Isolation of Wheat Germ Agglutinin-resistant Clones of Chinese Hamster Ovary Cells Deficient in Membrane Sialic Acid and Galactose*

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Several clones of Chinese hamster ovary cells have been selected for their resistance to the toxic effects of wheat germ agglutinin. The clones do not bind wheat germ agglutinin as well as parent cells and are 5- to 250-fold more resistant to the toxic effects of the lectin. Of three clones studied in detail, all exhibit a decrease in wheat germ agglutinin binding affinity. Two have normal numbers of wheat germ agglutinin binding sites, while one (Clone 13) has a 65% decrease in binding sites. Crude membrane preparations of the clones have a decrease in sialic acid content relative to parent cells, and Clone 13 membranes are also deficient in galactose, while the mannose and hexosamine contents of all three clones are normal. The membrane sugar deficiencies affect both glycoproteins and glycolipids. Sialyl-lactosylceramide is the major glycolipid in parent cells, while Clones 1 and 1021 have lactosylceramide and Clone 13 has glucosylceramide as the predominant glycolipid.

Labeling experiments with N-(G-3H)acetylmannosamine suggest that Clone 1021 cells have a block in the transfer of sialic acid from CMP-sialic acid to glycoprotein and glycolipid acceptors. Yet CMP-sialic acid:glycoprotein sialyltransferase activity in cell lysates of Clone 1021 cells is 80% of normal. While CMP-sialic acid:glycolipid sialyltransferase activity is only 25% of normal, it can be restored to normal or elevated levels by sodium butyrate induction without an associated increase in cellular sialyl-lactosylceramide content. Similarly, the galactose-deficient Clone 13 can synthesize UDP-galactose and has normal levels of UDP-galactose:glycoprotein galactosyltransferase and UDP-galactose:glycolipid galactosyltransferase when assayed in vitro. The glycosyltransferases of both these clones can utilize their own glycoproteins as sugar acceptors in vitro assays. These data suggest that the variant cells fail to carry out specific glycosyltransferase reactions in vitro despite the fact that they possess the appropriate nucleotide sugars, glycoprotein and glycolipid acceptors, and glycosyltransferases.

It has recently been demonstrated that plant lectins with cytotoxic properties can be used as selective agents for obtaining tissue culture cells with altered membrane oligosaccharide units (2-7). Several of these lines are deficient in specific glycosyltransferases which can account for the observed oligosaccharide alterations (3, 5-7). We now describe an entirely different group of variant cells selected for resistance to wheat germ agglutinin. These variant cells have biosynthetic defects which lead to altered membrane oligosaccharides, but they differ from the previously described variants in that they contain adequate levels of the appropriate glycosyltransferases.

EXPERIMENTAL PROCEDURES

Materials

MEM-alpha, name given to a tissue culture developed by C. C. Stewart (8), was obtained from Flow Laboratories, Rockville, Md. Fetal calf serum, heat inactivated fetal calf serum, trypsin, fetuin (Spiro method), penicillin, and streptomycin were purchased from Grand Island Biological Co. Plastic Petri dishes (tissue culture grade) and T-flasks were from Lux or Falcon. Na125I (reagent grade) was from Mallinckrodt. All other radioactive materials were obtained from New England Nuclear Corp. CMP-N-acetylneuraminic acid was synthesized by the procedure of Kean and Roseman (9). Pronase and Vibrio cholerae neuraminidase were purchased from Calbiochem. Jack bean β-galactosidase and β-N-acetylglucosaminidase were prepared by the method of Li (10). N-Palmitoyl-1-O-a-glucosyl-sphinganine and N-palmitoyl-1-O-a-lactosylphinganine were obtained from Miles-Yeda. Purified brain gangliosides were a gift of Dr. R. Burton, Washington University School of Medicine, St. Louis. Cultures were obtained from Fisher while bovine cardiolipin and ovalbumin were from Sigma. Triton X-100 was purchased from Packard. All other chemicals were of reagent grade and obtained from commercial sources.

Wheat germ agglutinin and the lectins of Triticus communis, ricanus communus agglutinin I, Agaricus bisporus, Lens culinaris, and Phaseolus vulgaris were prepared as previously described (11-13). Soybean agglutinin was prepared by a slight modification of the affinity chromatography method of Lis and Sharon (16). The lectins were labeled with 125I by the chloramine-T method using a 10-s exposure to chloramine-T (17).

Cells

Chinese hamster ovary cells, obtained from Dr. Potter Stewart, Washington University, St. Louis, were grown in MEM-alpha supple-

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mented with 10% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml). The cells were maintained either as suspension cultures in spinner bottles or as monolayers in T-flasks. Cells were harvested from suspension cultures during log phase growth (3 to 6 x 10^6 cells/ml) by centrifugation at 900 x g for 7 min, and washed two to three times with 40 to 100 volumes of either 0.9% NaCl, 0.01 mM NaHCO₃, or phosphate-buffered saline. Monolayer cultures were washed three times with 0.9% NaCl, 0.01 mM NaHCO₃, and the cells harvested routinely by scraping with a rubber policeman. For GM₁ synthesis, see below the monolayers were harvested by trypsinization (0.25% trypsin) at room temperature.

Methods

Determination of Wheat Germ Agglutinin Toxicity

To determine LD₅₀ replicate numbers of cells (usually 200) were seeded onto 60-mm plastic Petri dishes. After a few hours of incubation to allow adherence to the dish, the medium was removed and replaced with complete medium containing from 0.1 to 50 μg/ml of lectin. The dishes were then incubated for several days and the number of colonies per dish counted after staining with 0.5% methyl blue in 10% formaldehyde. The cloning efficiency for the various lectins used in these experiments was 75%. The LD₅₀ is the lectin concentration at which only 50% of the cells (relative to the control) give rise to colonies in complete growth media.

Lectin Binding Assays

The binding reactions were carried out in plastic test tubes that had been presoaked for 1 h with 5 mg/ml of bovine serum albumin. The reaction mixtures contained in 0.25 ml: 125I-labeled lectin (from 0.2 to 25 μg): 1 mg of bovine serum albumin; 0.9% NaCl, 0.01 mM NaHCO₃, and 0.5 to 2 x 10⁶ cells. After 1-h incubation at room temperature, the cells were washed three times with 2.5 ml of 0.9% NaCl, 0.01 mM NaHCO₃, and the amount of 125I-labeled lectin bound to the cells was measured in a scintillation counter. The number of binding sites per cell and the apparent association constant for lectin binding were determined by the method of Scatchard (18). A molecular weight of 23,500 was assumed for wheat germ agglutinin.

Cell Fractionation

All operations were carried out at 4°C. The washed cells were either resuspended in 4 to 5 volumes of distilled water and subjected to six cycles of rapid freezing and thawing or resuspended with 4 to 5 volumes of phosphate-buffered saline and disrupted by sonication with the microprobe of a Biosonik II sonicator (Bronwill Scientific, Inc.) at 20% intensity for 30 s. The cell suspensions were centrifuged at 100,000 x g for 90 min to obtain a crude "membrane" (particulate) and a "soluble" fraction. The particulate material was washed once with 6 ml of water or phosphate-buffered saline and resuspended in the same buffer. The distribution of protein between the particulate and soluble fractions was approximately 1:1.

Membrane Carbohydrate Analysis

Sialic acid, galactose, mannose, glucose, and total hexosamines were released from crude membranes by acid hydrolysis and analyzed as previously described (2), except that free hexosamine was determined by the method of Reissig et al. (19). In experiments with intact cells, sialic acid was released by incubation with V. cholerae neuraminidase (50 units/ml) at 37°C for 48 h, and the amount of released sialic acid was determined by the method of Reissig et al. (19). The amount of released sialic acid was determined by the method of Lowry et al. (20) using bovine serum albumin as the standard.

Extraction and Analysis of Glycolipids

Total cellular lipids were extracted from either crude membranes or washed whole cells with 20 volumes of chloroform:methanol (2:1) at room temperature. Non-lipid contaminants were removed by passage through Sephadex G-25-80 as described by Wells and Dittmer (21). Carbohydrate analysis of total cellular lipids was performed as described below for the analysis of crude membranes. Organic phosphorus was determined by the method of Ames (22). Radioactive glycolipids were extracted from cells grown in the presence of radioactive sugars as described in the legend to Fig. 3 and were separated by thin layer chromatography (Silica Gel G plates, 250-μm thickness, 9 cm; Solvent A, butanol:ammonium hydroxide:water (60:35:1.7); Solvent B, chloroform:methanol:water (65:30:4)). Segments of silica gel, 0.5 cm, were scraped off the plates and counted in a scintillation spectrophotometer in water:3a7o (Research Products, International) (2.5:1). Sugar and glycosyltransferase activity of the labeled glycolipids were analyzed, after acid hydrolysis, by descending paper chromatography with Whatman No. 3MM paper in pyridine:ethyl acetate:water (1:3:6.1:15, upper phase). GM₁ identification was based on the following criteria: (a) the material chromatographed as GM₁ in Solvent Systems A and B; (b) release of sialic acid by either neuraminidase or mild acid hydrolysis yielded a product which co-chromatographed with lacto-N-tetraosylceramide.

Preparation of Glycopeptides

Crude membranes (1 mg/ml) prepared from cells labeled with radioactive sugars as described in the legends to Figs. 4 and 5 were incubated with pronase (1 to 5 mg/ml) at 0.1 M TrisCl, pH 8.1, 0.01 mM CaCl₂ at 37°C under a toluene atmosphere. Additional pronase was added daily for 3 to 5 days.

Intracellular Pools of Nucleotide Sugars

All operations were carried out at 4°C. For CMP-sialic acid analysis, washed cells which had been stored at -20°C were thawed in distilled water, and an equal volume of ethanol was then added. Alternatively, washed monolayer cultures were scraped into 10% ethanol using a rubber policeman. The suspension was sonicated with the microprobe of a Biosonik II sonicator using two 10-s pulses at a probe intensity of 35. For UDP-hexose analysis, the soluble cell fractions (see "Cell Fractionation") were adjusted to 60% ethanol and boiled for 1 min. In each case, a tracer amount of the appropriate radiolabeled nucleotide sugar was added at the first step to correct for subsequent losses. Precipitated material was removed by centrifugation, and the supernatant fluids were concentrated at reduced pressure. The nucleotide sugars were separated from free sugars and sugar monophosphates by descending paper chromatography with Whatman No. 3 proposed paper in 95% ethanol:1 M ammonium acetate (7:3), and were eluted from the paper with distilled water. CMP-sialic acid was assayed directly by the thiobarbituric acid assay method (23). Zero to 10% hydrolysis of the tracer CMP-sialic acid was observed during the isolation procedure. The UDP-hexoses were hydrolyzed (0.1 N HCl, 100°C, 1 min) to release free galactose and glucose, which were then quantitated enzymatically (24).

Glycosyltransferase Assays

For measuring glycosyltransferase activities toward exogenous acceptors, cells were usually disrupted either by freezing and thawing in water or by sonication in phosphate-buffered saline (see "Cell Fractionation"). Assays were conducted at least once for each glycosyltransferase using 0.5 mg/ml of each acceptor by adding a suspension of intact cells to the reaction mixture containing acceptors.

CMP-NeuAc Glycopeptide Synthesis

For measuring glycosyltransferase activities toward exogenous acceptors, cells were usually disrupted either by freezing and thawing in water or by sonication in phosphate-buffered saline (see "Cell Fractionation"). Assays were conducted at least once for each glycosyltransferase using 0.5 mg/ml of each acceptor by adding a suspension of intact cells to the reaction mixture containing acceptors.

UDP-Galactose:Glycoprotein Galactosyltransferase

The reaction mixture contained the following in 0.1 ml of phosphate-buffered saline, pH 7.4: 0.05% Triton X-100, 0.5 mM CMP-N-[G-3H]galactosylamine (20 μCi/μmol), 0.65 mg asialofetuin (3), and 0.5 to 1.5 mg cell protein. After a 30-min incubation at 37°C, the reactions were terminated by the addition of 5 ml of ice-cold 1% phosphotungstic acid in 0.5 M HCl. The precipitates were collected by centrifugation, washed twice with 1% phosphotungstic acid in 0.5 M HCl, dissolved in 0.5 ml of NCS tissue solubilizer (Amersham/Searle), and counted in toluene containing 0.3% PPO and 0.1% POPP in a scintillation spectrophotometer. Control assays were terminated at 0 min. Reaction mixtures without added asialofetuin were included to detect incorporation into endogenous acceptors.

UDP-Galactose:Glycoprotein Galactosyltransferase

The reaction mixture contained the following in 0.1 ml of phosphate-buffered saline, pH 7.4: 0.05% Triton X-100, 0.5 mM CMP-N-[G-3H]galactosylamine (20 μCi/μmol), 0.3 to 0.5 mg of cell protein, and, for assaying activity toward UDP-Galactose:serum albumin acceptor, 0.5 to 1.0 mg of bovine serum albumin and 0.5 mg of asialo-fetuin (3). Incubations and terminations were as described above for CMP-NeuAc glycopeptide sialyltransferase.

CMP-NeuAc:Lactosylceramide Sialyltransferase

The reaction mixture contained the following in 0.1 ml: 1.5 mg of Cutsicum, 95 μg of cardiolipin, 20 μg of lactosylceramide, 0.5 mM CMP-N-[G-3H]galactosylamine (20 μCi/μmol), 60 μg asialofetuin (3), and 0.3 to 1.5 mg of cell protein. Lipids and detergent were added to the assay tubes in chloroform, which was evaporated at room temperature.
temperature prior to the addition of the other components. After a 30-min incubation at 37°C, the lipid products were either extracted with 20 volumes of chloroform:methanol (2:1), followed by removal of non-lipid contaminants (21), or they were precipitated with 5 ml of cold 1% phosphotungstic acid in 0.5 N HCl and then extracted with 5 ml of chloroform:methanol (2:1). The lipid extracts were evaporated in scintillation vials and counted in a β-counter. Both extraction procedures gave the same result. Control reaction mixtures were assayed at 0 min.

**UDP-Galactose:Glucosylceramide Galactosyltransferase**—The reaction mixtures contained in 0.1 ml: 0.75 mg of CuSO4, 40 μg of glucosylceramide, 0.5 mM UDP-[3H]-galactose (20 μCi/μmol), 50 mM cacodylate-HCl, pH 7.3, 12.5 mM MnCl2, and 1 mg of cell protein. Incubations and terminations were as described for GM3 synthetase assays.

**CMP-NeuAc:Endogenous Surface Acceptors Sialyltransferase**—These assays were performed using confluent monolayer cultures of intact cells on 35-mm Petri dishes. The cultures were incubated at 37°C in MEM-α containing 10% heat-inactivated fetal calf serum and 0.1 mM CMP-N-(G-3H)-acetylneuraminic acid (20 μCi/μmol). The heat-inactivated serum was free of sialyltransferase activity. The reactions were terminated by removing the incubation media and adding cold 10% trichloroacetic acid directly to the dishes. The precipitates were scraped from the dishes with a rubber policeman, washed twice with cold 5% trichloroacetic acid, and dissolved and counted as described above. Parent cell cultures were pretreated with 50 units/ml V. cholerae neuraminidase in phosphate-buffered saline, pH 6.9, for 40 min at 37°C. The neuraminidase was removed by extensive washing of the monolayer cultures with phosphate-buffered saline prior to the enzyme assays.

In each of the assays described above, the reactions were linear with time and proportional to cell protein concentration over the range employed. Glucosylceramide and lactosylceramide were present in saturating amounts. In each case, 85 to 95% of the incorporated radioactivity was recovered as the appropriate sugar. Greater than 85% of the [3H]-NeuAc incorporated into endogenous and exogenous lipid acceptors in the GM3 synthetase assays chromatographed with authentic GM3 on thin layer plates using Solvent A. Similarly, the [3H]-labeled product of the UDP-galactose-glucosylceramide galactosyltransferase assays co-chromatographed with lactosylceramide on thin layer plates in Solvent B. Greater than 85% of the labeled product of the UDP-galactose-ovalbumin galactosyltransferase assay was susceptible to degradation by β-galactosidase.

The sialyltransferase and galactosyltransferase activities of the cell lysates were totally recovered in the crude membrane fraction (see "Cell Fractionation").

**Glycosidase Assays**

These assays were performed on crude membrane and soluble cell fractions (see "Cell Fractionation").

**Neuraminidase Assays**—The reaction mixtures contained in 0.2 ml: 0.5% Triton X-100, 1 mM CaCl2, 40 mM sodium acetate, pH 4.7, 1.5 mg of fetuin, and 0.1 to 0.5 mg of cell protein. After incubation at 37°C for 30 min, the reaction mixtures were assayed for free sialic acid (22).

**β-Galactosidase Assays**—The reaction mixtures contained in 0.3 ml: 3 mM p-nitrophenyl-β-p-galactopyranoside, 17 mM sodium acetate, pH 4.7, and 0.06 to 0.35 mg of cell protein. After incubation at 37°C for 30 min, the reactions were terminated by the addition of 0.7 ml of 0.2 M Na2CO3. The mixtures were centrifuged and released p-nitrophenol was quantitated by measuring the absorbance of the supernatant at 420 nm.

**RESULTS**

**Selection of Wheat Germ Agglutinin-Resistant Clones**—Resistant cells were selected for their ability to grow in the presence of toxic concentrations of wheat germ agglutinin. In a typical selection experiment, 2 × 10⁵ cells were plated onto 60-mm Petri dishes, allowed to adhere to the dishes, and then incubated in the presence of 4 μg/ml of lectin in complete growth medium. This amount of lectin killed most of the cells, but usually one or two clones were found per dish after a 2-week incubation period. Cells from individual colonies were collected by trypsinization within a stainless steel cloning ring, and subcultured in nonselective medium. No prior mutagenic treatment was employed.

Nine clones were selected for further studies. These clones have growth rates similar to the parent population and have been stable with respect to both lectin resistance and morphological characteristics over many serial passages (in some cases, for nearly 2 years of continuous culture). While all nine clones have individual distinguishing characteristics, the subsequent discussion will focus primarily on three clones which were studied in detail (Clones 1, 13, and 1021).

**Morphological Appearance of Clones**—Although the parental Chinese hamster ovary cell population is morphologically somewhat heterogeneous, most of the cells give rise to moderately compact colonies of epithelioid cells (Fig. 1A). Several of

![Fig. 1](http://www.jbc.org/Downloaded from http://www.jbc.org)
Wheat Germ Agglutinin-resistant Cells

the wheat germ agglutinin-resistant clones are morphologically distinguishable from each other and from the parent line. Clone 1 cells are extremely rounded in monolayer cultures, and the cells within a colony are not in close contact with each other (Fig. 1B). As the cultures approach confluence, the cells become slightly elongated and flattened. These cells are easily removed from plastic substrata by vigorous agitation, mild trypsinization or EGTA treatment. Clone 1021 cells are more ellipsoidal than the parent cells (Fig. 1C). Neighboring cells adhere tightly to each other and frequently form whorl-like patterns. Clone 13 cells are morphologically indistinguishable from parent cells.

Another morphological variation is exemplified by Clone 5 (Fig. 1D). These cells flatten much more than parent cells in monolayer culture, covering a much larger surface area per cell. Unlike most of the other clones, Clone 5 cells do not grow readily in suspension culture.

Wheat Germ Agglutinin Toxicity and Binding of Lectins to the Variant Clones—The LD₅₀ of wheat germ agglutinin toward parent cells ranged from 0.3 to 2.0 μg/ml. Clone 1 cells are 5 times more resistant to the toxic effects of the lectin than the parent cells; Clone 1021 cells are 8.7 times more resistant and Clone 13 cells are 254 times more resistant. While the LD₅₀ concentration tended to vary in different experiments, the relative resistance of the clones did not change. All of the resistant clones studied do not bind wheat germ agglutinin as well as normal Chinese hamster ovary cells. Typical binding curves are shown in Fig. 2A. When the binding data are analyzed by the method of Scatchard (18), biphasic and perhaps even multiphasic curves are obtained (Fig. 2B). Nonlinear Scatchard plots of wheat germ agglutinin binding to human erythrocytes has previously been observed (12). The complexity of the curves makes it difficult to calculate meaningful association constants but approximate values can be obtained as well as estimates of the total number of lectin binding sites. In the case of Clones 1 (not shown) and 1021, the decreased binding at low lectin concentrations is due primarily to a decrease in binding affinity (Kₛ) for the lectin rather than to a loss of binding sites. In contrast, Clone 13 exhibits both a decrease in binding affinity and in the total number of binding sites (6 x 10⁷ binding sites per cell versus 17 x 10⁷ sites on parent cells).

Clones 1 and 1021 exhibit a consistent increase in Ricinus communis agglutinin I and ricin binding and a 100- to 200-fold increase in soybean agglutinin binding compared to parent cells. A typical experiment is shown in Table I. In contrast, Clone 13 binding of R. communis agglutinin I is only 20% of that of parent cells. The binding of Agaricus bisporus, Lens culinaris, and Phaseolus vulgaris lectins by these clones is essentially normal (data not shown).

Membrane Carbohydrate Content of Variant Clones—The lectin binding data suggest that Clones 1 and 1021 have a selective decrease in membrane sialic acid, since terminal sialic acid residues are known to block the binding of soybean agglutinin, R. communis agglutinin I and ricin (12, 25) while facilitating the binding of wheat germ agglutinin (12, 26). The data also suggest that Clone 13, by virtue of its decreased ability to bind R. communis agglutinin, is deficient in surface galactose residues as well as sialic acid residues. The actual carbohydrate content of crude membrane preparations of parent and variant cells is shown in Table II. As predicted, membranes from Clones 1 and 1021 have a selective decrease in sialic acid content while Clone 13 membranes are strikingly deficient in both sialic acid and galactose. Since most of the membrane sialic acid in Chinese hamster ovary cells is linked to galactose, the reduced sialic acid content of Clone 13 membranes is probably secondary to the galactose deficiency. Three other clones had a partial deficiency of sialic acid, ranging from 50 to 70% of wild type levels. Further characterization of the membranes of these clones was not performed.

The carbohydrate alterations of all the clones affect both glycolipids and glycoproteins.

Glycolipids of the Variant Clones—When total cellular lipids

| Table II

Carbohydrate composition of total membrane fractions

Crude membrane fractions were prepared and analyzed as described under "Experimental Procedures." The values are the average of at least two separate membrane preparations.

| Cell line | Sialic acid | Galactose | Mannose | Hexosamine |
|-----------|-------------|-----------|---------|------------|
| Parent    | 14.0        | 16.0      | 20.7    | 36.9       |
| Clone 1   | 2.4         | 14.7      | 19.5    | 30.4       |
| Clone 13  | 2.1         | 1.8       | 17.6    | 46.6       |
| Clone 1021| 2.5 (3.5)   | 14.1      | 22.2    | 30.0       |

The higher values were obtained when intact monolayer cells were incubated with 50 units/ml of Vibrio cholerae neuraminidase to release sialic acid. The reason for this discrepancy is not known, but it is possible that small amounts of sialic acid are lost when crude membranes are isolated.
the 14C label is neither glucose nor galactose. The material co-
amide. In all instances, the [14C]galactose-labeled material
detected. In contrast, Clone 1021 cells contain primarily lacto-
sylceramide, while Clone 13 cells contain largely glucosylcer-
deficient in both sialic acid and galactose (Table III). Fig. 3
depicts the thin layer chromatographic patterns of glycolipids
labeled by growing the cells in the presence of [14C]galactose
and N-[G-3H]acetyl-α-mannosamine. The major labeled glyco-
lipid in parental Chinese hamster ovary cells is sialyl-lactosyl-
ceramide (GM3) while lactosylceramide and glucosylceramide
are present as minor components. No higher gangliosides were
detected. In contrast, Clone 1021 cells contain primarily lacto-
sylceramide, while Clone 13 cells contain largely glucosylcer-
amic acid. In all instances, the [14C]galactose-labeled material
gave rise to an unidentified peak, termed X. Acid hydrolysis of
this material followed by paper chromatography revealed that
the 14C label is neither glucose nor galactose. The material co-
chromatographs with phosphatidylinositol in Solvent System
B. Acid hydrolysis of the other glycolipid peaks yielded only
labeled glucose and galactose. The labeled glycolipid pattern
of Clone 1 cells is the same as that of Clone 1021.

Glycoproteins of Variant Clones—In order to define differ-
ences in glycosylation of the membrane glycoproteins, the
various cell lines were grown in the presence of [3H]fucose to
label the oligosaccharide units of the glycoproteins. Glycopep-
tides were then prepared by pronase digestion of isolated crude
membrane fractions. As shown in Fig. 4, the major fucose-
containing glycopeptides of Clones 1021 and 13 are signifi-
cantly smaller than the glycopeptides of parent cells. The
estimated molecular weights of the major parental Clone 1021
and Clone 13 glycopeptides are 3500, 2550, and 2000, respec-
tively. Similar results were obtained when the glycopeptides were
labeled with [3H]glucosamine.

Evidence that the glycopeptides from the variant cell lines are
deficient in specific sugar residues at their nonreducing
termini is presented in Fig. 5. [3H]Glucosamine-labeled glyco-
peptides from parent and Clone 13 cells were treated with
various glycosidases and the release of radioactivity was deter-
bined by gel filtration on a Bio-Gel P-2 column. Treatment of
parent cell glycopeptides with β-N-acetylglucosaminidase
alone resulted in the release of only 15% of the radioactivity,
whereas treatment with neuraminidase followed by digestion
with both β-galactosidase and β-N-acetylglucosaminidase
cam 45% of the label to appear as small molecular weight
material (Fig. 5, upper panel). Under those chromatographic
conditions, sialic acid elutes with the void volume; all the
labeled low molecular weight material is therefore free N-
acetyllactosamine. Treatment of Clone 13 glycopeptides with
β-N-acetylglucosaminidase alone resulted in the release of
40% of the radioactivity, and treatment with all three glyco-
sidases did not significantly increase the amount of labeled
material released (Fig. 5, lower panel). We conclude that
Clone 13 glycopeptides, unlike those of parent cells, have N-
acetyllactosamine residues at their nonreducing termini.

Similar experiments with [14C]galactose-labeled glycopep-
tides indicated that the glycopeptides from Clone 1021 have

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**Table III**

**Composition of Glycolipids**

Total cellular lipids were extracted and analyzed for sugar and
phosphate content as described under "Experimental Procedures." The values have been normalized against glucose. Glycolipid glucose
levels ranged from 2 to 3 nmol/mg of cell protein.

| Glycolipid | Parent | Clone 13 | Clone 1021 |
|-----------|--------|----------|------------|
| Glucose   | 1.00   | 1.00     | 1.00       |
| Galactose | 1.24   | 0.15     | 1.00       |
| Sialic acid| 0.62   | 0.20     | 0.04       |
| Phosphate | 53.9   | 30.4     | 50.0       |

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**Fig. 3.** Thin layer chromatograms of glycolipids. Cells were
grown on 100-mm Petri dishes in the presence of [14C]galactose (5 to
10 μCi/12 ml) and N-[G-3H]acetyl-α-mannosamine (100 to 200 μCi/12
ml) (A, B), or [3H]galactose (20 to 40 μCi/12 ml) (C), for 3 days. The
cells were scraped into cold water, their glycolipids were extracted as
described under "Experimental Procedures", and aliquots chro-
matographed in Solvent System B. A, parent; B, Clone 1021; C, Clone 13. 

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**Fig. 4.** Elution patterns of [3H]fucose-labeled glycopeptides on
Bio-Gel P-6. Cells were grown on 100-mm Petri dishes in the pres-
ence of [3H]fucose (12 μCi/12 ml) for 3 days. Glycopeptides were
prepared as described under "Experimental Procedures" and ali-
quots applied to a Bio-Gel P-6 (100 to 200 mesh) column (1.4 x 88
cm) in 0.1 N HClO4. Two-milliliter fractions were collected. △—△, parent; ○—○, Clone 1021; ■—■, Clone 13. **Inset,** calibration curve for the Bio-Gel P-6 column. Standards used: Vm, bovine
serum albumin, Vm, fucose, A, stachyose (M, = 600), B, IgG glyco-
capeptide (M, = 1900) (24); C, fetuin glycopeptide (M, = 3180)
(27). Molecular weights (Mw) of the [3H]fucose-labeled glycopep-
tides 1, parent, 2, Clone 1021, 3, Clone 13, were estimated by
interpolation, using the linear relationship between molecular
weight and log (Vm/Vm (100) - Vm (x) x 100) (28).
galactose residues at their nonreducing termini, while parent cell glycopeptides have terminal sialic acid residues linked to penultimate galactose residues.

The membrane carbohydrate deficiencies observed in the variant clones could result from either impaired biosynthesis or excessive degradation of the affected oligosaccharides.

There is considerable evidence that the biosynthesis of the terminal trisaccharide units (which have the sequence sialic acid $\to$ galactose $\to$ N-acetylglucosamine) of serum-type glycoproteins and the synthesis of glycolipids occur by the stepwise addition of monosaccharide units from nucleotide sugars to appropriate acceptor oligosaccharides, in reactions catalyzed by specific glycosyltransferases. The composition studies presented above suggest that appropriate glycoprotein and glycolipid acceptors for sialyltransferase are present in Clone 1021, and appropriate galactosyltransferase acceptors are present in Clone 13.

**Intracellular Pool Sizes of Nucleotide Sugars** — Table IV compares the nucleotide sugar intracellular pools of parent cell and the variant clones. In each clone, the intracellular CMP-sialic acid content is markedly higher than that of the parent cells. The intracellular UDP-galactose level in Clone 13 is normal with respect to the parent line. The possibility that impaired nucleotide sugar synthesis is responsible for the observed nucleotide sugar deficiencies can therefore be discounted. The increased CMP-sialic acid content of the sialic acid-deficient clones suggests that a block exists in the utilization of this nucleotide sugar and that therefore the defect in these clones probably involves a biosynthetic step. A similar block in the utilization of UDP-galactose by Clone 13 might not be expected to lead to accumulation of UDP-galactose due to the activity of UDP-glucose-4-epimerase.

**Sialyltransferase Activities in Parent Chinese Hamster Ovary and Clone 1021 Cells** — Sialyltransferase activities of parent and Clone 1021 cells assayed using a variety of acceptors are shown in Table V. Monolayer cultures of both cell lines, when incubated in the presence of CMP-[$^3$H]NeuAc, have been used to prepare the glycolipids and glycoproteins from which acceptors are eluted by Bio-Gel P-2 chromatography.

**Table IV**

| Nucleotide sugars | Parent | Clone 1021 | Clone 1 | Clone 13 |
|-------------------|--------|------------|---------|----------|
| CMP-sialic acid   | 0.6    | 0.9        | 1.6     | 4.4      |
| UDP-glucose       | 0.8    | 0.9        | 1.6     | 4.4      |
| UDP-galactose     | 0.2    | 0.9        | 1.6     | 4.4      |

* N.D., not determined.

**Table V**

Glycosyltransferase activities of parent cells and the variant clones

Details concerning assay conditions are described under "Experimental Procedures." Monolayer cultures were confluent or near confluent. ( ) indicates the number of separate enzyme preparations.

| Enzyme          | Acceptor                  | Parent | Clone 1021 | Clone 1 | Clone 13 |
|-----------------|---------------------------|--------|------------|---------|----------|
| Sialyltransferase | Endogenous acceptors on intact cells | 0.06 ± 0.03 (7) | 0.09 ± 0.05 (7) |        |         |
|                 | Asialofetuin              | 5.0 ± 0.7 (7) | 3.9 ± 0.7 (7) | 7.4 (1) |
|                 | Lactosylceramide          | 0.17 ± 0.03 (8) | 0.04 ± 0.03 (8) |        |         |
| Sialyltransferase | Endogenous glycoprotein   | 0.09 ± 0.01 (4) | 0.04 ± 0.01 (3) | 0.12 (1) |
|                 | Fetuin (minus sialic acid and galactose) | 1.9 ± 0.6 (3) | 1.7 (3) | 2.7 ± 0.5 (9) |
|                 | Ovalbumin                 | 1.6 ± 0.1 (2) | 1.5 ± 0.5 (2) | 2.5 ± 0.6 (3) |
|                 | Glucosylceramide          | 0.25 ± 0.01 (2) | 0.18 ± 0.01 (2) | 0.24 ± 0.06 (2) |

* Monolayer cultures were incubated in growth medium containing 10% heat-inactivated fetal calf serum and 0.1% newborn calf serum for 3 days; membranes were isolated and digested with pronase as described under "Experimental Procedures." The incubation products were counted. The increased CMP-sialic acid content of the sialic acid-deficient clones suggests that a block exists in the utilization of this nucleotide sugar and that therefore the defect in these clones probably involves a biosynthetic step. A similar block in the utilization of UDP-galactose by Clone 13 might not be expected to lead to accumulation of UDP-galactose due to the activity of UDP-glucose-4-epimerase.

* Activity in cells grown in suspension culture.

+ Activity in cells grown in monolayer culture. Cells were harvested by trypsinization.
incorporate similar amounts of [3H]sialic acid into endogenous surface acceptors. That this activity is a surface activity is based on the following criteria: (a) Hydrolysis of the CMP-[3H]NeuAc prior to incubation with the cells completely abolished sialic acid incorporation; (b) 95% of the cells excluded trypan blue, suggesting that they were intact; (c) 90% of the incorporated counts are released by neuraminidase treatment of intact cells; (d) disruption of the cells by sonication resulted in reduced transfer of sialic acid to endogenous acceptors; (e) neuraminidase pretreatment of parent cells is required for detectable incorporation but has no effect on incorporation in Clone 1021 cells. Efforts to restore the surface sialic acid content of the sialic acid-deficient clones or neuraminidase-treated parent cells by incubating the cells for extended periods with CMP-NeuAc failed. At most, 2 nmol of sialic acid were incorporated per mg of membrane protein. Replenishment of the CMP-NeuAc-containing medium did not increase incorporation.

CMP-NeuAc:glycoprotein sialyltransferase activity in Clone 1021 cell lysates ranged from 50 to 100% of parent levels. The reason for this fluctuation is not clear. Activity in both the parent and Clone 1021 cells exhibited a broad pH optimum with the peak at pH 6.9. To rule out the possibility that proteases released at the time of cell lysis activated the transferases, the protease inhibitor phenylmethylsulfonylfluoride was added to the cell suspensions prior to cell lysis. The inhibitor had no effect on glycoprotein sialyltransferase in Clone 1021 or parent cells.

We considered the possibility that the sialyltransferase activity assayed in vitro using exogenous glycoprotein acceptors may not be representative of the predominant enzyme which functions in intact cells. Methylolation analysis of parent glycopeptides with or without neuraminidase digestion indicates that greater than 80% of the sialic acid residues linked to penultimate galactose residues are linked 2-6 while less than 20% are linked 2-3. However, Clone 1021 cell lysates lack the capability of synthesizing both sialyl(2-3)-lactose and sialyl(2-6)-lactose in amounts equivalent to that observed with parent cell lysates (data not shown).

The CMP-NeuAc: lactosylceramide sialyltransferase (GM$_1$ synthetase) activity of Clone 1021 cells is markedly decreased relative to parent cells, while Clone 1 cells exhibit wild type activity (Table V). GM$_1$ synthetase activity in parent cells grown in suspension culture is twice that of cells grown in monolayer, while no such difference was observed in Clone 1021. Clone 1021 extracts exhibit a 3-fold decrease in GM$_1$ synthetase activity as assayed in vitro in both cell lines but there was no significant effect on cellular GM$_1$ content. In parent cells, the limiting factor may be the low amount of endogenous lactosylceramide available in vivo (Fig. 3), but this is not the case for Clone 1021 cells since their major glycolipid is lactosylceramide. Absolute val-

![Fig. 6. Effect of CMP-NeuAc concentration on GM$_1$ synthetase. Assays were conducted as described under "Experimental Procedures" with crude membranes rather than cell lysates. The activity was normalized against total cell protein. \( \Delta \) - \( \Delta \), CHO (parent); o---o, Clone 1021. CHO, Chinese hamster ovary cells.](http://www.jbc.org/)

**TABLE VI**

| Enzyme                  | Cell line | Acceptor       | Apparent \( K_v \) |
|-------------------------|-----------|----------------|-------------------|
| Sialyltransferase       | Parent    | Endogenous     | 3.3 \times 10^{-4} M |
| surface activity        | Clone 1021|                | 3.8 \times 10^{-4} M |
| Sialyltransferase       | Parent    | Asialofetuin   | 6 \times 10^{-4} M |
|                        | Clone 1021|                | 4 \times 10^{-4} M |
| Sialyltransferase       | Parent    | Lactosylceramide | 7 \times 10^{-4} M |
|                        | Clone 1021|                | 5 \times 10^{-4} M |
| Galactosyltransferase   | Parent    | Ovalbumin      | 7 \times 10^{-4} M |
|                        | Clone 13  |                | 6.9 \times 10^{-5} M |

2 The major glycopeptide fraction was isolated from a pronase digest of chloroform:methanol (2:1)-extracted Chinese hamster ovary cell pellets by chromatography on a Bio-Gel P-6 column (see Fig. 4). This glycopeptide fraction, which contained 87% of the total membrane-bound sialic acid, was subjected to methylation analysis of intact cells by sonication resulted in reduced transfer of sialic acid to endogenous acceptors; (e) neuraminidase pretreatment of parent cells is required for detectable incorporation but has no effect on incorporation in Clone 1021 cells. Efforts to restore the surface sialic acid content of the sialic acid-deficient clones or neuraminidase-treated parent cells by incubating the cells for extended periods with CMP-NeuAc failed. At most, 2 nmol of sialic acid were incorporated per mg of membrane protein. Replenishment of the CMP-NeuAc-containing medium did not increase incorporation.

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The apparent $K_v$ values for CMP-NeuAc using various ac-
ues of GM₃ synthetase activity and GM₃ content varied within a 2-fold range over several experiments, but within each experiment butyrate treatment failed to change GM₃ levels with respect to controls, although in vitro enzyme activity was always induced. Butyrate did not affect CMP-NeuAc:glycoprotein sialyltransferase activities in either cell line.

These data suggest that the decreased GM₃ content of Clone 1021 is not due solely to the reduced GM₃ synthetase activity.

**Galactosyltransferase Activities of Parent and Clone 13 Cells** — Clone 13 cell lysates have normal galactosyltransferase activities toward ovalbumin, asialoagalactofetuin, and glucosylceramide (Table VI). The apparent $K_m$ for UDP-galactose using ovalbumin as acceptor was the same for parent and Clone 13 cells (Table VI). Furthermore, appreciable galactosyltransferase activity was observed in the absence of added exogenous acceptors, demonstrating that Clone 13 possesses enzymes capable of utilizing the cells' own endogenous acceptors in in vitro assays.

The time course of heat inactivation and the effect of varying detergent concentrations on the Clone 13 UDP-galactose:glycoprotein galactosyltransferase activity was investigated in an attempt to detect more subtle alterations in the enzyme. Heat inactivation of the enzymes from Clone 13 and parent cells followed an identical time course at 50°, with a half-life of 5 min. Similarly, the activation of the enzyme from both cell lines as a function of Triton X-100 concentrations was identical, with maximal stimulation of activity observed at 0.015% detergent.

No surface galactosyltransferase activities were observed in either Clone 13 or parent cells.

**Glycosidase Activities of Parent and Variant Cells** — Using cell extracts prepared by repeated freezing and thawing, approximately 90% of the neuraminidase activity is recovered in the soluble fraction while 10% is associated with the particulate material. No significant differences in total neuraminidase activity or distribution were found among parent cells and Clones 1, 1021 and 13 (data not shown). Also, no differences in β-galactosidase activities were found. Thus, it is unlikely that the observed membrane sugar deficiencies are due to enzymatic removal of the missing sugars from the oligosaccharides.

**N-[G-3H]Acetylmannosamine Incorporation into Membrane Sialic Acid** — N-Acetyl-d-mannosamine is selectively metabolized to CMP-sialic acid in mammalian cells and thus serves as a specific tracer for studying sialic acid incorporation into membrane oligosaccharide (33-35). The rate of incorporation of N-[G-3H]acetylmannosamine into CMP-sialic acid and trichloroacetic acid-insoluble material in parent and Clone 1021 cells was studied. Since the pool size of CMP-sialic acid is greatly increased in 1021 cells, the specific activity of the CMP-sialic acid was measured so that more accurate rates of sialic acid transfer to acid-insoluble material could be determined (Table VIII). The rate at which sialic acid was transferred from CMP-sialic acid to acid-insoluble material was reduced about 85% in Clone 1021. This estimation is in good agreement with the finding that the membrane sialic acid content of Clone 1021 is decreased 82% compared to parent cells. While the possibility that sialic acid residues are removed minutes or even seconds after incorporation into glycoprotein and glycolipid acceptors cannot be eliminated, this experiment, coupled with the marked elevation of CMP-sialic acid content, suggests that the defect in 1021 cells involves the transfer of sialic acid from CMP-sialic acid to appropriate acceptor molecules.

The incorporation of label into large molecular weight substances released into the medium is also reduced 90% in Clone 1021.

**Discussion**

Several previously described lectin-resistant cell line variants have membrane sugar alterations (2-7), but the wheat germ agglutinin-resistant clones are the first to have a selective deficiency in membrane sialic acid content or, in the case of Clone 13, in membrane sialic acid and galactose content. The resistance of most of the variant cell lines to wheat germ

**Table VII**

|          | GM₃ synthetase activity and GM₃ content of parent and clone 1021 cells grown in normal and butyrate-supplemented media |
|----------|-------------------------------------------------------------------------------------------------------------------|
|          | Cells were plated from suspension culture at 1 x 10⁶ cells/150 mm dish. After 24 h at 37°, the medium was replaced with fresh medium ± sodium butyrate (1 mm). The cells were incubated for another 48 h. GM₃ synthetase activity was assayed as described under "Experimental Procedures." Glycoproteins were extracted as described under "Experimental Procedures." GM₃ was determined by measuring the amount of sialic acid in the glycolipid fraction. In this experiment, the GM₃ content of parent control cells is lower than usually observed (9 to 3 nmol/mg of protein). |
|          |                                                                                                                   |
|          | **GM₃ synthetase activity** (nmol/mg protein)  **GM₃ content** (nmol/mg protein/30 min)                          |
|          |                                                                                                                   |
| Parent control | 1.4                                                                 | 0.073                                                                 |
| Parent + butyrate | 2.0                                                                 | 0.150                                                                 |
| Clone 1021 control | 0.4                                                                 | 0.026                                                                 |
| Clone 1021 + butyrate | 0.2                                                                 | 0.099                                                                 |

**Table VIII**

| Incorp. of [G-3H]Acetylmannosamine in monolayer cultures of parent and clone 1021 cells |
|--------------------------------------------------------------------------------------------|
| Confluent monolayer cultures of parent and 1021 cells on 60-mm Petri dishes (2 mg of cell protein/plate) were incubated in complete medium containing 50 μCi/ml [G-3H]Acetylmannosamine (2.5 Ci/mmol) at 37°. At the indicated times the media were removed, the cells scraped into cold 10% trichloroacetic acid and pelleted by centrifugation. The precipitates were washed twice with cold 5% trichloroacetic acid and then hydrolyzed in 0.1 M H₂SO₄ at 80° for 1 h to release bound sialic acid. Unlabeled sialic acid (1 μmol) was added to correct for subsequent losses and the released sialic acid was adsorbed onto a small Dowex 1-X8 formate column and eluted with 0.3 N formic acid. All the radioactivity recovered by this procedure chromatographed on descending paper chromatography with authentic N-acetyleneuraminic acid in n-butyl acetate:acetic acid:water (3:2:1). CMP-[3H]sialic acid was isolated as described under "Experimental Procedures" using a separate set of cultures from the same experiment. The values for incorporation of [3H]sialic acid into acid-insoluble material are averages of duplicate determinations. The average specific activity of CMP-[3H]sialic acid represents the arithmetic means of the specific activity measured at the beginning and the end of each time period. |
|                                                                                                                   |
| **Time** | **CPM/mg protein** | **CPM/mmol** | **nmol/mg/h** |
| Cl. 1021 |                                                                                                                   |
| 0-60     | 17                                                                   | 216         | 0.08          |
| 0-90     | 26                                                                   | 1/3         | 0.10          |
| 90-180   | 37                                                                   | 775         | 0.03          |
| Parent   |                                                                                                                   |
| 0-90     | 3,000                                                                | 5,800      | 0.36          |
| 90-180   | 10,850                                                               | 13,700     | 0.53          |
agglutinin can be explained on the basis of their decreased membrane sialic acid content, which results in a decreased affinity of the lectin for membrane oligosaccharides which serve as binding sites. The role of sialic acid residues in wheat germ agglutinin binding has been demonstrated in a number of systems (12, 26). However, the 250-fold resistance of Clone 13 to wheat germ agglutinin toxicity cannot be explained on this basis. Since Clone 13 has a 65% decrease in binding sites, it is possible that this clone has lost a special class of "productive" binding sites which are necessary for mediating wheat germ agglutinin toxicity at low lectin concentrations.

Stanley et al. have described a variant phenotype of Chinese hamster ovary cells characterized by resistance to wheat germ agglutinin and increased sensitivity to ricin cytotoxicity (36). Although no membrane carbohydrate analyses or lectin binding data have been reported for the clones with this phenotype, their characteristics are very similar to those of Clones 1 and 1021.

The mechanism by which the membrane sugar alterations occur in these variant cells is not clear. It appears that these cells fail to carry out specific glycosyltransferase reactions in vivo despite the fact that they possess the appropriate nucleotide sugars, glycoprotein and glycolipid acceptors, and glycosyltransferases. This situation is in contrast to several other lectin-resistant cell lines where membrane sugar alterations can be accounted for by deficiencies of particular glycosyltransferases. This situation is in contrast to several other examples where the in vitro assays of glycosyltransferase activities do not correlate with the measured changes in membrane carbohydrate composition. In baby hamster kidney cells transformed by a temperature-sensitive polyoma virus mutant, both GM₁ content and GM₁ synthetase activity are significantly decreased at the permissive temperature. At the non-permissive temperature, however, GM₁ content returns to normal but GM₁ synthetase activity remains low (37). In addition, Smith et al. have characterized a yeast mannann mutant which fails to transfer N-acetylglucosamine residues to mannosidase side chains, yet which exhibits normal N-acetylglucosaminyltransferase activity in cell-free extracts (38).

The synthesis of complex oligosaccharide chains of plasma membrane glycoproteins and glycolipids in mammalian cells takes place primarily in the membranes of the endoplasmic reticulum and the Golgi apparatus (39). Glycosylation, therefore, occurs within the two-dimensional framework of a membrane and the process is separated from events occurring in the cytoplasm and in other organelles by that membrane. While little is known about the topographical organization of the multiglycosyltransferase system, Arce et al. (40) have presented indirect evidence that the enzymes and lipid acceptors involved in ganglioside biosynthesis are in a rigid arrangement in the membrane. Winquist and Daliner (41) have presented evidence that enzymes are arranged in specialized patches in the microsomal membrane. Any defect affecting the organization of the multiglycosyltransferase system, such as an altered subcellular localization of an enzyme, abnormal insertion of a component enzyme in the membrane, or impaired transport of the nucleotide sugar from the cytoplasm to the Golgi apparatus could impair enzyme function and yet be missed by assays involving cell disruption and the use of detergents. It is also possible that glycosyltransferase activity may be subject to tight regulation in vivo. The biochemical defect in the variant clones could affect such regulation so that the glycosyltransferase is permanently in the "off" position in vivo although the regulatory mechanism may be inept in vitro assay conditions.

It is of interest that wheat germ agglutinin is extremely toxic to Chinese hamster ovary cells, with an LD₅₀ of approximately 1 µg/ml. The mechanism of wheat germ agglutinin toxicity is not known, although two recent reports have shown that it inhibits amino acid transport in tissue culture cells (42, 43), suggesting that the lectin may exert its toxic effects by altering plasma membrane function. We have confirmed this finding and are currently studying the mechanism whereby wheat germ agglutinin inhibits amino acid transport in Chinese hamster ovary cells.

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