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Diphtheria Toxin Does Not Enter Resistant Cells by Receptor-Mediated Endocytosis

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Diphtheria toxin (DT) and pseudomonas toxin are two distinct microbial toxins which inhibit protein synthesis in an identical manner, i.e., by the inactivation of cytoplasmic elongation factor 2. Although murine cells bind both toxins, they are 10,000-fold less sensitive to DT than to pseudomonas toxin. This suggests that the level of resistance resides at some event after binding. We have previously shown that pseudomonas toxin enters mouse LM fibroblasts by receptor-mediated endocytosis, a process in which ligand is internalized via specialized clathrin-coated pits and moves to the Golgi region and then to the lysosomes. Here, we visually follow the entry and trafficking of DT by resistant mouse fibroblasts. A biotinyl-toxin–avidin-gold system was used to visualize DT on the ultrastructural level. DT entered resistant cells through non-clathrin-coated regions of the plasma membrane and within 2.5 to 5 min was seen in lysosomes. Only rarely was DT seen in coated pits or in the Golgi region. Furthermore, the temperature dependence of internalization of ^{125}I-labeled horseradish peroxidase and ^{125}I-labeled DT by LM cells was similar. On the basis of these observations, we postulate that DT does not enter DT-resistant LM cells by receptor-mediated endocytosis and that receptor-mediated endocytosis is required for efficient expression of toxicity.

Diphtheria toxin (DT) inhibits protein synthesis in mammalian cells by the ADP-ribosylation of elongation factor 2 thereby inactivating it. Animals and cultured cells of most mammalian species, with the exception of mice and rats, exhibit a high level of sensitivity to DT (17). Since all naturally occurring elongation factor 2 is susceptible to ADP-ribosylation by DT, it is assumed that cells owe their resistance to the lack of a functional receptor for the toxin or to defects in the entry process (15). Heagy and Neville (9) reported that the DT-resistant mouse L-cell has a plasma membrane receptor of low affinity for the toxin; they propose that resistance is due to defects in internalization. Robbins et al. (19) have isolated a mutant of Chinese hamster ovary cells which is deficient in the uptake of several ligands including DT. Keen et al. (10) have shown that there are DT-specific receptors on resistant mouse cells, and these investigators suggest that DT entry in both sensitive and resistant cells is by receptor-mediated endocytosis. Recently, Didsbury et al. (4) suggested that LM cells have a defect in a step in the processing of toxin, subsequent to binding.

Receptor-mediated endocytosis has been implicated in the internalization of several peptide hormones, serum factors, viruses, and a bacterial toxin, pseudomonas exotoxin A (PE) (8, 16, 18, 21). In general terms, this process involves the binding of ligand to specific receptors on the cell, clustering of ligand-receptor complex over specialized clathrin-coated regions of the membrane, internalization in endosomes, and movement of the ligand to the Golgi region and then to lysosomes (8, 18). PE (6, 16) and DT (manuscript in preparation) enter sensitive mammalian cells by receptor-mediated endocytosis, and it is proposed that entry by this route is required for efficient expression of biological activity. Here we examine, on the ultrastructural level, the binding and internalization of DT by DT-resistant LM cells and propose that, in a resistant system, the majority of toxin enters through non-clathrin-coated regions of the membrane and is delivered directly to lysosomes, bypassing the Golgi region.

MATERIALS AND METHODS

Toxin and markers. Diphtheria toxin was obtained from Connaught Medical Research Laboratories and was purified by the method of Collier and Kandel (3). The final preparation ran as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. PE was prepared by a modification (16) of the method of Leppla (12). DT was iodinated by the method of

Roth (20) to a specific activity of 9.6×10^6 cpm/μg. Toxins were biotinated with biotin-*N*-hydroxysuccinimide ester and retained most of their biological activity (1, 16). Horseradish peroxidase (HRP) (HRP-gold; 18 nm) used to monitor fluid phase pinocytosis (22) was labeled by the method of Geoghegan and Ackerman (7). Egg white avidin (E-Y Laboratories, San Mateo, Calif.) was succinoylated and adsorbed onto the surface of 5.2-nm gold as described previously (16).

Cells. Mouse LM fibroblasts (ATCC CCL 1.2 LM, a derivative of L929 cells) were maintained in McCoy 5A medium (GIBCO Laboratories, Grand Island, N.Y.) containing heat-inactivated fetal bovine serum (5%), streptomycin (0.2 mg/ml), and penicillin (200 U/ml).

Electron microscopy. LM cells grown in glass Leighton tubes to 80% confluency were incubated for 90 min in Hanks balanced salt solution (HBSS) to exhaust exogenous biotin, cooled to 4°C and incubated with biotinyl-DT (25 μg/ml) overnight in the cold. All samples were washed three times with cold HBSS, incubated with avidin-stabilized 5.2-nm gold colloids for 30 min, and washed three times with cold HBSS. The zero time samples were fixed immediately for 30 min at 4°C with 0.5% glutaraldehyde in phosphate-buffered saline (0.10 M NaCl in 0.05 M PO_4 [pH 7.4]). Other samples were washed with warm HBSS (37°C) and incubated at 37°C as indicated before fixation. Samples were processed for electron microscopy, and sections were quantitated as previously described (6, 16). All experiments were repeated at least three times.

Internalization assay. Internalization of ^{125}I-labeled DT was followed by a modification of the method of Dorland et al. (5). Cells were seeded (5×10^5 cells per ml) into 24-well culture dishes. At the time of the experiment, monolayers and ^{125}I-labeled DT (500 ng/ml) were equilibrated to temperature, toxin was added, and cells were incubated at appropriate temperatures. Surface-associated and intracellular ^{125}I-labeled DT was differentiated on the basis of sensitivity (surface) or resistance (intracellular) to release with trypsin-pronase-EDTA. Monolayers were incubated with trypsin-pronase-EDTA (2.5% trypsin, 0.25 mg of pronase per ml, 0.002 M EDTA in HBSS without Ca^{2+} or Mg^{2+}) for 60 min at 4°C; the cells were collected by centrifugation, the pellet was washed and dissolved in 0.1 M NaOH, and radioactivity and protein concentration was determined.

Protein synthesis. Cells were incubated with DT for 5 h at 37°C; protein synthesis was then assayed by measuring the incorporation of [^{3}H]leucine (2 μCi/ml) into acid-precipitable material during a 60-min incubation at 37°C.

RESULTS

Initial protein synthesis inhibition studies established that 25 μg of DT per ml inhibited protein synthesis by 50%, and this concentration of toxin was used in all electron microscopy experiments. The amount of DT required to inhibit protein synthesis in a resistant cell is ca. 10,000-fold more than is required to achieve the same effect in a sensitive cell (Vero cell; unpublished data).

Early steps in internalization. The early stages in the interaction of DT with LM cells were examined on the ultrastructural level. Initial experiments established that binding was saturable and that similar amounts of toxin associated with cells, whether binding (4°C) was for 60 min or 15 h (data not given). In addition, preliminary experiments in which biotinyl-DT was incubated overnight in the cold in the presence of 50-fold excess native toxin reduced the level of surface DT-gold by 60% (7.5 or 3.0 sites per section in the absence or presence of native toxin). This level of nonspecific binding is similar to that reported for ^{125}I-labeled DT and LMTK$^-$ cells (4). The data suggest that at least some DT was bound to specific DT-binding proteins.

The kinetics of DT-gold internalization from a representative experiment are presented in Fig. 1. When LM monolayers were maintained in the cold, DT-gold was randomly distributed on the cell surface with 4 to 5% of the total sitings in coated areas. Upon warming monolayers to

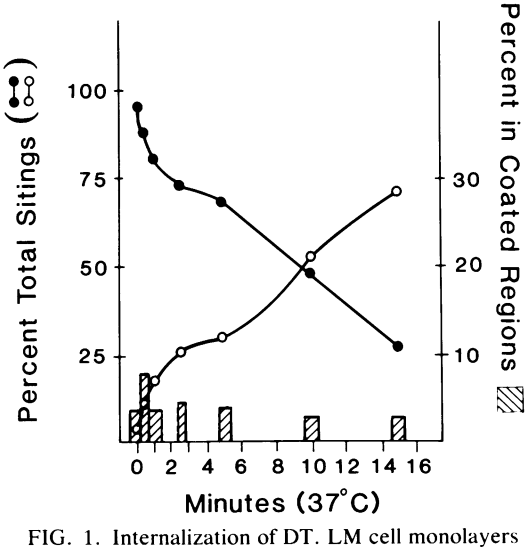

FIG. 1. Internalization of DT. LM cell monolayers were incubated at 37°C for 90 min to remove exogenous biotin, cooled to 4°C, and incubated for 15 h with 25 μg of biotinyl-DT per ml. Monolayers were washed, incubated 30 min at 4°C with avidin-gold, washed, and warmed to 37°C. At indicated times, monolayers were washed with phosphate-buffered saline, fixed with 0.5% glutaraldehyde, and processed for electron microscopy. DT-gold was scored according to the number of gold grains (singlet, doublet, multiple), location (surface, periphery, cytoplasm), whether in coated area or not, and if intracytoplasmic, type and location of vesicle and size of multiple. Symbols: ●, plasma-membrane associated; ○, periphery plus intracytoplasmic (data expressed as percent of total sitings); ▨, sitings in coated pits or coated vesicles (expressed as percent of surface plus periphery sitings).

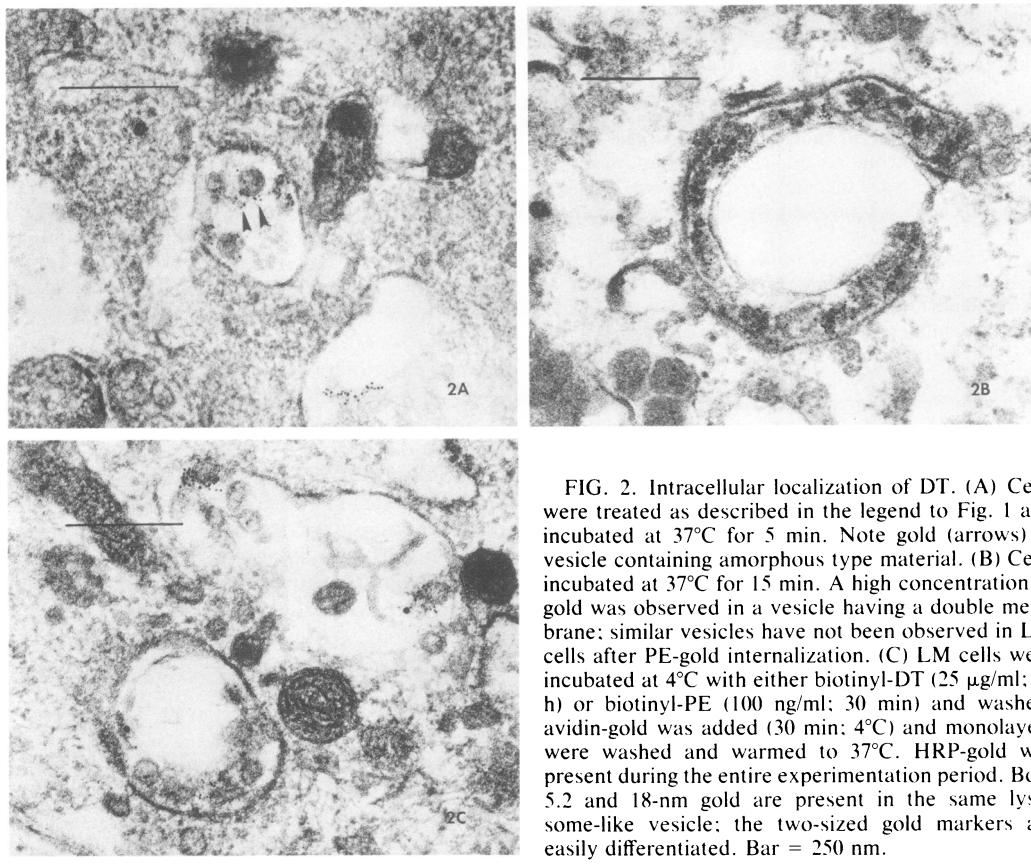

FIG. 2. Intracellular localization of DT. (A) Cells were treated as described in the legend to Fig. 1 and incubated at 37°C for 5 min. Note gold (arrows) in vesicle containing amorphous type material. (B) Cells incubated at 37°C for 15 min. A high concentration of gold was observed in a vesicle having a double membrane; similar vesicles have not been observed in LM cells after PE-gold internalization. (C) LM cells were incubated at 4°C with either biotinyl-DT (25 μg/ml; 15 h) or biotinyl-PE (100 ng/ml; 30 min) and washed; avidin-gold was added (30 min; 4°C) and monolayers were washed and warmed to 37°C. HRP-gold was present during the entire experimentation period. Both 5.2 and 18-nm gold are present in the same lysosome-like vesicle; the two-sized gold markers are easily differentiated. Bar = 250 nm.

37°C, DT-gold was cleared from the cell surface and was localized intracellularly in membrane-bound vesicles. In two of five experiments, a slight increase in DT-gold in coated areas was noted upon warming.

DT-gold was internalized with a half-time of ca. 10 min. As early as 2.5 min and consistently by 5 min, internalized DT-gold was observed in vesicles containing amorphous materials and identified morphologically as lysosomes (Fig. 2A). The number of gold grains in vesicles increased with time. DT-gold was also located in vesicles with the morphology shown in Fig. 2B. Only on rare occasions was DT-gold observed in vesicles in the Golgi region. Therefore, we conclude that the great majority of DT enters the toxin-resistant LM cell through non-clathrin-coated regions of the membrane, moves directly to lysosomes, and does not pass through the Golgi.

We next measured the simultaneous internalization of HRP, a standard marker of pinocytosis (22), and toxin by LM cells (Table 1). Both DT and PE were compared with HRP so that entry could be followed in a resistant (DT) and sensitive (PE) cell system. A double label electron microscopic technique with HRP adsorbed onto 18-nm gold sols and toxin identified with 5.2-nm gold was used. As shown in Fig. 2C, these two different sized markers are readily distinguished. Within 5 min of warming, nearly 20% of all intracellular sitings of DT-gold were coincident with HRP (i.e., both 5.2- and 18-nm gold were seen in the same vesicle). In contrast, in the same period only 3% of the internal sitings of PE-gold and HRP were coincident. By 30 min, the percentage of coincident sitings (toxin and HRP) was similar in both the toxin-sensitive and toxin-resistant cell system. These results would be expected, since PE moves to the Golgi region and then to lysosomes, whereas DT, in the same cell line, goes directly to lysosomes.

Effect of temperature on internalization. We have recently reported (M. Manhart, C. B. Saelinger, and R. E. Morris, Soc. Cell. Biol., **95:** 434a, 1982; submitted for publication) that entry of ^{125}I-labeled PE into LM cells (highly sensitive to PE) is temperature dependent, with a rate-limiting step occurring near 20°C. In contrast, internalization of ^{125}I-labeled HRP does not show this rate-limiting step. The temperature dependence of the internalization of ^{125}I-labeled

TABLE 1. Cointernalization of toxin and HRP by LM cells[a]

Toxin	Time (min)	Sites per section			% Coincident
		Toxin[b]	HRP[b]	Both[c]	
PE	5	3.5	0	0.1	2.8
	15	3.5	0.1	0.5	12.1
	30	2.2	0.1	1.0	30.3
	50	2.5	0.2	1.0	27.0
DT	5	2.0	0.2	0.5	18.5
	15	4.8	0	1.1	18.6
	30	2.8	0.2	1.9	38.0
	50	2.9	0	0.9	23.6

[a] Protocol as given in the legend to Fig. 2C.

[b] Intracellular sites per section containing only toxin (5.2-nm gold) or HRP (18 nm gold); 10 sections viewed per sample.

[c] Intracellular sites per section containing both 5.2 and 18-nm gold.

[d] Percentage of total intracellular sitings containing both 5.2- and 18-nm gold.

DT by LM cells was measured to determine whether it was similar to PE (probe for receptor-mediated endocytosis) or to HRP (probe for fluid-phase endocytosis). Internalization of ^{125}I-labeled DT (Fig. 3) was linear with time and showed no lag in uptake. Raising the incubation tmeperature gradually increased the rate of internalization over a range of 4 to 37°C without any break in uptake over the entire temperature range. From Arrenhius plots of the data (Fig. 3, inset) an energy of activation (Ea) of 16.8 Kcal/mol and a temperature coefficient (Q_{10}) of 3.52 were determined.

DISCUSSION

The resistance of mouse L-cells to DT has been attributed to an absence of high affinity toxin receptors (13, 17) or to defects in the internalization process (9, 10), with the toxicity seen in the presence of high toxin concentrations occurring by a nonspecific uptake process (2, 14). Heagy and Neville (9) reported that intoxication of L-cells by DT is a saturable process and that the saturable component may be a low affinity receptor for DT. They also suggest that the high level of DT resistance observed in L-cells is related to a deficiency in transport. The results presented here suggest that the association of DT with mouse LM cells is specific and that internalization of toxin by a resistant and sensitive cell is dramatically different.

The trafficking of PE in LM cells, which are very sensitive to PE, is different than for DT in the same cell. PE rapidly (30 s) moves to coated pits, is internalized through these clathrin-coated regions, moves in endosomes to the Golgi region of the cell (5 min), and finally appears in lysosomes (20 min; reference 16). A similar pathway has been observed for DT in a sensitive monkey kidney cell (Vero, in preparation). This is in direct contrast to the route DT takes in a resistant cell. Our results support the hypothesis of Didsbury et al. (4) that LM cells have a defect in the entry process between toxin binding and delivery of fragment A to the cytoplasm. Our data show that in a resistant cell DT moves in endocytic vesicles directly to the lysosomes. Presumably, the delivery step occurs in a vesicle which is bypassed when toxin does not enter cells via coated pits. Furthermore, entry via clathrin-coated pits appears to be required for efficient expression of toxicity. This hypothesis is supported by the fact that the weak base methylamine, which protects cells from PE, blocks entry via clathrin-coated pits and presumably directs toxin to lysosomes, bypassing the Golgi region (16). Therefore in the presence of methylamine, the toxin-sensitive cell takes on the characteristics of a resistant cell.

The results presented here are in conflict with those of Keen et al. (10) who reported that DT is internalized by receptor-mediated endocytosis in both sensitive and resistant cells. Their conclusions are based on viewing fluorescein-labeled α_2-macroglobulin, a ligand known to enter cells by receptor-mediated endocytosis (8, 18), and rhodamine-labeled DT in the same vesicles. Epifluorescence video intensification microscopy was used to detect ligand; this technique is not as sensitive as transmission electron microscopy (11). Also, Keen et al. did not examine time periods earlier than 15 min; by this time it would not be surprising to see ligands, internalized by two different mechanisms, together in the same lysosome.

The studies on internalization of ^{125}I-labeled DT at different temperatures also support fluid phase and not receptor-mediated endocytosis as the route of entry of DT in a resistant LM cell. DT, like HRP in the same cell line, exhibited a gradual decrease in the rate of internalization at all temperatures between 37 and 4°C. The Ea of 16.8 Kcal/mol and Q_{10} of 3.52 are in close agreement with the values reported by us (submitted for publication; for HRP in LM cells, Ea is 19.6 and Q_{10} is 2.3) and by Steinman et al. (23) for HRP. The uptake patterns for HRP and for DT in LM cells are different from the biphasic pattern of internalization observed with ligands such as PE (submitted for publication; for PE in LM cells between 37 and 23°C, the Ea is 16.9 Kcal/mol and Q_{10} is 2.5; below 16°C, the Ea is 49.1 Kcal/mol and Q_{10} is 22.9) and asialo-orosomucoid (24), which are internalized by receptor-mediated endocytosis.

In summary, we suggest that DT enters a

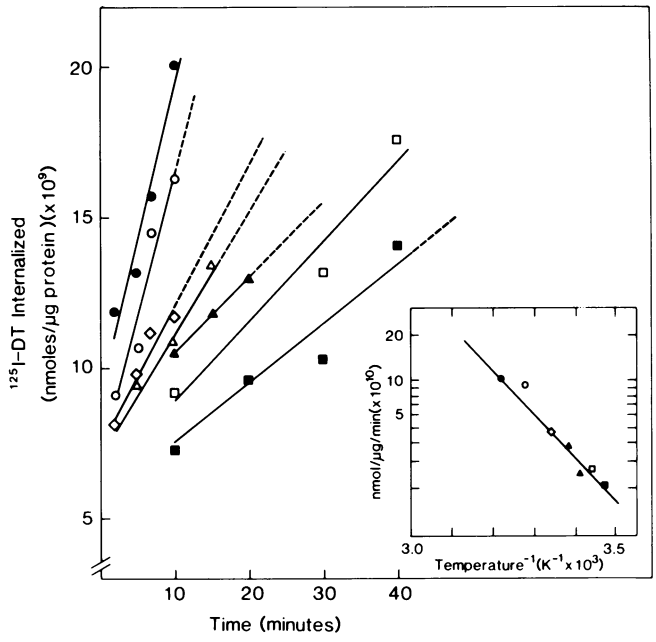

FIG. 3. Effect of temperature on internalization of ^{125}I-labeled DT by toxin-resistant LM cells. LM cell monolayers, equilibrated to temperature, were exposed to ^{125}I-labeled DT (500 ng/ml). At the times indicated, monolayers were quickly washed with ice-cold HBSS and exposed to trypsin-pronase-EDTA for 60 min at 4°C to remove surface-bound toxin. Cells were washed twice in HBSS, and pellets were dissolved in 0.1 M NaOH before counting in a Packard gamma counter. Each point is the mean of triplicate samples. Lines were fitted by linear regression. Inset: the slopes of the lines at each temperature are plotted against the inverse of absolute temperature. The best fit line was determined by linear regression and has a correlation coefficient of −0.960. Symbols: 37°C (●), 32°C (○), 26°C (◇), 23°C (▷), 20°C (▲), 18°C (□), 15°C (■).

resistant cell through non-clathrin-coated regions, moves very quickly to lysosomes, and presumably is degraded there. In a sensitive cell, toxin enters through clathrin-coated pits, moves first to the Golgi, and then moves to lysosomes (16). It is suggested that this latter route, i.e., receptor-mediated endocytosis, is required for efficient expression of biological activity.

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