Somatic Cell Mutants Resistant to Ricin, Diphtheria Toxin, and to Immunotoxins*

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The human B-cell line Namalwa expresses the common acute lymphoblastic leukemia antigen (CALLA). Frame-shift mutants in Namalwa cell cultures were generated with ICR-191, and mutants were then selected for resistance to ricin or resistance to a conjugate of ricin with the anti-CALLA antibody J5 in the presence of lactose. Three mutants were found that were resistant to ricin and were in addition shown to be resistant to diphtheria toxin, to a J5-ricin conjugate, and to a conjugate between ricin B-chain and gelonin. The mutants, however, were sensitive to a J5-gelonin conjugate. These mutants expressed high levels of CALLA and/or receptors for ricin, and their cell-free translation systems appeared to be as sensitive to the inhibitory action of ricin A-chain and of gelonin as the translation system of wild-type Namalwa cells. The behavior of these mutants was consistent with the hypothesis that these cells possess an alteration of their surface that impedes the passage of ricin and diphtheria toxin across the plasma membrane. A fourth mutant was found to bind reduced quantities of ricin and was resistant to ricin but was sensitive to J5-ricin. The properties of this cell line provide evidence that the binding of antibody-ricin conjugates to cells via the ricin moiety may be prevented without impeding the cytotoxicity of the conjugates.

Ricin, a lectin isolated from the seeds of Ricinus communis L., is extremely toxic to most eucaryotic cells (1). This toxin can be directed to kill target cells by conjugating it to monoclonal antibodies as targeting agents (2), and the hope is that conjugates of ricin with antibodies will be developed that are highly toxic and selective in their action (3).

In order to study the pathway(s) of the internalization of ricin and of ricin-antibody conjugates by cells, we employed the techniques of biochemical genetics: the analysis of somatic cell mutants that are resistant to ricin or ricin-antibody conjugates.

This paper describes the isolation and general characterisation of somatic cell mutants that are resistant to ricin or to a ricin-anti-CALLA1 antibody J5 conjugate. ICR-191, a potent and moderately toxic mutagen for human cells was used for the generation of the mutants (4). The CALLA-expressing human B-lymphoblast cell line Namalwa was used as the parental line for generation of mutants.

EXPERIMENTAL PROCEDURES AND RESULTS*

Resistance of Mutants to Protein Toxins, Immunotoxins, and Some Other Cytotoxic Drugs—All clones that are listed in Table II were resistant to both ricin and J5-ricin, when compared to the wild type cells. In the case of J5-ricin, this resistance was observed either with or without lactose. In order to understand better the mechanisms of resistance, the cytotoxicities of ricin (Fig. 3a), J5-ricin (Fig. 3b), diphtheria toxin (Fig. 3c), gelonin, and J5-gelonin (Table III) as well as three other cytotoxic agents (Table III) toward representative cell lines r23, J5r6, J5p11, and J5R12 were studied in detail. Gelonin is a protein which inhibits protein synthesis in cell-free translation systems as efficiently as ricin A-chain but is not very toxic to intact cells because it apparently cannot penetrate cell membranes (10, 18). The r23 cell line appeared to be 65-fold more resistant to ricin than the Namalwa line (Fig. 3a) but showed no increased resistance to J5-ricin. J5p6, J5p11, and J5R12 cell lines appeared to be resistant to ricin and to J5-ricin (Fig. 3, a and b). In addition, these three lines were resistant to diphtheria toxin (Fig. 3c). J5r6 cells were resistant to ricin B-gelonin, and J5r12 cells were resistant to J5-gelonin (Table III). The mutant J5R11 was resistant to ricin B-gelonin but sensitive to J5-gelonin (Table III). In their sensitivity to free gelonin, all four of these cell lines were similar to the parental Namalwa line (Table III).

We also tested whether the mutants acquired the phenotype of so-called "multidrug resistance," which is associated with an alteration of the plasma membrane that impedes the internalization of a number of cytotoxic drugs (19). However, none of the four mutants were resistant to actinomycin D, adriamycin, or colchicine (Table III), the agents to which the cells that acquired the multidrug resistance phenotype would become resistant (19).

DISCUSSION

Mutants with Reduced Expression of CALLA and/or of Receptors of Ricin—The resistance of mutants to ricin or to J5-ricin could originate from several factors: (i) reduced binding to the cell surface; (ii) reduced endocytosis; (iii) reduced...
transport of intact ricin or possibly of ricin A-chain across the cell membrane; (iv) more efficient intracellular degradation of the toxin molecules; (v) modification in the translation system rendering it resistant to ricin A-chain. Most of the rR and J5rR mutants belong to class (i). These mutant cell lines bind reduced amounts of ricin (rR) or of J5 (J5rR). Similar phenotypes have been reported previously (29, 32-37). The experiments with wild-type Namalwa cells also suggest that the level of binding of a toxin or an immunotoxin to the cell surface may determine the cytotoxicity of a toxin. Namalwa cells bind less J5 than ricin B-chain (Fig. 2) and, accordingly, J5-ricin in the presence of lactose (i.e. under conditions where conjugate can bind only to the cell's CALLA) is 15-fold less cytotoxic than ricin and 11-fold less cytotoxic than J5-ricin in the absence of lactose. Similarly, J5-gelonin was 7-fold less toxic to the Namalwa cells than ricin B-gelonin. The rR23 cell line appeared to be 65-fold more resistant to ricin than the Namalwa line (Fig. 3a) but was equally sensitive to J5-ricin. This mutant expresses less receptors for ricin but more CALLA than do Namalwa cells (Table II). Ribosomes of this cell line are as sensitive as the ribosomes of the wild-type cells toward ricin A-chain. These data suggest that reduced expression of receptors for ricin was the main factor responsible for the resistance of rR23 cells to ricin, which is consistent with previous reports that the binding of ricin-antibody conjugates to cells via the ricin moiety could be blocked without significant loss of cytotoxicity of the conjugates (2, 3, 38-40). Similar data were obtained in our experiments with J5-ricin and Namalwa cells (Fig. 1). These experiments showed that the cytotoxicity of this CALLA-directed immunoconjugate was the most potent retained in the presence of lactose while the cytotoxicity of ricin was abolished by lactose.

**Mutants with Reduced Transport of Toxins—Mutants J5rP6, J5P11, and J5rP12 expressed levels of CALLA and/or receptors for ricin that are comparable with those of wild-type Namalwa cells, and these mutants might, therefore, possess another type of resistance.**

J5rP12 cells internalized only about 40% as much ricin B-chain as Namalwa cells. This fact may indicate a deficiency in endocytosis of ricin and could contribute toward the resistance of these cells. In contrast, J5rP11 cells endocytosed 80% as much ricin B-chain and 120% as much diphtheria toxin as did Namalwa cells. No indication was found that the mutants had elevated levels of cytoplasmic proteases that might cause faster degradation of toxins than in the cytoplasm of the Namalwa cells. The cell-free translation systems of the four mutants rR23, J5rP6, J5rP11, J5rP12 and of the Namalwa cells are equally sensitive to ricin A-chain and to gelonin, and these mutants are, therefore, different from the mutants described by Ono et al. (35), which were resistant to ricin because of an alteration in their ribosomes. An explanation for the resistance of J5rP11 cells (and a possible contributing factor for the resistance of J5rP6 and J5rP12 cells) toward ricin and J5-ricin is that these cells may have an alteration on their surface that diminishes the transport of ricin, J5-ricin, and of ricin B-gelonin across the plasma or an intracellular membrane. This hypothesis is consistent with the finding that all three J5rR

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![Table III: ID50 cytotoxic agents in Namalwa cells and rR and J5rR mutants](table3.png)

| Cell line | Gelonin, a | J5-ricin, b | B-gelonin, b | Diphtheria toxin | Actinomycin D1, a | Adriamycin, a | Colchicine, a |
|-----------|------------|-------------|--------------|-----------------|-----------------|--------------|--------------|
| Namalwa   | 4          | 0.2         | ND           | ND              | 5               | 50           | 60           |
| rR23      | 2          | 0.04        | ND           | ND              | 4               | 90           | 90           |
| J5rP6     | 4          | 0.8         | 60           | 2               | 20              | 50           |              |
| J5rP11    | 5          | 0.2         | 100          | 3               | 40              | 60           |              |
| J5rP12    | 2          | 100         | ND           | 11              | 40              | 50           |              |

a Assayed by back-extrapolation from growth curves.
b Assayed by inhibition of 3H-labeled thymidine incorporation into cell DNA.
c ND, not done.
mutants are resistant to diphtheria toxin, while J5rR11 cells are able to endocytose the toxin as well as the Namalwa cells. Diphtheria toxin is quite different from ricin in its mechanism of action. It binds to different receptors on the cell surface, and, in contrast to ricin, requires internalization into an acidified intracellular compartment in order to exhibit toxicity (41–43). Further, diphtheria toxin inactivates a different target, elongation factor 2, while ricin inactivates the 6 S ribosomal subunit (1). The phenomena of ricin resistance and of diphtheria toxin resistance are not necessarily linked, since previously reported ricin-resistant somatic cell mutants were sensitive to diphtheria toxin (44). And diphtheria toxin-resistant mutants were sensitive to ricin (45). One similarity in the mechanism of action of diphtheria toxin and of ricin is that both protein toxins or at least their A-chains must penetrate a cellular membrane in order to reach the cytoplasm. The J5rR11 cells may possess an alteration in the plasma membrane area situated in the vicinity of these glycoproteins. The mechanism of the resistance may be linked to an alteration in the composition of the cell surface glycoproteins or an alteration in the plasma membrane area situated in the vicinity of these glycoproteins.

J5-R Mutants Are Resistant to Ricin—All J5-R mutants that are listed in Table II appeared to be resistant not only to J5-ricin in the presence of lactose, the agents they were selected with, but also to ricin and J5-ricin in the absence of lactose. Six out of eight mutants had decreased levels of receptors for ricin on their surface (Table II). Similar selection of ricin-resistant mutants was sensitive to diphtheria toxin (45). One similarity in the phenomena of ricin resistance and of diphtheria toxin resistance may be linked to an alteration in the mechanism of action of diphtheria toxin and of ricin is that both protein toxins or at least their A-chains must penetrate a cellular membrane in order to reach the cytoplasm.

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Mutants Resistant to Protein Toxins

EXPERIMENTAL PROCEDURES

Preparation of Immunoprecipitate: A disulfide-linked conjugate between 25 and gallamine was prepared for examination by SDS-polyacrylamide gel electrophoresis. The conjugate was prepared by reacting 25 that had been modified with iodoacetamide with gallamine (3). A conjugate of this nature was prepared by using 25 that had been modified with P-iodoacetyl-2-mercaptoethanol (P-IAE) in the presence of gallamine (30). All other conditions for the modification of 25 were the same as described previously in the preparation of 25 or gallamine (3). The level of 2-iodoacetamide was monitored for each specific preparation of conjugate by thin-layer chromatography with silica gel plates. Preparative Bio-Gel P-100 columns were used for isolation of 25 when necessary. The isolated conjugate was then used for immunoprecipitation through a system of Spinco ultracentrifuge rotors and centrifugal filters. The purified conjugate was then used to prepare antiserum.

Cells and cell culture materials: Mouse cells (C57BL/6J, 3T3L1) have been cultured as described previously (3).

Transfection of cells with 25RNA: A standard procedure was used to transfect the prostate cell line, the passage 30 cells, and the passage 60 cells with polyethyleneimine (PI). The plasmid-horseradish peroxidase (HRP) expression vector was used for transfection of the prostate cell line, the passage 30 cells, and the passage 60 cells with polyethyleneimine (PI). The plasmid-horseradish peroxidase (HRP) expression vector was used for transfection of the prostate cell line, the passage 30 cells, and the passage 60 cells with polyethyleneimine (PI). The plasmid-horseradish peroxidase (HRP) expression vector was used for transfection of the prostate cell line, the passage 30 cells, and the passage 60 cells with polyethyleneimine (PI).

Results

Survival of 25 and J5 (25-31)-resistant mutants: The stability of 25 and J5 (25-31)-resistant mutants was determined by measuring the degree of survival of the mutants at various concentrations of toxin. The degree of survival of the mutants was determined by measuring the degree of survival of the mutants at various concentrations of toxin. The degree of survival of the mutants was determined by measuring the degree of survival of the mutants at various concentrations of toxin. The degree of survival of the mutants was determined by measuring the degree of survival of the mutants at various concentrations of toxin. The degree of survival of the mutants was determined by measuring the degree of survival of the mutants at various concentrations of toxin.

LIGAND, BOUND PER CELL

MOLeCULES PER CaLL

Table 1

| Frequency of appearance of mutants | Cell population | Normalized with 100% (25) | 100% population |
|-----------------------------------|----------------|--------------------------|-----------------|
| Classical                          | 25             | 7 x 10^6                  | 2 x 10^6         |
| J5                                | 7 x 10^6       | 2 x 10^6                  | 1 x 10^6         |
| 25                                | 2 x 10^6       | 1 x 10^6                  | 1 x 10^6         |
| J5                                | 1 x 10^6       | 1 x 10^6                  | 1 x 10^6         |

Conclusion of study: The data presented here indicate that the binding of 25 to the prostate cell line is enhanced to the type of cell surface receptors, and that the conjugate may be a useful tool for the identification of potential therapeutic targets.
Mutants Resistant to Protein Toxins

Fig. 1. Inhibition of protein synthesis in the cell-free system by ricin A-chain or the D-globulin to

RICIN A-CHAIN, M

GELONIN, M

0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0

10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}

0

Further studies on the mechanism of action of these toxins are needed to understand their effects on protein synthesis in the cell-free system.

Interleukin-1 in the presence of ricin A-chain becomes active as a ribosomal inhibitor only after it stimulates the ribosomes in the cell-free system. This suggests that the inhibition of protein synthesis in the cell-free system is due to the ribosomal inhibitory activity of the ricin A-chain.

Degradation of interleukin-1 by processing - Interleukin-1 is degraded by a processing mechanism that involves the proteolytic degradation of its precursor, interleukin-1 precursor. This degradation process occurs in the endoplasmic reticulum and is mediated by a protease that is specific for interleukin-1. The degradation of interleukin-1 precursor is similar to the degradation of the proform of interleukin-1, which has been shown to be degraded by a protease that is specific for interleukin-1.

In conclusion, the results presented in this study demonstrate that ricin A-chain inhibits protein synthesis in the cell-free system by a mechanism that involves the inhibition of the ribosomal activity. Further studies are needed to understand the mechanism of action of this toxin and its potential use as a tool for studying the regulation of protein synthesis.