MOLECULAR AND CELLULAR BIOLOGY, July 1983, p. 1283–1294
0270-7306/83/071283-12$02.00/0
Copyright © 1983, American Society for Microbiology
Vol. 3, No. 7

Binding and Uptake of Diphtheria Toxin by Toxin-Resistant Chinese Hamster Ovary and Mouse Cells

JOHN R. DIDSBURY,† JOAN M. MOEHRING, AND THOMAS J. MOEHRING*

Department of Medical Microbiology; College of Medicine, University of Vermont, Burlington, Vermont 05405

Received 27 January 1983/Accepted 22 April 1983

We investigated two phenotypically distinct types of diphtheria toxin-resistant mutants of Chinese hamster cells and compared their resistance with that of naturally resistant mouse cells. All are resistant due to a defect in the process of internalization and delivery of toxin to its target in the cytosol, elongation factor 2. By cell hybridization studies, analysis of cross-resistance, and determination of specific binding sites for ^{125}I-labeled diphtheria toxin, we showed that these cell strains fall into two distinct complementation groups. The Dip^r group encompasses Chinese hamster strains that are resistant only to diphtheria toxin, as well as mouse LM cells. These strains possess a normal complement of high-affinity binding sites for diphtheria toxin, but these receptors are unable to deliver active toxin fragment A to the cytosol. Cells of the the DPV^r group have a broader spectrum of resistance, including *Pseudomonas* exotoxin A and several enveloped viruses as well as diphtheria toxin. In these studies, which investigate the resistance of these cells to diphtheria toxin, we demonstrate that they possess a reduced number of specific binding sites for this toxin and behave, phenotypically, like cells treated with the proton ionophore monensin. Their resistance is related to a defect in a mechanism required for release of active toxin from the endocytic vesicle.

Diphtheria toxin is a potent inhibitor of protein synthesis in most mammalian cells. The toxin is a protein (M_r = 62,000) that can be cleaved, by proteolysis and reduction, into two fragments, A and B. Fragment A is the enzymatically active fragment that causes inhibition of protein synthesis by catalyzing the ADP-ribosylation of elongation factor 2 (EF-2), thereby inactivating it (7, 41). Although the mechanism of action of fragment A has been well characterized, less is known about the receptor-mediated transport process that facilitates its entry into the cytosol of sensitive cells.

In recent years there has been increasing evidence that although diphtheria toxin is a protein foreign to mammalian cells, it shares a common route of entry into sensitive target cells with physiologically important proteins like low-density lipoprotein (1), α_2-macroglobulin (53), hormones (3, 30), growth factors (15, 16), and also some viruses (19, 51). Diphtheria toxin binds, through its B fragment (50), to specific receptors on the cell surface and then enters the cell in endocytic vesicles (12, 14). There is evidence that these vesicles become acidified (48), triggering translocation of a limited amount of active fragment A from the receptor-toxin complex to the cytosol where it catalyzes the ADP-ribosylation of EF-2 (40). The bulk of the toxin is degraded in secondary lysosomes, and the degradation products are released into the surrounding medium (12). Entry of the toxin can be blocked by treating cells either with the lysosomotropic agent ammonium chloride (13, 14, 24, 44) or with the carboxylic ionophore monensin (28, 44), agents which are known to interfere with acidification of intracellular vesicular compartments. The block can be circumvented by exposing toxin-treated cells to a low-pH environment that triggers events leading to the transport of fragment A directly across the plasma membrane into the cytosol (14, 28, 44).

To study the process of diphtheria intoxication and to provide a model for the study of receptor-mediated endocytosis, we have isolated a number of diphtheria toxin-resistant mutants, derived from sensitive cultured cells, and we have applied techniques of biochemical and genetic analysis to characterize the nature of their resistance to the toxin (34, 35, 37, 39). In this study two phenotypically distinct types of

† Present address: Department of Biochemistry, North Carolina State University, Raleigh, NC 27607.

entry mutants of Chinese hamster cells were compared with naturally resistant mouse LM cells with respect to the physiological factors responsible for their resistance. All are resistant due to some defect in the process of internalization of diphtheria toxin, and a purpose of our investigation was to ascertain the nature of these defects, i.e., whether they were related to reduced capacity to bind the toxin or to alterations at other steps in the entry of toxin. In the case of cultured mouse L cells, resistance has been attributed either to the lack of high-affinity toxin receptors (33, 41, 42) or to the lack of an efficient mechanism for transport of diphtherial fragment A into the cytosol (5, 6, 18, 23). We measured direct binding of toxin and demonstrated that the latter is the case.

MATERIALS AND METHODS

Cells, media, and culture conditions. CHO-K1 cells (Chinese hamster ovary cells, auxotrophic for proline) were obtained from the American Type Culture Collection. Diphtheria toxin-resistant mutants RE10.21 and RE.31 were selected from mutagenized cells by a single-step selection procedure, as previously described (35). Selection of RPE.28 and RPE.51 by a similar procedure using *Pseudomonas* exotoxin A will be described elsewhere (J. M. Moehring and T. J. Moehring, submitted for publication). The thymidine kinase-deficient mouse cell line LMTK$^-$ was obtained from the W. Alton Jones Cell Science Center (Lake Placid, N.Y.). Cells were routinely maintained in Ham nutrient mixture F12 (GIBCO Laboratories, Grand Island, N.Y.) containing 5% fetal bovine serum (referred to as complete F12) at 37°C in an atmosphere of 5% CO_2 in air. The cell strains used in these studies are described in Table 1.

Toxins. Two lots of diphtheria toxin were used. Concentrated and partially purified diphtheria toxin was obtained from Connaught Medical Research Laboratories (Toronto, Ontario, Canada). This toxin produced a single major band and several trace bands in acrylamide gel electrophoresis and had 19,000 guinea pig minimum lethal doses (MLD) per mg of protein. This toxin was used in dose-response assays on intact cells.

Column-purified diphtheria toxin, a gift from R. John Collier (University of California, Los Angeles), produced a single band in acrylamide gel electrophoresis in the absence of thiols and had 20,000 MLD per mg of protein. This toxin was used in toxin binding assays. The two toxins are nearly identical in toxicity when considered on an MLD per milliliter basis (34). Toxicity equivalent relationships are 1 MLD = 0.05 μg or 0.82 pmol of toxin.

Mutant toxins CRM 45 and CRM 197 were the gift of A. M. Pappenheimer, Jr. (Harvard University, Cambridge, Mass.).

Cell hybridization. Details of our hybridization procedure have been presented elsewhere (36). In each hybridization one of the two parental strains was Pro$^-$ TK$^+$ and the other was Pro$^+$ TK$^-$, or one was Pro$^-$ HPRT$^+$ and the other was Pro$^+$ HPRT$^-$. All parental cells could therefore be eliminated by use of proline-free hypoxanthine-aminopterin-thymidine selective medium (HAT medium) (26), and the hybrid nature of recovered clones was verified by their growth in the selective medium. Appropriate controls were included in each experiment, and the phenotype of parental strains was verified at the time of the experiment. Karyological analysis was performed as previously detailed (36) to show that the hybrids possessed a near-tetraploid modal chromosome number.

Intact-cell assay for inhibition of protein synthesis by toxins. This assay, which involves the exposure of log-phase cells to sequential concentrations of toxin for 24 h, followed by pulse-labeling for 30 min with medium containing 0.4 μCi of a ^{14}C-amino acid mixture (Amersham Corp., Arlington Heights, Ill.) per ml, is detailed elsewhere (35).

Assays for effect of ammonium chloride, monensin, and low pH on diphtheria toxin action. Log-phase cells were exposed to various concentrations of NH_4Cl or monensin (Sigma Chemical Co., St. Louis, Mo.) in complete F12 for 1 h at 37°C. Toxin was then added to the cultures for 1 h at either 4°C (monensin-treated cells) or 37°C (NH_4Cl-treated cells). The toxin medium was then removed, and 1 ml of Dulbecco phosphate-buffered saline either at pH 7.2 or reduced to pH 4.5 by addition of H_3PO_4 was added (with or without protective agent, as appropriate). The cells were incubated for 10 to 30 min at 37°C. The phosphate-buffered saline was removed and replaced with complete F12 (with or without protective agent), and incubation at 37°C was continued for 3 or 23 h, followed by pulse-labeling, as described above.

Radiolabeling of toxin. Diphtheria toxin was labeled with radioactive iodine by a modification of the method of Marchalonis (27). The lactoperoxidase-catalyzed

TABLE 1. Cell strains used and their origins

Strain	Phenotype[a]	Origin
CHO-K1	DT^s PT^s Pro$^-$	ATCC CCL 61 (Chinese hamster)
P1R2	DT^s PT^s Pro$^+$ HPRT$^-$	Selected from CHO-K1 (36)
RPE.28	DT^r PT^r Pro$^-$	Selected from CHO-K1
RPE.51	DT^r PT^r Pro$^-$	Selected from CHO-K1
P1R2.P50	DT^r PT^r Pro$^+$ HPRT$^-$	Selected from P1R2
RE.31	DT^r PT^s Pro$^-$	Selected from CHO-K1 (35)
RE10.21	DT^r PT^s Pro$^-$	Selected from CHO-K1 (35)
RE10.21A4P6	DT^r PT^s Pro$^+$ HPRT$^-$	Selected from RE10.21
LMTK$^-$	DT^r PT^s Pro$^+$ TK$^-$	Selected from LM cells (25) (mouse)

[a] DT, Diphtheria toxin; PT, *Pseudomonas aeruginosa* exotoxin A; Pro, proline; TK, thymidine kinase; HPRT, hypoxanthine phosphoribosyl transferase; s, sensitive; r, resistant.

reaction was carried out at room temperature. Each reaction mixture contained 40 μg of column-purified diphtheria toxin, 0.2 μg of lactoperoxidase (Sigma), 800 μCi of carrier-free $Na^{125}I$ (Amersham), and 4 μl of 8.8 mM H_2O_2, in a reaction buffer of 0.1 M sodium phosphate (pH 7.6). The H_2O_2 was added in 2-μl portions at 0 and 2.5 min during the reaction, and the final volume of the reaction mixture was 50 μl. The reaction was terminated after 5 min by the addition of 60 μl of cold 5 mM cysteine-hydrochloride. The products of the iodination were passed through a Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, N.J.) column equilibrated with reaction buffer containing 0.05% bovine serum albumin (Sigma). Two well-separated radioactive peaks were observed. The first peak, which eluted immediately after the void volume, contained the iodinated diphtheria toxin. More than 95% of the radioactivity in this peak was precipitable with 5% trichloroacetic acid and appeared as a single labeled band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The toxin was routinely labeled to an activity of 1×10^7 to 2×10^7 cpm per μg (0.1 to 0.2 mol of iodine per mol of toxin) and was used within 6 h of labeling.

Toxin binding assay. The assay procedure was carried out essentially as described by Middlebrook et al. (33), and our data for Vero monkey kidney cells approximated theirs (201,000 receptors per cell with an affinity of 1.5×10^{-9} M). Cells were grown in tissue culture multidishes with 16-mm wells (Costar) in 1 ml of complete F12 per well. The cells were grown until the monolayer was approximately two-thirds confluent. The growth medium was replaced with 1 ml of medium 199 in Hanks balanced salt solution containing 10% fetal bovine serum and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4), and the cells were incubated at 4°C for 30 min. The appropriate concentrations of ^{125}I-labeled diphtheria toxin were then added, and the cells were incubated at 4°C for 15 h. Each well was then washed four times with 1.8 ml of Hanks balanced salt solution, and the cells were dissolved by the addition of 0.9 ml of 0.1 M NaOH. The dissolved cells, and a subsequent 0.9-ml wash, were transferred to 4-ml Beckman Biovials and counted in a Beckman Biogamma counting system.

RESULTS

Response to diphtheria toxin, CRM 45, and CRM 197. The dose-responses to diphtheria toxin of four mutant strains of CHO-K1 cells are compared with those of wild-type cells and naturally resistant mouse $LMTK^-$ cells in Fig. 1. Strain RE10.21 has an increased resistance of approximately 4 logs over parental CHO-K1 and is equal in resistance to mouse $LMTK^-$ cells. The other CHO-K1 mutants are intermediate in resistance. From dose-response curves such as those in Fig. 1 we determine 50% inhibitory dose (ID_{50}) values for cell strains and use these values in comparisons of relative toxin sensitivity. We define the ID_{50} value as the concentration of toxin which inhibits protein synthesis 50% in a given time of exposure. When working with resistant cell strains, we use a 24-h exposure (unless otherwise indicated) and refer to $ID_{50}(24)$ concentrations.

Mutant diphtheria toxins that cross-react with diphtheria antitoxin but are altered in toxicity (CRMs) have been produced by *Corynebacterium diphtheriae* carrying β-phages with mutations in their *tox* genes (41). The study of the action of certain CRMs on mutant cell strains that are altered in binding or uptake of toxin has provided us with information on the nature of their resistance.

CRM 45 (M_r = 45,000) contains a normal fragment A that is able to catalyze ADP-ribosylation of EF-2, but it is missing the carboxy-terminal portion of fragment B and hence cannot bind to the toxin-specific receptors on sensitive cell membranes. It is, therefore, of low toxicity to intact cells (41). We found, however, that two of our CHO-K1 mutant strains, RPE.28 and RPE.51, were 8- to 10-fold more resistant to CRM 45 (Table 2) than either the parental CHO-K1 cells or the other toxin-resistant strains studied.

CRM 197 (M_r = 62,000) is a nontoxic analog of diphtheria toxin which has a normal fragment B but an inactive fragment A. Although it is nontoxic, it can bind normally to cell receptors and, hence, compete with active diphtheria toxin. In the experiments summarized in Table 2, cells were pretreated with CRM 197 for 1 h, and then toxin dilutions were added and the incubation was continued for 24 h. CRM 197 afforded considerable protection to parental CHO-K1 cells and to strains RPE.28 and RPE.51. It had little effect on diphtheria intoxication in $LMTK^-$ cells or in strains RE.31 and RE10.21.

Diphtheria toxin binding to specific cell surface receptors. To accurately measure toxin-specific binding to cell surface receptors, it is necessary to satisfy four biophysical criteria (9). Binding

TABLE 2. Protection against diphtheria toxin by CRM 197 and toxicity of CRM 45[a]

Strain	Diphtheria toxin $ID_{50}(24)$ concn (nM)		Increase in resistance	CRM 45 $ID_{50}(24)$ concn (nM)
	−CRM 197	+CRM 197		
CHO-K1	0.0026	0.576	222×	75
RE.31	12.0	24.0	2.0×	80
RE10.21	56.0	128.0	2.3×	70
RPE.28	0.051	16.0	314×	600
RPE.51	0.72	24.0	33×	660
$LMTK^-$	176.0	256.0	1.5×	60

[a] Diphtheria toxin was tested with (+) or without (−) 800 nM CRM 197. The $ID_{50}(24)$ value is the concentration of toxin that will inhibit protein synthesis by 50% in 24 hours.

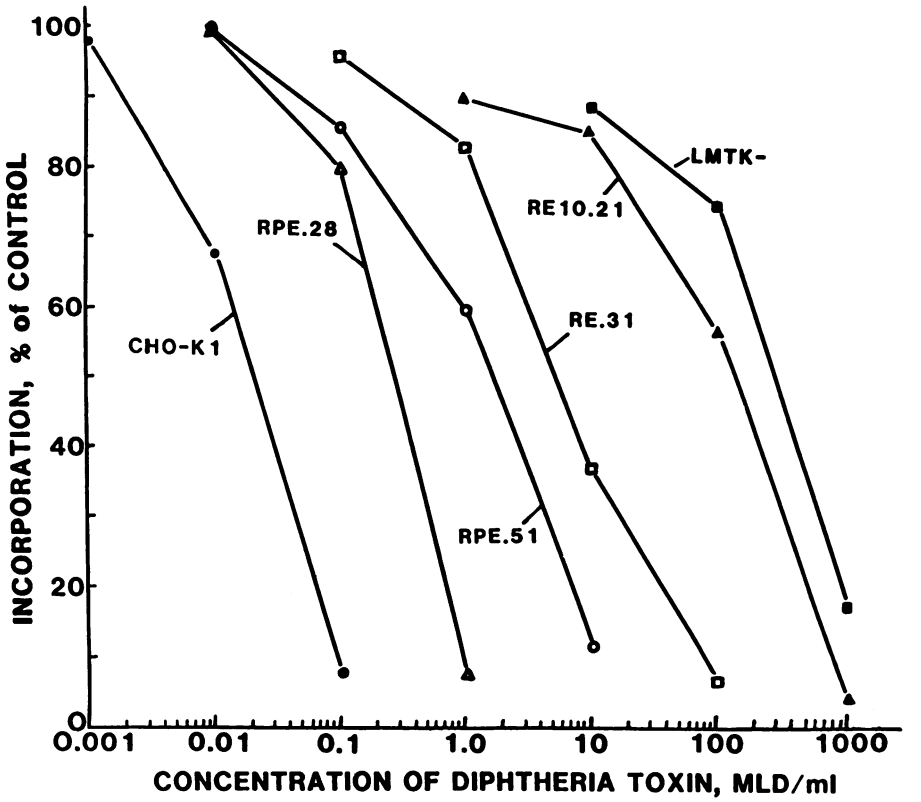

FIG. 1. Response to diphtheria toxin of toxin-resistant entry mutants compared with that of wild-type CHO-K1 and mouse LMTK$^-$ cells, as measured by the intact-cell assay. Cells were exposed to toxin for 24 h.

should exhibit saturability, high affinity, chemical specificity, and reversibility. Saturable toxin-specific binding was measured in two ways. First, increasing concentrations of labeled toxin were added to cells, and the amount bound was plotted versus the concentration of toxin added. From these data total binding curves were constructed. Toxin-specific saturable binding to CHO-K1 and mouse LMTK$^-$ cells, corrected for nonspecific binding by the method of Hamilton et al. (17), is presented in Fig. 2. Specific, saturable toxin binding was also determined by subtracting counts per minute of ^{125}I-labeled toxin bound in the presence of a 100-fold molar excess of unlabeled toxin from counts per minute of replicate samples containing labeled toxin alone. This method of correcting for nonspecific binding produced essentially the same saturation curves as the Hamilton method (data not shown). In these studies the Hamilton method of correction for nonspecific binding was used.

Data for specific toxin binding were analyzed according to the method of Scatchard (45). Scatchard plots for strain CHO-K1 and its mutants and for LMTK$^-$ cells were constructed. The affinity, measured as the dissociation constant (K_d = -1/slope), was equal to 1×10^{-9} to 3×10^{-9} M for these cells and was indicative of high affinity. The results of Scatchard analyses of the saturation binding data are summarized in Table 3. RE.31 and RE10.21 cells possess approximately the same number of binding sites as parental CHO-K1 cells, whereas mutant RPE.28 and RPE.51 cells have reduced numbers of binding sites. The number of toxin-specific binding sites on mouse LMTK$^-$ cells and their affinity for toxin are both similar to values obtained for the binding sites on CHO-K1 cells. Since the affinity for toxin of the receptors on mutant and parental CHO-K1 cells, as well as on LMTK$^-$ cells, was not significantly different, it is not likely that any of these cells are resistant because their receptors have an altered affinity for toxin.

The ability of compounds that bind specifically to cell surface receptors to compete with diphtheria toxin for binding to CHO-K1 and LMTK$^-$ cells was used as an additional measure of specific binding (Table 4). Only unlabeled diphtheria toxin and ATP interfered with specific binding of ^{125}I-labeled diphtheria toxin. ATP, which binds to the toxin molecule (32), also interfered with nonspecific binding.

To demonstrate reversibility of toxin binding,

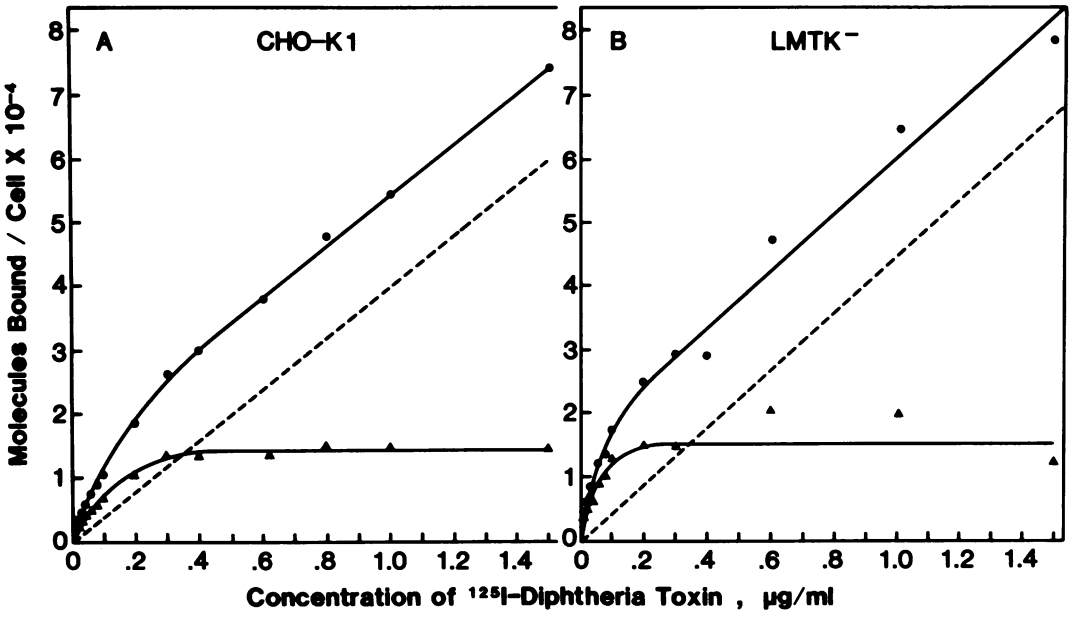

FIG. 2. Saturability of binding of ^{125}I-labeled diphtheria toxin. Nonsaturable binding (- - - -) was calculated by taking the final limiting slope of the total toxin binding curve (●) and extrapolating through the origin. High-affinity (specific) binding (▲) was calculated by taking the difference between the total and low-affinity binding curves (17).

CHO-K1 and LMTK$^-$ cells were first incubated with labeled toxin (0.2 µg/ml) in the presence or absence of a 100-fold excess of unlabeled toxin (20 µg/ml) at 4°C until equilibrium was attained (15 h). The cells were then washed four times with Hanks balanced salt solution to remove unbound toxin and incubated further at 4°C in the presence of a 100-fold excess of unlabeled toxin (20 µg/ml) for 7 h. The amount of radioactivity that remained bound to the cells over time was measured. The amount of bound labeled toxin decreased as a function of time. The decrease exhibited apparent first-order kinetics, indicating that the binding was reversible.

TABLE 3. Number and affinity of diphtheria toxin receptors on CHO-K1 and toxin-resistant cells[a]

Cell Strain	No. of receptors/ cell	Affinity (K_d) ($\times 10^{-9}$ M)
CHO-K1	15,873 ± 3,390	1.83 ± 0.64
RPE.28	8,018 ± 2,010	1.40 ± 0.20
RPE.51	5,184 ± 1,300	1.40 ± 0.50
RE.31	19,131 ± 2,303	1.42 ± 1.12
RE10.21	19,867 ± 580	2.22 ± 0.05
LMTK$^-$	15,631 ± 1,990	1.11 ± 0.39

[a] Summary of Scatchard plot analysis of binding data on wild-type CHO-K1 cells, four toxin-resistant mutants of CHO-K1, and LMTK$^-$ cells. Results are the average of two or more binding experiments on each cell strain ± standard deviation.

The kinetics of diphtheria toxin-induced inhibition of protein synthesis on CHO-K1 cells was examined. From data such as those shown in Fig. 2A it was determined that saturation of toxin binding occurred at a toxin concentration of approximately 0.4 µg/ml (6.4×10^{-9} M). Maximal inhibition of protein synthesis by diphtheria toxin in a minimal time was achieved at a

TABLE 4. Specific toxin binding in the presence of various compounds[a]

Added compound	Specific binding of ^{125}I-diphtheria toxin (% of control)	
	CHO-K1	LMTK$^-$
None	100	100
Unlabeled diphtheria toxin	54	52
ATP	13	17
Pseudomonas toxin	96	107
Cholera toxin	107	98
Ricin	102	107
Concanavalin A	104	108
Insulin	94	96
Transferrin	95	102
β-Glucuronidase	97	102
Mannose 6-phosphate	94	98
Epidermal growth factor	103	103

[a] Cells were incubated with ^{125}I-labeled diphtheria toxin (0.2 µg/ml) with or without a 100-fold molar excess of each compound, and samples were processed as described in the text.

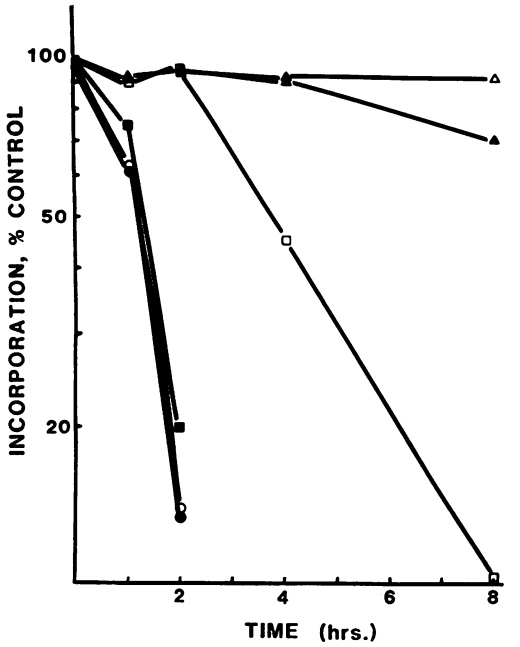

FIG. 3. Kinetics of diphtheria toxin-induced inhibition of protein synthesis in CHO-K1 cells. Cells were incubated with diphtheria toxin, at the indicated concentrations, for various times at 37°C, followed by a 30-min pulse with medium containing ^{14}C-amino acids. Counts per minute are expressed as a percentage of ^{14}C-amino acid incorporation in untreated control cells. Symbols: ●, 8×10^{-7} M; ○, 8×10^{-8} M; ■, 8×10^{-9} M; □, 8×10^{-10} M; ▲, 8×10^{-11} M; △, 8×10^{-12} M.

concentration of approximately 8.0×10^{-9} M (Fig. 3). The fact that these values were in close agreement further indicates that the toxin-specific binding we measured is biologically relevant.

Dominance hybridization and complementation analysis. To investigate further the nature of the toxin resistance of the CHO-K1 mutants, as well as the naturally occurring resistance of the LM cells, dominance hybridization tests and complementation analyses were carried out.

Hybrids were formed between the various cell strains, individual clones were selected and grown up, and the hybrid strains were assayed to determine their toxin sensitivity. Hybrids were analyzed by comparison of their $ID_{50}(24)$ concentrations of diphtheria toxin with those of wild-type and individual parental cells. Dominance hybridization analysis has shown that the mutations expressed in the resistant strains used in this study are recessive. In Table 5, data for two representative strains, hybridized with wild-type Chinese hamster strain P1R2, are given. It has previously been shown that the resistance of mouse L cells is recessive (8). Strains RPE.51 and RE.31 are members of different complementation groups.

Complementation analysis (Table 5) demonstrated that the strains used in this study fall into two complementation groups and that mouse LM cells may be assigned to the same group as two of the diphtheria toxin-resistant Chinese hamster strains (Table 5, footnote *c*). It has been shown that the genetic determinant for sensitivity to diphtheria toxin resides on Chinese hamster chromosome 2 in hybrids of CHO and mouse cells (46). We verified the presence of hamster chromosome 2, by G-banding and karyotypic analysis (36), in interspecific hybrids formed between RE10.21 and LMTK$^-$ cells. This assured us that the non-complementarity observed in these hybrids was not due to loss of hamster chromosome 2. We have designated these two groups the DPV^r group, as all of its members are resistant to diphtheria toxin, *Pseu-*

TABLE 5. Analysis of toxin resistance of mutants by cell hybridization

Strains	Diphtheria toxin $ID_{50}(24)$ conc (MLD/ml)[a]
Cell strains studied	
CHO-K1 (wt[b])	0.010
P1R2 (wt)	0.012
RPE.28	0.17
RPE.51	2.9
P1R2.P50	0.90
RE.31	9.8
RE10.21	163.0
RE10.21A4P6	160.0
LMTK$^-$	157.0
Dominance hybridization	
RPE.51 × P1R2	0.019
RE.31 × P1R2	0.038
Complementation analysis[c]	
RPE.28 × P1R2.P50	0.30
RPE.51 × P1R2.P50	0.29
RE.31 × RE10.21A4P6	59.0
RPE.51 × RE10.21A4P6	0.028
RPE.51 × LMTK$^-$	0.046
RE10.21 × LMTK$^-$	45.0

[a] $ID_{50}(24)$ concentrations were determined from three to six assays on individual cell strains or, in the case of hybrid crosses, from assays on four or five separately selected hybrid strains.

[b] wt, Wild type with respect to toxin sensitivity.

[c] Strains RPE.28, RPE.51, and P1R2.P50 belong to complementation group DPV^r, and strains RE.31, RE10.21, and LMTK$^-$ belong to complementation group Dip^r.

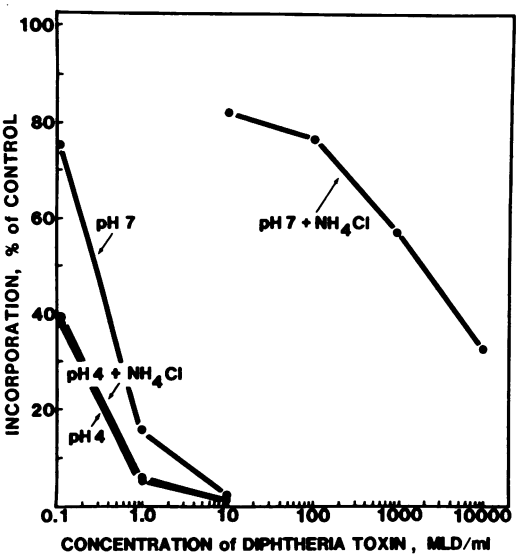

FIG. 4. Effect of pH and ammonium chloride on the response to diphtheria toxin of CHO-K1 cells. Cells were exposed to toxin with or without NH_4Cl (400 μg/ml) for 1 h at 37°C, exposed to buffers of pH 4 or pH 7 with or without NH_4Cl for 30 min at 37°C, and then incubated in toxin-free complete F12 for 24 h. Cells were pulsed for 30 min with ^{14}C-amino, acids and protein synthesis was measured as described in the text.

domonas exotoxin A, and certain viruses, and the Dip^r group, whose members are resistant to diphtheria toxin only. The ID_{50}(24) values for the mutants belonging to each of these two complementation groups varied by a log or more. These differences could be the result of mutations causing different amino acid substitutions within the active sites of the respective gene products. The degree of protection produced by each mutation would, then, be a reflection of the extent to which the functional properties of the gene product were altered.

Effects of ammonium chloride, monensin, and low pH. We investigated whether resistance of entry mutants in either the DPV^r or the Dip^r complementation group was similar to that of wild-type cells treated with NH_4Cl or monensin. We first determined that NH_4Cl protected parental CHO-K1 cells against diphtheria toxin and that this protection was reversed by exposing the cells to a pH of 4 (Fig. 4). Exposure of untreated cells to a low-pH environment did not significantly affect their sensitivity to the toxin. Likewise, exposure of CHO-K1 cells to monensin afforded them a high degree of protection against diphtheria toxin, and this protection was reversible by exposure to low pH (Fig. 5).

Representatives of the DPV^r and Dip^r groups were exposed to a series of concentrations of toxin and then to buffers of pH 4 to 7 (Fig. 6). In DPV^r strains (RPE.51 is shown), a progressive reversal of resistance to toxin was noted as the pH was lowered. At pHs below 5, the cells became nearly as sensitive to toxin as parental CHO-K1 cells (indicated by the broken line). The response of strains of the Dip^r group was markedly different. In strains RE10.21 and LMTK$^-$, some increase in sensitivity to toxin could be produced by exposure to buffers with pH of less than 5, but their resistance was still 2 logs or more greater than that of CHO-K1 cells.

These results indicated that resistance of the DPV^r strains might be similar to that conferred by exposure of cells to NH_4Cl or monensin. Because of our finding that these strains have a reduced number of diphtheria toxin binding sites (Table 3), we measured the number of specific binding sites on CHO-K1 cells treated with NH_4Cl or monensin. No reduction in toxin binding due to treatment with NH_4Cl was observed (data not shown). However, when cells were treated with monensin, a significant reduction in

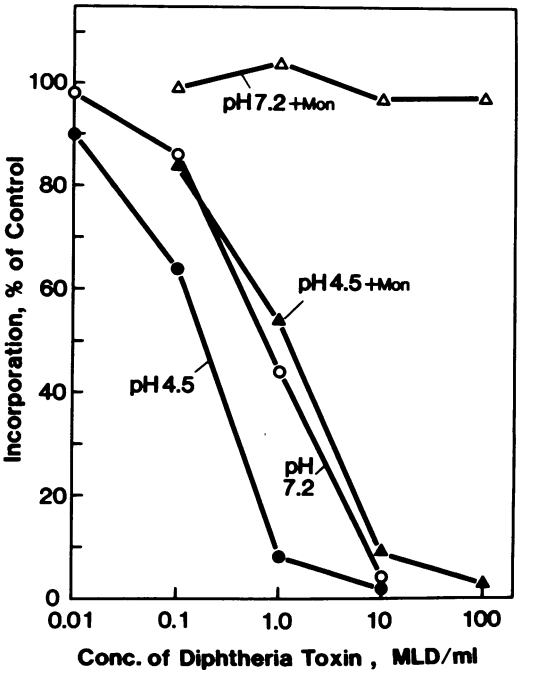

Fig. 5. Effect of monensin and low-pH treatment on the response to diphtheria toxin of CHO-K1 cells. Cells were treated with 1 μM monensin (Mon) in complete F12 for 1 h at 37°C and then exposed to diphtheria toxin (with or without monensin) for 1 h at 4°C, the medium was replaced with buffer at pH 7.2 or pH 4.5 (with or without monensin) for 10 min at 37°C and then growth medium (with or without monensin) was replaced for 24 h at 37°C. Cells were then pulse-labeled as described in the text.

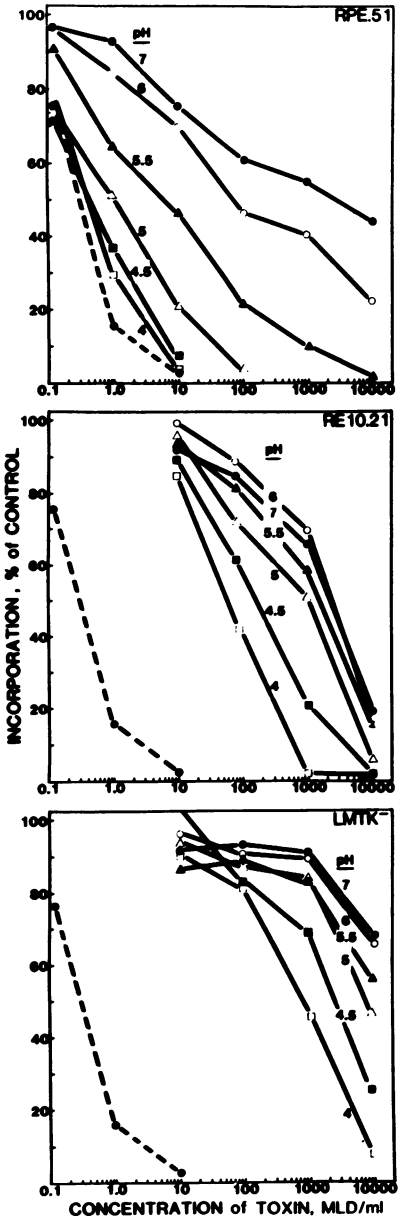

FIG. 6. Effect of pH on the response of RPE.51, RE10.21, and LMTK⁻ cells to diphtheria toxin. Cells were exposed to toxin for 1 h at 37°C, exposed to phosphate buffers adjusted to the indicated pH for 30 min, and incubated in toxin-free complete F12 for 24 h. Protein synthesis was measured as described in the text. Dose-response curves are compared with the dose-response curve of CHO-K1 cells exposed to pH 7 (- - - -).

the number of toxin binding sites was detectable over time (Table 6). After a 60-min exposure to monensin, CHO-K1 cells had lost approximately 75% of their specific binding capacity. We had determined that strains of the DPV^r group had 50 to 68% fewer binding sites than did CHO-K1 cells (Table 4). It thus appears that the mutation affecting toxin uptake in the DPV^r complementation group has an effect remarkably similar to that observed when CHO-K1 cells are treated with monensin.

DISCUSSION

The diphtheria toxin-resistant entry mutants described in this study were obtained by single-step selection with either diphtheria toxin or *Pseudomonas* exotoxin A. Resistance to diphtheria toxin behaved recessively in hybrids constructed between these mutants and wild-type cells. Testing for complementation, by measuring the resistance to diphtheria toxin of appropriate hybrids, allowed us to separate the mutants into two genotypically distinct complementation groups, Dip^r and DPV^r. DPV^r mutants possess a pleiotropic phenotype. They are resistant to diphtheria toxin, CRM 45, *Pseudomonas* toxin, and several enveloped viruses. A detailed characterization of the cross-resistance of the DPV^r group will be reported elsewhere (Moehring and Moehring, submitted for publication). Mutants in this group show decreased binding of ^{125}I-labeled diphtheria toxin, and their resistance to diphtheria toxin is overcome by exposure to low-pH media. Their resistance to viruses and *Pseudomonas* toxin is overcome by low-pH treatment as well (Moehring and Moehring, submitted for publication). Dip^r mutants were found to be uniquely resistant to diphtheria toxin and to bind normal amounts of ^{125}I-labeled diphtheria toxin when compared with wild-type cells. Their resistance to diphtheria toxin is not overcome by low pH. Both groups of entry mutants possess normal levels of ADP-ribosylatable EF-2 (35; Moehring and Moehring, submitted for publication).

Although the mutants of the DPV^r comple-

TABLE 6. Effect of monensin on binding of ^{125}I-labeled diphtheria toxin to CHO-K1 cells[a]

Time of exposure to monensin (min)	cpm/10^6 cells	% of cpm of untreated cells
0	8,159 ± 1,503	—
10	6,219 ± 947	76
20	7,086 ± 750	87
40	4,323 ± 193	53
60	1,840 ± 1,040	23

[a] Cells were treated with 25 μM monensin for the indicated times, and then specific binding was measured at 4°C by the addition of 0.1 μg of ^{125}I-labeled toxin per ml for 15 h. Values were corrected for nonspecific binding by subtracting counts per minute bound in the presence of a 100-fold molar excess of unlabeled toxin.

mentation group had a reduced number of binding sites for diphtheria toxin, the reduction in binding (50 to 68% less binding than with sensitive CHO-K1 cells) was not sufficient to fully account for the resistance of these cells, which was 1.5 to 2.5 logs greater than that of parental cells. The remaining binding sites were functional, binding toxin that ultimately reached the cytosol to inactivate EF-2. This was demonstrated with the nontoxic mutant toxin CRM 197, which competes with diphtheria toxin for binding to toxin-specific receptors (49, 50). CRM 197 effectively blocked the action of diphtheria toxin on cells of the DPV^r complementation group.

CRM 45 is a mutant toxin that possesses an active fragment A but is much less cytotoxic because it lacks the C-terminal polypeptide sequence of fragment B which recognizes toxin-specific binding sites on target cells (49, 50). CRM 45 does not bind to toxin-specific receptors, and, therefore, its fragment A does not enter cells by a toxin-specific receptor-mediated process (49). Cells of the DPV^r complementation group had an increased resistance to CRM 45, indicating that the pathway of toxin entry into these cells is blocked at a step subsequent to binding of the toxin.

The lysosomotropic amine NH_4Cl and the carboxylic ionophore monensin, which collapse proton gradients and raise the pH of acidic vacuoles in the endocytic pathway, block the action of diphtheria toxin on sensitive cells (13, 14, 28, 31, 43). This protective effect can be overcome by first exposing the cells to toxin and then incubating them briefly in a low-pH medium (14, 28, 43, 44). These observations formed the basis for suggesting that diphtheria toxin fragment A enters the cytosol from acidified endocytic vesicles. Ammonium chloride and monensin are believed to block the cytotoxic action of diphtheria toxin by interfering with acidification of endocytic vesicles and preventing the pH-dependent translocation of fragment A into the cytosol. Exposing cells to low pH, then, enables the toxin to bypass the block by activating the mechanism of translocation at the cell surface so that fragment A can enter directly across the plasma membrane into the cytosol.

DPV^r mutants behave, phenotypically, like monensin-treated wild-type cells. Their resistance to toxin is overcome by low pH, and they, like monensin-treated cells, possess a reduced number of diphtheria toxin binding sites. By analogy with the results of treating cells with monensin to protect them against diphtheria toxin, it appears that the mutation in DPV^r strains is responsible for a defect in acidification of endocytic vesicles and that failure to acidify these vesicles prevents translocation of fragment A into the cytosol. Likewise, a defect in acidification could also lead to a reduction in the number of toxin-specific cell surface receptors. The collapse of proton gradients could, by interfering with intracellular transport or vesicle fusion, disrupt receptor recycling or prevent delivery of newly synthesized receptors to the cell surface. Treatment of cells with monensin has been shown to inhibit receptor recycling (4) and transport of proteins from the Golgi apparatus to the plasma membrane (20, 21, 47).

Our findings with DPV^r strains, therefore, indicate that entry into the cytosol of both fragment A and A45 requires exposure to a low-pH environment. This pH-dependent step(s) in toxin entry subsequent to binding might involve either insertion of part of the toxin molecule into the membrane or transport of fragment A across the membrane of the endocytic vesicle, or both. Diphtheria toxin and CRM 45 have been shown to insert in planar lipid bilayers and form channels at acidic pH (10, 11, 22). Perhaps upon insertion of toxin or a CRM 45-like molecule into the membrane of an acidified endocytic vesicle a channel is formed through which fragment A then passes to reach the cytosol. Both insertion into planar lipid bilayers and channel formation require low pH, and the pH gradient across membranes of endocytic vesicles could provide the driving force for translocation of fragment A (10, 22). Thus, a defect in the mechanism for acidification of endocytic vesicles of DPV^r strains could, by interfering with any one or a combination of the events proposed, prevent fragment A from reaching the cytosol and inhibiting protein synthesis.

We have found that DPV^r strains are resistant to certain enveloped viruses, among them Sindbis and Semliki Forest virus (Moehring and Moehring, submitted for publication). These viruses enter cells by adsorptive endocytosis, and the release of their nucleocapsids into the cytosol is facilitated by low pH (6 or slightly below) (51, 52). This release can be blocked by amines or monensin, and the block can be overcome by exposure of cells to a low-pH environment, in a manner analogous to overcoming resistance to diphtheria toxin (19, 29, 52). It was recently reported that Semliki Forest virus begins to enter the cytosol from the endosome 4 to 6 min after leaving the cell surface (M. Marsh, J. Wellsteed, E. Bolzau, and A. Helenius, Abstr. Annu. Meet. Am. Soc. Cell Biol. 1982, abstr. no. 21030, p. 418). It was also reported that endosomes become acidified (to pH 5) within 5 min after internalization of ligand, before fusion with lysosomes, and that they possess a mechanism for regulation of their pH (47; F. R. Maxfield, B. Tycko, and S. Fluss, Abstr. Annu. Meet. Am. Soc. Cell Biol. 1982, abstr. no. 12123, p. 271).

In view of the concomitant resistance of the DPV^r strains to toxin and viruses, therefore, we postulate that they possess a mutation affecting this mechanism for regulation of endosomal pH. We recently obtained additional evidence that such an alteration exists in the endosomes of DPV^r strains. Endosomes and lysosomes were isolated by subcellular fractionation, and it was demonstrated that acidification of these vesicles is mediated by a Mg^{2+}- and ATP-dependent process. A comparison of acidification of endosomes from CHO-K1 cells and from DPV^r strains showed that DPV^r strains have a defect in the ATP-dependent acidification of their endosomes (M. Merion, P. Schlesinger, R. M. Brooks, T. J. Moehring, J. M. Moehring, and W. S. Sly, Proc. Natl. Acad. Sci. U.S.A., in press).

The evidence provided by these studies indicates that neither the toxins nor the viruses that we have studied require exposure to the lysosomal compartment for their active component to enter the cytosol. Indeed, it appears that in most cases when these agents do reach the secondary lysosomes, they are degraded and released from the cell (31, 42).

The results of our studies on LM cells and Dip^r mutants substantiate what we, and others, have previously postulated: that naturally resistant mouse cells and Dip^r strains are intoxicated only by toxin that is taken in by a nonspecific entry mechanism (5, 38), one which does not utilize specific toxin binding sites. Our data agree with recent reports showing that L cells possess toxin-specific receptors (6, 18, 23). However, our data indicate that the resistance is due to an impaired step in transport and not to a reduced affinity of binding, as was suggested by Heagy and Neville (18). Our studies, utilizing direct measurements of toxin binding to intact cells, indicated that the binding sites of LMTK$^-$ cells have an affinity equal to that of binding sites on toxin-sensitive CHO cells and to that previously reported for Vero cells (33). Since the determinations of Heagy and Neville (18) were based on the kinetics of diphtheria toxin inactivation of protein synthesis in L cells, it is possible that the K_d they determined was based on the activity of fragment A which entered the cytosol by a nonspecific mechanism.

Dip^r and LM cells are altered at a different genetic locus than are DPV^r mutants. While they bind normal amounts of diphtheria toxin, CRM 197 does not alter their response to toxin, suggesting that the toxin that is bound to specific receptors is effectively blocked and that its enzymatically active A fragment does not reach the cytoplasm. It is unlikely that Dip^r mutants or L cells are defective in acidification of intracellular vesicles since they show normal sensitivity to CRM 45 and their resistance to toxin is not overcome by low pH. Dip^r mutants and LM cells may be blocked at a step in entry between binding of toxin and the pH-dependent step which is blocked in DPV^r mutants.

One model of toxin entry proposes that when the toxin-receptor complex is exposed to a low pH, the toxin molecule undergoes a conformational change that facilitates translocation of fragment A into the cytosol (11). Analysis of Dip^r mutants and L cells suggests that while this may be a necessary condition for toxin entry, it alone is not sufficient to permit the fragment A of diphtheria toxin to reach the cytosol. Thus, the study of Dip^r mutants and naturally resistant L cells provides evidence for another step in the entry of diphtheria toxin that is unique to this toxin. It seems likely that either a second function of the receptor or some other component in the membrane which is alterable by mutation is involved in the transfer of fragment A from the receptor-toxin complex to the cytosol. Further, it appears that this function is specific for entry of fragment A and not required for entry of A45. One possible explanation is that the defect in LM cells and Dip^r mutants prevents cleavage of a peptide bond in fragment B which releases a CRM 45-like fragment of toxin from the highly charged C-terminal tail so that it can insert into the membrane with the proper orientation for translocation of fragment A (5, 10, 22). Another possibility is that under acidic conditions the receptor itself is induced to undergo a conformational change which is necessary for insertion of the toxin molecule into the membrane. Or, the receptor could be defective at a second active internalization site and fail to concentrate in coated pits (2). We consider the latter possibility unlikely, however, as Keen et al. (23) have shown that resistant mouse 3T3 cells internalize toxin bound on the cell surface. Whether the step in question requires an acidic pH remains to be determined.

ACKNOWLEDGMENTS

We thank Dennis Danley, Cheryl Albaugh, Peter Gehlbach, Janis Campbell, and Nancy Drown for technical assistance in preparing the cell hybrids, and Monique Phillips for assistance in preparation of the manuscript.

This investigation was supported by Public Health Service grant AI-09100 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Anderson, R. G. W., J. L. Goldstein, and M. S. Brown.** 1976. Localization of low density lipoprotein receptors on plasma membrane of normal human fibroblasts and their absence in cells from a familial hypercholesterolemia homozygote. Proc. Natl. Acad. Sci. U.S.A. **73**:2434–2438.
2. **Anderson, R. G. W., J. L. Goldstein, and M. S. Brown.** 1977. A mutation that impairs the ability of lipoprotein receptors to localize in coated pits on the cell surface of human fibroblasts. Nature (London) **270**:695–699.
3. **Barazzone, P., M. A. Lesniak, P. Gorden, E. van Ob-**

berghen, J.-L. Carpentier, and L. Orci. 1980. Binding, internalization, and lysosomal association of ^{125}I-human growth hormone in cultured human lymphocytes. A quantitative morphological and biochemical study. J. Cell Biol. 87:360–369.
4. Basu, S. K., J. L. Goldstein, R. G. W. Anderson, and M. S. Brown. 1981. Monensin interrupts the recycling of low density lipoprotein receptors in human fibroblasts. Cell 24:493–502.
5. Boquet, P., and A. M. Pappenheimer, Jr. 1976. Interaction of diphtheria toxin with mammalian cell membranes. J. Biol. Chem. 251:5770–5778.
6. Chang, T., and D. M. Neville, Jr. 1978. Demonstration of diphtheria toxin receptors on surface membranes from both toxin-sensitive and toxin-resistant species. J. Biol. Chem. 253:6866–6871.
7. Collier, R. J. 1975. Diphtheria toxin: mode of action and structure. Bacteriol. Rev. 39:54–85.
8. Creagan, R. P., S. Chen, and F. H. Ruddle. 1975. Genetic analysis of the cell surface: association of human chromosome 5 with sensitivity to diphtheria toxin in mouse-human somatic cell hybrids. Proc. Natl. Acad. Sci. U.S.A. 72:2237–2241.
9. Cuatrecasas, P. 1974. Membrane receptors. Annu. Rev. Biochem. 43:169–214.
10. Donovan, J. J., M. I. Simon, R. K. Draper, and M. Montal. 1981. Diphtheria toxin forms transmembrane channels in planar lipid bilayers. Proc. Natl. Acad. Sci. U.S.A. 78:172–176.
11. Donovan, J. J., M. I. Simon, and M. Montal. 1982. Insertion of diphtheria toxin into and across membranes: role of phosphoinositide asymmetry. Nature (London) 298:669–672.
12. Dorland, R. B., J. L. Middlebrook, and S. H. Leppla. 1979. Receptor-mediated internalization and degradation of diphtheria toxin by monkey kidney cells. J. Biol. Chem. 254:11337–11342.
13. Dorland, R. B., J. L. Middlebrook, and S. H. Leppla. 1981. Effect of ammonium chloride on receptor-mediated uptake of diphtheria toxin on Vero cells. Exp. Cell Res. 134:319–327.
14. Draper, R. K., and M. I. Simon. 1980. The entry of diphtheria toxin into the mammalian cell cytoplasm: evidence for lysosomal involvement. J. Cell Biol. 87:828–832.
15. Gorden, P., J.-L. Carpentier, S. Cohen, and L. Orci. 1978. Epidermal growth factor: morphological demonstration of binding, internalization and lysosomal association in human fibroblasts. Proc. Natl. Acad. Sci. U.S.A. 75:5025–5029.
16. Haigler, H., J. F. Ash, S. J. Singer, and S. Cohen. 1978. Visualization by fluorescence of the binding and internalization of epidermal growth factor in human carcinoma cells A-431. Proc. Natl. Acad. Sci. U.S.A. 75:3317–3321.
17. Hamilton, T. A., H. G. Wada, and H. H. Sussman. 1979. Identification of transferrin receptors on the surface of human cultured cells. Proc. Natl. Acad. Sci. U.S.A. 76:6406–6410.
18. Heagy, W. E., and D. M. Neville, Jr. 1981. Kinetics of protein synthesis inactivation by diphtheria toxin in toxin-resistant L cells. J. Biol. Chem. 256:12788–12792.
19. 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.
20. Johnson, D. C., and M. J. Schlesinger. 1980. Vesicular stomatitis virus and Sindbis virus glycoprotein transport to the cell surface is inhibited by ionophores. Virology 103:407–424.
21. Kääriäinen, L., K. Hashimoto, J. Saraste, I. Virtanen, and K. Penttinen. 1980. Monensin and FCCP inhibit the intracellular transport of alphavirus membrane glycoproteins. J. Cell Biol. 87:783–791.
22. Kagan, B. L., A. Finkelstein, and M. Colombini. 1981. Diphtheria toxin fragment forms large pores in phospholipid bilayer membranes. Proc. Natl. Acad. Sci. U.S.A. 78:4950–4954.
23. 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. U.S.A. 79:2912–2916.
24. Kim, K., and N. B. Groman. 1965. In vitro inhibition of diphtheria toxin action by ammonium salts and amines. J. Bacteriol. 90:1552–1556.
25. Kit, S., L. J. Piekarski, and D. R. Dubbs. 1963. Induction of thymidine kinase by vaccinia-infected mouse fibroblasts. J. Mol. Biol. 6:22–23.
26. Littlefield, J. W. 1964. Selection of hybrids from mating of fibroblasts *in vitro* and their presumed recombinants. Science 145:709–710.
27. Marchalonis, J. J. 1969. An enzymic method for the trace iodination of immunoglobulins and other proteins. Biochem. J. 113:299–305.
28. Marnell, M. H., M. Stookey, and R. K. Draper. 1982. Monensin blocks the transport of diphtheria toxin to the cell cytoplasm. J. Cell Biol. 93:57–62.
29. Marsh, M., J. Wellsteed, H. Kern, E. Harms, and A. Helenius. 1982. Monensin inhibits Semliki Forest virus penetration into culture cells. Proc. Natl. Acad. Sci. U.S.A. 79:5297–5301.
30. Maxfield, F. R., J. Schlesinger, Y. Schechter, I. Pastan, and M. C. Willingham. 1978. Collection of insulin, EGF, and α_2-macroglobulin in the same patches on the surface of cultured fibroblasts and common internalization. Cell 14:805–810.
31. Middlebrook, J. L., and R. B. Dorland. 1977. Differential chemical protection of mammalian cells from the exotoxins of *Corynebacterium diphtheriae* and *Pseudomonas aeruginosa*. Infect. Immun. 16:232–239.
32. Middlebrook, J. L., and R. B. Dorland. 1979. Protection of mammalian cells from diphtheria toxin by exogenous nucleotides. Can. J. Microbiol. 25:285–290.
33. Middlebrook, J. L., R. B. Dorland, and S. H. Leppla. 1978. Association of diphtheria toxin with Vero cells. Demonstration of a receptor. J. Biol. Chem. 253:7325–7330.
34. Moehring, J. M., and T. J. Moehring. 1976. Comparison of diphtheria intoxication in human and non-human cell lines and their resistant variants. Infect. Immun. 13:221–228.
35. Moehring, J. M., and T. J. Moehring. 1979. Characterization of diphtheria toxin-resistance system in Chinese hamster ovary cells. Somat. Cell Genet. 5:453–468.
36. Moehring, T. J., D. E. Danley, and J. M. Moehring. 1979. Codominant translational mutants of Chinese hamster ovary cells selected with diphtheria toxin. Somat. Cell Genet. 5:469–480.
37. Moehring, T. J., and J. M. Moehring. 1972. Response of cultured mammalian cells to diphtheria toxin. IV. Isolation of KB cells resistant to diphtheria toxin. Infect. Immun. 6:487–492.
38. Moehring, T. J., and J. M. Moehring. 1976. Interaction of diphtheria toxin and its active subunit, fragment A, with toxin-sensitive and toxin-resistant cells. Infect. Immun. 13:1426–1432.
39. Moehring, T. J., and J. M. Moehring. 1977. Selection and characterization of cells resistant to diphtheria toxin and *Pseudomonas* exotoxin A: presumptive translational mutants. Cell 11:447–454.
40. Moynihan, M. R., and A. M. Pappenheimer, Jr. 1981. Kinetics of adenosine diphosphoribosylation of elongation factor 2 in cells exposed to diphtheria toxin. Infect. Immun. 32:575–582.
41. Pappenheimer, A. M., Jr. 1977. Diphtheria toxin. Annu. Rev. Biochem. 46:69–94.
42. 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. U.S.A. 76:685–689.
43. Sandvig, K., and S. Olsnes. 1980. Diphtheria toxin entry into cells is facilitated by low pH. J. Cell Biol. 87:828–832.

44. **Sandvig, K., and S. Olsnes.** 1982. Entry of the toxic proteins abrin, modeccin, ricin, and diphtheria toxin into cells. II. Effect of pH, metabolic inhibitors, and ionophores and evidence for toxin penetration from endocytotic vesicles. J. Biol. Chem. **257**:7504–7513.
45. **Scatchard, G.** 1949. The attraction of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. **51**:660–672.
46. **Stallings, R. L., M. J. Siciliano, G. M. Adair, and R. M. Humphrey.** 1982. Structural and functional hemi- and dizygous Chinese hamster chromosome 2 gene loci in CHO cells. Somat. Cell Genet. **8**:413–422.
47. **Strous, G. J. A. M., and H. F. Lodish.** 1980. Intracellular transport of secretory and membrane proteins in hepatoma cells infected by vesicular stomatitis virus. Cell **22**:709–717.
48. **Tycko, B., and F. R. Maxfield.** 1982. Rapid acidification of endocytic vesicles containing α_2-macroglobulin. Cell **28**:643–651.
49. **Uchida, T., A. M. Pappenheimer, Jr., and R. Greany.** 1973. Diphtheria toxin and related proteins. I. Isolation and properties of mutant proteins serologically related to diphtheria toxin. J. Biol. Chem. **248**:3838–3844.
50. **Uchida, T., A. M. Pappenheimer, Jr., and A. A. Harper.** 1972. Reconstitution of diphtheria toxin from two nontoxic cross-reacting mutant proteins. Science **175**:901–903.
51. **White, J., and A. Helenius.** 1980. pH dependent fusion between the Semliki Forest virus membrane and liposomes. Proc. Natl. Acad. Sci. U.S.A. **77**:3273–3277.
52. **White, J., J. Kartenbeck, and A. Helenius.** 1980. Fusion of Semliki Forest virus with the plasma membrane can be induced by low pH. J. Cell Biol. **87**:264–272.
53. **Willingham, M. C., F. R. Maxfield, and I. Pastan.** 1979. α_2-macroglobulin binding to the plasma membrane of cultured fibroblasts. J. Cell Biol. **82**:614–625.