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Altered Glycosylation in Tumor Cells 177–186

Changes in Glycoprotein Fucosylation in a Concanavalin A-Resistant Variant of a Human Leukemia Cell Line (K562)

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Previous studies from this and other laboratories have shown that variants of tumor cell lines can be selected for resistance to the lytic action of natural killer (NK) cells. One of these (K562-Clone I), when made resistant to the toxic effects of Concanavalin A (Con A-R1), regained its sensitivity to NK. Comparing the plasma membranes of Clone I and Con A-R1, we observed 1) a very similar electrophoretic pattern of membrane glycoproteins identified by binding to the lectins Con A, WGA, PNA, and SBA; 2) an increase in binding of *Ulex europaeus* lectin to a group of glycoproteins from Con A-R1 that were sensitive to treatment with fucosidase and N-glycanase and that had a diffuse mobility ranging in apparent molecular weight from 30 to 200 kDa; and 3) a marked decrease in binding of an antibody reactive with the lactoneofucopentaose III antigen (Lewis x). This constellation of changes is an unusual pattern to follow Con A resistance and may point to a pathway of glycosylation that a leukemic cell might use to modify its recognition by the NK mechanism.

Key words: plasma membranes, glycoproteins, leukemia, lectin resistance, natural killer (NK) cells

Natural killer cells (NK), a population of large granular lymphocytes, are able to recognize and lyse in vitro certain normal and tumor targets [1,2]. Although the molecular components of the reaction have not been identified in detail, reports showing that the NK lytic reaction can be inhibited by monosaccharides [3–5], certain N-linked oligosaccharides [6,7], and some antibodies directed to the Lewis x determinant [8] all indicate that oligosaccharides may be involved at one or more steps.

Another approach has been to derive and analyse cultured cell lines selected for relative resistance to NK-mediated lysis. Young et al. [9] and Yogeeswaran et al. [10] have shown that murine cells made resistant to both unstimulated and activated NK lack asialo GM_2, but they concluded that these glycolipids were not the targets themselves. We have derived a variant (K562-Clone I) of the human leukemic line K562 that is partially resistant to killing by fresh peripheral blood lymphoid cells but is equally sensitive to complement-mediated cytotoxicity, antibody-dependent cellular cytotoxicity,

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and interferon-activated effectors [11]. Because this subline was selected primarily for decreased binding and because it competed less well with the parent line in the cold target assay, we proposed that it might bear fewer or deficient target structures. The plasma membrane proteins of the two lines were almost identical, but K562-Clone I showed increased incorporation of fucose into a minor population of glycolipids. From Clone I we derived another variant subclone, selected for resistance to toxicity by Concanavalin A (K562-Clone I-ConA-R1), which concurrently regained sensitivity to NK lysis and showed a parental (K562) pattern of fucose incorporation into glycolipid [12]. The work reported here describes our initial studies on the characteristics of the plasma membrane glycoproteins of these cells.

MATERIALS AND METHODS

Chemicals and Enzymes

Biotinylated lectins (soybean, peanut, and wheat germ agglutinins: SBA, PNA, WGA) and the Vectastain ABC kit were obtained from Vector Laboratories (Burlingame, CA), unconjugated Concanavalin A (Con A) from P.L. Biochemicals (Milwaukee, WI), and peroxidase-conjugated *Ulex europaeus* agglutinin (UEA-1) from E.Y. Labs (San Mateo, CA). The enzyme N-glycanase (peptide: N-glycosidase F) was obtained from Genzyme (Boston, MA), fucosidase (bovine kidney) [EC 3.2.1.51], from Sigma Chemicals (St Louis, MO), and neuraminidase (*Vibrio cholerae*) [EC 3.2.1.18], from Calbiochem (San Diego, CA). All unconjugated lectins were obtained from E.Y. Labs, except for Con A, which was obtained from P.L. Biochemicals. *Ricinus communis* lectin (RCA-1) was obtained as a 1 mg/ml solution in galactose and required dialysis against PBS before use. The monosaccharides α-methyl mannoside, N-acetyl-D-galactosamine, and L-fucose were obtained from Aldrich Chemicals (Milwaukee, WI), N-acetyl-D-glucosamine and β-lactose, from Sigma Chemicals.

Manipulation of Cells and Derivation of Cell Lines

Cell-culture methods, techniques for flow cytometry, and characteristics of the NK-resistant (Clone I), and Con A-resistant (Con A-R1) cell lines all have been described in detail elsewhere [11,12].

Radiolabeling and Cell Fractionation

Radioiodination of cell-surface proteins was done according to Hubbard and Cohn [13], as modified by Peyman et al. [14]. For isolation of the plasma membrane-enriched fraction, approximately 10^8 cells were harvested and washed three times in PBS. The pellet was suspended in 8 ml of disruption buffer (10 mM HEPES, 5 mM $MgCl_2$, 150 mM NaCl, pH 7.4). The cells were then disrupted by nitrogen cavitation [15] at 4°C for 20 min at 400 psi. The resulting suspension was centrifuged for 10 min at 1,500 g; its supernatant centrifuged for another 15 min at 4,500 g, and that supernatant centrifuged for 1 hr at 100,000 g at 4°C. The membrane-enriched pellet was frozen at −20°C for later analysis.

Analysis of Membrane Glycoproteins

The membrane pellets were dissolved in "sample buffer" (2% [w/v] sodium dodecyl sulfate, 10% [v/v] glycerol, 0.1 M dithiothreitol in 0.01 M Tris-HCl, pH 6.8) and heated in a boiling water bath for 1 min. An aliquot was taken for measurement of

protein content by the method of Lowry [16], and 10 μg were electrophoresed in polyacrylamide gels (PAGE) [17]. The proteins were then transferred from the gels to 0.1-μm-pore nitrocellulose sheets (Schleicher and Schuell, Keene, NH) in a BIO-RAD transblot apparatus at 60 V overnight. With this small pore size, an extended period of electrophoresis allowed maximum transfer of high molecular weight proteins, and minimized leakage of the smaller species. Strips were cut from the membranes, preincubated for 30 min at room temperature in PBS/0.2% (v/v) Tween 20 to block nonspecific adherence of lectin, and incubated in solutions of the different biotinylated lectins (5 μg/mL inPBS/0.2% Tween) for 60 min at room temperature. After being washed, the strips were incubated with the avidin-biotin complex (1/1,000 in PBS, prepared at least 30 min previously to allow formation of the complex), washed another three times with PBS, and developed with the DAB solution (0.3 mg/ml 3,3′ diaminobenzidine tetra-HCl in 0.05 M Tris-Cl, 0.03% H_2O_2, pH 7.4). For visualization of UEA-1, the preblocking agent was PBS/0.05% (w/v) casein; incubation with the ABC complex was omitted.

Incubation of Glycoproteins With Enzymes

Neuraminidase. Membrane vesicles were suspended in 400 μl of 0.05 M sodium acetate buffer at pH 5.5 with a syringe and a 27-ga needle. After addition of 100 μl of enzyme (100 mU), the mixture was incubated at 37°C for 30 min and the reaction stopped by cooling on ice and centrifuging at 100,000 g for 1 hr at 4°C. The membrane pellet was dissolved in sample buffer and analysed by gel electrophoresis.

Fucosidase. Membrane vesicles were suspended in 253 μl of sodium acetate (0.2 M, pH 6.0) as previously described. After addition of the enzyme (147 μl, 250 mU/mL), the mixture was incubated at 37°C for 18 hr, and the reaction stopped by cooling on ice and centrifuging at 100,000 g for 1 hr at 4°C. The membrane pellet was dissolved and electrophoresed.

N-glycanase. Membrane pellets were dissolved in 0.5% SDS/0.1 M DTT and an aliquot was taken for protein measurement. Fifty μg of protein in 10 μl were added to 88 μl of Tris-HCl (125 mM in 0.6% [v/v] NP-40, pH 8.6). To this mixture, 2 μl of enzyme were added (0.25 U/μl, final concentration 5.0 U/ml) prior to incubation at 37°C for 18 hr. At this time another 2 μl of enzyme were added, and the mixture was incubated for another 18 hr. The reaction was then stopped by adding 100 μl of sample buffer and boiling. Forty μl (10 μg of protein) were loaded onto a gel and electrophoresed as previously described.

Analysis of Lectin Resistance

The pattern of lectin resistance was determined as described by Stanley [18]. In brief, 4×10^3 cells in 100 μl of media were added to each well of a 96-well culture dish (Nunc, Roskilde, Denmark) containing 20 μl of a sixfold concentrated solution of lectin in RPMI 1640. The dishes were incubated at 37°C in a 5% CO_2 humidified atmosphere for 72 hr prior to addition of tritiated thymidine (1 μCi, Amersham, 5 Ci/mM, Oakville, Ontario) and further overnight incubation. The cells were then disrupted by freeze/thaw, and the lysates were recovered on filters with a cell harvester.

RESULTS

Major Surface Peptides

Because peripheral surface structures are those most likely to effect initial lectin binding and to mediate intercellular events, we compared the patterns resulting from Coomassie blue staining of gels from PAGE of plasma membranes from the Con A-sensitive and resistant lines. The electrophoretic profile in Figure 1A shows that neither qualitative nor reproducible quantitative differences were evident among the major bands of the three variants. Radioiodination by the lactoperoxidase technique did not reveal any other differences (not shown). This indicates that the phenotypic changes resulting in return to NK sensitivity did not lie in a major alteration of the plasma membrane protein composition.

Membrane Glycoproteins

To analyse the major groups of membrane glycoproteins, the polypeptides transferred to nitrocellulose strips were visualized with enzyme-conjugated lectins selected for binding to different sugars. The patterns shown in Figure 1B–D, stained with Con A (mannose), WGA (N-acetylglucosamine/sialic acid), and SBA (N-acetylgalactosamine/galactose), did not reveal any major reproducible differences between parent K562, Clone I, and its Con A-resistant subclone; PNA (galactose) gave a pattern similar to that of SBA (not shown). Treatment of the membranes with neuraminidase prior to

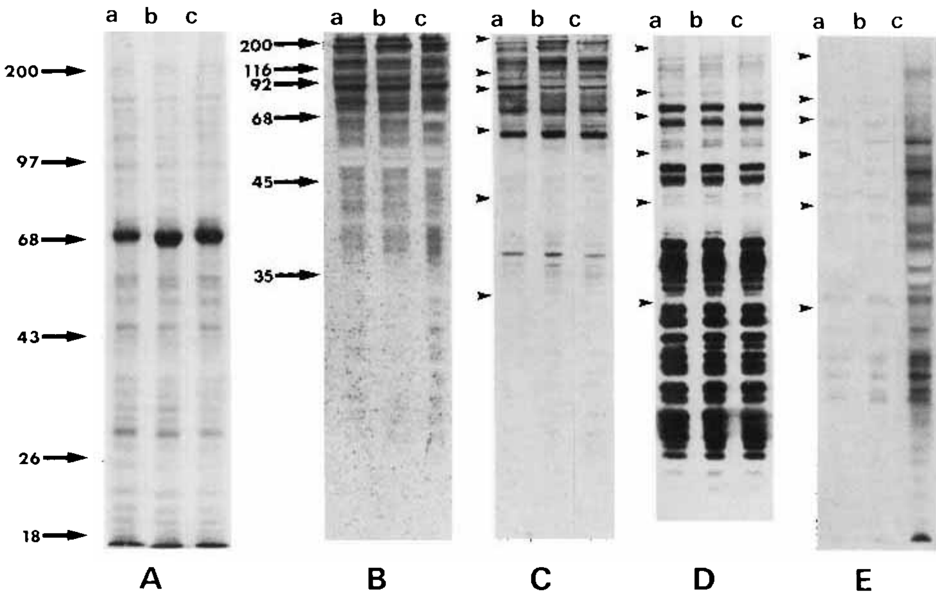

Fig. 1. **Panel A**. Coomassie blue-stained gel (7.5% acrylamide) of a SDS-PAGE electrophoresis of the plasma membrane fraction of the different cell lines. Lane a, K562; b, Clone I; c, Con A-R1. Equal quantities of protein (10 μg) were loaded onto each lane. **Panels B–E**. Lectin-stained nitrocellulose blots from electrophoretic gels (10% acrylamide) of plasma membranes incubated with unbiotinylated Con A (5 μg/ml) (B) (unbiotinylated Con A was used because this lectin has a binding site for peroxidase), biotinylated WGA (C), biotinylated SBA (D), and peroxidase UEA-1 (E). All were developed with DAB. Equal quantities of protein (10 μg) were loaded onto each lane. Lane designation is the same as in panel A.

electrophoresis did not unmask any further differences. Binding of these lectins could be inhibited completely by their respective free monosaccharide at 0.2 M concentration. In contrast, UEA-1 bound to a population of proteins from Con A-R1, ranging in apparent molecular weight from 30 to 200 kDa, which were not found in extracts of the other lines (Fig. 1E). Treatment of membranes of these cells with neuramidase did not result in increased *Ulex* binding.

Specificity of the *Ulex*-Binding Polypeptides

To assess the specificity of UEA binding, the lectin solution was preincubated with 0.2 M L-fucose (in PBS/0.2% Tween 20) prior to application to the nitrocellulose. Although some fainter bands remained even after 1 M fucose, this manipulation prevented binding to the broad bands (Fig. 2A), indicating that probably there was a specific sugar interaction. In support of this idea, it can be seen that treatment with fucosidase eliminated UEA binding (Fig. 2B) but did not alter the staining pattern of Con A or Coomassie blue (not shown), minimizing the possibility that this was the result of contaminating proteases.

To determine which form of linkage might attach the UEA-binding oligosaccharides to their peptides, the membranes were incubated with N-glycanase prior to electropho-

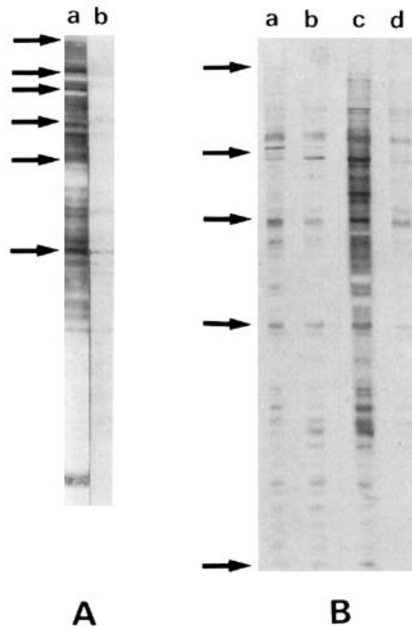

Fig. 2. **Panel A**: Lectin-stained blots of electrophoresed (10% acrylamide) plasma membrane proteins of Con A-R1 cells. Lane a: nitrocellulose incubated with UEA-1 peroxidase. Lane b: incubated with UEA-1 + L-fucose 0.2M. Equal quantities of protein (10 μg) were loaded onto each lane. Molecular weight standards and 200, 116, 92, 68, 45, and 35 kDa. **Panel B**: UEA-stained blot from 7.5% acrylamide gel showing effect of preincubation of the membranes with fucosidase. Lanes a/b: Clone I. Lanes c/d: Con A-R1. Lanes a/c: membranes not exposed to enzyme. Lanes b/d: membranes incubated with fucosidase (250 mU/ml). Equal quantities of protein (10 μg) were loaded onto each lane. Molecular weight standards are 200, 97, 68, 43, and 18 kDa.

resis. As shown in Figure 3A, this treatment eliminated most of the binding of Con A and UEA (Fig. 3B) but did not alter that of SBA (Fig. 3C), suggesting that the major fucose-bearing oligosaccharides are N-linked. However, a minor population of UEA-binding proteins of apparent molecular weight 30 kDa remained even after 36 hr of incubation with the enzyme. Possibly, these lower-molecular-weight proteins could bear O-linked, or N-linked structures not accessible to N-glycanase.

Immunological Characterization of the Cell Lines

Many human hemopoietic-derived cells, including both K562 and Clone I, bear the Lewis x (SSEA-1) antigen [12,19]. Surprisingly, the Con A-R1 line bound significantly less of the H36/71 antibody than did the K562 parents, as assessed by flow cytometry (Fig. 4), and showed only 5% visible staining by fluorescence microscopy. Treatment with neuraminidase prior to antibody incubation did not alter this pattern, which suggests that blocking by sialylation was not the reason for the change in antibody binding [20].

Lectin Cross-Resistance Analysis

Another parameter of alteration in the glycoprotein composition can be assessed by measuring the pattern of cross-resistance to other lectins [18]. When the survival of the Con A-R1 line in toxic concentrations of lectins was assessed by thymidine incor-

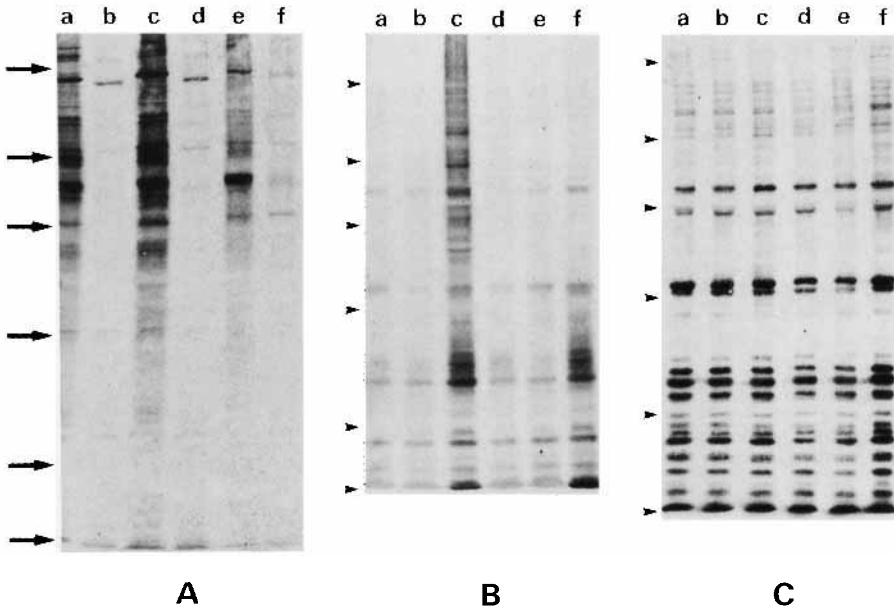

Fig. 3. Effect of N-glycanase treatment of plasma membranes on subsequent lectin binding to blotted proteins. **Panel A**: Stained with Con A. **Panel B**: Stained with UEA. **Panel C**: Stained with SBA. Panels A and C, lanes a/b: K562. Lanes c/d: Clone I. Lanes e/f: Con A-R1. Lanes a/c/e: not treated with enzyme. Lanes b/d/f: treated with N-glycanase. Panel B, lanes a/d: K562. Lanes b/e: Clone I. Lanes c/f: Con A R-1. Lanes a/b/c: not treated with enzyme. Lanes d/e/f: treated with N-glycanase. Equal quantities of protein (10 μg) were loaded onto each track of the running gel (7.5% acrylamide). Molecular weight standards are 200, 97, 68, 43, 26, and 18 kDa.

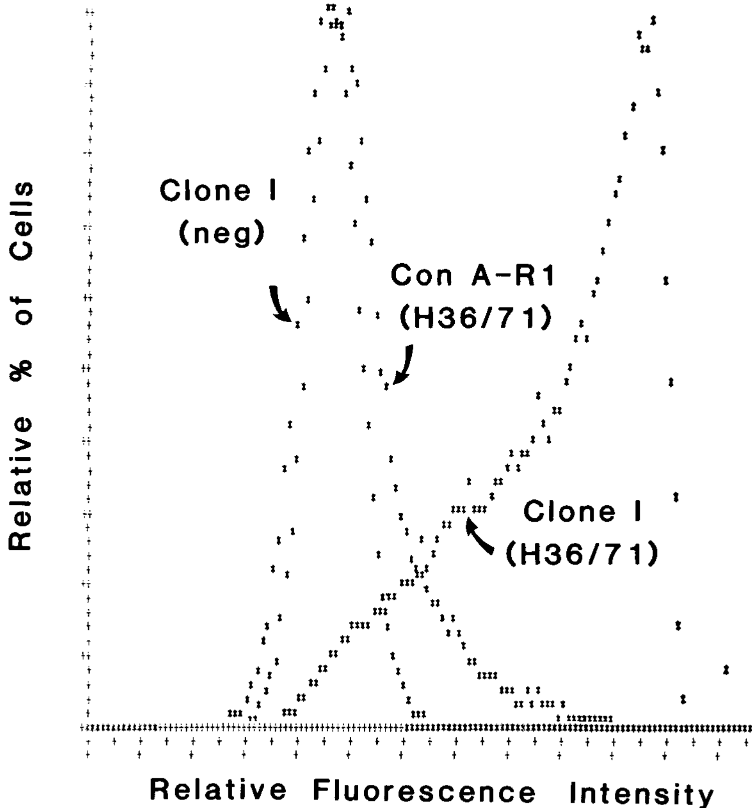

Fig. 4. Analysis by flow cytometry of immunofluorescent staining of Clone I and Con A-R1 cells by antibody H36/71 (IgM) reactive with the lactoneofucopentaose III structure. Horizontal axis is the log fluorescence intensity expressed so that ten of the smallest divisions represent a doubling of the intensity. The vertical axis is the relative cell number, normalized to percentage of the total. The negative control was a nonreactive IgM; channel of peak intensity was for K562: no. 45; Clone I: no. 47 (shown in graph above); and Con A-R1: no. 47. Channel of peak intensity for the H36/71 antibody was for K562: no. 91; Clone I: no. 89; and Con A-R1 no. 53 (nos. 89, 53 shown in graph above).

poration, it was found to be greater than two times that of the parent, even after more than 1 year in continuous culture in the absence of Con A (Table I). Results obtained from measuring the plating efficiency in agar confirmed this pattern. On the other hand, this cell line was more sensitive to UEA-1 but was less sensitive than its Clone I parent to RCA.

DISCUSSION

These experiments show that the Con A-R1 cell line, selected for resistance to toxicity by Concanavalin A, expressed a new population of fucose-bearing proteins and failed to react with an antibody to a fucosylated lactosamine structure (Lewis x). Concurrently, it regained sensitivity to NK-mediated lysis over that of its resistant parent (K562-Clone I) [12], selected from the human erythroleukemia line K562 on the basis of decreased binding to lymphoid cells. The changes composing this constellation may

TABLE I. Lectin Resistance Properties of K562 Variants

	Lectin resistance phenotype (D50, μg/mL)[a]			
Cell line	Con A	WGA	RCA	UEA
K562	100	6	<0.075	400
Clone I	100	4	<0.075	300
ConA R-1	250	5	3	90

[a]D50 is the concentration of lectin in μg/mL required to reduce the thymidine incorporation of each cell line to 50%.

or may not be interrelated. However, the fact that differences in the binding of lectins with affinity for mannose (Con A), N-acetylgalactosamine (SBA and PNA), and N-acetylglucosamine/sialic acid (WGA) were not detected suggests that the initiating event may be a very specific metabolic lesion.

Although the fucose-directed specificity of UEA was confirmed by monosaccharide inhibition and fucosidase digestion, we cannot be certain which types of linkage might form the attachment of this sugar to the proximal residues. The human blood group H substance, with fucose in the α(1→2)-linkage, is a potent inhibitor, 900-fold greater than free fucose [21]. However, in some complex oligosaccharides, where repeating units can increase avidity, the α(1→3)-form found in X-hapten can be a ligand [22]. These considerations also might explain why we could not completely inhibit UEA binding to all of the bands of the blots with free fucose. Likewise, the specificity of bovine kidney fucosidase has not been sufficiently characterized to give clear information on linkage. Because the pathways for biosynthesis of N- and O-linked glycoproteins are different, it is of interest that the new broad bands of UEA staining were sensitive to digestion by N-glycanase, indicating that most of the UEA-binding oligosaccharides are N-linked to polypeptide. A minority of bands appeared to be resistant, and other methods will be required to determine if they are O-linked.

A similar pattern of staining by UEA-1 has been described by Matsushita et al. [23] for glycoproteins obtained from human colon carcinoma tissue. They described the appearance of a "smear" of glycoproteins of molecular weight ranging from 30 to 200 kDa, which could be visualized also by antibodies to carcinoembryonic antigen; they suggested that it might be a single protein with very heterogenous glycosylation. Experiments are in progress to determine whether the structures identified here in K562 cells correspond to one or to several proteins of diffuse mobility in polyacrylamide gels.

Although we did not detect a decrease in binding of Con A to any specific protein ligand, we directly confirmed the resistance of the Con A-R1 line by measuring both inhibition of cell division and colony formation in the presence of Con A. Failure to detect decreased binding of a lectin to resistant cells has been noted also by Stanley et al. in some WGA-resistant lines [24]. The concurrent increase in UEA sensitivity is consistent with the new pattern of fucose binding. The observation of increased resistance to RCA, directed to galactose, might be explained by a general decrease in the number of galactose-containing oligosaccharides. Alternatively, because this increased resistance was not reflected in decreased PNA binding, it might be the result of decreased accessibility of galactose resulting from further conjugation to fucose. Since UEA recognizes the H blood group determinant [fucose(α1→2)Gal] [21], it is possible that the additional fucose found in Con A-R1 could be bound to galactose in this manner.

Several lectin-resistant lines have been described for which a specific lesion has

been found, including enzymatic defects in the conversion of mannose to fucose [25] and the inability to translocate nucleotide sugars across the Golgi membrane [26,27]. Of interest to the discussion here are the LEC 11 and LEC 12 WGA-resistant mutants reported by Campbell and Stanley [28], in which the SSEA-1 antigen was induced. This was explained by an up-regulation of a $\alpha(1,3)$ fucosyltransferase resulting in the appearance of the antigen on the membrane. Were the $\alpha(1,2)$ transferase up-regulated in a similar fashion in Con A-R1 so that the galactose of the X-determinant were blocked by another fucose, it could explain both the decreased sensitivity to RCA and the loss of binding to the H36/71 antibody [20].

Other cell lines that show altered interaction with natural killers and characteristics different from the human K562 variants described here have been derived from rodent cells cultured in the presence of lectins. A Con A-resistant CHO line (CR-7) observed also to have sensitivity to NK above that of the parent [29] showed several membrane alterations, including new structures radiolabeled by galactose oxidase/borohydride tritiation and after glucosamine incubation, slower incorporation of GDP-mannose into glycolipid and glycoprotein, and a slight increase in the conversion of mannose to fucose [30,31]. Although it is difficult to compare their measurements of incorporated radioactivity in slices of acrylamide gels with the patterns of lectin-stained bands shown here, it appears from the general similarity of the profiles of Con A-R1 and Clone I that the membrane changes are different from those induced in CHO cells. Ahrens and Ankel, using lectin-resistant CHO mutants with well-characterized lesions, showed that cells with less complex surface oligosaccharides were better targets for human interferon-activated effectors [32]. Comparison of this system with the one described here is difficult because the recognition process for activated effectors may not be identical to that for NK cells not exposed to interferon or other lymphokines [33]. Furthermore, the effectors that kill such anchorage-dependent targets as CHO may be different from those most active against such hemopoietic-derived cells as K562 [34 and references therein]. Dennis and Laferte found that WGA-resistant mutants of MDAY-D2 (metastatic/NK-resistant) murine lymphoma cells became NK-sensitive and less metastatic [6]. This behavioral phenotype correlated with truncation of more complex oligosaccharides into a simpler high mannose form, capable of binding to immobilized Con A. They proposed that structures of this type might be able to function as target molecules for activated NK cells. This idea was supported by the work of Pospisil et al., which showed that defined triantennary oligosaccharides insome, but not all, conformations can block activity of pig NK against K562 cells [7]. Again, the similarities among all three human K562 lines, in all of the lectin-staining patterns other than UEA, suggest that the biochemical basis for their differences in NK-sensitivity probably results from another mechanism. Also, these data do not support the proposal [8] that X-hapten structures are the major NK recognition molecule, because the K562 parent and Clone I, with different NK sensitivities, bear almost identical quantities of immunoreactive antigen, but Con A-R1 bears much less.

All of these changes observed in sequence in the progeny of a single cell line (K562) were the result of selection in the absence of a mutagen. If a tumor cell can respond in vivo to environmental pressures and rearrange its constellation of carbohydrate antigens as readily as it does in culture, then it might be able to escape immune surveillance without necessarily mutating any of its surface peptides. Further study of the regulation of such mechanisms may provide a rationale for pharmacological manipulation designed to render resistant cells sensitive.

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