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Properties and Action Mechanism of the Toxic Lectin Modeccin: Interaction With Cell Lines Resistant to Modeccin, Abrin, and Ricin

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The toxic lectin modeccin, which inhibits protein synthesis in eukaryotic cells, is cleaved upon treatment with 2-mercaptoethanol into two peptide chains which move in polyacrylamide gels at rates corresponding to molecular weights 28,000 and 38,000. After reduction, the toxin loses its effect on cells, while its ability to inhibit cell-free protein synthesis increases. Like abrin and ricin it inhibits protein synthesis by inactivating the 60S ribosomal subunits.

Modeccin binds to surface receptors containing terminal galactose residues. Competition experiments with various glycoproteins indicate that the modeccin receptors are different from the abrin receptors. In addition, they were present on HeLa cells in much smaller numbers. Moreover, mutant lines resistant to abrin and ricin were not resistant to modeccin and vice-versa.

The toxin resistance of various mutant cell lines could not be accounted for by a reduced number of binding sites on cells. The data are consistent with the view that the cells possesss different populations of binding sites with differences in ability to facilitate the uptake of the toxins and that in the resistant lines the most active receptors have been reduced or eliminated.

Key words: modeccin, abrin, ricin, toxin, lectin, mutant cell, receptor, sialic acid, glycoprotein, ribosomes, enzyme, inhibitor of protein synthesis

One of the models available for the study of transmembrane processes is the interaction of toxic lectins with eukaryotic cells. For several years we have been studying in our laboratory the interaction of the toxic lectins abrin and ricin with mammalian cells. The mechanism of action of these toxins is now rather well understood (for review, see Olsnes and Pihl [1]). The toxins consist of two polypeptide chains which have different functions. The B chain binds the toxin to cell surface receptors, a process which is necessary for the toxic action. Subsequently, the toxin is internalized by a process which is as yet poorly understood. The toxic action is exerted in the cytoplasm by the A chain, which is an

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enzyme inactivating the 60S ribosomal subunit, thus inhibiting cellular protein synthesis. The binding of the toxins to the cells can readily be measured, and the penetration into the interior of the cell can be gauged by measuring the inhibition of protein synthesis. Thus, by correlation of the binding and toxicity under different experimental conditions information can be obtained on the factors influencing the penetration of the proteins through the membrane.

Here we wish to report on the properties and mechanism of action of another toxin, modeccin, which is present in the roots of an African plant, Adenia digitata [2]. The data indicate that modeccin, although it binds to other receptors, is very similar to abrin and ricin in properties and mechanism of action. By testing the binding and toxicity of modeccin in normal cell lines and in variants resistant to modeccin, abrin, and ricin, we have attempted to obtain information on the specificity of the receptors and on the factors influencing the transmembrane transport.

MATERIALS AND METHODS

The gangliosides were given to us by Dr W.E. van Heyningen, Oxford. T antigen (desialylated O, NN-antigen) was a gift from Dr G.F. Springer, Evanston, Illinois. α_1-acid glycoprotein was obtained from Behring-Werke, Marburg. Fetuin and mucin from bovine serum submaxillary gland were obtained from Sigma Chemical Co, St Louis.

Modeccin was extracted from the root of Adenia digitata and purified as described earlier [2] and by affinity chromatography on a column containing desialylated fetuin. Diphtheria toxin was a gift from Professor A.M. Pappenheimer, Jr, Harvard. Abrin and ricin were isolated as earlier described [3, 4]. The antitoxins were prepared by immunizing rabbits with formaldehyde-treated toxins [5]. Modeccin-resistant cells were obtained from cells irradiated with a dose of ultraviolet light which killed two-thirds of the cells. Two days after the irradiation 60 ng/ml of modeccin was added to the growth medium and a surviving clone was selected. Cell culture was carried out as earlier described [6]. Cells resistant to abrin and ricin were isolated as surviving clones of X-ray irradiated HeLa cells grown in the presence of 1 μg/ml of abrin or ricin [7].

RESULTS

Pure modeccin is highly toxic to cells in culture. Thus, when HeLa cells were plated out in the presence of increasing toxin concentrations, as little as about 1 pg/ml inhibited by 50% the formation of colonies. In the same system 0.1–0.5 ng/ml of diphtheria toxin, abrin, and ricin was required (Fig 1).

In polyacrylamide gels containing sodium dodecyl sulfate modeccin moves at a rate corresponding to an apparent molecular weight of 63,000. Upon treatment with 2-mercaptoethanol it is cleaved into two peptide chains with molecular weights of about 28,000 and 38,000 (Fig 2). When the toxin is treated with 2-mercaptoethanol is loses its ability to inhibit protein synthesis in HeLa cells (Fig 3). After removal of the reducing agent a considerable fraction of the toxicity is recovered. Apparently only the intact toxin is able to intoxicate living cells. These results are all analogous to those previously obtained with abrin and ricin [1].

Previous studies in several laboratories have shown that abrin and ricin bind to carbohydrates containing terminal galactose residues [1], and this also appears to be the case with modeccin. Thus, galactose, lactose, and melibiose inhibit the toxic effect of modeccin on HeLa cells, whereas a variety of other common sugars have no inhibitory

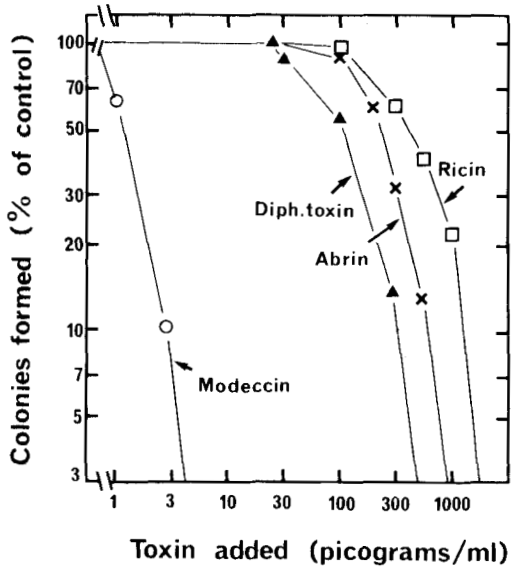

Fig 1. Ability of different toxins to inhibit colony formation of HeLa cells. Two hundred cells were plated out in the presence of increasing concentrations of toxins. After two weeks the medium was removed, and the colonies were fixed and stained.

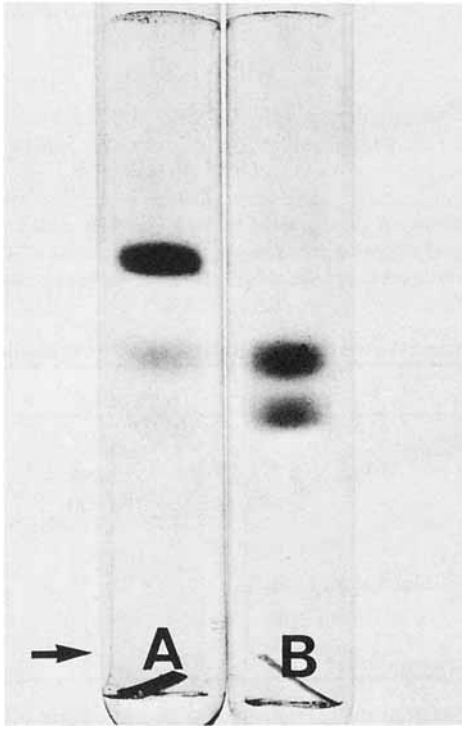

Fig 2. Polyacrylamide gel electrophoresis of modeccin. Modeccin was treated with 1% sodium dodecyl sulfate, boiled for 1 min in the absence (A) and presence (B) of 1% 2-mercaptoethanol, and then submitted to electrophoresis in 7% polyacrylamide gels containing sodium dodecyl sulfate as earlier described [3]. The arrow indicates the position of the tracking dye (bromphenol-blue) at the end of the electrophoresis.

effect (K. Refsnes, unpublished). The data in Table I show that four glycoproteins known to contain terminal galactose residues inhibit the binding of modeccin to HeLa cells. Based on the molar amount of galactose residues present [8–10] T antigen is the best inhibitor, but also neuraminidase-treated fetuin, mucin from the bovine submaxillary gland, and α_1-acid glycoprotein are more efficient inhibitors than free galactose. In the case of abrin the glycoproteins tested were all much more efficient inhibitors than free galactose, and,

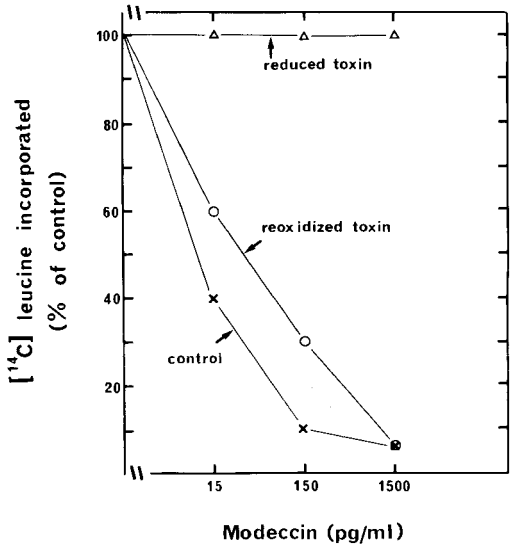

Fig 3. Effect of 2-mercaptoethanol treatment of modeccin on its ability to inhibit protein synthesis in HeLa cells. A solution of modeccin (50 μg/ml in 10 mM sodium phosphate– 0.14 M NaCl) was made up to contain 14 mM 2-mercaptoethanol and incubated over night at room temperature. Part of the material was then dialyzed for four days against several shifts of distilled water at 4° to allow reoxidation to take place. Increasing amounts of the different preparations were added to HeLa cells growing in Linbro trays FB-16-24-TC, and the incorporation of [^{14}C]-leucine was measured after 14 h as described previously [2]. △) reduced modeccin; ○) reduced and dialyzed modeccin; X) untreated modeccin.

TABLE I. Ability of Sugars and Glycoproteins to Inhibit by 50% the Binding of Toxins to HeLa Cells[a]

Inhibitor	Modeccin	Abrin	Ricin
	μM	μM	μM
D-galactose	850	1,500	2,000
L-fucose	> 100,000	> 100,000	> 100,000
N-acetyl-D-glucosamine		> 100,000	> 100,000
D-mannose	> 100,000	> 100,000	> 100,000
T-antigen (desialylated O, NN blood group antigen)	27	2.4	2.4
Fetuin[b]	110	0.2	3.3
α_1-acid glycoprotein[b]	562	11.9	85
Mucin (from bovine submaxillary gland)[b]	164	2	

[a]The binding of ^{125}I-labelled toxins to neuraminidase-treated HeLa cells (6) was measured at 0° in the absence and presence of various concentrations of inhibitors. The concentrations required to reduce the amount of bound toxin to 50% of the control value (no inhibitor present) are given. In the case of glycoprotein inhibitors the values were normalized to the molar galactose content.
[b]The glycoprotein (1–10 mg/ml) was preincubated with 50 units/ml of neuraminidase overnight at 37°C.

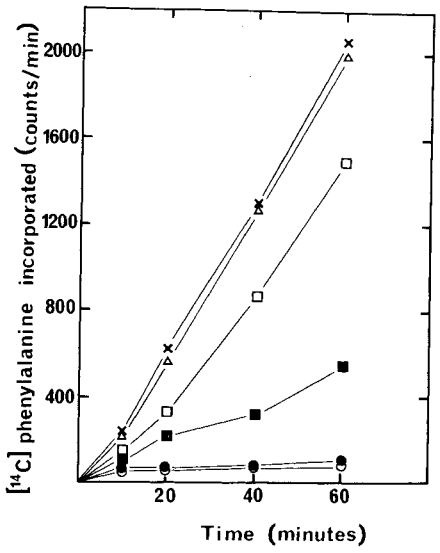

Fig 4. Ability of modeccin-treated ribosomes and supernatant factors to support poly(U)-directed polymerization of phenylalanine. Two-ml samples of rabbit reticulocyte lysate were incubated for 30 min at 28° in the absence and presence of 0.2 μg modeccin pretreated for 1 h at room temperature with 0.3 M 2-mercaptoethanol. The samples were than layered onto 3 ml of 10% (w/v) sucrose in 10 mM triethanolamine (pH 7.5), 0.15 M KCl, and 7 mM $MgCl_2$ and centrifuged at 234,000 g_{av} in rotor SW-50.1 for 2 h. The upper 1 ml of the supernatant was carefully sucked off (supernatant fraction); the tubes were then decanted and the ribosome pellet was dissolved in 0.5 ml of the above buffer (without sucrose). Cell-free systems were prepared and poly(U)-dependent polymerization of [^{14}C]-phenylalanine was measured as earlier described [12]. ×) Ribosomes and supernatant untreated; ■) ribosomes treated with modeccin, supernatant untreated; □) supernatant treated with modeccin ribosomes untreated; △) supernatant treated with modeccin, ribosomes untreated, 5 μl antimodeccin added; ○) untreated supernatant alone; ●) untreated ribosomes alone.

interestingly, neuraminidase-treated fetuin was a better inhibitor than the T antigen. This was also the case in experiments with ricin. It is therefore clear that the specificity of modeccin is different from that of abrin and ricin. Gangliosides were not very efficient inhibitors in the binding of any of the toxins (not demonstrated).

In cell-free systems modeccin is a potent inhibitor of protein synthesis [2, 11]. To see whether modeccin acts in a similar way as abrin and ricin a cell-free system from rabbit reticulocyte lysate was treated with modeccin and then separated into ribosomes and supernatant fraction. The treated ribosomes and supernatant fractions were then combined with untreated supernatant and ribosomes respectively, and the ability of the reconstituted system to support poly(U)-directed polymerization of phenylalanine was measured (Fig 4). The results show that modeccin-treated ribosomes had low activity when combined with modeccin-treated or untreated supernatant. In contrast, the supernatant fraction was fully active provided antimodeccin had been added to inactivate remaining toxin. If antimodeccin was omitted from the toxin-treated supernatant the incorporation was reduced, indicating that the remaining modeccin present in the supernatant fraction inhibited the ribosomes in the reconstituted system.

To study which of the ribosomal subunits are targets for the toxin, modeccin-treated ribosomes were separated into 40S and 60S subunits and then combined with untreated 60S and 40S subunits, respectively. The data in Table II show that 40S subunits derived

TABLE II. Ability of Modeccin-Treated and Untreated Ribosomal Subunits to Support Poly(U)-Directed Synthesis of Polyphenylalanine

Untreated subunits	Modeccin-treated subunits	[^{14}C]-phenylalanine incorporated (counts/min)
40A, 60S		197
40S	60S	30
60S	40S	225
	40S, 60S	38
40S	40S	11
60S	60S	44

Samples of rabbit reticulocyte lysate were incubated in the absence and presence of modeccin as in Figure 4. Ribosomal subunits were isolated [12] and tested for their ability to support poly(U)-directed synthesis of polyphenylalanine as described [13].

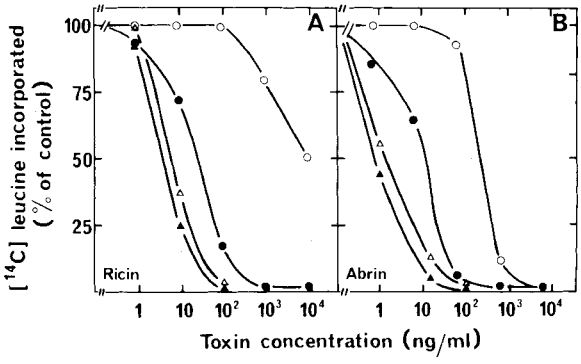

Fig 5. Effect of neuraminidase treatment on the toxin sensitivity of wild-type HeLa cells and of the ricin-resistant variant R^{R}III. Cells growing as monolayers in Linbro FB-16-24-TC plates were incubated with and without neuraminidase. Untreated cells (open symbols) and neuraminidase-treated cells (closed symbols) in serum-free medium were preincubated with the indicated amounts of toxin for 3 h. Then [^{14}C]-leucine was added to each sample and after 1 h the trichloroacetic acid-precipitable radioactivity was measured as earlier described [6]. △, ▲) HeLa cells; ○, ●) R^{R}III.

from modeccin-treated ribosomes gave reconstituted ribosomes with activity as high as that of untreated subunits. In contrast, when the 60S subunits were derived from modeccin-treated ribosomes the activity in the reconstituted system was very low. This indicates that modeccin, like abrin and ricin, acts by inactivating the 60S ribosomal subunits. It is therefore likely that also modeccin must enter the cytoplasm to exert its effect.

HeLa cells possess a markedly high number of receptors, both for modeccin and for abrin and ricin (Table III). The question arises whether all these receptors are equally effective in the uptake of the toxin or whether the biologic uptake is primarily associated with a small subpopulation of receptors. Evidence that the latter may be the case was obtained in work with various toxin-resistant variants of established cell lines. Such variants have been isolated in several laboratories [7, 14–19]. In some cases the resistance can be accounted for by a decreased number of toxin-binding sites, while other resistant variants

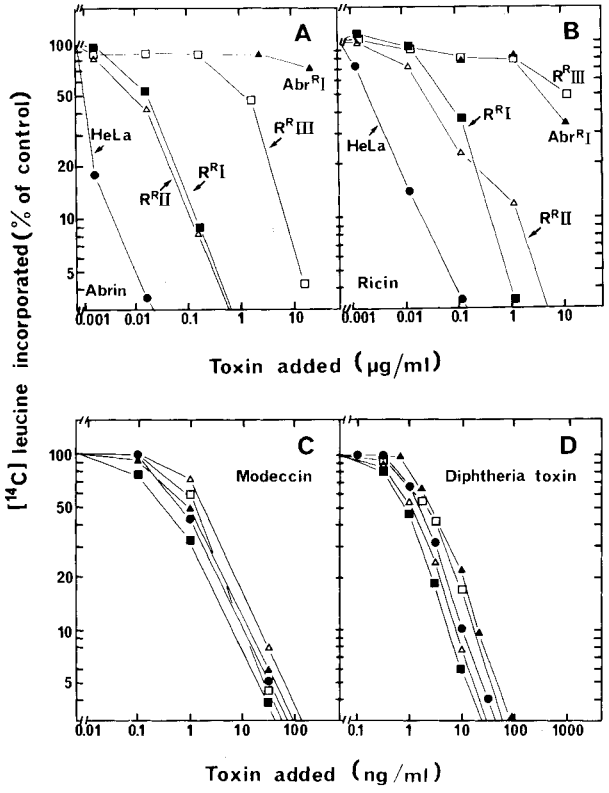

Fig 6. Toxin sensitivity of HeLa cells and abrin- and ricin-resistant variants. Cells were grown in Linbro FB-16-24-TC trays overnight, then changed to serum-free medium and increasing amounts of toxin were added to the wells (in duplicate). The plates were incubated at 37°C for 3 h. Then [^{14}C]-leucine was added, the incubation was continued for 1 h more, and trichloroacetic acid-precipitable radioactivity was measured. •) HeLa cells; ▪) HeLa R^RI; △) HeLa R^RII; □) HeLa R^RIII; ▲) HeLa Abr^RI.

possess a normal number of toxin-binding sites. In the case of a HeLa cell variant, R^RIII, isolated in our laboratory, the number of binding sites is strongly reduced, due to the fact that the majority of the galactose residues have been masked by terminal sialic acid. However, the reduction in the total number of receptors cannot explain the resistance, since the R^RIII cells tolerated 5,000 times more toxin in the medium than HeLa wild-type cells under the conditions used, while the total number of receptors was reduced only by a factor of 6 [6]. After treatment with neuraminidase the number of binding sites for abrin and ricin was increased to a similar value as that found in the parent cells. Nevertheless, the neuraminidase-treated R^RIII cells still tolerated 5–10 times as much abrin and ricin (Fig 5).

The possibility was considered that the resistance might largely be due to a general change in the cell membrane. In this event the resistant line would be expected to be resistant also to other toxic proteins acting on intracellular targets. To test this possibility the sensitivity of R^RIII to modeccin and diphtheria toxin [20] was studied. The data in Figure 6 show that R^RIII was as sensitive to these toxins as was the wild-type HeLa cells.

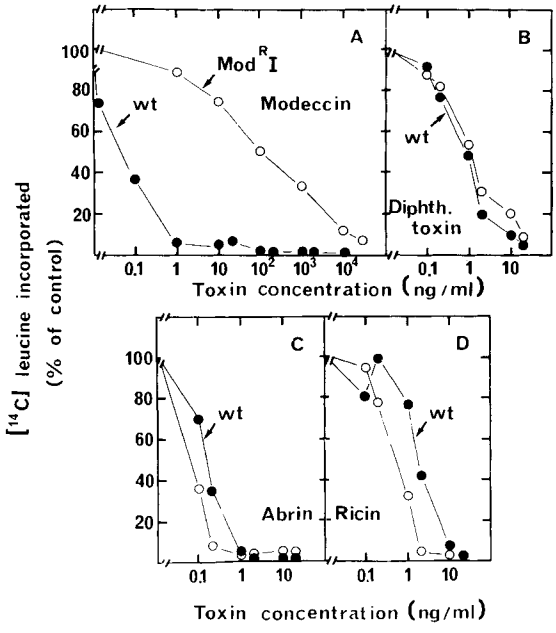

Fig 7. Toxin sensitivity of HeLa S3 and the modeccin-resistant variant Mod ^{R}I. Cells were grown as in Figure 5 in Eagle's medium containing 10% calf serum. Without change of medium, increasing amounts of modeccin (A), diphtheria toxin (B), abrin (C), or ricin (D) were added and the plates were incubated at 37° over night in an atmosphere containing 5% CO_2. Then the medium was removed, 1 ml of medium containing HEPES, one-tenth the usual leucine concentration, and 0.5 μCi [^{14}C]-leucine were added and the incorporation during 1 h was measured. •) HeLa S3, wild-type; ○) HeLa, Mod^{R}I.

Another oversialylated variant, R^{R}II, as well as a variant isolated in the presence of abrin, Abr^{R}I, were studied in the same way. Again it was found that the cells showed cross-resistance to abrin and ricin, but not to modeccin and diphtheria toxin. We obtained similar results with baby hamster kidney cells and with six ricin-resistant variants isolated by Meager, Ungkitchanukit, and Hughes [15]. Some of these variants possessed a normal number of ricin-binding sites. Also, when the HeLa variants were tested in a colony assay, there was no evidence of cross-resistance to modeccin and diphtheria toxin (not demonstrated). Altogether, the data indicate that the resistance may be due to loss of a small population of particularly efficient receptors, the absence of which is not revealed when the overall binding capacity of the cells is measured.

Recently we have obtained similar results with a modeccin-resistant variant of HeLa cells. In this variant about 10^5 times more modeccin was required to inhibit protein synthesis by 50% after 17 hours than in the parent cell line. As shown in Figure 7 the modeccin-resistant line showed the same sensitivity to diphtheria toxin as the parent cell line, and the sensitivity to abrin and ricin was increased compared to that of the parent cells. The results show that the alteration responsible for the resistance to modeccin is specific for this toxin. The increased sensitivity to abrin and ricin was associated with an increase in the total number of binding sites for abrin (Table III). We have earlier shown that neuraminidase treatment of the parent HeLa cells increased their sensitivity to modeccin about 10-fold [2]. The data in Table III show that the number of binding sites on these cells increased by the same factor. Treatment of Mod^{R}I with neuraminidase increased the number

TABLE III. Number of Binding Sites for Modeccin and Abrin on Modeccin-Sensitive and Resistant HeLa Cells

Cell line	Treatment	Modeccin ($\times 10^5$)	Abrin ($\times 10^7$)
HeLa	None	2	3
HeLa	Neuraminidase	20	7
Mod^RI	None	4	7
Mod^RI	Neuraminidase	20	

HeLa cells in concentrations varying between 5×10^5 and 10^7 per milliliter were mixed with increasing amounts of ^{125}I-labeled modeccin or abrin in 0.5 ml serum-free medium and incubated 1 h at 0°. The cells were sedimented and the radioactivity present in the supernatant was measured. The amount of toxin bound at saturation was estimated. When so indicated, the cells had previously been treated with neuraminidase as earlier described [6]. The saturating concentrations of toxins varied between 0.6 and 3 μg/ml.

TABLE IV. Toxin Sensitivity of Resistant Cells in a Colony Assay System and in a System Measuring Protein Synthesis

		Toxin concentration[a]		
Cell line	Toxin	Inhibition of colony formation A	Inhibition of [^{14}C]-leucine incorporation B	Ratio B/A
HeLa		1	1	1
R^RI	Ricin	10	20	2
R^RII	Ricin	10	10	1
R^RIII	Ricin	267	3,300	12
Abr^RI	Abrin	330	30,000	90
Mod^RI	Modeccin	25	3,300	133

[a]The concentration required to inhibit colony formation after 10 days and to inhibit by 50% leucine incorporation after incubation for 3 h (abrin and ricin) and 17 h (modeccin). The concentration required for the parent HeLa cells is set equal to 1. In the colony assay system this concentration was 0.3 ng/ml abrin, 0.3 ng/ml ricin, and 2 pg/ml modeccin. In the experiment measuring [^{14}C]-leucine incorporation the value was 1 ng/ml abrin, 3 ng/ml ricin, and 30 pg/ml modeccin.

of binding sites for modeccin by a factor of 5, but these cells did not become more sensitive to the toxin (data not shown). Clearly, the resistance of Mod^RI to modeccin is not due to oversialylation.

It is conceivable that cells may contain several kinds of receptors – highly efficient ones which internalize the toxins rapidly and efficiently and less efficient receptors. The toxin resistance present in the variants R^RIII, Abr^RI, and Mod^RI could thus be largely due to loss of the former kind of receptors. If this is the case, the resistance could be expected to be more easily demonstrable in the system measuring protein synthesis inhibition than in the colony-formation assay, where the cells are exposed to the toxins for about 10 days. In the latter case the more inefficient receptors might have time to internalize sufficient toxin to kill cells. The data in Table IV, which show that R^RIII, Abr^RI, and Mod^RI, were 12–133 times more resistant in the short-time experiment where protein synthesis inhibition was measured than in the colony assay, are consistent with this possibility.

DISCUSSION

The data here presented provide evidence that modeccin is very similar to abrin and ricin in its structure and mechanism of action. Thus, all three toxins consist of two dissimilar peptide chains joined by SS-bonds which are required for the toxins to act on cells and animals. Reduction of the SS-bonds increases the effect of the toxins in cell-free systems, where they exert their action by inactivating the 60S ribosomal subunit. It is well established that abrin and ricin A chain inactivate ribosomes enzymically [1]. Also modeccin appears to act catalytically. Thus, modeccin was inhibitory in cell-free protein-synthesizing systems containing more than 100 ribosomes per toxin molecule. The data in Figure 4 showing that modeccin-treated supernatant fraction had no inhibitory effect on protein synthesis provided antimodeccin is added indicates that the toxin does not activate an inhibitor which then acts on the ribosomes. Altogether the data indicate that modeccin, like abrin and ricin, must penetrate into the cytoplasm to exert its effect.

Although terminal galactose appears to be involved in the receptors for all three toxins, the carbohydrate specificity of modeccin is clearly different from that of abrin and ricin. Thus, on HeLa cells the number of modeccin receptors is about one-hundredth that of abrin receptors. Furthermore, experiments with different glycoproteins revealed clear differences between abrin and modeccin. It is particularly interesting that in several abrin- and ricin-resistant cell lines no cross-resistance to modeccin was found and that, conversely, a modeccin-resistant cell line was not resistant to abrin and ricin.

The finding that the resistance of mutant cells cannot be explained only by a reduced number of surface receptors is most easily explained by assuming the presence of different kinds of receptors, of which some are active in toxin internalization and others are less active or completely unable to facilitate the toxin internalization [17–19]. This might in fact be anticipated, since the toxin receptors appear to consist in oligosaccharide chains attached to a variety of proteins and lipids.

We have earlier presented evidence that in the case of HeLa wild-type cells the toxic effect of abrin and ricin is closely correlated with the total number of binding sites [21]. In toxin-resistant cells this is not always the case. Possibly the mutation has affected preferentially the most efficient receptors. Thus, it is conceivable that in the oversialylated mutant, $R^{R}III$, the sialyl transferase involved catalyzes the addition of terminal sialic acid most actively to the most efficient ricin receptors. Interestingly, no sialylation of modeccin receptors appears to take place.

It is clear that binding of toxin to the cell surface is not sufficient to achieve internalization in spite of the fact that it increases the local toxin concentration at the cell membrane and thereby the chances that the toxin is engulfed into pinocytotic vesicles.

It is possible that all receptors on the cell surface may internalize the toxin, but that some receptors do this much more rapidly than other ones. The difference observed in toxin resistance when the different cell lines were tested in a colony assay system and in a system measuring protein synthesis inhibition after a few hours is in accordance with this interpretation. In any case, it may be misleading to correlate overall receptor number with toxin sensitivity, particularly in toxin-resistant mutants.

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