Endocytosis from Coated Pits of Shiga Toxin:  
A Glycolipid-binding Protein from Shigella dysenteriae 1

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Abstract. Evidence is presented that endocytosis is involved in the transport to the cytosol of the cytotoxin from Shigella dysenteriae 1, Shiga toxin, which acts by removal of a single adenine residue in 28-S ribosomal RNA. Inhibition of endocytosis by ATP depletion of the cells prevented toxin uptake. Exposure of HeLa S3 and Vero cells to toxin at low extracellular pH, where translocation to the cytosol, but not endocytosis is inhibited, allowed the toxin to accumulate in a compartment where it was protected against antibodies to the toxin. Upon transfer of the cells to normal medium endocytosed toxin entered the cytosol. Electron microscopical studies of cells exposed at 0°C to a toxin–horseradish peroxidase (HRP) conjugate, or to unconjugated toxin followed by horse antitoxin antibodies and then protein G-gold, revealed that the Shiga toxin binding sites were randomly distributed on the cell surface, without any preference to, for example, coated pits. In contrast, when cells were exposed to toxin at 37°C, the binding sites were preferentially localized in coated pits. The Shiga–HRP conjugate was also seen in endosomes, lysosomes, and in the Golgi region. Endocytosis by the coated pit/coated vesicle pathway was selectively inhibited by acidification of the cytosol. Under these conditions, both the uptake of toxin–HRP conjugates and intoxication of the cells were inhibited. Evidence from the literature as well as our own results suggest that Shiga toxin binding sites are glycolipids. Thus, Shiga toxin appears to be the first example of a lipid-binding ligand that is endocytosed from coated pits.

During recent years endocytic uptake of a large number of ligands has been studied. Most of these ligands are bound to protein receptors, and they are usually taken up by the coated pit/coated vesicle pathway (5, 13, 15, 21, 22, 32, 34). However, there has been an ongoing discussion as to whether alternative pathways of endocytosis also exist. Thus, ultrastructural data indicated that the glycolipid-binding toxins, cholera toxin and tetanus toxin (24, 46), as well as monoclonal antibodies directed against a cell surface glycolipid (45) are endocytosed from uncoated areas of the membrane. Furthermore, fluid-phase endocytosis appeared to continue even when receptor-mediated endocytosis was inhibited by high osmolarity in the medium (4).

Also, the toxic protein ricin, which binds not only to glycoproteins, but also to glycolipids with terminal galactose residues (for review, see reference 28), continued to be taken up when endocytosis from coated pits was blocked by potassium depletion of the cells (25), or by acidification of the cytosol (37, 38). Similar results were obtained with human rhinovirus 2 (20). Also insulin, which binds to protein receptors, has been reported to be endocytosed from uncoated areas of the membrane in some cell types (11, 12, 42), suggesting that the alternative pathway of endocytosis is not limited to ligands binding to glycolipids.

Shiga toxin is an extremely toxic protein produced by Shigella dysenteriae 1, which is considered the most virulent agent of bacillary dysentery (8, 27, 51). Shiga-like toxins produced by Escherichia coli are associated with hemorrhagic colitis and hemolytic uremic syndrome (17, 26). Shiga toxin consists of an enzymatically active A-chain and a pentameric B-subunit that binds to glycolipids containing the Galα1-4Gal sequence (3, 16, 18, 19). The A-chain penetrates to the cytosol and inactivates the 60-S ribosomal subunits (33) by depurination of a single adenosine residue in 28-S RNA (9). It has been assumed that endocytosis is involved in the penetration of the toxin to the cytosol (28, 35). In the present paper we present evidence that this is the case and that the endocytosis occurs by the coated pit/coated vesicle pathway despite the glycolipid nature of the Shiga toxin receptor.

Materials and Methods

Materials

Horseradish peroxidase type VI (HRP),1 transferrin, pronase, SPDP (3-[2 pyridylthio]-propionic acid N-hydroxysuccinimideester), Hepes, and Tris

1. Abbreviations used in this paper: HRP, horseradish peroxidase; PGG, protein G-gold.
were obtained from Sigma Chemical Co., St. Louis, MO. [3H]leucine was from the Radiochemical Centre, Amersham, UK. Recombinant protein G was obtained from Zymed Laboratories Inc., San Francisco, CA. Horse anti-Shiga toxin serum was obtained from Bureau of Biologics, Food and Drug Administration, Bethesda, MD. Shiga toxin was purified as previously described (2). Monovalent conjugates of Shiga toxin and HRP were prepared by the SPDP method as previously described (50).

**Cells**

HeLa S3 cells and Vero cells were maintained as monolayer cultures in minimum essential medium complemented with penicillin, streptomycin, and 10% (vol/vol) fetal calf serum in an atmosphere containing 5% CO2. The day before use the cells were seeded out into 24-well disposable trays or T-25 flasks.

**Formaldehyde Fixation and Isobutanol Extraction of Cells**

Cells growing as monolayers were incubated for 30 min at 4°C with 1% formaldehyde in PBS containing 2 mM CaCl2. The cells were then washed twice in PBS and incubated for 10 min at 25°C with 50 mM NaBH4 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.

**Measurement of Cytotoxic Effect**

After incubation of cells with toxin as described in legends to figures, the medium or buffer was removed, and the cells were incubated in the same medium or buffer (no unlabeled leucine) for 10 min at 37°C with 1 µCi [3H]leucine per milliliter. 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 differences between duplicates were <10% of the average value.

**Application of Shiga-HRP Conjugate**

HeLa S3 cells grown in T-25 flasks were washed and incubated at 0°C with Shiga-HRP, fixed, and processed for electron microscopy. Alternatively, after preincubation at 0°C the cells were washed and further incubated for 15–60 min at 37°C before fixation. Other cells were washed and incubated directly (without preincubation) for 2, 5, or 15 min at 37°C in the presence of Shiga-HRP before fixation.

**Immunocytochemical Detection of Shiga Toxin**

HeLa S3 cells in T-25 flasks were washed and kept on ice in minimal essential medium containing Hepes instead of bicarbonate for 10 min, then incubated with precooled medium containing Shiga toxin (10 µg/ml; 1 ml per flask) at 0°C for 30 min. Other cells were washed with prewarmed medium and then Shiga toxin (10 µg/ml; 1 ml per flask) was added. The cells were incubated in the presence of Shiga toxin for 15 min at 37°C.

After these incubations the cells were washed with ice-cold PBS and incubated with horse anti-Shiga toxin serum for 60 min on ice before washing, incubation for 60 min at 0°C with protein G–gold (PGG), washing, fixation, and processing for electron microscopy. Recombinant protein G, which has a high binding affinity to horse IgG, was conjugated to 6 nm colloidal gold particles (I). The gold particles were prepared according to Slot and Geuze (41).

In control experiments carried out at 0°C, either Shiga toxin or anti-Shiga toxin serum were omitted from the protocol before incubation with PGG. The amount of unspecific (background) gold labeling in these controls was virtually zero and can be ignored.

**Processing for Electron Microscopy**

HeLa cells treated as described above, were fixed in the monolayer with 2% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.2, for 60 min at room temperature. In experiments with Shiga-HRP the cells were then carefully washed with PBS and incubated with diaminobenzidine-H2O2 as previously described (50). In experiments with Shiga-HRP and PGG labeling cells were thereafter scraped off the flasks, pelleted, 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 as previously described (50).

**Results**

**Evidence That Shiga Toxin Binds to Glycolipid Receptors**

There is now good evidence from several laboratories that Shiga toxin binds to glycolipids in a specific manner (3, 16, 18, 19, 23). To test whether glycolipids in fact represent the cell surface receptors for the toxin, we studied the effect of lipid extraction on the ability of Vero and HeLa cells to bind the toxin. To maintain the integrity of the cells after the lipid extraction, we used formaldehyde-fixed cells in this experiment. Control experiments showed that the formaldehyde treatment had little or no effect on the ability of the cells to bind the toxin in a specific way (Table I).

As shown in Table I, 80–90% of the binding activity of formaldehyde-fixed cells was removed by extraction with water saturated with isobutanol. This supports the view that the cellular receptors for Shiga toxin are indeed lipids.

To further test if Shiga toxin was bound to protein receptors, cells in which the surface proteins had been labeled by lactoperoxidase catalyzed radiiodination were incubated with Shiga toxin. After solubilization of the cells with Triton X-100, we carried out immunoprecipitation of cell surface-bound toxin with anti–Shiga toxin antiserum. We did not find any evidence for coprecipitation of a putative protein receptor (data not shown).

Although the data above are consistent with the contention that glycolipids represent the major part of the toxin receptors, the possibility could not be excluded that protein receptors were involved in the binding of small amounts of toxin. If this was the case, only the binding to protein receptors could be relevant for intoxication, while binding to the glycolipid receptors could be nonproductive. To test this possibility we treated Vero cells and HeLa cells with trypsin and then tested the toxin sensitivity of the cells. We found that the protease treatment had little or no effect on the sensitivity of the cells to the toxin (Table II).

A protein receptor for Shiga toxin would most likely be a

| Treatment | Vero cells | HeLa cells |
|-----------|------------|------------|
| None      | 100        | 100        |
| Fixed cells | 105      | 84         |
| Fixed and isobutanol-treated cells | 14 | 17 |

Cells growing in 24-well disposable trays were fixed with formaldehyde and extracted with isobutanol as described in Materials and Methods. Both untreated and treated cells were then incubated with 125I-labeled Shiga toxin (200 ng/ml, sp act 2,110 cpm/ng) for 1 h at 0°C in Hepes medium, pH 7.2. The cells were then washed three times with PBS, dissolved in 0.1 M KOH, and the radioactivity associated with the cells was measured. The amount of radioactivity associated with the cells in the presence of 20 µg/ml unlabeled Shiga toxin was subtracted in all cases.
Table II. Effect of Treatment of Cells with Trypsin, Swainsonine, and Tunicamycin on Their Sensitivity to Shiga Toxin

| Treatment      | Sensitivity (ID50 toxic/ID50 control) |
|---------------|---------------------------------------|
| None          | 1.0                                   |
| Trypsin§      | 0.8                                   |
| Swainsonine‖  | 1.9                                   |
| Tunicamycin‖  | 1.5                                   |

* Vero cells were incubated with increasing concentrations of Shiga toxin, and the rate of protein synthesis was measured as indicated below.
§ Vero cells were incubated for 20 min at 37°C with 50 μg/ml trypsin. Control cells were incubated without trypsin. The cells were then washed, increasing concentrations of Shiga toxin were added, and, after 15 min further incubation, unbound Shiga toxin was removed by washing. The cells were incubated overnight in Hepes medium containing 5% serum to allow bound toxin time to intoxicate the cells. The rate of protein synthesis was then measured.
‖ Vero cells were incubated for 72 h with 3 μg/ml swainsonine or for 24 h with 0.25 μM tunicamycin. Increasing concentrations of Shiga toxin were then added, and the cells were incubated for 3 h in the presence of toxin. Finally, the rate of protein synthesis was measured.

Evidence That Endocytosis Is Involved in Shiga Toxin Action

Endocytosis is involved in the penetration into the cytosol of a number of protein toxins which act on cytosolic targets (for review, see reference 28). To test if this is also the case with Shiga toxin, we exposed the cells to the toxin under conditions where toxin translocation to the cytosol, but not endocytosis, was prevented. We then inactivated with anti-Shiga toxin serum any toxin remaining at the cell surface, and finally incubated the cells overnight to allow toxin that was shielded from the antibodies (i.e., endocytosed toxin) time to exert its toxic effect on the cells.

These experiments depend on the ability of the antitoxin to neutralize surface-bound toxin. We therefore tested to what extent the horse antiserum here used was able to protect cells against the toxic effect of surface-bound toxin. Cells were exposed to increasing concentration of Shiga toxin at 0°C to allow binding, but not endocytosis to take place. Then the cells were washed and treated with different concentrations of anti-Shiga toxin serum. Subsequently, the cells were incubated at 37°C to allow cell-bound toxin not inactivated by the antibodies time to exert its toxic effect. Finally, the rate of protein synthesis was measured. The data in Fig. 1 show unlikely that a protein receptor is involved in the binding and internalization of Shiga toxin.

![Figure 1](image1.png)

Figure 1. Ability of horse anti-Shiga serum to inactivate cell-bound Shiga toxin. Vero cells growing in 24-well disposable trays were incubated for 1 h at 0°C with increasing concentrations of toxin. The cells were then washed and ice-cold growth medium containing the indicated amounts of antitoxin was added. The cells were transferred to a CO₂ incubator and incubated overnight at 37°C. Finally, the rate of protein synthesis was measured.

![Figure 2](image2.png)

Figure 2. Ability of Shiga toxin endocytosed at low pH to intoxicate cells. Vero cells in 24-well disposable trays were incubated at 37°C in Hepes medium adjusted to pH 6.1 or 7.0. Increasing concentrations of toxin were then added. (A) The rate of protein synthesis was measured after 1 h. (B) The medium was removed after 1 h and cold growth medium containing horse antitoxin was added. The cells were transferred to a CO₂ incubator and incubated for 18 h as described in Fig. 1. Finally, the rate of protein synthesis was measured.
that addition of 10 µl/ml horse antiserum was highly efficient in neutralizing cell-bound toxin. Smaller amounts of antitoxin protected the cells to a lesser extent.

We have previously shown that when cells are incubated with Shiga toxin in medium with low pH, the toxin is unable to inhibit protein synthesis, suggesting that it is not translocated to the cytosol (8). Similar results were obtained with abrin and ricin (36). We could show that abrin and ricin were endocytosed under such conditions, and that the endocytosed toxins were able to inhibit protein synthesis upon transfer of the cells to neutral medium.

To carry out a similar experiment with Shiga toxin, Vero cells were incubated with the toxin for 1 h at pH 6.1 or 7.0. In some cases, protein synthesis was measured immediately after this incubation. As shown in Fig. 2 A, the cells that were exposed to the toxin at low pH were strongly protected. (It should be noted that in this experiment we varied the pH in the medium, not in the cytosol. As will be discussed below, acidification of the cytosol inhibits endocytosis from coated pits.)

In other cells, horse anti-Shiga toxin was added after the 1-h exposure to the toxin to inactivate any toxin remaining at the cell surface. Then the cells were incubated overnight in neutral medium to allow toxin that was endocytosed and therefore shielded against the antiserum time to express its effect. Protein synthesis was measured the next day. As shown in Fig. 2 B, in this case the cells were intoxicated to the same extent whether they had been exposed to the toxin at pH 6.1 or 7.0. Similar results were obtained when the exposure to the toxin lasted only 15 min and the cells were then incubated with antitoxin overnight. The data indicate that Shiga toxin taken up by endocytosis is fully capable of being translocated to the cytosol.

Depletion of cells for ATP induces a general inhibition of endocytosis (36, 40). To test whether such inhibition prevents the transfer of Shiga toxin to a compartment where it is shielded against antitoxin, we incubated cells with a combination of azide and 2-deoxyglucose to inhibit cellular ATP production, and then Shiga toxin was added. Cells exposed to toxin in the absence of metabolic inhibitors were used as a control. After 15 min, surface-bound toxin was neutralized with antitoxin and the cells were incubated overnight. Finally the rate of protein synthesis was measured. As shown in Fig. 3, much higher toxin concentrations were required to intoxicate the ATP-depleted cells than the control cells. This indicates that upon inhibition of endocytosis, Shiga toxin remained at the cell surface where it was subsequently neutralized by antitoxin.

To reduce strongly the ATP level in the cells, both NaN3 and 2-deoxyglucose were required. The presence of either azide or 2-deoxyglucose alone did not inhibit transfer of the toxin to a location where it was shielded against antitoxin (data not shown). Although the data obtained do not exclude the possibility of a direct transport of Shiga toxin through the plasma membrane, they show that endocytosed Shiga toxin can be efficiently transported to the cytosol.

Figure 3. Ability of metabolic inhibitors to prevent shielding of Shiga toxin from antitoxin. Vero cells in 24-well disposable trays were incubated for 10 min at 37°C in Hepes medium with and without 10 mM NaN3 and 50 mM 2-deoxyglucose. Then increasing concentrations of toxin were added, and, after 15-min further incubation at 37°C, the metabolic inhibitors were removed, and the cells were transferred to growth medium containing neutralizing amounts of antitoxin. Protein synthesis was measured after 18-h incubation at 37°C.

Figure 4. Internalization rate of cell surface-bound Shiga toxin. HeLa S3 cells growing in 24-well disposable trays were exposed for 1 h at 0°C to increasing concentrations of toxin. The cells were washed and then incubated at 37°C. Growth medium with or without antiserum to Shiga toxin was added after the indicated periods of time. The cells were then incubated overnight at 37°C, and finally the rate of protein synthesis was measured.
Figure 5. Distribution of binding sites for Shiga toxin on the surface of HeLa S3 cells. Cells were incubated at 0°C with Shiga toxin followed by anti-Shiga toxin serum and PGG. The cells were then fixed and processed for electron microscopy. The binding sites are distributed randomly over the cell surface (a) and are mostly excluded from coated pits (arrows) as seen in a–c, although a few coated pits do contain binding sites (d–e). Bars, 0.25 µm.

Rate of Endocytosis of Surface-bound Shiga Toxin

To study the internalization rate of cell-bound Shiga toxin, cells were first exposed to toxin for 1 h at 0°C to allow binding of the toxin to the cell surface to take place in the absence of endocytosis. Unbound toxin was removed by washing, and then the cells were incubated at 37°C to allow endocytosis of surface-bound toxin to take place. After different periods of time, toxin uptake was interrupted by addition of antitoxin, and then the cells were incubated overnight to allow endocytosed toxin time to exert its effect on the cells.

The data in Fig. 4 show that antiserum added after 30 min was essentially unable to protect against the toxin, indicating that most of the toxin was internalized by this time. Also when the antiserum was added after 15 min, the cells were strongly intoxicated, indicating that the surface-bound toxin is internalized rapidly.

Ultrastructural Studies of Surface Binding and Endocytosis of Shiga Toxin

To visualize cell surface binding of Shiga toxin two approaches were used: (a) immunocytochemical detection of the ligand using PGG, and (b) incubation with a monovalent Shiga-HRP conjugate. These two approaches support each other although certain differences were noticed because of the particulate and enzymatic nature of the labels. In the immunocytochemical experiments the cell surface was randomly labeled with gold after incubation with Shiga toxin at 0°C (Fig. 5). However, marked variations in the amount of gold particles were obtained from cell to cell. More than 99% of the gold particles were localized to nonspecialized membrane. Only a few coated pits (<5%) were gold labeled as judged from random individual sectioning (Fig. 5, d and e). Control experiments showed that the immunogold labeling was specific (see Materials and Methods).
When cells were incubated at 0°C with Shiga-HRP, many cells showed a marked labeling of their entire surface and of many coated pits (Fig. 6, a–c). The fact that more coated pits appeared labeled here than with the immunogold technique can most likely be ascribed to the enzymatic nature of the Shiga-HRP conjugate. Also some small uncoated pits were labeled. Preincubation at 0°C with excess unconjugated Shiga toxin prevented the binding of Shiga-HRP (Fig. 6, d and e), thus demonstrating that the conjugate was bound to the cells in a specific manner.

Figure 7. Redistribution of binding sites for Shiga toxin at 37°C in the presence of ligand conjugate. a–d show that after 15 min of incubation at 37°C in the presence of Shiga toxin, the toxin is predominantly localized close to or within coated pits (arrow) as revealed by immunogold (PGG) labeling. This distribution of binding at 37°C is even more marked when using Shiga-HRP: (e) 2 min at 37°C; (f) 5 min at 37°C; (g–i) 15 min at 37°C in the presence of Shiga-HRP. In e and h uncoated pits (arrowheads) are also labeled, and in i a labeled multivesicular body is shown. Bars, 0.25 μm.
When the cells were incubated for 15 min at 37°C in the presence of Shiga toxin, the labeling pattern as revealed with the immunogold technique changed from the random one seen at 0°C to a selective sequestration into, or close to, many of the coated pits (10–20% of the total population of coated pits as counted on random, individual sections of gold-labeled cells) (Fig. 7, a–d). On occasion gold labeling of small uncoated (flask shaped) pits was also noticed. When cells prelabeled with Shiga-HRP at 0°C were washed and warmed to 37°C (not shown), or cells were incubated directly at 37°C in the presence of Shiga-HRP (for 2, 5, or 15 min; Fig. 7, e–i), the change from the random labeling pattern at 0°C was more dramatic than seen with the immunogold technique. Hence, the peroxidase reaction product appeared to a very high degree located selectively to coated pits (20–40% of the total amount of coated pits on labeled cells). Some uncoated pits were also labeled (Fig. 7, e and h).

To test whether the change in localization of Shiga toxin which occurs upon transfer of cells with bound Shiga-HRP from 0 to 37°C could be due to selective dissociation of toxin from uncoated areas of the membrane, we measured the dissociation of 125I-labeled toxin from cells under similar conditions. The experiments which involved prebinding of 125I-labeled Shiga toxin at 0°C, washing and subsequent incubation at 37°C, clearly showed that dissociation of toxin from the cell surface could not account for the disappearance of Shiga toxin from uncoated areas of the cell membrane (data not shown).

In conclusion, the experiments with immunogold detection of Shiga toxin and with the Shiga-HRP conjugate strongly suggest that at 0°C the Shiga binding sites (receptors) are randomly distributed on the cell surface with no preferential binding to, for example, coated pits, whereas at 37°C the receptors with bound toxin are localized in coated pits.

After 15-min incubation at 37°C Shiga-HRP was found to label various tubular and vacuolar endosomal structures (Fig. 7 i). On rare occasions, also elements of the Golgi complex contained some Shiga-HRP. After 60 min of incubation at 37°C, endosomal and lysosomal structures throughout the cytoplasm, but most frequently in the Golgi region, were heavily labeled (Fig. 8, a and b). Moreover, stacked Golgi elements (presumptive Golgi cisternae; 47–50) contained Shiga-HRP (Fig. 9). Interestingly, not only elements on one side of the Golgi stack were labeled, but also what appeared to be medial cisternae were occasionally labeled (Fig. 9 c). Whether such cisternae are truly medial or actually in continuity with the trans-cisternae as reported in a serial section study by Orci et al. (29) remains uncertain. Similar observations on internalization of Shiga toxin were made when the cells were incubated in the medium at pH 6 rather than at pH 7. This demonstrates that the observation that the toxic effect is inhibited at low pH in the medium (see above) is not due to inhibition of endocytosis or transport to the Golgi region.

### Biochemical Evidence That Shiga Toxin Is Endocytosed from Coated Pits

Endocytosis of ligands from coated pits is strongly inhibited at cytosolic pH <6.5, whereas endocytic uptake of ricin, possibly from uncoated areas of the cell surface, is inhibited to a much lesser extent (37, 38, 49). To test whether the endocytosis of Shiga toxin that leads to intoxication of cells takes place predominantly from coated or from uncoated pits, we
lowered the cytosolic pH by the NH₄Cl prepulsing technique (37) and exposed the cells to Shiga toxin for 15 min. Then cell surface-bound toxin was neutralized with antitoxin while the internal pH was still low. Subsequently, the cells were incubated overnight in normal medium to allow any internalized toxin time to intoxicate the cells. The results in Fig. 10A show that when acidified cells were exposed to the toxin, they were protected against Shiga toxin. In a control experiment where addition of antitoxin was omitted, the cells were intoxicated to the same extent whether they were acidified or not during the exposure to the toxin (Fig. 10B) demonstrating that the acidification period did not have any long lasting effect interfering with toxin entry.

The experiment here demonstrated was carried out with HeLa cells, but similar results were also obtained with Vero cells (data not shown). The data indicate that not only endocytosis of Shiga-HRP as shown by electron microscopy, but also endocytosis of Shiga toxin relevant for intoxication occurs from coated pits.

**Ultrastructural Observations on Acidified Cells**

We have shown elsewhere that acidification of the cytosol...
Figure 11. Details of an experiment where HeLa S3 cells were acidified by preincubation for 30 min with 30 mM \( \text{NH}_{4}\text{Cl} \) which was then removed and replaced by medium without \( \text{NH}_{4}\text{Cl} \) and \( \text{Na}^{+} \). This resulted in a reduction in the uptake of \(^{125}\text{I}\)-transferrin from 62% of total cell bound to 6% as measured after 10 min incubation at 37°C. Nevertheless, distinct coated pits at the cell surface are present, both with Shiga-HRP (a, arrow) and without Shiga-HRP (b and c). It is clearly seen that the coated structures are not just empty cages, but represent coated membrane profiles (vesicles or pits). Bars, 0.1 \( \mu \text{m} \).

Inhibits endocytosis of transferrin, although coated pits are still present at the surface of the acidified cells (37, 38). However, when peroxidase conjugates or immunoperoxidase cytochemistry are used to localize, for example, transferrin (37) and Shiga toxin receptors in coated pits, it is often difficult to demonstrate the clathrin coat and the membrane of a coated pit at the same time in a convincing manner (Fig. 11 a). The demonstration of a distinct coated pit membrane in continuity with the plasma membrane (or a vesicular profile membrane) is necessary to conclude that the coated structure of interest is really a pit (or a vesicle) and not an empty clathrin cage. In the present study we have therefore paid special attention to show unequivocally the presence of coated pits at the surface of acidified cells in which biochemical measurements on parallel cultures (experiments carried out the same day with the same \( \text{NH}_{4}\text{Cl} \) concentration, etc.) showed marked inhibition in endocytosis of transferrin. Coated pits from one of these experiments are shown in Fig. 11. The two coated profiles in Fig. 11 a as well as those in c may represent coated vesicles rather than pits. Such vesicles may have pinched off immediately before the acidification exerted its effect on the coated pits. However, serial section analysis has revealed that most coated vesicular profiles close to the cell surface are, in fact, coated pits (31).

In acidified cells very little internalized Shiga-HRP was observed after 15 min of incubation at 37°C. Shiga-HRP was present on the cell surface and in particular in coated pits (Fig. 11 a). These data strongly support our biochemical observations on the lack of Shiga toxin internalization in
acidified cells (Fig. 10) and also support our notion that acidification of the cytosol somehow immobilizes coated pits at the cell surface, thereby preventing endocytosis of ligands that normally use this pathway (37, 49). Also, our combined biochemical and morphological findings indicate that Shiga toxin is predominantly taken up via coated pits and vesicles.

Effect of Temperature on the Ability of Shiga Toxin to Inhibit Cellular Protein Synthesis

Since the ultrastructural studies indicated that Shiga toxin is transferred to the Golgi apparatus, and since we have earlier found that such transport is inhibited at 18°C (39, 47), we decided to study the effect of temperature on the ability of Shiga toxin to inhibit cellular protein synthesis. It was earlier reported that when HeLa cells were incubated with Shiga toxin for a short period of time at 18°C, the toxic effect was much less than when the incubation was at 37°C (8). Since the reduced toxicity could be due to slow transfer of toxin to the compartment from which it enters the cytosol, we incubated HeLa cells with Shiga toxin at 37 and 18°C for different periods of time and then measured the toxic effect. As shown in Fig. 12A, there was a large difference in the toxic effects obtained at the two temperatures. It is clear that increased incubation time did not diminish this difference.

To study if the low toxic effect at 18°C was due to low binding or endocytosis of toxin at this temperature, we allowed toxin to bind to the cells for 30 min at either 18 or 37°C. Then the cells were washed and incubated overnight at 37°C to allow the bound toxin time to exert its effect on the cells. The results showed that at 18°C it was necessary to expose the cells to approximately twice the toxin concentration required at 37°C to obtain the same toxic effect (Fig. 12B). This indicates that at low toxin concentration approximately twice as much is bound at 37 as at 18°C.

Essentially, the same difference in protein synthesis inhibition was obtained in an experiment where the cells were exposed to toxin for 30 min at the two temperatures, then treated with antitoxin to inactivate surface-bound toxin and subsequently incubated overnight at 37°C in the absence of toxin (Fig. 12B). This indicates that approximately the same amount of toxin is endocytosed at the two temperatures. Clearly therefore, the twofold difference in binding is far too small to account for the >100-fold difference in toxin sensitivity of the cells at the two temperatures. The data are consistent with the idea that transport of Shiga toxin to the Golgi apparatus is required for intoxication of cells.

Discussion

The most important finding here described is that a glycolipid-binding ligand, Shiga toxin, is endocytosed from coated pits. This is in contrast to other glycolipid-binding ligands, such as tetanus toxin and cholera toxin, which have been reported to be endocytosed from uncoated areas of the cell membrane (24, 46). To our knowledge, Shiga toxin is the first glycolipid-binding ligand that has been found to be endocytosed from coated pits.

Glycolipid Nature of the Receptor for Shiga Toxin

It is now well established that Shiga toxin, and the almost identical Shiga-like I toxin, bind to glycolipids containing Galβ1-4 Gal sequences (3, 16, 18, 19, 23). Not only does Shiga toxin bind to the glycolipids, but there is evidence that naturally resistant cells practically lack glycolipids with Galβ1-4 Gal sequence (18). Furthermore, a mutant cell line selected from Daudi cells for resistance to Shiga-like toxin I, was deficient in these glycolipids and did not bind the toxin (3).

We here show that lipid extraction removes 80–90% of the binding sites. However, since Vero and Hela cells contain a high number (~106/cell) of Shiga toxin binding sites (8), the experiment did not exclude the possibility that a considerable number of glycoprotein receptors were present as well. The findings here reported that trypsin treatment did not reduce significantly the toxin-sensitivity of the cells, and that it did not remove 125I-labeled Shiga toxin bound to cells,
argue against the possibility of a protein receptor. Furthermore, immunoprecipitation of toxin that was bound to 125I-labeled cells did not result in precipitation of a labeled protein. Finally, swainsonine and tunicamycin that interfere with glycosylation and processing of glycoproteins did not affect the sensitivity of cells. Altogether, several lines of evidence argue against the involvement of a glycoprotein receptor.

**Endocytic Uptake of Shiga Toxin**

The evidence presented here that endocytic uptake is involved in the action of Shiga toxin is in accordance with earlier findings with most other toxins that exert their action in the cytosol (28). We found that when cells were allowed to endocytose toxin under conditions where the translocation to the cytosol was blocked as estimated from the lack of protein synthesis inhibition, the toxin was accumulated in a location where it was shielded against antitoxin, probably in compartments of the endocytic pathway. When the translocation block was removed, the shielded toxin was highly efficient in intoxicating the cells. This intoxication must occur from within intracellular vesicles since any toxin that is recycled back to the plasma membrane would be inactivated by the anti–Shiga toxin that remained in the medium for the rest of the experiment.

On the other hand, when ATP-depleted cells were exposed to the toxin, they were unable to accumulate toxin in a compartment where it was shielded against the antibody. This is in accordance with the observation that endocytosis is inhibited in ATP-depleted cells (36, 40).

Ultrastructural studies strongly suggest that Shiga-HRP was endocytosed from coated pits. Shiga-HRP was observed mainly in coated pits, even after a short incubation at 37°C. In agreement with this, acidification of the cytosol, which inhibits pinching off of coated vesicles (37), inhibited this entry.

In addition to coated pits, we also found Shiga toxin in some uncoated pits or caveolae which may be involved in endocytosis as well (11, 12, 24, 46). The fact that acidification of the cytosol inhibits uptake of Shiga toxin almost completely means that either (1) the uncoated pits or caveolae are not involved in endocytosis, or (2) they are involved in internalization, but (a) were inhibited as were the clathrin coated pits in internalization, or (b) they still take up Shiga toxin, but the uptake kinetics are so slow that it makes no difference in 15 min (a notion that fits with the data of Tran et al. [46]).

Both the electron microscopical studies, and the experiments where entry was estimated from toxicity measurements, indicate that there is a rapid entry of Shiga toxin into an endocytic compartment. The kinetics of endocytosis are similar to those of other ligands, such as transferrin and epidermal growth factor, which are rapidly removed from the cell surface by the coated pit/coated vesicle pathway. In contrast, ricin that seems to enter both from coated as well as from uncoated areas of the cell membrane, and cholera toxin that is reported to enter exclusively from uncoated areas, are endocytosed much more slowly (36, 46).

Coated pits are involved in endocytosis of transferrin, low density lipoprotein, and epidermal growth factor, which all bind to protein receptors. It is widely accepted that the cytoplasmic domains of these receptors are required for transport to the coated pits (5, 32, 34). While our results strongly indicate that Shiga toxin is taken up by coated pits, it remains uncertain whether a direct clustering of Shiga toxin receptors takes place at 37°C. However, our observation that at 0°C <5% of the coated pits contained receptor-bound Shiga toxin as determined by immunogold labeling, whereas at 37°C 10–20% of the coated pits were labeled, suggests that some clustering takes place.

It is not clear how clustering of glycolipids in coated pits could occur, but interactions of the glycolipids with integral membrane proteins would most likely be involved. Glycosphingolipids, in contrast to glycerolipids, are in fact known to form intermolecular hydrogen bonds (30). It should also be noted that the composition of phospholipids in coated pits differs from that in the rest of the plasma membrane (6), suggesting that selective migration to or retention of certain lipids in these structures does take place.

**Intracellular Transport of Shiga Toxin**

The electron microscopical studies showed that Shiga toxin is transported to the Golgi region. Transport to the Golgi apparatus has been demonstrated with other ligands that are endocytosed from coated pits. Thus, both the transferrin and mannose 6-phosphate receptors as well as cell surface molecules in general are transferred to different compartments in the Golgi apparatus (7, 10, 43, 44).

Interestingly also ricin, which is largely endocytosed by a mechanism not involving coated pits, is transported to the Golgi apparatus (14, 39, 47–50). This transport is inhibited at 18°C (39, 47). Under the same conditions the toxic effect is strongly reduced, suggesting that transport of ricin to the Golgi apparatus is required for translocation to the cytosol. We here show that also intoxication of cells with Shiga toxin is strongly reduced at 18°C as compared with 37°C. It is therefore possible that Shiga toxin must be transferred to the Golgi apparatus before it can subsequently be translocated to the cytosol.

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