

Internalization and Degradation of Cholera Toxin by Cultured Cells: Relationship to Toxin Action

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ABSTRACT Using anticholera antibodies and ^{125}I -protein A, we developed a specific and quantitative assay for measuring cholera toxin on the surfaces of cultured cells. When neuroblastoma cells containing bound toxin were incubated at 37°C , surface toxin disappeared with a half-life of ~ 2 h and a significant loss was detected by 10 min. When cells were incubated with ^{125}I -cholera toxin in order to measure toxin degradation, cell-associated radioactivity disappeared with time and a corresponding amount of TCA-soluble label appeared in the culture medium with a half-life of 4–6 h. No degradation was detected until 45 min. Although there was a lag of 15 min before bound cholera toxin activated adenylate cyclase, the enzyme became maximally activated between 45 and 60 min. Similar results were obtained with Friend erythroleukemia cells. Internalization, degradation, and activation all were blocked when the cells were maintained at 4°C . At 22°C , internalization and activation occurred, albeit at a slower rate, whereas degradation was effectively inhibited. These results indicated that cholera toxin does not have to be degraded by intact cells in order for it to activate adenylate cyclase. Some internalization of the toxin, however, appears to precede the activation process.

Cholera toxin (CT), the active agent of cholera, is composed of two components, A and B (4). The B component binds to specific receptors on the cell surface that are believed to be the ganglioside GM_1 (see reference 6 for a recent review). The A component consists of two polypeptide chains, A1 and A2, connected by a single disulfide bond. The A1 peptide is an ADP-ribosyltransferase (23) and catalyzes the transfer of ADP-ribose from nicotinamide adenine dinucleotide (NAD) to the regulatory component of adenylate cyclase (2, 15). This modification results in a persistent activation of adenylate cyclase. With intact cells, there is a definite lag period before cyclase activity begins to rise in response to the toxin (1, 5). When membranes are incubated with A1 plus NAD, there is no lag (14). It is believed that during the lag period CT or some part of it is translocated across the membrane, that A1 is formed, and that adenylate cyclase is then activated by A1 (6, 9, 12, 16). Several groups, using immunocytochemistry or cytochemistry and electron microscopy, have reported that CT becomes internalized in a time- and temperature-dependent manner (17, 19, 20). These observations, however, were not correlated with the activation of adenylate cyclase. Other groups have suggested that CT must first be degraded by the cell in order to generate a fragment that can then activate the cyclase (18, 21, 22).

The present studies were initiated in order to develop a specific and quantitative method for assaying surface bound CT and to follow the kinetics of CT internalization, degradation, and activation of adenylate cyclase.

MATERIALS AND METHODS

Cells and Cell Culture

Mouse neuroblastoma clone NB41A3 was obtained from the American Type Culture Collection (Rockville, MD) and grown in Dulbecco's modified Eagle's medium containing 0.45% glucose, 10% fetal calf serum, and $50\ \mu\text{g}/\text{ml}$ gentamicin. Friend erythroleukemic cells and rat glioma C6 cells were cultured as described previously (7). The NB and C6 cells were grown attached to 35-mm wells of multi-cluster dishes and the Friend cells were grown in suspension. Where indicated, C6 cells were treated with GM_1 , as described previously (5, 11). Routinely, the cells were washed once with phosphate-buffered saline (PBS) and incubated in serum-free medium buffered with 25 mM HEPES and containing 0.01% bovine serum albumin and 10 nM CT or ^{125}I -CT for 30 min at 4°C . The cells then were washed three times with ice-cold PBS, incubated in fresh serum-free medium for the indicated times at the appropriate temperature and assayed for surface CT, CT degradation, or adenylate cyclase activity as described below.

Assay for Surface Toxin

The incubation medium was removed and 1 ml of the HEPES-buffered medium (ice-cold) was added with and without $10\ \mu\text{l}$ of anticholera antibodies.

After 40 min at 4°C, the cells were washed twice with ice-cold PBS and incubated with 1 ml of the above medium containing 0.01% bovine serum albumin and 50 nM ¹²⁵I-protein A for 40 min at 4°C. Finally, the cells were washed three times with ice-cold PBS, dissolved in 1 ml of 1 M NaOH, and counted in a Beckman Gamma 4000 (Beckman Instruments, Inc., Fullerton, CA). Nonspecific binding was determined by carrying cells not treated with CT through the same procedure or by omitting the antibodies. Values reported represent specific binding and are the mean of triplicate determinations; standard deviations of the means were 5% or less.

In separate studies, the concentrations of protein A and antitoxin as well as the incubation times were varied. When NB cells containing saturating amounts of bound CT were incubated with a constant amount of anti-CT (10 μl for 1 h) and increasing amounts of protein A for 1 h, maximum binding was observed with 30 nM protein A. When the anti-CT was varied and the protein A kept constant (50 nM), binding was essentially maximal with 5 μl of antibodies and little or no further increase was observed with more anti-CT. When the time of the two incubations was varied, maximum binding was observed in each case between 30 and 45 min. Thus, optimum conditions for the assay appeared to be 10 μl of anti-CT for 40 min followed by 50 nM ¹²⁵I-protein A for 40 min as described above.

Degradation of Toxin

After the cells containing bound ¹²⁵I-CT were incubated in fresh medium for different times, the medium was removed and the cells dissolved in 1 ml of 1 M NaOH. The medium was cooled to 4°C and adjusted to 0.1% bovine serum albumin and 10% TCA. The samples were centrifuged at 2,000 g for 10 min. The supernatants, pellets, and digested cells were then counted for ¹²⁵I. Cell-associated radioactivity was further analyzed by SDS PAGE. Cells were scraped in 2 ml of 10% TCA and collected by centrifugation. The precipitates were dissolved by boiling for 2 min in a solution containing 2% SDS, 5 mM dithiothreitol, 10% glycerol, 0.1 M Tris-Cl (pH 7.4) and 0.005% bromophenol blue. Portions were analyzed on 12.5% slab gels (16) and radioactivity was detected by slicing and counting the gels (16) or by autoradiography.

Assay for Cyclic AMP and Adenylate Cyclase

The activation of adenylate cyclase by CT was determined in two ways: by the increase in intracellular cyclic AMP in intact cells or by the increase in adenylate cyclase activity in membranes prepared from the cells. For the cyclic AMP method, the cells were incubated in HEPES-buffered medium containing 1 mM isomethylbutylxanthine for the appropriate times. After the medium was aspirated, cyclic AMP was extracted from the cells and assayed as described previously (5). In the case of the Friend cells, the cell suspensions were boiled for 5 min and assayed for cyclic AMP. For the cyclase method, the cells were incubated for the appropriate times and lysed in 2 mM Tris-HCl (pH 7.4)/1 mM EDTA/0.2 mM dithiothreitol by homogenizing for 5 s with a Brinkmann Polytron (Brinkman Instruments, Inc., Westbury, NY) (8). The lysates were centrifuged at 30,000 g for 15 min and the membranes were assayed for cyclase activity in presence of 50 μM GTP (10).

Materials

Cholera toxin and cholera toxinogen were obtained from Schwarz/Mann (Becton, Dickinson & Co., Orangeburg, NY). Anticholera toxin antiserum was raised in a burro and was a generous gift of Dr. W. Habig, Bureau of Biological Standards. In a quantitative precipitin assay, 1 ml precipitated 140 μg of CT. CT was iodinated essentially as described by Cuatrecasas (3). Protein A was purchased from Pharmacia Fine Chemicals (Piscataway, NJ) and ¹²⁵I-protein A from either Amersham Corp. (Arlington Heights, IL) or New England Nuclear (Boston, MA).

RESULTS

Assay for Surface Cholera Toxin

When NB cells were incubated with CT and then anti-CT, there was a large increase in ¹²⁵I-protein A binding compared with cells not treated with CT (Table I). The cells could be lightly fixed before adding the anti-CT with little effect on the interaction of antibodies and protein A with the CT-treated cells. In the absence of anti-CT, protein A did not specifically bind to CT-treated cells and there was little nonspecific binding of the antibodies to control cells (Table I).

When NB cells were incubated with increasing concentra-

tions of CT and assayed for surface CT as described above, there was increasing binding of ¹²⁵I-protein A which paralleled the amounts of ¹²⁵I-CT bound at these same concentrations (Fig. 1a). When corrected for their respective specific activities, the ratio of protein A to CT bound at saturation was 0.92. There was only a small amount of specific binding of protein A to rat glioma C6 cells (Table II) consistent with the few toxin receptors present on these cells (5, 11). When the cells were treated first with GM₁, the amount of protein A specifically bound increased (Table II). When the concentration of GM₁ was increased, protein A binding increased in parallel up to 1 μM GM₁ (Fig. 1b). In previous studies, it was shown that GM₁

TABLE I
Effect of CT and Anti-CT on Binding of ¹²⁵I-Protein A to NB Cells

Additions			¹²⁵ I-protein A bound cpm/dish
CT	Anti-CT	Fixative*	
-	+	-	320 ± 2
+	+	-	4,967 ± 127
-	-	+	220 ± 13
-	+	+	549 ± 35
+	-	+	211 ± 26
+	+	+	5,932 ± 232

Cells were incubated for 30 min at 37°C in the presence and absence of 12 nM CT, washed and incubated in fresh medium for 45 min at 4°C in the presence and absence of 10 μl of anti-CT. The cells were then washed, incubated with 50 nM ¹²⁵I-protein A (1,235 cpm/pmol) for 45 min at 4°C and assayed for bound protein A as described under Materials and Methods. Each value is the mean ± SD of binding to triplicate dishes.

* Before incubating the cells with anti-CT, the washed cells were incubated with 1 ml of fixative (4% formaldehyde and 5% sucrose in Dulbecco's phosphate-buffered saline with Ca²⁺ and Mg²⁺) for 1 h at 4°C.

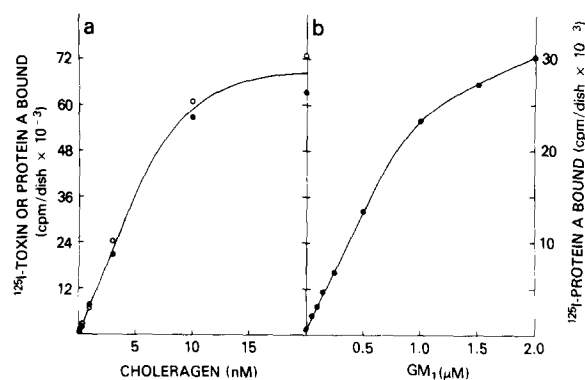


FIGURE 1 Assay of surface cholera toxin (CT) as a function of bound CT. (a) NB cells were incubated with the indicated concentrations of CT (○) or ¹²⁵I-CT (●) for 30 min at 4°C and washed. The cells then were assayed for bound ¹²⁵I-CT or incubated further with 10 μl/ml of anti-CT for 40 min and 50 nM ¹²⁵I-protein A for 40 min and assayed for bound ¹²⁵I-protein A (○). Values for ¹²⁵I-CT binding have been corrected for nonspecific binding to cells treated with 0.2 μM unlabeled CT. Values for ¹²⁵I-protein A binding have been corrected for nonspecific binding to cells not treated with CT. (b) Rat glioma C6 cells were incubated at 37°C for 40 min with the indicated concentration of GM₁, washed, and incubated for 30 min at 4°C with 20 nM CT. The cells then were washed and incubated with anti-CT and ¹²⁵I-protein A as described in panel a. Binding has been corrected for nonspecific binding to cells not treated with CT.

uptake and thus subsequent CT binding increased in proportion to the GM₁ concentration (5, 11). These results indicated that the assay could be used to quantify the amount of CT on the cell surface.

Internalization and Degradation of Toxin

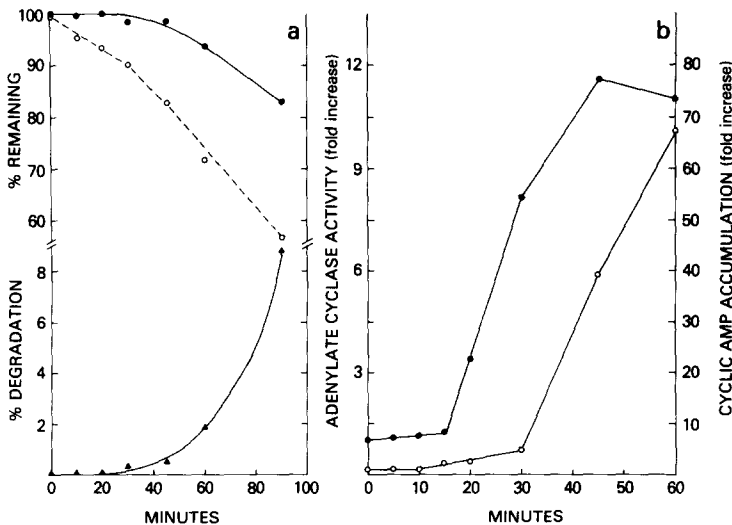
When NB cells were incubated for 30 min at 37°C with CT or ¹²⁵I-CT, washed and incubated in fresh medium for different times, the amount of surface CT disappeared rapidly with a half-life of 2 h (Fig. 2). The amount of cell-associated ¹²⁵I-CT also decreased and there was a corresponding increase of TCA-soluble radioactivity in the medium. Only a very small fraction of the label in the medium was precipitated by TCA; even after 46 h, this accounted for <10% of CT initially bound to the cells. Thus once bound, the toxin did not spontaneously dissociate from the cell surface. The half-life of CT degradation was ~5 h.

When the NB cells were first incubated with CT or ¹²⁵I-CT at 4°C, washed, and shifted to 37°C, disappearance of surface CT was detected as early as 10 min, whereas loss of cell-associated ¹²⁵I-CT and appearance of TCA-soluble label in the medium occurred between 45 and 60 min (Fig. 3a). Cells containing bound ¹²⁵I-CT were shifted to 37°C and at 15-min intervals scraped in 10% TCA. At each time point for up to 60 min, 98–100% (98.8 ± 0.8%) of the cell-associated radioactivity was TCA insoluble. Analysis of this material by SDS PAGE indicated that all of the label migrated as A1 or dissociated B

TABLE II
Effect of Treatment of Rat Glioma C6 Cells with GM₁ on Assay of Surface CT

Additions		¹²⁵ I-protein A bound	
CT	Anti-CT	Control cells	GM ₁ -treated cells
cpm/dish			
-	-	615	632
+	-	699	493
-	+	900	529
+	+	969	7,627

Cells were incubated at 37°C for 1 h in the presence and absence of 1 μM GM₁ (5, 11), washed, and assayed for surface CT. The specific activity of the ¹²⁵I-protein A was 4,000 cpm/pmol.



subunits with no evidence of any other labeled peptides (data not shown).

Kinetics of Toxin Action and Degradation

Under these conditions, activation of adenylate cyclase and accumulation of intracellular cyclic AMP in response to CT began only after a lag of 15 min (Fig. 3b). Cyclase activation was complete between 45 and 60 min. Similar results were obtained when the NB cells were detached from the culture vessels and incubated in suspension with CT or ¹²⁵I-CT. The rise in cyclase activity began after 15 min and there was no increase in the amount of TCA-soluble radioactivity in the total cell suspension until after 45 min. The relation between CT action and degradation also was examined in Friend erythroleukemic cells that grow in suspension. The lag period appeared to be even shorter in these cells compared to NB cells yet no CT degradation was detected until 1 h (Fig. 4).

Similar results were obtained with GM₁-treated C6 cells; the studies, however were complicated by the fact that 10–20% of the CT initially bound to the cells rapidly dissociated and appeared as TCA-precipitated material in the medium. When corrected for this loss, the disappearance of surface CT was

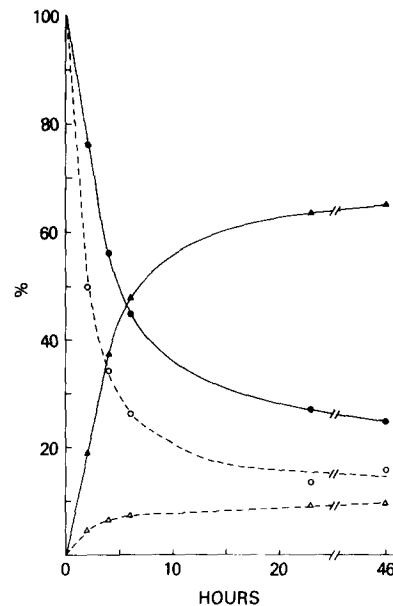


FIGURE 2 Effect of time on surface and cell-associated CT. NB cells were incubated at 37°C for 30 min with 10 nM CT or ¹²⁵I-CT, washed, and incubated in fresh medium at 37°C for the indicated times. The cells were then assayed for surface CT (○), cell-associated ¹²⁵I-CT (●), and TCA-precipitated (△) and TCA-soluble (▲) ¹²⁵I in the medium as described under Materials and Methods.

FIGURE 3 Effect of time on activation of adenylate cyclase and loss of surface and cell-associated CT. NB cells were incubated at 4°C for 30 min without or with 10 nM CT or ¹²⁵I-CT, washed, and incubated in fresh medium at 37°C for the indicated times. (a) The cells were assayed for surface CT (○), cell-associated ¹²⁵I-CT (●) or TCA-soluble ¹²⁵I in the medium (▲). (b) The cells were assayed for adenylate cyclase activity (●) or accumulation of intracellular cyclic AMP (○).

more rapid than toxin degradation at 30 and 60 min (Table III). During this same time period, adenylate cyclase was completely activated by CT (5).

Effects of Temperature

When NB cells containing bound CT were incubated at 4°C for up to 4 h, there was no loss of surface CT (data not shown). As indicated in Table IV, activation of adenylate cyclase and degradation of ¹²⁵I-CT were effectively blocked at 4°C. At 22°C, however, some internalization and activation did occur without a substantial amount of degradation (Table IV). As reported previously (5), CT was able to activate adenylate cyclase of GM₁-treated C6 cells at 22°C but at a slower rate than at 37°C. At 22°C, NB cells did not begin to accumulate cyclic AMP in response to CT until at least 60 min compared with 20 min at 37°C. Similar results were obtained with Friend

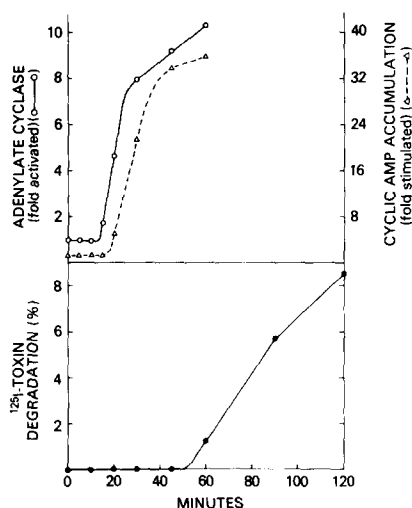


FIGURE 4 Effect of time on activation of adenylate cyclase and degradation of CT in Friend erythroleukemic cells. Friend cells were incubated at 4°C for 30 min with and without 10 nM CT or ¹²⁵I-CT. The cells were then washed, incubated at 37°C for the indicated times, and assayed for adenylate cyclase activity (O), cyclic AMP accumulation (Δ), and TCA-soluble ¹²⁵I (●).

TABLE III
Loss of Surface and Cell-associated CT in GM₁-treated Rat Glioma C6 Cells

Time min	Surface CT*	TCA-soluble ¹²⁵ I in medium†
	% remaining	% of total
30	88.1 ± 2.1	1.84 ± 0.33
60	73.9 ± 4.4	7.65 ± 0.47

Cells were incubated for 30 min with 0.5 μM GM₁, washed, and incubated with 10 nM CT or ¹²⁵I-CT (38,000 cpm/pmol) for 30 min at 4°C. The cells were then washed and assayed for surface CT or ¹²⁵I-CT bound or incubated at 37°C for the indicated times and then assayed for surface CT or TCA-soluble label in the medium.

* Values have been corrected for the amount of ¹²⁵I-CT (10%) that dissociated from the cells and appeared in the medium as TCA-precipitated label. In a separate kinetic experiment, this value remained constant between 30 min and 24 h. The cells initially bound 14,000 cpm of ¹²⁵I-protein A specifically.

† The cells initially bound 73,000 cpm of ¹²⁵I-CT compared with 2,000 cpm by C6 cells not treated with GM₁. Values represent the TCA-soluble label in the medium as a percentage of the total label in the medium plus the cells at each time.

cells. At 4°C, no degradation was observed for up to 6 h (data not shown). Low temperatures also completely blocked activation of adenylate cyclase by CT; after 90 min, activities were 20.5 and 21.7 pmol/min per mg of protein for control and for CT-treated cells, respectively.

Internalization of B and Absence of Recycling

Because the anticholera antibodies were raised against the holotoxin and can bind to both the A and B components, an attempt was made to determine whether there was any

TABLE IV
Effect of Temperature on Internalization, Degradation, and Action of CT

Temperature °C	Surface CT* % remaining	¹²⁵ I-CT degradation‡ % TCA-soluble in medium	Cyclic AMP§	
			-CT	+CT
4	100 ± 7.4	0.10 ± 0.02	23.3 ± 0.5	23.8 ± 0.8
22	83.4 ± 3.2	0.63 ± 0.02	53.9 ± 3.4	78.7 ± 1.1
37	39.9 ± 6.2	17.1 ± 0.32	136 ± 14.6	4,070 ± 184

* NB cells were incubated with 10 nM CT for 30 min at 4°C, washed, assayed for surface CT, or incubated for 2 h at the indicated temperature and then assayed. Initially, 70,300 cpm of ¹²⁵I-protein A specifically bound to the cells.

‡ Cells were incubated with 10 nM ¹²⁵I-CT for 30 min at 4°C, washed, incubated for 2 h at the indicated temperature, and assayed for TCA-soluble label in the medium. Values are expressed as a percent of the total label in each culture (165,000 cpm).

§ Cells were incubated with and without 10 nM CT for 30 min at 4°C, washed, incubated for 1 h at the indicated temperatures, and assayed for cyclic AMP. Values are in pmol/mg of protein. After 2 h at 22°C, cells treated with CT had accumulated 225 pmol/mg of protein.

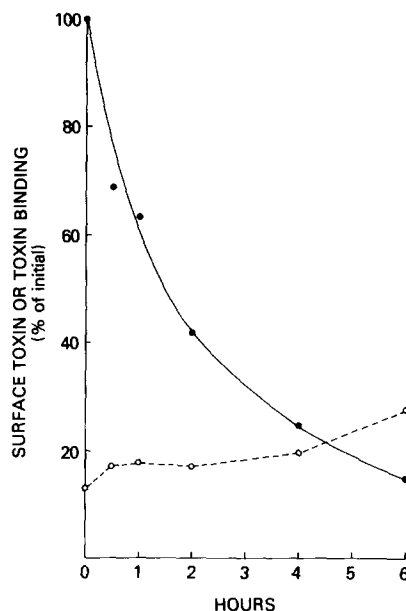


FIGURE 5 Effect of time on disappearance of surface CT and reappearance of CT receptors. NB cells were incubated at 4°C for 30 min with 10 nM CT, washed, and incubated in fresh medium at 37°C for the indicated times. The cells were then assayed for surface CT (●) and ¹²⁵I-CT binding (○) at 4°C as described under Materials and Methods. Naive cells incubated with 10 nM ¹²⁵I-CT in the presence and absence of 0.2 μM unlabeled CT bound 86,000 and 1,690 cpm, respectively.

difference in the rate of disappearance of the two components from the cell surface. NB cells were incubated with CT or the purified B component (cholera toxin) at 4°C for 30 min, washed, and incubated for different times at 37°C. The cells were then incubated with anti-CT and assayed for protein A binding. There appeared to be little, if any, difference in the rate of loss of surface B compared with that of CT. The decrease in protein A binding after 60, 120, and 210 min was 43, 64, and 87%, respectively, for CT-treated cells and 42, 62, and 85%, respectively, for B-treated cells.

These results suggested that the holotoxin-receptor complex was being internalized. The possibility that the CT receptors were being recycled was examined. CT-treated NB cells were incubated at 37°C for different times and assayed for surface CT and ¹²⁵I-CT binding (Fig. 5). Because the amount of CT to which the cells were initially exposed was close to saturating (see Fig. 1a), <13% of the surface receptors were still accessible to ¹²⁵I-CT. Although the amount of surface CT decreased steadily over the course of the 6-h incubation to 15% of the initial value, there was only a small increase in the amount of ¹²⁵I-CT binding.

DISCUSSION

A specific and quantitative assay was developed for the detection of CT on the cell surface based on its ability to bind anticholera antibodies which in turn can bind protein A through their Fc region. In the absence of CT and/or anti-CT, there was negligible binding of ¹²⁵I-protein A to the cells. Binding was proportional to the amount of CT bound. In addition, rat glioma C6 cells, which bind only trace amounts of CT, bound very little protein A; by treating the cells with GM₁, both CT binding and protein A binding were increased.

Using this assay, CT was observed to disappear from the surface of NB cells with a half-life of 2 h or less. As there was only a negligible amount of CT detected in the culture medium (as measured by TCA-precipitated label from ¹²⁵I-CT-treated cells), this loss can only be explained by internalization of the toxin or a change in the surface-bound toxin that renders its antigenic sites inaccessible to the anti-CT. Since CT (as measured with ¹²⁵I-CT) is eventually degraded by the cells, it would appear that CT is internalized. This conclusion is supported by the electron microscope studies described below.

Both CT and its B component appeared to be internalized by NB cells at the same rate. In addition, there appeared to be no recycling of toxin receptors, as NB cells treated with CT were unable to bind much ¹²⁵I-CT after most of the surface CT had disappeared. These results suggest that the holotoxin-receptor complex is being internalized. When neuroblastoma N2a cells were incubated with ³H-GM₁ followed by horseradish peroxidase-conjugated cholera toxin and analyzed by electron microscope autoradiography and cytochemistry, both labels underwent partial endocytosis at 37°C with time. There was a similar distribution of both labels on the plasma membrane and internal sites corresponding to the Golgi-associated vesicles and lysosomes (N. K. Gonatas, A. Stieber, J. Gonatas, T. Momoi, and P. H. Fishman, manuscript in preparation).

Others, using electron microscopy, have also described the internalization of CT (17, 19, 20). Hansson et al. (17), using horseradish peroxidase coupled to anticholera antibodies, reported that CT remained confined to the cell surface when the temperature was 4° or 18°C. At 37°C, some penetration of CT into the plasma membrane was observed after 30 but not 15 min. The antiserum used in these studies precipitated both

A and B components of CT (17). Using peroxidase conjugated to CT and to B, Joseph et al. (20) observed that both conjugates were internalized by neuroblastoma cells in culture. Again, the process was dependent on time and temperature. Endocytosis of the conjugates was observed within 30 min at 37°C and they were detected in internal organelles corresponding to Golgi apparatus-endoplasmic reticulum-lysosomes (GERL) (20). Whether the holotoxin conjugate contained peroxidase coupled to A or B or both was not determined.

Using ¹²⁵I-CT, we also followed the kinetics of toxin degradation. In both NB and Friend cells, there was no detectable degradation of CT at 37°C until between 45 and 60 min. In both cells, activation of adenylate cyclase by CT is essentially completed by then. Thus, degradation of CT does not appear to be required for its action. This is supported by the studies at different temperatures. At 4°C, both activation and degradation were blocked. At 22°C, however, activation occurred, although at a slower rate than at 37°C; degradation was still effectively inhibited. These observations do not support previous studies that implicated toxin degradation with toxin action. Matuo et al. (22) described the generation from CT of active fragments by proteases in sarcoma cell membranes. These fragments were as small as 1,400 daltons and reported to be capable of activating adenylate cyclase. Others, based on the inhibition of CT action by alkyl amines and other lysosomotropic agents, concluded that endocytosis and lysosomal processing of the toxin was an essentially step for its action on intact cells (18, 21).

There was a significant disappearance of surface CT from NB cells during the lag period before cyclase activation. Both internalization and activation were affected similarly at different temperatures. Since only a small fraction of the bound toxin may be involved in the activation of adenylate cyclase (5, 13), the possibility of a small pool of surface CT following different kinetics or a separate pathway can not be excluded at this time. The results do indicate that there are several discrete steps in the interaction of CT with intact cells. Initially, the toxin binds rapidly to specific receptors on the cell surface. Then, the toxin becomes internalized during which time adenylate cyclase is activated. Finally, the toxin undergoes degradation presumably in the lysosomes.

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