Identification of Diphtheria Toxin Receptor and a Nonproteinous Diphtheria Toxin-binding Molecule in *Vero* Cell Membrane

Eisuke Mekada, Yoshio Okada, and Tsuyoshi Uchida

Institute for Molecular and Cellular Biology, Osaka University, Suita, Osaka 565, Japan

Abstract. Two subStances possessing the ability to bind to diphtheria toxin (DT) were found to be present in a membrane fraction from DT-sensitive *Vero* cells. One of these substances was found on the basis of its ability to bind DT and inhibit its cytotoxic effect. This inhibitory substance competitively inhibited the binding of DT to *Vero* cells. However this inhibitor could not bind to CRM197, the product of a missense mutation in the DT gene, and did not inhibit the binding of CRM197 to *Vero* cells. Moreover, similar levels of the inhibitory activity were observed in membrane fractions from DT-insensitive mouse cells, suggesting the inhibitor is not the DT receptor which is specifically present in DT-sensitive cells. The second DT-binding substance was found in the same *Vero* cell membrane preparation by assaying the binding of ^{125}I-labeled CRM197. Such DT-binding activity could not be observed in membrane preparation from mouse L cells. From competition studies using labeled DT and CRM proteins, we conclude that this binding activity is due to the surface receptor for DT. Treatment of these substances with several enzymes revealed that the inhibitor was sensitive to certain RNases but resistant to proteases, whereas the DT receptor was resistant to RNase but sensitive to proteases. The receptor was solubilized and partially purified by chromatography on CM-Sepharose column. Immunoprecipitation and Western blotting analysis of the partially purified receptor revealed that a 14.5-kD protein is the DT receptor, or at least a component of it.

DIPHTHERIA toxin (DT)[1] is a cytotoxic protein which inhibits cellular protein synthesis (4, 40) in eukaryotes by catalyzing the ADP-ribosylation of EF-2, which results in its inactivation (9, 12). The first step of intoxication by DT is binding of the toxin to a susceptible cell. A specific receptor for DT is believed to be involved in this step (13, 43). Cells from a number of mammals including humans and monkeys are sensitive to DT, but mouse and rat cells are insensitive (29). Several lines of evidence show that the difference in sensitivity to DT between species is primarily determined by the presence or absence of a cell surface receptor (18, 28, 31, 48). However, this receptor has not been isolated. The toxins bound to cell surface are then internalized by endocytosis (33), and the toxins, or at least their A fragments, enter the cytoplasm to exert its effects. Like in the case of Semliki Forest virus (SFV) (11), intravesicular low pH is required for the penetration of the toxin into cytoplasm (6, 17, 20, 27, 36).

Some information has been obtained on the biochemical properties of DT receptor. The treatment of DT-sensitive cells with some proteases or phospholipase C reduces the sensitivity to DT (32), whereas treatment with neuraminidase increases the sensitivity (26). Although these studies

give a clue to the chemical nature of DT receptor, the possibility that extensive alteration of cell surface affecting the sensitivity of cells can not be neglected. Some of the most specific information on the DT receptor was obtained from immunoprecipitation studies, after surface iodination of DT-sensitive cells and addition of DT and anti–DT antibody to the cell lysates. A glycoprotein of 160 kD was shown to be associated with DT binding upon SDS–PAGE (35). However, this protein has not been isolated in biologically active form, thus it has been uncertain whether it has a function in the binding of DT to cells and/or its internalization.

A strategy to isolate the DT receptor has been to explore its binding activity for DT in isolated membrane or using solubilized membrane fractions. It has been shown that there is a correlation between the amount of the association of labeled toxin and the sensitivity of the cells to the toxin (28, 31). However, the specific binding of labeled DT to isolated membrane has not been observed as yet. When binding studies were performed with labeled toxin and an isolated membrane fraction (2) or with intact cells at relative high toxin concentrations (16), a significant association of toxin with DT-insensitive cells was observed, and the amount of toxin associated with cells was similar for DT-insensitive and DT-sensitive cells. These results have led to the interpretation that DT-insensitive cells also bear DT receptors.

In this paper we describe two DT-binding molecules that

---

1. *Abbreviations used in this paper*: CRMs, cross reacting materials related to diphtheria toxin; DT, diphtheria toxin.

are present in isolated *Vero* cell membranes. One of these substances is referred to as inhibitor, because it inhibited the cytotoxic effects of DT and it is found on both DT-sensitive cells and DT-insensitive cells. The other substance, which is the DT receptor, was found to bind to both DT and CRM197 with high affinity and is present only on DT-sensitive cells. Using the CRM197 it was possible to identify the DT receptor as a single protein band after SDS-PAGE.

Materials and Methods

Enzymes

RNase A from bovine pancreas, RNase T1 from Aspergillus, DNase I from bovine pancreas, DNase II from bovine spleen and α-chymotrypsin from bovine pancreas were purchased from Sigma Chemical Co. (St. Louis, MO). Nuclease P1 from *Penicillium citrinum* was obtained from Yamasa Shoyu Co. (Choshi, Japan). RNase T2 from *Aspergillus orizae* and RNase U2 from *Ustilego sphaerogem* were obtained from Sankyo Co. (Tokyo, Japan). Trypsin (TPCK-treated) was from Worthington Biochemical Corp. (Freehold, NJ).

Buffers

PBS (150 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.2); Buffer A (130 mM NaCl, 50 mM Hepes, 10 mM KCl, 0.5 mM CaCl$_2$, 1 mM NaN$_3$, 1 mg/ml BSA, pH 7.0); Buffer B (10 mM phosphate buffer, 30 mM β-D-octylglucoside, pH 7.2); Buffer C (150 mM NaCl, 10 mM phosphate buffer, 20 μg/ml antipain, 20 μg/ml leupeptin, 10 μg/ml chymostatin, pH 7.2); Buffer D (130 mM NaCl, 50 mM MES [2-(N-morpholino)ethane-sulfonic acid] 10 mM KCl, 0.5 mM CaCl$_2$, 1 mM NaN$_3$, 1 mg/ml BSA, pH 6.1).

Assays of Protein Content and RNA Content

The protein contents of membranes were determined by microbiuret method (14) with BSA as standard. The protein contents of samples containing octylglucoside were measured by the method described previously (38). The RNA contents of membranes were determined by orcinol reaction (24) as described previously (39). The RNA content of samples containing octylglucoside was determined after removing the detergent by ethanol precipitation.

DT and Related Proteins

DT, CRM45, CRM176, CRM197, and CRM228 were produced as described previously (44). The nicked form of CRM197 was prepared by treatment with trypsin (7). Fragment A of diphtheria toxin was purified from the culture fluid of the C7(β-22) strain (45).

Preparation of Membranes from Cultured Cells

Vero cells and other cultured cells were grown on plastic dishes (150-mm wide), collected with rubber policemen, and stored at −80°C until use. Ehrlich ascites tumor cells were grown in and harvested from mouse abdomen. The membrane fractions were obtained by an alkali-extraction method essentially as described by Thom (42). In our study, cells were extracted with 20 mM borate buffer, pH 10.2, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF). The plasma membrane-rich pellet were resuspended and repelleted twice in Buffer C.

Inhibition of the Cytotoxicity of DT

Cytotoxicity of DT was measured by assaying the rate of protein synthesis in cultured cells as described previously (27). The inhibitory activity, [I], was calculated by the following formula,

$$[I] = \frac{(Ci - Ct)}{(Co - Ct)} \times 100,$$

where *Co* is the radioactivity incorporated by cells without toxin without the inhibitor, *Ct* is the count incorporated by cells with toxin without the inhibitor, and *Ci* is the count incorporated by cells with both toxin and the inhibitor. We defined one unit of inhibitory activity as the amount which

gives an inhibitory activity of 50 under the assay conditions described above. The inhibitory activity of samples containing octylglucoside was measured after removing the detergent by dialysis.

Effect of the Inhibitor on the Binding of DT or CRMs to Vero Cells

Indirect assay of binding of DT or CRMs was carried out using ^{125}I-labeled anti-DT monoclonal antibody as described previously (25).

Solubilization of the Inhibitor from Vero Cell Membrane and Chromatography on DEAE-Cellulose

Vero cell membrane suspended in buffer C (15 ml containing 60 mg protein) was mixed with 15 ml of 10 mM phosphate buffer, pH 7.2, and with 1.2 ml of 1 M octylglucoside, then the mixture was put on ice for 30 min, followed by centrifugation at 80,000 g for 60 min. The supernatant was applied to a DE52 (Whatman Inc., Clifton, NJ) column. The column was washed with Buffer B containing 0.2 M NaCl, and then eluted with a linear gradient of NaCl (0.2–0.7 M) in Buffer B. The inhibitory activity of each fraction was assayed after dialysis.

Treatment of the Inhibitor with Enzymes

For treatment with nucleases, the inhibitor purified by DE52 chromatography (10 U of the inhibitory activity) was dialyzed against PBS, then incubated at 37°C for 1 h with each enzyme in the presence of 1 mg/ml BSA. For treatment with RNase U2 and DNase II, the enzyme reaction was carried out in the presence of 50 mM acetate buffer, pH 5.0. For treatment with protease, the dialyzed inhibitor and each enzyme were incubated at 37°C for 60 min, and the reaction was stopped by addition of 1 mM PMSF, 50 U/ml aprotinin and 2 mg/ml BSA.

### Binding of [^{125}I]CRM197 to Isolated Membranes

Nicked CRM197 was labeled with Na^{125}I using Enzymobeads (Bio-Rad Laboratories, Richmond, CA) as described (25). Membrane fractions were prepared from *Vero* cells or L cells by the alkali-extraction method. Membranes were washed twice and resuspended with Buffer D by repeated aspiration through a 26-gauge needle. The binding reaction was carried out in a 200 μl reaction volume (0.5–1 mg of protein) with 2–20 ng of [^{125}I]CRM197 at 24°C for 4 h unless otherwise stated, with gentle shaking. Then the mixture was rapidly filtered on Millipore GVWP filters, and each filter was washed with 10 ml of cold Buffer D. The amount of radioactivity retained on each filter was counted in a γ-counter. Nonspecific binding was assessed in the presence of a 1,000-fold excess of unlabeled CRM197.

Solubilization of CRM197-binding Activity from Isolated Membrane and a Column Assay

Vero cell membranes were suspended with Buffer A at a protein concentration of 10 mg/ml, and solubilized by addition of 1 M octylglucoside to a final concentration of 60 mM. The mixture was incubated for 30 min on ice and then centrifuged at 80,000 g for 30 min to remove insoluble material. The supernatant (100 μl) was incubated with 3 μl of [^{125}I]CRM197 (15 ng, 1.5–2 × 10^7 cpm/μg) at 4°C for 6–14 h. An aliquot (70 μl) of the mixture was applied to a Sephadex G-150 column (190 × 7 mm), eluted with Buffer A containing 15 mM octylglucoside, and 110-μl fractions were collected. The radioactivity in 50 μl of each fraction was determined with a γ-counter. The amount of nonspecific binding was determined by the addition of 1,300-fold excess unlabeled CRM197. To determine total binding and nonspecific binding under the same conditions, we carried out the experiments at the same time using two columns of the same size.

Dot Blot Assay of CRM197-binding Activity

Each fraction of solubilized *Vero* cell membrane eluted from CM-Sepharose was diluted to 10% with buffer containing 0.1 M Tris and 0.19 M glycine, pH 9.0, and blotted onto nitrocellulose filters. Two identical blot sets were set. The filters were soaked in blocking solution (0.15 M NaCl, 10 mM phosphate buffer, 10 mg/ml BSA, 4.5 mg/ml fish gelatin, 5 mg/ml lysozyme, pH 7.2) at room temperature overnight, and then in Buffer D containing 0.05% Tween-20 at 37°C for 1 h. One of the filters was treated with 100 ng/ml of [^{125}I]CRM197 in Buffer D containing 0.5% Tween-20, while the other filter was treated with the same amount of [^{125}I]CRM197 and
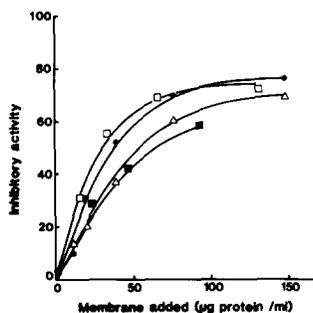
Figure 1. Inhibition of DT-cytotoxicity by membrane fractions of DT-sensitive and DT-insensitive cells. FL cells were incubated for 2 h at 37°C with DT (40 ng/ml) and various amounts of membrane fractions from cultured cells. Then the cells were labeled with [³H]leucine for 1 h at 37°C, and the rate of protein synthesis was measured. The inhibitory activity was calculated as described in Materials and Methods. The control values of protein synthesis with DT and without DT were 1,200 and 12,500 cpm, respectively. The membrane fractions with 1 mg protein from *Vero* cells, L cells, HeLa cells and Ehrlich's ascites tumor cells contained 16.6 µg, 12.0 µg, 29.0 µg and 10.6 µg of RNA, respectively. (●) *Vero* cell membrane; (□) HeLa cell membrane; (△) L cell membrane; (■) Ehrlich's ascites tumor cell membrane.

100 µg/ml of unlabeled CRM197 in the same buffer. After incubation at 24°C for 5 h, the filters were washed five times with buffer containing 0.3 M NaCl, 10 mM MES, 0.05% Tween-20, pH 6.2. The filters were dried and autoradiographed using Fuji X-ray film.

Radioiodination and Immunoprecipitation of DT Receptor

A fraction (200 µl) of DT receptor eluted from CM-Sepharose was radioiodinated by 0.5 mCi Bolton-Hunter reagent with 50 mM borate buffer, pH 9.1. The labeled sample (1.1 × 10⁶ cpm) was incubated at 4°C for 14 h with DT or one of the CRM proteins at the concentration indicated dissolved in immunoprecipitation buffer (0.3 M NaCl, 50 mM MES, 10 mM KCl, 0.5 mM CaCl₂, 1 mg/ml BSA, 1 mg/ml ovalbumin, 1 mg/ml lysozyme, 30 mM octylglucoside, 0.01% SDS, 0.5 mM PMSF, pH 6.1). Followed by the addition of horse anti-DT antibody conjugated with CNBr-activated Sepharose beads (20 µl). The mixture was incubated at 24°C for 5 h with gentle shaking. The Sepharose beads were pelleted, washed with buffer: 0.3 M NaCl, 10 mM phosphate buffer, 30 mM octylglucoside, 0.01% SDS, pH 6.1. The radioactivity of the pellet was counted by a γ-counter. For analysis by SDS-PAGE, the pellet was suspended with 50 µl of SDS gel sample buffer, boiled for 5 min. The supernatant was subjected by SDS-PAGE under reducing or nonreducing condition as described (19). After electrophoresis the gels were fixed, dried and autoradiographed.

Western Blot Analysis of DT Receptor

The DT receptor fraction from CM-Sepharose was concentrated ~10 times of the original volume and the buffer was replaced to SDS-gel sample buffer using a Molcut concentrator (Millipore, Bedford, MA). After incubation at 37°C for 3 h, two sets of protein samples were run on 15% SDS-PAGE and then transferred to Durapore filters (type GVHP, Millipore) using a electroblotting equipment. After treatment with the blocking solution, one of the filters was treated with [¹²⁵I]CRM197 and the other with [¹²⁵I]CRM-197 plus 1,000 times excess unlabeled CRM197 according to the same conditions as described in dot blot assay. The filters were washed, dried, and autoradiographed.

Results

Cell Membranes Contain an Inhibitor of DT Cytotoxicity

The *Vero* cell line is highly sensitive to DT (29). We found that a membrane fraction isolated from *Vero* cells inhibited the cytotoxicity of DT. Addition of DT at 40 ng/ml to FL cell cultures reduced protein synthesis to ~10% of the value in control cultures. When the membrane fraction was added with DT to FL cell cultures, the rate of cellular protein syn-

thesis was increased in a dose-dependent manner, indicating that the cytotoxicity of DT was blocked (Fig. 1). The membrane fraction per se had no effect on cellular protein synthesis. The inhibitory activity of the membrane was also observed when *Vero* cells were used in cytotoxicity assay, but ~twofold higher amounts of membrane were required for a similar inhibitory effect. Thus, we used FL cells in the cytotoxicity assay of DT in the following studies.

We next tested whether membrane fractions isolated from other cell lines with different sensitivities to DT show inhibition of the cytotoxicity. HeLa cells are ~100 times and mouse L cells ~10⁵ times less sensitive than *Vero* cells (28). Ehrlich ascites tumor cells are more resistant than L cells (15). Fig. 1 shows that membrane fractions from all these cell lines inhibited the cytotoxicity of DT, indicating the inhibitor was present both in DT-sensitive and in DT-insensitive cell lines.

Release of the Inhibitor from Isolated Membrane and Chromatography on a DEAE-Ion Exchange Column

The membrane fraction of *Vero* cells was treated with various concentrations of octylglucoside, and the inhibitory activity recovered in the supernatant after centrifugation was determined. The inhibitory activity was partially released with octylglucoside at a concentration 15 mM, and maximal release was found at concentrations greater than 30 mM.

The solubilized inhibitor was chromatographed on a DEAE-cellulose ionexchange column in the presence of 30 mM octylglucoside. Inhibitory activity was observed in the fractions with NaCl concentrations between 300 and 500 mM. The fractions with inhibitory activity were mixed and used for further characterization. This inhibitor fraction with one unit of inhibitory activity contained 0.38 µg protein and 1.84 µg RNA.

DT Inhibitor Binds to DT but not to CRM197

We examined whether the inhibitor could bind to DT. The inhibitor was incubated with Sepharose beads conjugated with DT or BSA. The gels were washed and the inhibitor bound to gels was eluted with 4 M KSCN. When the inhibitor was incubated with DT-beads, ~95% of the initial inhibitory activity was bound to the beads. With BSA-beads only a trace amount was adsorbed to the beads (Table I).

Next we determined the region of DT responsible for the binding of the inhibitor. To do this, we used DT fragment A and CRMs. CRM45 is a premature termination protein of DT containing enzymatically active fragment A and about half of the B fragment (43). As shown in Table I, the inhibitor was not bound to beads conjugated with fragment A or with CRM45, indicating that at least the COOH-terminal 15-kd region of DT is required for binding of the inhibitor. CRM197 is a product of a missense mutation in the DT gene (44) and differs from wild type toxin only in one amino acid residue in fragment A (8). CRM197 is known to bind to DT-sensitive cells with an affinity similar to or higher than that of native toxin (25). Interestingly, the inhibitor was not adsorbed with CRM197-beads. Therefore, not only fragment B but also fragment A is required for the binding of the inhibitor.

Effect of the Inhibitor on the Binding of DT and CRMs to Vero Cells

The effects of the inhibitor on the binding of DT and CRM

Table I. Adsorption of the Inhibitor onto Sepharose beads Conjugated with DT or Related Proteins

Protein conjugated to beads	Percentage of total inhibitory activity recovered	
	Not retained by gel	Retained by gel
DT	4.9	95.1
BSA	99.5	0.5
DT	1.0	99.0
CRM45	96.8	3.2
Fragment A	96.4	3.6
DT	11.6	88.4
CRM197	90.1	9.9

DT inhibitor purified using a DEAE-cellulose column (1 ml, 20 U of the inhibitory activity) was incubated with Sepharose beads conjugated with DT or the related proteins for 2.5 h at 37°C. After washing with Buffer B containing 0.3 M NaCl, the inhibitor bound to gels was eluted with 4 M KSCN. After dialysis, samples of Sepharose-unbound fraction and Sepharose-bound fraction were serially diluted, and the inhibitory activity in each diluted sample was determined.

proteins to *Vero* cells were examined by an indirect binding assay using a ^{125}I-labeled monoclonal antibody against DT (25). This antibody, referred to as No. 2, binds to DT but does not inhibit the binding of DT to cells (10). As shown in Fig. 2, the inhibitor blocked the binding of DT to *Vero* cells but not the binding of CRM197. This was also confirmed by a direct binding assay using [125I]DT or [125I]CRM197 (data not shown). Moreover, the indirect binding assay showed that the inhibitor blocked the binding of CRM176 and CRM228 to the cells (Fig. 2). It is known that several nucleotides containing ATP bind to DT (1, 5, 21) and inhibit the binding of DT to cells (31, 34). However, ATP does not bind to CRM45 or CRM197 (22). We have shown (25) that ATP inhibited the binding of CRM176 and CRM228 to *Vero* cells, but not the binding of CRM197. The effects of the inhibitor on binding of DT and CRMs to *Vero* cells are very similar to those of ATP.

Inhibitory Activity Is Destroyed by RNase

To investigate the chemical properties of the inhibitor, we treated the inhibitor with various enzymes, and measured the remaining inhibitory activity. The similarity between the effects of the inhibitor and those of ATP or other nucleotides on the binding of DT and CRMs suggested that the inhibitor might have a nucleotide-like structure. Therefore, we treated the inhibitor with several RNases and the remaining inhibitory activity was measured. As no suitable inhibitor for RNase was available, we also carried out control experiments for each enzyme in which the same amount of enzyme

Figure 2. Effect of DT inhibitor on the binding of DT or CRMs to *Vero* cells measured by an indirect binding assay using ^{125}I-anti-DT antibody. ^{125}I-Antibody was incubated with DT or CRMs. The mixture and various amounts of DT inhibitor were added to *Vero* cells, and the cells were incubated for 90 min at 37°C. Then specific cell-associated radioactivity was measured. (●) DT; (▲) CRM197; (■) CRM176; (□) CRM228.
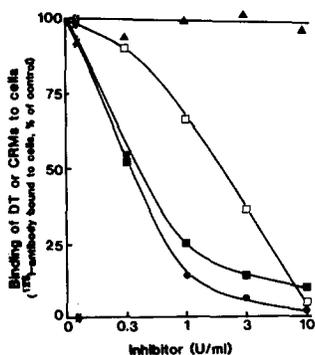
alone was added to FL cell cultures in the DT cytotoxicity assay. The results are shown in Table II. The inhibitory activity was not affected by RNase T1, but was inactivated by RNase T2, RNase U2 or nuclease P1. RNase A was slightly effective. DNase I, II and *Escherichia coli* alkaline phosphatase had no effect (data not shown). None of these enzymes alone influenced either cellular protein synthesis per se or the sensitivity of FL cells to DT. These results indicate that the inhibitor contains a poly-ribonucleotide structure that is required for inhibitory activity. The inhibitor was also treated with trypsin or chymotrypsin, but these enzymes did not affect the inhibitory activity (Table II).

As shown in Fig. 2, addition of the inhibitor at 0.3 U/ml (containing RNA at 0.55 µg/ml) shows 50% inhibition on the binding of DT. ATP was required at ~165 µg/ml for the same effect (25). Therefore, although the effects of the inhibitor and those of ATP on the binding of DT and CRMs are very similar, the inhibitor described here is much more effective than ATP.

[125I]CRM197 Binds to Vero Cell Membrane but not to L Cell Membrane

The inhibitor was present even in the membrane fractions of toxin-insensitive cells. Moreover, although CRM197 binds to *Vero* cells with an affinity similar to, or greater than that of DT (25), the inhibitor did not bind to CRM197 and did not inhibit the binding of CRM197 to *Vero* cells. These facts suggest that the inhibitor is not the receptor responsible for the difference in sensitivity to DT between species, and that another DT-binding substance would be present in the membrane of DT-sensitive cells. If the membrane fraction from *Vero* cells contains two DT-binding substances, the inhibitor and the receptor, the inhibitor could prevent the binding of DT to DT receptor when the binding assay is performed using the membrane fraction. CRM197 did not bind the inhibitor, so the effect of the inhibitor on the binding of CRM197 to the DT receptor would be minimal. Thus, we used CRM197 to look for a DT receptor in *Vero* cell membrane preparations.

Vero cell membrane was incubated with [125I]CRM197 and then the radioactivity associated with the membrane was measured as described under Materials and Methods. As shown in Fig. 3, saturable specific binding of [125I]CRM197 was observed. Specific binding was increased with time and

514

Table II. Effects of Treatment of Inhibitor with Various Enzymes

Experiment	Enzyme	Concentration	Protein synthesis, percentage of control		
			−Toxin −inhibitor	+Toxin −inhibitor	+Toxin +inhibitor
1	No enzyme		100	11.9	89.1
	RNase T1	19 U/ml	104	10.6	82.1
	RNase A	30 μg/ml	111	16.2	63.4
	RNA T2	5 U/ml	114	10.2	19.9
	Nuclease P1	20 μg/ml	108	8.5	23.6
2	No enzyme		100	19.4	78.9
	RNase T2	0.1 U/ml	ND	ND	50.0
		1 U/ml	ND	ND	28.1
		10 U/ml	ND	19.4	16.1
3	No enzyme		100	9.6	66.0
	RNase U2	82 U/ml	ND	10.0	12.8
4	No enzyme		100	15.6	84.6
	Trypsin	100 μg/ml	ND	16.1	79.8
	chymotrypsin	100 μg/ml	ND	16.3	71.6

DT inhibitor purified by DEAE-cellulose column was dialyzed against PBS. The detergent-free inhibitor (100 μl, 10 units of the inhibitory activity) was incubated with each of the enzymes at the concentration indicated for 1 h at 37°C. Remaining inhibitory activity was determined by the addition of 30 μl of enzyme-treated sample and DT to FL cell cultures. To measure the effect of the enzymes alone on cellular protein synthesis and sensitivity to DT, the equivalent concentration of the enzyme was added in the absence of the inhibitor. ND, not done.

reached a maximum in 4 h. Analysis by Scatchard plots (37) indicates a single class of binding sites with a K_a value of 2.1×10^9 liter/mol (Fig. 3 B). This value is consistent with the value determined by us in intact Vero cells (25).

We also examined the binding of [^{125}I]CRM197 to mouse L cell membrane. Over the range of CRM197 concentration used for Vero cells, no specific binding was observed (data not shown). Thus, the sites specific for CRM197 binding are either not present on DT-insensitive L cells, or exist in very low density to be detected in our binding assay. These results clearly show that the molecule which binds CRM197 differs

from the inhibitor, because the inhibitor exists on L cell membrane in densities similar to those on Vero cell membrane.

To examine whether the observed binding of [^{125}I]CRM-197 to Vero cell membrane is truly specific for DT, we performed a competition assay using unlabeled DT or CRMs. As shown in Fig. 4, the binding of [^{125}I]CRM197 was most strongly inhibited by nicked CRM197. Native DT was about three times less potent and CRM228 was ~200 times less potent than nicked CRM197. CRM45 did not inhibit the binding of labeled CRM197 (data not shown). These results strongly suggest that the binding sites for CRM197 observed in membrane fractions were DT receptors.

Binding of [^{125}I]CRM197 to Solubilized Cell Membrane Components

We next examined the binding of [^{125}I]CRM197 to solubilized membrane components using a gel filtration method.
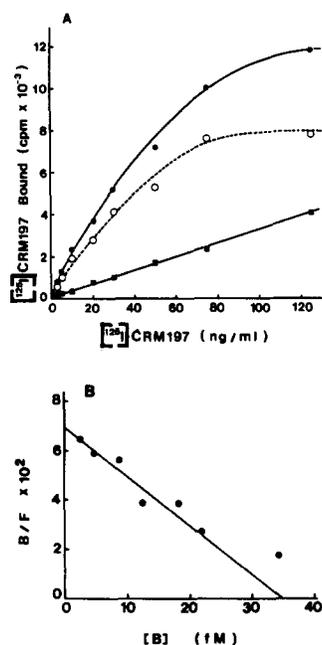

Figure 3. Binding of various concentrations of [^{125}I]CRM197 to Vero cell membrane. (A) Vero cell membrane (200 μl containing 125 μg protein) was incubated at 24°C for 4 h with various concentrations of [^{125}I]CRM197 (2.5 × 10^7 cpm/ μg). Then 150 μl of the mixture was removed and the radioactivity associated with the membranes determined. (●) [^{125}I]CRM197; (■) [^{125}I]CRM-197 + unlabeled CRM197; (○) difference between ● and ■. (B) Scatchard plot of the specific binding of [^{125}I]CRM-197 to Vero cell membrane. The lines were fitted by regression analysis.
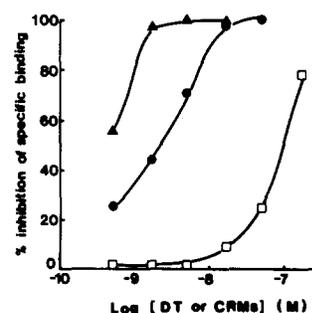

Figure 4. Competition for binding of [^{125}I]CRM197 to Vero cell membranes by unlabeled DT or unlabeled CRMs. Vero cell membranes (200 μl containing 100 μg protein) was incubated at 24°C for 4 h with [^{125}I]CRM197 at 30 ng/ml (2.5 × 10^7 cpm/μg) and various concentrations of one of the unlabeled protein. The specific radioactivity associated with Vero cell membranes was measured. The data are expressed as a percentage of the inhibition of the specific binding, which was 6,000 cpm. (▲) Nicked CRM197; (●) nicked DT; (□) CRM228.
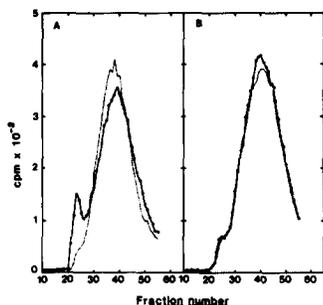
Figure 5. Column assay for the binding of [^{125}I]CRM197 to solubilized membrane components. [^{125}I]CRM197 was incubated with solubilized material from *Vero* cell membrane (A) or L cell membrane (B) for 6 h at 4°C. The mixture was applied to a Sephadex G-150 column. The radioactivity of each fraction was determined. The broken line is the radioactivity obtained by incubation with [^{125}I]CRM197 and excess unlabeled CRM197.

Figure 6. Dot-blot analysis of CRM197-binding activity in CM-Sepharose fractions. The solubilized *Vero* cell membrane materials (300 mg protein) were applied to CM-Sepharose column (1.1 × 8 cm). The proteins were eluted with a linear gradient of NaCl (0.2–1 M) in 10 mM phosphate buffer, pH 7.0, containing 0.5 mM CaCl$_2$, 30 mM octylglucoside. Each fraction was spotted on nitrocellulose filters. The filter was probed with 100 ng/ml [^{125}I]-CRM197 (A), or 100 ng/ml [^{125}I]CRM197 plus 100 µg/ml unlabeled CRM197 (B). The specific CRM197-binding activity was seen in fractions 6 to 9.
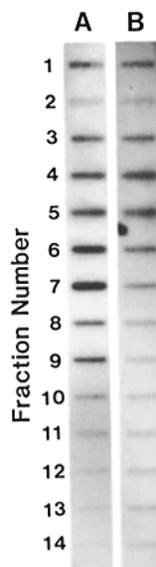
Solubilized material from *Vero* cell membrane treated with 60 mM octylglucoside was incubated with [^{125}I]CRM197. The mixture was applied to a Sephadex G-150 column in the presence of detergent, and the radioactivity of each fraction was counted. The results are shown in Fig. 5 a. Two radioactive peaks were observed: the first at fraction No. 23 and the second at fraction No. 39. When excess unlabeled CRM197 was added with [^{125}I]CRM197, the first peak did not appear (Fig. 5, *broken line*). When [^{125}I]CRM197 was applied to the column in the absence of membrane components, the first peak did not appear (data not shown). We carried out the same experiment on solubilized material from L cell membranes. Although a tiny peak appeared around fraction No. 25, significant differences were not observed when the membrane material was incubated with [^{125}I]CRM197 only or with [^{125}I]CRM197 and excess unlabeled CRM197 (Fig. 5 b). Therefore, we concluded that the first peak seen in the *Vero* cell experiments was [^{125}I]CRM197 bound to the receptor and the second was unbound CRM197. When *Vero* cell membranes were treated with 30 mM octylglucoside, the receptor activity in soluble fraction was less. This is additional evidence that the inhibitor and the receptor are different substances because the inhibitor was released fully by treatment of membranes with 30 mM octylglucoside. This observation also indicates that the DT receptor can bind to CRM197 in the presence of detergent.

Treatment of the Receptor with Trypsin or RNase T2

As shown above, the inhibitory activity was destroyed by some RNases, but not by protease. To compare the CRM197-binding substance, that is the receptor, with the inhibitor, we tested the effects of trypsin and RNase T2 on the CRM197-binding activity using the column assay. The results showed that trypsin destroyed the CRM197 binding activity, but RNase T2 had no effect. This is a striking difference from the case of the inhibitor, and shows that the DT receptor has a proteinaceous structure.

Partial Purification of DT Receptor

The solubilized *Vero* cell membrane material was chromatographed on a ion-exchange column. When the membrane material was applied on a DEAE-cellulose column under conditions similar to that for the purification of DT inhibitor, CRM197-binding activity was detected in the flow-through fraction. On a CM-Sepharose column, CRM197-

binding activity was retained on the column and found to be eluted with 0.4–0.6 M NaCl (Fig. 6). For this sample dot-blot assay was performed for the measurement of CRM197-binding activity. The specific CRM197-binding activity was seen in fractions 6–9, corresponding to eluates with 0.4–0.6 M NaCl. These fractions were mixed and used for further studies.

Protection of DT Cytotoxicity by DT Receptor

The protective effect of soluble DT receptor was tested by addition of the excess amounts of receptor to a cell and DT mixture. We tested this using the CM-Sepharose fraction of DT receptor, because this fraction did not contain the inhibitor. As shown in Table III, DT receptor fraction protected the toxicity of DT.

Identification of DT Receptor

The CM-Sepharose fraction containing DT receptor was radioiodinated and immunoprecipitated using either DT or other CRM proteins and immobilized anti-DT antibody. When the iodinated material was incubated with CRM197

Table III. Protection of DT Cytotoxicity by DT Receptor

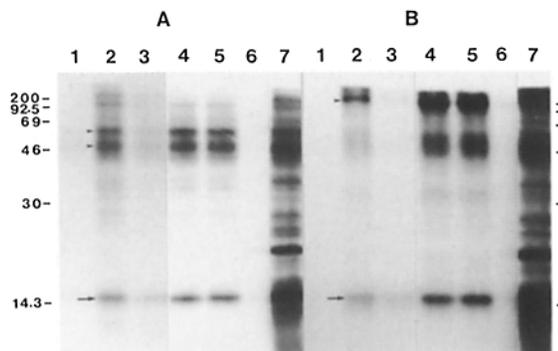| | The rate of protein synthesis (Percentage of control) | | |
| | Receptor sample added | | |
Experiments	0	100 µl	250 µl
1	9.5	28	49
2	38	63	83

Partially purified DT receptor by CM-Sepharose was mixed with 1 mg/ml BSA and the mixture was dialyzed against leucine-diminished MEM. 500 µl of MEM containing 10% leucine and 1 mg/ml BSA and the indicated volume of dialyzed receptor fraction was incubated with DT (1 ng/ml) at 37°C for 30 min. The mixtures were added to *Vero* cell cultures and incubated at 37°C for 2 h, followed by incubation with 2 µCi/ml [^{3}H]leucine at 37°C for 1 h. The radioactivity incorporated in proteins was determined and the rate of protein synthesis was expressed as a percentage of that in cultures containing same amounts of the receptor without DT.
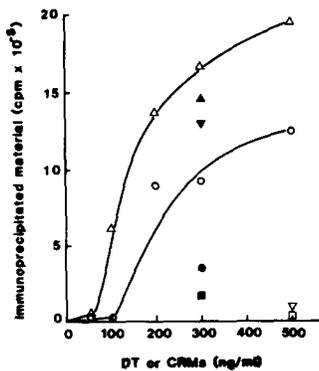
Figure 7. DT-specific precipitation of radioiodinated membrane proteins. The radioiodinated material containing DT receptor was incubated with DT or one of the CRM proteins at the concentrations indicated, and then further incubated with anti–DT antibody conjugated with Sepharose gels. In some samples, ATP (2 mM) or the inhibitor isolated here (15 U/ml) was added with DT and CRM197. The radioactivity associated to gels was counted. The results are expressed as a percentage of total radioactivity added. The symbols indicate: CRM197 (△); DT (○): CRM45 (▽); fragment A (□): CRM197 with ATP (▲); DT with ATP (●); CRM197 with the inhibitor (▼) and DT with the inhibitor (■).

and anti–DT antibody, the radioactive material was precipitated by CRM197 in a dose-dependent manner (Fig. 7). A similar result was obtained using DT and anti–DT antibody, but the precipitate of the radioactive materials was lower than that in the case of CRM197. With CRM45 or with fragment A of DT no significant precipitation was observed even at a concentration of 500 ng/ml. To test whether the immunoprecipitation is properly specific for DT receptor, we used ATP or DT inhibitor. The addition of DT inhibitor (15 U/ml) strongly decrease the specific precipitation by DT, while the precipitation by CRM197 was slightly affected with the inhibitor. ATP also showed a similar result. These findings strongly suggest that the precipitation is DT receptor specific.

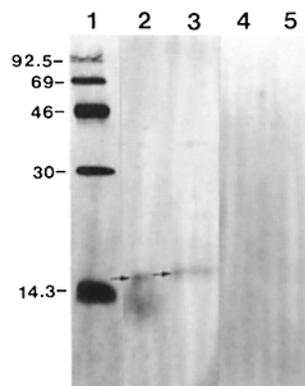The immunoprecipitated materials were analyzed by SDS–PAGE. In the samples precipitated with DT or

CRM197 and anti–DT antibody, three major bands with molecular masses of 14.5, 47, and 62 kD and several minor bands are seen in a reducing condition (Fig. 8 *A*, lanes *2* and *4*, respectively). When neither DT nor CRM197 was added (lane *1*), or DT and a nonspecific antibody was added (lane *6*), such major three bands were not seen. By the addition of ATP in the precipitation procedure with DT, the three major bands and some of the minor bands were greatly diminished (lane *3*). However, ATP did not affect the precipitation with CRM197 (lane *5*). Similar results were obtained in the case of DT inhibitor. Under nonreducing conditions, a band with more than 100 kD appeared, the density of the 47-kD and the 62-kD bands was diminished. The mobility of 14.5-kD protein did not change under nonreducing conditions.

Although the results of immunoprecipitation study suggest that the three major proteins seen on SDS–PAGE are related to DT receptor, there is the possibility that the receptor co-precipitated with contaminating cellular materials. As addition of ATP or the inhibitor caused global reduction of the immunoprecipitates with DT, we could not identify specific protein band(s) responsible for DT binding. To identify which molecule on SDS-PAGE has the property to bind to DT, we carried out a Western blot analysis of the receptor fraction eluted from CM-Sepharose using [^{125}I]CRM197 as probe. As shown in Fig. 9, only a single band with a molecular mass of 14.5 kD was observed. This band was identical to the 14.5-kD band seen in immunoprecipitation study of Fig. 8, judged by its mobility on an SDS gel. When the probe contained an excess amount of unlabeled CRM197, no significant bands were observed. Thus we concluded that this 14.5-kD band is the DT receptor or at least a component of it. This finding also suggested that 14.5-kD DT receptor is associated with the 47- and the 62-kD proteins, and these proteins were co-precipitated by the addition of DT or CRM197 and the antibody.

Discussion

We have shown that an inhibitor present in a membrane fraction of cultured cells blocks the cytotoxicity or DT by preventing its binding to target cells. RNase treatment of the inhibitor revealed that the ribonucleotide structure is in-

Figure 8. Immunoprecipitates of the partially purified DT receptor fraction were analyzed by SDS–PAGE. DT receptor fraction was radioiodinated and precipitated with DT or CRM proteins and anti–DT antibody. The precipitated proteins were run on SDS–PAGE (15% gel) in the presence (*A*) or absence (*B*) of dithiothreitol (DTT) and autoradiographed. (Lane *1*) Anti–DT antibody only; (lane *2*) DT (300 ng/ml) and anti–DT antibody; (lane *3*) ATP (2 mM) with DT and anti–DT antibody; (lane *4*) CRM197 (300 ng/ml) and anti–DT antibody; (lane *5*) ATP (2 mM) with CRM197 and anti–DT antibody; (lane *6*) DT and control antibody; (lane *7*) total ^{125}I-labeled material. The 14.5-kD bands are shown by arrows and the 47- and 62-kD bands are shown by arrowheads.

Figure 9. Western blot analysis of DT receptor. The samples of partially purified DT receptor (used 10 μl for lanes *2* and *4*, and 30 μl for lanes *3* and *5*) were run on SDS–PAGE (15% gel) and transferred to two sets of filters. One filter was probed with 100 ng/ml ^{125}I-CRM197 (lanes *2* and *3*), the other with 100 ng/ml ^{125}I-CRM197 in the presence of 100 μg/ml of unlabeled CRM197 (lanes *4* and *5*). The filters were dried and autoradiographed. Lane *1* is molecular markers containing lysozyme (14,300), carbonic anhydrase (30,000), ovalbumin (46,000), BSA (69,000) and phosphorylase b (92,500). The 14.5-kD bands are shown by arrows.

volved in its activity. In fact, it has been reported that some dinucleotides, such as adenyl-(3′,5′)-uridine-3′-monophosphate (ApUp), bind to DT (5). ATP is known to bind to DT (21) and inhibit its cytotoxic activity (30). Thus the inhibitor may inhibit the cytotoxicity of DT by a mechanism similar to that of the above mentioned nucleotides. We suggest that the inhibitor is not a molecule with a specific role in the binding DT to the surface of DT-sensitive cell, the reasons are as follows: (a) the inhibitor was found in DT-insensitive cells, (b) the inhibitor did not bind to CRM197 and (c) the affinity of the inhibitor for DT appeared to be lower than the affinity of DT receptor for DT.

Does the inhibitor exist on the cell surface? The plasma membrane-rich fraction used in this paper contained inner membranes, and RNA from the cytoplasm may associate with inner membranes. To clarify whether the inhibitor comes from the surface of the plasma membrane or from inner membrane components, we treated Ehrlich ascites tumor cells with RNase T2 or RNase U2 and then isolated the membrane fractions. Membranes isolated from such RNase-treated and untreated cells showed similar inhibitory activities for DT-mediated cytotoxicity. Although this finding may indicate that the inhibitory substance mainly exists on inner membranes, it was not clear whether RNases used were effective for the inhibitor on the cell surface of intact cells. Therefore, we could not show any direct evidence on location of the inhibitor. However, Keen et al. (16) observed that fluorescently labeled DT binds to and is internalized into mouse cells when the toxin is added at relatively high concentrations, indicating that binding sites for DT are present on the surface of DT-insensitive cells. Weiss and Mayhew (23, 47) reported that RNA is a structural component of the cell surface membrane based on observations made on the mobility of cells after RNase treatment and electrophoresis. Terasaki et al. (41) using antibody against single-stranded RNA confirmed the existence of RNA on the cell surface. These facts support the existence of the inhibitor on the cell surface. The inhibitor reported in this study may be derived from both the inside and the outside of cells.

The second DT-binding molecule present in the membrane fraction has characteristics of the surface receptor for DT. This molecule actually defines the sensitivity of cells to DT in different species. In fact, (a) this substance is present only in DT-sensitive cells, (b) it binds to CRM197, and its binding affinity is similar to that obtained with intact Vero cells. Furthermore, the results of the competition experiments in which binding of [125I]CRM197 to Vero cell membrane was measured in the presence of DT and various CRMs are consistent with CRM197 binding experiments with intact Vero cells. Therefore, we conclude that this binding activity is due to the DT receptor present in DT-sensitive cells. Specific binding of DT to the receptor has not been reported using isolated membrane or a solubilized membrane fraction. The presence of the inhibitor might have interfered in the binding of DT to its receptor and made the interpretation of such experiments hard. Using 125I-labeled CRM197 we could demonstrate DT receptor in a crude solubilized membrane fraction by a column assay and in a partially purified fraction by a dot blot assay. The immunoprecipitation study and Western blot analysis revealed a protein of 14.5 kD in SDS-PAGE, we think this molecule is the DT receptor or a component of it. Furthermore, it was clear by Western blot analysis

probed with the labeled CRM197 that the 14.5-kD protein is the molecule directly binding DT. This conclusion is supported by other evidence: using cross-linking agents, Cieplak et al. (3) recently suggested that a 10–20-kD cell surface protein(s) is, or constitutes a portion of DT receptor. Is this 14.5-kD protein a functional DT receptor? As DT-DT receptor complexes appeared in the void fraction of the column assay, using Sephadex G-150 column and crude solubilized membranes (Fig. 5), it is possible that the 14.5-kD proteins form aggregates in membrane prior to solubilization. Of course, the possibility that 14.5-kD protein was a processed form of the precursor or a native molecule cannot be ruled out. As shown in Fig. 8, two proteins with molecular weights of 47 and 62 kD precipitated prominently with 14.5 kD protein when separated under reducing conditions. These molecules are seen to form one bigger molecule under nonreducing conditions. These proteins may also be related to DT receptor, but much more extensive studies will be required to elucidate the relationship of these components to the DT receptor.

It is unclear whether the inhibitor plays any role in DT intoxication. However, we think it may. First, the inhibitor may act as a second receptor on DT-sensitive cells. It may serve to draw the DT molecule more closely to lipid bilayer, or the binding of DT to the second receptor may lead to a change in the conformation of the DT molecule to expose a hydrophobic region. The receptor site and inhibitor site on the DT molecule may be close together or overlapped. If the inhibitor is added exogenously, the inhibitor binds and covers the receptor site or causes a conformational change, resulting in inhibition of the binding to the receptor. The second possible role of the inhibitor is in the passage of the toxin from an endocytotic vesicle to the cytoplasm through the lipid bilayer. A RNA-protein complex, called signal recognition particle, is known to be essential for the translocation of newly synthesized proteins to the luminal sides of rough endoplasmic reticulum (46). Like the signal recognition particle the inhibitor may play some role in translocation of macromolecules into the cytoplasm. As CRM197 does not bind to the inhibitor, the function of the inhibitor may be revealed when the fate of CRM197 is analyzed more precisely. Finally, even if the inhibitor has no function in DT entry, the fact that this DT-binding molecule is present in membrane fractions must be taken into account in attempts to isolate the DT receptor. As CRM197 does not bind to the inhibitor, CRM197 may be useful for the purification of the DT receptor.

References

1. Barbieri, J. T., S. F. Carroll, R. J. Collier, and J. A. McCloskey. 1981. An endogenous dinucleotide bound to diphtheria toxin. Adenylyl-(3′,5′)-uridine 3′-monophosphate. J. Biol. Chem. 256:12247–12251.
2. Chang, T., and D. M. Neville Jr. 1978. Demonstration of diphtheria toxin receptor on surface membranes from both toxin-sensitive and toxin-resistant species. J. Biol. Chem. 253:6866–6871.
3. Cieplak, W., H. M. Gaudin, and L. Eidels. 1987. Diphtheria toxin receptor. Identification of specific diphtheria toxin-binding proteins on the surface of Vero and BS-C-1 cells. J. Biol. Chem. 262:13246–13253.
4. Collier, R. J., and A. M. Pappenheimer Jr. 1964. Studies on the mode of action of diphtheria toxin. II. Effect of toxin on amino acid incorporation in cell free systems. J. Exp. Med. 120:1019–1039.
5. Collins, C. M., and R. J. Collier. 1984. Interaction of diphtheria toxin with adenylyl-(3′,5′)-uridine 3′-monophosphate. II. The NAD-binding site and determinants of dinucleotide affinity. J. Biol. Chem. 259:15159–15162.
6. Draper, R. K., and M. I. Simon. 1980. The entry of diphtheria toxin into mammalian cell cytoplasm: evidence for lysosomal involvement. J. Cell

Biol. 87:849–854.

7. Drazin, R., J. Kandel, and R. J. Collier. 1971. Structure and activity of diphtheria toxin. II. Attack by trypsin at a specific site within the intact molecule. *J. Biol. Chem.* 246:1504–1510.

8. Giannini, G., R. Rappuoli, and G. Ratti. 1984. The amino-acid sequence of two non-toxic mutants of diphtheria toxin:CRM45 and CRM197. *Nucl. Acids Res.* 12:4063–4069.

9. Gill, D. M., A. M. Pappenheimer Jr., R. Brown, and J. J. Kurnik. 1969. Studies on the mode of action of diphtheria toxin. VII. Toxin-stimulated hydrolysis of nicotinamide adenine dinucleotide in mammalian cell extracts. *J. Exp. Med.* 129:1–21.

10. Hayakawa, S., T. Uchida, E. Mekada, M. R. Moynihan, and Y. Okada. 1983. Monoclonal antibody against diphtheria toxin. Effect of toxin binding and entry into cells. *J. Biol. Chem.* 258:4311–4317.

11. Helenius, A., J. Kartenbeck, K. Simons, and E. Fries. 1980. On the entry of Semliki Forest virus into BHK-21 cells. *J. Cell Biol.* 84:404–420.

12. Honjo, T., Y. Nishizuka, O. Hayaishi, and I. Kato. 1968. Diphtheria-toxin-dependent adenosine diphosphate ribosylation of aminoacyl transferase II and inhibition of protein synthesis. *J. Biol. Chem.* 243:3553–3555.

13. Ittelson, T. R., and D. M. Gill. 1973. Diphtheria toxin: Specific competition for cell receptors. *Nature (Lond.).* 242:330–332.

14. Itzhaki, R. F., and D. M. Gill. 1964. A micro-biuret method for estimating proteins. *Anal. Biochem.* 9:401.

15. Kaneda, Y., T. Uchida, E. Mekada, M. Nakanishi, and Y. Okada. 1984. Entry of diphtheria toxin into cells: possible existence of cellular factor(s) for entry of diphtheria toxin into cells was studied in somatic cell hybrids and hybrid toxins. *J. Cell Biol.* 98:466–472.

16. Keen, J. H., F. R. Maxfield, M. C. Hardegree, and W. H. Habig. 1982. Receptor-mediated endocytosis of diphtheria toxin by cells in culture. *Proc. Natl. Acad. Sci. USA.* 79:2912–2916.

17. Kim, K., and N. B. Groman. 1965. Inhibition of diphtheria toxin action by ammonium salts and amines. *J. Bacteriol.* 90:1552–1556.

18. Kohno, K., T. Uchida, E. Mekada, and Y. Okada. 1985. Characterization of diphtheria-toxin-resistant mutants lacking receptor function or containing nonribosylatable elongation factor 2. *Somat. Cell Genet.* 11:421–431.

19. Laemmli, U. K. 1971. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

20. Leppla, S. H., R. B. Dorland, and J. L. Middlebrook. 1980. Inhibition of diphtheria toxin degradation and cytotoxic action by chloroquine. *J. Biol. Chem.* 255:2247–2250.

21. Lory, S., and R. J. Collier. 1980. Diphtheria toxin: Nucleotide binding and toxin heterogeneity. *Proc. Natl. Acad. Sci. USA.* 77:267–271.

22. Lory, S., S. F. Carroll, and R. J. Collier. 1980. Ligand interactions of diphtheria toxin. II. Relationships between the NAD site and the P site. *J. Biol. Chem.* 255:12016–12019.

23. Mayhew, E., and L. Weiss. 1968. Ribonucleic acid at the periphery of different cell types, and effect of growth rate on ionogenic groups in the periphery of cultured cells. *Exp. Cell Res.* 50:441–453.

24. Mejbaum, W. 1939. Uber die Bestimmung kleiner Pentosemengen, insbesondere in Derivaten der Adenylsaure. *Z. Physiol. Chem.* 258:117.

25. Mekada, E., and T. Uchida. 1985. Binding properties of diphtheria toxin to cells are altered by mutation in the fragment A domain. *J. Biol. Chem.* 260:12148–12153.

26. Mekada, E., T. Uchida, and Y. Okada. 1979. Modification of the cell surface with neuraminidase increases the sensitivities of cells to diphtheria toxin and *Pseudomonas aeruginosa* exotoxin. *Exp. Cell Res.* 123:137–146.

27. Mekada, E., T. Uchida, and Y. Okada. 1981. Methylamine stimulates the action of ricin toxin but inhibits that of diphtheria toxin. *J. Biol. Chem.* 256:1225–1228.

28. Mekada, E., K. Kohno, M. Ishiura, T. Uchida, and Y. Okada. 1982. Methylamine facilitates demonstration of specific uptake of diphtheria

toxin by CHO cell and toxin-resistant CHO cell mutants. *Biochem. Biophys. Res. Commun.* 109:792–799.

29. Middlebrook, J. L., and R. B. Dorland. 1977. Response of cultured mammalian cells to the exotoxins of *Pseudomonas aeruginosa* and *Corynebacterium diphtheriae*: differential cytotoxicity. *Can. J. Microbiol.* 23:183–189.

30. Middlebrook, J. L., and R. B. Dorland. 1979. Protection of mammalian cells from diphtheria toxin by exogenous nucleotides. *Can. J. Microbiol.* 25:285–290.

31. Middlebrook, J. L., R. B. Dorland, and S. H. Leppla. 1978. Association of diphtheria toxin with *Vero* cells. *J. Biol. Chem.* 253:7325–7330.

32. Moehring, T. J., and J. P. Crispell. 1974. Enzyme treatment of KB cells altered effect of diphtheria toxin. *Biochem. Biophys. Res. Commun.* 60:1446–1452.

33. Moya, M., A. Dautry-Varsat, B. Goud, D. Louvard, and P. Boquet. 1985. Inhibition of coated pit formation in Hep₂ cells blocks cytotoxicity of diphtheria toxin but not that of ricin toxin. *J. Cell Biol.* 101:548–559.

34. Proia, R. L., D. A. Hart, and L. Eidels. 1979. Interaction of diphtheria toxin with phosphorylated molecules. *Infect. Immunity.* 26:942–948.

35. Proia, R. L., D. A. Hart, R. K. Holmes, K. V. Holmes, and L. Eidels. 1979. Immunoprecipitation and partial characterization of diphtheria toxin-binding glycoproteins from surface of guinea pig cells. *Proc. Natl. Acad. Sci. USA.* 76:685–689.

36. Sandvig, K., and S. Olsnes. 1980. Diphtheria toxin entry into cells is facilitated by low pH. *J. Cell Biol.* 87:828–832.

37. Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* 51:660–672.

38. Schneider, W. J., J. L. Goldstein, and M. S. Brown. 1980. Partial purification and characterization of the low density lipoprotein receptor from bovine adrenal cortex. *J. Biol. Chem.* 255:11442–11447.

39. Schmidt, G., and S. J. Thannhauser. 1945. A method for the determination of desoxyribonucleic acid, ribonucleic acid, and phosphoproteins in animal tissues. *J. Biol. Chem.* 161:83–89.

40. Strauss, N., and E. D. Hendee. 1959. The effect of diphtheria toxin on the metabolism of HeLa cells. *J. Exp. Med.* 109:144–163.

41. Terasaki, T., Y. Shimosato, M. Izawa, and K. Miyamoto. 1983. Negatively charged RNase-susceptible molecules on the surface of ascites tumor cells. *Cell Struct. Funct.* 8:233–244.

42. Thom, D., A. J. Powell, C. W. Lloyd, and D. A. Rees. 1977. Rapid isolation of plasma membranes in high yield from cultured fibroblasts. *Biochem. J.* 168:187–194.

43. Uchida, T., A. M. Pappenheimer Jr., and A. A. Harper. 1972. Reconstitution of diphtheria toxin from two nontoxic cross-reacting mutant toxins. *Science (Wash. DC).* 175:901–903.

44. Uchida, T., A. M. Pappenheimer Jr., and R. Greany. 1973. Diphtheria toxin and related proteins. I. Isolation and some properties of mutant proteins serologically related to diphtheria toxin. *J. Biol. Chem.* 248:3838–3844.

45. Uchida, T., J. Kim, M. Yamaizumi, Y. Miyake, and Y. Okada. 1979. Reconstitution of lipid vesicles associated with HVJ (Sendai virus) spikes. Purification and some properties of vesicles containing nontoxic fragment A of diphtheria toxin. *J. Cell Biol.* 80:10–20.

46. Walter, P., and G. Blobel. 1982. Signal recognition particle contains a 7s RNA essential for protein translocation across the endoplasmic reticulum. *Nature. (Lond.).* 299:691–698.

47. Weiss, L., and E. Mayhew. 1969. Ribonuclease-susceptible charged groups at the surface of Ehrlich ascites tumor cells. *Int. J. Cancer* 4:626–635.

48. Yamaizumi, M., E. Mekada, T. Uchida, and Y. Okada. 1978. One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. *Cell.* 15:245–250.