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Isolation of a Clone of Chinese Hamster Ovary Cells Deficient in Plant Lectin-Binding Sites

(ricin/glycoproteins/membranes)

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ABSTRACT **Ricin, a galactose-binding lectin with potent cytotoxic activity, was used to select a clone of Chinese hamster ovary cells with altered plant lectin-binding properties. The clone (15B) is 80-fold less sensitive to the toxic action of ricin than the parent line. In the absence of ricin, it grows both in monolayer and suspension culture with a normal generation time. Plating efficiency, however, is significantly reduced. Relative to the parent cells, its binding of the *Ricinus communis* lectins, *Phaseolus vulgaris* erythroagglutinating phytohemagglutinin, *Abrus precatorius* phytohemagglutinin, and soybean phytohemagglutinin is less than 7%, while binding of lentil phytohemagglutinin, wheat-germ agglutinin, and mushroom phytohemagglutinin is 17%, 40%, and 109%, respectively. In contrast, its concanavalin A binding is increased by 70%. Consistent with these alterations, crude membrane preparations of the 15B cells were found to contain the same sugars as the parent-cell membranes but in different proportions. The 15B membranes have 28% less sialic acid, 38% less *N*-acetylglucosamine, 49% less galactose, the same amount of *N*-acetylgalactosamine, and 53% more mannose than the membranes of the parent cells.**

A number of basic questions remain unanswered about the structure, function, and biosynthesis of cell-membrane glycoproteins and their oligosaccharide units. Many of these questions have proved difficult to approach with standard biochemical techniques. For instance, it is extremely difficult to remove sugars other than terminal sialic acid residues from the surfaces of intact cells. Consequently, it has not been possible to degrade cell-surface oligosaccharides and determine what effect their removal may have on various biologic functions of the cell. In considering this problem, it seemed to us that if cell lines with altered surface carbohydrates could be isolated, they might provide a unique opportunity to study the biologic role of membrane glycoproteins. To achieve this, we have used ricin, the toxic lectin of *Ricinus communis*, as a selective agent.* This lectin binds to a galactose-containing membrane receptor (1–3) and subsequently causes an irreversible inhibition of protein and DNA synthesis leading to cell death (4–7). Our approach was to add ricin to cells growing in tissue culture and test surviving cells for their ability to bind various lectins. Using this technique we have isolated a clone of Chinese hamster cells that displays a marked alteration in lectin-binding properties. The carbohydrate composition of the membranes of this cell line is strikingly different from that of the parent-cell membranes.

MATERIALS AND METHODS

Materials. α-MEM, a modified Eagle's minimal essential medium supplemented with nonessential amino acids, pyruvate, glutamine, and additional vitamins (9), was obtained from Flow Laboratories, Rockville, Md. Fetal-calf serum, trypsin, penicillin, and streptomycin were purchased from Grand Island Biological Co. Plastic petri dishes and T-flasks were products of Falcon Plastic. $Na^{125}I$ (Reagent Grade) was purchased from Mallinckrodt, and [^{3}H]thymidine and [^{3}H]leucine were from New England Nuclear Corp. Ovomucoid and wheat germ were obtained from Sigma Chemical Corp.

Cells. Chinese hamster ovary (CHO) cells were obtained from Dr. Potter Stewart. The cells were grown in suspension culture and monolayer in α-MEM supplemented with 10% fetal-calf serum, 50 units/ml of penicillin, and 50 μg/ml of streptomycin. Cells were freed from the monolayer dishes with 0.25% trypsin.

Lectins. The *Ricinus communis* lectins were prepared from castor beans as described (7). Ricin has an estimated molecular weight of 60,000 and is 200 times more toxic than *Ricinus* phytohemagglutinin (PHA) IV. *Phaseolus vulgaris* erythroagglutinating phytohemagglutinin (E-PHA), lentil PHA, and mushroom PHA were purified as described (10–12). Wheat-germ agglutinin (WGA) was purified from crude wheat germ by affinity chromatography on ovomucoid–Sepharose (13). The ovomucoid was conjugated to Sepharose activated with cyanogen bromide, as described by Cuatrecasas (14). The *Abrus precatorius* lectin was also purified by affinity chromatography on ovomucoid–Sepharose. Soybean agglutinin was prepared by the method of Lis and Sharon (15). Concanavalin A (Con-A) was purchased from Nutritional Biochemical Corp. Each of the lectins gave a single band on disc-gel electrophoresis.

Iodination of Lectins. The lectins were iodinated with ^{125}I by the method of Hunter (16), by a 10-sec exposure to the chloramine-T. Iodination of Con-A was performed in the presence of 0.2 M α-methyl-D-mannose, which was subsequently removed by gel filtration.

Preparation of Crude Membrane Fractions. Cells (100 to 300 $\times 10^6$) were suspended in 3 ml of H_2O and subjected to freeze-

Abbreviations: PHA, phytohemagglutinin; WGA, wheat-germ agglutinin; CHO cells, Chinese hamster ovary cells; E-PHA, erythroagglutinating phytohemagglutinin; Con-A, concanavalin A.

* The toxic lectin of *Ricinus communis* has been called ricin by most investigators (3–5, 8). In our previous publication we referred to this substance as *Ricinus* PHA II (7), but will use the term ricin in this paper.

TABLE 1. *Effect of ricin on the plating efficiency of parent CHO and clone 15B cells*

No. of cells plated	Ricin (μg/ml)	No. of colonies formed	Plating-efficiency (%)
CHO			
10^2	—	56	56
10^2	0.01	0	0
10^3	—	512	51
10^3	0.01	28	2.8
10^3	0.1	0	0
15B			
10^2	—	26	26
10^2	0.1	16	16
10^3	—	364	36
10^3	0.1	153	15

Plastic petri dishes (5.2 cm in diameter) were plated with either 10^2 or 10^3 cells growing in logarithmic phase in suspension culture. On day 2, ricin was added at the indicated concentrations. On day 7, the colonies were stained with 3% methylene blue–10% formaldehyde and counted. The values represent the average of triplicate plates.

thawing five times in a dry ice–acetone bath. The membranes were sedimented at 100,000 $\times$ g for 60 min, resuspended in 3 ml of water, and sedimented again. The crude membrane pellet was then suspended in H_2O.

Chemical Analyses. Protein was determined by the method of Lowry *et al.* (17). Sialic acid was assayed by the thiobarbituric acid method of Warren (18) after hydrolysis in 1 N HCl for 90 sec at 100°. Mannose and galactose were determined enzymatically after hydrolysis in 2 *N* H_2SO_4 for 4 hrs at 100° and deionization on Amberlite MB-3 (19). Hexosamine was determined with an automatic amino-acid analyzer after hydrolysis in 4 N HCl for 4 hr at 100° under reduced pressure followed by lyophilization to remove HCl.

RESULTS

Toxicity of Ricin Toward CHO Cells. Previous work has demonstrated that ricin is extremely toxic to a number of cell types. The toxicity to CHO cells is reflected in the different plating efficiencies of this cell line in the presence and absence of ricin. Thus, 0.01 μg/ml of ricin caused a 95% decrease in plating efficiency while 0.1 μg/ml caused a greater than 99.5% decrease (Table 1).

Isolation of a Clone of CHO Cells Resistant to Ricin. On the basis of data obtained from the plating efficiency experiment with CHO cells, we plated petri dishes with either 10^4 or 10^5 cells and grew the cells in complete media containing 0.1 μg/ml of ricin. Only the petri dishes plated with 10^5 cells developed colonies. After 14 days, the dish containing clone 15B contained four to six loose colonies and by day 18 the dish containing 15B contained a confluent monolayer. Since these cells proved to be deficient in ricin-binding sites, their characteristics will be described in detail.

The 15B cell line grew in monolayer and in suspension culture with a generation time of 16–18 hr, which is very similar to the generation time of the parent line. The cells grown in monolayer looked similar to the parent line. However, at very low densities the 15B cells appeared rounded while the parent cells were elongated (Fig. 1). In several experiments it was noted that even in the absence of ricin, the plating efficiency of the 15B line was less than that of the parent line (Table 1). When the 15B cells were grown in the presence of 0.1 μg/ml of ricin, the generation time was prolonged to 54 hr and the plating efficiency was decreased about 50% (Table 1). Compared

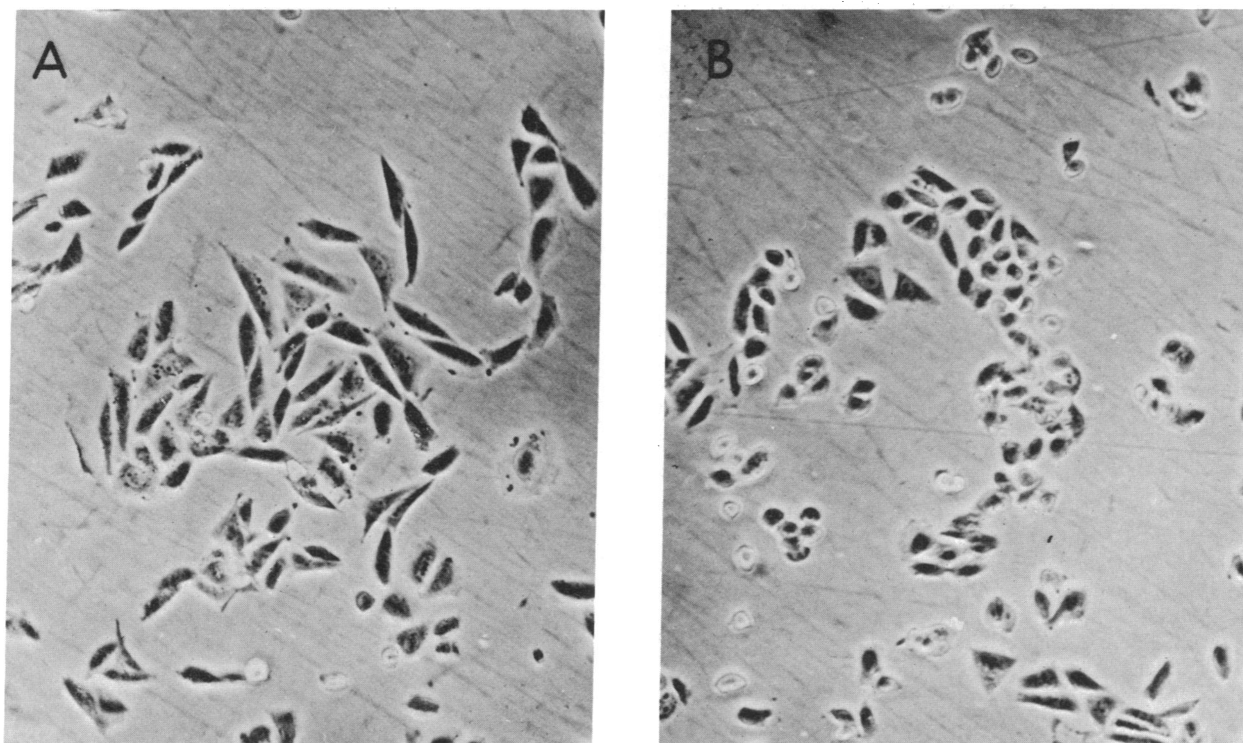

FIG. 1. Appearance of the CHO parent cells (*left*) and 15B cells (*right*) when grown in monolayer at comparably low densities.

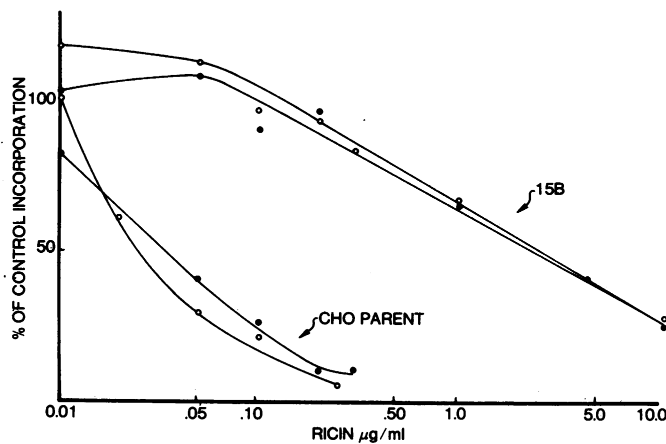

FIG. 2. Inhibition of protein and DNA synthesis by ricin. CHO and 15B cells (1.2×10^6) were taken from logarithmic phase suspension cultures, washed once with α-MEM and incubated in serum-free α-MEM with various concentrations of ricin, as shown, in a total volume of 2 ml. The cells were incubated for 3 hr at 37° in 5% CO_2–95% air, and then 3 µCi of either [^{3}H]thymidine or [^{3}H]leucine was added for 30 min. The incorporation of radioactivity into 5% trichloroacetic acid-precipitable material was then determined. The results are expressed as the percentage of the control cultures. ●——●, [^{3}H]thymidine; ○——○, [^{3}H]leucine.

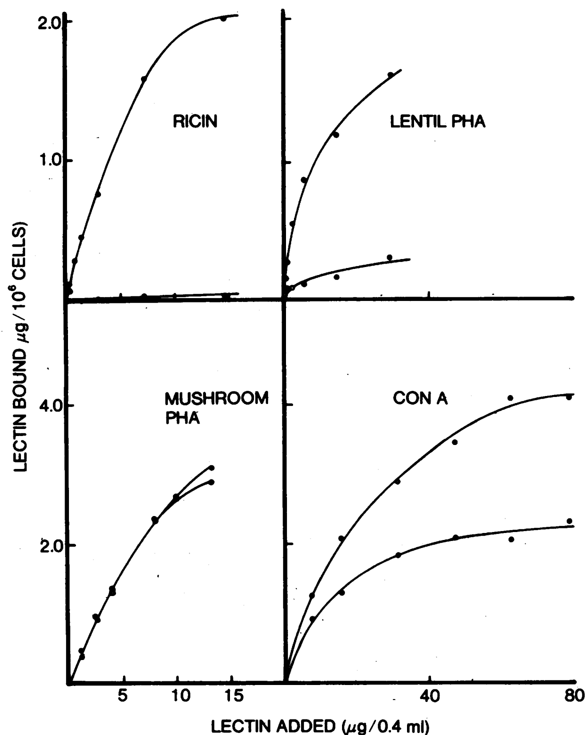

FIG. 3. Binding of ricin, lentil PHA, mushroom PHA, and Con-A to the CHO parent and 15B cells. The binding reactions were carried out in plastic counting tubes that had been soaked for 1 hr with 5 mg/ml of albumin. The incubation mixtures contained in 0.4 ml: from 0.5 to 80 µg of ^{125}I-labeled lectin, 1 mg of bovine-serum albumin, and 1 to 2×10^6 CHO or 15B cells derived from logarithmic phase suspension cultures. After 60 min of incubation at room temperature, the cells were washed twice with 5 ml of 0.9% NaCl, and the amount of bound ^{125}I-labeled lectin was determined in a Packard autogamma counter. ●——●, CHO parent cells; ○——○, 15B cells.

TABLE 2. *Binding sites per cell for various lectins*

Lectin	CHO parent	15B (sites/cell*)	15B/ parent (%)
Ricin	26×10^6	0.33×10^6	1.3
Ricinus communis PHA IV	45×10^6	0.87×10^6	1.9
Abrus precatorius PHA	33×10^6	2.20×10^6	6.7
P. vulgaris E-PHA	14×10^6	$<0.14 \times 10^6$†	<1
Lentil PHA	21×10^6	3.60×10^6	17
WGA	130×10^6	49.0×10^6	38
Soybean PHA‡	2×10^6	$<0.02 \times 10^6$†	<1
Mushroom PHA	34×10^6	37.0×10^6	109
Con-A	33×10^6	55.0×10^6	167

* The sites per cell were determined by plotting the data from the binding curves (Fig. 3) according to the method of Steck and Wallack (20).

† There was too little binding to permit calculation of an exact value from a double-reciprocal plot.

‡ Binding studies performed after neuraminidase treatment of cells.

to the parent line, the 15B clone was about 80-fold more resistant to the toxic effects of ricin (Fig. 2).

Binding of Lectins to the 15B Clone. The ability of the 15B clone to bind a selected panel of iodinated lectins was tested and compared to the parent cell line. Fig. 3 illustrates the types of alterations seen, ranging from marked and moderate decreases to an actual increase of different lectin-binding sites. Double-reciprocal plots of the data in Fig. 3 (not shown) revealed that the ricin bound to both cell types with approximately the same affinity, but that there was an absolute decrease in the number of binding sites on the 15B cells. The ricin binding was completely blocked by the presence of 0.01 M lactose. The data in Table 2 summarize the results of a number of binding studies with different lectins. Binding of the *Ricinus* lectins, *P. vulgaris* E-PHA, *Abrus precatorius* PHA, and soybean PHA to the 15B cells was decreased by 93% or more, while lentil PHA binding was decreased 83%, WGA binding was decreased 60%, and mushroom PHA binding was essentially unchanged. In contrast to these findings, 15B cells bound 70% more Con-A than the parent cells.

After continuous growth in the absence of ricin for over 50 generations, 15B cells showed no increase in the binding of this toxin. The possibility that the 15B line was secreting a substance that selectively inactivated certain lectins was ruled out by the fact that preincubation of ricin with 15B cells for 1 hr did not alter the subsequent binding of the ricin to the parent cells. Since the original 15B cell line may not have been derived from a single cell, we isolated 7 clones from the 15B line and tested each one for its lectin-binding ability. In each case ricin binding was less than 10% of that obtained with the parent line.

Carbohydrate Composition of the 15B Membranes. The carbohydrate composition of crude membrane preparations of the 15B and parent CHO cells is shown in Table 3. Compared to the parent cells, there is a 28% decrease in sialic acid, a 49% decrease in galactose, and a 38% decrease in *N*-acetylglucosamine content, while the mannose content of the 15B

TABLE 3. *Carbohydrate composition of CHO and 15B membranes*

	CHO parent	15B clone	15B/parent
	(mg/10^8 cells)		(%)
Membrane protein →	6.3	6.4	100
Carbohydrate	(nmol/mg of protein)		(%)
Sialic acid	5.3	3.8	72
Galactose	15.0	7.7	51
Mannose	19.0	29.0	153
N-Acetylglucosamine	17.0	10.5	62
N-Acetylgalactosamine	2.4	2.7	112

The preparation of the membranes and the methods for carbohydrate analysis are described in *Methods*.

membranes is actually increased by 53% over the parent line. The *N*-acetylgalactosamine content is similar in both cell lines.

DISCUSSION

These data demonstrate that the toxic lectin of *Ricinus communis* can be used to select a clone of cells with altered membrane oligosaccharides. One striking consequence of this membrane alteration is a marked decrease in the number of binding sites for some, but not all, lectins. This pattern of lectin binding to the 15B cells becomes more understandable when we consider the available information concerning the structure of lectin receptors on human erythrocyte membranes (11, 12, 21). In that cell type, mushroom PHA binds to the Type II oligosaccharide chain while *P. vulgaris* E-PHA, lentil PHA, and probably Con-A bind to the Type I oligosaccharide (Fig. 4). The *Ricinus communis* and *Abrus precatorius* lectins bind to a third type of oligosaccharide unit that contains predominately galactose and *N*-acetylglucosamine residues (L. Adair and S. Kornfeld, *J. Biol. Chem.*, in press). The finding that mushroom PHA-binding to the 15B line is essentially normal and that the *N*-acetylgalactosamine content of the membranes of these cells is about the same as the parent line suggests that the Type II oligosaccharide units of the 15B cells are intact. It has also been shown for human erythrocytes that removal of the galactose residues from the erythrocyte Type I chains results in a 90% loss of *P. vulgaris* E-PHA binding while removal of the outer *N*-acetylglucosamine residues leads to a 70% decrease in lentil PHA binding (11, 21). The mannose residues in the core account for the residual lentil PHA binding and probably account for all of the Con-A binding since Con-A is known to bind to α-linked mannose residues (22). Therefore, in 15B cells a block in the synthesis of Type I-like units distal to the mannose residues could account for the observed alterations in the pattern of lectin binding as well as for the finding of a decreased content of sialic acid, galactose, and *N*-acetylglucosamine in the membranes. Since galactose residues also serve as the determinant sugars for the *Ricinus communis* and *Abrus precatorius* lectin receptors (1–3, 6–7), we postulate that the 15B cells may have a defect in membrane oligosaccharide biosynthesis which affects the synthesis of at least two types of galactose- and *N*-acetylglucosamine-containing units. The explanation for the increased mannose content of the 15B cells is not clear. One possibility is that additional mannose residues are added to the Type I-like oligosaccharide units as a consequence of the

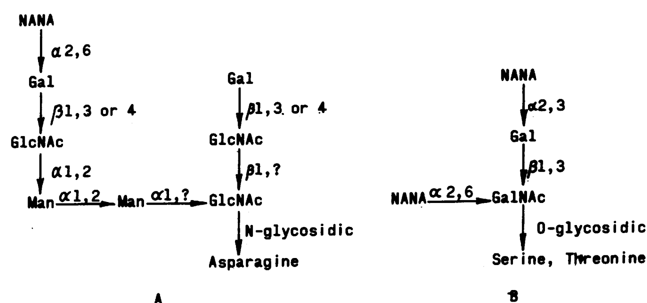

FIG. 4. Structures of the oligosaccharide units of human erythrocyte trypsin-released glycopeptides. (*A*) Type I glycopeptide (21); (*B*) Type II glycopeptide (12).

failure to add the outer *N*-acetylglucosamine residues. Another possibility is that the 15B cells contain either a new or increased amount of a normally occurring oligosaccharide that is rich in mannose.

The 15B clone represents just one mechanism for escaping the toxic effects of ricin. In addition to the loss of lectin receptors, resistant cells could lack the ability to transport the lectin into the cells or contain an altered protein-synthesizing system that is resistant to ricin. Wright has isolated clones of CHO cells that are resistant to the toxic effects caused by very high concentrations of Con-A and *P. vulgaris* PHA (23). These cell lines bind the lectins in a normal fashion, indicating that the resistance to lectin toxicity must result from some other change in the cell membrane or another organelle. Hyman *et al.* have recently used immunoselection with lectin–anti-lectin and complement to isolated murine lymphoma-cell variants that are 10- to 100-fold less sensitive to the direct cytotoxic action of ricin and WGA and (24). These variant cell lines differ from the 15B clone in that they possess 40–70% the lectin binding capacity of the parental cell line. The mechanism of lectin resistance in these cell lines is not clear.

The question of whether the 15B clone represents a true genetic mutant or a serially propagated epigenetic change cannot be resolved at this time (25, 26). The fact that the 15B clone retains its altered lectin-binding properties when grown for many generations in the absence of ricin demonstrates that the membrane alteration is stable, but does not distinguish between a genetic or an epigenetic phenomenon.

The availability of cell lines with altered surface oligosaccharides should provide an opportunity to perform a variety of studies on the structure, biosynthesis, and function of cell-membrane glycoproteins and their oligosaccharide units. Just as bacterial cell-wall mutants have proved to be extraordinarily useful in the elucidation of the structure and biosynthesis of the bacterial cell wall, it seems reasonable to suggest that mammalian cell lines with altered membrane oligosaccharides may play a major role in future investigations in this area.

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