INFECTION AND IMMUNITY, Sept. 1983, p. 998–1009
0019-9567/83/090998-12$02.00/0

Vol. 41, No. 3

Strains of CHO-K1 Cells Resistant to *Pseudomonas* Exotoxin A and Cross-Resistant to Diphtheria Toxin and Viruses

JOAN M. MOEHRING* AND THOMAS J. MOEHRING

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

Received 4 April 1983/Accepted 13 June 1983

We have investigated two phenotypically distinct types of mutants of CHO-K1 cells that are resistant to *Pseudomonas* exotoxin A due to a defect in the delivery of active toxin to the target site in the cell, elongation factor 2. Both types contain normal levels of toxin-sensitive elongation factor-2. Hybridization studies have shown that these cells fall into two distinct complementation groups. One group, designated DPV^r, is resistant to *Pseudomonas* toxin, diphtheria toxin, and four enveloped RNA viruses. This group is also hypersensitive to ricin. The resistance of this group is apparently related to a defect in a mechanism for the acidification of endocytic vesicles. The other group, designated PV^r, is resistant to *Pseudomonas* toxin and to three enveloped RNA viruses. The resistance of this group appears to be related to a defect in a cellular mechanism required for the maturation of Sindbis virus that is likewise required for the entry of active *Pseudomonas* toxin.

Pseudomonas exotoxin A, like diphtheria toxin, inhibits protein synthesis in sensitive cells by catalyzing the transfer of the ADP-ribose moiety of NAD^+ to elongation factor 2 (EF-2), thereby inactivating it (14, 15). Although these two toxins have the same mode of action in the cell, they do not utilize the same cell receptors or mechanism of uptake (14, 27, 29, 37, 45). The selection, from sensitive cell lines, of several toxin-resistant phenotypes with resistance to the action of diphtheria toxin has been reported (6, 11, 29, 33). We now report the isolation of mutants of Chinese hamster ovary cells that possess different degrees of resistance to *Pseudomonas* exotoxin A. These mutants, like the diphtheria toxin-resistant mutants, may be separated into two general classes.

In one class, resistance is at the level of the target enzyme EF-2. The resistant cell strains of this class possess EF-2 that is resistant to toxin-catalyzed ADP ribosylation. All of the cell strains of this class that we have isolated using *Pseudomonas* toxin are also resistant to diphtheria toxin. They are indistinguishable from strains isolated by diphtheria toxin selection, and we have discussed this class of mutants elsewhere (30, 32, 35).

In this report, we will present only studies on mutant cell strains that are resistant to *Pseudomonas* toxin due to an alteration in the binding, uptake, or delivery of the toxin to the target site in the cell. These strains possess a normal complement of sensitive EF-2. We have identified strains that are altered at different steps in the uptake of the toxin. Some are resistant to *Pseudomonas* toxin, their response to other toxins is not altered, but they are resistant to certain enveloped viruses. Other strains are resistant to *Pseudomonas* and diphtheria toxins, as well as several enveloped viruses, and possess an enhanced sensitivity to the plant toxin ricin.

MATERIALS AND METHODS

Cells, media, and culture conditions. CHO-K1, Chinese hamster ovary cells, auxotrophic for proline, were obtained from the American Type Culture Collection. *Pseudomonas* toxin-resistant mutants were selected from this line as described below. The cell strains used in these studies and their origins are described in Table 1. The cells were routinely maintained in monolayer cultures 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. This medium was used in all assays unless otherwise stated.

Toxins and lectins. Purified *Pseudomonas* exotoxin A was the gift of S. H. Leppla (U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Md.) (18). Diphtheria toxin was obtained from Connaught Medical Research Laboratories, Toronto, Ontario, Canada (29). Ricin was the gift of S. Olsnes (Norsk Hydro's Institute for Cancer Research, Oslo, Norway). The sources of other plant lectins were: wheat germ agglutinin, Sigma Chemical Co., St. Louis, Mo.; concanavalin A, Miles Laboratories, Inc.,

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 (32)
RPE.8	DT^s, PT^r, PRO^-	Selected from CHO-K1
RPE.23	DT^s, PT^r, PRO^-	Selected from CHO-K1
RPE.40	DT^s, PT^r, PRO^-	Selected from CHO-K1
RPE.28	DT^r, PT^r, PRO^-	Selected from CHO-K1
RPE.44	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

[a] DT, Diphtheria toxin; PT, *P. aeruginosa* exotoxin A; PRO, proline; HPRT, hypoxanthine phosphoribosyl transferase; s, sensitive; r, resistant.

[b] ATCC, American Type Culture Collection.

Elkhart, Ind.; phytohemagglutinin (PHA), Wellcome Research Laboratories, Beckenham, England. The toxins were stored at −70°C in small portions, and their activity was verified regularly by assays on wild-type cells.

Selection of *Pseudomonas* toxin-resistant cells. CHO-K1 or P1R2 cells were exposed to 300 μg of ethyl methane sulfonate per ml of complete F12 for 16 to 18 h. This treatment caused 30 to 50% killing of the cells. The mutagenized cells were then maintained in complete F12 for an expression period of 5 to 6 days.

The cells were then seeded in 60-mm plastic tissue culture dishes, in medium containing 0.7 to 1.4 μg of *Pseudomonas* toxin per ml of complete F12, at 2×10^5 to 5×10^5 cells per dish. The toxin-containing medium was replaced with complete F12 after 5 to 7 days. The plates were examined, and when the clones developed 25 to 50 cells, they were transferred to 16-mm well plates. When sufficient cells were generated, the strains were tested for sensitivity to toxins in the intact cell assay for the inhibition of protein synthesis, as described below.

Intact-cell assay for inhibition of protein synthesis by toxins and determination of ID_{50} (24). The cells were seeded in 8-ml flint glass vials (ICN Pharmaceuticals, Cleveland, Ohio) at 2×10^4 cells per vial and incubated at 37°C for 40 to 48 h. The medium was then changed to toxin-containing or control medium (duplicate or triplicate samples), and incubation was continued for 24 h, unless otherwise stated. The medium was then replaced with serum-free Eagle basal medium, buffered to pH 7.4 with 0.05 M Tris containing a 1:20 dilution of amino acids and 0.4 μCi of a ^{14}C-labeled amino acid mixture per ml (15 amino acids; 100 μCi/ml; New England Nuclear Corp., Boston, Mass.). The incubation was continued for 30 min, after which the labeling medium was removed, and the cells were precipitated and washed in the vial with 5% trichloroacetic acid. After drying, 5 ml of Econofluor (New England Nuclear) was added to each vial, and the cells were counted in a scintillation spectrometer. All data are representative of at least three repetitions of similar assays. From the dose-response curves generated from these assays (see Fig. 1 to 3), the concentration of a given toxin that inhibited protein synthesis 50% in a 24-h exposure [ID_{50} (24)] was determined.

Assays for effect of monensin and ammonium chloride. Log-phase cells grown as described above were exposed to various concentrations of monensin (Sigma) or NH_4Cl in complete F12 for 1 h at 37°C. The toxins were then added to the cultures in complete F12 with or without monensin or NH_4Cl. The cultures were incubated for 3 h at 37°C before pulse-labeling, as described above.

Assay for effect of low pH treatment on intoxication by *Pseudomonas* toxin. Monensin-treated (see above) or untreated cells were cooled to 4°C, and various concentrations of *Pseudomonas* toxin in complete F12, with or without monensin, were added. The toxin was bound to the cells for 1 h at 4°C. The toxin-containing medium was then removed and 1 ml of Dulbecco phosphate-buffered saline at pH 7.2 (or phosphate-buffered saline reduced to pH 4.5 by the addition of H_3PO_4) was added (with or without monensin, as appropriate). The cells were then incubated 10 to 30 min at 37°C. The phosphate-buffered saline was removed, and the cultures were incubated for 23 h at 37°C in complete F12, with or without monensin, followed by pulse-labeling, as described above.

Preparation of cell extracts and ADP ribosylation assay. Our procedure for the preparation of cell extracts and the measurement of EF-2 by the diphtheria toxin-catalyzed transfer of [^{14}C]ADP-ribose from [^{14}C]NAD has been described in detail elsewhere (29).

Virus production and infectivity assays. Stocks of Sindbis virus (SbV), Semliki Forest virus (SFV), Chikungunya virus (ChV), and vesicular stomatitis virus (VSV) were all grown in monolayers of CHO-K1 cells and stored at −80°C. Virus titers were determined by plaque assay on monolayers of CHO-K1 cells, by a standard agar overlay procedure (31). For endpoint titrations, wild-type and mutant cell strains were seeded in 16-mm well plates at a concentration of 2×10^4 to 5×10^4 cells per well in complete F12 and allowed to grow for 42 to 48 h. Dilutions of virus in complete F12 were then added to the wells, and the cultures were incubated at 37°C. The endpoints were scored at the time required for the lowest multiplicity of infection (MOI) to completely destroy all parental CHO-K1 cells in a well, i.e., 3 days for VSV, SFV, and SbV, and 4 days for ChV. Cytopathic effect was observed microscopically, and at the end of incubation, a solution of 0.05% 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (Aldrich Chemical Co., Milwaukee, Wis.) in complete F12 was added. Only the living cells were stained, and the endpoints were verified.

Measurement of viral RNA synthesis. Virus penetration was measured by the synthesis of viral RNA, according to the method of Helenius et al. (13, 47), with the modification that the cells were seeded in 8-ml flint glass vials (ICN) (5×10^4 cells per vial), and a binding/incubation medium of Eagle minimum essential medium with nonessential amino acids, 1% fetal bovine serum, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer was used. Viral RNA produced in actinomycin D-treated cells was measured by the incorporation of [5,6-^3H]uridine (ICN), adjusted to 20 Ci/mmol. The trichloroacetic acid-fixed cells were dried in the vial and counted in 5

ml of Econofluor (New England Nuclear) in a Beckman LS-7500 scintillation spectrometer.

Cell hybridization. The details of the hybridization procedure that we employed in these studies have been presented elsewhere (32). In each hybridization, one of the two parental strains was PRO^-, $HPRT^+$, and the other was PRO^+, $HPRT^-$. All parental cells could therefore be eliminated by culture in proline-free hypoxanthine-aminopterin-thymidine medium (20), and the hybrid nature of the recovered clones was verified by their growth in this selective medium.

RESULTS

Characterization of *Pseudomonas* toxin-resistant strains. Protein synthesis in CHO-K1 cells was inhibited by exposure to concentrations of *Pseudomonas* toxin above 0.001 μg/ml. The maximum inhibitory effect was achieved with concentrations of 1.0 μg/ml and above. In selection experiments, the cells that survived exposure to 1.0 μg of *Pseudomonas* exotoxin A per ml developed into colonies that had a stable resistance greater than that of the parental cells even after many generations in culture without reexposure to the toxin. As was the case with the selection of diphtheria toxin-resistant mutants (29, 32), we were able to identify two general classes of mutant. We have designated these class I (in which resistance can be completely overcome by increased concentrations of *Pseudomonas* toxin) and class II (in which resistance cannot be overcome by the highest concentrations of toxin applied, in this case 100 μg/ml).

The class II strains selected by the exposure of cells to *Pseudomonas* toxin were indistinguishable from those selected with diphtheria toxin and occurred with comparable frequency. All were cross resistant to diphtheria toxin and could be further subdivided on the basis of their content of resistant EF-2, as we previously reported (29). Class IIa strains possess no toxin-sensitive EF-2, whereas class IIb strains produce both resistant and sensitive (or normal) EF-2. Studies on some of these mutants have been reported elsewhere (30, 32, 35). This report will deal primarily with class I strains, in which resistance is due to cellular alterations that affect the uptake and delivery of active toxin to its target site.

Pseudomonas toxin-resistant class I strains occurred with a lower frequency in CHO-K1 than did diphtheria toxin-resistant class I strains. For example, when 2×10^5 to 4×10^5 recently cloned CHO-K1 cells were treated with concentrations of the two toxins that cause approximately the same percent killing in a given time (0.05 μg of diphtheria toxin per ml and 1.4 μg of *Pseudomonas* toxin per ml), the frequency of class I diphtheria toxin-resistant mutants in ethyl methane sulfonate-mutagenized cells was 1.1×10^{-4}, whereas the frequency of class I *Pseudomonas* toxin-resistant mutants was only 1.2×10^{-5}.

Pseudomonas toxin-resistant class I strains were first tested in an intact cell assay demonstrating the inhibition of protein synthesis by increasing concentrations of toxin. The response of several strains is compared with that of parental cells in Fig. 1. The class I mutants fell into two general groups: one in which resistance to *Pseudomonas* toxin was increased 1.5 to 2 orders of magnitude above that of parental cells and one in which resistance was increased 3 to 4 orders of magnitude.

Response to diphtheria toxin. When these strains were tested in the same way against diphtheria toxin, the two groups were defined further (Fig. 2). The group with higher resistance to *Pseudomonas* toxin had no increased resistance to diphtheria toxin, whereas the other group had an increased resistance of 1 to 2 orders of magnitude to diphtheria toxin, as well as to *Pseudomonas* toxin. We have designated the strains resistant to *Pseudomonas* toxin and diphtheria toxin (typified by strains RPE.28, RPE.44, and RPE.51) DPV^r mutants and the strains resistant to *Pseudomonas* toxin (typified by RPE.8 and RPE.40) PV^r mutants.

Extracts of these mutant strains were prepared, and their content of ADP-ribosylatable EF-2 was measured by diphtheria toxin-catalyzed transfer of ADP-ribose from NAD^+ to EF-

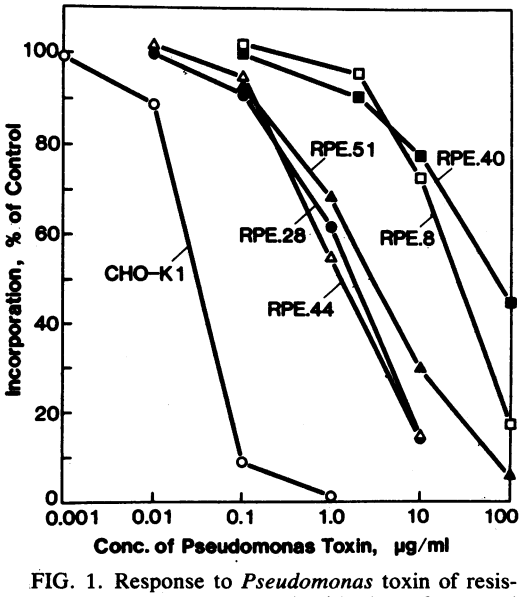

FIG. 1. Response to *Pseudomonas* toxin of resistant entry mutants compared with that of parental CHO-K1 cells, as measured by the intact-cell assay for inhibition of protein synthesis. The cells were exposed to toxin for 24 h.

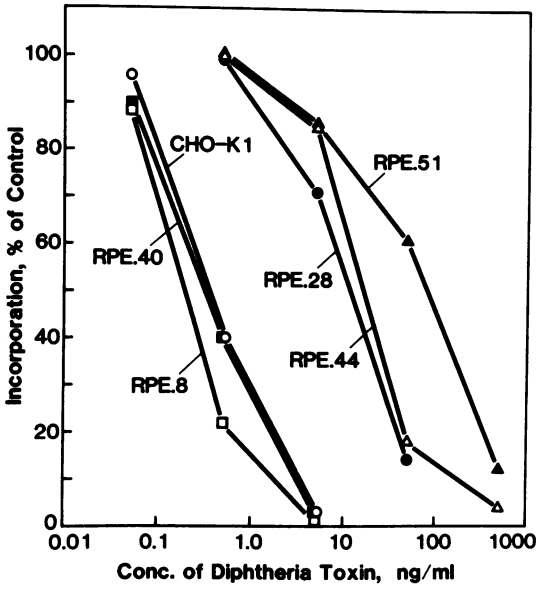

FIG. 2. Response to diphtheria toxin of resistant entry mutants selected with *Pseudomonas* toxin compared with that of CHO-K1 cells as measured by the intact-cell assay; the cells were exposed to toxin for 24 h.

2, as we have previously described (29). These strains all possessed an amount of ADP-ribosylatable EF-2 in the normal range for their cell line. Specifically, the average amount of ADP-ribosylatable EF-2 per milligram of extract protein for CHO-K1 cells (calculated from six extract preparations) was 93 ± 7 pmol. The average from extracts of three PV^r strains was 89 ± 6 pmol, and the average from extracts of four DPV^r strains was 77 ± 6 pmol of EF-2 per mg of protein.

Response to ricin and other lectins. When class I strains were exposed to lethal concentrations of the plant toxin ricin, the DPV^r and PV^r groups again responded differently (Fig. 3). The PV^r strains were as sensitive to this lectin as were the parental cells. None of these strains displayed a resistance; however, the DPV^r strains displayed an enhanced sensitivity to ricin. A concentration of approximately 1 order of magnitude less ricin was required to achieve an equal inhibition of protein synthesis. The response of these strains to the lectin abrin was identical to their response to ricin (data not shown).

We also measured the response of mutant and parental strains to the toxic lectins concanavalin A, PHA, and wheat germ agglutinin, using the inhibition of the incorporation of radioactive amino acids into protein as an indicator of toxicity. From dose-response curves, such as those in Fig. 1 and 2, the concentration of lectin required to inhibit protein synthesis by 50% after an exposure time of 24 h (ID_{50} [24]) was determined (Table 2). The parental cells and all mutants responded similarly to concanavalin A. The mutants in the DPV^r group were slightly more sensitive to PHA and wheat germ agglutinin than were the parental cells or PV^r cells, but the differences were less than 1 order of magnitude.

Monensin and ammonium chloride treatment of CHO-K1 cells. It is well known that NH_4Cl treatment of cells protects them from diphtheria toxin (5, 7, 40), but differing results have been reported regarding the protective effect of this compound against *Pseudomonas* toxin (8, 26, 27). The proton ionophore monensin likewise protects against diphtheria toxin (22, 40), but it has been reported to potentiate the toxicity of *Pseudomonas* toxin, as well as that of ricin (37). We tested the effect of increasing concentrations of these compounds on the intoxication of CHO-K1 cells by diphtheria toxin, *Pseudomonas* toxin, and ricin. Our results demonstrate that the concentration of monensin or NH_4Cl used is very important and, indeed, can determine whether reduction or enhancement of toxic effects is produced (Fig. 4). In general, monensin and NH_4Cl protect CHO-K1 cells against diph-

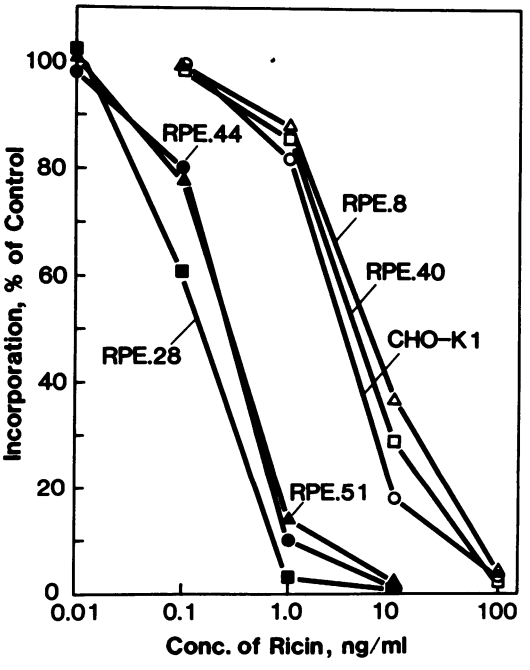

FIG. 3. Response to ricin of resistant entry mutants selected with *Pseudomonas* toxin compared with that of CHO-K1 cells as measured by the intact-cell assay; the cells were exposed to ricin for 24 h.

TABLE 2. Response of *Pseudomonas* toxin-resistant strains to three toxic lectins

Strain	Classification	ID_{50} (24) (μg/ml)[a] of:		
		PHA	WGA[b]	ConA[b]
CHO-K1	Wild type	22	5.0	9.0
RPE.23	PV^r	32	5.5	8.0
RPE.40	PV^r	30	4.0	8.0
RPE.28	DPV^r	9	1.5	6.5
RPE.44	DPV^r	ND[c]	1.5	ND
RPE.51	DPV^r	11	2.5	6.5

[a] ID_{50} (24), the concentration of a toxic compound that inhibits protein synthesis by 50% in 24 h.

[b] WGA, Wheat germ agglutinin; ConA, concanavalin A.

[c] ND, Not determined.

theria toxin at all active concentrations and enhance the action of ricin to a greater or lesser extent. But both of these agents can either potentiate the toxicity of *Pseudomonas* toxin (lower active concentrations of monensin or NH_4Cl) or protect cells to a high degree (if higher concentrations are used).

Effect of low pH treatment on *Pseudomonas* intoxication. It has been shown that the protective effect of NH_4Cl or monensin can be bypassed, or overcome, when diphtheria toxin-treated cells are exposed briefly to a medium of low pH immediately after the toxin has been allowed to bind to the cells (7, 22, 40). We found that the same was true when CHO-K1 cells were treated with monensin and *Pseudomonas* toxin. The protective effect of monensin may be overcome by the exposure of toxin-treated cells to a buffer of pH 4.5 (Fig. 5). This treatment causes monensin-treated cells to respond to the toxin approximately as do untreated cells. Experimental evidence indicated that the DPV^r mutant strains were phenotypically similar to cells exposed to 1 μM monensin (i.e., protected against diphtheria and *Pseudomonas* toxins and sensitized to ricin [Fig. 4A]). This analogy was further borne out by the fact that their resistance to *Pseudomonas* toxin may be overcome by the exposure of toxin-treated DPV^r cells to pH 4.5 (Fig. 5B). The resistance of DPV^r strains RPE.28 and RPE.51 was reduced to approximately the level of untreated parental CHO-K1 cells by low pH treatment. The resistance of PV^r strain RPE.40, however, was unaffected (Fig. 5C).

Hybridization analysis. Representative *Pseudomonas* toxin-resistant strains were hybridized with cell strain P1R2, a derivative of CHO-K1 that is $HPRT^-$ and does not require proline

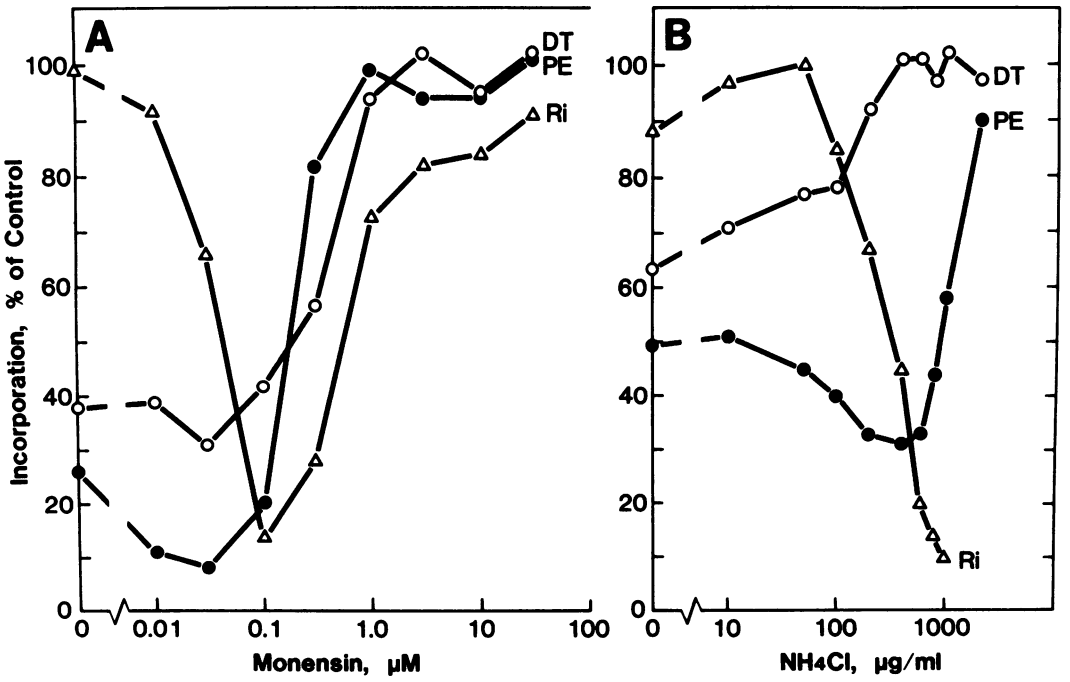

FIG. 4. Effect of increasing concentration of (A) monensin and (B) ammonium chloride on inhibition of protein synthesis in CHO-K1 cells by *Pseudomonas* toxin (PE; 1.0 μg/ml), diphtheria toxin (DT; 25 ng/ml), and ricin (Ri; 10 ng/ml) as measured by the intact-cell assay; a 1-h pretreatment was followed by a 3-h exposure to toxin plus a lysosomotropic agent.

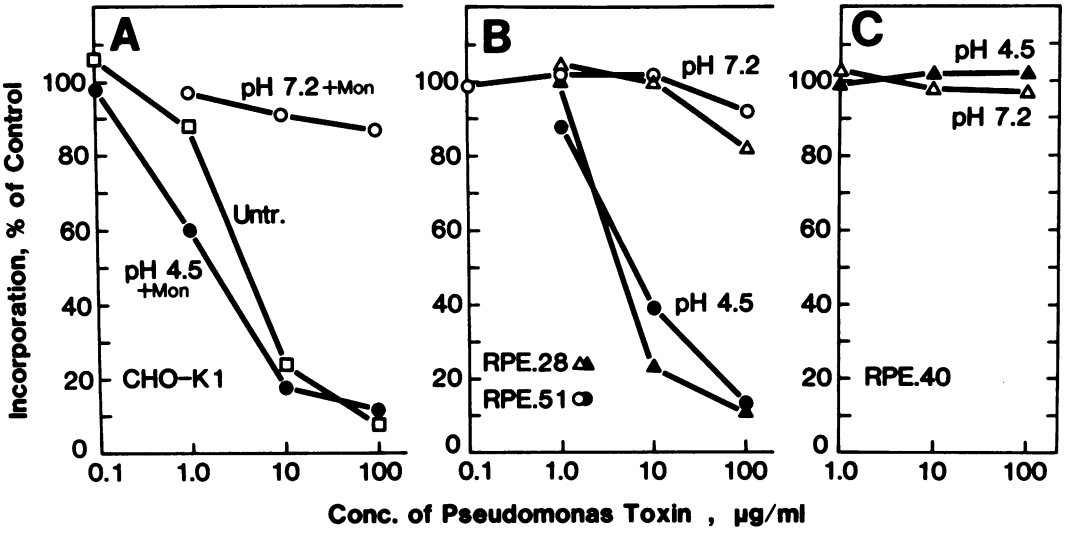

FIG. 5. Effect of low-pH treatment on inhibition of protein synthesis by *Pseudomonas* toxin in monensin-treated CHO-K1 cells and resistant mutant strains. For a description of the procedure used, see the text. (A) Monensin-treated (1 μM) and untreated CHO-K1 cells. (B) DPV^r mutants RPE.28 and RPE.51. (C) PV^r mutant RPE.40.

(Table 3). In all cases, resistance to *Pseudomonas* toxin proved to be a recessive characteristic. In the case of DPV^r strains, resistance to diphtheria toxin and increased sensitivity to ricin were likewise lost upon hybridization with P1R2 (data not shown). Complementation analysis showed that the DPV^r strains P1R2.P50, RPE.28, RPE.44, and RPE.51 were all members of the same complementation group, and RPE.40, as expected, represented a different complementation group.

TABLE 3. Analysis of resistance to *Pseudomonas* toxin by cell hybridization

Strain	Classification	*Pseudomonas* toxin ID_{50} (24) (μg/ml)[a]
CHO-K1	Wild type	0.027
P1R2	Wild type[b]	0.055
RPE.40	PV^r	35.0
RPE.28	DPV^r	1.8
RPE.44	DPV^r	1.3
RPE.51	DPV^r	3.0
P1R2.P50	DPV^r	1.2
Dominance hybridization		
P1R2 × RPE.40		0.020
P1R2 × RPE.28		0.028
P1R2 × RPE.44		0.022
P1R2 × RPE.51		0.022
Complementation analysis		
P1R2.P50 × RPE.40		0.032
P1R2.P50 × RPE.28		1.0
P1R2.P50 × RPE.44		1.2
P1R2.P50 × RPE.51		1.9

[a] The concentration of toxin that will inhibit protein synthesis by 50% in 24 h, determined from three assays on individual cell strains or from assays on three separately selected hybrid strains, in the case of hybrid crosses.

[b] With respect to toxin sensitivity.

Response to enveloped RNA viruses. In contrast to what we have found in studies of resistant CHO-K1 cells selected with diphtheria toxin, both PV^r and DPV^r strains had increased resistance to two or more enveloped RNA viruses. In Table 4 we present the results of endpoint titrations of four viruses on six cell strains. The PV^r strains had a very pronounced resistance to ChV and SbV (2 to 4 orders of magnitude), a lesser resistance to SFV (1 order of magnitude), and no resistance to VSV. The DPV^r strains were most resistant to SbV (2 to 3 orders of magnitude) but had a resistance of 1 to 2 orders of magnitude to each of the other three viruses.

Studies of the production of specific viral RNA provided a critical measure of productive infection of the strains by SbV. The cells were infected with a high multiplicity of virus (20 to 100 PFU/cell) at 4°C. They were then exposed briefly to buffers of slightly alkaline (7.2) or low (5.5 to 5.7) pH at 37°C and then incubated in complete medium for 4 to 8 h. Viral RNA production was measured by the incorporation of [^{3}H]uridine. The production of viral RNA was greatly reduced in DPV^r strains, compared with wild-type or PV^r strains, when slightly alkaline conditions were maintained (Table 5; Fig. 6). However, when the adsorption of virus was

TABLE 4. MOIs required to produce cytopathic effect

Strain	Classification	MOI[a] of:			
		VSV	SFV	SbV	ChV
CHO-K1	Wild type	10^{-5}	10^{-5}	10^{-4}	10^{-4}
RPE.8	PV^r	10^{-5}	10^{-4}	10^{-2}	10^{0}
RPE.40	PV^r	10^{-5}	10^{-4}	10^{-2}	10^{-1}
RPE.28	DPV^r	10^{-3}	10^{-4}	10^{-2}	10^{-3}
RPE.44	DPV^r	10^{-4}	10^{-4}	10^{-1}	10^{-3}
RPE.51	DPV^r	10^{-4}	10^{-3}	10^{-2}	10^{-3}

[a] The endpoints were read at the time required for the lowest MOI to destroy 100% of CHO-K1 cells in a culture, i.e., 3 days for VSV, SFV, and SbV and 4 days for ChV. The lowest MOI to destroy at least 50% of mutant cells in a culture is recorded.

followed by a brief (1.5 to 3 min) exposure to low pH buffer, the production of viral RNA increased markedly in all DPV^r strains, but was little changed in wild-type or PV^r strains.

In this respect as well, the cells of the DPV^r group behaved as do wild-type cells treated with lysosomotropic agents, such as NH_4Cl and monensin. BHK-21 cells can be protected by monensin treatment from productive infection by SFV, and this protection can be overcome by low pH treatment (23). The same was true for CHO-K1 cells and SbV (Fig. 6C and D). DPV^r mutant strain RPE.51 responded as do monensin-treated CHO-K1 cells (Fig. 6E and F). When the viruses were bound to RPE.51 cells and a near-neutral pH was maintained, little virus-specific RNA was produced in 8 h, compared with CHO-K1 cells. This block was overcome, and substantially more RNA was produced when virus-treated RPE.51 cells were briefly exposed to pH 5.5.

The yield of infectious SbV particles produced by parental CHO-K1 and mutant cells was measured by plaque assay (Fig. 7). Under the condi-

TABLE 5. Effect of low-pH treatment on production of SbV RNA[a]

Strain	Classification	cpm (10^4) per 10^6 cells at pH:	
		7.2	5.7
P1R2	Wild type[b]	11.9	7.7
RPE.40	PV^r	9.5	7.7
RPE.28	DPV^r	0.4	4.0
RPE.44	DPV^r	1.2	3.2
RPE.51	DPV^r	0.3	2.5
P1R2.P50	DPV^r	0.9	3.8

[a] The cells were infected with an MOI of 20, and the RNA was measured 4.5 h postinfection. The procedure is outlined in the legend to Fig. 6.

[b] With respect to toxin and virus sensitivity.

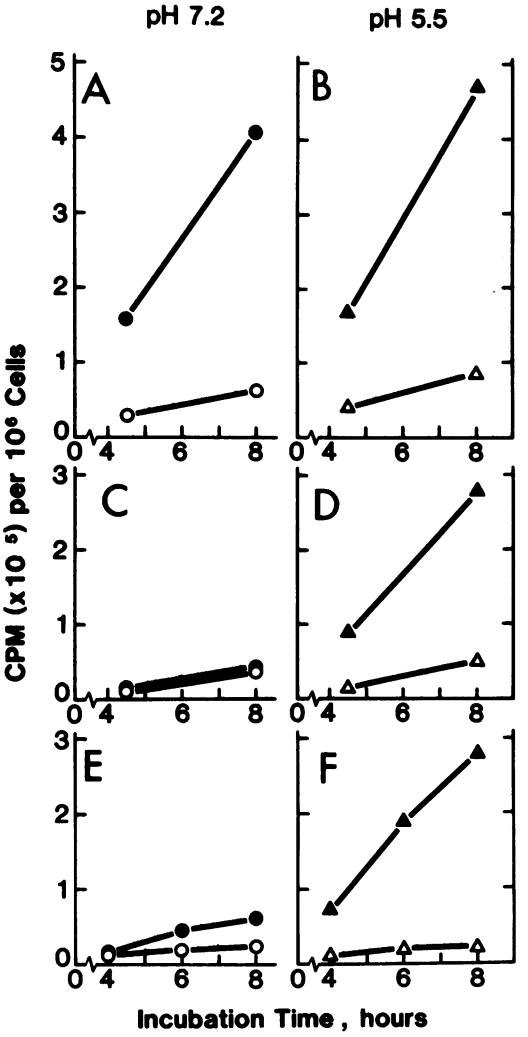

FIG. 6. Production of SbV RNA by untreated and monensin-treated CHO-K1 cells and DPV^r mutant strain RPE.51. The cells were exposed to an MOI of 50 infectious units of virus for 10 min at 4°C. They were then exposed to phosphate-buffered saline (pH 7.2 or 4.5) for 90 s at 37°C, followed by incubation in incubation medium for 90 min at 37°C. Actinomycin D at 4 μg/ml was added for 30 min, and the medium was replaced with incubation medium containing actinomycin D and [^{3}H]uridine at 10 μCi/ml for further incubation for 2 to 6 h. The counts per minute of [^{3}H]uridine incorporated into the RNA of virus-treated and untreated control cells was then determined. (A and B) CHO-K1 cells, no monensin. (C and D) CHO-K1 cells, monensin treated (cells were pretreated with 10 μM monensin for 1 h, and this concentration of monensin was retained throughout the entire procedure). (E and F) DPV^r strain RPE.51. Symbols: ● and ▲, virus-infected cells; and ○ and △, uninfected cells.

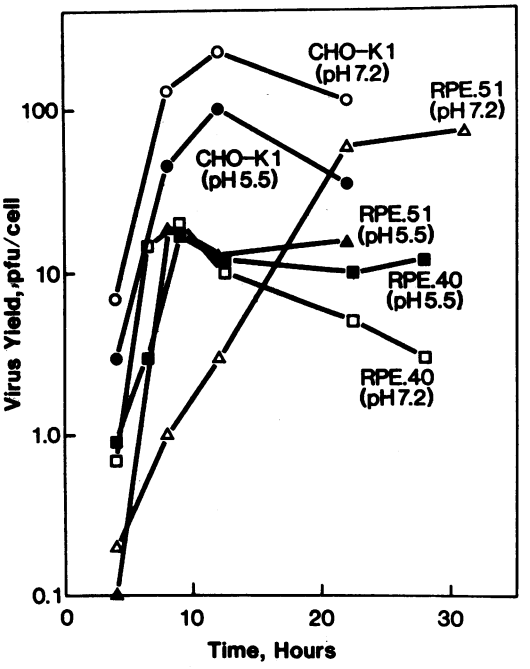

FIG. 7. Infectious SbV produced by CHO-K1 cells and mutant strains after a brief exposure to near-neutral or low pH. The procedure was as outlined in the legend to Fig. 6 except that actinomycin D and [^{3}H]uridine were omitted. PFU were determined on monolayers of CHO-K1 cells.

tions of our experiments, the maximum number of infectious virus was released from CHO-K1 cells 12 h after infection. The brief treatment of these cells with pH 5.5 buffer generally depressed the yields somewhat. DPV^r strains (represented by RPE.51) when infected with a high MOI (20 to 50 PFU/cell) produced RNA and virus at a reduced rate and did not reach their maximum yield until 24 h postinfection. When these cells were briefly exposed to pH 5.5 after the adsorption of virus, they produced their maximum yield of infectious particles rapidly (in 8 h), but the yield was much lower than that from CHO-K1. Although PV^r strains (represented by RPE.40) produced normal amounts of viral RNA, they produced low numbers of infectious virus, approximately 1 order of magnitude less than CHO-K1 cells, whether treated with low pH buffer or not. The maximum amounts of SbV RNA and infectious virus produced by CHO-K1 and RPE.40 cells when infected at an MOI of 100 infectious SbV particles were compared. The maximum production of cell-associated [^{3}H]uridine-labeled virus-specific RNA was 3.6×10^5 cpm/10^6 CHO-K1 cells and 3.4×10^5 cpm/10^6 RPE.40 cells measured at the time of maximum cell-associated viral RNA. The infectious virus produced was 270 per CHO-K1 cell and 18 per RPE.40 cell, as measured at the time of the maximum release of infectious virus.

DISCUSSION

The toxin-resistant mutants described in this study were obtained from mutagenized CHO-K1 cells by single-step selection with *Pseudomonas* exotoxin A. This is the first report of mutant cells selected with *Pseudomonas* toxin that are resistant to this toxin at the level of binding or uptake, rather than at the level of the target enzyme EF-2. Two distinct phenotypes were recognized, and complementation analysis confirmed that they fell into two genetic complementation groups. We have designated one group DPV^r, as the strains of this group are resistant to diphtheria toxin, *Pseudomonas* toxin, and four enveloped RNA viruses. These strains also have an increased sensitivity to the plant toxin ricin. We designated the second group PV^r, as its strains are resistant to *Pseudomonas* toxin and to three enveloped RNA viruses. In contrast to what we have found to be the case in studies of diphtheria toxin entry mutants, all of the *Pseudomonas* toxin entry mutants described in this report have a decreased sensitivity to virus. We have now examined 12 strains of entry mutants selected with diphtheria toxin and resistant only to this toxin (we designated them Dip^r), and none has had resistance to the viruses used in this study—VSV, SFV, SbV, and ChV.

Pseudomonas toxin-resistant entry mutants occurred at a frequency of at least 1 order of magnitude less than diphtheria toxin entry mutants in our CHO-K1 line. Furthermore, most (at least 80%) of them proved to be of the DPV^r type, and few were of the PV^r type. Indeed, we have so far been unable to isolate any PV^r strains from our PRO$^+$, HPRT$^-$ Chinese hamster strain P1R2, and for this reason a complete complementation analysis of this mutant type remains to be done. The phenotypes of DPV^r and PV^r strains have proven to be stable in culture for over 100 generations with no re-exposure to toxin. Both the DPV^r and the PV^r phenotypes proved to be recessive in hybrids constructed between these mutants and wild-type cells.

We previously reported on a strain of diphtheria toxin-resistant KB (human carcinoma) cells that was cross resistant to a number of viruses (28, 34). The KB-R2 strain was selected by the exposure of wild-type cells to low concentrations of diphtheria toxin over a long period of time and, therefore, more than one mutation may be responsible for its altered phenotype. Until this report, no other studies of toxin-resistant mutants that are cross resistant to

viruses had been published, but Mento and Siminovitch have reported the isolation of CHO strains resistant to SbV that are cross resistant to diphtheria toxin (25). They did not examine the response of their mutants to *Pseudomonas* toxin or ricin, but they did report enhanced sensitivity to PHA and cross-resistance to VSV, similar to that which we found in our DPV^r strains. We found only a slight increase in PHA sensitivity in DPV^r strains, but it is likely that assay by the inhibition of colony formation, the method used by Mento and Siminovitch, would amplify this sensitivity. They found, however, that viral RNA was produced as efficiently in their mutant as in the wild-type cells, and this is quite different from our findings with DPV^r strains.

Ray and Wu (38) have reported the isolation of CHO mutants that are simultaneously resistant to ricin and to *Pseudomonas* toxin and that were selected with a mixture of these two toxins. These strains comprise a type different from any of ours, as we have found none with increased resistance to ricin, only increased sensitivity (in the case of DPV^r strains). They postulated a defect in a common step in the internalization of the two toxins and showed that ricin remained at or near the cell surface.

Intact-cell assays of the response of DPV^r cells to *Pseudomonas* toxin, diphtheria toxin, and ricin revealed that these cells existed in a state found in wild-type cells treated with certain lysosomotropic agents, i.e., resistant to the bacterial toxins and hypersensitive to ricin. To address this, we examined the effect of the treatment of CHO-K1 cells with increasing concentrations of ammonium chloride and monensin. Reports in the literature on the effect of NH_4Cl on the action of *Pseudomonas* toxin have differed. It is well known that the treatment of cells with this compound protects them from the action of diphtheria toxin (5, 7, 27, 40). However, it has been reported both that NH_4Cl is without effect on *Pseudomonas* toxin action (27, 37) and that it protects cells from this toxin (8, 26). Likewise, it was reported that monensin substantially enhanced the activity of *Pseudomonas* toxin (37), but we found that, at concentrations known to protect cells from diphtheria toxin (22, 40), monensin protected CHO-K1 cells from *Pseudomonas* toxin as well. Our results show that the activity of these compounds against *Pseudomonas* toxin is very concentration dependent indeed (Fig. 4). Both NH_4Cl and monensin can cause a slight enhancement of *Pseudomonas* toxin toxicity at certain lower concentrations, and both can be increased to the point where they will protect the cell from this toxin (while still maintaining the cell in an active, protein-synthesizing state). Low concentrations (<1 μM) of monensin, when added to cells, cause the inhibition of membrane protein recycling (2) and the inhibition of the transport of membrane proteins from the Golgi to the cell surface (16, 17, 43, 44). Increasing concentrations of carboxylic ionophores, such as monensin, raise the intralysosomal pH (36), and perhaps the endosomal pH (23), above normal levels. The protection of cells from diphtheria toxin and from viral infection by monensin treatment can be explained by this elevation in pH (23, 39, 40), and this protection may be overcome by binding diphtheria toxin or SFV to monensin-treated cells at 4°C and then exposing the cells to a low-pH medium at 37°C (22, 23). It appears that monensin protects cells from *Pseudomonas* toxin by the same mechanism, since we can overcome this protection by a similar (low pH) treatment. This has allowed us to identify a step in the uptake of *Pseudomonas* toxin that is dependent upon contact with a low-pH environment. The slightly enhanced toxicity of *Pseudomonas* toxin and the very pronounced enhancement of ricin toxicity found with low concentrations of monensin may have to do with changes in protein exchange between different intracellular compartments (40), perhaps due to a reduction in the rates of degradative processes. The enhancement of ricin toxicity at low concentrations of carboxylic ionophores and protection from it at higher concentrations have been reported by Sandvig and Olsnes (40), using Vero cells.

Marsh et al. (23) have concluded that monensin and NH_4Cl inhibit viral fusion and the entry of viral RNA into the cytoplasm by the same mechanism. Marnell et al. (22) have come to the same conclusion for diphtheria toxin entry into cells, and we agree with their findings. It appears, however, that monensin and NH_4Cl do not inhibit the action of *Pseudomonas* toxin by the same mechanism. We found that NH_4Cl at a concentration that afforded maximum protection to CHO-K1 cells from diphtheria toxin (400 μg/ml) caused a pronounced enhancement of *Pseudomonas* toxin toxicity, and protection was elicited only at concentrations of NH_4Cl above 1 mg/ml. The enhancement of ricin toxicity was observed at all concentrations of NH_4Cl above 100 μg/ml. Mekada et al. (24) have observed very similar effects of methylamine on these three toxins.

At concentrations above 1 mg/ml, NH_4Cl blocks the uptake of *Pseudomonas* toxin (8, 26) and causes it to remain on the surface of the cell; this may account for the protection that we observed at 2 mg/ml. Although we have identified the need for exposure to a low-pH environment at some point in the delivery of active *Pseudomonas* toxin to the cytosol, we have not

yet determined in which intracellular compartment the toxin encounters the low pH. Our studies suggest that diphtheria toxin may enter the cytosol directly from an acidified endosome (4). After internalization, *Pseudomonas* toxin rapidly associates with a subcellular fraction rich in GERL marker enzyme (D. M. Manhart, C. B. Saelinger, and R. E. Morris, Abstr. Annu. Meet. Am. Soc. Cell Biol. 1982, abstr. no. 21091, p. 434). Native *Pseudomonas* toxin must undergo a processing, perhaps involving proteolysis, to release or expose the enzymatically active portion of the molecule (3, 19, 21, 46). This toxin may require exposure to the GERL region in conjunction with exposure to a low pH to be delivered to the cytosol in an active form. The system that normally delivers diphtheria toxin and viral RNA (12) to the cytosol appears to be more sensitive to changes in the intravesicular pH caused by lower concentrations of NH_4Cl than is the system that delivers *Pseudomonas* toxin.

Our findings on the resistance of DPV^r mutant strains to *Pseudomonas* and diphtheria toxin, their reduced and slow production of viral RNA, and even their increased sensitivity to ricin are consistent with an alteration in some system required for the acidification of an intracellular compartment. The resistance of DPV^r strains to *Pseudomonas* toxin and to diphtheria toxin (4) can be completely overcome by binding the toxins to the cell surface and then exposing the cells to a low-pH medium. DPV^r mutants have an increased resistance to VSV, SFV, SbV, and ChV that is characterized by a much slower development of cytopathic effect than that seen in wild-type cells, in addition to the requirement for at least 1 order of magnitude more infectious particles to completely destroy a culture. The production of SbV RNA was reduced and slowed compared with that of wild-type cells. Although with a high MOI (>20), yields of infectious viral particles that were almost as high as those of wild-type cells could eventually be recovered, more than twice the time was required to reach this peak. However, if viruses were bound to the cells and fusion was induced by low-pH treatment, the peak production of viral RNA and infectious particles was reached as rapidly as with wild-type cells, although the yields produced by this artificial procedure were not as high. The failure to maintain a normally low pH in endocytic vesicles could also explain the increased sensitivity to ricin observed in DPV^r strains, as Sandvig and Olsnes (40) have shown that this toxin is most efficiently transported into the cytosol at higher pHs. This defect in acidification has now been identified. Subcellular fractionation studies have shown that DPV^r mutants are defective in an ATP-dependent mechanism for the acidification of endosomes that is demonstrable in endosomes from parental CHO-K1 cells (M. Merion, P. Schlesinger, R. M. Brooks, J. M. Moehring, T. J. Moehring, and W. S. Sly, Proc. Natl. Acad. Sci. U.S.A., in press). This may indicate a mutation in DPV^r cells that affects an ATP-dependent proton pump (10).

The resistance of PV^r mutants is apparently unrelated to any defect in the acidification of endocytic vesicles. The best evidence for this is the fact that SbV RNA is delivered to the cytosol and replicated as efficiently in PV^r mutants as it is in CHO-K1 cells. In addition, low-pH treatment of PV^r cells does not overcome their resistance to *Pseudomonas* toxin.

As previously mentioned, native *Pseudomonas* toxin is a proenzyme that must undergo processing, perhaps involving proteolysis, to become enzymatically active in the ADP ribosylation of EF-2 (3, 19, 21, 46). It is not known when or where this processing takes place in the course of the normal intoxication of a sensitive mammalian cell, but we might assume that it would occur in an endocytic vesicle, in the cisternae of the Golgi complex, in secondary lysosomes, or perhaps in the cytosol itself. Many host cell functions are involved in the production and maturation of SbV virus components (1). Proteolytic processing is required to cleave the single precursor polyprotein into the capsid protein, envelope protein E_1, and a precursor to the second envelope protein (PE_2) (41, 42). PE_2 and E_1 are integrated into the cell membranes, and a final cleavage of PE_2 to envelope protein E_2 takes place immediately before the envelopment and release of the virion (1). PV^r strains clearly possess a mutation that causes a block in the assembly, maturation, or release of infectious virus. The binding or uncoating of virus is unaffected, and normal amounts of RNA are synthesized, although we do not, at present, know whether this is entirely normal RNA. It is possible that the mutation in PV^r cells involves an enzyme required both for some step in the activation of *Pseudomonas* toxin and for the proteolytic processing of alphavirus structural proteins. Alternatively, the postulated enzyme might be required for a step in the processing of the *Pseudomonas* toxin receptor. These possibilities are currently under study in our laboratory.

Good evidence has been presented that *Pseudomonas* toxin enters cells by receptor-mediated endocytosis (8, 9). It is unfortunate that we have not been able to measure any alterations in the specific binding sites for *Pseudomonas* toxin on our mutant strains, but we found that the nonspecific binding of ^{125}I-labeled *Pseudomonas* toxin to cells was too great, compared with

specific binding, to obtain reproducible measurements. The binding assay described elsewhere (4) was used in these studies. Apparently, the number of receptor sites for *Pseudomonas* toxin is very low on all cells, even the most sensitive mouse cells. We have found that *Pseudomonas* toxin is nearly 2 orders of magnitude less toxic than is diphtheria toxin for wild-type KB (human), Vero (monkey), or CHO-K1 cells, when compared on a molar basis. Mouse cells, of course, cannot be compared since they are naturally highly resistant to diphtheria toxin. This may be an indication of fewer specific receptor sites per cell or of less efficient transport across the cell membrane, or it may simply mean that more molecules of *Pseudomonas* toxin are degraded or inactivated en route through the cell.

ACKNOWLEDGMENTS

We thank Cheryl Albaugh and Peter Gehlbach for technical assistance in preparing the cell hybrids and Monique Phillips for assistance in the preparation of the manuscript.

This investigation was supported by Public Health Service grant AI-09100 from the National Institute of Allergy and Infectious Diseases, and one of us (JMM) received partial support from Public Health Service grant S07 RR 05429.

LITERATURE CITED

1. **Adams, R. H., and D. T. Brown.** 1982. Inhibition of Sindbis virus maturation after treatment of infected cells with trypsin. J. Virol. **41**:692–702.
2. **Basu, S. K., J. L. Goldstein, R. G. W. Anderson, and M. S. Brown.** 1981. Monensin interupts the recycling of low density lipoprotein receptors in human fibroblasts. Cell **24**:493–502.
3. **Chung, D. W., and R. J. Collier.** 1977. Enzymatically active peptide from the adenosine diphosphate-ribosylating toxin of *Pseudomonas aeruginosa*. Infect. Immun. **16**:832–841.
4. **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.
5. **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.
6. **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.
7. **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.
8. **FitzGerald, D., R. E. Morris, and C. B. Saelinger.** 1980. Receptor-mediated internalization of Pseudomonas toxin by mouse fibroblasts. Cell **21**:867–873.
9. **FitzGerald, D., R. E. Morris, and C. B. Saelinger.** 1982. Essential role of calcium in cellular internalization of *Pseudomonas* toxin. Infect. Immun. **35**:715–720.
10. **Forgac, M., L. Cantley, B. Wiedenmann, L. Altstiel, and D. Branton.** 1983. Clathrin-coated vesicles contain an ATP-dependent proton pump. Proc. Natl. Acad. Sci. U.S.A. **80**:1300–1303.
11. **Gupta, R. S., and L. Siminovitch.** 1978. Diphtheria-toxin-resistant mutants of CHO cells affected in protein synthesis: a novel phenotype. Somat. Cell Genet. **4**:553–571.
12. **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.
13. **Helenius, A., M. Marsh, and J. White.** 1982. Inhibition of Semliki Forest virus penetration by lysosomotropic weak bases. J. Gen. Virol. **58**:47–61.
14. **Iglewski, B. H., and D. Kabat.** 1975. NAD-dependent inhibition of protein synthesis by *Pseudomonas aeruginosa* toxin. Proc. Natl. Acad. Sci. U.S.A. **72**:2284–2288.
15. **Iglewski, B. H., P. V. Liu, and D. Kabat.** 1977. Mechanism of action of *Pseudomonas aeruginosa* exotoxin A: adenosine diphosphate-ribosylation of mammalian elongation factor 2 in vitro and in vivo. Infect. Immun. **15**:138–144.
16. **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.
17. **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.
18. **Leppla, S. H.** 1976. Large-scale purification and characterization of the exotoxin of *Pseudomonas aeruginosa*. Infect. Immun. **14**:1077–1086.
19. **Leppla, S. H., O. C. Martin, and L. A. Muehl.** 1978. The exotoxin of *P. aeruginosa*: a proenzyme having an unusual mode of activation. Biochem. Biophys. Res. Commun. **81**:532–538.
20. **Littlefield, J. W.** 1964. Selection of hybrids from mating of fibroblasts *in vitro* and their presumed recombinants. Science **145**:709–710.
21. **Lory, S., and R. J. Collier.** 1980. Expression of enzymic activity by exotoxin A from *Pseudomonas aeruginosa*. Infect. Immun. **28**:494–501.
22. **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.
23. **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.
24. **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.
25. **Mento, S. J., and L. Siminovitch.** 1981. Isolation and preliminary characterization of Sindbis virus-resistant Chinese hamster ovary cells. Virology **111**:320–330.
26. **Michael, M., and C. Saelinger.** 1979. Toxicity of Pseudomonas toxin for mouse LM cell fibroblasts. Curr. Microbiol. **2**:103–108.
27. **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.
28. **Moehring, J. M., and T. J. Moehring.** 1976. The spectrum of virus resistance of a KB cell strain resistant to diphtheria toxin. Virology **69**:786–788.
29. **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.
30. **Moehring, J. M., T. J. Moehring, and D. E. Danley.** 1980. Posttranslational modification of elongation factor 2 in diphtheria toxin-resistant mutants of CHO-K1 cells. Proc. Natl. Acad. Sci. U.S.A. **77**:1010–1014.
31. **Moehring, J. M., W. R. Stinebring, and D. J. Merchant.** 1971. Survey of interferon production and sensitivity in human cell lines. Appl. Microbiol. **22**:102–105.
32. **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.
33. **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.

34. **Moehring, T. J., and J. M. Moehring.** 1972. Response of cultured mammalian cells to diphtheria toxin. V. Concurrent resistance to ribonucleic acid viruses in diphtheria toxin-resistant KB cell strains. Infect. Immun. **6**:493–500.
35. **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.
36. **Ohkuma, S., and B. Poole.** 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl. Acad. Sci. U.S.A. **75**:3327–3331.
37. **Ray, B., and H. C. Wu.** 1981. Enhancement of cytotoxicities of ricin and Pseudomonas toxin in Chinese hamster ovary cells by monensin. Mol. Cell. Biol. **1**:552–559.
38. **Ray, B., and H. C. Wu.** 1982. Chinese hamster ovary cell mutants defective in the internalization of ricin. Mol. Cell. Biol. **2**:535–544.
39. **Sandvig, K., and S. Olsnes.** 1980. Diphtheria toxin entry into cells is facilitated by low pH. J. Cell Biol. **87**:828–832.
40. **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.
41. **Schlesinger, M. J., and S. Schlesinger.** 1973. Large-molecular-weight precursors of Sindbis virus proteins. J. Virol. **11**:1013–1016.
42. **Schlesinger, S., and M. J. Schlesinger.** 1972. Formation of Sindbis virus proteins: identification of a precursor for one of the envelope proteins. J. Virol. **10**:925–932.
43. **Srinivas, R. V., L. R. Melsen, and R. W. Compans.** 1982. Effects of monensin on morphogenesis and infectivity of Friend murine leukemia virus. J. Virol. **42**:1067–1075.
44. **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.
45. **Vasil, M. L., and B. H. Iglewski.** 1978. Comparative toxicities of diphtherial toxin and *Pseudomonas aeruginosa* exotoxin A: evidence for different cell receptors. J. Gen. Microbiol. **108**:333–337.
46. **Vasil, M. L., D. Kabat, and B. H. Iglewski.** 1977. Structure-activity relationships of an exotoxin of *Pseudomonas aeruginosa*. Infect. Immun. **16**:353–361.
47. **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.