acid treated cells were revealed (data not shown), a finding that could be due to low affinity of the antibody to carbohydrates coupled to ceramides with short fatty acids (see Discussion). The HPTLC overlay assay clearly shows that butyric acid changes the composition of the Shiga toxin-binding sites in A431 cells.

Structural information on the toxin-binding glycolipids was obtained by sugar analysis and FAB MS. Toxin-binding glycolipids from closely migrating bands on the HPTLC were extracted and analyzed together. Sugar analysis revealed identical patterns of glucose and galactose in the preparations from both untreated and butyric acid-treated A431 cells as well as from standard Gb3. No other sugar residue was detected. The FAB MS spectrum of underivatized glycolipids from butyric acid-treated A431 cells, run in the negative mode, demonstrated molecular ions at m/z 1023 and 1133/1135 (Fig. 6). The fragment ions m/z 861, 699, 537 and m/z 971, 809, 647, originating from the loss of hexose residues (162 mass units), confirmed that the major glycolipids contained three hexoses and a ceramide group. Fragment ions at m/z 398, 426, 452/454, and 482 are proposed to be derived from hex3-ceramides with short fatty acids (C6-C12). Their molecular ions were not detected in these spectra.

The data suggest that the ceramide of the lower molecular weight glycolipid (m/z 1023, middle band in HPTLC, Fig. 5) is composed of either a C18:0 base with C16:1 fatty acid or a C18:1 base with a C16:0 fatty acid. The glycolipid of higher molecular weight is assumed to be composed of a C18:1 base with C24:1/C24:0 fatty acids. Permethylation of the material implies exchange of all OH- and NH- groups to O-methyl- and *N*-methyl groups, respectively. The FAB MS spectrum of permethylated samples (data not shown), run in the positive mode, demonstrated sodiated molecular ions at m/z 1325/1327 and 1215. This represents an addition of 12 methyl groups to m/z 1133/1135 and 1023 upon methylation. Two methyl groups belong to the ceramide and 10 methyl groups belong to the three hexoses, thus indicating that the ceramide part of the glycolipids has one hydroxyl group. We also observed in this spectrum a molecular ion at m/z 1104 which is assumed to correspond to a hex3-ceramide with a C18:0 base and a C8:0 fatty acid. The spectra of the two lipid preparations revealed the same ions, indicating the presence of the same glycolipids. However, the intensities of the ions differed. In A431 cells the molecular ions corresponding to the hex3-ceramides with short-chain fatty acids were much more abundant than those corresponding to the hex3-ceramides with longer fatty acids, which were almost absent. This is in agreement with the HPTLC autoradiogram, where

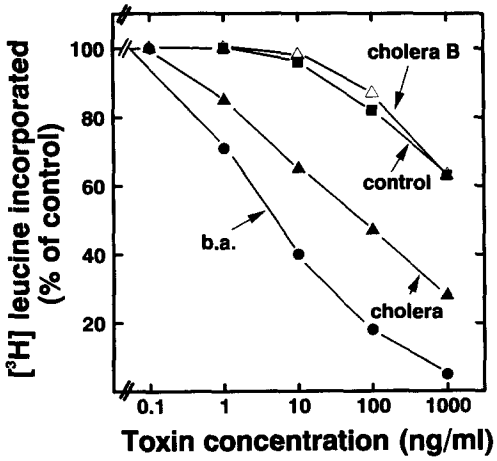

Figure 8. Effect of cholera toxin treatment of A431 cells on the sensitivity to Shiga toxin. A431 cells growing in 24-well plates were incubated for 48 h with and without cholera toxin (2 μg/ml), cholera toxin B subunit (2 μg/ml) or butyric acid (*b.a.*; 2 mM). Then increasing concentrations of Shiga toxin were added, and the protein synthesis was measured after 3 h as described under Materials and Methods.

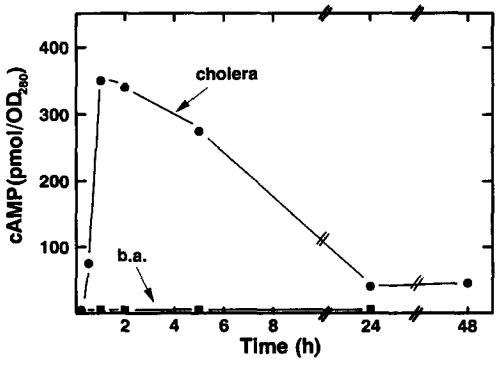

Figure 9. Effect of butyric acid and cholera toxin on the level of cAMP in A431 cells. A431 cells were incubated with and without butyric acid (*b.a.*; 2 mM) or cholera toxin (2 μg/ml). After increasing periods of time the cells were harvested for analysis of cAMP as described under Materials and Methods.

the lower bands (slowly migrating lipids) thus correspond to the glycolipids with the short fatty acids.

Cholera Toxin and cAMP Sensitize A431 Cells to Shiga Toxin and Induce Retrograde Transport

Since butyric acid is known to cause differentiation of cells (8, 30, 34), we tested whether other differentiating agents (25, 26, 36, 43, 53) could sensitize A431 cells to Shiga toxin. Neither retinoic acid (10^{-5}–5×10^{-5} M), DMSO (1%), TPA (0.1–1000 ng/ml), TNFα (10–1000 ng/ml), nor vitamin D (10 nM–10 μM) had any sensitizing effect (data not shown). Since dibutyryl-cAMP has been used to induce differentiation of HL60 cells (26, 30), we tested whether 8-Bromoadenosine-3′,5′-cyclic monophosphate (8-Br-cAMP) could sensitize A431 cells to Shiga toxin. We did not use dibutyryl-cAMP since butyric acid in itself sensitizes the A431 cells. As shown in Fig. 7, incubation of cells with 8-Br-cAMP had a strong sensitizing effect. Also, as shown in Fig. 8, incubation of A431 cells with cholera toxin made the cells sensitive to Shiga toxin, and as shown in Fig. 9, cholera toxin, in contrast to butyric acid, strongly increased the level of cAMP in these cells. Cholera toxin B subunit alone was without any effect. Similarly as found for butyric acid-induced sensitization, a long incubation (48 h) in the presence of 8-Br-cAMP or cholera toxin was required to obtain maximal sensitivity to Shiga toxin, and, as also found for the butyric acid-induced sensitization, presence of cycloheximide during the 48 h prevented the cells from becoming sensitive, suggesting that protein synthesis is required for the sensitization (data not shown). Also in these experiments cycloheximide was removed before the sensitivity of the cells to the toxin was tested. Electron microscopy of 8-Br-cAMP-treated A431 cells incubated with HRP conjugated to Shiga toxin or to a mutant of Shiga toxin (see Materials and Methods) revealed distinct labeling of ER cisterns in some cells (Fig. 10).

Discussion

The results described here show that retrograde transport of Shiga toxin to the endoplasmic reticulum is a process that

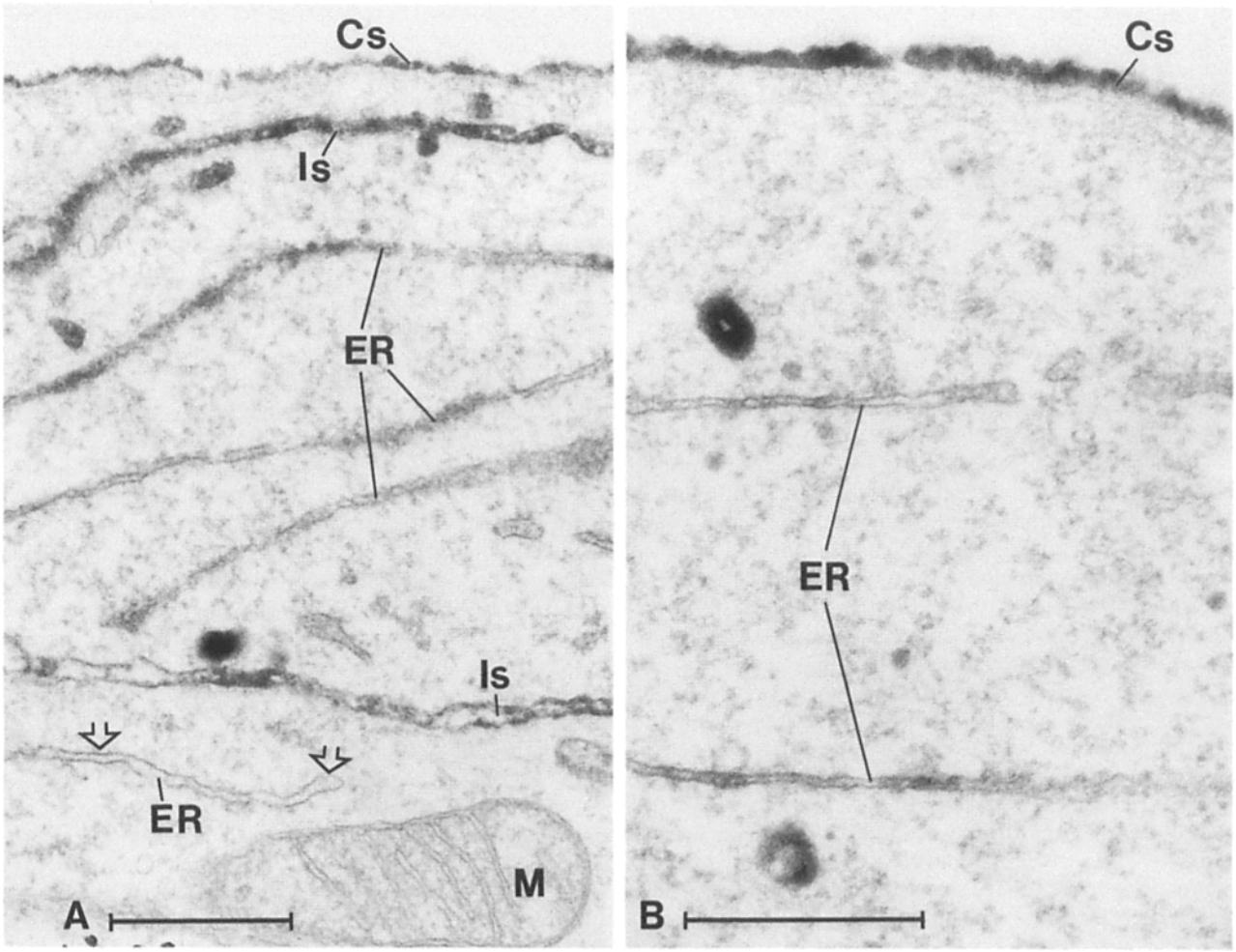

Figure 10. cAMP induces retrograde transport of Shiga toxin to the ER. A431 cells were incubated with 2 mM 8-Br-cAMP for 48 h at 37°C and then with a Shiga (His-mutant)–HRP conjugate for 75 min at 37°C before fixation and processing for EM. The toxin is seen on the cell surface (*Cs*), in the intracellular spaces (*Is*) of overlapping cells, and in ER cisterns. Note the apparently unlabeled ER cistern (*open arrows*) in *A*. Bars, 0.5 μm.

can be regulated both by butyric acid and cAMP. A number of physiologically active ligands are able to increase the concentration of cAMP in cells (73), and it is therefore possible that a change in retrograde transport could be part of a physiological response. Interestingly, it was recently reported that constitutive secretory traffic can be modulated by membrane receptors and second messengers (12), suggesting that net retrograde transport could be affected also by a regulation of the rate of secretion.

Butyric acid is able to change gene transcription in a number of cells (3, 16, 17, 49, 55, 56), and exposure of cells to butyric acid does occur also in vivo. The concentration of butyric acid in the intestine varies, but can be ~20 mM (19), a concentration that might certainly affect the epithelial cells in the intestine. Whether this exposure will change retrograde transport in these cells and thereby sensitize them to Shiga toxin, thus making a person more susceptible to disease during infection with *E. coli*-producing Shiga-like toxins, is an open question. Intestinal cells can clearly be sensitive to Shiga toxin (40, 42). However, although human colonic and ileal epithelial cells in primary culture are sensitive to the toxin (40), it is not known to which extent these cells are influenced by agents they have been exposed to before the isolation. On the other hand, two human colonic carcinoma cell lines, polarized CaCo-2 cells grown on filters and T-84 cells, are quite resistant to Shiga toxin (reference 35 and unpublished data). The production of butyric acid from dietary fiber may, on the other hand, protect against colon cancer (8, 34).

Incubation of A431 cells with cholera toxin sensitized the cells to Shiga toxin. However, since cholera-B in some systems has physiological effects that are independent of cAMP (37), we checked that cholera-B was unable to sensitize the cells to Shiga toxin. Since also 8-Br-cAMP had a sensitizing effect, we believe that increased levels of cAMP are responsible for the observed sensitization to Shiga toxin also after incubation with cholera toxin.

Not only Shiga toxin, but also the B fragment alone, could be transported to the ER. Although the B fragment does not contain a KDEL sequence (26, 67, 70), it may contain another signal of importance for retrograde transport. However, it is possible that the receptors themselves, even in the absence of toxin, are transported from the Golgi to the ER after treatment with butyric acid and cAMP. Of the ligands here tested we can visualize only Shiga toxin and the binding subunit (the B fragment) of this toxin in the ER. As shown in the present study butyric acid treatment caused a dramatic change in the fatty acid composition of the Shiga toxin receptor, Gb3 (9, 21, 32). The data suggest that the butyric acid-treated cells contain Gb3 with longer fatty acid chain lengths than the untreated cells, and this change in receptor composition may be important for the observed retrograde transport in butyric acid-treated cells. It has previously been reported that variations in the ceramide composition can affect sphingolipid sorting in epithelial cells (81). Changes in the fatty acid chain in glucosylceramide and sphingomyelin affected the apical to basolateral polarity of these molecules in MDCK and Caco-2 cells. Similarly, ceramide composition might affect sorting also from the cell surface to the Golgi apparatus and to the ER. Not only could the interaction of the receptor with other molecules in the lipid bilayer be changed, but also the interaction of the glycosphingolipid sugar moiety (and bound Shiga toxin) with other molecules at the cell surface could be different since long fatty acids in the ceramide moiety seem to increase the surface exposure of the carbohydrate chain (10). Antibodies with low affinity can recognize a glycolipid incorporated into liposomes when the fatty acid chain length is increased (10), and the ceramide composition also influences the antigenicity of glycolipids in lymphoma cells (24). Furthermore, in studies where mammalian glycolipids were separated by HPTLC or microtiter wells were coated with glycolipids, it was shown that the ceramide composition of lactosylceramide is important for recognition of different subspecies of *Propionobacterium* (71). Also, other studies indicate that lipid composition may affect carbohydrate conformation on TLC (52). An effect of the lipids on the carbohydrate presentation could be the reason why the antibody to galα1-4galβ1-4glc only recognized the upper two of the three bands recognized by Shiga toxin on the HPTLC. The sugar analysis excluded that the lower band contains other sugars than galactose and glucose, and the possibility that the lowest band contains a four-hexose glycolipid is unlikely since a third molecular ion would then have been detected in the FAB MS. Also, a four-hexose glycolipid would migrate even slower on HPTLC (32). Although at the present time we can not conclude that a change in lipid composition is responsible for the retrograde transport of Shiga toxin, it is certainly a possibility which should be tested in future studies.

It is difficult to make any absolute statements about the generality of the retrograde transport. Ricin–HRP may not be detectable in the ER due to a low concentration of the conjugate. Although the effect on sensitivity to ricin is much smaller than the sensitization to Shiga toxin, butyric acid treatment makes the cells more sensitive also to this toxin. It should be noted that ricin binds to both glycoproteins and glycolipids with terminal galactose (46), and a changed routing of glycolipids might therefore also affect ricin toxicity. Similarly to ricin, modeccin also binds to both glycoproteins and glycolipids with terminal galactose, although this toxin binds to a subgroup of the ricin-binding sites at the cell surface (46). Thus, a change in the binding sites might therefore also explain the increased toxicity of this molecule. It should be stressed that the peroxidase technique is not a quantitative approach. However, under the actual experimental conditions where only relatively small amounts of toxin may be transported retrogradely, the peroxidase technique appears more sensitive than immunogold labeling on ultracryosections (we have so far been unable to detect retrograde toxin transport to the ER convincingly with the latter approach).

The fact that the protein synthesis is completely inhibited in A431 cultures after butyric acid treatment and exposure to high concentrations of Shiga toxin suggests that if translocation to the ER is required for intoxication, Shiga toxin is present in the ER cisterns of virtually all cells even though the toxin is morphologically detectable only in a fraction of the cells. Thus, it is possible that also cholera toxin B, although not visible in the ER, could be present at low concentrations. It is remarkable that all Golgi cisterns are often distinctly labeled by cholera-B–HRP without any visible reaction product in the ER. If the volume of the ER is considerably larger than that of the Golgi apparatus, one could get a dilution of conjugates transported retrogradely, thus making visualization more difficult or even impossible. It has

earlier been reported that the cisterns of the Golgi apparatus were unstained by a cholera toxin conjugate (22, 23). Whether this is due to the cell type studied is not clear.

The fact that BFA inhibits appearance of the Shiga toxin–HRP conjugate in the ER, suggests that there is no direct transport of Shiga toxin to the ER from the cell surface. Also, if there had been a direct route from the cell surface to the ER in the butyric acid-treated cells, one would expect to see transport also of fluid phase markers (HRP) to the ER. This was however not the case, supporting the view that the regulated retrograde transport to the ER occurs via the Golgi apparatus.

There is a large variation in the sensitivity of cells to protein toxins (for review, see 46). This can in some cases be explained by a variation in the number of receptors for the toxin at the cell surface. However, in the case of Shiga toxin, several cell lines are almost resistant to the toxin in spite of being able to bind the toxin (14, 60). We have shown that not only do A431 cells bind the toxin, they also endocytose the toxin. One possible explanation for the variation in sensitivity between cell lines could be that there is a different extent of retrograde transport in these cells. In spite of the increasing evidence for a link between retrograde transport and the transport of some of the protein toxins to the cytosol, it is still premature to conclude that transport to the ER is an obligatory step for intoxication. Recently Bau and Draper (1) showed that cell mutants having an aberrant Golgi complex were still intoxicated with ricin. On the basis of these studies, they suggested that ricin entry into the cytosol might be independent on transport through the Golgi stacks. Clearly more work is required to clarify these questions.

By learning more about factors that are involved in the regulation of retrograde transport we might be able to modulate the intracellular transport not only of protein toxins, but perhaps also of immunotoxins (47) and molecules of physiological importance, like growth factors (5, 38, 54, 85) and cytokines (11, 28, 29, 39), which in some cases also seem to be translocated to the cytosol and the nucleus in the cell.

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