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Enhancement of Cytotoxicities of Ricin and *Pseudomonas* Toxin in Chinese Hamster Ovary Cells by Nigericin

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Nigericin and monensin, ionophores for Na^+ and K^+, have been found to enhance the cytotoxicities of abrin, ricin, and *Pseudomonas aeruginosa* exotoxin A in Chinese hamster ovary (CHO) cells. They do not affect the cytotoxicity of diphtheria toxin in the same cell line. Maximal sensitization of the CHO cells toward ricin and *Pseudomonas* toxin requires preculture of CHO cells in the presence of nigericin. Inhibition of protein synthesis in CHO cells by ricin or *Pseudomonas* toxin is also enhanced by preculture of CHO cells in the presence of nigericin. These results suggest a common step in the intoxication process of ricin and *Pseudomonas* toxin, the rate of which is facilitated by pretreatment with nigericin. This step is, however, not shared by the intoxication of CHO cells with diphtheria toxin.

Proteins of bacterial or plant origin are potent cytotoxins both in vivo and in vitro (2, 6, 11, 13). Many of these toxins share common structural and functional features. They consist of two structural domains, A and B subunits which correspond to enzymatic and receptor-binding activities of the toxin molecules, respectively (1, 3, 12). Toxins may have similar enzymatic activities of their A subunits (or domains) but different B subunits (or domains) which are involved in the binding of toxins to the surface receptors of the susceptible cells. Diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A are examples of such a relationship (2). Plant cytotoxins such as ricin and abrin have a mode of action different from that of diphtheria and *Pseudomonas* toxins, even though they are also potent inhibitors of cellular protein synthesis. Both the A and B subunits of ricin and abrin are thus structurally and functionally distinct from the corresponding subunits (or domains) in diphtheria and *Pseudomonas* toxins. Although the receptors and the intracellular biochemical targets for these microbial and plant toxins are different, they may still share a common component in the overall intoxication process. It is generally assumed that these toxins, or their subunits, must enter the cell to exert their biological and enzymatic activities (13). It is conceivable, therefore, that there exists a common step in the internalization process of these unrelated toxins into the target cells.

Vesicles have been involved in the transport of macromolecules into and out of the cell by the process of endocytosis and exocytosis, respectively. Recent studies have indicated that certain proteins such as lysosomal enzymes or surface receptors may be recycled by a combination of these two processes (5, 9). Na^+ K^+ ionophores have been shown to interfere with the secretions of certain proteins such as acetylcholinesterase, collagen, fibronectin, etc. (15-17). Therefore, we undertook the study of the effect of ionophores on the uptake of proteins into the cell.

In this paper, we report the enhancement of cytotoxicities of ricin and *Pseudomonas* toxin by preculture of Chinese hamster ovary (CHO) cells in the presence of nigericin. Under the same conditions, the cytotoxicity of diphtheria toxin was not affected. Our results suggest a common step in the intoxication of CHO cells with ricin and *Pseudomonas* toxin which is not shared by diphtheria toxin.

MATERIALS AND METHODS

Cell culture. The cell line used in the present study was a CHO cell line auxotrophic for proline (*pro*$^-$), a gift of L. Siminovitch, Toronto, Canada. The cells were grown in 100-mm plastic petri dishes containing 15 ml of α-minimal essential medium (α-MEM) supplemented with 10% fetal calf serum. For spinner culture, α-MEM suspension media supplemented with 10% fetal calf serum and 40 μg of proline per ml were used. Cell culture was carried out at 37°C under 5% CO_2.

Cytotoxicity assay. The cytotoxicities of abrin, ricin, diphtheria toxin, and *Pseudomonas* toxin were measured by the determination of plating efficiencies

of CHO cells in the presence of various concentrations of these toxins. Cells were plated on 60-mm or 100-mm petri dishes 1 day before the addition of cytotoxins. Distinct and visible colonies were counted after staining with 0.2% methylene blue in 50% methanol.

In vivo protein synthesis. The spinner culture was harvested and incubated in serum-free α-MEM without leucine for 1 h. Cells were treated with various concentrations of toxins for 90 min (for ricin) or 180 min (for *P. aeruginosa* exotoxin A) at 37°C. After the incubation with toxin, the cells were washed and suspended in serum-free, leucine-free α-MEM and labeled with [^{3}H]leucine (0.5 μCi/ml; specific activity, 39 Ci/mmol) for 2 h at 37°C. The cells were centrifuged and washed with the medium. To the cell pellet, 0.1 ml of 1% bovine serum albumin dissolved in 1 N NaOH was added. After incubation at 37°C for 10 min, 5 ml of 10% trichloroacetic acid was added, mixed, and kept in the ice bath for 30 min. The trichloroacetic acid precipitate was collected on glass fiber paper (GF/C) and washed with 5% cold trichloroacetic acid solution. The filter paper was then dried and counted with 4 ml of toluene-Liquifluor scintillation solution in a Beckman LS 230 liquid scintillation counter.

Chemicals and radiochemicals. The following chemicals were purchased from Sigma Chemical Co.: chloroquine phosphate, cytochalasin B and D, colchicine, glycylglycine, wheat germ agglutinin, cysteamine, putrescine, dansylcadavarine, galactose, pronase, proline, ouabain, lactoperoxidase, and bovine serum albumin. Ionophores A23187, X537A, nigericin, and monensin were obtained from Eli Lilly & Co. Ricin and phytohemagglutinin were the products of Miles Laboratories, Inc. Abrin and concanavalin A were purchased from Calbiochem. Diphtheria toxin was purchased from Connaught Laboratories, Ontario, Canada. *P. aeruginosa* exotoxin A was a gift from S. Leppla (U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Md.). [^{3}H]leucine was purchased 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

Effect of various chemicals on the cytotoxicities of ricin, *Pseudomonas* toxin, and diphtheria toxin. Using Linbro plates to assay the relative cytotoxicity of ricin, diphtheria toxin, and *Pseudomonas* toxin, we have screened a large number of chemicals to see whether they enhance or reduce the cytotoxicities of these unrelated toxins. The agents we have tested include nigericin, monensin, ionophores A23187 and X537A, ammonium chloride, cytochalasin B and D, chloroquine phosphate, K^+, Na^+, methylamine, dansylcadaverine, cysteamine, putrescine, etc. (Table 1). Of special interest were the strikingly different effects of

TABLE 1. *Effect of different agents on the cytotoxicities of ricin, P. aeruginosa exotoxin A, and diphtheria toxin*[a]

Agent	Concn	Cytotoxicity (LD_{50}) of:		
		Ricin (ng/ml)	*Pseudomonas* toxin (ng/ml)	Diphtheria toxin (LF/ml)
None		16	9	1.5×10^{-4}
Nigericin	10 nM	1.5	1	1.7×10^{-4}
Monensin	50 nM	1.3	0.8	1.3×10^{-4}
Ionophore A23187	0.1 μM	14	10	2×10^{-4}
Ionophore X537A	0.1 μM	15	8	1.5×10^{-4}
Ouabain	5 μM	12	6	ND
Ammonium chloride	0.2 mg/ml	3.5	8	2×10^{-2}
Sodium fluoride	0.5 mM	14	4	5×10^{-2}
Cytochalasin B	2 μg/ml	14	8	ND
Cytochalasin D	2 μg/ml	16	7	ND
Chloroquine phosphate	20 μM	8	7.5	6×10^{-2}
Colchicine	20 nM	14	7	ND
Glycylglycine	50 mM	20	8	1.0×10^{-4}
Cysteamine	50 μM	18	9.5	ND
Methylamine	0.5 mM	19	8	2×10^{-4}
Putrescine	1 mM	18	ND	ND
Dansylcadavarine	1 μM	17	ND	ND
NaCl	0.1 M	12	9	1.3×10^{-4}
Na_2SO_4	0.1 M	14	5	2.3×10^{-4}
KCl	0.1 M	17	8	2.5×10^{-4}
Nigericin and NH_4Cl	5 nM and 0.1 mg/ml	0.8	0.8	ND

[a] All of the incubations were done in 24 Linbro wells with 500 cells in each well. The cells were seeded 1 day before the addition of various agents and toxins. The chemical agents were added 2 h before the addition of toxins. The colonies were stained 7 days later with 0.2% methylene blue in 50% methanol. LF, Flocculating units; ND, not determined.

NH_4Cl on the toxicity of diphtheria toxin and ricin. As previously reported by other investigators (4, 8), NH_4Cl protects cells from the cytotoxicity of diphtheria toxin. In contrast, it enhances the toxicity of ricin in CHO cells. It has no effect on toxicity of *Pseudomonas* toxin. Likewise, chloroquine phosphate reduces toxicity of diphtheria toxin but enhances that of ricin (7). NaF protects CHO cells from diphtheria toxin, but slightly enhances the cytotoxicity of *Pseudomonas* toxin (3).

The second group of compounds of special interest is the monovalent ionophores, monensin and nigericin. When either of these compounds was present, the cytotoxicities of ricin, abrin, and *Pseudomonas* toxin were significantly enhanced whereas those of diphtheria toxin, concanavalin A, wheat germ agglutinin, and phytohemagglutinin remained unchanged (Tables 1 and 2). Quantitative measurement of cytotoxicity was carried out in the presence or absence of 10 nM nigericin, a concentration of nigericin having no effect on the growth and plating efficiency of CHO cells (data not shown). The 50% lethal dose (LD_{50}) of ricin was reduced from 16 ng/ml to 1.5 ng/ml by the presence of nigericin (Fig. 1). Likewise, the LD_{50} of *Pseudomonas* toxin was reduced from 9 ng/ml to 1 ng/ml in the presence of the ionophore. The LD_{50} of diphtheria toxin was not affected by nigericin. The differential effects of nigericin on the cytotoxicities of ricin, *Pseudomonas* toxin, and diphtheria toxin are not restricted to CHO cells; similar results have been obtained with HeLa cells (Ray and Wu, unpublished observations). Other ionophores (A23187 and X537A) had no effect on the toxicities of ricin and *Pseudomonas* toxin, nor could various cations or anions reverse the effects of nigericin or monensin (data not shown). The effects of monensin and nigericin are not shared by valinomycin, which does not alter the cytotoxicity of ricin (data not shown).

TABLE 2. *Effect of nigericin on the cytotoxicities of various lectins*[a]

Lectin	Cytotoxicity (LD_{50})	
	No nigericin	With nigericin (10 nM)
Ricin	16 ng/ml	1.5 ng/ml
Abrin	12 ng/ml	2.0 ng/ml
Concanavalin A	10 μg/ml	7 μg/ml
Wheat germ agglutinin	2 μg/ml	2 μg/ml
Phytohemagglutinin	2 μg/ml	1.2 μg/ml

[a] All of the incubations were carried out in 24 Linbro wells with 500 cells in each well. The cells were seeded 1 day before the addition of nigericin and lectins. The colonies were stained 7 days later with 0.2% methylene blue in 50% methanol.

Sensitization of CHO cells toward the cytotoxicities of ricin and *Pseudomonas* toxin required the pretreatment of the CHO cells with the ionophore (Fig. 2a). Cotreatment of toxin and ionophore for 10 h followed by removal of both toxin and ionophore did not affect the cytotoxicity of the toxins (data not shown). Moreover, pretreatment of CHO cells with nigericin sensitizes the cells to ricin in such a way that the ionophore is not needed during the subsequent addition of ricin to CHO cells (Fig. 2a). In contrast, the effect of NH_4Cl on cytotoxicity of ricin did not require pretreatment. It required, instead, the presence of NH_4Cl during the toxin treatment (Fig. 3). The effect of NH_4Cl appeared specific, and other primary amines (e.g., methylamine and dansylcadavarine) did not appreciably alter the LD_{50} of ricin even at concentrations of 0.5 mM and 1 μM, respectively. Unlike the monovalent ionophores, NH_4Cl did not affect the cytotoxicity of *Pseudomonas* toxin in CHO cells. NH_4Cl and nigericin appeared to act synergistically in enhancing the cytotoxicity of ricin, but not that of *Pseudomonas* toxin (Table 1). These results suggest that these two compounds have different sites of action.

Enhancement of cytotoxicity of ricin and *Pseudomonas* toxin by nigericin was also reflected in the greater inhibition of cellular protein synthesis by ricin and *Pseudomonas* toxin in cells pretreated with 10 nM nigericin for 60 h (Fig. 4). Nigericin treatment alone at this low concentration did not affect the rate of protein synthesis. This result suggests that the decreased survival of CHO cells in the presence of both toxin and nigericin is due to a more efficient inhibition of cellular protein synthesis by these two toxins in nigericin-treated cells. Maximal enhancement of inhibition of protein synthesis by either of these two toxins requires pretreatment of CHO cells with nigericin for 60 to 80 h (Fig. 2b). Increased concentration of nigericin from 10 nM to 0.1 μM did not significantly reduce the time of preincubation for the enhancement of cytotoxicity of ricin (data not shown).

Figure 5 shows the effect of nigericin on ricin cytotoxicity in a CHO mutant cell line which is resistant to abrin and ricin due to decreased number of receptors for the toxins. This cell line, CHO *pro*$^-$ *ric*^{r} *ts*-1-1, isolated in this laboratory, was shown to have a reduced level of one of the uridine diphosphate *N*-acetylglucosamine:glycoprotein *N*-acetylglucosamine transferases (S. Narashimhara, G. Upreti, and H. C. Wu, unpublished data). Due to a decreased binding of [^{125}I]ricin to this mutant, this mutant cell line was approximately eight times more resistant to ricin as compared with its parental wild type.

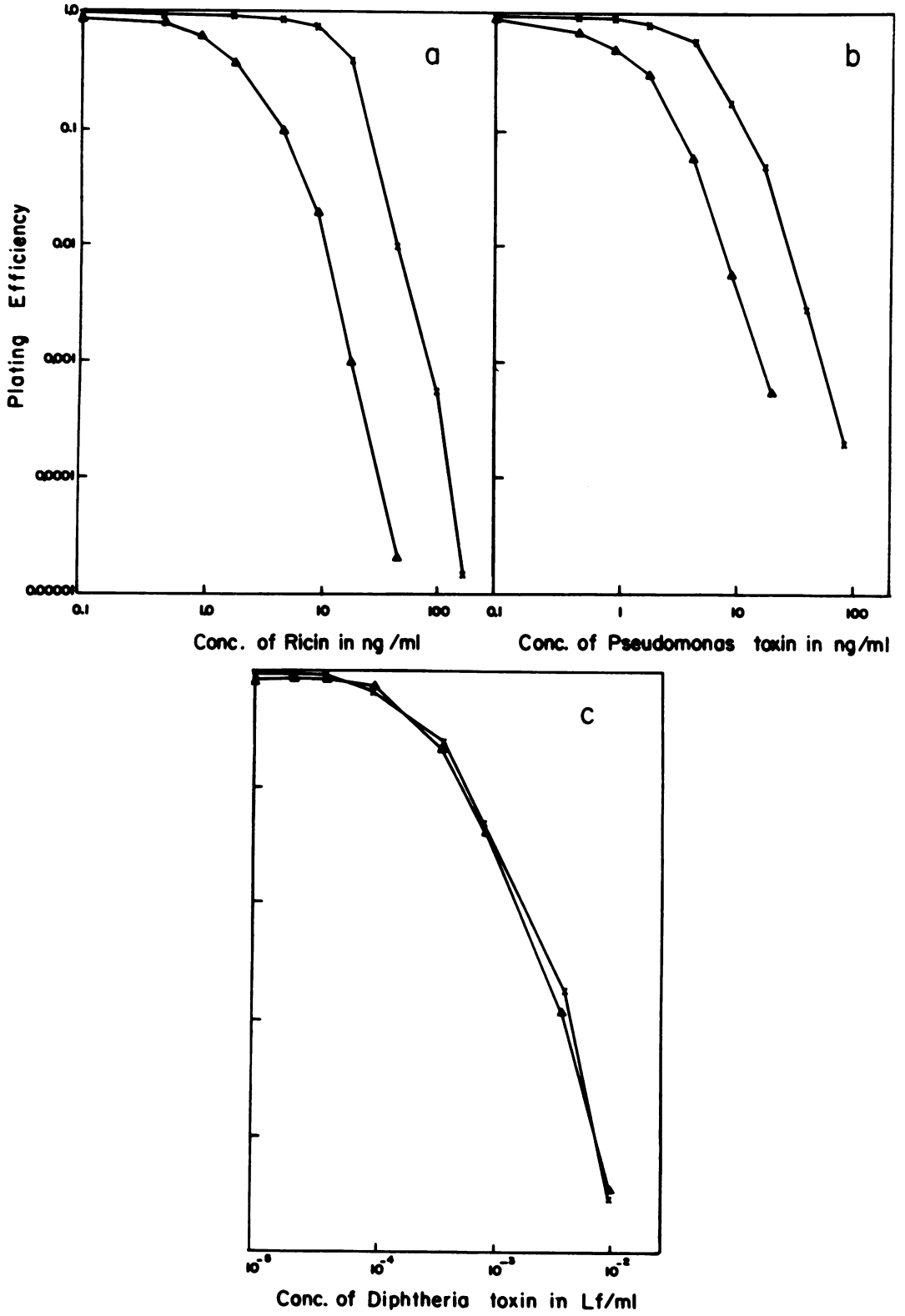

FIG. 1. *The dose-response curve of wild-type CHO pro⁻ cells with (a) ricin, (b) P. aeruginosa exotoxin A, and (c) diphtheria toxin in the absence or presence of nigericin (10 nM). Symbols: ×, plating efficiency in the absence of nigericin; ▲, plating efficiency in the presence of nigericin.*

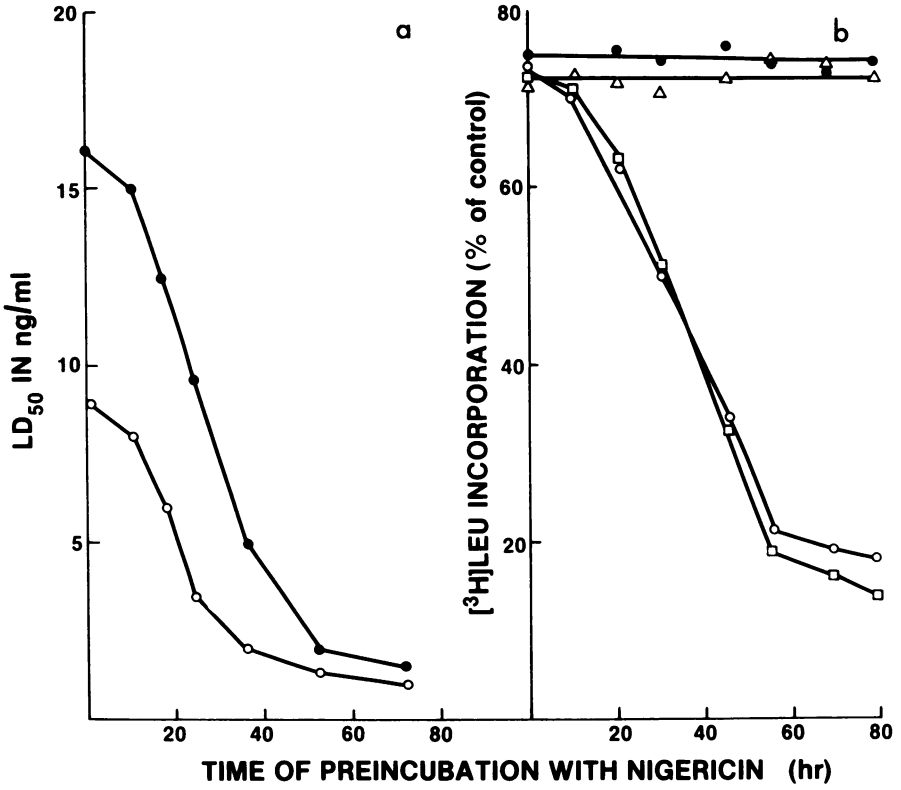

FIG. 2. *Effect of nigericin pretreatment (10 nM) on the sensitization of CHO pro$^-$ cells toward the cytotoxicities of ricin and Pseudomonas toxin. (a) Symbols: ●, LD_{50} of ricin in nigericin-pretreated cells; ○, LD_{50} of Pseudomonas toxin in nigericin-pretreated cells. The cells were first pretreated for different intervals of time with nigericin (10 nM). Pretreated cells were washed, and the plating efficiency of the cells was determined in the presence of toxins without nigericin. (b) Effect of pretreatment of CHO cells with nigericin on the inhibition of protein synthesis by ricin or Pseudomonas toxin. The concentrations of ricin and Pseudomonas toxin were 10 ng/ml and 5 ng/ml, respectively; these doses resulted in an inhibition of protein synthesis by approximately 20 to 25% in control cells after 90 min. Symbols: ●, control cells in the presence of ricin (10 ng/ml); △, control cells in the presence of Pseudomonas toxin (5 ng/ml); ○, nigericin (10 nM)-pretreated cells in the presence of ricin but no nigericin; □, nigericin (10 nM)-pretreated cells in the presence of Pseudomonas toxin but no nigericin.*

Nigericin-treated mutant cells were also more resistant to ricin than were wild-type cells treated with the same ionophore. However, nigericin pretreatment did result in a fivefold enhancement of ricin toxicity in mutant *ts*-1-1, a sensitization of similar magnitude to that in the wild-type cell. Furthermore, the cytotoxicity of *Pseudomonas* toxin and its enhancement by nigericin were unaltered in *ts*-1-1 as compared to the parental cell line (data not shown).

DISCUSSION

Although the cytotoxicity of ricin, diphtheria toxin, and *P. aeruginosa* exotoxin A can all be attributed to the inhibition of cellular protein synthesis, they have distinctly different modes of actions both in terms of surface receptors and biochemical targets (2, 10, 11). The A subunit of ricin inactivates the 60S subunits of ribosomes, whereas the A domains or fragments of both *Pseudomonas* toxin and diphtheria toxin have adenosine diphosphate ribosyl transferase activities capable of inactivating elongation factor 2 of the cellular protein-synthesizing machinery (11). The receptors for these three toxins also appear to be different. In view of the current knowledge of the nature of surface receptors and intracellular targets, the results reported in this paper are of considerable interest.

The enhancement of cytotoxicities in CHO cells of both ricin and *Pseudomonas* toxin by monovalent cation ionophores strongly suggests that there exists a step common to the intoxication processes of both toxins which is affected

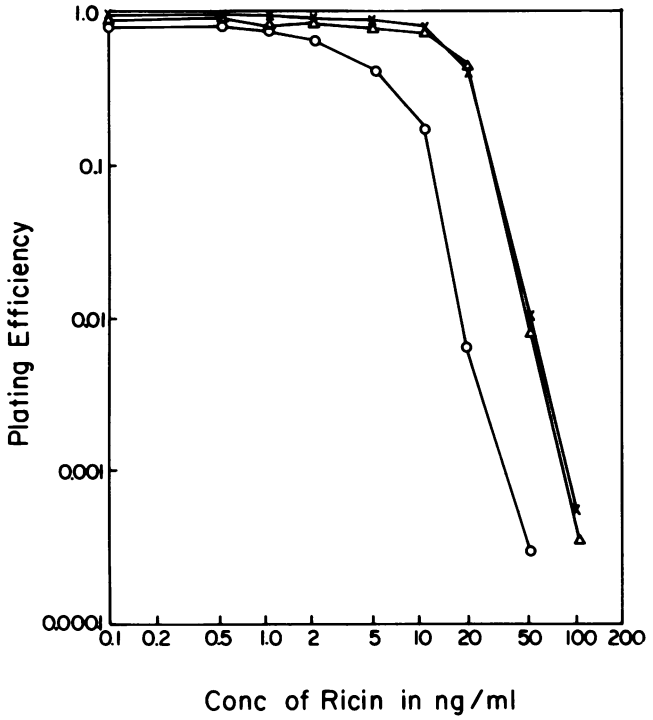

FIG. 3. *Effect of NH_4Cl on the cytotoxicity of ricin in CHO cells. Symbols: ×, control; △, cells pretreated with NH_4Cl (0.1 mg/ml) for 48 h and washed and cytotoxicity of ricin measured in the absence of NH_4Cl; ○, cells treated with ricin in the presence of NH_4Cl (0.2 mg/ml) for 5 h and the medium changed and the incubation continued with the fresh medium containing no toxin or NH_4Cl.*

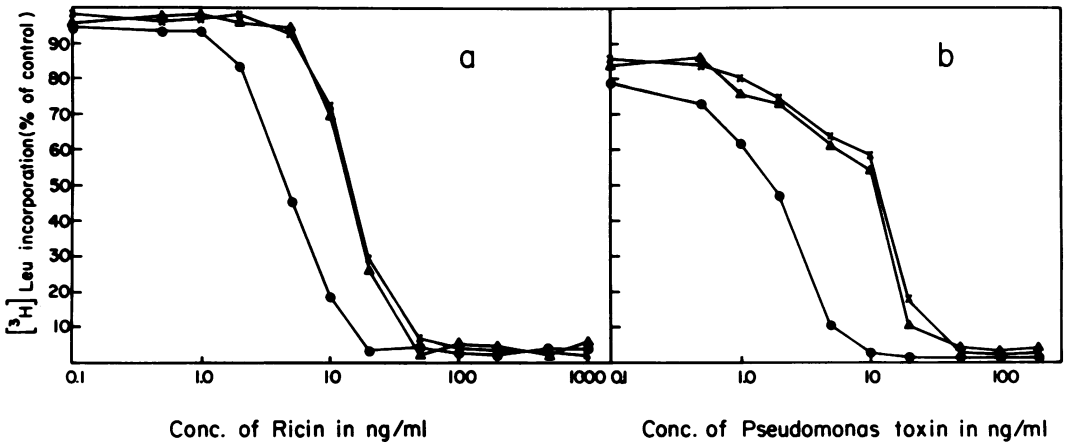

FIG. 4. *Effect of nigericin on the inhibition of protein synthesis by ricin or Pseudomonas toxin. (a) Ricin treatment, 90 min; (b) Pseudomonas toxin treatment, 180 min. Symbols: ×, control cells without nigericin; ●, cells pretreated with nigericin for 60 h and toxins added to the washed cells without nigericin; ▲, toxins and nigericin (10 nM) added together for 90 and 180 min, respectively.*

by pretreatment of CHO cells with nigericin or monensin. The studies with ricin receptor-deficient mutant *ts*-1-1 strongly suggest that the cytotoxicity of ricin in nigericin-pretreated cells is mediated by the same glycoprotein receptors used in the intoxication of wild-type CHO cells by ricin. Furthermore, the receptors for ricin appear distinct from those for *Pseudomonas*

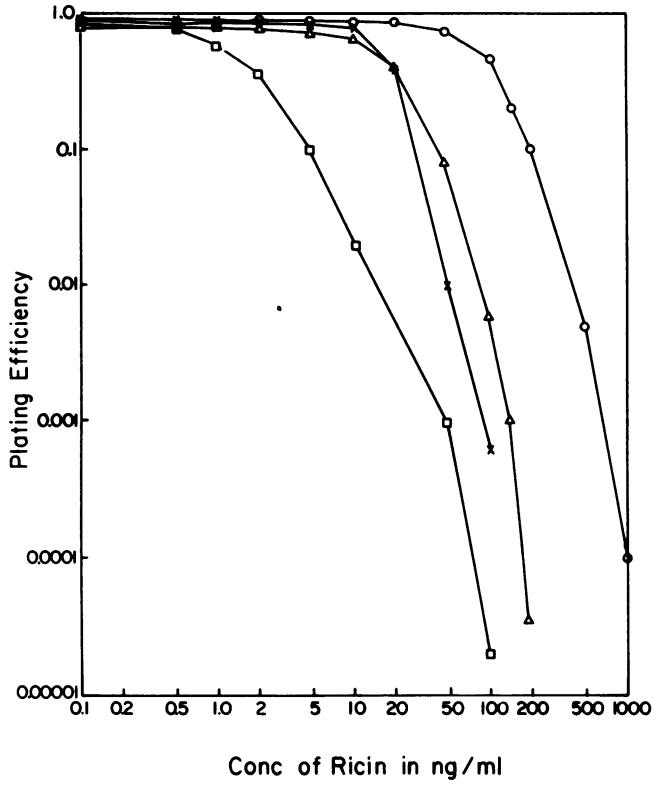

FIG. 5. *Effect of nigericin on the response curve of ricin in wild-type CHO pro⁻ and CHO ts-1-1 mutant cells. Symbols: ×, wild-type cells in the absence of nigericin; □, wild-type cells in the presence of nigericin (10 nM); ○, ts-1-1 mutant cells in the absence of nigericin; △, ts-1-1 mutant cells in the presence of nigericin (10 nM).*

toxin. These results further suggest the possibility that nigericin treatment affects a step common to the entry processes of these two toxins but beyond the initial binding to the specific receptors. The observed enhancement of internalization of [^{125}I]ricin in nigericin-pretreated cells reported in the accompanying paper (14) is consistent with this speculation. These results suggest that ricin and *Pseudomonas* toxin are internalized into the CHO cells by a similar mechanism, whereas the mechanism of entry of diphtheria toxin into CHO cells may be totally different. Different pathways for the entry of diphtheria toxin, *Pseudomonas* toxin, and ricin into CHO cells are further indicated by the differential effects of chloroquine phosphate, NaF, and NH_4Cl on the cytotoxicities of these three toxins.

The mechanism by which nigericin potentiates the cytotoxicities of ricin and *Pseudomonas* toxin remains unknown. The fact that this effect requires preculture of CHO cells in medium containing nigericin strongly suggests that the potentiation is an indirect effect of nigericin as an ionophore. This conclusion is also supported by the failure of cations and anions to reverse the effect of nigericin on the cytotoxicity of ricin (data not shown) and by the lack of enhancement of cytotoxicity by valinomycin. Nor is it likely that the effect of nigericin or monensin can be attributed to an alteration in pH. The effect of pH on the cytotoxicity of ricin has been measured, and the pH profile of cytotoxicity as measured by inhibition of protein synthesis follows closely that of binding and internalization of [^{125}I]ricin into CHO cells, with the maximal cytotoxicity at pH 7.5 (data not shown). The requirement of preculture of CHO cells in nigericin-containing medium suggests an alteration in the surface architecture or cell surface-cytoskeleton interaction so that internalization of receptor-bound ricin and *Pseudomonas* toxin, but not diphtheria toxin, is facilitated. This is in contrast with the potentiation of ricin toxicity by NH_4Cl; the latter compound does not require preculture.

Evidence supporting our working hypothesis that nigericin pretreatment results in an enhanced internalization of ricin into CHO cells is documented in the accompanying paper (14).

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