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Chinese Hamster Ovary Cell Mutants Defective in the Internalization of Ricin

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Chinese hamster ovary mutants simultaneously resistant to ricin and *Pseudomonas* toxin have been isolated. Two mutant cell lines (4-10 and 11-2) were found to retain normal levels of binding of both ricin and *Pseudomonas* toxin. They were defective in the internalization of [^{125}I]ricin into the mutant cells, as measured by both a biochemical assay for ricin internalization and electron microscopic autoradiographic studies. Although pretreatment of Chinese hamster ovary cells with a Na^+/K^+ ionophore, nigericin, resulted in an enhancement of the cytotoxicities of ricin and *Pseudomonas* toxin in the wild-type Chinese hamster ovary cells, preculture of the mutant cells did not alter the susceptibility of the mutant cells to either toxin. These results provide further evidence that there is a common step in the internalization process for ricin and *Pseudomonas* toxin.

We have recently shown that the cytotoxicities of both ricin and *Pseudomonas aeruginosa* exotoxin A in Chinese hamster ovary (CHO) cells are enhanced by pretreatment of CHO cells with the monovalent cation ionophores nigericin or monensin (7). The cytotoxicity of diphtheria toxin is not affected in nigericin-pretreated CHO cells. Inasmuch as both the surface receptor and the intracellular biochemical target for ricin differ from those of *Pseudomonas* toxin, these observations strongly suggest that nigericin pretreatment has affected a rate-limiting step in the intoxication process common to both ricin and *Pseudomonas* toxin. This step presumably lies between the binding of toxins to the surface receptors and the expression of their enzymatic activities of inactivating the protein-synthesizing machinery in the cytosol. We have demonstrated that the extent of internalization of ricin into CHO cells is enhanced by nigericin pretreatment (8).

The presumption of a common step in the internalization of ricin and *Pseudomonas* toxin provides the rationale for the isolation of CHO mutant cell lines simultaneously resistant to both toxins by a single-step selection. In this paper we report the isolation and characterization of two CHO mutant cell lines which exhibit increased resistance towards both ricin and *Pseudomonas* toxin. Furthermore, we present evidence that the increased resistance of both mutants to ricin and *Pseudomonas* toxin is not due to changes in the binding of toxins to the cells, but rather results from a defect in a subsequent step of the internalization process.

MATERIALS AND METHODS

Cell culture. The cell lines used in the present study included a CHO *pro* strain obtained from L. Siminovitch, Toronto, Ont., Canada, and a ricin-resistant mutant (ts 1-1) deficient in ricin receptor isolated in this laboratory (7). The cells were grown in 100-mm plastic petri dishes containing 15 ml of α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum. Cells were incubated at 37°C in 5% CO_2.

Cytotoxicity assay. The cytotoxicities of ricin, *Pseudomonas* toxin, and diphtheria toxin were measured by the determination of the plating efficiencies of CHO cells in the presence of various concentrations of these toxins. Cytotoxicities were also measured by the effects of these toxins on cellular protein synthesis as described previously (7).

Binding and internalization of [^{125}I]ricin into CHO cells. [^{125}I]ricin was prepared as described previously (6). The specific radioactivity of [^{125}I]ricin was 3×10^6 cpm/μg. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a single radioactive band; reduction with 2-mercaptoethanol gave rise to two bands, corresponding to the A and B subunits (6). [^{125}I]ricin retained more than 80% of the cytotoxicity of the unlabeled ricin based on a plating efficiency assay. Binding and internalization of [^{125}I]ricin into CHO cells were assayed by the procedure described previously (6). Internalization of [^{125}I]ricin into CHO cells was also studied by the electron microscopic autoradiographic techniques, as described previously (8, 9).

Preparation of *Pseudomonas* ^{125}I-labeled toxin. A 200-μg portion of *Pseudomonas* toxin (a gift of S. Leppla) was dialyzed overnight against 0.05 M phosphate buffer (pH 7.2). The dialyzed toxin was radioiodinated by lactoperoxidase-catalyzed iodination as described previously (6). Free ^{125}I was removed by

Sephadex G-25 gel filtration and by repeated dialysis of iodinated toxin against 0.05 M phosphate buffer (pH 7.2). The dialyzed ^{125}I-labeled toxin was purified by affinity chromatography with a modification of the procedure described by Cukor et al. for the purification of diphtheria toxin (1). Bio-Gel P-150 was activated by hydrazine and then coupled with 6-aminocaproic acid. The free carboxyl group was coupled to NAD^+ with 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide as the coupler. The covalently linked NAD^+-polyacrylamide gel was used as the affinity adsorbent. The dialyzed ^{125}I-labeled *Pseudomonas* toxin was passed through the NAD^+-polyacrylamide gel column equilibrated with 0.02 M phosphate buffer (pH 7.2). The column was washed extensively with the same buffer, and ^{125}I-labeled toxin was eluted with 0.02 M phosphate buffer (pH 7.2) containing 0.5 M NaCl. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified ^{125}I-labeled toxin showed that ^{125}I radioactivity migrated as a single band corresponding to the mobility of *Pseudomonas* toxin under the same condition. Reduction of ^{125}I-labeled toxin with 0.1% β-mercaptoethanol revealed no alteration in the toxin's electrophoretic profile. The cytotoxicity of ^{125}I-labeled *Pseudomonas* toxin was 65 to 70% of the unlabeled toxin based on a plating efficiency assay. The specific radioactivity of ^{125}I-labeled toxin was 1.4×10^6 cpm/μg.

Binding of ^{125}I-labeled *Pseudomonas* toxin to CHO cells. Spinner cultured CHO cells were washed with serum-free α-MEM and resuspended in this medium to a final cell density of 2×10^6 cells per ml in silicone-coated Erlenmeyer flasks. After a preincubation at the given temperature for 15 min, the cell suspension was mixed with an equal volume of serum-free medium containing 2×10^{-8} M ^{125}I-labeled *Pseudomonas* toxin. Samples, 1 ml, were taken at regular intervals into Eppendorf tubes precoated with 0.5% bovine serum albumin in phosphate-buffered saline. After centrifugation, the pellet was washed with cold medium, and the ^{125}I radioactivity in the washed pellet was measured in a gamma counter.

Isolation of CHO mutants simultaneously resistant to ricin and *Pseudomonas* toxin. Spontaneous mutants of the CHO cell line simultaneously resistant to ricin and *Pseudomonas* toxin were isolated by treatment of a nonmutagenized culture with 400 ng of ricin and 200 ng of *Pseudomonas* toxin per ml for 7 days. After the removal of the dead cells, fresh medium without toxins was added, and the incubation was continued until visible colonies were formed. These mutants were cloned in 96-well Linbro plates repeatedly. Frequencies for ricin and *Pseudomonas* toxin doubly resistant mutants were about 2×10^{-7} to 3×10^{-7}. Frequency for singly ricin-resistant mutants was about 5×10^{-6} to 1×10^{-5} under the same conditions. Mutants 4-10 and 11-2 were chosen for the present study because they were found to retain normal levels of binding of [^{125}I]ricin (see data below). These two mutants were independent isolates obtained from separate cultures in different experiments. They have been maintained under nonselective conditions for over 2 years in the laboratory, and the ricin-resistant, *Pseudomonas* toxin-resistant phenotype has remained unchanged. The mean chromosome numbers of mutants 4-10 and 11-2 were 20.4 ± 0.19 and 20.7 ± 0.18, respectively.

Chemicals and radiochemicals. Ricin was purchased from Miles Laboratories, Inc. Pronase, galactose, lactoperoxidase, proline, NAD^+, 6-aminocaproic acid, and 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide were obtained from Sigma Chemical Co. Nigericin was a gift from Eli Lilly Co. Concanavalin A was purchased from Calbiochem. Diphtheria toxin was obtained from Connaught Laboratories. *P. aeruginosa* exotoxin A was a gift from S. Leppla (U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Md.). [^{125}I]Na (carrier-free; specific activity, 4 mCi/nmol) was purchased from Amersham Corp. [^{3}H]leucine was obtained from ICN Chemicals and Radioisotope Division. Powdered α-MEM and α-MEM suspension culture media were purchased from Flow Laboratories. Fetal bovine serum was obtained from Microbiological Associates.

RESULTS

Cytotoxicities of ricin, *Pseudomonas* toxin, and diphtheria toxin in CHO wild-type and mutant cell lines. The cytotoxicities of ricin, *Pseudomonas* toxin, and diphtheria toxin in CHO wild-type and mutant cell lines 4-10 and 11-2 were ascertained by measuring the plating efficiencies of wild-type and mutant cells in the presence of various toxin concentrations. Both mutants showed increased resistance to ricin and *Pseudomonas* toxin (Fig. 1a and b), as compared with the parental cell line. The susceptibilities of both mutants (4-10 and 11-2) towards diphtheria toxin remained indistinguishable from that of the wild-type cell line (Fig. 2).

The lack of concomitant resistance towards diphtheria toxin in mutants 4-10 and 11-2 strongly suggests that the increased resistance of these two mutants towards *Pseudomonas* toxin is not attributable to an alteration in elongation factor 2 or to a defect in the post-translational modification of histidine residue in elongation factor 2 to form diphthamide (2, 11). Furthermore, the increased resistance of these two mutants to ricin and *Pseudomonas* toxin is not likely due to a defect in cell surface receptors shared by both toxins since mutant cell line ts 1-1, known to be deficient in ricin receptors due to a defective GlcNAc transferase, exhibited normal sensitivity towards *Pseudomonas* toxin (Fig. 1a and b).

Evidence of increased resistance of the mutant cell lines 4-10 and 11-2 to ricin and *Pseudomonas* toxin was also obtained by measuring cellular protein synthesis in the presence of increasing concentrations of either toxin. Toxin treatment resulted in a greater inhibition of protein synthesis in the wild-type cell as compared with that in either mutant cell line (Fig. 3a and b).

Nigericin pretreatment does not enhance the cytotoxicities of ricin and *Pseudomonas* toxin in mutants 4-10 and 11-2. We have previously shown that preculture of CHO cells in the presence of 10^{-8} M nigericin enhances the cytotoxicities of ricin and *Pseudomonas* toxin by virtue

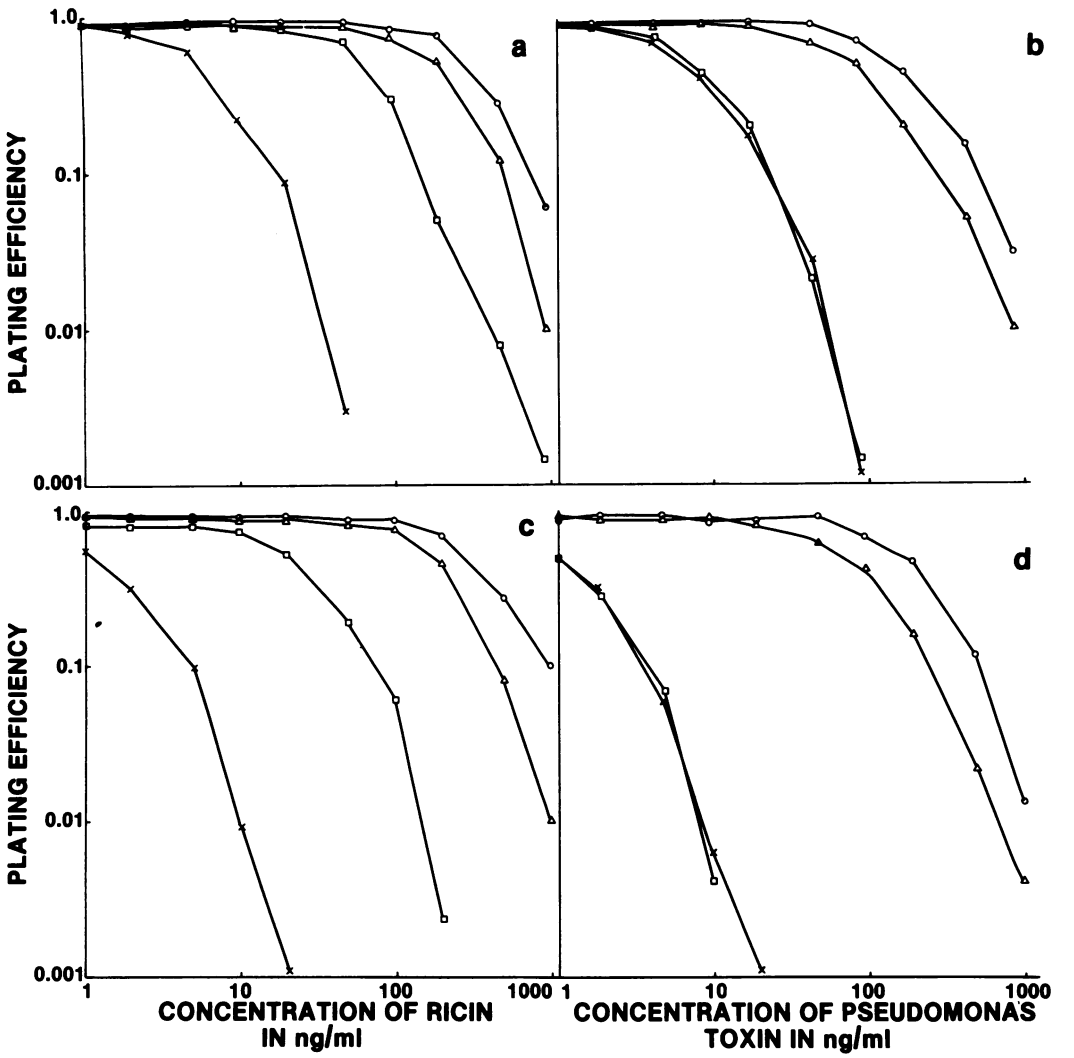

FIG. 1. Cytotoxicities of ricin and *Pseudomonas* toxin in wild-type and mutant CHO cells based on a plating efficiency assay. (a) Ricin; (b) *Pseudomonas* toxin; (c) ricin in the presence of nigericin (10^{-8} M); (d) *Pseudomonas* toxin in the presence of nigericin (10^{-8} M). Symbols: ×, wild type; ○, 4-10; △, 11-2; □, ts 1-1.

of increased efficiency of internalization of ricin and presumably *Pseudomonas* toxin into CHO cells (7, 8). The effect of nigericin pretreatment on the cytotoxicities of ricin and *Pseudomonas* toxin in mutant cell lines 4-10 and 11-2 was examined. Nigericin-pretreated mutant cells exhibited the same dose-response curves for ricin and *Pseudomonas* toxin as did the untreated mutant cells (Fig. 1a and d, 3c and d). In contrast, nigericin pretreatment of both the parental CHO cells and the ricin-resistant mutant ts 1-1, deficient in ricin receptors, resulted in enhanced sensitivities to both toxins. These results strongly suggest that the mutations in 4-10 and 11-2 have altered the effect of nigericin in potentiating the cytotoxicities of these two toxins. This is not due to an increased resistance to nigericin per se since the dose-response curves of the effect of nigericin on the viability of mutant cells were found to be indistinguishable from that of the wild-type cells (data not shown).

Comparison of cytotoxicity assays based on plating efficiency and cellular protein synthesis. The 50% lethal doses (LD_{50}s) of ricin and *Pseudomonas* toxin, as measured by the loss of viability of CHO wild-type and mutant cell lines, and the 50% inhibitory doses (ID_{50}s) based on inhibition of cellular protein synthesis are sum-

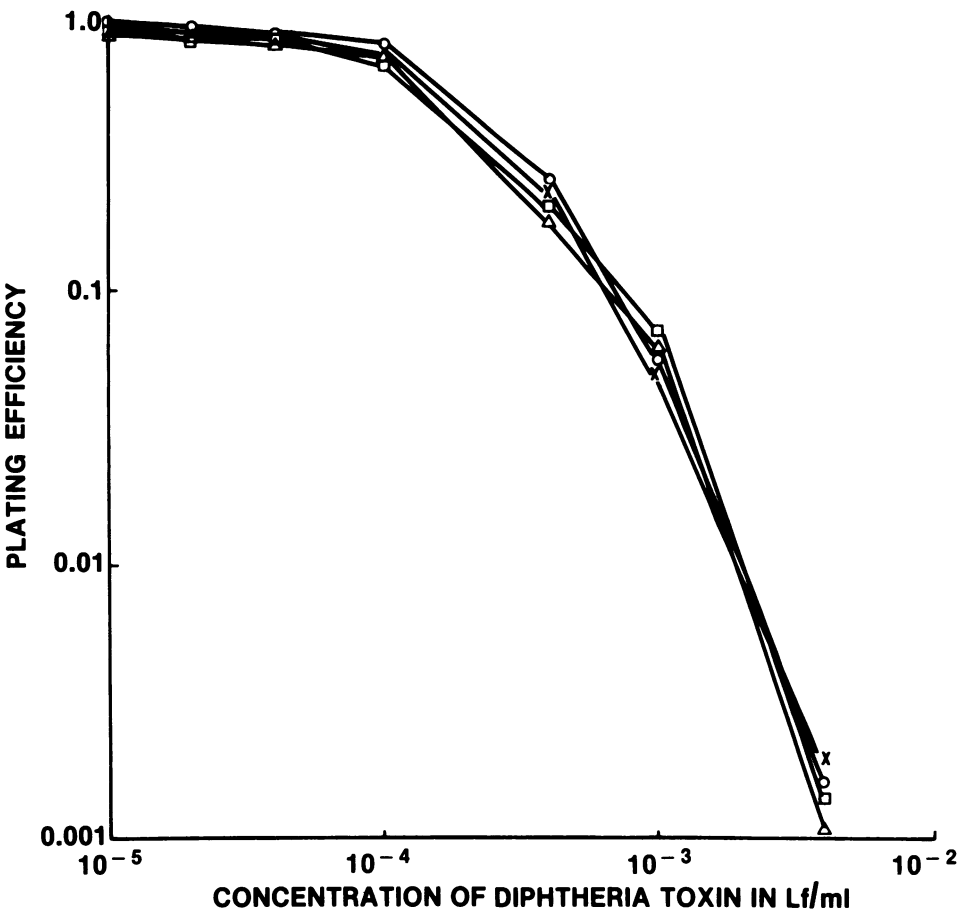

FIG. 2. Dose-response curves of wild-type and mutant cells with diphtheria toxin as measured by a plating efficiency assay. Symbols: ×, wild type; ○, 4-10; △, 11-2; □ ts 1-1.

marized in Table 1. Analysis of these data revealed an interesting difference between the mutants (4-10 and 11-2) and the wild-type cell line.

(i) The ID_{50}/LD_{50} ratios of wild-type CHO cells are 1 to 2 for both ricin and *Pseudomonas* toxin. The same is true for ID_{50}/LD_{50} ratios of both toxins in mutant ts 1-1 even though there is an approximately 10-fold increase in resistance to ricin in this receptor-deficient mutant. Nigericin pretreatment of wild-type CHO cells and mutant ts 1-1 cells resulted in a 5- to 10-fold increase in their susceptibilities towards ricin and *Pseudomonas* toxin. However, the ID_{50}/LD_{50} ratios for either toxin remained unchanged in nigericin-pretreated cells.

(ii) The ID_{50}/LD_{50} ratios for ricin and *Pseudomonas* toxin were 10-fold higher in mutants 4-10 and 11-2 than in the parental cell line. These ratios remained high in nigericin-pretreated mutant cells.

One major difference between the plating efficiency assay and measurement of protein synthesis concerned the time at which these assays were performed. An increase in ID_{50}/LD_{50} ratio may reflect a delayed appearance of active toxin molecules in the cytosol of mutants 4-10 and 11-2.

Binding and internalization of [^{125}I]ricin in CHO wild-type and mutant cell lines. The binding of [^{125}I]ricin to wild-type and mutant CHO cells at 0 and 37°C is shown in Fig. 4. Cells were preincubated at 0 or 37°C for 30 min before the binding assay. As expected for a receptor-deficient mutant, ts 1-1 cells bound much less [^{125}I]ricin at both 0 and 37°C. Mutant ts 1-1 is resistant to ricin at both 33 and 37°C, and the receptor deficiency in ts 1-1 is not temperature dependent. In contrast, binding of [^{125}I]ricin to mutants 4-10 and 11-2 were normal at both temperatures. These results provide direct evidence that neither mutant is deficient in ricin

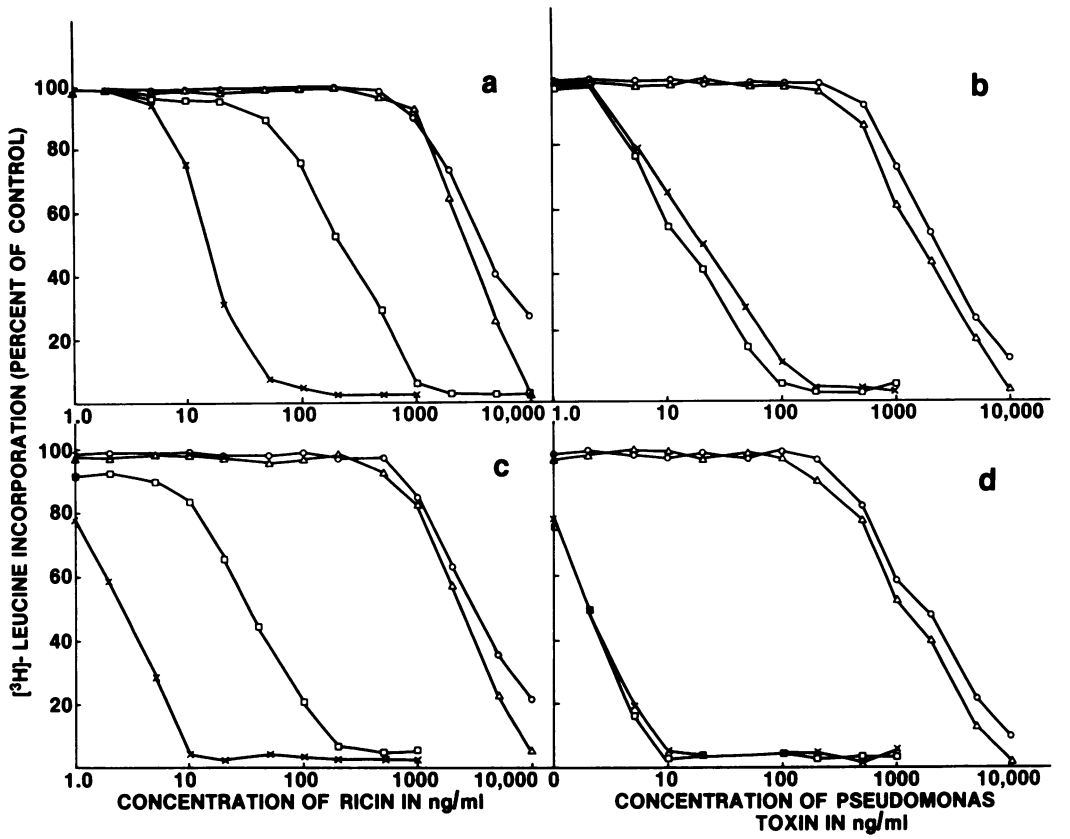

FIG. 3. Inhibition of protein synthesis by ricin or *Pseudomonas* toxin in CHO wild-type and mutant cells. (a) Treatment of CHO cells with ricin for 90 min before the assay; (b) treatment of CHO cells with *Pseudomonas* toxin for 180 min before the assay; (c) dose-response curves for ricin in nigericin-pretreated cells; (d) dose-response curves for *Pseudomonas* toxin in nigericin-pretreated cells. In (c) and (d), cells were pretreated with 10^{-8} M nigericin for 2 to 3 days, and toxin was added to the washed cells without the continuous presence of nigericin. Symbols: ×, wild type; O, 4-10; △, 11-2; □, ts 1-1.

receptors. This finding is further supported by the results shown in Table 2. As reported previously, ricin-resistant CHO mutants defective in GlcNAc transferase show increased sensitivity towards concanavalin A, presumably due to the unmasking of concanavalin A receptors on the cell surfaces (10). Whereas mutant ts 1-1 was hypersensitive to concanavalin A, the concanavalin A sensitivities of both mutants 4-10 and 11-2 were unaltered.

TABLE 1. Comparison of cytotoxicities of ricin and *Pseudomonas* toxin as measured by ID_{50} of cellular protein synthesis and LD_{50} of cellular viability in CHO wild-type and mutant cells

Cell line	Ricin (ng/ml)						*Pseudomonas* toxin (ng/ml)					
	Without nigericin pretreatment			With nigericin pretreatment[a]			Without nigericin pretreatment			With nigericin pretreatment[a]		
	ID_{50}[b]	LD_{50}[c]	ID_{50}/LD_{50}	ID_{50}	LD_{50}	ID_{50}/LD_{50}	ID_{50}	LD_{50}	ID_{50}/LD_{50}	ID_{50}	LD_{50}	ID_{50}/LD_{50}
Wild type	18	16	1.1	33	1.5	2	14	9	1.6	2	1	2
4-10	3,800	330	11.8	3,200	290	11	2,100	150	14	1,800	130	14
11-2	2,800	220	12.8	2,400	190	12.6	1,500	100	15	1,100	85	13
ts 1-1	210	150	1.4	36	22	1.6	11	8	1.4	2	1	2

[a] Cells were pretreated with 10^{-8} M nigericin for 48 to 56 h before the addition of ricin or *Pseudomonas* toxin.
[b] ID_{50} was measured as described in the legend to Fig. 3.
[c] LD_{50} was determined as described in the legend to Fig. 1.

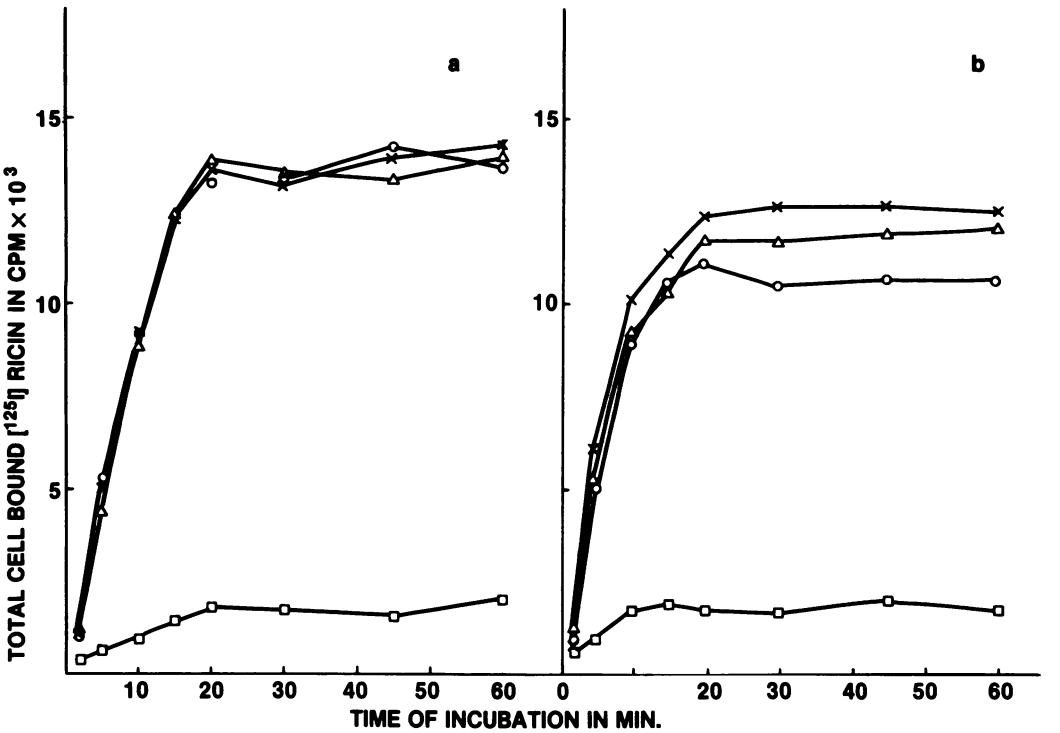

FIG. 4. Binding of [^{125}I]ricin to CHO wild-type and mutant cells at 0°C (a) and 37°C (b), respectively. Details for the binding assay are given in the text. Symbols: ×, wild type; ○, 4-10; △, 11-2; □, ts 1-1.

Internalization of [^{125}I]ricin into mutants 4-10 and 11-2 at 37°C was measured. Mutants 4-10 and 11-2 internalized 15 to 18% of total cell-bound [^{125}I]ricin, whereas both the wild-type and mutant ts 1-1 internalized 40 to 45% of cell-bound ricin (Fig. 5). Throughout the wide range of ricin concentrations (1 to 1,000 ng/ml) examined, the efficiency of internalization of [^{125}I]ricin in mutants 4-10 and 11-2 was about 30 to 40% of that found in the wild-type cells (data not shown).

Figure 6a and b shows the Scatchard analysis of the binding and internalization of [^{125}I]ricin in wild-type and mutant cell lines over a range of ricin concentrations, 1 ng/ml to 1 μg/ml. It is clear that mutants ts 1-1 has a reduced number of binding sites for ricin and consequently a reduced number of internalization sites for ricin as well. In contrast, mutants 4-10 and 11-2 have normal numbers of binding sites for ricin but reduced numbers of internalization sites for ricin. The apparent affinities of the binding sites and internalization sites for ricin remained unchanged in mutants 4-10 and 11-2 (data not shown). (The equation of Klotz and Hunston [5] was used for these calculations.)

TABLE 2. Concanavalin A sensitivity of CHO wild-type and mutant cells

Cell line	LD_{50} (μg/ml)[a]
Wild type	10
4-10	9
11-2	11
ts 1-1	2

[a] Cytotoxicity of concanavalin A was measured by plating 500 cells per well in 24-well Linbro plates. Cells were seeded 1 day before the addition of various concentrations of concanavalin A. Colonies were stained 7 days later with 0.2% methylene blue in 50% methanol.

Electron microscopic autoradiographic studies of [^{125}I]ricin in wild-type and mutant 4-10 cells. We have used thin-section electron microscopy and autoradiography to study the internalization of [^{125}I]ricin into CHO cells. The total numbers of grains per cell were similar between the wild-type and mutant 4-10 cells at both 0 and 37°C (Table 3, lines 1 to 4). This observation is consistent with the nearly identical binding of [^{125}I]ricin in both the wild-type and mutant 4-10 cells, as measured by [^{125}I]ricin binding studies (Fig. 4). After the binding of [^{125}I]ricin, subsequent treatment of CHO cells with galactose and pronase at 0°C resulted in the removal of surface-bound ricin in the wild-type cells. Thus, 90% of grains in the wild-type cells which had

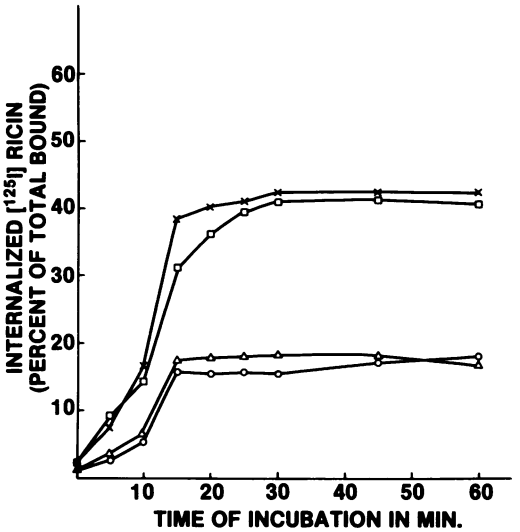

FIG. 5. Time course of internalization of [^{125}I]ricin in wild-type and mutant cells. Cells were suspended in serum-free α-MEM and preincubated for 15 min at 37°C. [^{125}I]ricin was added and the cell suspension was incubated at 37°C. At the given intervals, 0.5 ml of the cell suspension (2 × 10^6 cells per ml) was centrifuged and washed. Radioactivity in the washed pellet was measured and represented the total cell-bound toxin. To determine the internalized ricin, cells were treated at 0°C for 15 min with a mixture of 0.1 M galactose and 0.5 μg of pronase per ml, and the radioactivity remaining in the cells after washing was measured. Symbols: ×, wild type; ○, 4-10; △, 11-2; □, ts 1-1.

been treated with galactose and pronase was found to be inside the cells, at both 0 and 37°C (Table 3, lines 5 and 7). These results provide direct evidence for the validity of our biochemical assay of [^{125}I]ricin internalization in CHO cells (6). Mutant 4-10 cells showed interesting differences from the wild-type cells with regard to both the efficiency of internalization and the distribution of grains after the galactose-pronase treatment. A total of 65% of the grains were found inside the wild-type cells at 37°C, whereas 37% were judged to be inside the mutant cells (Table 3, lines 3 and 4). After the galactose-pronase treatment, less total grains were found per mutant cell compared with the wild-type cells (Table 3, lines 7 and 8). This is consistent with a decreased efficiency of ricin internalization in mutant 4-10 (Fig. 5). More interesting, only 60% of the grains were found inside the mutant cells, even after galactose-pronase treatment, with 30% remaining within two half-distances from the plasma membrane (Table 3, line 8). This result suggests that a significant fraction of [^{125}I]ricin may remain at the cell surface of mutant 4-10 at 37°C even though [^{125}I]ricin molecules appear refractory to the combined action of galactose and pronase treatment.

Binding of *Pseudomonas* [^{125}I]toxin to CHO wild-type and mutant 4-10 cells. The bindings of *Pseudomonas* [^{125}I]toxin to CHO wild-type and mutant 4-10 cells were measured at 0 and 37°C, respectively. Mutant 4-10 cells bound as much *Pseudomonas* [^{125}I]toxin as the wild-type cells (Fig. 7), and the kinetics of binding at 0°C was identical. At 37°C, mutant 4-10 cells bound the same amount of [^{125}I]toxin as that at 0°C, and a plateau was reached by 15 to 20 min. The wild-type cells showed the same kinetics of binding at 37°C for the first 15 min. In the wild-type cells, binding increased gradually for up to 120 min at 37°C, whereas the binding in the mutant 4-10 cells remained unchanged under the same condition (Fig. 7b). Inasmuch as the nature of surface receptors for *Pseudomonas* toxin is unknown, we have not been able to remove the surface-bound toxin and specifically examine the internalization of this toxin. However, it is reasonable to assume that the second and slow phase of association of *Pseudomonas* [^{125}I]toxin with the wild-type cells at 37°C represents internalization of this toxin. Our results are consistent with the working hypothesis that mutant 4-10 is defective in the internalization of *Pseudomonas* toxin as well as that of ricin.

DISCUSSION

Based on our previous observations that nigericin pretreatment of CHO cells results in an increased sensitivity towards ricin, abrin, and *Pseudomonas* toxin but not diphtheria toxin, we have postulated that there is a common step in the internalization processes for ricin and *Pseudomonas* toxin in CHO cells (7). The isolation of spontaneous mutants of CHO cell lines (4-10 and 11-2) by a single-step selection with ricin and *Pseudomonas* toxin provides further support of our hypothesis.

The results presented in this paper strongly suggest that mutants 4-10 and 11-2 are defective in one of the steps in the overall process of transporting surface-bound toxin molecules into the cytosol. This conclusion is based on the following observations: (i) binding of ^{125}I-labeled ricin and *Pseudomonas* toxin to the mutant cells at 0°C is indistinguishable from that of the wild-type cells; (ii) internalization of [^{125}I]ricin into the mutant cells is less efficient as judged by both biochemical and electron microscopic autoradiographic studies; (iii) second-phase and gradual increase in the association of ^{125}I-labeled *Pseudomonas* toxin with the mutant 4-10 cells at 37°C is diminished compared with the wild-type cells; (iv) mutants 4-10 and 11-2 require 100 to 200 times more toxin to elicit 50% inhibition of protein synthesis by either toxin than the wild-type cells when the assays are

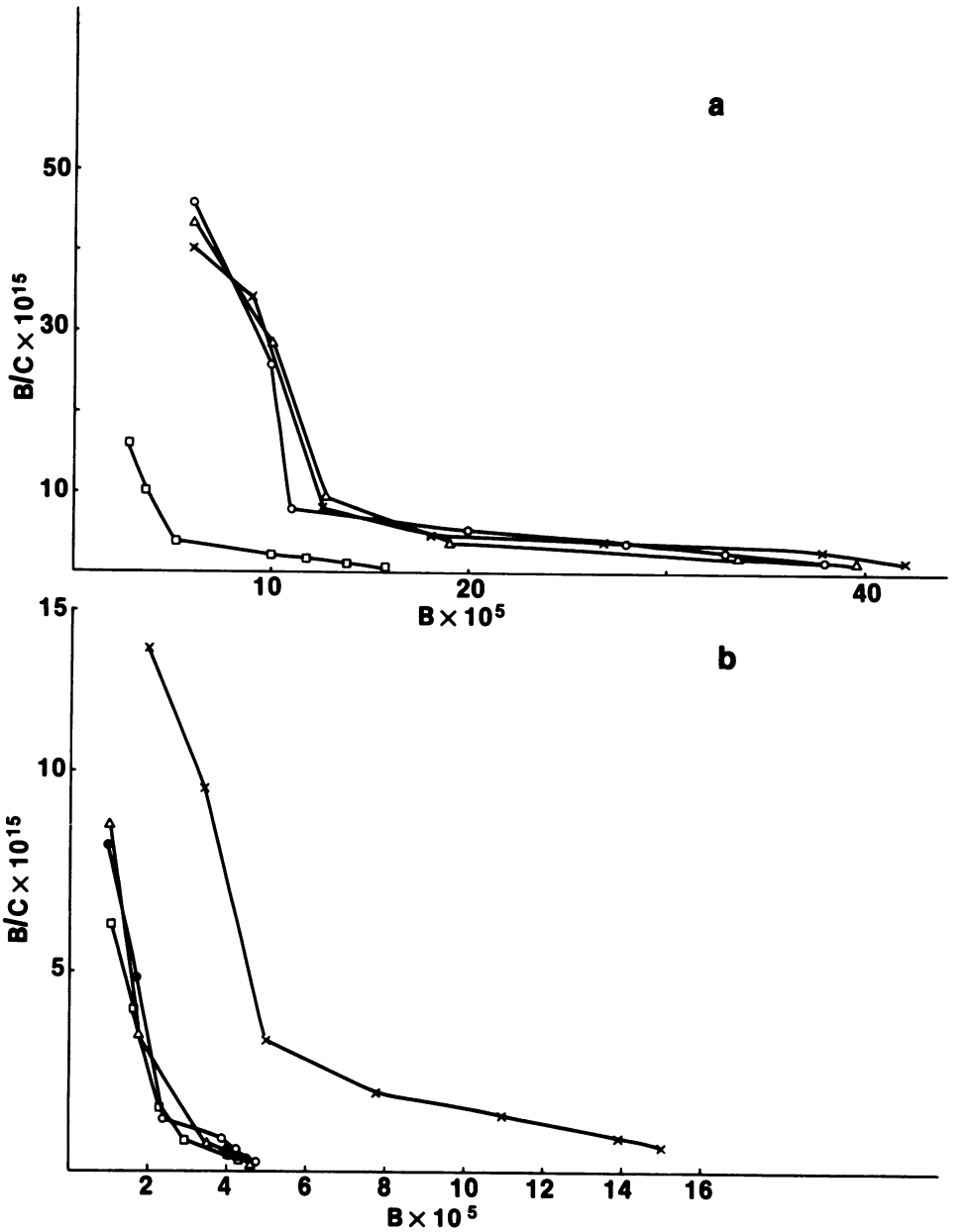

FIG. 6. Scatchard analysis of the binding (a) and internalization (b) of [^{125}I]ricin in CHO wild-type and mutant cells. Symbols: ×, wild-type; ○, 4-10; △, 11-2; □, ts 1-1. The formula used was $B/C = K(n - B)$, where B is the number of ricin molecules bound or internalized per cell, C is the number of free ricin molecules, n is the number of binding or internalization sites per cell, and K is the apparent association constant.

performed after a 90- or 180-min treatment with ricin or *Pseudomonas* toxin, respectively. On the other hand, the LD_{50} for either toxin based on a plating efficiency assay, which measures the cumulative effect of toxin treatment over 7 to 10 days, is increased about 15- to 20-fold in both mutants. This greater discrepancy between ID_{50} and LD_{50} in the mutants compared with the wild-type cells is consistent with the possibility that these two mutants are defective in the rapid transport of surface-bound toxins into the cytosol, where they exert their enzymatic activities of inactivating 60S ribosomes or elongation factor 2, respectively (3, 4).

TABLE 3. Binding and internalization of [^{125}I]ricin in wild-type and mutant 4-10 cells as measured by electron microscopic autoradiography[a]

Cell line	Incubation temp, 30 min (°C)	Pronase-galactose treatment, 15 min at 0°C	No. of grains per cell	Distribution of grains from surface membrane (% of total)[b]	
				≤2 HD	>2 HD
1. Wild type	0	−	7.9 ± 1.5	90	10
2. 4-10	0	−	7.6 ± 1.9	90	10
3. Wild type	37	−	8.2 ± 1.3	35	65
4. 4-10	37	−	7.6 ± 1.2	63	37
5. Wild type	0	+	0.3 ± 0.02	11	89
6. 4-10	0	+	0.2 ± 0.03	27	73
7. Wild type	37	+	4.1 ± 0.45	9	91
8. 4-10	37	+	2.2 ± 0.16	40	60

[a] A total of 200 to 400 grains were counted for lines 1 to 4 and 7 to 8; 25 to 30 grains were counted for lines 5 and 6.

[b] 2 HD, Two half-distances of the grains (200 nm). Grains located ≤2 HD from the plasma membrane are considered to be at the cell surface; those >2 HD from the cell boundaries are presumed to be inside the cells.

Biochemical studies of the internalization of [^{125}I]ricin have failed to reveal a significant difference in the kinetics of internalization between wild-type and mutant cells, even though the overall efficiency is reduced from 40 to 18%. The biochemical measurement of internalization is obtained by measuring the [^{125}I]ricin which is not removed by the combined treatment of galactose and pronase (6). This assay does not distinguish internalized ricin from ricin molecules which remain at the cell surface and are sequestered from the galactose-pronase treatment. The latter possibility may indeed be the case for the mutants reported here since a significant fraction of silver grains are found within two half-distances from the plasma membrane after the galactose-pronase treatment. It is conceivable that surface-bound [^{125}I]ricin is vesicularized in mutant cells, and the defect in the internalization process lies in the subsequent transport of ricin into the cell or in the release of ricin from the endocytotic vesicles into the cytosol. Whereas it is difficult to define the molecular meaning of the effective number of internalization sites for ricin as measured by Scatchard analysis (8), it is interesting to note that for two distinct groups of ricin-resistant mutants (receptor-deficient ts 1-1 and internalization-defective mutants 4-10 and 11-2), there is an excellent correlation between LD_{50} (Table 1) and the

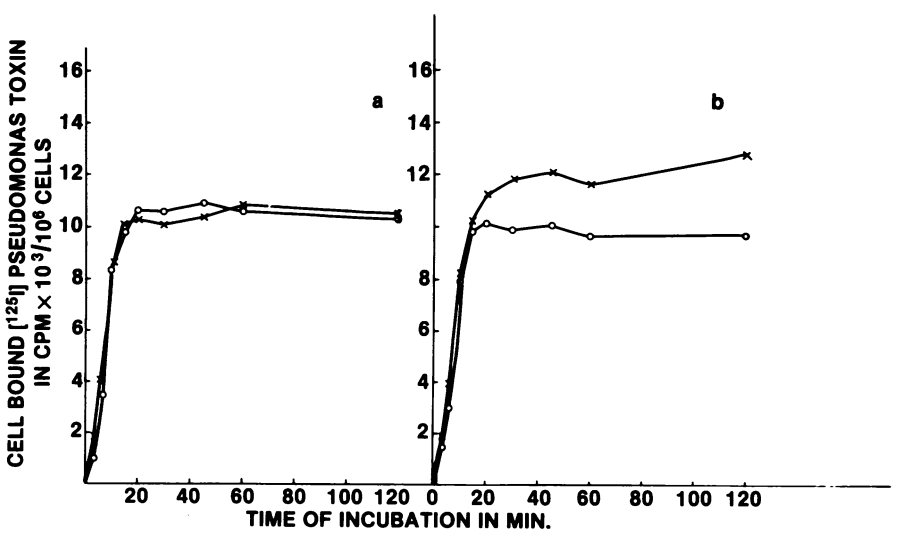

FIG. 7. Time course of binding of ^{125}I-labeled *Pseudomonas* toxin in wild-type and mutant cells at 0°C (a) and 37°C (b), respectively. Symbols: ×, wild type; ○, 4-10.

number of internalized ricin molecules (Fig. 6b). This correlation strongly argues in favor of the validity of the internalization assay. Had the bulk of internalized [^{125}I]ricin been physiologically irrelevant to ricin cytotoxicity, such a correlation would not have been observed.

Unlike the wild-type cells, mutants 4-10 and 11-2 are refractory to the potentiation of cytotoxicities of ricin and *Pseudomonas* toxin by pretreatment of the mutant cells with nigericin. This observation is of considerable interest since our previous studies have suggested that nigericin pretreatment enhances ricin cytotoxicity by facilitating the internalization of surface-bound ricin. The mutations affecting the transport of ricin and *Pseudomonas* toxin into the cytosol of mutants 4-10 and 11-2 may have altered the machinery for internalization in such a way that preculture in nigericin could not bring about either an increase in the number of the putative internalization sites or an increase in its efficacy.

Ricin and *Pseudomonas* toxin are foreign to CHO cells, and the transport of these toxins into the cells probably utilizes receptors and internalization processes which function in the uptake of physiologically relevant polypeptides. We can now use three different criteria to ascertain whether a given polypeptide utilizes the same internalization process as those used by ricin and *Pseudomonas* toxin: (i) potentiation of the biological effects of the ligand protein by preculture in media containing nigericin or monensin; (ii) reduced biological responses in mutants described in this paper; and (iii) competition of internalization of [^{125}I]ricin by the ligand protein.

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