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Receptor-Mediated Entry of Diphtheria Toxin into Monkey Kidney (Vero) Cells: Electron Microscopic Evaluation

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To express toxicity in living cells, diphtheria toxin (DT) must cross a membrane barrier and reach its target in the cytosol. Here we examine the entry of DT into the toxin-sensitive monkey kidney (Vero) cells. Using electron microscopy we directly demonstrated for the first time that DT is internalized by receptor-mediated endocytosis, i.e., via clathrin-coated pits, and enters the endosomal system. Methylamine, which is known to protect cells from DT, stopped the movement of toxin to coated areas of the cell membrane. In the presence of amine, prebound biotinyl-DT was internalized, but toxicity was inhibited. Biochemical evidence revealed that methylamine maintained toxin molecules at a site accessible to neutralization by antitoxin. The data suggest that DT entering Vero cells in the presence of methylamine is sequestered within the cell and does not express toxicity.

Diphtheria toxin (DT) entry into mammalian cells of DT-susceptible species is a receptor-mediated process, i.e., toxin-mediated events in the cell cytoplasm leading to cell death are preceded by the binding of DT to specific toxin receptors on the cell surface (10, 26). The biochemical mechanism of action of DT has been well characterized (33). Fragment A, the enzymatically active portion of DT, catalyzes the transfer of adenosine diphosphoribose from NAD to cellular elongation factor 2. Depletion of functional elongation factor 2 results in cessation of cellular protein synthesis. The intervening step(s) between DT binding and inactivation of elongation factor 2 is not completely understood. It is evident that DT may be internalized by several endocytic mechanisms (36). The wide range in sensitivity to DT exhibited by different animal species and cell lines may be determined by the endocytic pathway taken (29).

Previous studies from our laboratory established that *Pseudomonas* exotoxin A (PE) is internalized by receptor-mediated endocytosis (12, 13, 21, 27). PE is a bacterial toxin having an enzyme activity identical to that of DT. After PE binding to mouse LM fibroblasts, the toxin rapidly migrates to specialized clathrin-coated areas of the membrane, is internalized in membrane-bound vesicles (endosomes), moves to the Golgi region, and ultimately is delivered to lysosomes. A similar internalization process has been described for peptide hormones, serum proteins, and transport proteins (e.g., insulin, epidermal growth factor, transferrin, low-density lipoprotein, and alpha 2-macroglobulin; reviewed in references 3, 14, and 40).

It can be postulated that DT enters cells by several different routes, including (i) entry by fluid-phase endocytosis with direct delivery of toxin to lysosomes; (ii) entry by a receptor-mediated event followed by direct delivery to lysosomes; (iii) entry by a receptor-mediated event, with the ligand-receptor complex returned to the cell surface; and (iv) entry by a receptor-mediated event through coated pits followed by movement in endosomes to the Golgi region and ultimately to lysosomes. We suggest that although all four mechanisms of entry may be operative, only the fourth possibility results in a "productive intoxication" (17). The data we present suggest that a small population of the toxin molecules originally bound to Vero cell DT receptors enter by this productive route. Additionally, we provide evidence that methylamine protects Vero cells from DT at two levels: by inhibiting entry through clathrin-coated regions and by sequestration of DT within organelles at a pH unfavorable for DT escape into the cytoplasm.

MATERIALS AND METHODS

Toxin. DT was obtained from Connaught Laboratories, Willowdale, Ontario, Canada, and was purified by chromatography over DEAE-cellulose. The final preparation ran as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. DT was biotinated by the method of Bayer et al. (2) with biotin-*N*-hydroxysuccinimide ester (Calbiochem-Behring, La Jolla, Calif.). Biotinyl-DT retained at least 80% of its biological activity, as measured by inhibition of protein synthesis. Antitoxin was prepared by hyperimmunization of rabbits with formalinized toxoid.

Cells. Monkey kidney cells, Vero (ATCC CCL81) and BS-C-1 (ATCC CCL26) were maintained as monolayers in McCoy 5A medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (200 U/ml), and streptomycin (0.2 mg/ml).

Protein synthesis studies. Protein synthesis was measured by determining [^{3}H]leucine incorporation into acid-precipitable material during a 30-min pulse period, as described previously (12, 13). Inhibition of protein synthesis was determined by comparing the incorporation of [^{3}H]leucine in DT-treated cells with that in control cells. All experiments were carried out two to four times with each point done in triplicate, and results are expressed as counts per minute per microgram of protein ± one standard deviation.

Electron microscopy. Initial studies to quantitate DT binding were done by using the antibody-bridging technique as described by Morris and Saelinger (28) in conjunction with

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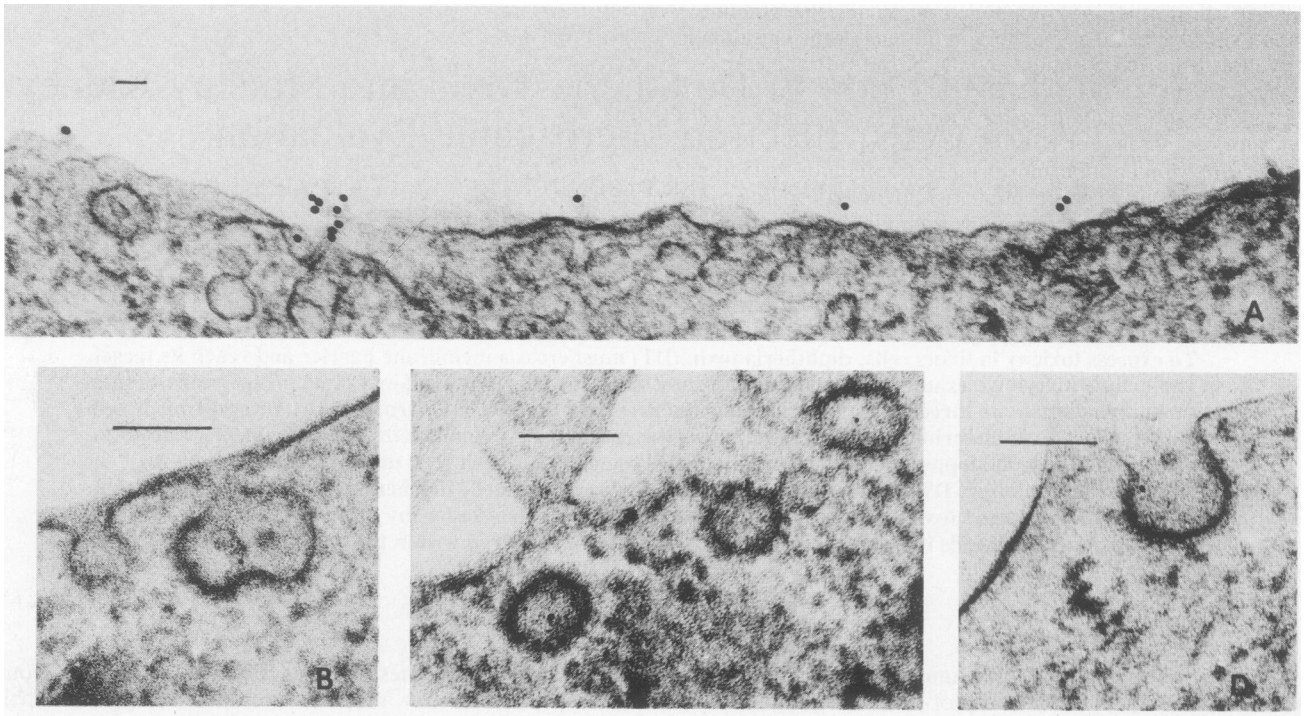


FIG. 1. Intracellular trafficking of biotinyl-DT (DT-gold). (A) Distribution of DT-binding sites along the plasma membrane, zero time sample; assayed by the antibody-bridging technique. All other figures were done by the biotinyl-DT–avidin-gold method. (B to D) Samples at 60 s. Note DT-gold within clathrin-coated pits and vesicles. (E) Sample at 5 min. Note DT-gold within an amorphic vesicle, presumably an endosome, near the nucleus. (F) Sample at 5 min. Note DT-gold within vesicles (arrows) near cisternae of the Golgi complex (GC). (G) Sample at 30 min. Note numerous DT-gold sitings within a multivesicular body. (H) Sample at 30 min incubated in the presence of 20 mM methylamine. Bars, 100 nm.

the immunoglobulin G fraction of rabbit anti-DT. The electron dense marker was colloidal gold (diameter, 18 nm) conjugated to rabbit immunoglobulin G. Subsequent studies, characterizing internalization and intracellular trafficking, involved the use of biotinyl-DT and avidin-gold colloids (30). Here monolayers were exhausted of exogenous biotin by incubation in Hanks balanced salt solution for 2 h at 37°C, cooled to 4°C, and incubated sequentially with biotinyl-DT (150 ng/ml) and avidin-gold colloids (diameter, 5.2 nm). Cells were then washed extensively and warmed to 37°C for the indicated times. The cells were fixed in glutaraldehyde (0.5%) and processed for electron microscopy (30). Avidin-gold formed complexes with surface-bound biotinyl-DT to allow direct visualization of toxin; these complexes are designated DT-gold.

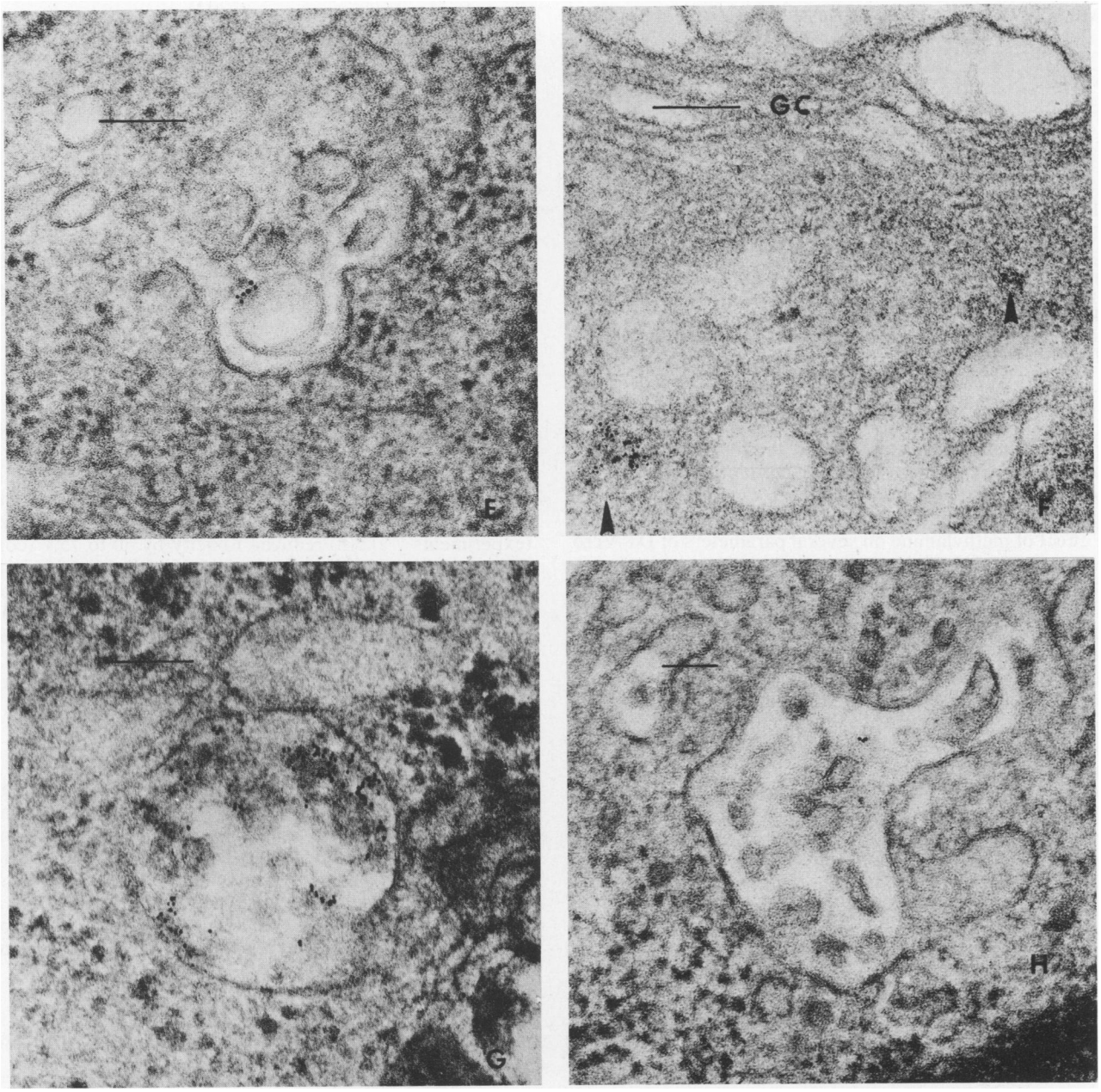
Since Vero cells typically have a large amount of glycocalyx on their cell surface and tend to grow juxtaposed to one another, quantitative morphologic evaluation was difficult; therefore, a qualitative scoring procedure (0 to 4+) was used. The glycocalyx is minimal on BS-C-1 cells, and thus direct quantitation was possible. DT-gold sitings were scored as to location (surface, periphery, intracytoplasmic) and place of residence (coated pit, coated vesicle); if intracytoplasmic, the size and electron density of the vesicles and the number of gold grains were recorded. A minimum of 10 cells were scored at each time point. Each experiment was repeated two or three times.

RESULTS

Specific binding of iodinated DT to Vero cells was described previously by Middlebrook and co-workers (10, 26). We have demonstrated similar specific binding of iodinated DT to Vero cells (data not presented). In addition, we have found that the association of biotinyl-DT with the Vero cell surface also was specific, saturable, and competitively inhibited by excess native DT as judged by electron microscopy (data not presented).

Visualization of DT-Vero cell interactions by electron microscopy. Initial studies, involving the use of an antibody-bridging technique and electron microscopy, revealed considerable binding of DT to Vero cells at low temperature with approximately 5% of the total DT sitings occurring in coated areas of the cell surface. Figure 1A shows the random distribution of DT binding at 4°C. When the monolayers were warmed to 37°C (1 to 5 min) there was a reduction in the number of surface-associated toxin sitings and a small increase in DT sitings in coated areas.

The antibody-bridging technique may be used to localize ligand on the cell surface, but is not useful for localization of DT within cells. Therefore, to follow intracellular movement of DT, we used a recently developed biotinyl-DT–avidin-gold system (30). Entry of PE via clathrin-coated areas of the membrane is required for expression of toxicity (27); we therefore first determined whether DT entered Vero cells by the same route (Table 1). Initially biotinyl-DT was diffusely distributed over the entire cell surface; however, when the cells were warmed to 37°C, a rapid movement (60 s) of DT molecules into coated areas was observed (Table 1; Fig. 1B to D). The level of sitings in coated areas returned to background with continued incubation at 37°C. Methylamine, an acidotropic agent which has been shown to stop the movement of certain ligands into coated pits (27) and which totally blocks the toxicity of DT (9, 25), also inhibited



the clustering of biotinyl-DT into coated regions of the plasma membrane (Table 1). In addition, similar experiments with BS-C-1 cells, another monkey kidney cell line highly sensitive to DT (26), indicated a rapid movement of biotinyl-DT into coated areas; this movement was blocked in the presence of methylamine (Table 2). These ultrastructural data provide the first direct evidence that DT enters toxin-sensitive cells via receptor-mediated endocytosis.

The kinetics of DT internalization by Vero cells also were followed by electron microscopy (Table 3). At 4°C, DT-gold was present at significant levels on the cell surface. However, when the cells were warmed to 37°C, an estimated 70% of the DT-gold initially bound was no longer localized on the surface. Within 5 min, DT-gold was visualized within small membrane-bound vesicles (Fig. 1E). The morphology of these vesicles suggests that they are endosomes (16). Occasionally, DT-gold was seen in the Golgi region between 5 and 10 min postinternalization (Fig. 1F). In contrast to the interactions of PE and LM cells (27), DT-gold was not seen within Golgi cisternae. After 15 min at 37°C, approximately 50% of the internalized DT was located within structures morphologically consistent with multivesicular bodies (Fig. 1G). After internalization in the presence of methylamine, DT-gold frequently was seen in large electron-translucent vesicles (Fig. 1H). Although the DT-containing vesicles cannot be precisely identified, the fact that they appear swollen would suggest that they contain trapped methylamine and thus can be assumed to have been acidified vesicles before treatment of cells with the basic amine (35).

Route of DT internalization leading to cell death. Although

TABLE 1. Reduction by methylamine of movement of biotinyl-DT to coated areas on Vero cells[a]

Time (min)	DT sitings in coated areas (%)[b]	
	Without methylamine	With methylamine[c]
0	6	0
0.5	4	6
1.0	26	5
8	10	5
15	0	8

[a] Vero cell monolayers were depleted of excess biotin, cooled, incubated with biotinyl-DT (150 ng/ml) for 18 h at 4°C, washed, incubated with avidin-gold (30 min at 4°C), washed, and warmed to 37°C for the times indicated.
[b] Represents approximate percentage of total surface DT-gold sitings which were located in coated areas.
[c] Methylamine (20 mM) was present from 15 min before cooling to the end of the experimental period.

TABLE 3. Quantitative estimate of DT internalization by Vero cells as determined by electron microscopy[a]

Time (min) at 37°C	Amt of gold on surface[b]	% Intracellular sitings[c]
0	4+	0
0.5	2+	0.7
1.0	1+	3
7.5	1+	10
15	1+	30
30	±	40

[a] Vero cell monolayers were treated as described in Table 1.
[b] Amount of gold on cell surface at zero time was arbitrarily scored as 4+; this represents 30% of surface labeled. ± Represents 1 to 5% of surface labeled.
[c] Approximate percentage of total sitings which were intracellular.

current technology does not permit the fate of internalized protein molecules to be traced with certainty, we attempted to obtain evidence for the putative productive route of DT entry and intracellular movement. To do this we examined the effect of methylamine on several parameters of DT-Vero cell interaction. Methylamine unequivocally protects toxin-sensitive mammalian cells from the deleterious effects of DT (9, 25). We confirmed that methylamine (10 mM) fully protected cells against previously bound DT up to concentrations of 10 μg/ml (1,000-fold shift in 50% tissue culture infective dose). In addition, as reported by Mekada et al. (25), methylamine did not alter the initial binding of ^{125}I-labeled DT to Vero cells. However, it did prevent the release of internalized ^{125}I-DT from these cells for at least 6 h (9, 25; unpublished observations).

Additional information was obtained by measuring the capacity of antitoxin to neutralize DT on the Vero cell surface in the presence and absence of methylamine. In these experiments Vero cells were incubated with 10 ng of DT per ml for 45 min at 37°C. This protocol results in complete shutdown of protein synthesis after the monolayers were washed and incubated an additional 4.5 h at 37°C in DT-free medium. The data presented in Table 4 show that the presence of methylamine caused the retention of potentially productive DT molecules at sites where they were accessible to antitoxin neutralization at both 4 and 37°C. When methylamine was omitted during the initial 45 min of incubation with DT at 37°C, subsequent addition of antitoxin did not protect Vero cells against toxicity (experiment 2). In contrast, the presence of methylamine during the initial incubation with DT permitted absolute neutralization with antitoxin (experiment 6). Short exposure of cells to antitoxin (experiments 7 and 8) reduced toxicity by 50 to 75% (compared with that in cells not treated with antitoxin). A 5-min exposure to antitoxin (experiment 9) resulted in a significant but lesser protection. Electron microscopic observations done in tandem with these experiments showed that although DT was internalized by Vero cells in the presence of methylamine, DT did not enter Vero cells through coated areas of the membrane (Table 1).

TABLE 2. Cessation of movement of biotinyl-DT into coated areas on BS-C-1 cells as a result of treatment with methylamine[a]

Time (min)	DT sitings in coated areas[b]					
	Without methylamine			With methylamine[c]		
	Total no.	No. in coated areas	% in coated areas	Total no.	No. in coated areas	% in coated areas
0	380	13	3.4	471	23	4.8
0.5	503	62	12.3	222	9	4.0
1.0	205	29	14.2	316	16	5.1
2.5	226	22	9.7	142	6	4.2
5.0	91	8	8.8	157	11	7.0

[a] Experiment as for Table 1, except that BS-C-1 cells were used.
[b] Data are presented as total number of surface sitings and number of surface sitings in coated areas on 10 representative cell sections.
[c] Methylamine (20 mM) was present from 15 min before cooling to the end of the experimental period.

DISCUSSION

In this paper direct evidence for the entry of DT into Vero and BS-C-1 cells via coated pits is presented for the first time. Previous evidence for such a mechanism of entry into sensitive cells was circumstantial. Keen et al. (19) reported that rhodamine-labeled DT was bound to human fibroblasts in a punctate manner and accumulated in the same regions as did fluorescein-labeled α-2 macroglobulin. From these observations, the authors inferred that DT and α-2 macroglob-

TABLE 4. Maintenance of productive DT on Vero cell surface by methylamine[a]

Expt	Initial incubation substrates (45 min at 37°C)	Treatment	Inhibition (%)
1	DT	Medium	94
2	DT	Antitoxin[b]	82
3	DT + antitoxin	Antitoxin[b]	0
4	DT + methylamine	Methylamine	28
5	DT + methylamine	Medium	83
6	DT + methylamine	Antitoxin[b]	0
7	DT + methylamine	Antitoxin,[c] 15 min, 37°C	20
8	DT + methylamine	Antitoxin,[d] 15 min, 4°C	39
9	DT + methylamine	Antitoxin,[c] 5 min, 37°C	47

[a] Cell monolayers were incubated with DT (10 ng/ml) for 45 min at 37°C in the presence or absence of 20 mM methylamine. Monolayers were washed and reincubated at 37°C under the treatment conditions given for 4.5 h. Protein synthesis was then measured.
[b] Antitoxin present for 4.5 h at 37°C.
[c] Antitoxin present for 5 or 15 min at 37°C; monolayers washed and reincubated in medium at 37°C.
[d] Antitoxin present for 15 min at 4°C; monolayers washed and reincubated in medium at 37°C for the remainder of the 4.5-h period.

ulin had been internalized via coated areas of the cell membrane. More recently, Moya et al. (31) reported that depletion of the intracellular potassium level in Hep2 cells leads to inhibition of coated-pit formation and also protects cells from DT. From these data the authors inferred that DT cytotoxicity depends on the presence of coated pits on the surface of the cell. In similar experiments, Sandvig et al. (39) showed that potassium depletion protects Vero cells against DT cytotoxicity. In this study, we directly visualized the movement of biotinyl-DT to coated pits with subsequent entry into endosomes and movement to the perinuclear region of the cell. Methylamine stopped the movement to coated areas and sequestered DT in vesicles in which activity was not expressed.

One surprising observation in these experiments was that only a small portion of the biotinyl-DT initially bound to Vero cells was internalized. A plausible explanation for the rapid loss of cold-bound DT on warming of the Vero cells is the dissociation of DT, weakly bound through ionic interactions, into the culture medium. The abrupt change in the temperature of cells prebound with DT may cause conformational changes which no longer favor binding. Prebound insulin also is rapidly dissociated from human hepatoma cell surface receptors after a transition from 4 to 37°C (5). More recently, Critchley et al. (6) showed that much of the tetanus toxin initially bound to cultured neurons at 4°C dissociated when the temperature was raised to 37°C. Although tetanus toxin dissociated more slowly than did DT at 37°C, the results of other investigators suggest that our observation is not unique to our system.

How DT crosses intracellular membrane barriers to reach the cytoplasm is not yet clearly defined. In fact, it may be technically impossible to trace the small number of DT molecules which successfully do so. It is known that under conditions in which a large number of DT molecules (ca. 50,000) are bound to the surface of a sensitive cell, only a minor fraction (ca. 100 molecules) participate in the inactivation of cytoplasmic elongation factor 2 (32). Furthermore, Yamaizumi et al. (42) have estimated that a single molecule of DT introduced directly into the mammalian cell cytoplasm is theoretically capable of preventing cell division. Therefore, tracing the putative DT molecules responsible for cell death is technically out of reach at present.

There is information, however, which allows speculation of the events after DT entry. Several investigators have demonstrated a direct penetration of DT through membranes at low pH (8, 11, 18, 37). We have observed DT-gold in endosomes shortly after endocytosis is initiated. Endosomes are recognized as organelles wherein dissociation of ligand and receptor occurs and where the ultimate destination of the ligand and receptor is determined; i.e., delivery to lysosomes, return to the cell surface, or release into the cell cytoplasm (1, 7, 16, 34). Endosomes, like lysosomes, possess an ATP-dependent proton pump and therefore are acidified. However, endosomes lack acid hydrolases, and therefore degradation of ligand does not occur within these organelles (20, 23, 41). Therefore, the acidic nature of endosomes provides an appropriate environment for DT entry into the cytoplasm. In fact Marnell et al. (22) have shown that internalized DT reaches a nonlysosomal acidic environment within 3 to 4 min after uptake. Our data suggest that this intracellular compartment is probably the endosome. Interestingly, Marnell et al. (22) observed a 15-min lag after acidification and before a reduction in protein synthesis was measurable. Our own observations prompt the suggestion that this lag in expression of toxicity may represent the time required for movement through several prelysosomal acidic organelles. A reasonable hypothesis is that the routing of DT within endosomes through the perinuclear region provides sufficient time in an acidic environment for pore formation and subsequent escape of DT or A fragment into the cell cytoplasm to occur (8, 18, 37, 38).

Although we are not able to state unequivocally that DT entering through coated pits is the DT responsible for cell death, several lines of evidence suggest that this is the case. First, as reported previously (29), there is no evidence that DT enters the DT-resistant mouse LM fibroblast via coated pits. Instead, the evidence is that DT enters LM cells through noncoated areas and is delivered rapidly to lysosomes. Second, methylamine, which blocks movement of DT to coated pits (Tables 1 and 2) and alters the subsequent trafficking of DT in Vero cells, also provides solid protection (9, 25). The protection is afforded even though DT binds normally to Vero cells in the presence of methylamine (25) and is subsequently internalized (9, 25). Quantitation by electron microscopy revealed that comparable levels of prebound biotinyl-DT were internalized in the presence and absence of methylamine; therefore, it may be that receptor-bound DT, in the presence of the amine, is cleared from the cell surface through noncoated regions and is subsequently sequestered in methylamine-laden vesicles. The biochemical data corroborate the morphologic observations. Methylamine is concentrated both in endosomes and lysosomes where the weak base raises intraorganelle pH (24, 35). Thus the acidic environment required for direct membrane penetration of DT is not available. Alkaline conditions, in addition, may not favor receptor-ligand dissociation (4, 15), endosome-lysosome fusion, or conversion of DT to the enzyme active state. Thus, the net effect of methylamine may be to accumulate receptor-ligand complexes within organelles at an elevated pH, thereby preventing transport of DT to the cytoplasm.

The antitoxin data (Table 4) also favor the hypotheses presented. After removal of methylamine from the medium, it is likely that intracellular vesicles containing nondissociated receptor-ligand complexes were recycled to the cell surface. The receptor-ligand complexes, in the absence of the amine, were then permitted to reenter the cell by a normal route (i.e., through clathrin-coated regions), ultimately causing cell death (Table 4, experiment 5). However, when antitoxin was present during the initial incubation period and during the subsequent treatment period (experiment 3), or only during the treatment period after initial incubation in the presence of methylamine (experiment 6), antitoxin protected cells by neutralizing DT, which recycled to the cell surface after removal of the amine block. The movement of receptor-DT complexes is apparently a rapid and dynamic process (experiments 7 through 9). Antitoxin added for 5 min at 37°C provided 50% protection (experiment 9); if present for 15 min at 37°C, antitoxin provided about 80% protection (experiment 7). Treatment of cells with antitoxin for 15 min at 4°C after removal of methylamine provided 60% protection (experiment 8). The different levels of protection afforded by the addition of antitoxin at 37 and 4°C (80 versus 60% protection) may reflect intracellular receptor-DT complexes not initially accessible to antibody. At 37°C, complexes may be cycled to the cell surface, where DT can be neutralized by antitoxin; recycling of complexes at 4°C does not occur. Mekada et al. (25) also reported the rapid loss of cell-associated ^{125}I-labeled DT on removal of methylamine. Thus, the cumulative evidence suggests that methylamine permits sequestration of DT within the cell and

that on release of the amine block, DT is returned to the cell surface and reenters via coated areas; i.e., via receptor-mediated endocytosis.

Our data suggest that the productive pathway involves specific binding of DT to receptors followed by internalization via clathrin-coated pits, after which DT appears to migrate within endosomes to the perinuclear region of the cell. Ultimately, residual DT is transported to the lysosomal compartment. During the transport process, a few DT molecules reach the cytoplasm, presumably by direct penetration of a prelysosomal vesicle membrane, which is facilitated by the acid environment. The route described allows for multiple interendosomal fusion events which would result in an increase in DT concentration. It also permits a prolonged residence in an acidic compartment. Both of these factors afford a greater opportunity for DT to enter the cytoplasm.

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