Surface Carbohydrates of Hamster Fibroblasts

1. CHEMICAL CHARACTERIZATION OF SURFACE-LABELED GLYCOSPHINGOLIPIDS AND A SPECIFIC CERAMIDE TETRASACCHARIDE FOR TRANSFORMANTS*

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SUMMARY

1. Neutral glycosphingolipids of hamster fibroblast NIL cells have been characterized as follows: glucosylceramide, lactosylceramide (βGal1→4Glc→Cer), a digalactosylceramide (αGal1→4βGal1→4Glc→Cer), a trilexosylceramide (αGal1→4βGal1→4Glc→Cer), two kinds of ceramide tetrasaccharides (A: αGalNAc1→3βGalNAc1→3αGal1→4βGal1→4Glc→Cer), a new type of Forssman active glycolipid; B: globoide, βGalNAc1→3αGal1→4βGal1→4Glc→Cer), and a ceramide pentasaccharide having a classical structure for Forssman antigen (αGalNAc1→3βGalNAc1→3αGal1→4βGal1→4Glc→Cer).

2. Neutral glycosphingolipids of polyoma virus-transformed NIL cells (NILpy) have been characterized as having an additional ceramide tetrasaccharide which was absent in normal NIL cells. The structure of this specific glycolipid was identified as lacto-N-neotetraosylceramide (βGal1→4βGlcNAc1→3βGalNAc1→3αGal1→4βGal1→4Glc→Cer). Chemical quantities of ceramide tetra- and pentasaccharides in NILpy cells were much lower than in NIL cells.

3. All of these glycolipids, except glucosylceramide and lactosylceramide, were labeled externally by galactose oxidase and tritiated borohydride according to the method previously described (Gahmberg, C. G., and Hakomori, S. (1973) J. Biol. Chem. 248, 4311–4317). The specific activities of the label in glycolipid of NILpy cells were much greater than that in NIL cells, i.e. reactivity of glycolipids with galactose oxidase in NILpy cells was much higher for NIL cells. The surface label in glycolipids was cell cycle-dependent in NIL cells, and a remarkable exposure of a galactosyl residue of a ceramide tetrasaccharide was demonstrated only on the surface of NILpy cells, due to the presence of lacto-N-neotetraosylceramide.

Of the various phenotypes expressed in malignant cells, the change of chemical composition and synthesis of glycolipids and the organization of glycolipids in membranes have been studied by a number of investigators during the past few years. Simplification of glycolipids, possibly due to blocked synthesis of higher ganglioside and neutral glycolipids, has been the major change noticed in various transformed cells in vitro as well as in vivo, regardless of the agent of transformation (see Refs. 1 and 2 for review). Some, but not all, glycolipid changes observed in transformed cells can be reverted to a normal state when phenotypes of transformed cells with thermosensitive mutants of tumor viruses are suppressed at a nonpermissive temperature (3, 4), suggesting that the changes in glycolipids are related to the loss of growth control seen in transformed cells.

Change in neutral glycolipids upon cell-to-cell interaction (i.e., cell contact-dependent enhancement of glycolipid synthesis) and loss of this response in transformed cells have been well documented by a number of studies with the use of hamster NIL1 or BHK fibroblasts (5–12). Most of these studies were based merely on the change in the intensities of glycolipid spots after thin layer chromatography, as revealed by chemical reaction, or by the radioactivities incorporated from radioactive precursor sugars or fatty acids added to the culture medium. The structures of the glycolipids of NIL or NILpy cells have not been studied carefully, and it is not known whether the chemical structures of each glycolipid found in transformed cells are qualitatively identical with those found in normal cells, although the quantity of higher neutral glycolipids is greatly reduced after transformation (6–10).

Recently we developed an external labeling technique utilizing galactose oxidase to label surface-exposed glycolipids and glycoproteins (13–15). Galactose oxidase is not able to penetrate into the cell and it can oxidize terminal galactosyl and N-acetylgalactosaminyi residues of surface-exposed glycolipid and glycoproteins to their corresponding 6-O-alkylides which are then reduced by tritiated borohydride. These techniques can distinguish subtle changes in cell surface carbohydrate structures associated with transformation.

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1 The abbreviations used are: NIL, hamster embryo fibroblasts first described by Diamond (17); NILpy, NIL cells transformed by polyoma virus; Cer, ceramide; PBS, phosphate-buffered saline; GL2, 3, 4, and 5, neutral glycosylceramide with di-, tri-, tetra-, and pentasaccharides.
change in the growth behavior of cells (15) and with different phases of the cell cycle (16).

This paper describes two major items: (a) thorough fractionation and chemical characterization of surface-labeled glycolipids of hamster NIL and NILpy cells, the presence of a ceramide tetrasaccharide specific for transformed NILpy cells, and a new type of Forssman glycolipid with a ceramide tetrasaccharide; and (b) the extent of exposure of various glycolipids in terms of specific activities when cells are surface-labeled, and a comparison of these activities between normal and transformed cells in various phases of growth.

MATERIALS AND METHODS

Cells and Cell Culture—Hamster NIL 2E fibroblasts, originally from Dr. Leila Diamond, Wistar Institute, Philadelphia (17), were cloned in this laboratory and designated as NIL-2K. The NIL-2K cells were transformed with small plaque polyoma virus and the transformed clones were isolated. These transformed cells (NILpy) were used throughout these experiments. NILpy cells were passaged into hamsters and were designated as NILPT. Injection of NILpy cells (5 × 10^6) into 7- to 8-week-old female hamsters induced tumors (tumor size 0.5 × 0.5 cm) after 2 weeks. They grew to 4 × 5 cm after 5 weeks. Injection of NIL cells (5 × 10^6) into 7- to 8-week-old female hamsters did not induce tumors after 2 weeks. A small tumor (0.2 × 0.2 cm) appeared after 8 weeks.

The cells were cultured in Eagle's medium with 10% fetal calf serum or in Dulbecco's medium with 10% fetal calf serum in a 5% CO_2 atmosphere. The cells were checked for mycoplasma infection but were found negative. Unless otherwise specified, cells were harvested at an early confluent stage with rubber policeman; they were washed twice with phosphate-buffered saline, pH 7.0, 100 units per ml (Sigma Biochemical, type III, or Glucosidase prepared from hog liver according to the procedure of Weissman and Hinrichsen (30), and α-galactosidase prepared from ficin (General Biochemicals) according to the procedure of Li et al. (31). Some enzymes were donated by Dr. Y-T. Li, Department of Biochemistry, Tulane University, New Orleans. Degradation of glycolipids by glycosyl hydrolases and examination of hydrolysis products were carried out according to the procedure previously described. Glycolipids were dissolved in citrate buffer at optimal pH, containing 1 mg of sodium taurocholate per ml, with brief sonication; the enzyme was added and the samples were incubated at 37° overnight to a few days. The incubation mixture was extracted with chloroform-methanol (2:1). The lower phase was subjected to thin layer chromatography with silica Gel G with the solvents described previously (19).

Extraction and Isolation of Glycolipids—Cells or tissues were homogenized with at least 20 volumes of chloroform-methanol (2:1) in an Omni-Mixer (Sorvall Instruments) for 3 min; the cell debris was re-extracted and filtered through a Buchner funnel with suction. The extract was filtered again, and the filtrates were combined and evaporated in a vacuum rotary evaporator to complete dryness. The dried residue was dissolved in a minimum quantity of chloroform-methanol; the glycolipid fraction was obtained according to the procedure of Saito and Hacman (32). After desacytelylation in 0.1% sodium methoxide in chloroform-methanol (2:1), the glycolipid fraction was analyzed on thin layer chromatography on precoated silica Gel G plates (Analtech). The solvent system used for separation of neutral glycolipids was chloroform-methanol-water (65:30:8) (lower phase).

Separation of glucosylceramide and galactosyleramide or separation between ceramide tetrasaccharides was carried out by chromatography on a borate-impregnated silica Gel H plate, according to the procedure previously described (33). Silica Gel H (30 g) was mixed with 75 ml of 1% sodium tetraborate and the slurry was spread over five plates (20 × 20 cm). The plates were activated before use. One component closely associated with galactose and a Forssman glycolipid on the tetrasaccharide, as appeared in the extract of NILpy cells, was best separated on such a plate with the solvent system chloroform-methanol-water-ammonia (60:35:7:1).

Procedure for Determination of Chemical Quantity of Glycolipids—The total glycolipid fraction was prepared according to the procedure of Saito and Hacman (32), and the glycolipids were separated by chromatography on a thin layer plate; each glycolipid was lightly stained with iodine and extracted with chloroform-methanol-water (10:1:1), followed by elution with 50% acetic acid in methanol. The residue was transferred into a Pyrex tube with Teflon-lined screw cap (12 × 1 cm), dried under nitrogen, and placed in a vacuum desiccator over phosphorus pentoxide. To each tube 50 µl of 5 µg/ml oligosaccharides, and 5 µg/ml was denaturated in 2 ml of 1 N methanolic HCl at 80° overnight. The tri-methylsilyl derivatives of galactose, glucose, galactosamine, and glucosamine were compared with the peak of the internal standard, inositol, and the quantities of glycolipids were calculated.

Procedure for Cell Surface Labeling—Cells on plastic dishes were washed with phosphate-buffered saline, pH 7.0, twice, and then washed with phosphate-buffered saline, pH 7.0, 100 units per ml with suction. Galactose oxidase (100 µl) was dissolved in phosphate-buffered saline, pH 7.0, 100 units per ml (Sigma Biochemical, type III, or Kabi, Stockholm) per plate were added, and the cell sheets were incubated at room temperature for 30 min to 2 hours. The galactose oxidase did not contain any measurable proteolytic or neuraminidase activity. In some experiments galactose oxidase was purified by affinity chromatography on Sepharose 4B column, as shown in the legend to Fig. 1. After incubation the cells were de-
Characterization of Glycosphingolipids

The following glycosphingolipids were characterized: glucosylceramide, lactosylceramide, digalactosylceramide, trihexosylceramide, three kinds of ceramide tetrasaccharides, and two chromatographically distinct ceramide pentasaccharides differing in fatty acid moieties. After surface labeling with galactose oxidase and tritiated sodium borohydride, the lower spot glycolipid obtained from the lower spot area of NILpy cells in addition contained glucosamine. The lower spot glycolipid obtained from “surface-labeled” NIL cells gave, on methanolysis, only a radioactive peak corresponding to galactose in addition to a radioactive galactosamine peak. The lower spot glycolipid obtained from both NIL and NILpy cells gave a strong reaction with anti-Forssman glycolipid antiserum, but only that obtained from NILpy cells gave a strong reaction with anti-pneumococcal type XIV polysaccharide antiserum. These results are summarized in Table I.

The results indicate that three types of glycolipids are present...
Fig. 2. Schematic drawing of glycolipids of hamster NIL cells and derived polyoma transformants. Total glycolipid fractions were prepared by the acetylation procedure (32). A, simple Silica Gel G plate developed with chloroform-methanol-water (65:30:8) (lower phase); B, borate-impregnated Silica Gel H plate developed with chloroform-methanol-water-concentrated ammonia (60:35:7:1). Lane 1, reference glycolipids: CM, glucosylceramide; CD, lactosylceramide; CT, αGal-βGal-βGlc-Cer; CTT, globoside; CP, Forssman-active glycolipid of sheep erythrocytes; Hem., hematoside. Lane 2, glycolipids of NIL cells. Lane 3, glycolipids of NILpy cells; spots corresponding to CT, CTT, and CP were greatly reduced in NILpy cells (shown in dotted circle). Lane 4, glycolipid extracted from Spot 2 of CTT. Lane 5, paragloboside (20). Spots a and b separated only on Plate B and were identified as lacto-N-neotetraosylceramide and a new Forssman-active ceramide tetrasaccharide, respectively (see the text).

Table I

Distinctive properties of lower spot ceramide tetrasaccharide (Spot 2 of Fig. 1) of NILpy cells from that of NIL cells

<table>
<thead>
<tr>
<th>Sugar detected</th>
<th>NIL</th>
<th>NILpy</th>
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<tbody>
<tr>
<td>Galactosamine</td>
<td>Galactosamine</td>
<td>Galactosamine</td>
</tr>
<tr>
<td>Galactose</td>
<td>Galactose</td>
<td>Galactosamine</td>
</tr>
<tr>
<td>Glucose (small quantity)</td>
<td>Galactosamine</td>
<td>Galactosamine</td>
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<thead>
<tr>
<th>3H Radioactivity found in glycolipid obtained from “surface-labeled” cells</th>
<th>NIL</th>
<th>NILpy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactosamine exclusively</td>
<td>Galactosamine and high activity in galactose</td>
<td></td>
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<table>
<thead>
<tr>
<th>Immunological reactivity</th>
<th>NIL</th>
<th>NILpy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forssman antibody</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anti-type XIV</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glycolipid identified</th>
<th>NIL</th>
<th>NILpy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Forssman-active ceramide tetrasaccharide: αGalNac1→βGalNac1→βGal1→Glc/ or Gal-Cer</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2. Ceramide tetrasaccharide cross-reacting with anti-type XIV pneumococcal antiserum: βGal1→αGalNac1→βGal1→4Glc→Cer</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

*−, absent; +, present; ++, present in large quantity.

in the “lower spot” glycolipid fraction derived from NILpy cells; one type of glycolipid was found to be separated from the other types by thin layer chromatography on a borate-impregnated plate. The lower spot ceramide tetrasaccharide obtained by large scale preparative thin layer chromatography was then further separated into components.

3. The faster migrating component was separated from the lower spot ceramide tetrasaccharide (Spot a of Lane 4, Fig. 2) on a borate-impregnated Silica Gel H plate developed with solvents chloroform-methanol-water-concentrated ammonia (60:35:7:1). The component was identified as having most of the [3H]galactosyl label from the surface label and gave a reaction with anti-type XIV pneumococcal antiserum. This glycolipid was found to consist of galactose, glucosamine, and glucose; it was hydrolyzed only by β-galactosidase, not by other enzymes, and was converted to a ceramide trisaccharide. The resulting ceramide trisaccharide was further degraded by jack bean β-N-acetylgalactosaminidase. The degradation product was identified as hexosylceramide on thin layer chromatography and was further degraded by β-galactosidase (see Fig. 3A). Permethylated glycolipid gave 2,3,4,6-tetra-O-methylgalactitol, 2,4,6-tri-O-methylgalactitol, 3,6-di-O-methyl(2-deoxy-2-N-methyl-2-acetamido-glucitol) and 2,4,6-tri-O-methylglucitol. Thus, the structure was identified as 3βGal1→αGalNAc1→4Gall→αGlc→Cer, which is identical with that of "paragloboside" previously isolated from human erythrocytes (20). The glycolipid gave a strong precipitin reaction with anti-type XIV pneumococcal polysaccharide antiserum and with anti-paragloboside antiserum (20) (see Fig. 4A).

4. The slow migrating glycolipid was separated from lower spot ceramide tetrasaccharide (Spot b of Lane 4, Fig. 2) by chromatography on a borate-impregnated plate. This glycolipid was partially hydrolyzed and converted to a ceramide trisaccharide by hog liver α-N-acetylgalactosaminidase of Weissmann and Hin-
Fig. 4. A, Reactivity of Spot a ceramide tetrasaccharide with anti-lacto-N-neotetraosylceramide ("paragloboside") (20) and with anti-type XIV pneumococcal polysaccharide antiserum. Center, Spot a ceramide tetrasaccharide; 1, anti-type XIV pneumococcal polysaccharide horse antiserum; 2, anti-paragloboside rabbit antiserum. B, reactivity of Spot b ceramide tetrasaccharide with anti-Forssman glycolipid antiserum in comparison with the reactivities of other Forssman-active ceramide pentasaccharides. Center, anti-Forssman ceramide pentasaccharide of horse spleen; 1, standard Forssman glycolipid (ceramide pentasaccharide of horse spleen (21); 2, NIL cells Spot b ceramide tetrasaccharide (Fig. 2, Lanes 2 and 3); 3, NIL cells Spot 3 ceramide pentasaccharide (Fig. 2, Lanes 2 and 3). Richsen (30), and the resulting ceramide trisaccharide was further degraded by β-N-acetylhexosaminidase by jack bean. Interestingly, the resulting ceramide disaccharide was not hydrolyzed by β-galactosidase (jack bean), but was readily hydrolyzed by α-galactosidase (fiein) to yield ceramide monohexoside (see Fig. 3B). The resulting ceramide monohexoside was a mixture of galactosylceramide and a glucosylceramide. The permethylated glycolipid gave on hydrolysis, followed by reduction, 3,4,6-tri-O-methyl-2-deoxy-2-N-methylacetamidogalactitol, 4,6-di-O-methyl-2-deoxy-2-N-methylacetamidogalactitol, 2,4,6-tri-O-methylglaucitol, and 2,3,6-tri-O-methylglucitol. These results indicated that the glycolipid fraction contains two similar carbohydrate chains; one is αGalNAc1→3βGalNAc1→3αGal→4Gal→Cer and the other is αGalNAc1→3βGalNAc1→3αGal→4Glc→Cer. These two glycolipids were inseparable and showed a strong precipitin reaction with anti-Forssman antiserum (Fig. 4B). Fatty acid analysis of this Forsmann-active ceramide tetrasaccharide showed a predominance of α-hydroxy fatty acids.

Ceramide Pentasaccharides—Two discrete bands were separated on thin layer chromatography (Spots 3 and 4, Fig. 2). The upper spot (Spot 3) corresponds to a ceramide pentasaccharide of horse spleen and sheep erythrocytes. Both the upper and lower spot glycolipids showed Forsmann activity (see Fig. 4B) and had the same carbohydrate composition as the horse ceramide pentasaccharide antigen that contained 2 mol of galactosamine, 2 mol of galactose, and 1 mol of glucose. Both glycolipids were degraded by α-N-acetylhexosaminidase of hog liver, and the degradation product showed the same migration rate as globoside, which was further degraded by incubation with β-hexosaminidase of jack bean. The resulting ceramide trihexoside showed the same migration rate as ceramide trihexoside of human erythrocyte membrane and was degraded further by incubation with α-galactosidase of fiein; the degradation product was identical with lactosylceramide. Both glycolipids gave, after permethylation, followed by acetylation, hydrolysis, reduction, and acetylation, acetates of 3,4,6-tri-O-methyl-2-deoxy-2-N-methylacetamidogalactitol, 4,6-di-O-methyl-2-deoxy-2-N-methylacetamidogalactitol, 2,4,6-tri-O-methylglaucitol, and 2,3,6-tri-O-methylglucitol. Thus, the carbohydrate moieties of these two glycolipids were identical. Fatty acid analysis showed that the lower spot ceramide pentasaccharide had a large proportion of α-hydroxy fatty acids. The structure of each glycolipid component is summarized in Table II.

### Table II

<table>
<thead>
<tr>
<th>Structures and chemical quantities of neutral glycolipids found in NIL and NILpy cells</th>
</tr>
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<tbody>
<tr>
<td>Presen or absence</td>
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<tr>
<td>-------------------</td>
</tr>
<tr>
<td><strong>GL1</strong></td>
</tr>
<tr>
<td><strong>GL2</strong></td>
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<tr>
<td><strong>GL3</strong></td>
</tr>
<tr>
<td><strong>GL4</strong></td>
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<tr>
<td><strong>GL5</strong></td>
</tr>
<tr>
<td><strong>GL6</strong></td>
</tr>
</tbody>
</table>

*— absent; +, present in trace quantity; ++, present in large quantity.

*Calculated on the quantity of galactose appeared in gas chromatography after methanolysis, ND, not determined.

The chemical quantity of glycolipids as determined by gas chromatography is also shown in Table II. There was a significant decrease of ceramide penta-, tetra-, and trihexosides in NILpy cells as compared to NIL cells, whereas there was a significant increase of ceramide dihexoside in NILpy cells (Table II). The change of chemical quantity observed between NIL and NILpy cells essentially agrees with the results previously described (8-11). [14C]Galactose incorporated into glycolipids is shown in...
**Table III**

Incorporation of \(^{14}C\)galactose into glycolipids

<table>
<thead>
<tr>
<th></th>
<th>cpm/mg dry weight during 36 hrs</th>
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<tbody>
<tr>
<td></td>
<td>GL5</td>
</tr>
<tr>
<td>NILpy</td>
<td>1272</td>
</tr>
<tr>
<td>NIL confluent</td>
<td>5004</td>
</tr>
<tr>
<td>NIL sparse</td>
<td>4931</td>
</tr>
</tbody>
</table>

**Figure 5.** \(^3H\) Radioactivity in each glycolipid of NIL cells labeled by treating cells with galactose oxidase and tritiated sodium borohydride. Radioactivities in counts per min \(\times 10^{-3}\) per mg dry weight residue acquired at different lapses of the reaction time (in minutes) with galactose oxidase.  

\(\rightarrow \), GL 2;  
\(\Delta \rightarrow \), GL 4;  
\(\circ \rightarrow \), GL 3;  
\(\bigcirc \rightarrow \), GL 5. Note that GL 2 was not labeled.

Table III. Here again, there was a significant increase in activity of ceramide dihexoside in NILpy cells, whereas a significant decrease in activities of ceramide penta- and tetrasaccharides was observed in NILpy cells, as compared to NIL cells. There was a consistent difference between chemical quantity and isotope incorporation into glycolipids, reflecting a significant difference of turnover rate of each glycolipid.

**Surface-labeling Pattern and Rate of Exposure of Glycolipids in NIL and NILpy Cells**

The time course of surface labeling in glycolipids when cells were treated with galactose oxidase and tritiated sodium borohydride is shown in Fig. 5. The highest label was found in ceramide tetrasaccharide, whereas essentially no label was found in ceramide dihexoside. The rate of exposure of glycolipids on cell surfaces was determined by the activities of each glycolipid obtained by the surface label procedure divided by the chemical quantities of each glycolipid (see Table IV). There was significantly higher specific activities in labels of NILpy cells, as observed in ceramide tri-, tetra-, and pentasaccharides, whereas ceramide dihexoside was not exposed or could not be labeled by this method, which is in agreement with previous results of studies on human erythrocytes (13) and BHK fibroblasts (3). As shown in Table II, the chemical quantity of ceramide tetrasaccharide and ceramide tetracosaccharide was found to be nearly constant irrespective of the different phases of the cell cycle, although ceramide di- and trihexoside increased significantly at the G1 and mitotic phases (see Table V). Since a large variation of the surface label occurred in ceramide penta- and tetrasaccharide, the observed change of the variation in surface label should depend on the rate of exposure of these glycolipids on the cell surface.

The label of glycolipids in NILpy cells did not vary much during the phases of the cell cycle; i.e. no maximum label was found in the G1 phase (see Fig. 6III). The chemical quantity of ceramide penta- and tetrasaccharide in NILpy cells was much lower than that of NIL cells, as shown in Table II, whereas the surface label in these glycolipids was not much reduced, but rather enhanced. This finding agrees with the labeling efficiency of glycolipids in NILpy cells being much higher than that of NIL cells. The label in these glycolipids of NILpy cells was invariant during the cell cycle (Fig. 6III).

**Discussion**

The present studies show a distinct difference between the surface-labeled glycolipids of NIL cells and their transformants. The following points are of particular interest.

1. Ceramide tetrasaccharides of NIL cells contain two distinguishable components and those of NILpy cells contain three distinguishable components, one of which is a new variant of Forssman hapten.

2. One component of NILpy cells with a ceramide tetrasaccharide structure was distinctly labeled at the galactosyl moiety and was not detectable in nontransformed NIL cells. It

<table>
<thead>
<tr>
<th></th>
<th>GL5</th>
<th>GL4</th>
<th>GL3</th>
<th>GL2</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1L</td>
<td>89</td>
<td>196</td>
<td>163</td>
<td>20</td>
</tr>
<tr>
<td>NILpy</td>
<td>178</td>
<td>480</td>
<td>288</td>
<td>43</td>
</tr>
</tbody>
</table>

Expressed on specific activities: cpm/\(\mu\text{mol}\) glycolipid \(\times 10^{-4}\)

<table>
<thead>
<tr>
<th></th>
<th>GL5</th>
<th>GL4</th>
<th>GL3</th>
<th>GL2</th>
</tr>
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<tbody>
<tr>
<td>N1L</td>
<td>261</td>
<td>297</td>
<td>543</td>
<td>36</td>
</tr>
<tr>
<td>NILpy</td>
<td>4944</td>
<td>3255</td>
<td>1811</td>
<td>44</td>
</tr>
</tbody>
</table>
FIG. 6. Cell cycle-dependent changes of surface-exposed glycolipids. I, variation of surface label in glycolipids of synchronized NIL cells started from confluent trypsinized cells; II, variation of surface label in glycolipids of synchronized NIL cells; synchronization was carried out by double thymidine block method (18). Chart A shows the change in cell number (O), surface label in glycolipids of synchronized NIL cells; synchronization carried out by double thymidine block method (18). Chart B shows the activities of label in each glycolipid fraction.

TABLE V

<table>
<thead>
<tr>
<th>Chemical concentration of glycolipids of NIL cells at different phases of cell cycle (determined by gas chromatography)*</th>
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<tbody>
<tr>
<td>Glycolipids**</td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td>GL5</td>
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<tr>
<td>GL4</td>
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<tr>
<td>GL3</td>
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<td>GL2</td>
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</tbody>
</table>

* Synchronized cells started from trypsinized confluent cell cultures.
** For structures, see Table II.

was found both in NILpy cells grown in vitro or in vivo as tumors. The structure of this ceramide tetrasaccharide was identified as lacto-N-neotetraosylceramide.

3. The chemical quantity of ceramide penta-, tetra-, and tri-saccharides in NILpy cells was much lower than that in NIL cells. However, the reactivity of these glycolipids to externally applied galactose oxidase was much higher in NILpy cells than in NIL cells, i.e. these glycolipids in NILpy cells are in a more "exposed state" or show higher reactivity.

Recently, glycolipid composition of cultured cells as related to cellular function and transformation has been studied with increasing interest. Usually conclusions have been drawn from comparison of thin layer chromatograms. It is now apparent that a single spot can contain more than one glycolipid component with different carbohydrate structures. For example, ceramide pentasaccharides of human erythrocytes, which show a single spot under normal thin layer chromatography conditions, contain at least three components: Leα-glycolipid (lacto-N-fucopentaose-II-ceramide), H-active glycolipid (lacto-N-fucopentaose-IV-ceramide), and an additional glycolipid with two galactosyl residues (β-galactosyl-"lacto-N-neotetraosyl"-ceramide) (40, 41). Although this is technically difficult, chemical characterization of each component is therefore desired and sometimes offers new and valuable information.

Ceramide tetrasaccharide of NIL and NILpy cells was identified as globoside simply because it migrated in thin layer chromatograms at the same rate as globoside and showed a similar chemical composition (6-10). Radiochemical and immunohistochemical analysis of the ceramide tetrasaccharide fraction of surface-labeled cells showed that the fraction is a mixture with different radioactive labels and with three immunologically distinct components. One component showed a reaction with anti-globoside, the second component reacted to anti-Forssman antibody, and the third component reacted to anti-lacto-N-neotetraosylceramide antiserum, as well as to anti-type XIV pneumococcal polysaccharide antiserum. The last component was detectable only in NILpy cells, and the radioactive label was exclusively found in the terminal galactosyl residue. This glycolipid component could be a tumor-specific antigen.

The hapten of Forssman antigen has been known as a glycolipid since Brunis first described the hapten as a hexosamine-containing lipid (42). Studies by Papirmeister and Mallette (43) and by Yamakawa et al. (43) and Makita et al. (45) indicated that the hapten is a galactosamine-containing glycolipid similar to globoside. Eventually, the hapten has been characterized as a ceramide pentasaccharide with the structure αGalNAc1→3βGalNAc1→3αGal1→4βGal1→4Glc→Cer (21). This structure is
common for the hapten isolated from various sources (47, 48). Forssman antigen activity has been detected in various cultured cells, including NIL cells (49-55), but the antigen has not been isolated nor chemically characterized from cultured hamster cells. The present study indicates that the structure of the antigen in both NIL and NILpy cells contains at least two polymorphic variations with regard to its internal carbohydrate chain, although they share the common terminal structure composed of 3 sugar residues, αGalNAc1-3βGalNAc1-3αGal.

Reduction of the chemical quantity of ceramide penta- and tetrasaccharides in NILpy cells as compared with that of NIL cells was observed in agreement with the observations previously reported (6-10), possibly due to blocked synthesis of ceramide trihexoside (11, 56). Nevertheless, their reactivities in NILpy cells with galactose oxidase were much greater than that in NIL cells. Two possibilities are considered to explain this phenomenon: (a) Increased reactivity is due to a deleted steric hindrance. The glycolipids on normal NIL cell membranes could be cryptic as they are imbedded in the lipid bilayer of cell membrane among ectoprotein or ektoglycoprotein bushes that extend on the surface of cells to mask a part of lipid bilayer. These cryptic glycolipids sites could be exposed by deletion of ectoprotein or ektoglycoprotein bushes, or by their dislocation. Deletion of at least one ectoprotein or ektoglycoprotein on the tumor cell surface has been increasingly apparent (3, 15, 57-59). (b) Increased reactivity is due to increased motility of glycolipid on transformed cells. It is likely that at least certain components on the tumor cell surface are more mobile than on normal cells (60-62). If glycolipid residues in the lipid bilayer of tumor cells are more mobile, this might account for the higher reactivity of glycolipids with surface label reagents, such as galactose oxidase.

Increased reactivity of glycolipid residues in virally transformed BHK and 3T3 cells with antibodies directed against glycolipid was demonstrated several years ago (63). "Exposure" of Forssman antigen site in transformed cells was claimed by Fogel and Sachs (49), O'Neill (50), Makita and Seyama (51), and Burger (53), and the finding is now essentially substantiated by the surface labeling method. A preliminary study with indirect immunofluorescence also indicates that membrane fluorescence by anti-globoside, anti-Forssman, and anti-lacto-N-neotetraosylceramide in the average cell population of NILpy cells is much greater than that of NIL cells.1

Organizational change of glycolipid during the cell cycle in terms of "exposure extent" of glycolipid can easily be detected by surface labeling. The finding that ceramide penta- and tetrasaccharides are markedly exposed during Gl phase and decrease during S phase in NIL cells agreed with the idea that some surface antigens, such as Forssman antigen and H2 histocompatibility antigens, are expressed maximally at Gl phase (55, 64). A dramatic increase in the incorporation of [14C]galactose into various glycolipids at Gl and M-phase of the cell cycle has been observed recently by Chatterjee et al. (65). It is increasingly apparent that both synthesis and organization of glycolipids on cell surface may determine cell cycle. Interestingly, the cell cycle dependent change of surface label in various glycolipids was minimum in NILpy cells, which supports the view that transitional change of cell surface function is in a "frozen state" in transformed cells (66, 67). The accompanying paper shows that the exposure of the surface glycoprotein and glycolipids can be modified by lectins.2


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