

# Endocytosis and Intracellular Transport of the Glycolipid-Binding Ligand Shiga Toxin in Polarized MDCK Cells

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**Abstract.** The glycolipid-binding cytotoxin produced by *Shigella dysenteriae* 1, Shiga toxin, binds to MDCK cells (strain 1) only after treatment with short-chain fatty acids like butyric acid or with the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate. The induced binding sites were found to be functional with respect to endocytosis and translocation of toxin to the cytosol. Glycolipids that bind Shiga toxin appeared at both the apical and the basolateral surface of polarized MDCK cells grown on filters, and Shiga toxin was found to be endocytosed from both sides of the cells. This was demonstrated by EM of cells incubated with Shiga-HRP and by subcellular fractionation of cells in-

cubated with <sup>125</sup>I-labeled Shiga toxin. The data indicated that toxin molecules are endocytosed from coated pits, and that some internalized Shiga toxin is transported to the Golgi apparatus. Fractionation of polarized cells incubated with <sup>125</sup>I-Shiga toxin showed that the transport of toxin to the Golgi apparatus was equally efficient from both poles of the cells. After 1-h incubation at 37°C ~10% of the internalized toxin was found in the Golgi fractions. The results thus suggest that glycolipids can be efficiently transported to the Golgi apparatus from both sides of polarized MDCK cell monolayers.

SHIGA toxin binds to glycolipids with the structure gal $\alpha$ 1-4gal expressed on some cell types (9, 20, 23; Mobassaleh, M., A. Donohue-Rolfe, R. Montgomery, G. Keusch, and R. Grand. 1986. *Pediatr. Res.* 20:a245.) Ligand binding apparently induces redistribution of the glycolipids into coated pits, and the toxin-glycolipid complex is subsequently internalized (40). Since Shiga toxin is firmly bound to glycolipids and does not dissociate at low pH, it may serve as a marker of glycolipid uptake and intracellular transport. Compared to the large number of studies on uptake, sorting, and transport of proteins, relatively little is known about trafficking of glycolipids and glycolipid-binding ligands. We therefore wished to extend our studies on Shiga toxin transport from cells grown on impermeable substrates (40) to polarized epithelial cells.

We have previously used MDCK cells grown on permeable filters to study the uptake and transport of the toxic plant protein ricin (49). MDCK cells do, however, normally not bind detectable amounts of Shiga toxin. As we show in this study, treatment with butyric acid and the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) induced the ap-

pearance of significant amounts of Shiga toxin-binding sites, and the cells became sensitive to the toxin. This has allowed us to study uptake and transport of Shiga toxin in polarized MDCK cells. The results demonstrate that butyric acid-induced binding sites for Shiga toxin appear at both the apical and basolateral side of the MDCK cells and that the toxin is efficiently internalized from both sides. Furthermore, this glycolipid-binding ligand is rapidly transported to the Golgi apparatus from both cell surface domains. To our knowledge this is the first quantitative study of the transport of a glycolipid-binding ligand to the Golgi apparatus.

## Materials and Methods

### Materials

HRP, pronase, diaminobenzidine, SPDP (3-[2-pyridyldithio]-propionic acid *N*-hydroxysuccinimideester), asialofetuin, Hepes, and Tris were obtained from Sigma Chemical Co., St. Louis, MO. Nycodenz was obtained from NYCOMED (Oslo, Norway); CMP[<sup>14</sup>C]sialic acid, [<sup>3</sup>H]leucine and Na<sup>125</sup>I were from the Radiochemical Centre (Amersham, UK). Neuraminidase (from *Vibrio cholera*) was obtained from Behring, (Marburg, Germany). High-performance thin-layer chromatography (HPTLC) plates coated with Silica Gel 60 were purchased from E. Merck (Darmstadt, Germany). Standard mixture of pure neutral glycolipids containing Gal $\beta$ 1-Ceramide (Cer) (CMH), Gal $\beta$ 1-4Glc $\beta$ 1-Cer(CDH), Gal $\alpha$ 1-4Gal $\beta$ 1-4Glc $\beta$ 1-Cer(Gb<sub>3</sub>[CTH]), GalNAc $\beta$ 1-3Gal $\alpha$ 1-4Gal $\beta$ 1-4Glc $\beta$ 1-Cer(Gb<sub>4</sub>[GL4]), GalNAc $\alpha$ 1-3GalNAc $\beta$ 1-3Gal $\alpha$ 1-4Gal $\beta$ 1-4Glc $\beta$ 1-Cer (Forssman pentasaccharide) was from BioCarb AB (Lund, Sweden). Polyisobutylmethacrylate was obtained from Plexigum (Polysciences, Inc., Warrington, PA). Horse anti-Shiga toxin serum

1. *Abbreviations used in this paper:* CMP sialic acid, cytidine monophosphate sialic acid; H-buffer, homogenization buffer; HPTLC, high-performance thin-layer chromatography; PNS, postnuclear supernatant; SPDP, (3-[2-pyridyldithio]-propionic acid *N*-hydroxysuccinimideester); TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

was obtained from Bureau of Biologics, FDA, (Bethesda, MD). Shiga toxin was purified as previously described (7), and was a generous gift from Dr. J. E. Brown. Conjugates of Shiga toxin and HRP were prepared by the SPDP method as previously described (46).

## Cells

MDCK cells, strain I, were grown in Costar 3,000 flasks (Costar, Badhoevedorp/The Netherlands), or on polycarbonate filters (Costar Transwell, pore size 0.4  $\mu\text{m}$ , 24.5-mm diam). The cells were routinely seeded at a density of  $10^6$  per filter and used for experiments 2 to 5 d later (49). All filters used for experiments had a transepithelial resistance of at least 1,000  $\Omega\text{ cm}^2$  as measured with the Millicell-ERS equipment (Millipore Continental Water Systems, Bedford, MA, USA). The medium used was DME (3.7 g/l sodium bicarbonate) (Flow Laboratories, Irvine, Ayrshire, Scotland) containing 5% FCS (Gibco Laboratories, Ltd., Paisly, Scotland) and 2 mM L-glutamine (Gibco Laboratories).

## Formaldehyde Fixation and Isobutanol Extraction of Cells

Cell monolayers were incubated for 30 min at 4°C with 1% formaldehyde in PBS with 2 mM  $\text{CaCl}_2$ . The cells were then washed twice in PBS and incubated for 10 min at 25°C with 50 mM  $\text{NaBH}_4$  in PBS. The cells were again washed in PBS, and when indicated, the cells were incubated for 10 min at 25°C with water saturated with isobutanol to extract lipids. At the end of this incubation the cells were again washed with PBS.

## Binding of Shiga Toxin to Isolated Glycolipids

Total neutral and acidic glycolipids from MDCK cells ( $1 \times 10^{12}$  cells) grown as monolayers in plastic flasks were prepared as described by Hakamori and Kanfer (18). The cells were lyophilized and extracted twice with 10 vol (50 ml) of chloroform:methanol:water (4:8:3) overnight. Cell debris were removed by filtration through Whatman filter papers, the filtrates were pooled, and neutral and sialic acid containing glycolipids were separated using Folch partition (15). Samples were separated on Silica Gel 60 HPTLC plates using chloroform:methanol:water (60:35:8) for neutral glycolipids and chloroform:methanol:0.2%  $\text{CaCl}_2$  (60:40:9) for acidic glycolipids, as mobile phases. Bands were visualized with 10% sulfuric acid in ethanol. Highly purified  $^{125}\text{I}$ -labeled Shiga toxin was tested for binding to the prepared glycolipids (3). The air-dried chromatogram was soaked in 100 ml 0.05% polyisobutylmethacrylate in hexane  $3 \times 1$  min, followed by incubation in PBS supplemented with 1% BSA and 0.05% Tween 20 for 15 min. The plate was then incubated with 50 ml 20  $\mu\text{g/ml}$   $^{125}\text{I}$ -labeled Shiga toxin (280 cpm/ng) for 1 h, washed 5 times with 0.05% Tween 20 in PBS, air dried and exposed to X-OMAT X-ray film (Eastman Kodak Co., Rochester, NY) for 48 h. All incubations were performed at 20°C.

## 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  $\mu\text{Ci}$  of [ $^3\text{H}$ ]leucine per ml. Then the solution was removed, the cells were washed twice with 5% (wt/vol) TCA 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 <10% of the average value.

## Measurement of Receptor-mediated Endocytosis of $^{125}\text{I}$ -Transferrin and $^{125}\text{I}$ -Ricin

The amount of internalized transferrin was measured as described by Ciechanover (8). Endocytosis of  $^{125}\text{I}$ -labeled ricin was measured as the amount of toxin that could not be removed with lactose as previously described (36).

## Subcellular Fractionation of Polarized Cells Incubated with Shiga Toxin

$^{125}\text{I}$ -Shigella toxin (1  $\mu\text{g/ml}$ ) was administered either to the apical or the basolateral medium of filter-grown MDCK I cell monolayers treated with butyric acid as described in the legend to Fig. 3. The toxin was continuously present during the incubation, and HRP was usually also added to label endosomal/lysosomal compartments. The incubations were carried out technically as described by Bomsel et al. (5). The incubations were terminated

by cooling the cells, and all subsequent steps were carried out at 0–4°C. The filters were cut out of their plastic holders, and washed  $3 \times 10$  min in cold PBS/0.2% BSA with shaking. To each filter was then added 0.5 ml homogenization buffer (H-buffer; 0.3 M sucrose, 3 mM imidazole, pH 7.4), and the cells were scraped off the filters with a rubber policeman. Each filter was subsequently scraped once more and washed with  $2 \times 1$  ml of H-buffer. Cells from 3–6 filters were pooled for each experimental point. The pooled cells were pelleted by centrifugation for 10 min at 100 g. To the pellet was added H-buffer to 1 ml, it was resuspended and homogenized by passing it 10 times up and down through a 1-ml blue tip on a pipette (Gilson Co., Inc., Worthington, OH) followed by six times through a 1-ml syringe with a 22G 1/4 needle. The homogenate was centrifuged at 2,500 rpm for 10 min in 1.5-ml tubes (Brinkman Instruments Inc., Westbury, NY) in a centrifuge (model 5415; Beckman Instruments, Inc.) to obtain a nuclear pellet and a postnuclear supernatant (PNS). The PNS was subjected to discontinuous gradient centrifugation in a system similar to one applied by Sandberg et al. (35) to separate rat liver Golgi fractions from rough and smooth ER. In the bottom of SW 40 tubes, gradients were made of 4.5-ml light solution (1.15 M sucrose, 15 mM CsCl) and 1.5 ml heavy solution (1.15 M sucrose, 15 mM CsCl, 15% Nycodenz [wt/vol]). The gradients were made in a Bio-comp Gradient Master (Nycomed, Oslo, Norway) (angle 74, speed 16, time 2 min 45 s). PNS (5.6 parts) was mixed with 2 M sucrose, 10 mM CsCl (4.4 parts), usually a total of 1.5 ml, and layered on top of the gradient. This was again overlaid with 3 ml 0.9 M sucrose, and finally 1–2 ml of 0.3 M sucrose. After 4.5 h at 33,000 rpm, the gradients were fractionated (25–30 fractions) and analyzed with respect to marker distribution. The behavior of MDCK cells in this system was characterized.

## Enzyme Analysis

HRP was measured according to Steinman et al. (45), UDP-galactose:glycoprotein galactosyl transferase according to Brändli et al. (6), esterase and  $\beta$ -N-acetyl-glucosaminidase according to Beaufay et al. (1), and sialyltransferase according to Dall'Olio et al. (10).

## Processing for EM

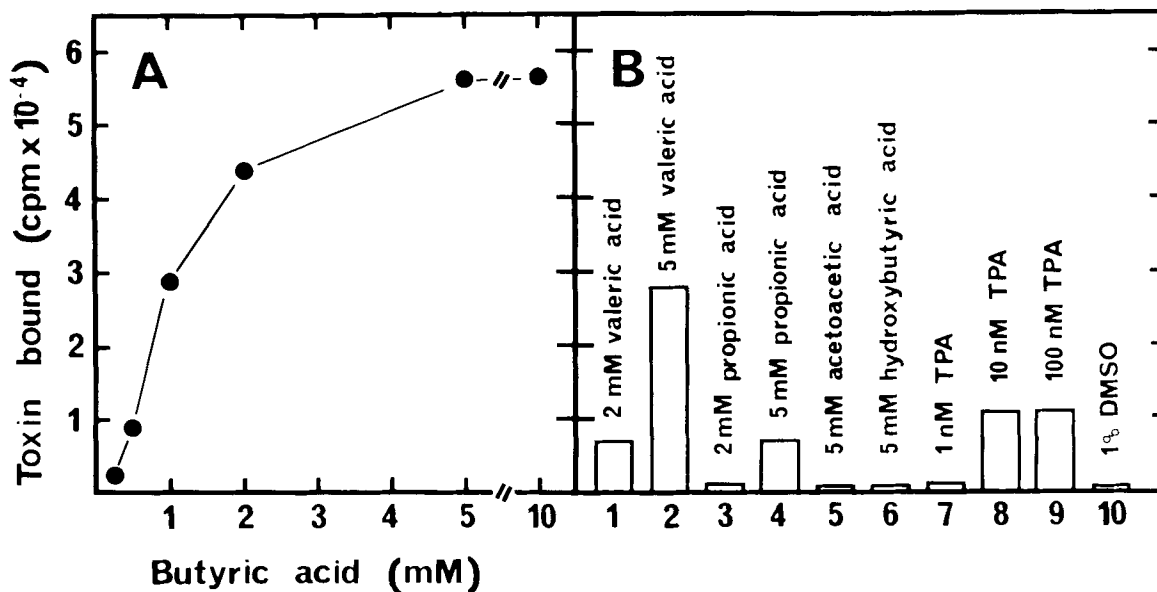
MDCK cells grown in monolayers in T-25 flasks or on filters, were treated as described in the text, and fixed with 2% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.2, for 60 min at room temperature. The cells were then carefully washed with PBS and incubated with diaminobenzidine- $\text{H}_2\text{O}_2$  as previously described (46). The cells were postfixated with  $\text{OsO}_4$ , treated with 1% uranyl acetate in distilled water, embedded in Epon, cut at 50 nm, and examined in an electron microscope (model 100 CX; JEOL USA, Electron Optics Div., Peabody, MA) without further contrasting as previously described (46).

## Results

### Induction of Binding Sites for Shiga Toxin on MDCK Cells Grown on Impermeable Substrate

Differentiation agents such as butyric acid, phorbol esters, retinoic acid and dimethylsulfoxide have been shown to produce changes in the carbohydrate pattern at the cell surface (14, 31). We therefore tested whether such agents were able to induce synthesis of Shiga toxin-binding sites in MDCK cells. When MDCK cells grown on impermeable substrate were incubated with increasing concentrations of butyric acid for 48 h, the butyric acid-treated cells were able to bind  $^{125}\text{I}$ -labeled Shiga toxin whereas untreated cells did not possess this ability (Fig. 1 A). In agreement with this we found that while a Shiga-HRP conjugate (40) did not bind to control MDCK cells, it distinctly labeled MDCK cells treated with 5 mM butyric acid (see Fig. 6 a). Maximal binding ( $\sim 6 \times 10^6$  toxin molecules/cell) was obtained with 5 mM butyric acid.

The appearance of Shiga toxin receptors at the cell surface appears to be dependent on the synthesis of new receptors since cycloheximide blocked the butyric acid-mediated in-



**Figure 1.** Effect of short-chain fatty acids, TPA, and DMSO on the binding of  $^{125}\text{I}$ -labeled Shiga toxin to MDCK cells. MDCK cells growing in 24-well disposable trays were incubated for 48 h in bicarbonate-containing medium with 10% FCS in the presence of increasing concentrations of butyric acid (A), or with valeric and propionic acid, and with different concentrations of TPA and DMSO (B). The cells were then incubated with  $^{125}\text{I}$ -labeled Shiga toxin (0.2 ml with 120 ng/ml; 5900 cpm/ng) for 20 min at 37°C in a Hepes-containing medium with 5% serum. Then the cells were washed three times in ice-cold PBS, they were dissolved in KOH (0.1 M), and the radioactivity was measured. The deviations between duplicate measurements were <10%.

duction of Shiga toxin binding (data not shown). Probably cycloheximide inhibited synthesis of enzymes required for glycolipid synthesis. Also, treatment of noninduced MDCK cells with pronase to expose possible "cryptic" glycolipids at the cell surface did not reveal any binding sites for the toxin.

Also the fatty acids valeric acid and propionic acid induced the appearance of binding sites for Shiga toxin, although to a lesser extent than butyric acid. Butyric acid, in contrast to hydroxybutyric and acetoacetic acid, is reported to induce hyperacetylation of histones (42), a phenomenon which may be related to some of the effects of butyric acid. We therefore tested whether these related compounds were able to induce appearance of Shiga toxin binding sites. As shown in Fig. 1 B that was not the case.

Of the other differentiation agents tested, only the phorbol ester TPA induced binding of  $^{125}\text{I}$ -Shiga toxin, although to a smaller extent than butyric acid. TPA and butyric acid together gave no additive effect (data not shown), suggesting that the compounds act at least partially through the same pathway. The inactive phorbol ester phorbol 12-myristate, 13-acetate, 4-*O*-methylether, and the compounds dimethylsulfoxide (1%), retinoic acid ( $10^{-5}$  M), and dexamethasone (20–250  $\mu\text{M}$ ) did not induce any binding of Shiga toxin to MDCK cells (Fig. 1 B and data not shown).

We next measured the time course for the butyric acid and TPA-induced appearance of Shiga toxin receptors. As shown in Fig. 2, no binding could be measured until 18–20 h after addition of either of the two compounds regardless of the concentrations used. The binding then increased rapidly and leveled off after  $\sim 36$  h.

#### **Binding of Shiga Toxin to Polarized MDCK Cells Grown on Filters**

As described for MDCK cells grown on a plastic surface,

filter-grown MDCK cells did not bind detectable amounts of Shiga toxin. We therefore decided to test whether Shiga toxin-binding sites could be induced in filter-grown MDCK cells after they had formed tight junctions (resistance higher than 1,000  $\Omega \text{ cm}^2$ ), and whether the receptors appeared on both sides of the cells. The experiments revealed that when 1–2 mM butyric acid was added shortly after formation of a cell layer with the required electrical resistance, the cells did express binding sites for Shiga toxin both apically and basolaterally without loss of electrical resistance (data not shown). At higher concentrations of butyric acid the electrical resistance was strongly reduced. When the cells were incubated with Shiga toxin (2  $\mu\text{g}/\text{ml}$ ) for 1 h at 0°C or for 20 min at 37°C,  $\sim 2 \times 10^5$  molecules were bound per cell. The amount of toxin bound basolaterally was 1.6 times higher than the amount bound apically (data not shown). Considering the larger surface area of the basolateral side (a factor of 3–6) (30), this implies a higher density of binding sites at the apical side. When MDCK cells were incubated with butyric acid before they were plated on filters (Fig. 3), similar amounts of Shiga toxin was bound as when butyric acid was added to filter-grown cells. This method was used to obtain binding sites for Shiga toxin in the fractionation studies shown below, since it was more reproducible. The phorbol ester TPA can not be used to induce synthesis of binding sites for Shiga toxin in filter-grown cells since it rapidly abolishes the electrical resistance across the cell layer (28).

#### **Properties of the Shiga Toxin Binding Sites on MDCK Cells**

The functional Shiga toxin binding sites on sensitive cells studied so far have proved to be glycolipids (9, 40; Mobasaleh, M., A. Donohue-Rolfe, R. Montgomery, G. Keusch, and R. Grand. 1986. *Pediatr. Res.* 20:a245.). To investigate

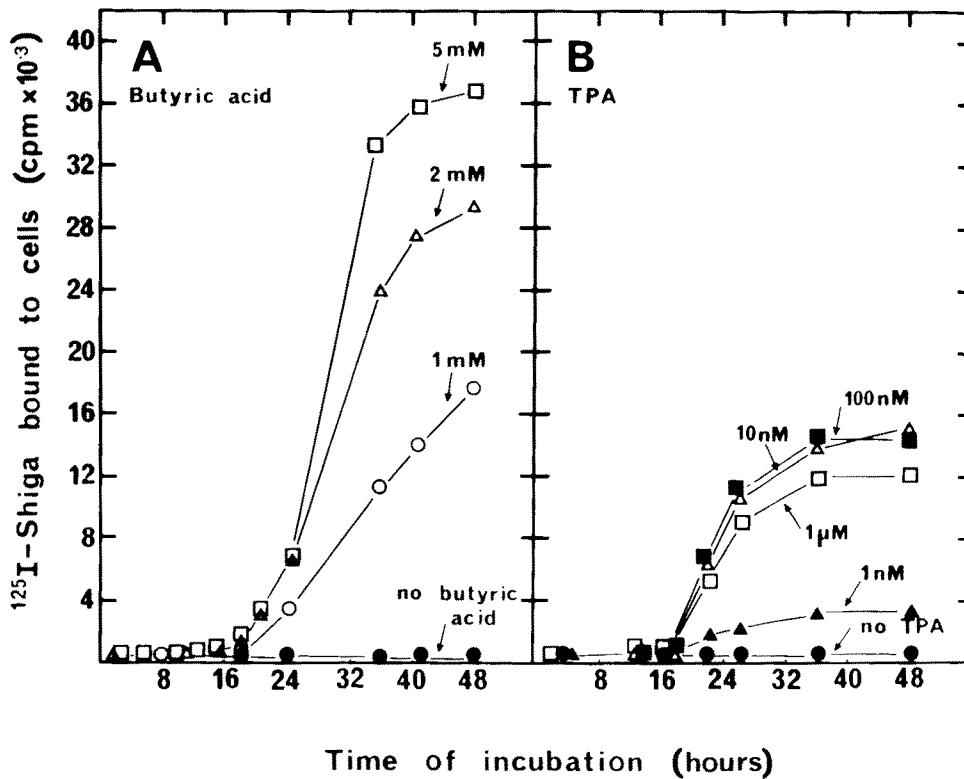


Figure 2. Time course for the appearance of Shiga toxin binding sites upon addition of butyric acid and TPA. MDCK cells growing in 24-well disposable trays were incubated in a bicarbonate-containing medium with 10% FCS with and without the indicated concentrations of butyric acid (A) or TPA (B). After the indicated periods of time the binding of  $^{125}\text{I}$ -labeled Shiga toxin to the cells was measured as described in the legend to Fig. 1.

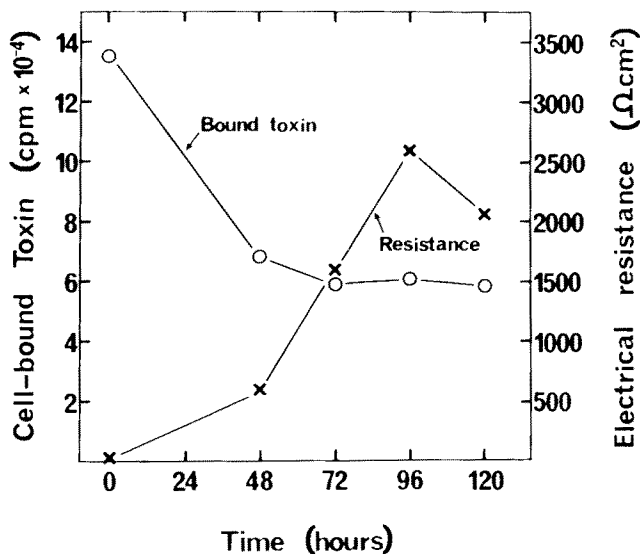


Figure 3. Development of electrical resistance and binding of Shiga toxin to butyric acid-treated MDCK cells growing on polycarbonate filters. MDCK cells were incubated with 2 mM butyric acid in flasks for 48 h. The cells were then trypsinized, transferred to fresh medium containing 1 mM butyric acid, and plated on filters. Binding of  $^{125}\text{I}$ -labeled Shiga toxin to the cells was measured both at the time when the cells were transferred to the filters and after increasing time when growing on the filters. The cells were incubated for 20 min at  $37^\circ\text{C}$  in the presence of toxin (1,500 ng/ml; 685 cpm/ng), they were then washed three times in ice-cold PBS and the cell-bound radioactivity was measured. Also the electrical resistance across the filters was measured after different periods of incubation. (O), Cell-bound toxin; (X), electrical resistance.

if that was the case also in MDCK cells, we treated cells with butyric acid-induced binding sites with pronase. If the binding sites were glycoproteins, the pronase treatment should abolish the binding. However, as shown in Table I, pronase treatment did not decrease, but rather slightly increased the binding of  $^{125}\text{I}$ -labeled Shiga toxin to the cells, suggesting that also on MDCK cells the toxin is bound to glycolipids. Similar results were obtained with filter-grown MDCK cells. Furthermore, extraction of glycolipids from formaldehyde-fixed cells almost completely removed the toxin binding sites (Table I). We were also able to demonstrate that  $^{125}\text{I}$ -Shiga toxin binds to glycolipids extracted from MDCK cells only after treatment of the cells with butyric acid (Fig. 4).

The lipids were separated on a thin-layer chromatogram, and binding of toxin was revealed by autoradiography. In the neutral glycolipid fraction from butyric acid-treated cells Shiga toxin was found to bind to two closely migrating glycolipids with approximately the same  $R_f$  value as for globotriallyceramide (Fig. 4 b, lane 3). Globotriallyceramide was the only glycolipid in the standard mixture of neutral glycolipids which bound the toxin. No binding of toxin to sialic acid-containing glycolipids was detected (data not shown).

Shiga toxin binds the structure gal  $\alpha 1-4$  gal (9, Mobasaleh, M., A. Donohue-Rolfe, R. Montgomery, G. Keusch, and R. Grand. 1986. *Pediatr. Res.* 20:a245). Sialylation of this structure might reduce the binding of toxin. We therefore treated butyric acid-induced MDCK cells with neuraminidase. As shown in Table I the enzyme treatment almost doubled the binding of toxin to the cell surface, suggesting that removal of sialic acid from galactose indeed revealed additional binding sites. Control experiments showed that

**Table I. Effect of Neuraminidase, Pronase, and Lipid-extraction on the Binding of <sup>125</sup>I-Shiga Toxin to Butyric Acid-Treated MDCK Cells**

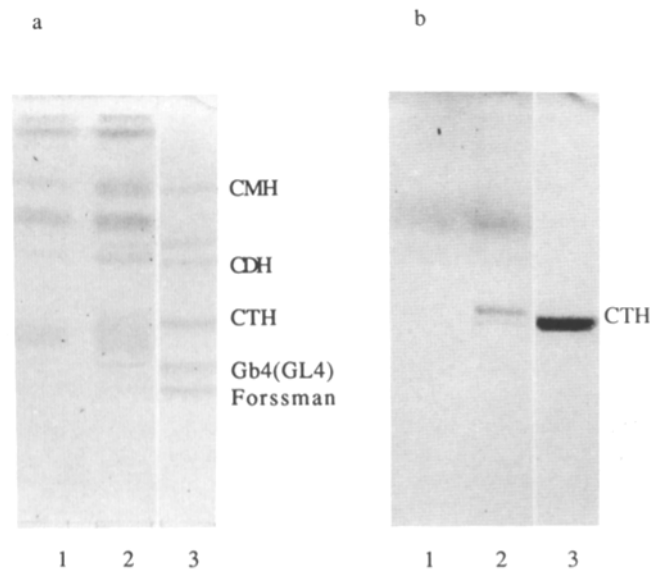
Treatment	Toxin bound (% of control)
None	100
Pronase*	179
Fixed and isobutanol-treated cells†	4
Neuraminidase‡	188

\* MDCK cells growing on plastic and incubated with butyric acid (5 mM) for 48 h were incubated with pronase (1 mg/ml) for 15 min at 37°C. The cells were then transferred to Eppendorf tubes (Brinkman Instruments, Inc.) spun down, and resuspended in 1 ml HEPES medium with 10% serum and <sup>125</sup>I-Shiga toxin. After 20-min incubation at 37°C, the cells were washed twice and the cell-associated radioactivity was measured.

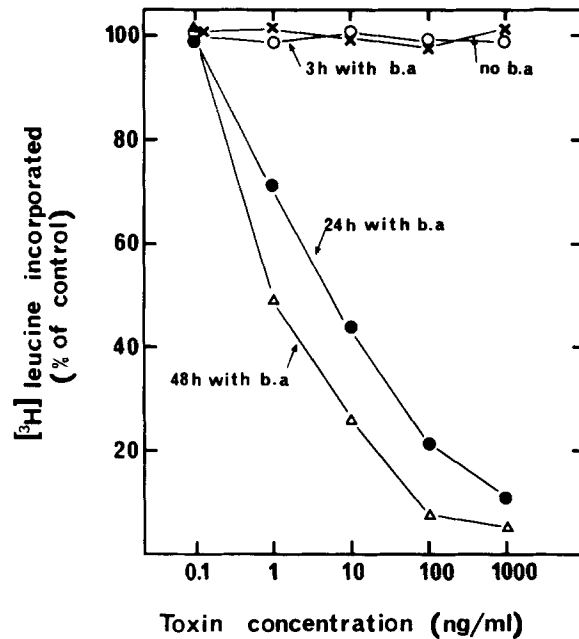
† Cells growing in 24-well disposable trays were incubated for 48 h with butyric acid (5 mM), fixed with formaldehyde, and extracted with isobutanol as described in Materials and Methods. Both untreated and treated cells were then incubated with <sup>125</sup>I-Shiga toxin for 20 min at 37°C, washed twice with PBS, and finally the amount of bound toxin was measured.

‡ Cells incubated with butyric acid (5 mM) for 48 h were treated with neuraminidase (0.1 U) for 30 min at 37°C. Then binding of <sup>125</sup>I-Shiga toxin was measured as described above.

neuraminidase treatment did not reveal any binding sites for Shiga toxin on MDCK cells not incubated with butyric acid. That the butyric acid-induced synthesis of Shiga toxin receptors also in MDCK cells involves addition of galactose to surface structures is supported by the finding that there is no induction of Shiga toxin receptors in a mutant MDCK cell line with a reduced number of galactose residues on the cell surface (data not shown).



**Figure 4. Binding of <sup>125</sup>I-Shiga toxin to glycolipids.** Glycolipids from MDCK cells were extracted and separated on HPTLC plates as described in Materials and Methods. (a) HPTLC pattern of total neutral glycolipids from MDCK cells grown without butyric acid (lane 1), and with 5 mM butyric acid for 48 h (lane 2). Lane 3 shows a standard mixture of glycolipids containing pure monohexylceramide (CMH), galabiosylceramide (CDH), globotriacylceramide (CTH), Globoside (Gb<sub>4</sub> (GL<sub>4</sub>)) and Forssman pentasaccharide. In b is shown an autoradiogram of the binding of <sup>125</sup>I-Shiga toxin to the same glycolipids. The binding experiment was performed as described in Materials and Methods.



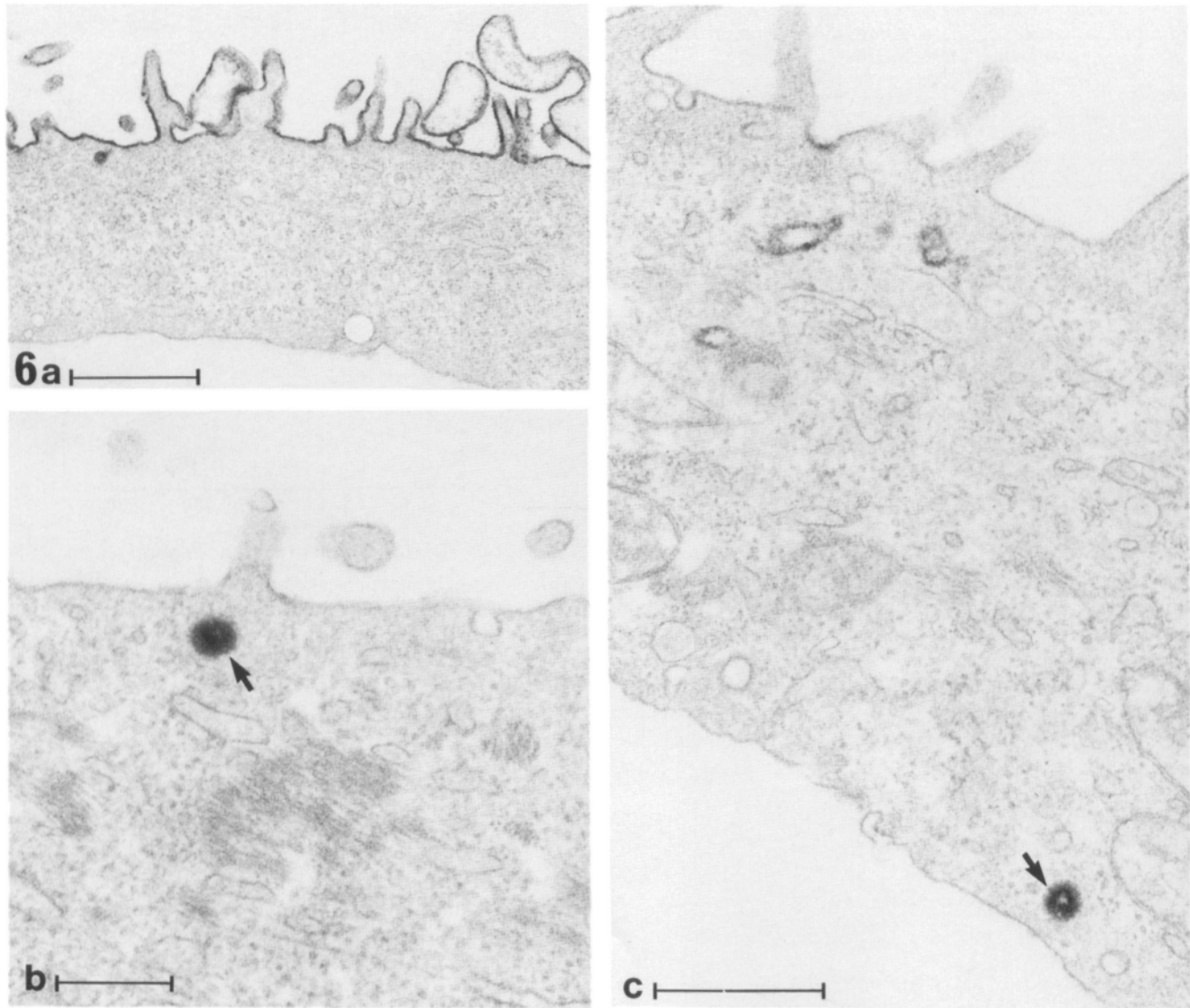
**Figure 5. Effect of butyric acid on the sensitivity of MDCK cells to Shiga toxin.** MDCK cells growing in 24-well disposable trays in bicarbonate-containing medium with 10% FCS were incubated with and without butyric acid (5 mM) for the indicated periods of time. Then increasing concentrations of Shiga toxin were added and the protein synthesis was measured as described in Materials and Methods 3 h later.

#### **Intoxication of Butyric Acid-treated MDCK Cells**

Shiga toxin inhibits protein synthesis in a limited number of cell lines. Even cells that possess binding sites for the toxin may be completely resistant (12). To test whether MDCK cells treated with butyric acid are sensitive to Shiga toxin, we incubated the cells with 5 mM butyric acid and then added Shiga toxin after increasing periods of time. After 3 h in the presence of toxin we measured the protein synthesis. As shown in Fig. 5, the cells were resistant when toxin and butyric acid were added simultaneously. Also, when the toxin was added 3 h later than butyric acid, the cells were still resistant. However, when the cells had been incubated for 24 or 48 h with butyric acid they were intoxicated upon addition of toxin. The sensitizing effect of butyric acid was reversible. Cells treated with butyric acid became resistant to the toxin 24 h after removal of the fatty acid (data not shown). The results therefore indicate that the new binding sites obtained are functional in the sense that they mediate the translocation of Shiga toxin to the cytosol.

#### **Endocytosis and Intracellular Traffic of Shiga Toxin in MDCK Cells**

We have previously shown that Shiga toxin in toxin-sensitive HeLa cells is internalized by the coated pit/coated vesicle pathway in spite of being bound to glycolipid receptors in sensitive cells (40). The internalization of Shiga-HRP in MDCK cells treated with butyric acid appears to occur in a similar way. Thus, as shown in Fig. 6, Shiga-HRP was evenly distributed at the cell surface at 4°C. Upon warming the cells to 37°C, a marked redistribution of Shiga-HRP occurred. The toxin was rapidly internalized in coated pits and



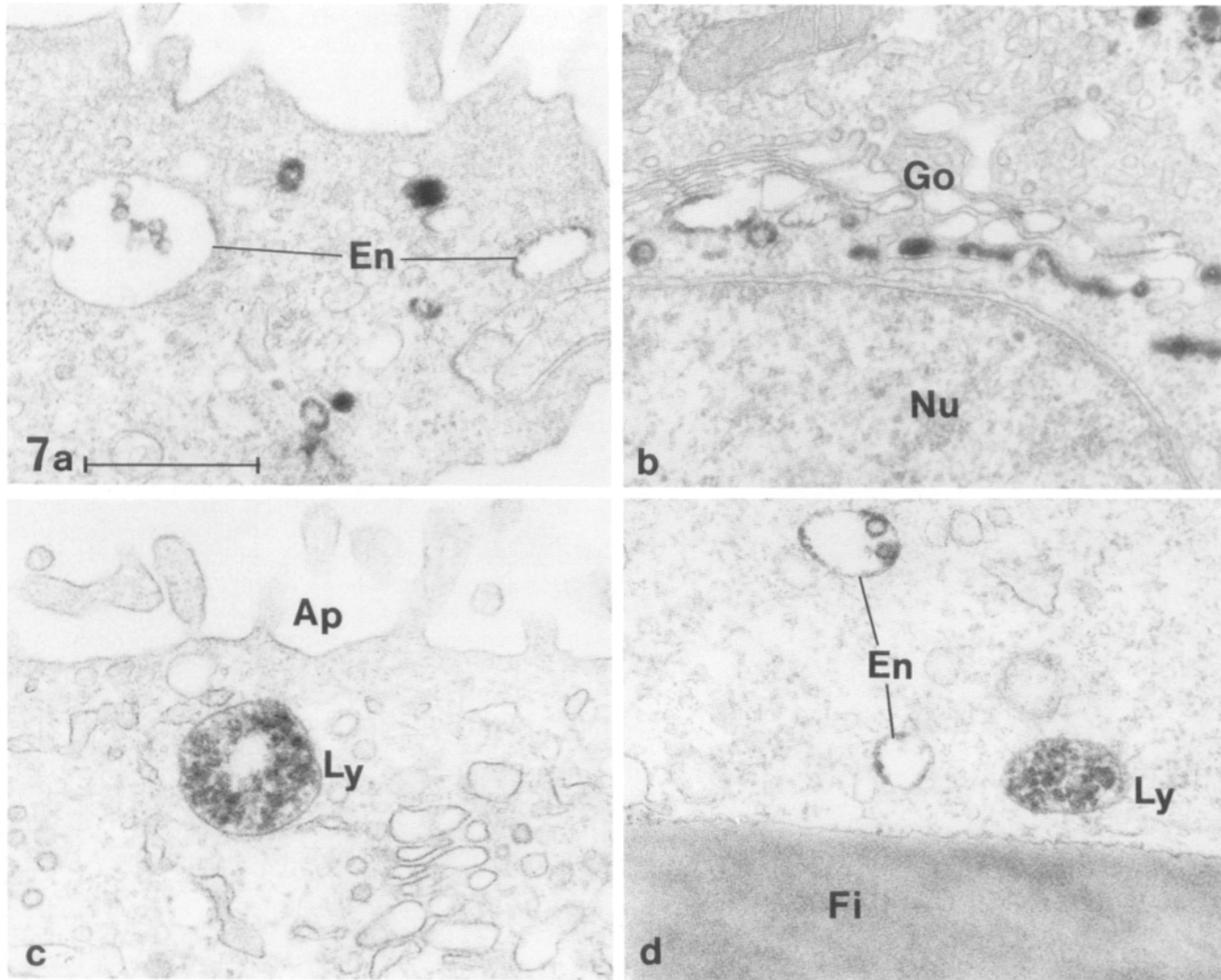
**Figure 6.** Labeling of butyric acid (5 mM)-treated MDCK cells with Shiga-HRP. In *a* cells were incubated with Shiga-HRP at 4°C, and a uniform labeling (HRP reaction product) is seen on the cell surface. In *b* and *c* cells were incubated with Shiga-HRP at 4°C, and thereafter washed and further incubated for 15 min at 37°C. In this situation surface labeling is reduced or absent whereas coated pits and/or vesicles (arrows) are heavily labeled. The cells were grown on plastic. Bars: (a) 1  $\mu\text{m}$ ; (b) 0.25  $\mu\text{m}$ ; and (c) 0.5  $\mu\text{m}$ .

vesicles (Fig. 6). That Shiga toxin was endocytosed from coated pits also in MDCK cells was as earlier shown for HeLa cells (40) supported by the finding that acidification of the cytosol, which inhibits endocytosis from coated pits (38), protected the cells against the toxin (data not shown). After 15–60-min incubation with Shiga-HRP at 37°C, reaction product was distinct in endosomes and lysosomes (Fig. 7). Also Golgi profiles with distinct reaction product could be observed (Fig. 7).

Butyric acid treatment of cells has been reported to cause morphological changes (17, 22). We therefore tested whether treatment of MDCK cells with butyric acid had any effect on the rate of clathrin-dependent and clathrin-independent endocytosis. To monitor these two processes we measured the uptake of transferrin, ricin, and HRP. Transferrin is known to be internalized from coated pits, whereas ricin and fluid phase markers seem to be endocytosed also by a clathrin-

independent mechanism (38, 39, 41). HRP uptake was measured both at the apical and the basolateral side of filter-grown MDCK cells. We observed no changes in the uptake of these markers for endocytosis (data not shown), suggesting that butyric acid does not affect endocytosis as such.

To study the intracellular traffic of  $^{125}\text{I}$ -Shiga toxin in a quantitative way, we applied subcellular fractionation methods to polarized cells incubated with the  $^{125}\text{I}$ -labeled toxin and measured the amount of toxin associated with the various intracellular organelles. In this discontinuous gradient system the PNS is mixed with a heavy solution and is positioned in the middle of the tube before centrifugation. The load zone is overlaid by a 0.9 M sucrose layer, which is again overlaid by a 0.3 M sucrose layer (see Materials and Methods). Organelle markers separated in three reproducible peaks. Most of the galactosyl- and sialyltransferase activity moved upward in the gradient to the 0.9 M/0.3 M sucrose interphase



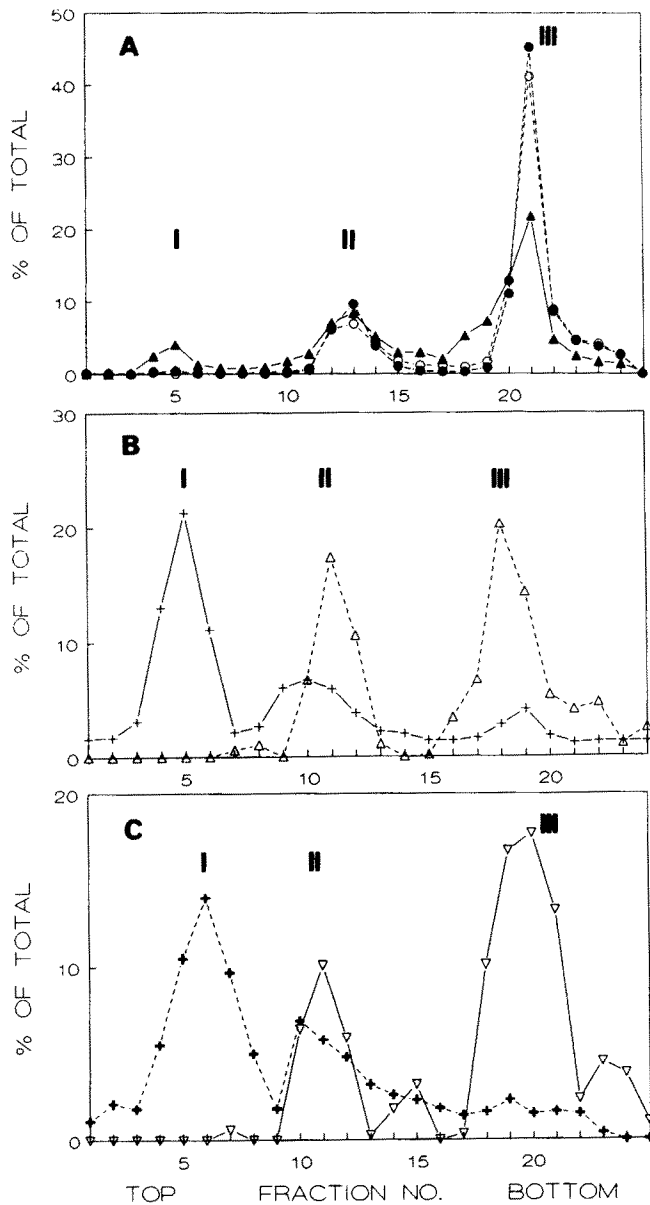
**Figure 7.** Endocytosis of Shiga-HRP in butyric acid (5 mM)-treated MDCK cells. In *a*, labeled endosome-like vacuoles (En) are seen, after 15 min at 37°C, and in *b* is shown a labeled Golgi complex (Go) next to the nucleus (Nu), after 60 min at 37°C. *a* and *b*, cells grown on plastic. In *c* butyric acid-treated cells grown on a filter have been incubated with Shiga-HRP from the apical surface for 90 min. The apical (Ap) plasma membrane is unlabeled whereas a lysosome-like vacuole (Ly) is heavily labeled. In *d* butyric acid-treated MDCK cells on a filter were incubated for 90 min with Shiga-HRP from the basal side. A portion of the filter is seen (Fi). Both endosome-like (En) and lysosome-like (Ly) vacuoles are present. Bar, 0.5  $\mu\text{m}$ .

(peak I in Fig. 8), indicating that the Golgi apparatus components could be recovered from this part of the gradient.

A second peak of enzyme activity (peak II in Fig. 8) remained in the load zone. This part of the gradient contained markers released from disrupted endosomes and lysosomes during homogenization (HRP from endosomes and  $\beta$ -N-acetyl-galactosaminidase from lysosomes) as well as plasma membrane-bound toxin (verified by fractionation of cells with toxin bound at 0°C). From these experiments it could be calculated that only 0.2% of the plasma membrane-bound toxin was recovered in the Golgi-rich fractions. Thus, our measurements of Shiga toxin transport to the Golgi apparatus are not affected by contamination by plasma membrane.

Peak III in Fig. 8 contained a number of organelles that had moved downward into the heavy gradient. Endosomes (loaded with HRP), lysosomes ( $\beta$ -N-acetyl-galactosaminidase), and ER (esterase) were found in this peak. All classes

of endosomes were found in this position (Fig. 8), early apical endosomes as revealed by the location of HRP in cells incubated with this enzyme for 10 min at 37°C, early basolateral endosomes (labeled in the same way), and late endosomes (the cells were incubated with HRP for 10 min at the apical or basolateral side followed by a 40-min chase without HRP). Also after 2-h continuous incubation with HRP at 37°C this peak was labeled. Typically, the recovery of HRP within organelles was 60% after homogenization and centrifugation, while 80% of the lysosomes were intact. The maximal contamination of the Golgi enriched fractions of these gradients was 0.4% of the total cell-associated HRP and 0.8% of the total cell-associated marker  $\beta$ -N-acetyl-galactosaminidase. These contaminations could either represent molecules released during fractionation and sticking to Golgi membranes, or they could represent intact organelles. In any case, the contaminations are too small to affect our quan-



**Figure 8.** Distribution of Shiga toxin, HRP, and organelle markers in a discontinuous sucrose/Nycodenz gradient system. Filter-grown, butyric acid-treated (see legend to Fig. 3) MDCK cells were incubated apically with  $^{125}\text{I}$ -Shiga toxin ( $1\ \mu\text{g}/\text{ml}$ ;  $10,000\ \text{cpm}/\text{ng}$ ) and HRP ( $3\ \text{mg}/\text{ml}$ ) for 60 min at  $37^\circ\text{C}$  (A), basolaterally for 10 min with HRP (B), or basolaterally for 10 min with HRP followed by a 40-min chase in HRP-free medium (C). (A): ( $\blacktriangle$ ),  $^{125}\text{I}$ -Shiga toxin; ( $\bullet$ ),  $\beta$ -*N*-Acetylglucosaminidase; ( $\circ$ ), HRP, 60 min at  $37^\circ\text{C}$  apically. (B): ( $\Delta$ ), HRP, 10 min basolaterally; (+), UDP-galactosyltransferase. (C): ( $\nabla$ ), HRP, 10 min plus 40-min chase basolaterally; (+), sialyltransferase.

titative results. We verified that incubation of filter-grown cells with butyric acid had no effect on the localization of the different organelles in the gradients.

By using this system we were able not only to measure the amount of Shiga toxin transported to the Golgi apparatus after different periods of time, but also to study whether this glycolipid-binding ligand is transported to the same extent

to the Golgi apparatus when added at the apical and the basolateral side of the cells. A typical experiment where the cells were incubated with Shiga toxin and HRP simultaneously for 1 h at  $37^\circ\text{C}$  is shown in Fig. 8. The experiment shows that Shiga toxin was found at the plasma membrane, in endosomes/lysosomes and in the Golgi fractions, whereas no HRP was detected in the Golgi apparatus. Furthermore, the experiments showed that Shiga toxin was transported rapidly to the Golgi compartment from the apical and the basolateral side of the cell monolayer, the transport being equally efficient from the two sides (Fig. 9). As shown, Shiga toxin was found in the Golgi apparatus already after 15 min, and the fraction associated with this organelle increased with time. The fraction of endocytosed toxin transported to the Golgi apparatus can be estimated by subtracting the amount of toxin found in the load zone, presumably membrane-bound toxin. Expressed in this way the fraction of toxin transported to the Golgi apparatus after 1 h at  $37^\circ\text{C}$  was 10% after basolateral addition and 11% after apical addition.

It has earlier been shown for several cell types that there is little or no transport from the cell surface to the Golgi apparatus at temperatures below  $18$ – $20^\circ\text{C}$  (37, 47, 48). In agreement with this, we found a strongly reduced transport of Shiga toxin to the Golgi apparatus at low temperatures (Fig. 10). The amount of Shiga toxin in the gradients represents essentially all the internalized toxin since there is almost no degradation of toxin (measured as nonprecipitable radioactivity) during a 2-h incubation period (data not shown).

## Discussion

The results show that functional binding sites for Shiga toxin in MDCK cells can be induced by incubation of the cells with butyric acid and TPA. As earlier shown for other cell types (9, 20, 23, 40; Mobassaleh, M., A. Donohue-Rolfe, R. Montgomery, G. Keusch, and R. Grand. 1986. *Pediatr. Res.* 20: a245.) also the binding sites in MDCK cells seem to be glycolipids. We were able to show that butyric acid induced synthesis of neutral glycolipids that bind Shiga toxin. Furthermore, the binding sites were removed upon extraction of glycolipids with butanol but not by pronase treatment. The importance of the galactose residues was supported by the finding that neuraminidase treatment increases the binding and that a mutant cell line with a reduced number of terminal galactose residues was unable to synthesize binding sites for the toxin upon addition of butyric acid. Interestingly, the binding sites were able to mediate transport of the toxin to the cytosol where the toxin inhibits the protein synthesis enzymatically and thereby kills the cell (33).

It has been shown that differentiation agents such as butyric acid, phorbol esters, and retinoic acid may change the glycosylation pattern at the cell surface (2, 13, 14, 31). As shown here, only butyric acid and TPA induced synthesis of Shiga toxin-binding sites in MDCK cells. Retinoic acid and DMSO (27) had no effect. Also dexamethasone has been shown to affect glycosyltransferase activities (51) and to interfere with the action of butyrate and TPA (16, 32, 44), but it was unable to induce synthesis of Shiga toxin-binding sites in MDCK cells. Butyric acid has been found to modulate the expression of a number of genes (13, 14, 19, 25, 31, 32, 34). This could be because of its ability to cause changes in phosphorylation of nuclear proteins (4, 11), changes in DNA



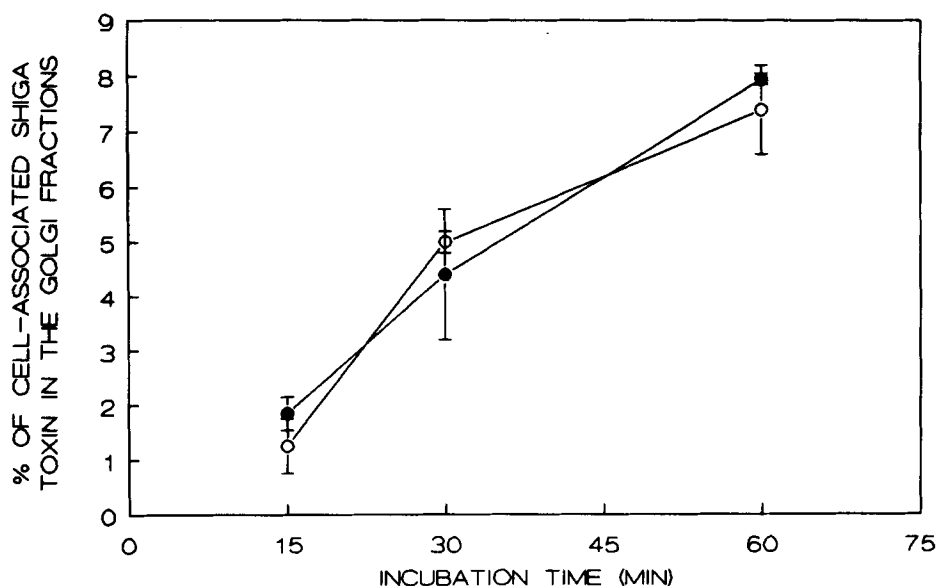


Figure 9. Transport rate of Shiga toxin from the apical and basolateral side of MDCK cells to the Golgi apparatus.  $^{125}\text{I}$ -labeled Shiga toxin was added to the basolateral or the apical side of butyric acid-treated MDCK cells, and the fraction of labeled toxin in the Golgi apparatus (peak I, Fig. 6) was measured after increasing periods of time as described in Materials and Methods. (○), apical toxin addition; (●), basolateral toxin addition. The bars represent the range of variation between three different experiments.

methylation (29), or hyperacetylation of histones (42). It is not clear whether such changes are involved in the induced synthesis of Shiga toxin-binding sites here observed, but hydroxybutyric acid and acetoacetic acid which are both unable to induce hyperacetylation of histones were also unable to induce synthesis of Shiga toxin-binding sites. As shown, both propionic acid and valeric acid which also in other systems affect cells in a similar way as butyric acid (25) induced synthesis of Shiga toxin-binding sites.

We have earlier shown that Shiga toxin in spite of being a glycolipid-binding ligand is internalized by the coated pit/coated vesicle pathway (40). As shown here this appears also to be the case in MDCK cells. It is not understood how glycolipids can be induced to aggregate in coated pits. It was recently shown that also glucosylceramide seems to be internalized from clathrin-coated pits (21), suggesting that lipids can be internalized by this pathway also without being cross-linked with a ligand such as Shiga toxin.

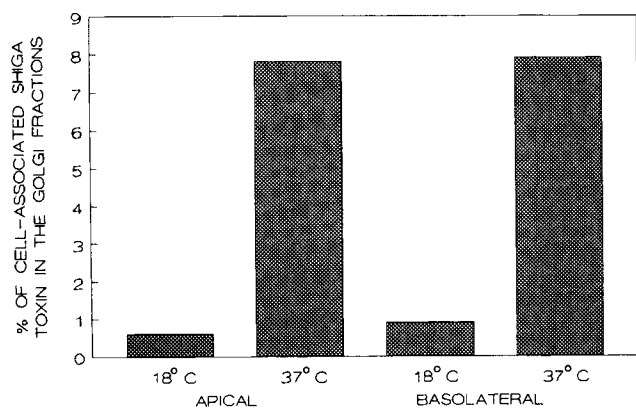


Figure 10. Effect of temperature on the amount of Shiga toxin transported to the Golgi apparatus. Butyric acid-treated MDCK cells (see Fig. 3) were incubated with  $^{125}\text{I}$ -Shiga toxin (1  $\mu\text{g}/\text{ml}$ ; 10,000 cpm/ng) added apically or basolaterally for 60 min at the indicated temperature. The cells were subsequently washed and homogenized as described in Materials and Methods.

We have earlier observed that a Shiga-HRP conjugate is transported to the Golgi apparatus of sensitive cells (40). As shown here this was also the case after induction of Shiga toxin-binding sites in MDCK cells. Similarly, a fluorescent analogue of glucosylceramide was found to be transported to the Golgi apparatus in BHK cells (21). However, in none of these studies was the amount of lipid transported to the Golgi apparatus quantified. Since Shiga toxin can be used as a marker for the transport of its glycolipid-binding sites, we therefore used  $^{125}\text{I}$ -labeled Shiga toxin and subcellular fractionation to determine the amount of toxin transported to the Golgi apparatus. Furthermore, we used polarized cells to test whether toxin internalization and the subsequent transport to the Golgi apparatus were equally efficient when the toxin was added apically and basolaterally. Interestingly our data demonstrate that Shiga toxin not only bound to both sides of the polarized cells, but it was rapidly internalized and transported to the Golgi apparatus from both the apical and the basolateral surface of the cells. Our quantitative studies with cell fractionation revealed that ca. 10% of the cell-associated Shiga toxin reached the Golgi complex after 60-min incubation at 37°C. We have previously found a similar figure, ca. 5%, for ricin reaching the Golgi complex in BHK cells (48). However, in contrast to Shiga toxin which binds only to glycolipids, ricin binds to both glycoproteins and glycolipids with terminal galactose.

That a higher amount of Shiga toxin was bound per surface area at the apical than at the basolateral side of the cells is in agreement with earlier measurements of the distribution of glycolipids in these cells (26, 43, 50). Whether glycolipids are normally excluded from coated pits to preserve the uneven distribution at the two poles of the cell is not known. However, our data strongly suggest that glycolipids can be efficiently transported to the Golgi apparatus from both sides of a polarized epithelial cell.

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