

Chinese Hamster Cell Variants Resistant to the A Chain of Ricin Carry Altered Ribosome Function

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Ricin, a toxic lectin from *Ricinus communis*, is composed of two different polypeptide chains, A and B, and the ricin A chain (RA) blocks protein synthesis. We studied cell lines resistant to cytotoxic action of RA. One low-RA-resistant cell line, AR10, isolated from Chinese hamster ovary (CHO) cells, was resistant to a low dose of RA (1 $\mu\text{g/ml}$) and showed a 10-fold-higher resistance to RA and ricin than that of CHO. We further mutagenized AR10 to isolate high-RA-resistant cell lines AR100-6, AR100-9, and AR100-13, which were resistant to higher doses of RA and ricin (100- to 1,000-fold) than CHO was. The binding of [¹²⁵I]ricin to AR10, AR100-6, AR100-9, and AR100-13 cells was decreased to about 30% of that of CHO. The internalization of [¹²⁵I]ricin in AR10 cells and in the high-RA-resistant clones was the same. Polyuridylylate-dependent polyphenylalanine synthesis, using S-30 extracts from either AR100-9 or AR100-13, was about 100-fold more resistant to the inhibitory action of RA than when CHO, AR10, and AR100-6 cells extracts were used. The protein synthesis with ribosomes (80S) from AR100-9 or AR100-13 was 10- to 100-fold more resistant to RA than it was with parental ribosomes when combined with the S-100 fraction of CHO cells. The polyphenylalanine synthesis assay using the ribosomes constituted from the 60S subunit of AR100-9 and the 40S subunit of CHO indicated that the resistant phenotype of AR100-9 cells is due to an alteration of the 60S ribosomal subunit.

Ricin is one of the toxic lectins isolated from the seeds of *Ricinus communis* (6, 7, 28, 32). Ricin consists of two different polypeptide chains, A and B, which are linked with a disulfide bond (8, 9). The B chain, or "haptomer," binds to galactose-containing cell surface receptors, and the A chain catalytically inactivates the 60S ribosomal subunit (25-27). The cytotoxic effect of ricin is thought to be due to its inhibitory effect of protein synthesis (5, 27). Somatic cell genetics has provided a powerful tool for examining the mechanism of ricin action (38). The somatic cell mutants resistant to ricin are expected to be altered in any one of three steps that lead to the cytotoxic action of ricin: cell surface binding, endocytosis (or internalization), and protein synthesis. So far, ricin-resistant clones with altered binding or endocytosis mechanisms for ricin have been reported, but none of these ricin-resistant clones showed alterations in ribosomal function, that is, a presumed target site for the ricin A chain (RA) (for review see [38]). The first class of ricin-resistant clones exhibited a decreased level of ricin binding because of either reduced galactose (or *N*-acetyl-galactosamine) residues on the cell surface (11, 12, 17, 22, 23, 36) or increased sialic acid in the terminal regions of glycoproteins (10,

33). The phenotype of the second class of ricin-resistant cell lines is caused by an impairment in the targeting of RA to ribosomes (35). Nicolson and his colleagues (24, 34) have isolated cell lines defective in the internalization of ricin.

Various ribosomal mutants which have been invaluable in examining the structure and function of eucaryotic ribosomes have been isolated (38). Attempts to isolate more ricin-resistant mutants should provide new mutants with altered ribosome functions; this will be an important step in understanding the mechanism of ricin action as well as the genetics of eucaryotic ribosomes. Several ribosomal mutants have been isolated from Chinese hamster cells as either emetine- or cycloheximide-resistant (13, 29) or temperature-sensitive clones (15). Blastocidin S-resistant mouse cell lines with altered ribosome function have also been isolated (19, 21). In this report, we describe the isolation and the characterization of mutants resistant to RA.

MATERIALS AND METHODS

Cell lines and cell culture. Chinese hamster ovary (CHO) cells and the variant cells were routinely grown at 37°C in minimal essential medium (MEM) (Nissui Seiyaku Co., Tokyo) containing 10% newborn calf serum (Flow Laboratories, New South Wales, Australia).

lia), 1 mg of peptone (Difco Laboratories, Detroit, Mich.) per ml, 0.929 mg of L-glutamine per ml, and 100 U of penicillin G per ml, as described previously (1, 20).

Isolation of RA-resistant variants from CHO cells. RA-resistant clones were isolated in a single-step procedure after ethyl methane sulfonate (EMS) mutagenesis. CHO cells at 5×10^5 cells per ml in 10 ml of medium were treated with 300 μg of EMS per ml for 18 h and then were incubated in the absence of the drug for 4 days; this kills about 50% of the cells. The mutagenized cells (5×10^5 cells) were plated in a 100-mm plastic dish and incubated for 18 h at 37°C. RA (10 $\mu\text{g}/\text{ml}$) was then added, and 7 to 10 days later, a few colonies were observed on the plates. A clone, AR10, was isolated as described previously (20). To isolate mutants which are resistant to a higher dose of RA, we mutagenized AR10 cells by treatment with 300 μg of EMS per ml for 18 h, and the surviving cells, about 50% of the initial population, were incubated in the absence of mutagen for 4 days. The mutagenized cells (10^6 cells) were plated in a 100-mm dish with 100 μg of RA per ml; 7 to 10 days later, a few colonies were observed on the plate. RA was added to the medium every day. Three independently isolated clones, AR100-6, AR100-9, and AR100-13, were picked for further study. The resistance phenotype was stable during culture for 6 months in the absence of ricin or RA.

Chemicals. Colchicine (Boehringer Mannheim Co., West Germany), phytohemagglutinin (PHA) and concanavalin A (ConA) (E-Y Laboratories, San Mateo, Calif.), cycloheximide (Ishizu Pharmaceutical Co., Osaka), emetine (Nakarai Chemical Co., Tokyo), blastidicin S (Kaken Chemical Co., Tokyo), and EMS (Eastman Kodak Co., Rochester, N.Y.) were used. The isotopic compounds L- ^3H ring-2,6-phenylalanine (46.7 Ci/mmol) and Na ^{125}I (30 mCi/ml) were obtained from New England Nuclear Corp., Boston, Mass.

Purification of RA. Ricin (ricin D) was prepared as described previously (14). RA was isolated from reduced ricin by affinity chromatography on Sepharose 4B as described previously (8) and further purified by carboxymethyl (CM)-cellulose column chromatography (pH 6.5). The fraction possessing only a slight affinity for Sepharose was dialyzed against 10 mM phosphate buffer (pH 6.5) containing 0.1% β -mercaptoethanol, 0.01% EDTA, and 50 mM lactose at 5°C and applied to a column of CM-cellulose equilibrated with the same buffer. The adsorbed protein was eluted by a linear gradient of NaCl from 0 to 0.1 M in the same buffer. The elution pattern is shown in Fig. 1. Fraction F-2 was found to correspond to RA. After being treated with β -mercaptoethanol and after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the F-2 fraction gave a single band which corresponded to a molecular weight of 31,000. The N-terminal amino acid of the purified RA was found to be only isoleucine (data not shown).

Growth curve and colony formation. CHO and its RA-resistant variants (2×10^4 cells per ml) were plated in 60-mm dishes. After 18 h of incubation at 37°C, the cells were treated with various doses of ricin or RA. At the indicated times, the number of viable cells was counted by trypan blue dye exclusion. To assay colony formation, the cells (300 cells per 60-mm dish) were plated in the absence of the drug and incubated at 37°C

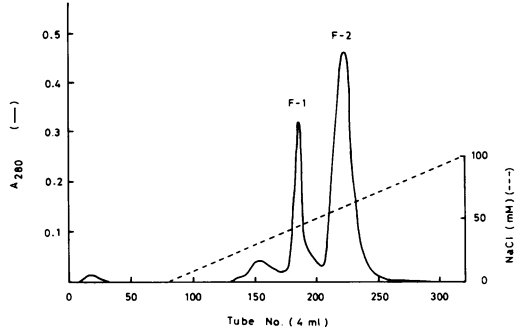


FIG. 1. Purification of RA on a CM-cellulose column. The column (1.8 by 27 cm) was pre-equilibrated with 10 mM phosphate buffer (pH 6.5) containing 0.1% β -mercaptoethanol, 0.01% EDTA, and 50 mM lactose and washed with the same buffer. Adsorbed protein was then eluted with a linear gradient of NaCl from 0 to 100 mM in the same buffer as described in the text.

for 18 h. Drugs were then added, and the plates were incubated for 7 more days as described previously (20).

Iodination of ricin. [^{125}I]ricin was made by the chloramine-T method of Bolton and Hunter (3). The [^{125}I]ricin was purified by applying it to a Sepharose G-75 column. The specific activity of our [^{125}I]ricin was in the range of 0.9×10^5 to 1.1×10^5 cpm per μg of ricin.

Binding and internalization assay. Approximately 4×10^5 cells in a 35-mm dish were washed twice with phosphate-buffered saline (PBS) and incubated with [^{125}I]ricin in 1 ml of MEM medium without serum at 0 or 37°C in a CO_2 incubator. To assay binding activity, the cells incubated at 0°C were washed three times with a cold PBS solution and treated with trypsin; the amount of cell-bound radioactivity was then counted with a gamma counter. To measure the amount of [^{125}I]ricin internalized in the cells, the cells incubated with [^{125}I]ricin at 37°C were incubated for an additional 10 min in 1 ml of PBS containing 0.1 M lactose and washed three times with the same buffer. The amount of radioactivity inaccessible to the action of lactose was counted. In these binding and internalizing assays, specific binding was calculated by subtracting the radioactivity of [^{125}I]ricin bound to the cells when the cells were incubated with MEM containing [^{125}I]ricin and 0.1 M lactose.

Preparation of S-30 extracts, S-100 supernatant, 80S ribosomes, and ribosomal subunits. The S-30 extracts, S-100 supernatant, and 80S ribosomes were prepared from approximately 2×10^8 to 10×10^8 cells of various cell lines by the procedure of Haralson and Roufa (15). Ribosomal subunits were prepared from CHO and AR100-9, respectively, by centrifugation of 35 to 40 units of absorbancy at 260 nm (A_{260} units) of ribosomes in a 10 to 30% sucrose gradient containing 20 mM Tris-hydrochloride (pH 7.8), 500 mM KCl, 3 mM MgCl_2 , and 20 mM β -mercaptoethanol, according to Haralson and Roufa (15).

Polyphenylalanine synthesis assay. A standard reaction mixture in a volume of 50 μl contained the following: 45 mM Tris-hydrochloride (pH 7.5), 9.0 mM

magnesium acetate, 128 mM KCl, 10 mM β -mercaptoethanol, 1 mM ATP, 0.5 mM GTP, 10 μ g of polyuridylic acid, 10 μ M creatine phosphate, 30 μ g of creatine kinase, 5 μ Ci of [3 H]phenylalanine, and 150 to 200 μ g of S-30 extracts. When the S-100 and ribosome fractions were used in the 50- μ l-volume assay, the S-100 fraction (40 to 60 μ g of protein) was combined either with 0.40 A_{260} unit of 80S ribosomes or with 0.23 A_{260} unit of 40S and 0.35 A_{260} unit of 60S ribosomal subunits. After incubation at 35°C, the reaction was terminated by adding 0.1 N KOH, and the 10% acid-insoluble fraction of [3 H]polyphenylalanine was counted.

RESULTS

The cellular level of the resistance in RA-resistant variants. The cellular sensitivity of the clone resistant to low doses of RA (AR10) and the high-RA-resistant clones (AR100-6, AR100-9, and AR100-13) to ricin and RA was compared with that of the parental CHO cell. The plating efficiencies of AR10 and AR100-13 were both 10^{-2} in the presence of 10 and 100 μ g of RA per ml, respectively, whereas the plating efficiency of CHO was less than 10^{-2} , even in the presence of only 1 μ g of RA per ml (Fig. 2A). In contrast, the plating efficiencies of AR100-6 and AR100-9 were 3×10^{-1} to 6×10^{-1} in the presence of 500 μ g of RA per ml. However, the plating efficiencies of CHO, AR10, and AR100-13 were less than 10^{-2} in the presence of 10, 100, and 1,000 ng of ricin per ml, respectively (Fig. 2B). The plating efficiencies of AR100-6 and AR100-9

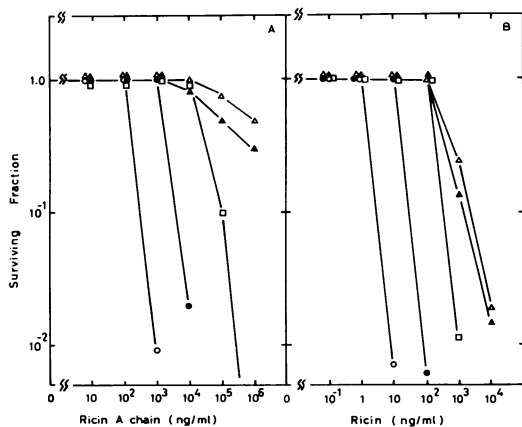


FIG. 2. Dose-response curves of CHO and its RA-resistant clones to RA (A) and to ricin (B) assayed by colony formation. The cells (300 cells per plate) were plated at 37°C, and 1 day later, various doses of RA or ricin were added, followed by incubation for 7 days. The curves drawn for each cell line show the survival values obtained from duplicate experiments. The number of colonies of these clones ranged from 150 to 285 in the absence of any drug. Cells: CHO (○), AR10 (●), AR100-6 (△), AR100-9 (▲) and AR100-13 (□).

were 10^{-2} in the presence of 10 μ g of ricin per ml. Thus, the low-RA-resistant clone AR10 was about 10 times and the high-RA-resistant clones were from 100 to 1,000 times more resistant to RA or ricin than the parental CHO cells.

We then examined the effect of RA or ricin on the growth curves of the low- and high-RA-resistant variants as compared with that of CHO (Fig. 3 and 4). One day after 2×10^4 cells per ml were inoculated, they were exposed to various concentrations of either RA (Fig. 3) or ricin (Fig. 4). The number of viable cells was counted at the indicated times. The growth of the three high-RA-resistant clones was affected only slightly, if at all, by RA at 100 μ g/ml or by ricin at 10 μ g/ml, whereas RA at 100 μ g/ml or ricin at 10 μ g/ml significantly inhibited the growth of CHO and AR10. The growth of CHO was almost completely inhibited by 1 μ g of ricin per ml. These results are in agreement with the colony formation data in Fig. 2.

Cross-resistance of RA-resistant clones to other agents. Somatic cell mutants resistant to one drug are often resistant to other drugs due to a common mutation (1, 16, 21). Since ricin recognizes β -D-galactose and *N*-acetylgalactosamine on the cell surface (32), we examined whether RA-resistant clones were cross-resistant to PHA, which exhibits sugar specificity that includes *N*-acetylgalactosamine and β -D-galactose (4, 18), and ConA, which exhibits a high affinity for β -D-mannose (30). AR10, AR100-6, AR100-9, and AR100-13 all showed greater than a 50-fold-higher resistance to PHA and approximately a 10-fold-higher sensitivity to ConA than CHO did (Fig. 5). The sensitivity to other lectins, like PHA and ConA, was similar in the low- and high-RA-resistant clones. However, the response to colchicine, which alters membrane permeability, was not different between CHO and the RA-resistant clones (data not shown). We also tested whether antibiotics which inhibit protein synthesis, such as cycloheximide, blasticidin S, and emetine, inhibited colony formation of RA-resistant clones. The dose-response curves of CHO and RA-resistant clones to emetine were almost similar (Fig. 5C). CHO, AR10, and AR100-6 cells showed similar sensitivity to blasticidin S, which interacts with the 60S ribosome subunit of mammalian cells (21). However, AR100-9 and AR100-13 cells showed slightly higher sensitivity to the cytotoxic action of blasticidin S (Fig. 5D). CHO, AR10, AR100-6, AR100-9, and AR100-13, however, showed similar sensitivity to cycloheximide, an inhibitor of the 60S ribosome subunit function (data not shown).

Binding and internalization of ricin in CHO and RA-resistant cells. We tested whether the binding of [125 I]ricin to these variants was al-

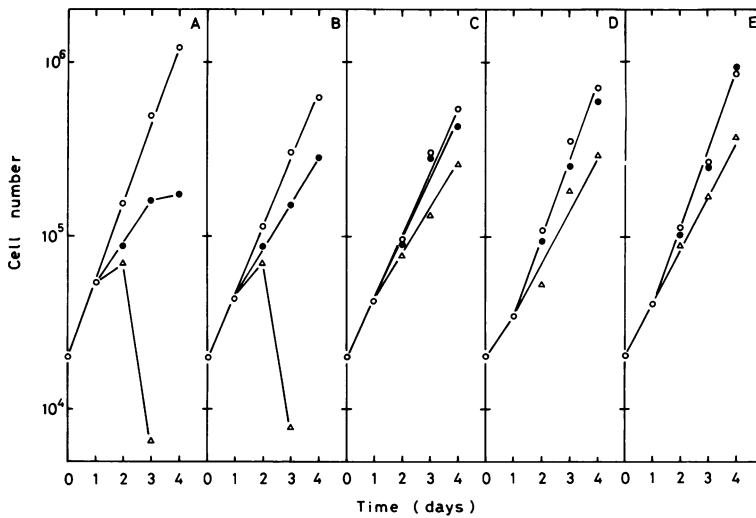


FIG. 3. Effect of RA on growth of CHO and its RA-resistant clones. Exponentially growing cells (2×10^4) were plated, and 10 or 100 μg of RA per ml was added 1 day after inoculation of the cells (indicated by arrow). Viable cells were counted after trypan blue exclusion. Cells were incubated without (○) or with (●) 10 μg of RA per ml or with (△) 100 μg of RA per ml. Cells: CHO (A), AR10 (B), AR100-6 (C), AR100-9 (D) and AR100-13 (E).

tered or not. In our binding assay system, the addition of lactose (0.1 M) almost completely (more than 95%) inhibited [^{125}I]ricin binding to CHO cells at 0°C (data not shown). The binding assay was carried out at 0°C as a function of the dose of [^{125}I]ricin. The binding activity of AR10 as well as cell lines resistant to high doses of RA was reduced to one third that of CHO (Fig. 6).

We could find no further reduction in the ricin binding of AR100-6, AR100-9, and AR100-13 compared with that of AR10.

We then tested whether the process of internalization of ricin is altered in the RA-resistant clones. After the cells were incubated at 37°C for 90 min and the cell surface-bound ricin was removed by the addition of 0.1 M lactose to the

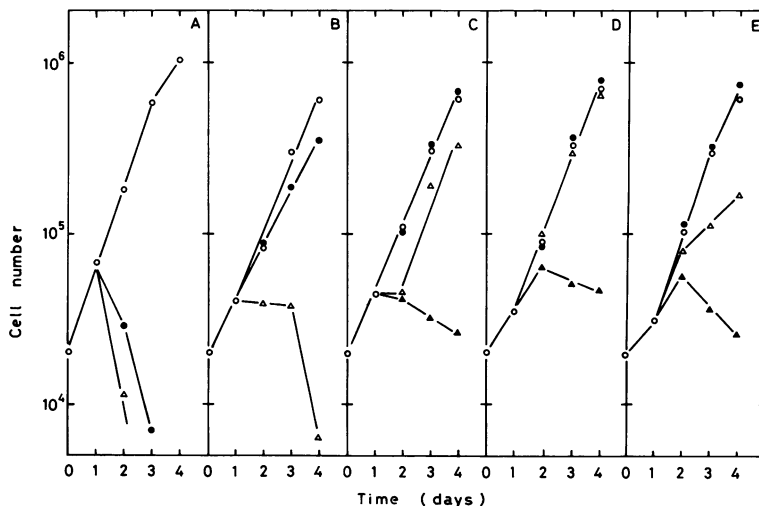


FIG. 4. Effect of ricin on the growth of CHO and its RA-resistant clones. Exponentially growing cells (2×10^4) were plated; 1 day later (indicated by arrow), concentrations of ricin (1 $\mu\text{g/ml}$, ●; 10 $\mu\text{g/ml}$, △; and 100 $\mu\text{g/ml}$, ▲) were added, and the control sample without ricin was observed (○). Cells: CHO (A), AR10 (B), AR100-6 (C), AR100-9 (D), and AR100-13 (E).

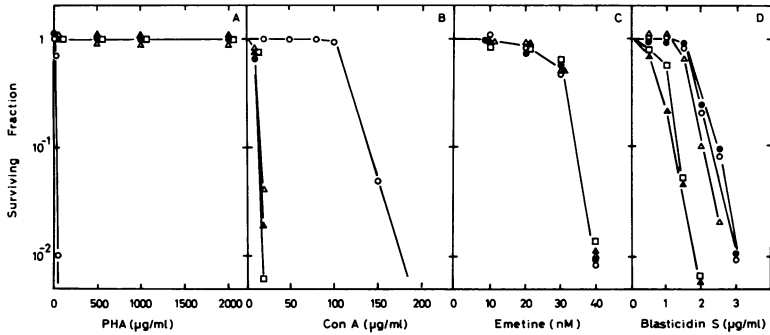


FIG. 5. Dose response of CHO and RA-resistant clones to PHA (A), ConA (B), emetine (C), and blasticidin S (D). The number of colonies for each cell line was counted as described in Fig. 2. Cells: CHO (○), AR10 (●), AR100-6 (△), AR100-9 (▲), and AR100-13 (□).

medium, the radioactivity of the intracellular [¹²⁵I]ricin was assayed. The radioactivity inaccessible to the action of lactose was increased as a function of the added [¹²⁵I]ricin concentration (Fig. 7). The amount of [¹²⁵I]ricin internalized into all low- and high-RA-resistant clones was one-third that seen in CHO cells. The reduced internalization in AR100-6, AR100-9, and AR100-13 appeared to be due to the reduced binding in AR10, from which three high-RA-resistant variants were derived.

Altered protein synthesis machinery is involved in the resistant phenotype of some of the high-RA-resistant clones. To identify lesion in the high-RA-resistant cell lines, we carried out the *in vitro* polyuridylylate-directed polyphenylalanine synthesis assay, using S-30 extracts from CHO, AR10, AR100-6, AR100-9, and AR100-13. Polyphenylalanine synthesis with S-30 extracts from CHO, AR10, and AR100-6 cells was strongly inhibited (over 60% of the control activity) by 100 ng of RA per ml, whereas the protein synthesis with S-30 extracts from AR100-9 and AR100-13 cells was only slightly inhibited by the same concentration of RA (Fig. 8A). Thus, the *in vitro* protein synthesis, using AR100-9 and AR100-13 extracts, showed about 100-fold more resistance to RA than did the extracts of the parental CHO, AR10, and AR100-6 cells.

We also tested whether the ribosome fraction of the extracts was responsible for the resistant phenotype of AR100-9 or AR100-13. Ribosomes prepared from CHO, AR100-9, and AR100-13 cells had been treated with various doses of RA. The ribosomes were combined with the S-100 fraction from CHO cells and used for assaying polyphenylalanine synthesis. The polyphenylalanine synthesis was resistant to higher doses of RA with the ribosome fractions derived from AR100-9 and AR100-13 cells than the protein synthesis with ribosome from CHO, which was relatively sensitive to the toxin (Fig. 8B). We

also found that the protein synthesis was sensitive to the inhibitory action of RA when the ribosome was derived from AR10 (data not shown). Protein synthesis with AR100-13 ribosomes showed about 10-fold resistance, whereas that with AR100-9 ribosomes showed about 100-fold resistance to RA. It is likely, then, that the lesion of AR100-9 and AR100-13 cells involves ribosome function, but the altered lesion in AR100-6 remains to be studied. Ribosomes of

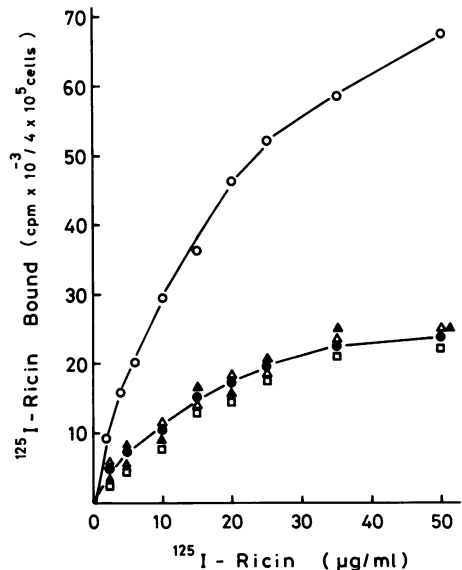


FIG. 6. Binding of [¹²⁵I]ricin to CHO and its RA-resistant clones. The cells (4×10^5) were incubated in 1 ml of MEM with different doses of [¹²⁵I]ricin for 30 min at 0°C. The cells were then washed twice with cold PBS buffer, trypsinized, and counted in a gamma counter. From each count, background radioactivity in the presence of 0.1 M lactose was subtracted. Cells: CHO (○), AR10 (●), AR100-6 (△), AR100-9 (▲), and AR100-13 (□).

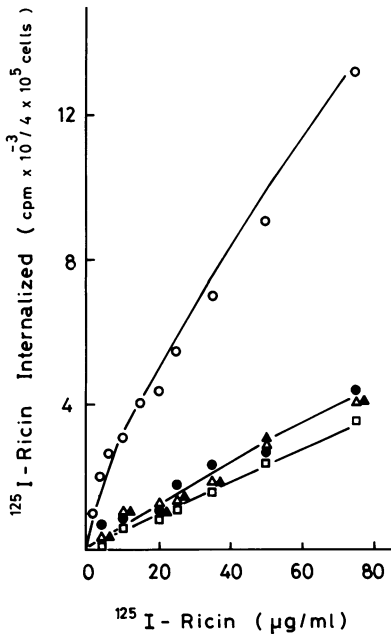


FIG. 7. Internalization of [125 I]ricin into CHO and its RA-resistant clones. The amount of [125 I]ricin internalized into the cells was counted after incubating the cells with various doses of [125 I]ricin for 90 min at 37°C, followed by incubation for 10 min with 0.1 M lactose in PBS buffer. Cells: CHO (○), AR10 (●), AR100-6 (△), AR100-9 (▲), and AR100-13 (□).

CHO and AR100-9 were then dissociated into the 40S and 60S subunits. Protein synthesis in which both subunits were obtained from either CHO or AR100-9 cells was sensitive or resistant, respectively, to RA (Table 1). When the 60S subunit from AR100-9 cells was combined with the wild-type CHO 40S subunit, the protein synthesis was resistant to the toxin (Table 1). These data indicate that a lesion in the high-RA-resistant AR100-9 clone is in the 60S ribosomal subunit.

DISCUSSION

Although much work on ricin-resistant cells with altered ricin binding or with altered endocytosis activity of ricin has been described, a class of variants with altered protein synthesis machinery has not been identified (38). In this report, two high-RA-resistant clones, AR100-9 and AR100-13, were isolated after two-step mutagenesis and appeared to carry ribosomes with altered sensitivity to RA. Our present study might suggest that the two-step mutagenesis is effective for selecting toxin-resistant clones with altered ribosome function. However, we failed to isolate high-resistant clones such as AR100-9 or AR100-13 when selected in the presence of a

large dose of ricin molecules (data not shown). The ribosomal subunit exchange test assayed by polyphenylalanine synthesis suggests that an alteration in the 60S subunit is responsible for the high-resistance phenotype of one variant (AR100-9). The isolation of an RA-resistant variant with a lesion in the 60S ribosomal subunit supports biochemical evidence that RA blocks ribosomal function by acting on the 60S subunit (25, 27). Somatic cell genetics on mammalian ribosomes have been developed by the selection of clones resistant to drugs that inhibit protein synthesis. In particular, emetine-resistant mutants from CHO cells have been exhaustively characterized and were found to carry an aberrant 40S ribosomal subunit (13). Roufa and his colleagues have further linked the emetine resistance of the mutant with an altered S20 protein in the 40S subunit (2). Genetics studies on the emetine-resistant cell lines have mapped an emetine-resistant locus on the long arm of chromosome 2 in hamster cells (37). Further study is necessary to analyze the altered lesion

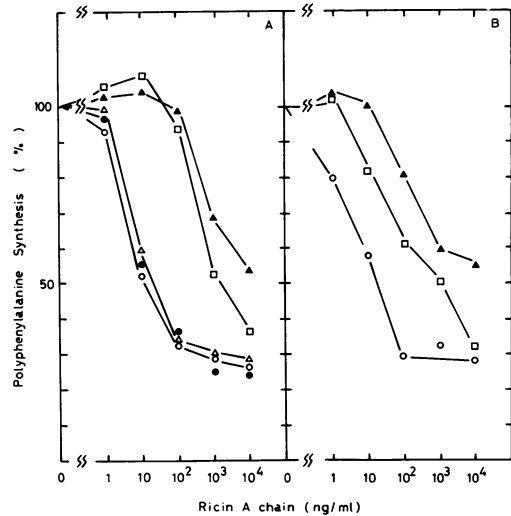


FIG. 8. Dose response of CHO and its RA-resistant clones in polyphenylalanine synthesis in vitro. (A) The S-30 extracts from CHO (○), AR10 (●), AR100-6 (△), AR100-9 (▲), and AR100-13 (□) were treated for 10 min with various doses of RA followed by protein synthesis assay. The values (counts per minute) for 100% activity in each cell line were 87,348 (CHO), 80,236 (AR10), 56,318 (AR100-6), 48,112 (AR100-9), and 74,856 (AR100-13) when background radioactivity was 3,000 to 4,000 cpm. (B) The 80S ribosomes from CHO (○), AR100-9 (▲), and AR100-13 (□) were combined with S-100 from CHO and treated with various doses of RA for 10 min at 37°C followed by protein synthesis assay. The values (counts per minute) for 100% activity were 66,085 (CHO), 64,793 (AR100-9), and 64,560 (AR100-13) when background radioactivity was 2,500 to 4,000 cpm.

TABLE 1. Reconstitution of CHO and AR100-9 ribosomal subunits

Source of ribosomal subunit		³ H]Phenylalanine incorporated (cpm) ^a		
40S	60S	None	RA (0.1 μg/ml)	RA (1.0 μg/ml)
CHO	CHO	17,838 (100%)	11,158 (63%)	7,909 (44%)
AR100-9	CHO	20,358 (100%)	9,010 (44%)	7,101 (35%)
CHO	AR100-9	17,133 (100%)	16,649 (97%)	13,309 (78%)
AR100-9	AR100-9	14,355 (100%)	12,921 (90%)	9,506 (66%)

^a The percentages in parentheses show protein synthesis activity in the presence of RA; 100% corresponds to the activity in the absence of RA under each combination of ribosomal subunits.

in the ribosome architecture causing RA resistance.

Ricin is known to be taken up by receptor-mediated endocytosis (5, 24), but a non-endocytotic mechanism for ricin uptake was discussed for toxin-resistant variants isolated from baby hamster cells (35). The initial step for the internalization of ricin into CHO cells was recently shown to require neither reduction nor proteolytic processing of the ricin (31). The ¹²⁵I-labeled RA uptake that is not affected by the presence of lactose was found to proceed for 2 h at 37°C in the parental CHO (data not shown). However, it is unclear how RA, which has no binding sites, can enter the cells and exert its toxic effect. The low-RA-resistant clone AR10, from which AR100-9 or AR100-13 was derived, showed decreased affinity for [¹²⁵I]ricin, and AR10 was cross-resistant to PHA and was highly sensitive to ConA (see Fig. 5). The ricin-resistant variants with high mannose levels in the glycopeptides are also resistant to PHA and sensitive to ConA (12). The loss of *N*-acetyl-glucosaminyltransferase activity in those ricin-resistant clones of CHO was found to lead to the production of altered carbohydrate moieties at the cell surface (36). It is peculiar that the low-RA-resistant clone AR10 has a phenotype similar to that of other variants selected by the resistance to ricin as reported by others (12, 17, 22, 23). The cytosolic activity of our RA sample was found to be about one-hundredth that of ricin (see Fig. 2, 3, and 4), which is comparable to data reported by another group (27). Although the homogeneity of our RA could be confirmed by sodium dodecyl sulfate gel electrophoresis and by testing the agglutination activity (8, 9), it is still possible that our RA sample is contaminated with ricin at a biologically significant level.

Therefore, we could not exclude the possibility that the contaminated ricin, if any, might be effective in selecting AR10 from CHO with a lower affinity for ricin.

Nicolson and his colleagues (24, 34) have isolated a ricin-resistant variant from murine lymphoma cells which is partly defective in the ricin internalization process. The transport of ferritin-[¹²⁵I]ricin at a low dose (1 to 3 μg/ml) can be observed in the parental mouse cell, but not in the resistant variant. By contrast, at ricin doses higher than 7 μg/ml, [¹²⁵I]ricin is transported in both cell lines (24). The difference in the internalization activity of the CHO and RA-resistant clones (Fig. 5 and 6) is comparable to that observed in the binding activity. Thus, it appears that our high-RA-resistant clones do not alter their ricin internalization process. Since the mechanisms of internalization of a toxin like ricin are not fully understood, the isolation of variants which are defective in the internalization of ricin is necessary to explore this process.

ACKNOWLEDGMENTS

We thank N. Shimizu (Arizona University) for fruitful discussion and also for critical reading of this manuscript. We also thank M. Andoh for her help with the experiments.

This work was supported by a grant-in-aid from the Ministry of Education, Science and Culture, Japan.

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