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

YASUFUMI KANEDA, TSUYOSHI UCHIDA, EISUKE MEKADA, MAHITO NAKANISHI, and YOSHIO OKADA
Institute for Molecular and Cellular Biology, Osaka University, Suita, Osaka 565, Japan

ABSTRACT Ehrlich ascites tumor cells were found to be very insensitive to diphtheria toxin. We formed 37 hybrids from Ehrlich tumor cells and diphtheria toxin–sensitive human fibroblasts. The effects of diphtheria toxin on protein synthesis in those hybrids were examined. The hybrids were divided into three groups on the basis of toxin sensitivity. Group A hybrids were as sensitive to diphtheria toxin as human fibroblasts, Group C were as resistant as Ehrlich tumor cells, and Group B had intermediate sensitivity. Group A hybrids had diphtheria toxin-binding sites but Group B and C had no detectable binding sites. Elongation factor-2 of all the hybrids was susceptible to ADP-ribosylation by fragment A of diphtheria toxin. Cells of Group A and B became more sensitive to CRM 45 (cross-reacting material 45 of diphtheria toxin) after they were exposed to low pH (pH = 4.5). The resistance of Group C to CRM 45 was not affected by the same treatment. Group A and B hybrids and human fibroblasts had similar sensitivities to a hybrid toxin composed of wheat germ agglutinin and fragment A of diphtheria toxin, but Group C and Ehrlich tumor cells were resistant to this hybrid toxin. All the hybrids and Ehrlich tumor cells were more sensitive to a hybrid toxin composed of wheat germ agglutinin and subunit A of ricin than were human fibroblasts. On subcloning of Group B hybrids, one Group C hybrid was obtained, but no Group A hybrid. These facts suggest that Ehrlich ascites tumor cells differ from human fibroblasts in the expression of a factor(s) that is involved in entry of fragment A of diphtheria toxin into the cytoplasm after the toxin binds to its surface receptors.

It is well known that fragment A of diphtheria toxin inhibits protein synthesis in the cytoplasm of mammalian cells by ADP-ribosylation of elongation factor-2 (EF-2)[1] (8, 10). The question of how fragment A reaches the cytoplasm is very interesting because it provides some suggestions about entry of biologically active macromolecules into cells and about membrane functions related to entry. Recent work has provided a detailed model of the mechanism of diphtheria toxin entry, but much remains to be known. There are two approaches to the study of this problem. First, what structural feature of diphtheria toxin is essential for entry of at least fragment A of the toxin? Second, what environmental and cellular factors are required for entry of the toxin?

With regard to the first question, two regions of diphtheria toxin fragment B have been shown to be involved in entry of the diphtheria toxin molecule. The C-terminal portion of the protein is required for binding to receptors on the surface of toxin-sensitive cells. The portion between 30,000 and 45,000 from the N-terminus of the toxin contains hydrophobic regions (1), and polypeptides containing this segment of the toxin can participate in entry of toxin into cells (9). With regard to the second approach, amines such as chloroquine

[1] *Abbreviations used in this paper:* CRM 45, cross-reacting material 45 of diphtheria toxin; EF-2, elongation factor-2; MEM, Eagle's minimum essential medium; WGA, wheat germ agglutinin; WGA-DA and WGA-RA, hybrid toxins containing WGA and fragment A of diphtheria toxin or subunit A of ricin, respectively.

THE JOURNAL OF CELL BIOLOGY · VOLUME 98 FEBRUARY 1984 466–472
© The Rockefeller University Press · 0021-9525/84/02/0466/07 $1.00

(15), methylamine (17), and ammonium chloride (14) protect cells against the action of diphtheria toxin, but exposure of cells to low pH prevents the inhibitory effects of amines on the cytotoxic activity of the toxin (26). At neutral pH, diphtheria toxin bound to cell surfaces is internalized by endocytosis, the pH of the endocytotic vesicles decreases, and then fragment A passes through the membrane of the vesicles to enter the cytoplasm. Recently, receptor-mediated endocytosis of diphtheria toxin was demonstrated by video intensification microscopy (13). It is now generally accepted that a low pH environment is essential for passage of diphtheria toxin through the membrane. Diphtheria toxin (6) and toxin fragments (12) alone have been shown to make channels in artificial lipid bilayers at low pH. However, the membranes of living cells are much more complicated than artificial lipid membranes. Thus, for entry of the toxin into living cells, it is likely that an additional cellular factor(s) plays a role. To study a cellular factor(s) required for entry of diphtheria toxin, we formed somatic cell hybrids from toxin-sensitive and -insensitive strains. The responses of the hybrids to diphtheria toxin suggest that the parent cells differ in the expression of a cellular factor(s) involved in entry of diphtheria toxin after it binds to its receptors.

MATERIALS AND METHODS

Cells: Human primary fibroblasts were derived from the forearm skin of a healthy young male. Ehrlich ascites tumor cells selected for 8-azaguanine resistance were passaged in the abdomen of ddO mice for >10 y and then cultured in vitro for more than 30 generations. Human fibroblasts and Ehrlich tumor cells were fused with UV-irradiated HVJ (Sendai virus, 500 hemagglutinating units [HAU] per milliliter), cultured in hypoxanthine-aminopterin-thymidine selection medium for 14 d, and then cloned in soft agar. All cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 10% new born calf serum.

The division of time of human fibroblasts was ~36 h, that of Ehrlich tumor cells was ~20 h, and that of hybrid cells was 20–24 h.

Toxins: Diphtheria toxin was purified by chromatography on DEAE cellulose (31). Cross-reacting material 45 of diphtheria toxin (CRM 45) (M_r ~45,000, a nontoxic mutant protein consisting of fragment A and an incomplete fragment B) was produced and purified as described previously (29). Fragment A of diphtheria toxin was prepared as described previously (30). *Pseudomonas aeruginosa* exotoxin A was kindly provided by Dr. B. H. Iglewski, University of Oregon Medical School. Ricin was extracted from decorticated ricinus seeds by a slight modification of the method described previously (21). A hybrid toxin containing fragment A of diphtheria toxin and wheat germ agglutinin (WGA-DA) was prepared from 0.5 mg of fragment A and 2 mg of WGA modified by *N*-succinimidyl-3-(2-pyridyl) propionate (Pharmacia Inc., Piscataway, NJ) according to the method of Collier et al. (2). A similar hybrid toxin (WGA-RA) was prepared from 0.5 mg of subunit A of ricin and 2 mg of WGA by the same method (2).

Virus: HVJ, Z strain, was propagated in embryonated eggs. The virus was purified from the allantoic fluid by differential centrifugation as described previously (22).

Monoclonal Anti–Diphtheria Toxin Antibody: Monoclonal anti–diphtheria toxin antibodies were isolated as described previously (9). Antibody #2 which we used here binds to diphtheria toxin but does not inhibit the toxin-receptor binding.

Assay of the Rate of Protein Synthesis in Cells Cultured with Toxins: Protein synthesis was measured as described by Mekada et al. (16), except that cells were incubated with each toxin for 24 h. The rate of protein synthesis of all cells was almost completely inhibited after 24 h exposure to 200 μg/ml of cycloheximide. Enhancement of cytotoxicity by low pH was carried out as described by Sandvig and Olsnes (26), except that we used CRM 45 instead of whole diphtheria toxin and the total incubation time with CRM 45 was 24 h.

Association of Iodinated Diphtheria Toxin with Cells: Diphtheria toxin (25 μg) was labeled with ^{125}I using chloramine T (25). The labeled toxin had a specific activity of 3.1×10^7 cpm/μg. The association of iodinated toxin with cells was examined in the presence of methylamine as reported by Mekada et al. (17).

Assay of Diphtheria Toxin Binding to Cells Using Iodinated Monoclonal Anti–Diphtheria Toxin Antibody: Monoclonal antibody (~20 μg) was labeled with ^{125}I using chloramine T (25). The labeled antibody had a specific activity of 1.18×10^7 cpm/μg. Cells were plated in 35-mm plastic dishes and cultured for 24 h at 37°C. Diphtheria toxin (0.5 μg) and the labeled antibody (3.0 μg) were mixed and incubated for 30 min at 37°C. Cells were washed and 0.5 ml of the mixture was added to each dish so that the final concentration of diphtheria toxin was 0.1 μg/ml and that of the antibody was 0.6 μg/ml. Each dish was kept at 4°C for 9 h. Cells were washed and then dissolved in 0.5 ml of 0.1 N NaOH. The wells were rinsed with an additional 0.5 ml of 0.1 N NaOH, and the radioactivity in the lysate was determined.

Assay of Iodinated WGA to Cell Surfaces: WGA (100 μg) was labeled with ^{125}I using Iodo-beads (Pierce Chemical Co., Rockford, IL). The specific activity of the labeled WGA was 1.7×10^5 cpm/μg. Cells were plated in 24-well trays and cultured overnight at 37°C. The cells were washed and 5.8 μg/ml of the labeled WGA was added to cells with or without 2.1 mg/ml of unlabeled WGA. Then the cells were chilled and incubated at 4°C for 10 h. The amount of ^{125}I-WGA associated with the cells was determined as described in the assay for diphtheria toxin binding.

ADP-Ribosylation of EF-2 in Cell Extracts: This was carried out using a slight modification of the method described previously (20).

Introduction of Diphtheria Toxin into Cells using HVJ: Cells were washed with PBS, and 3×10^6 cells were suspended in 0.5 ml of balanced salt solution (140 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4 buffered with 10 mM Tris-HCl at pH 7.6) containing 2 mM $CaCl_2$ and 5 μg/ml of cytochalasin D (dissolved in dimethylsulfoxide at a concentration 1 mg/ml) to inhibit cell-to-cell fusion (19). Then 2,000 HAU of UV-irradiated HVJ was added to the cell suspension. The mixture was kept at 4°C for 10 min and then incubated at 37°C for 40 min with shaking in a water-bath. After incubation, MEM supplemented with 10% new born calf serum was added to the mixture, and cells were washed twice. Cells were inoculated to 24-well plates at a density of 10^5 cells/well, and diphtheria toxin was added. After incubation for 24 h, the rate of protein synthesis was measured.

Isozyme and Karyotype Analyses of Each Hybrid: Isozyme analyses were carried out as described by Someren et al. (27) and Deluca et al. (4). Chromosomes in each hybrid were identified after 33258 Hoechst-quinacrine mustard staining (33).

RESULTS

Sensitivity of Various Cells to Diphtheria Toxin

The rate of protein synthesis of human fibroblasts was almost completely inhibited after 24-h exposure to 2×10^{-3} μg/ml of diphtheria toxin, but the rate of protein synthesis of Ehrlich tumor cells was >85% of the control rate even at 20 μg/ml of diphtheria toxin (Fig. 1). Fig. 1 also shows the effects of diphtheria toxin on protein synthesis of several hybrid clones. We could divide the hybrids into three groups on the basis of diphtheria toxin sensitivity. Group A hybrids, represented by N-E 8 in Fig. 1, were as sensitive to diphtheria toxin as human fibroblasts, and the rate of protein synthesis was <10% of the control value after 24-h exposure to 2×10^{-3} μg/ml of diphtheria toxin. In Group B hybrids, represented by N-E 32, the rate of protein synthesis was not inhibited by 2×10^{-3} μg/ml of diphtheria toxin, but inhibition was observed at 2×10^{-2} μg/ml and was almost complete at 2×10^{-1} μg/ml of the toxin. Group C hybrids, represented by N-E 26, were as resistant as Ehrlich tumor cells, and protein synthesis was scarcely inhibited even at 2 μg/ml of the toxin. Of 37 hybrids isolated, 10 hybrids were in Group A, 24 in Group B, and 3 in Group C. N-E 32 was subcloned and 84 subclones were isolated. One Group C hybrid, N-E 32–70, was obtained, but no Group A hybrid was found. Karyotype analyses revealed that all the hybrids retained some human chromosomes. Next, we examined the toxin binding sites of the hybrid cells using ^{125}I-diphtheria toxin or ^{125}I-antibody #2.

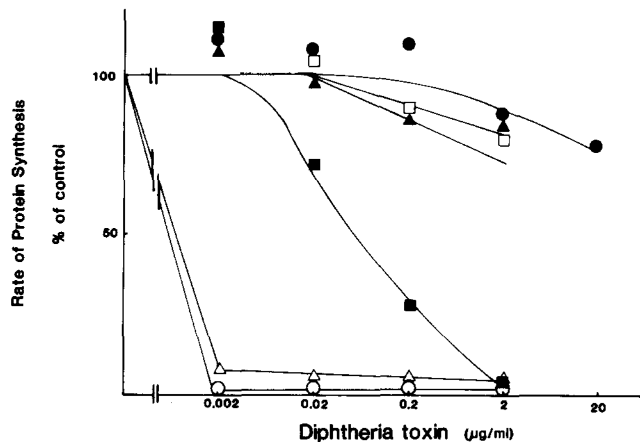


FIGURE 1 Effect of diphtheria toxin on the rate of protein synthesis of various cell lines. Cells were inoculated in 24-well trays at a density of 10^5 per well and cultured overnight. The medium was removed and normal MEM containing various concentrations of diphtheria toxin and 10% new born calf serum was added. After incubation for 24 h at 37°C, the medium was removed and cells were washed with PBS. Then 0.5 ml of growth medium with one-tenth of the normal concentration of leucine and 1 μCi of [^{3}H] leucine was added to each well. After incubation for 1 h at 37°C, the medium was removed and 0.1 N NaOH was added. The lysates were collected and treated with 20% trichloroacetic acid, and the precipitates were collected on a glass filter, dried, and counted in a liquid scintillation system. The rate of protein synthesis in each culture is expressed as a percentage of the value obtained in control cultures without toxin. O, human fibroblasts; ●, Ehrlich tumor cells; △, N-E 8 (Group A); ■, N-E 32 (Group B); ▲, N-E 26 (Group C); □, N-E 32-70 (Group C, a subclone of N-E 32).

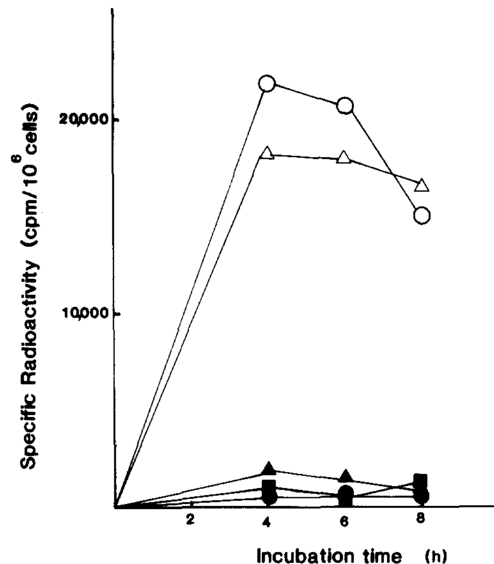


FIGURE 2 The association kinetics of ^{125}I-diphtheria toxin with the cells at 37°C. Cells were inoculated in 60-mm petri dishes and cultured for 2 d. The medium was replaced by MEM containing 10% newborn calf serum. ^{125}I-toxin (0.1 μg/ml) and methylamine (20 mM) were added, and cells were incubated at 37°C for the indicated times. Then, the cells were washed three times with PBS containing 0.2 mM $CaCl_2$ and 0.2 mM $MgCl_2$ and dissolved in 0.1 N NaOH, and the cell lysates were counted in a γ counter. In this experiment, values are shown as specific counts per 10^6 cells, determined by subtracting counts obtained in the presence of a 250-fold excess of unlabeled toxin. O, human fibroblasts; ●, Ehrlich tumor cells; △, N-E 8; ▲, N-E 26; ■, N-E 32.

Binding of Diphtheria Toxin to Cell Surfaces

First we studied the association of ^{125}I-diphtheria toxin with cells in the presence of methylamine. Since the number of toxin binding sites is quite low on most cells, we measured the accumulation of toxin both on cell surfaces and within vesicles in the presence of methylamine, which blocks both the inhibitory effect of the toxin on protein synthesis and degradation of radioactive toxin. Mekada et al. reported that the maximum association of radiolabeled toxin to Vero cells in the presence of methylamine is about seven to eight times that in the absence of methylamine (17). As shown in Fig. 2, in human fibroblasts and hybrid N-E 8 a significant amount of radioactivity was associated with the cells, while in Ehrlich tumor cells and hybrid N-E 26 and N-E 32 the amount of radioactivity was <2,000 cpm/10^6 cells.

The association of ^{125}I-toxin measured in the presence of methylamine is the result of the accumulation of the toxin both on the surface of the cells and within endocytotic vesicles. Therefore, if cells that possess toxin-receptors have low endocytotic activity, the association of ^{125}I-toxin with the cells may be reduced. To measure the specific binding of the toxin to cells in the absence of endocytosis, we used an ^{125}I-labeled monoclonal anti–diphtheria toxin antibody. ^{125}I-monoclonal antibody #2 can bind to Vero cells in the presence of the toxin but can not bind in the absence of the toxin (9). At 4°C the radioactivity specifically associated with cells using radioactive antibody and unlabeled diphtheria toxin was about five times greater than the radioactivity associated with cells in radioactive diphtheria toxin–binding experiments. As seen in Table I, hybrid N-E 8 and human fibroblasts bind a significant amount of diphtheria toxin, but hybrid N-E 26 and N-E 32 and Ehrlich tumor cells have little binding activity.

TABLE I
Comparison of the Levels of Diphtheria Toxin Binding Activity to Cells Using Monoclonal Anti–Diphtheria Toxin Antibody

Cell	^{125}I-radioactivity	No. of cells	^{125}I-radioactivity	% binding activity*
	cpm	*×10⁵*	*cpm/10⁶ cells*	
Human fibroblasts	4,424 ± 500	3.23	13,697	100
Ehrlich tumor cells	1,629 ± 347	6.23	2,615	0
N-E 8	2,964 ± 152	3.45	8,591	53.9
N-E 26	2,104 ± 301	6.60	3,188	5.2
N-E 32	1,890 ± 224	6.53	2,894	2.5

This experiment was carried out as described under Materials and Methods. The number of cells was counted after incubation for 9 h at 4°C and the final value was expressed as ^{125}I radioactivity per 10^6 cells. The binding activity of each cell type is also expressed as a percentage of the difference between the value for human fibroblasts and that for Ehrlich tumor cells.
* Since Ehrlich tumor cells bind little ^{125}I-diphtheria toxin, as shown in Fig. 2, the binding activity of Ehrlich tumor cells was taken as 0, and the difference between the binding activity of human fibroblasts and that of Ehrlich tumor cells was taken as 100%.

Diphtheria toxin sensitivity was mapped to human chromosome 5 by Creagan, Chen, and Ruddle (3). We analyzed the karyotype of our hybrids and identified human arylsulfatase A and hexosaminidase B, which are located on human chromosome 5 (data not shown). Hybrid N-E 8 retained human chromosome 5, but N-E 26 and N-E 32 had lost the chromosome. These facts suggest that the difference in the

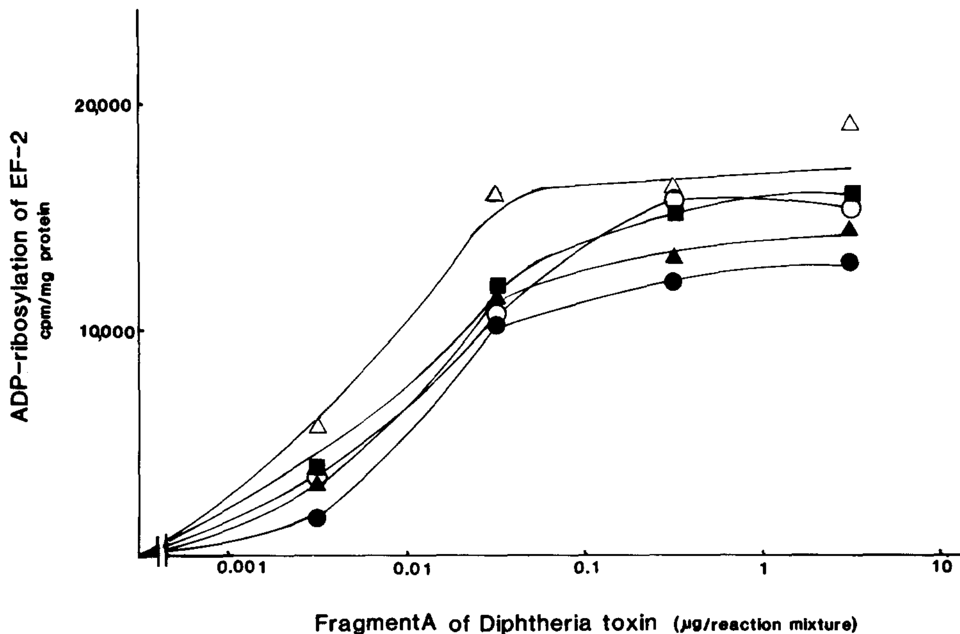


FIGURE 3 ADP-ribosylation of EF-2 in cell extracts by various concentrations of fragment A of diphtheria toxin. About 10^7 cells were collected and washed with hypotonic buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$). After being frozen and thawed, cells were suspended in 1.6 ml hypotonic buffer containing 1 mM phenylmethylsulfonyl fluoride, 7.5 mM dithiothreitol, and 0.75% Nonidet P-40 and the lysate was clarified by centrifugation at 2,000 g for 10 min. The lysate was dialyzed against buffer A (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 2.5 mM EDTA, 5% glycerol, and 1.5 mM dithiotreitol) for 12 h at 4°C. Then 180 μl of NAD mixture (^{14}C-NAD, 30 mM dithiotreitol, and 10 mM thymidine) was added to 0.9 ml of dialyzed lysate. Various concentrations of fragment A (3 μl) were added to 86-μl portions of each reaction mixture. After incubation for 15 min at 37°C, 0.2 ml of 10% trichloroacetic acid was added and the precipitate was trapped on a glass filter, dried, and counted in a liquid scintillation system. Protein content of each lysate was measured by the method of Lowry et al. ○, human fibroblasts; ●, Ehrlich tumor cells; △, N-E 8; ▲, N-E 26; ■, N-E 32.

sensitivity to diphtheria toxin between N-E 8 and N-E 32 is due to the activity of the gene(s) coded on chromosome 5, which was suggested to specify diphtheria toxin binding. The difference between N-E 26 and N-E 32 may be attributed to a different cellular factor(s).

ADP-Ribosylation of EF-2 in Cell Extracts

Since Ehrlich tumor cells and N-E 26 were resistant to diphtheria toxin, we determined whether EF-2 of these cells was ADP-ribosylated by fragment A of diphtheria toxin. Fragment A of diphtheria toxin has an extremely specific NAD:EF-2-ADP ribose transferase activity (8, 10). Fig. 3 shows fragment A-dependent ADP-ribosylation in cell extracts treated with various concentrations of fragment A. EF-2 of all cell types was similarly ADP-ribosylated by fragment A.

To get further confirmation that the EF-2 of the resistant cell lines was susceptible to ADP-ribosylation in the cells, we introduced diphtheria toxin into Ehrlich tumor cells and hybrid N-E 26 using HVJ, and measured the rate of protein synthesis. Proteins can be introduced directly into the cytoplasm of cells using HVJ (28, 32). Fig. 4 shows that the protein synthesis of both cell lines began to be inhibited at 2×10^{-3} μg/ml and that the rate of protein synthesis became <10% at 20 μg/ml of the toxin. These results reveal that all the cells are susceptible to diphtheria toxin that is introduced directly into the cytoplasm.

Therefore, the resistance of Ehrlich tumor cells and hybrid N-E 26 to diphtheria toxin is probably due to defects in the uptake of diphtheria toxin, not to defects in the machinery of protein synthesis. Next, we examined the effects of pH on the sensitivity of the hybrid cells to CRM 45.

Effect of pH on the Sensitivity to CRM 45

Sandvig and Olsnes (26) reported that diphtheria toxin entry into cells is facilitated by low pH even in the presence of ammonium chloride. They suggested that at low pH diphtheria toxin penetrates directly through the surface membrane of the cell, while at neutral pH diphtheria toxin penetrates through the membrane of endocytotic vesicles after the pH in the vesicle is reduced. To determine whether the differences in sensitivity to diphtheria toxin were due to differences in the exposure of toxin to low pH, we examined whether entry of diphtheria toxin into the cytoplasm of the resistant cells was facilitated by low pH (Table II).

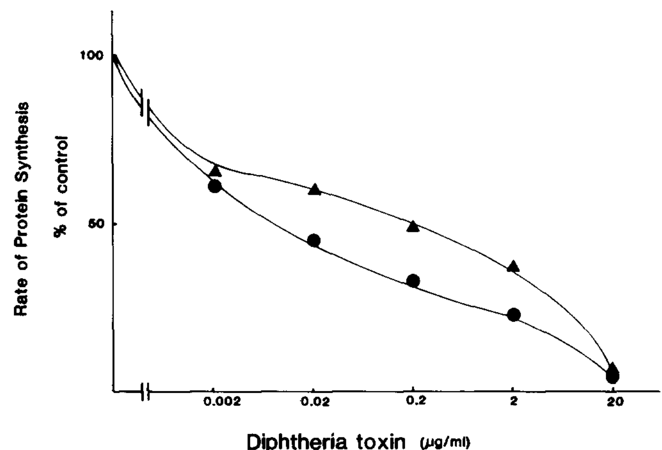


FIGURE 4 Effect of diphtheria toxin on the rate of protein synthesis of cells after treatment with HVJ. The treatment with HVJ was carried out as described under Materials and Methods. The rate of protein synthesis of cells was measured as described in the legend of Fig. 1. ▲, N-E 26; ●, Ehrlich tumor cells.

To exclude the effects of receptor-mediated endocytosis, we used CRM 45 instead of whole diphtheria toxin. CRM 45 does not bind diphtheria toxin receptors because of its incomplete B fragment (29). A 30-min shift to low pH (pH = 4.5) increased the sensitivity to CRM 45 of hybrids N-E 8 and N-E 32 and human fibroblasts, but hybrid N-E 26 and Ehrlich

tumor cells were still resistant to CRM 45 even at low pH. The resistance at low pH was almost the same as that at neutral pH.

These results suggest that Ehrlich tumor cells and hybrid N-E 26 lack a cellular factor(s) involved in entry of fragment A after diphtheria toxin binds to its surface receptors, while hybrids N-E 8 and N-E 32 and human fibroblasts retain it. Such a cellular factor(s) may be required for the uptake of a variety of macromolecules, or only of fragment A of diphtheria toxin. We therefore investigated sensitivity of these hybrids to *Pseudomonas* exotoxin A, ricin toxin, and hybrid toxins.

Sensitivity of Cells to Pseudomonas Toxin and Ricin Toxin

Pseudomonas toxin inhibits cellular protein synthesis by ADP-ribosylation of EF-2 in the same manner as diphtheria toxin (11), but it has different receptors. Sensitivity to *Pseudomonas* toxin is not correlated with sensitivity to diphtheria toxin (18). Fig. 5 *a* shows that hybrids N-E 8 and N-E 32 and human fibroblasts were equally sensitive to *Pseudomonas* toxin, but hybrid N-E 26 and Ehrlich tumor cells were as resistant to *Pseudomonas* toxin as to diphtheria toxin.

Fig. 5 *b* shows the sensitivity of the cells to ricin toxin. It is known that ricin toxin binds to galactose residues on cell surfaces and that the A subunit of the toxin penetrates into the cytoplasm where it inhibits protein synthesis (23). All the cells were sensitive to ricin toxin (Fig. 5 *b*), so that entry of ricin toxin is obviously different from entry of diphtheria toxin and *Pseudomonas* toxin.

Sensitivity of Cells to Hybrid Toxins

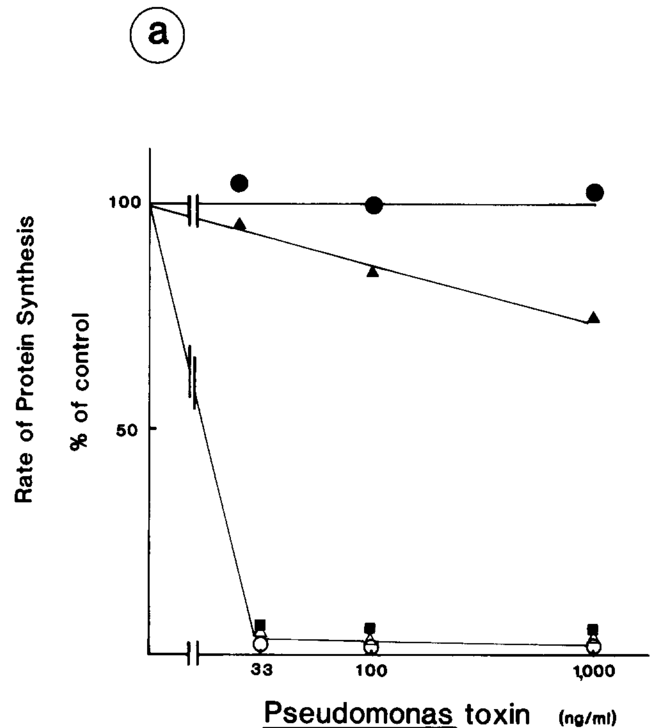
First, we used WGA-DA hybrid toxin to analyze the uptake of diphtheria toxin into cells without the participation of its receptors (Fig. 6). Human fibroblasts and hybrids N-E 8 and N-E 32 had similar sensitivities to WGA-DA hybrid toxin, but hybrid N-E 26 and Ehrlich tumor cells were insensitive, and the rate of protein synthesis was >60% of the control value even at 5 μg/ml of WGA-DA. Although this result suggests that entry of fragment A into these cells is relatively inefficient regardless of the binding moiety of the toxin, it is possible that the effect is due to low endocytotic activity, and that reduced endocytosis causes the resistance to WGA-DA hybrid toxin in N-E 26 and Ehrlich tumor cells. The following experiments were carried out to exclude the possibility of reduced endocytosis in N-E 26 and Ehrlich tumor cells.

The binding of ^{125}I-labeled WGA to cell surfaces was mea-

TABLE II
Effect of pH on the Cytotoxicity of CRM 45

Cell	CRM 45 conc.	Rate of protein synthesis	
		pH 7.5	pH 4.5
	µg/ml	%	
Human fibroblasts	0.5	98.6	13.0
Ehrlich tumor cells	50	74.1	71.8
N-E 8	5.0	72.3	44.2
N-E 26	50	86.8	85.0
N-E 32	5.0	72.8	30.4

Various concentrations of CRM 45 were added to cells in 24-well trays. After incubation for 4 h at 37°C, the medium was changed to phosphate buffer adjusted to pH 4.5 or 7.5 (the adjustment was made by adding sufficient H_3PO_4 to a buffer containing 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 3.2 mM Na_2HPO_4, and 137 mM NaCl). The appropriate amounts of CRM 45 were added and the cells were further incubated at 37°C for 30 min. The buffer was then removed, and growth medium containing the same concentrations of CRM 45 was added. After incubation for 19.5 h at 37°C, the rate of protein synthesis was measured as described in the legend of Fig. 1.



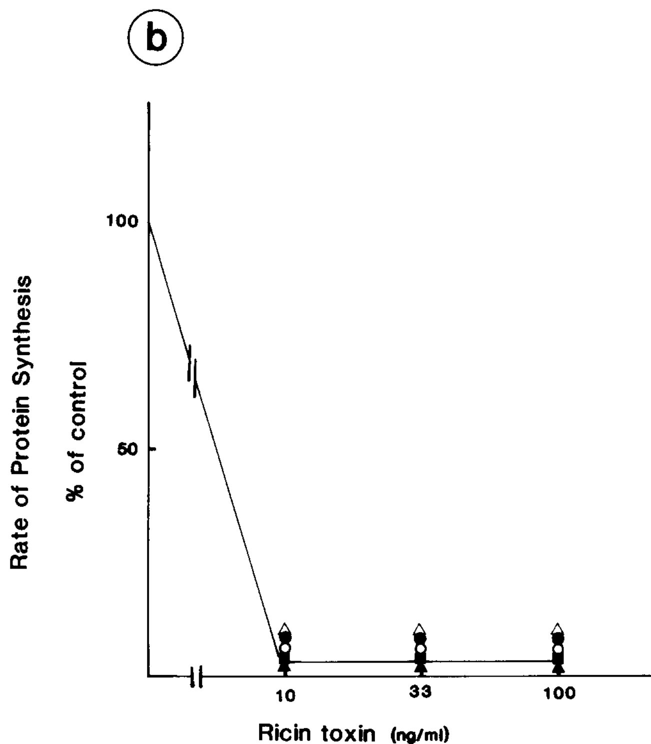


FIGURE 5 Effect of *Pseudomonas* toxin (*a*) or ricin toxin (*b*) on the rate of protein synthesis of cells. Various concentrations of each toxin were added to cells in 24-well trays, and cells were incubated for 24 h at 37°C. Then the rate of protein synthesis was measured as described in the legend of Fig. 1. ○, human fibroblasts; ●, Ehrlich tumor cells; △, N-E 8; ▲, N-E 26; ■, N-E 32.

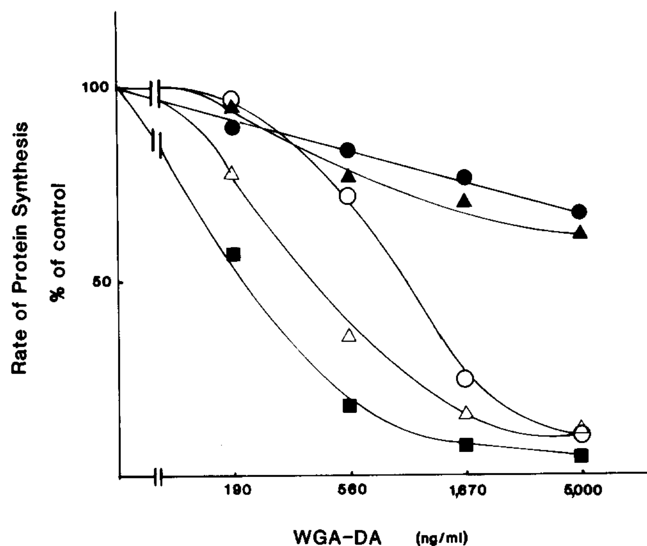


FIGURE 6 Effect of WGA-DA hybrid toxin on the rate of protein synthesis of various cells. Various concentrations of WGA-DA hybrid toxin were added to cells in 24-well trays and cells were incubated for 24 h at 37°C. Then the rate of protein synthesis was measured as described in the legend of Fig. 1. ○, human fibroblasts; ●, Ehrlich tumor cells; △, N-E 8; ▲, N-E 26; ■, N-E 32.

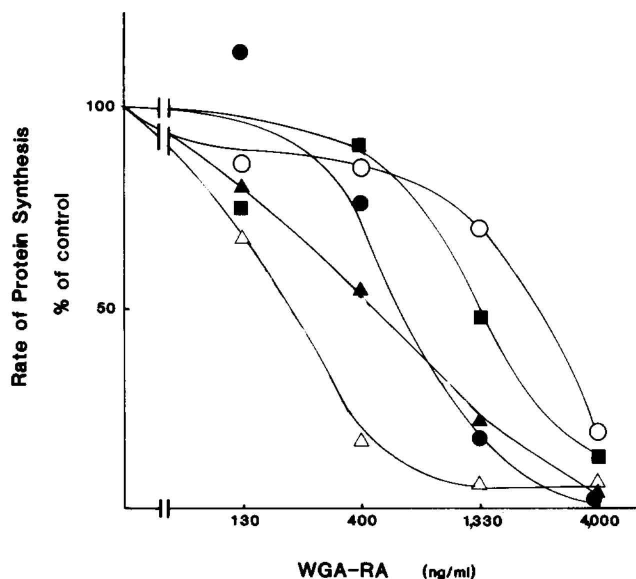


FIGURE 7 Effect of WGA-RA hybrid toxin on the rate of protein synthesis of various cells. All procedures were the same as in the case of WGA-DA hybrid toxin. ○, human fibroblasts; ●, Ehrlich tumor cells; △, N-E 8; ▲, N-E 26; ■, N-E 32.

TABLE III
Binding Activity of ^{125}I-WGA to Cell Surfaces

	^{125}I-WGA bound to cells
	cpm/10^5 cells
Human fibroblasts	5,130
Ehrlich tumor cells	8,453

^{125}I-WGA bound to cell surfaces was expressed as the difference between the radioactivity in the presence and absence of unlabeled WGA. In this table, the binding activity of ^{125}I-WGA is expressed as cpm per 10^5 cells.

sured in Ehrlich ascites tumor cells and human fibroblasts. The values are shown in the Table III. The resistance of Ehrlich ascites tumor cells and N-E 26 to WGA-DA hybrid toxin is not due to a deficiency of WGA receptors.

We then investigated the sensitivity of these cells to another hybrid toxin, WGA-RA (Fig. 7). The rate of protein synthesis of hybrids N-E 8 or N-E 26 or Ehrlich ascites tumor cells was reduced to <10% of control by 400 μg/ml of WGA-RA. All the hybrids and Ehrlich ascites tumor cells were more sensitive to the WGA-RA than human fibroblasts were. This fact suggests that the endocytotic activity via the WGA receptors was not lower in N-E 26 and Ehrlich ascites tumor cells than in human fibroblasts.

Therefore, these results suggest that a cellular factor(s) other than diphtheria toxin receptors may be involved in entry of diphtheria toxin into the cytoplasm and Ehrlich ascites tumor cells and that hybrid N-E 26 may have lost such a cellular factor(s).

DISCUSSION

Diphtheria toxin–resistant cells were divided into two main classes and their subclasses by Moehring and Moehring (20) and Draper et al. (7). Class I mutants have a mutation in the uptake of toxin, whereas Class II mutants have a mutation in the machinery of protein synthesis. The EF-2 of Class I mutants is susceptible to ADP-ribosylation by fragment A of diphtheria toxin, and that of Class II mutants is not ADP-ribosylatable. In this report we described three different groups of hybrids obtained from human fibroblasts and Ehrlich ascites tumor cells. Group A hybrids are relatively sensitive to diphtheria toxin, retain toxin binding sites, and are thus similar to wild-type human cells. The EF-2 of Group B and C hybrids is ADP-ribosylatable, so these hybrids resemble Class I mutants. The difference in resistance between Group B and C hybrids does not seem to be due to a difference in diphtheria toxin binding sites. This suggests that some factor(s) other than binding sites is involved in entry of diphtheria toxin into the cytoplasm after initial binding of the toxin to the surface of sensitive cells, and that Group B hybrids possess such a factor(s) but Group C hybrids have lost it. As described in Results, hybrid N-E 32 was subcloned and one hybrid of 84 subclones, N-E 32–70, was found to belong to Group C (Fig. 1). This suggests that hybrid N-E 32–70 lost the factor(s) involved in diphtheria toxin entry and that the factor(s) may be under genetic control.

When cells were treated with a hybrid toxin composed of diphtheria toxin fragment A and WGA, so that entry of fragment A was independent of diphtheria toxin receptors, Group A and B were similarly sensitive to the hybrid toxin (Fig. 6), but Group C was insensitive. This result supported our hypothesis that the resistance of Group B hybrids is due to a lack of toxin receptors, while the resistance of Group C hybrids can not be completely accounted for in this manner.

If Group C cells simply have a low rate of endocytosis, the same result could be obtained. But, as shown in Fig. 7, all the hybrids and Ehrlich tumor cells are more sensitive to another hybrid toxin, WGA linked to subunit A of ricin toxin, than are human fibroblasts. These results indicate that the resistance of N-E 26 and Ehrlich tumor cells to WGA-DA is due to the loss of a cellular factor(s) that is somewhat specific for entry of fragment A through plasma membrane into the cytoplasm.

Another possibility is that Group C hybrids and Ehrlich tumor cells are more resistant because the toxin is not exposed

to low pH. However the sensitivity of Ehrlich tumor cells and hybrid N-E 26 to CRM 45 was not increased by exposure to low pH. Sandvig and Olsnes (26) suggested that diphtheria toxin may penetrate directly through plasma membrane when cells are exposed to low pH. If this is the case, the participation of endocytotic uptake can be excluded at low pH. Thus this provides further evidence that the greater resistance of Ehrlich tumor cells and hybrid N-E 26 to diphtheria toxin can not be explained by reduced endocytosis.

Recently, Robbins et al. (24) reported that some diphtheria toxin-resistant Chinese hamster ovary cell mutants are deficient in the uptake of lysosomal enzymes and resistant to Sindvis virus. They suggested that since the mutants have diphtheria toxin receptors, the defect in the mutants stems from an inability to deliver virus, diphtheria toxin, and lysosomal hydrolases to an acidic compartment. The sensitivity of these mutants to diphtheria toxin was increased by exposure to pH 4.5 for 30 min. The mutants thus differ from the Group C hybrids. The Chinese hamster ovary cell mutants were resistant to diphtheria toxin and sensitive to *Pseudomonas* toxin and ricin toxin, while the Group C hybrids are resistant to both diphtheria toxin and *Pseudomonas* toxin, but remain sensitive to ricin. The receptors for diphtheria toxin and *Pseudomonas* toxin differ, so Ehrlich tumor cells and hybrid N-E 26 must have a defect in the receptors for *Pseudomonas* toxin as well as in diphtheria toxin receptors, or some cellular factor(s) involved in entry of *Pseudomonas* toxin. Didsbury et al. (5) also isolated two groups of diphtheria toxin-resistant mutants which had defects in some steps subsequent to binding of the toxin. The possibility that the defect is in a cellular factor(s) involved in entry of both *Pseudomonas* toxin and diphtheria toxin is interesting in that the two toxins have the same enzymic activity. Such a cellular factor(s) may be located in plasma membrane and bound to fragment A of diphtheria toxin by its enzymic activity.

Entry of diphtheria toxin into the cytoplasm can be divided into at least three steps. The first step is binding of toxin to the cell surface; the second, receptor-mediated endocytosis; and the third, passage of fragment A into the cytoplasm through lipid bilayer. It should be possible to obtain mutants that have a defect in each step. Compared with normal human cells, hybrid N-E 32 has a defect in the first step. The Chinese hamster ovary cell mutants described by Robbins et al. have a defect in the second step. Hybrid N-E 26 and Ehrlich ascites tumor cells have defects in at least the first and the third step, but it is not clear whether they have a defect in the second step.

The authors thank Dr. M. R. Moynihan for his helpful suggestions concerning this work and his critical reading of the manuscript and Dr. M. C. Yoshida for his kind advice about chromosomal analyses.

Received for publication 29 July 1983, and in revised form 1 September 1983.

REFERENCES

1. Boquet, P., M. S. Silverman, A. M. Pappenheimer, Jr., and W. Vernon. 1976. Binding of Triton X-100 to diphtheria toxin cross-reacting material 45, and their fragments. *Proc. Natl. Acad. Sci. USA.* 73:4449–4453.
2. Cowley, D. B., H. R. Hershman, D. G. Gilliland, and R. J. Collier. 1980. Epidermal growth factor-toxin A chain conjugate: EGF-ricin A is a potent toxin while EGF-diphtheria fragment A is non toxic. *Cell.* 22:563–570.
3. 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. USA.* 72:2237–2241.
4. Deluca, C., J. A. Brown, and T. B. Shows. 1979. Lysosomal arylsulfatase deficiencies in humans: chromosome assignments for arylsulfatase A and B. *Proc. Natl. Acad. Sci. USA.* 76:1957–1961.
5. Didsbury, J. R., J. M. Moehring, and T. J. Moehring. 1983. Binding and uptake of diphtheria toxin by toxin-resistant Chinese hamster ovary and mouse cells. *Mol. Cell Biol.* 3:1283–1294.
6. Donovan, J. J., M. I. Simon, R. K. Draper, and M. Montal. 1981. Diphtheria toxin forms transmembrane channels in planer lipid bilayers. *Proc. Natl. Acad. Sci. USA.* 78:172–176.
7. Draper, R. K., D. Chin, D. Eurey-Owens, I. E. Scheffler, and M. I. Simon. 1979. Biochemical and genetic characterization of three hamster cell mutants resistant to diphtheria toxin. *J. Cell Biol.* 83:116–125.
8. Gill, D. M., A. M. Pappenheimer, Jr., R. Brown, and J. J. Kurnick. 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.
9. Hayakawa, S., T. Uchida, E. Mekada, M. R. Moynihan, and Y. Okada. 1983. Monoclonal antibody against diphtheria toxin: effects on toxin binding and entry into cells. *J. Biol. Chem.* 258:4311–4317.
10. 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.
11. Iglewski, B. H., and D. Kabat. 1975. NAD-dependent inhibition of protein synthesis by *Pseudomonas aeruginosa* toxin. *Proc. Natl. Acad. Sci. USA.* 72:2284–2288.
12. Kagan, B. L., and A. Finkelstein. 1979. Channels formed in planer lipid membrane by mutant diphtheria toxin molecules. *Biophys. J.* 25:181*a*. (Abstr.)
13. 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.
14. Kim, K., and N. B. Groman. 1965. *In vitro* inhibition of diphtheria toxin action by ammonium salts and amines. *J. Bacteriol.* 90:1552–1556.
15. 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.
16. 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.
17. 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.
18. 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.
19. Miyake, Y., J. Kim, and Y. Okada. 1978. Effects of Cytochalasin D on fusion of cells by HVJ (Sendai virus): cell-cell fusion is separable from cell-virus fusion. *Exp. Cell Res.* 116:167–178.
20. Moehring, J. M., and T. J. Moehring. 1979. Characterization of the diphtheria toxin-resistance system in Chinese hamster ovary cells. *Somat. Cell Genet.* 5:453–468.
21. Nicolson, G. L., and J. Plausteins. 1972. The interaction of ricinus communis agglutinin with normal and tumor cell surfaces. *Biochem. Biophys. Acta.* 266:543–547.
22. Okada, Y., and F. Murayama. 1966. Requirement of calcium ions for cell fusion reaction of animal cells by HVJ. *Exp. Cell Res.* 44:527–551.
23. Olsnes, S., K. Refsnes, and A. Pihl. 1974. Mechanism of action of the toxic lectins abrin and ricin. *Nature (Lond.).* 249:627–631.
24. Robbins, A. R., S. S. Peng, and J. L. Marshall. 1983. Mutant Chinese hamster ovary cells pleiotropically defective in receptor-mediated endocytosis. *J. Cell Biol.* 96: 1064–1071.
25. Roth, J. 1975. Methods for assessing immunologic and biologic properties of iodinated peptide hormones. *Methods Enzymol.* 37B:223–233.
26. Sandvig, K., and S. Olsnes. 1980. Diphtheria toxin entry into cells is facilitated by low pH. *J. Cell Biol.* 87:828–832.
27. Someren, H., H. B. Henegouwen, W. Los, E. Wurzer-Figurelli, B. Doppert, M. Vervloet, and P. M. Khan. 1974. Enzyme electrophoresis on cellulose acetate gel. II. Zymogram patterns in man-Chinese hamster somatic cell hybrids. *Humangenetik.* 25:189–201.
28. Tanaka, K., M. Sekiguchi, and Y. Okada. 1975. Restoration of ultraviolet-induced unscheduled DNA synthesis of Xeroderma Pigmentosum cells by the concomitant treatment with T4 endonuclease V and HVJ (Sendai virus). *Proc. Natl. Acad. Sci. USA.* 72:4071–4075.
29. Uchida, T., D. M. Gill, and A. M. Pappenheimer, Jr., 1971. Mutation in the structural gene for diphtheria toxin carried by temperate phage beta. *Nature New Biology.* 233:8–11.
30. 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.
31. 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.
32. Yamaizumi, M., T. Uchida, and Y. Okada. 1979. Macromolecules can penetrate the host cell membrane during the early period of incubation with HVJ (Sendai virus). *Virology.* 95:218–221.
33. Yoshida, M. C., T. Ikeuchi, and M. Sasaki. 1975. Differential staining of parental chromosomes in interspecific cell hybrids with a combined quinacrine and 33258 Hoechst technique. *Proc. Jap. Academy.* 51:184–187.