Brief Definitive Report

INCREASED CYTOTOXICITY OF NORMAL RABBIT SERUM FOR LECTIN-RESISTANT MUTANTS OF ANIMAL CELLS

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Many purified plant lectins specifically interact with the carbohydrate moieties of glycoproteins and glycolipids at the cell surface and are cytotoxic (1, 2). Lectin-resistant mutants of animal cells have been isolated which have alterations in cell membrane molecules (3–6). It was found that a series of lectin-resistant mutants we isolated were much more sensitive to the cytotoxic activity of normal rabbit serum than was the parental Chinese hamster ovary (CHO) cell line. The hypothesis that naturally occurring antibodies in rabbit serum are reacting with incomplete carbohydrate chains on the lectin-resistant mutants to cause this increased sensitivity is explored.

Materials and Methods

The procedures for culturing Chinese hamster ovary (CHO-K1) cell mutants and hybrids were the same as previously described (7).

The cells used in this study were J1, a human/Chinese hamster hybrid that contains human chromosome 11 as its only human chromosome, expresses human cell surface antigens, and requires glycine for growth (8), and two CHO-K1 auxotrophic mutants that show requirements for asparagine and uridine, respectively (9, 10). Mutagenesis experiments were carried out using standard procedures and lectin-resistant clones were obtained by isolating those mutagenized cells that survived exposure to the specific lectin that was added to the cell culture medium. Lectins used in these experiments included phytohemagglutinin (PHA) (Difco Laboratory, Detroit, MI), concanavalin A (Con A), ricin (RIC), and wheat germ agglutinin (WGA) (Sigma Chemical Co., St. Louis, MO), and lentil (LCA) (Miles Laboratories, Elkhart, IN).

Normal rabbit serum (NRS) was obtained from The Jackson Laboratory, Bar Harbor, ME; Charles River Breeding Laboratories, Wilmington, MA; Colorado Serum Co., Denver, CO; Cedarlane Laboratories (Low-Tox Rabbit Complement), Hornby, Ontario, Canada; and Dutchland Laboratories Inc., Denver, PA. Cells were assayed for their lectin sensitivity by single-cell plating a constant number of cells in increasing concentrations of lectin and scoring the number of colonies formed after 6–7 d. The concentration needed to give 10% survival was determined from the survival curve. Complementation analysis was carried out as previously described (11). Hybrids were selected using the nutritional markers available on these cells. The hybrids were isolated and tested for lectin resistance by standard methods.

Results and Discussion

Table I summarizes properties of the eight clones with altered lectin sensitivity

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TABLE I

Lectin Resistance of Each of the Three Phenotype Classes

Cell	Lectin used for selection	Mutant class	Lectin phenotype				
			PHA	D_{10} (μg/ml) Con A	WGA	RIC	LCA
CHO-K1	—	(Parental	WT	WT	WT	WT	WT
J1	—	cells)	(75–100)	(25)	(5)	(0.01)	(10)
313-16A	PHA						
313-17B	PHA	A	R	S	R	R	R
313-22B	WGA		(250)	(2)	(40)	(0.3)	(100)
413-D	RIC						
313-19A	WGA	B	R	S	R	S	S
453-9A	WGA		(200)	(10)	(30)	(0.0003)	(2)
333-1C3	PHA						
313-21B	WGA	C	WT	WT	R	S	WT
			(100)	(25)	(10)	(0.0001)	(10)

The dose of each lectin required to reduce the survival of the cells to 10% was determined using lectin stock purchased from the suppliers listed in the Materials and Methods section. Lectins purchased from other suppliers gave the same relative pattern of resistance and sensitivity but the absolute D_{10} values were altered in some cases. Different lots from the same supplier gave some differences in absolute killing values for certain lectins.

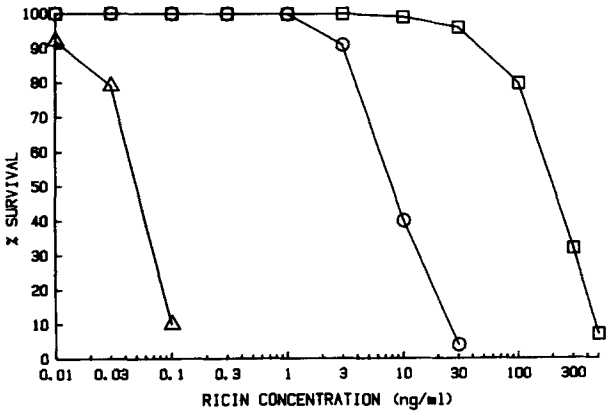

FIGURE 1. Comparison of the survival of the parental J1 cell (○) and two Lectin mutants, 313-21B (△) and 313-17B (□) with increasing concentrations of ricin.

isolated and characterized in the course of these experiments. All of the 313 clones were isolated from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-treated J1 cells. The other mutants were isolated from CHO auxotrophs after ethylmethanesulfonate (EMS) treatment. By an examination of the data in Table I, it appears that at least three phenotype classes exist: class A: 313-16A, 313-17B, 313-22B, 413-D, which are resistant to PHA, WGA, RIC, and LCA but sensitive to Con A; class B: 313-19A, 333-1C3, and 453-9a, which are resistant to PHA and WGA but sensitive to Con A, RIC and LCA; class C: 313-21B, which has wild type behavior to PHA, Con A and LCA and is resistant to WGA and sensitive to RIC.

Fig. 1 compares the survival of 313-17, one of the class A clones, and 313-21B, a class C clone, when plated in increasing amounts of ricin. The J1 parent is included as a control. This illustrates the large differences that exist among these clones in their sensitivity to the toxic action of ricin.

TABLE II

Toxicity of Different Samples of Normal Rabbit Serum

NRS Source	Clone	Relative plating efficiency in the presence of designated concentrations of NRS (%)						
		0.2	0.5	0.75	1.0	2.0	3.0	5.0
Charles River	J1	124	113	108	88	114	130	97
	313-17B	132	87	93	29	0	0	0
	313-19A	86	0	0	0	0	0	0
	313-21B	113	128	109	57	0	0	0
Jackson Laboratory	J1	105	101	91	103	117	94	91
	313-17B	115	0	0	0	0	0	0
	313-19A	14	0	0	0	0	0	0
	313-21B	125	97	98	102	10	0	0
Cedarlane Laboratories	J1	106	106	92	95	98	96	82
	313-17B	115	0	0	0	0	0	0
	313-19A	89	0	0	0	0	0	0
	313-21B	92	105	95	105	95	19	0

Complementation experiments showed that hybrids formed by fusing 313-17B and 413-D, both mutants of class A, had the same lectin sensitivities as the parental mutants, demonstrating that complementation did not occur and that they have the same genotype. However, when 413-D, a class A mutant, was fused with 313-19A, a class B mutant, the resulting hybrids had wild type sensitivity to all five of the test lectins used. Therefore, they have different genotypes.

It was found that amounts of normal rabbit serum (NRS) that we routinely used as a complement source for antibody-mediated cytotoxicity tests and which by themselves showed no cell killing for the J1 hybrid or CHO, completely killed the lectin mutants. Table II illustrates this increased toxicity with three different sources of NRS for a cell from each mutant class. These NRS samples were from rabbits that were free of common pathogens or from samples prepared to have low cytotoxicity. Other samples of NRS at high concentrations did exhibit toxicity for the parental cells, but in these cases the lectin clones were killed at correspondingly lower concentrations. This sensitivity to NRS was not unique to the lectin clones of J1, but was also true of the lectin clones isolated from CHO cells. In addition, it was found that members of each mutant class had similar behaviors when their survival to increasing concentrations of NRS was compared. In general, mutants of class B were the most sensitive and the mutant of class C the least sensitive.

It has been suggested that natural antibodies directed against carbohydrate determinants are the sources of some of the cytotoxicity of NRS (12). Other workers have shown the involvement of antibody-independent killing by activation of the alternate pathway of complement (13, 14). If complement is involved, heating the NRS should remove the toxicity. When the NRS was heated at 56°C for 10 min, its toxic reaction for the lectin mutants was removed, as is illustrated for one of the mutants in Table III.

If antibodies are involved, it should be possible to remove them by adsorption with the cells, and this was shown to occur as illustrated for two of the mutants in Table IV. Adsorption of NRS by a class B mutant (313-19A) removed killing activity for that mutant, but did not remove the toxicity for a class A mutant (313-16A).

TABLE III

Relative Plating Efficiency of Lectin-resistant Mutant 313-16A in Normal Rabbit Serum

	% NRS					
	0	0.2	0.5	1.0	2.5	5.0
Unheated	100	97	1	0	0	0
Heated	100	112	126	126	108	116

TABLE IV

Relative Plating Efficiency in NRS after Adsorption

Test cell	Cells used to adsorb NRS	Relative plating efficiency in the presence of designated concentrations of NRS (%)					
		0.1	0.25	0.5	1	1.5	2.0
313-19A	None	95	53	0	0	0	0
	313-19A	93	82	72	—	82	53
313-16A	None	94	70	0	0	0	0
	313-19A	113	80	0	0	0	0

A 10% solution of NRS was adsorbed with 1.3×10^7 cells/ml for 1 h at 4°C. The NRS used for the control in this experiment was also kept at 4°C for 1 h before use.

The alternate pathway of complement activation has been shown to be dependent on Mg^{++} but not Ca^{++}, and 0.008 M EGTA with 0.002 M Mg^{++} added has sufficient Mg^{++} to allow alternate pathway activity while still blocking the antibody-dependent classical pathway (14). Killing of the lectin mutants was compared with and without EGTA-Mg^{++}. These experiments were done with preplated cells and results were scored after 2 and 5 h. The lectin cells were clearly lysed by 3% NRS in the control wells, but no lysis was observed in wells where EGTA and Mg^{++} were present even at 8% NRS, suggesting that the alternate pathway of complement activation is not primarily responsible for the increased cytotoxicity of NRS for the lectin mutants.

It is known that lectin-binding sites can be competed for by certain monosaccharides (1, 2). We tested the ability of mannose and galactose to inhibit the killing action of NRS on our lectin mutants. Fig. 2 demonstrates that these sugars were successful in reversing the killing of a class A mutant, 313-17B, by NRS. Mannose and galactose also reversed NRS killing for a class B mutant, 313-19A, and the class C mutant, 313-21B. Further experiments are needed to establish possible sugar specificities involved in these reactions. A preparation of mannan, a mannose-rich oligosaccharide from yeast, was also effective in inhibiting the toxicity of NRS for a class A and a class B mutant, as shown in Table V. The class C mutant has not been tested.

An additional experiment was done to provide further evidence that the different sensitivity to lectins is responsible for the increased sensitivity to the toxic action of NRS. One of the mutants, 313-19A, was treated with 300 μg/ml EMS for 20 h. These mutagenized cells were plated in 5% NRS, and one surviving colony was isolated from 10^6 cells. This colony was compared with its lectin parent and the J1 wild type cell for killing by lectins and NRS and was shown to have the same behavior as J1. By selecting a cell that is no longer sensitive to NRS, we have been able to also select a revertant with wild type lectin sensitivity.

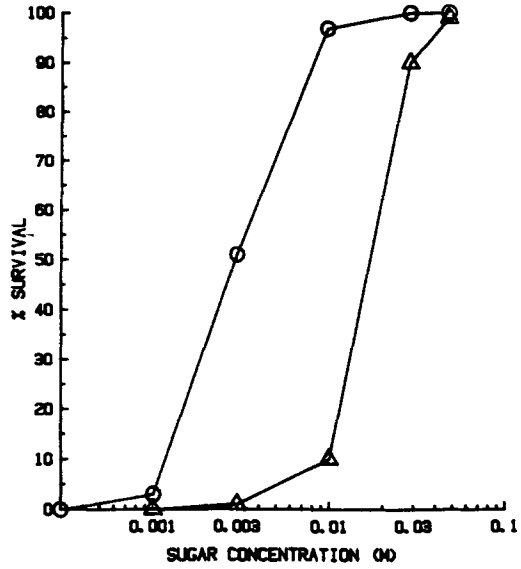

FIGURE 2. Demonstration of the inhibition of NRS toxicity for lectin mutant 313-17B by increasing concentration of mannose (○) and galactose (△).

TABLE V

Relative Plating Efficiency of Lectin Mutants in NRS and Increasing Concentrations of Mannan (mg/ml)

	0	0.001	0.003	0.01	0.03	0.1
313-17B + 1.5% NRS	1	88	95	116	103	104
313-19A + 0.3% NRS	5	80	111	103	93	118

Lectin mutants with phenotypes similar to those isolated here have been isolated by other workers. Mutants with the phenotype similar to our class A mutants are frequently isolated from CHO cells and are classified as Lec 1 mutants by Stanley (15), whose experiments have confirmed that our class A mutants belong to the Lec 1 complementation group. In addition, Dr. Stanley's analyses showed that class C mutants belong to Lec 3 complementation group.

While the mechanism(s) of cell lysis of the lectin mutants by NRS remains to be clarified, our evidence is consistent with the involvement of natural antibodies that may react with the incomplete carbohydrate chains on the cell surfaces of these mutants.

Summary

Plant lectins are cytotoxic and can be used to select for mutants of animal cells that exhibit structural changes in cell surface carbohydrates reflecting glycosylation defects. We isolated eight lectin mutants of Chinese hamster ovary (CHO) cells that appear to represent three different phenotype classes. These lectin mutants were much more sensitive to the cytotoxic action of normal rabbit serum (NRS) than were the parental cells. This increased cytotoxicity was heat sensitive, specifically absorbed, and inhibited by simple and complex carbohydrates. No

killing was observed under conditions in which only the alternate complement pathway was active. An NRS-resistant subclone that was isolated from one lectin mutant was shown to have also regained wild type behavior when tested with the lectins. The possibility that naturally occurring antibodies in rabbit serum are reacting with incomplete carbohydrate chains on the surface of the lectin mutants is discussed.

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