Glycolipids in a cultured human teratocarcinoma cell line (2102Ep) were investigated. The major glycolipids in these cells are globoseries glycolipids having the following structures:

\[
\begin{align*}
\text{GalNAc}\rightarrow\text{Gal}\rightarrow\text{Gal}\rightarrow\text{Glc} & \rightarrow \text{Cer} \\
\text{GalNAc}\rightarrow\text{Gal}\rightarrow\text{Gal}\rightarrow\text{Glc} & \rightarrow \text{Cer} \\
\text{GalNAc}\rightarrow\text{Gal}\rightarrow\text{Gal}\rightarrow\text{Glc} & \rightarrow \text{Cer} \\
\text{GalNAc}\rightarrow\text{Gal}\rightarrow\text{Gal}\rightarrow\text{Glc} & \rightarrow \text{Cer} \\
\end{align*}
\]

Synthesis of these structures by serial addition of galactose, fucose, and N-acetylgalactosamine to globo-side (Gb) in this teratocarcinoma is obvious, although further elongation of Gb in human cells and tissues has not been previously found with the exception of the presence of a small quantity of Forssman glycolipid in some tissues in the human population (F' group) and in some human cancers. The latter four glycolipids (b-e), with the common internal structure R→3GalNAc1→4Galal→4R', were all reactive to a monoclonal antibody directed to the 4- to 8-cell stage of murine embryos, known as the stage-specific embryonic antigen 3 (SSEA-3) (Shively, L. H., Knowles, B. B., Damjanov, I., and Solter, D. (1982) Cell 30, 697-705); structure (e) showed the strongest reactivity. These findings, together with the demonstration of the glycolipid nature of SSEA-1 antigens (Kannagi, R., Nudelman, E., Levery, S. B., and Hakomori, S. (1982) J. Biol. Chem. 257, 14865-14874), indicate that cell surface glycolipids play significant roles as differentiation antigens during the course of embryogenesis.

Changes in cell surface molecules have been observed during the process of embryogenesis and/or differentiation of cultured teratocarcinoma cells (see Refs. 1 and 2 for review). Many of these developmentally regulated antigens are carbohydrate in nature and include ABH, Forssman, globo-side, fi, and SSEA-1 (3-9). Such antigenic determinants may be carried by lipids and/or by protein molecules. Precise understanding of the glycoconjugates at the surface of embryos and teratocarcinoma cells is of increasing interest as these molecules may be important to the developmental potential of the cells. This paper describes characterization of the glycolipids in a cultured human teratocarcinoma, 2102Ep, cell line.

It has been previously reported that human teratocarcinoma cell lines express an embryonic antigen, SSEA-3, detected by a monoclonal antibody raised against 4- to 8-cell stage mouse embryos (10). The antigen is expressed in a stage-specific manner during early mouse embryogenesis and a change in the expression of SSEA-3 is also detected during the course of differentiation of human teratocarcinoma cells (11). The presence of an embryonic antigen common to mouse oocytes, mouse embryos, and human teratocarcinoma cell lines is of interest because of the possibility of conserved expression of such an antigenic determinant on functionally related cells from different species. The SSEA-3 antigenic determinant appears to be carbohydrate in nature and carried by both membrane glycolipids and glycoproteins (10). We have now purified glycolipids from human teratocarcinoma cells and studied their structure and reactivity with this monoclonal antibody.

MATERIALS AND METHODS

Cells and Antibodies—2102Ep cells were derived from a surgical specimen of a primary testicular germ-cell tumor containing embryonic carcinoma and yolk sac elements (11, 12). The cells were maintained in Dulbecco's modified minimal essential medium supplemented with 10% fetal bovine serum. For glycolipid analysis, 100 ml of packed cells were prepared from multiple harvests of cells grown in 15-cm plastic culture dishes. The monoclonal antibody to SSEA-3, the product of a rat spleen cell line fused with mouse myeloma cells (rat IgM) was prepared as described previously (10). A monoclonal antibody to Pk was a gift from J. Wiels, M. Lipinski, and T. Tursz, Institut Gustave-Roussy, Villejuif, France (13).

Received for publication, February 8, 1983)
Extraction and Purification of Glycolipids—Packed cells were homogenized and extracted with 20 volumes of chloroform/methanol (2:1, 1:1, and 1:2, v/v). After Folch’s partition (14), the lower layer glycolipids were freed from phospholipid contamination by acetylation (15). Upper layer and lower layer glycolipids were pooled and subjected to DEAE-Sephadex column chromatography to separate neutral and acidic glycolipids (16). Further purification of the glycolipids was performed by high performance liquid chromatography with a Varian HPLC system (model 5000, Varian Associates Inc., Walnut Creek, CA), using a column (1 × 50 cm) of Iatrobeads (IRS 8010, 10-μm diameter, Iatron, Tokyo) and eluted with a gradient of isopropanol/alcohol/hexane/water (17). The solvent composition for gradient elution is shown in the legend to Fig. 1.

Carbohydrate Analysis—Purified glycolipid was permethylated (18) and isolated by gel filtration on a column of Sephadex LH-20 in gradient elution is shown in the legend to Fig. 1. The neutral glycolipid fraction apparently contained only one glycolipid showing double spots (GL-7), which was eluted in fractions 53–57 with the same gradient system (Fig. 1b). Purified glycolipids GL-1 to GL-4, GL-6, and GL-7 showed a single spot on TLC after acetylation. Acetylated GL-5 was separated into three spots having very similar Rf values, 0.67, 0.64, and 0.61 (solvent system, dichloroethane/acetic acid/water, 60:40:0.1, v/v) and tentatively termed GL-5a, 5b, and 5c. GL-5b was a contained six glycolipids (each appeared as double spots on TLC), tentatively named GL-1 to GL-6 according to their mobility on TLC. Each glycolipid was purified to homogeneity by HPLC using a shallow gradient system (Fig. 1a) as shown in Fig. 2. GL-1 eluted in fractions 7–9, GL-2 in fractions 12–14, GL-3 in fractions 21–29, GL-4 in fractions 37–42, GL-5 in fractions 45–48, and GL-6 in fractions 51–54. The acidic glycolipid fraction apparently contained only one glycolipid showing double spots (GL-7), which was eluted in fractions 53–57 with the same gradient system (Fig. 1b). Purified glycolipids GL-1 to GL-4, GL-6, and GL-7 showed a single spot on TLC after acetylation. Acetylated GL-5 was separated into three spots having very similar Rf values, 0.67, 0.64, and 0.61 (solvent system, dichloroethane/acetic acid/water, 60:40:0.1, v/v) and tentatively termed GL-5a, 5b, and 5c.
Chemical Characterization of GL-1 to 4—GL-1 and 2 showed the same TLC mobilities as HexCer and LacCer standards prepared from human erythrocytes. Based on this finding and the following results on GL-3 to 7, the structure of these two glycolipids must be Glcβ1→1Cer and Galβ1→4Glcβ1→1Cer.

GL-3 was the major glycolipid in these cells, comprising about 60% of total glycolipids. GL-3 had the same TLC mobility as that of standard Gb4 (CTH) of human erythrocytes, both in the free form and in an acetylated form. Direct probe mass spectrometry showed characteristic ions for Hex- (m/z 219, 187, 155), Hex-O-Hex- (m/z 423, 391), and Hex-O-Hex-O-Hex- (m/z 627, 595, data not shown). Methylation analysis showed the presence of 2,3,4,6-O-Me4-Gal (terminal Gal), 2,3,6-O-Me4-Gal (→Gal1→1), and 2,3,6-O-Me4-Glc (→Glc1→1) as shown in Fig. 3a. GL-3 was cleaved by fig α-galactosidase, yielding a glycolipid having the same TLC mobility as GL-2. GL-3 was strongly reactive with a monoclonal Pk antibody (Fig. 4b). From these findings, the structure of GL-3 is identified as Galβ1→4Galβ1→4Glcβ1→1Cer (Gb4).

GL-4 comigrated with a Gb4 standard prepared from human erythrocytes on TLC. Direct probe mass spectrometry showed the characteristic ions for HexNAc- (m/z 260, 228), HexNAc-O-Hex- (m/z 464, 432), and HexNAc-O-Hex-O-Hex- (m/z 668, 636, data not shown). Methylation analysis (Fig. 3) showed minor component and GL-5a and c were the major glycolipids, comprising about 55 and 30% of the GL-5 glycolipid, respectively. Only GL-5a and c (Fig. 2, lanes 6 and 7) were further analyzed.

GL-6 showed the same TLC mobilities as HexCer and LacCer standards prepared from human erythrocytes. Based on this finding and the following results on GL-3 to 7, the structure of these two glycolipids must be Galβ1→4Glcβ1→1Cer and Galβ1→4Glcβ1→1Cer.

GL-5a and c were the major glycolipids, comprising about 55 and 30% of the GL-5 glycolipid, respectively. Only GL-5a and c (Fig. 2, lanes 6 and 7) were further analyzed.

**FIG. 3** Limited mass chromatogram of partially O-methylated hexitol and hexosaminitol acetates obtained from hydrolysis of permethylated human teratocarcinoma 2102Ep minor component and GL-5a and c were the major glycolipids, comprising about 55 and 30% of the GL-5 glycolipid, respectively. Only GL-5a and c (Fig. 2, lanes 6 and 7) were further analyzed.

**FIG. 4** Immunostaining of human teratocarcinoma glycolipids with monoclonal (a) SSEA-3 and (b) Pk antibodies. Total neutral glycolipids from teratocarcinoma 2102Ep cells were chromatographed on a HPTLC plate with solvent system of chloroform/methanol/water (60:35:8, v/v/v) and stained by TLC-immunostaining technique (see under “Materials and Methods”). For orcinol staining pattern of total neutral glycolipids, see Fig. 2, lane 1.
FIG. 5. Direct probe mass spectra of permethylated human teratocarcinoma glycolipids. Recorded with Finnigan 3300 mass spectrometer with 6110 data system after permethylation. a, GL-5c; b, GL-6; c, GL-7. Abscissa, m/z; ordinate, relative intensity (%).
the presence of 3,4,6-O-Me₆-GalNac (terminal GalNac), 2,4,6-O-Me₆-Gal (→3Gal→), 2,3,6-O-Me₆-Gal (→4Gal→), and 2,3,6-O-Me₆-Glc (→4Glc→). GL-4 was completely cleaved by β-N-acetylhexosaminidase from jack bean and yielded glycolipid spots having the same mobility as GL-3 doublets on TLC. Thus, the structure of GL-4 is identified as GalNacβ1→3Galβ1→4Glcβ1→1Cer (Gb₄).

Chemical Characterization of GL-5—GL-5 had a TLC mobility very similar to the standard asialo GM₁, prepared from human erythrocytes. Direct probe mass spectrometry of GL-5c (Fig. 5a) showed the characteristic ions for the structure Hex (m/z 219, 187, 155), Hex-O-HexNac (m/z 464, 432), and Hex-O-HexNac-O-Hex (m/z 872, 840). The other ions which can arise from the internal structure, O-HexNac-O-Hex- (m/z 450, 418), were also observed. The strong m/z 228 ion is the characteristic product from R→4HexNac1→ structure as reported (23, 24). The other ions observed are derived from the ceramide portion (m/z 364 from sphingosine, and others from various species of fatty acyl chain, m/z 294, 322, 350, 549, 577). GL-5a showed essentially the same results on direct probe mass spectrometry (data not shown) as GL-5c. The only difference observed between GL-5c and GL-5a is that the ions m/z 661, 659, 406, and 404 for ceramides containing C₂₄:₀ and C₂₄:₁ fatty acid were dominant in GL-5a and the ions m/z 549 and 294, which are characteristic for ceramides having C₁₆:₀ fatty acid, were dominant in GL-5c.

Methylation analysis of GL-5c showed the presence of 2,3,4,6-O-Me₆-Gal (terminal Gal), 4,6-O-Me₆-GalNac (→3GalNac1→), 2,4,6-O-Me₆-Gal (→3Gal1→), 2,3,6-O-Me₆-Gal (→4Gal1→), and 2,3,6-O-Me₆-Glc (→4Glc1→) as shown in Fig. 3c. The same neutral sugar derivatives were detected by GC-MS using an OV-225 capillary column (amino sugar derivatives do not elute from OV-225 capillary). Permethylation of GL-5a yielded exactly the same partially methylated alditol acetates as GL-5c on GC-MS analysis (data not shown). These results indicate that the subtle difference in TLC mobilities between GL-5a and GL-5c is due to the difference in the fatty acid composition of ceramide moieties. From these findings, the structure of GL-5a and GL-5c must be identical with galactosyl 1→3 Gb₄.

The anomeric structure of the terminal galactose residue in GL-5a and GL-5c was difficult to determine. Neither glycolipid was cleaved with jack bean β-galactosidase, although a nLc₄ standard bearing the Galβ1→4GlcNAC-terminus and a Gg₄ standard having a Galβ1→3GalNac-terminus were completely cleaved under the same conditions. Purified α-galactosidase from fig and β-galactosidase from A. niger also failed to cleave GL-5a and GL-5c. The only enzyme that cleaved GL-5a and GL-5c was β-galactosidase from C. lampas, as shown in Fig. 6. The complete hydrolysis of 20 µg of GL-5a or GL-5c was obtained with 20 µl of enzyme (1 unit/µl) after overnight incubation, yielding glycolipids having the same TLC mobility as the upper and lower spots of GL-4. Since this enzyme has so far not been utilized to determine the anomeric structure of glycolipid carbohydrates, a strict control study was performed. Under the same conditions, the enzyme preparation degraded nLc₄ into Lc₄, Galβ1→3nLc₄ into Lc₄, and Gg₄ to Gg₄, Gb₄, prepared from human erythrocytes having a Galα1→4Gal terminus, and Gb₄ having a Galα1→3Gal terminus, prepared by enzymatic degradation of Gb₄ (cytolipin-R) obtained from rat kidney, were not cleaved by the enzyme preparation. These findings confirm the specificity of the enzyme toward the β-galactose terminus. Based on these findings, we conclude that the terminal galactose in GL-5a and GL-5c is the β-anomer. The finding that GL-5a and GL-5c react with PNA lectin can be taken as additional evidence for the β-Gal terminus (data not shown). Thus, the entire structure of GL-5a and GL-5c must be Galβ1→3GalNacβ1→3Galα1→4Galβ1→4Glcβ1→1Cer (Gb₄). This structure was further confirmed by the results of 1H-NMR study for the anomeric resonances of underivatized GL-5a (Fig. 7a and Table 1). Three resonances corresponded to the first three saccharide units of Gb₄ given by Dabrowski et al. (25): 4.80 ppm, Galβ1→4; 4.26 ppm, Galβ1→3; 4.17 ppm, Glcβ1→1Cer. In their work and in ours, these three resonances were demonstrated to be shifted very little by further elongation of the saccharide chain, for the compounds tested. In addition, the β-Glc doublet can be seen to be broadened and distorted, most probably by the presence of the α-galac-

**Fig. 6.** HPTLC pattern of GL-5 after hydrolysis with C. lampas β-galactosidase. Lane 1, control GL-5a; lane 2, GL-5α cleaved with the enzyme; lane 3, control GL-5c; lane 4, GL-5c cleaved with the enzyme. Each glycolipid (5 µg) was incubated with 0.02 unit of enzyme in 20 µl of 0.1 M sodium citrate buffer, pH 4.0, with 0.02 ml of sodium taurodesoxycholate at 37 °C for overnight. Solvent system, chloroform/methanol/water (60:35:8, v/v/v); stained with orcinol/H₂SO₄ reagent. TC, sodium taurodesoxycholate; Gb₄, globo-side.

**Fig. 7.** Anomeric region proton NMR spectra of human teratocarcinoma glycolipids. a, GL-5a; b, GL-6; c, GL-7. Each glycolipid in 0.4 ml of dimethyl sulfoxide-d₄ (25); 1000 pulse (GL-5a and 7) or 500 pulse (GL-6) at 29 °C. A line-broadening program (LB = −1.0 Hz) was applied before transformation of free induction decays.
The structure of GL-6 was further confirmed by the 'H-NMR study. As shown in Fig. 7b, the spectrum of GL-6 contained six anomeric resonances, two of which coincide at 4.46 ppm. Three resonances from internal αGal, βGal, and βGlc were unchanged from their positions in the spectrum of GL-5 and were assigned similarly. The additional signal at 4.95 ppm had the extreme downfield position and vicinal coupling constant (J1,2 = 2.4 Hz), which is compatible with a terminal Fucα1→2 residue. The proton signals at 4.46 ppm, therefore, correspond to the GaINacβ1→3H-1 which has shifted upfield (Δδ = -0.15 ppm) and the Galβ1→3H-1 which has shifted downfield (Δδ = 0.26 ppm) from their positions in the GL-5a spectrum (Table I). The exact reason for the large changes in chemical shifts for these residues is not clear at present, but an analogous effect of terminal fucosylation on the anomeric resonances of internal sugar residues is reported with a type 1 chain H-active glycolipid and has been ascribed to the effect of sugar crowding upon fucosylation at the terminus (26). The type 1 chain H-terminal trisaccharide differs from that of GL-6 only at the C-4 configuration of the internal HexNAc. This difference should not alter the gross relative stereochemistry of the substituents. The analogy is supported by the presence of a quartet at 4.07 ppm, the position assigned for H-5 of Fucα1→2 of the type 1 chain H-terminal. For the type 2 chain H-terminal, this resonance was found at 4.00 ppm (26). The other signal in this region (Fig. 7b), a triplet centered at 4.10 ppm, is most probably the H-5 of α-galactose resonance, shifted upfield (Δδ = -0.06) from its position in GL-5. This shift can be taken as evidence for a very long range effect of fucosylation on the steric alignment of the glycosyl chain.

**Chemical Characterization of Ganglioside GL-7**—The ganglioside GL-7 showed a similar mobility to GM2α standard on TLC. GM1a, was prepared from mouse lymphoma L5178Y. Direct probe mass spectrometry showed characteristic ions for the structure NeuAc-O-Hex- (m/z 189, 157), NeuAc-O-Hex-O-Hex- (m/z 239, 361), NeuAc-O-Hex-O-HexNAc- (m/z 659, 607) and NeuAc-O-Hex-O-HexNAc-O-Hex- (m/z 843, 811) as shown in Fig. 5b. The strong m/z 228 ion was also observed. The other ions which can arise from the internal structures, -O-HexNAc-O- Hex- and/or -O-Hex-0-HexNAc-O- Hex- (m/z 450, 418) and -O-HexNAc-O-Hex-O-Hex- and/or -O-Hex-O-HexNAc-O-Hex- (m/z 654) were also observed.

Methylation analysis showed the presence of 2,4,6-O-Me6-Fuc (terminal Fuc), 4,6-O-Me6-GalNAc (α3Gallα→) and 2,3,6-O-Me6-Glc (α4Glcα→) (Fig. 3d). As to the derivatives of the galactose residues, GL-6 contained 2,3,6-O-Me6-Gal and twice the amount of 2,4,6-O-Me6-Gal and/or 3,4,6-O-Me6-Gal as shown in Fig. 3d-1. Since the peaks of 2,4,6-O-Me6-Gal and 3,4,6-O-Me6-Gal overlapped on the DB-5 column, an obvious that GL-6 contains equimolar amounts of 2,4,6-O-Me3-Gal, as shown in Fig. 3d-2. From these findings, it is obvious that GL-6 contains equimolar amounts of 2,4,6-O-Me6-Gal, 2,3,6-O-Me6-Gal, and 3,4,6-O-Me6-Gal (α3Gall→, α4Gall→, and α2Gall→), respectively. Thus, GL-6 yielded the same partially methylated alditol acetates as those from GL-5a or 5c; the only differences are the presence of terminal fucose and 2-substituted galactose residues, with the concomitant disappearance of the terminal galactose residue which was present in GL-5a and 5c. After defucosylation, GL-6 yielded glycolipids having the same mobility as GL-5a and 5c. The conversion of GL-6 to GL-5 by defucosylation in 0.1 N trichloroacetic acid was quantitative. Based on these findings, the structure of GL-6 is proposed to be a fucosylated GL-5, Fucα1→2Galβ1→3GaINacβ1→3Galα1→4Galβ1→4Glcβ1→1Cer.

<table>
<thead>
<tr>
<th>Glycolipid</th>
<th>Fucα1→2</th>
<th>Galβ1→3</th>
<th>GaINacβ1→3</th>
<th>Galα1→4</th>
<th>Galβ1→4</th>
<th>Glcβ1→1Cer</th>
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<tr>
<td>GL-6</td>
<td>4.52 (8.1)</td>
<td>4.81 (3.6)</td>
<td>4.26 (7.7)</td>
<td>4.16 (7.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GL-5</td>
<td>4.20 (7.3)</td>
<td>4.61 (8.3)</td>
<td>4.30 (7.3)</td>
<td>4.28 (7.3)</td>
<td>4.17 (7.3)</td>
<td></td>
</tr>
<tr>
<td>GL-7</td>
<td>4.95 (2.4)</td>
<td>4.46 (7.3)</td>
<td>4.80 (3.5)</td>
<td>4.26 (6.3)</td>
<td>4.17 (7.8)</td>
<td></td>
</tr>
</tbody>
</table>

*Data on GL (globoside) was taken from Dabrowski et al. (25).
The antibody does not seem to react with the terminal structure of GL-5, which is Galβ1→3GalNAcβ1→R, since Gg₄ having the same terminus did not cross-react with the antibody. In addition, GL-4, GL-6, GL-7, and Forssman antigen, which have entirely different terminal structures than that of GL-5, clearly cross-reacted with the antibody. The antibody seems to recognize the internal structures of these glycolipids, most probably the R→α2GalNAcβ1→3Galβ1→4Galβ1→R', the common internal sequence of these glycolipids. That GL-5 exhibited higher reactivity than the other glycolipids indicates that a favorable conformation of the internal determinant, R→α3GalNAcβ1→3Galβ1→4Galβ1→R', may be obtained by the Galβ1→3 substitution at the GalNAc residue in the determinant. The finding that the antibody did not react with Gg₄ which has the GalNAcβ1→3Galβ1→R terminal or IV²βGalNAcL₄ (X₂ glycolipid, Ref. 22) which has a GalNAcβ1→3Galβ1→R terminal indicates the importance of the 1→3 linkage between IV²βGalNAc and IIIαGal and the α-anomeric structure of the III-Gal. The 1→4 linkage between IIIαGal and IIβGal also seems important, since the reactivity of Gg₄ was significantly weaker than that of Gg₄.

**DISCUSSION**

This study was initiated to elucidate the structure of the glycolipid antigen reactive with the monoclonal antibody directed to SSEA-3 in human teratocarcinoma. Glycolipids in the teratocarcinoma 2102Ep cells were thoroughly analyzed by direct probe mass spectrometry, methylation analysis, enzymatic digestion, and nuclear magnetic resonance spectroscopy after extensive purification by HPLC. Almost all glycolipids which were visible on TLC by orcinol reaction were characterized, with the exception of one minor glycolipid comigrating with GL-5a and 5c. The proposed carbohydrate structures of these glycolipids are summarized in Table III. The human teratocarcinoma cell showed a characteristic glycolipid composition. All of the glycolipids characterized belonged exclusively to the globoseries glycolipids; no appreciable amounts of ganglio- or lactoseries glycolipids were detected. The synthetic pathway of these glycolipids in this cell line appears obvious from the carbohydrate structure of these glycolipids, the sequential conversion of each precursor glycolipid to a higher glycolipid by the stepwise addition of one terminal sugar residue.

The major terminal product of the synthetic pathway of globoseries glycolipids in human tissue was thought to be Gb₄ (globoside). The presence of these new structures revises the concept of the globoseries glycolipids in humans and makes the possibility that "extended globoseries" glycolipids, such as GL-5, 6, and 7, could be expressed in undifferentiated human tissues or embryos.

The presence of a large quantity of "extended globoseries" glycolipids detected in this cell line, including the novel structures GL-5, 6, and 7, may be unique for human teratocarcinoma and embryo; their chemical concentration in adult human cells and tissues must be very low or undetectable.

**TABLE II**

<table>
<thead>
<tr>
<th>Glycolipid</th>
<th>Binding of ¹²⁵I-Protein A</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL-3 (Gg₄)</td>
<td>0.6 ng</td>
</tr>
<tr>
<td>GL-4 (Gg₄)</td>
<td>6.3 ng</td>
</tr>
<tr>
<td>GL-6</td>
<td>50 ng</td>
</tr>
</tbody>
</table>

GL-5a showed essentially the same reactivity in other experiments.

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**Fig. 8. Inhibition of binding of the monoclonal SSEA-3 antibody to human teratocarcinoma 2102Ep cells by liposomes containing various glycolipids.**

1. GL-5a: O, GL-4: □, GL-3: ▲, iGb₄ (cytolipin R); ■, Gg₄ (asialo GM₃).

2. GL-5a contains various glycolipids.

3. Gg₄, (asialo GM₃).

4. GL-5a showed essentially the same reactivity in other experiments.

**Proton resonances as found in GL-5 (Fig. 7c and Table I).** The Galβ1→3 H-1 moved downfield by 0.04 ppm, as would be expected upon sialylation of this residue; the GalNAcβ1→3 H-1 moved upfield -0.04 ppm. The characteristic doublet of doublets for the H-3, of sialic acid was found at 2.77 ppm (not shown), which is within the region between 2.77-2.75 ppm. This signal is found for all terminal α2-3 sialylated glycolipids we have tested. The position of this resonance also has been used extensively in studies of oligosaccharides and glycopeptides for determination of sialic acid linkage with a high degree of reliability (see for example, Vliegenthart et al. (41) and references cited therein). The triplet at 4.13 ppm is again assigned to the H-5 of α-galactose which is shifted upfield (Δδ = 0.03) less than for GL-6.

**Immunological Reactivity of Teratocarcinoma Glycolipids to SSEA-3 Antibody.—As shown in Table II, GL-4 to 7 all reacted with the antibody to SSEA-3 in the solid state radioimmunoassay; the Forssman antigen was also weakly reactive. GL-5 showed the highest reactivity. Significant reactivity of GL-4 and GL-5 with the antibody was detected by TLC immunostaining using a 1:250 dilution of the antibody (Fig. 4a). However, when a 1:1000 dilution was used, only GL-5 was significantly reactive (data not shown). Results of binding inhibition test with 2102Ep cells also showed that GL-5 contained a higher affinity for the antibody than GL-4 (Fig. 8). A weak cross-reaction was observed with iGb₄ (cytolipin-R) purified from rat kidney, the isomer of Gg₄. A 50% inhibition of binding was obtained with 180 ng of GL-5c, 375 ng of GL-4, and 8.6 µg of cytolipin R per tube, respectively.

4. Previously we described a gangioside (G5) (39) which has very similar properties to teratocarcinoma GL-7 presented in this study. The TLC mobility of erythrocyte G5 was the same as a standard IV³αNeuAcGg₄, and desialylated G5 had a TLC mobility identical with Gg₄, similar to the teratocarcinoma GL-7 and GL-5 described in this study. At that time, erythrocyte G5 was tentatively identified as IV³αNeuAcGg₄, since the desialylated G5 reacted with a conventional anti-Gg₄ (asialo GM₃) antibody. The only difference between erythrocyte G5 and IV³αNeuAcGg₄ (GM₃) was that the desialylated G5 (supposed to be Gg₄ at that time) was not cleaved with any exoglycosidases tested, including the β-galactosidase from jack bean, which readily degraded a standard Gg₄ prepared from bovine brain under the same condition (see footnote of Ref. 39). The behavior of desialy-
Previously, Kundu et al. (40) described a dialysol derivative of a glycolipid with a similar sugar sequence to GL-5 as a very minor component of human erythrocyte membranes. The position of carbohydrate linkages and anomeric structure remain to be elucidated. Other examples of the presence of "extended globoseries" glycolipids in human tissue are Fursman antigen in tissues of a small Fursman positive population (27) and "para-Fursman antigen" as a very minor component of human erythrocytes (28).

GL-5 carries the terminal sugar sequence, Galβ1→3GalNAcβ1→Galβ1→3Gal1→4Glc1→1Cer, which is identical with the terminal sequence of Gaβ (asialo GM). Some of the asialo GM-reactive antibodies may cross-react with GL-5. Because of this terminal sugar sequence, Gaβ can react with PNA lectin and has been regarded as the glycolipid receptor for PNA lectin. GL-5 can be another PNA receptor glycolipid, which is carried by the globoseries core structure. GL-6 carries an H-active terminus. It is known that the H-active terminus in erythrocytes and/or intestinal tissue is carried by lactoseries and/or neolactoseries core structures (29); the H-terminus carried by gangliosides of glycolipids has also been reported (30). GL-6 is the first example of a globoseries glycolipid which carries the H-terminal structure. The terminal structure of GL-7 is identical with that of IVαNeuAcGg, (GMβ). It would be of interest to test if the glycosyltransferases involved in the synthesis of the terminal structures carried by the globoseries glycolipids are identical with those active in the synthesis of the same terminal structures which are ordinarily found in gangliosides in other human cells and tissues. A glycolipid having the same sugar sequence as GL-5 has been suggested to be present in cultured green monkey cells (31). However, the anomeric structure and/or linkage of sugar residues have not been fully elucidated. The anomeric structure of the terminal Gaβ in pentaglycosylceramide isolated from green monkey kidney cells was tentatively assigned as α because the glycolipid did not have any blood group B or P activity. The assignment of the anomeric structure by NMR was difficult because of a shortage of material (31). The chemical basis of the structure of a similar glycolipid to GL-7 detected in chick muscle has not been described so far (32, 33).

Since the antibody defining SSEA-3 seems to react with the sequence R→3GalNAcβ1→3Galα1→R', the terminal structure of GL-4 (globoside) and the internal structure of GL-5, 6, and 7, it is a useful reagent to detect the globoseries glycolipids. Most carbohydrate-reactive antibodies are directed to the terminal sugar structures; however, antibodies reacting with an internal sequence are known; a monoclonal IgM antibody reactive with both globoside and Fursman (34) and various types of Ii-reactive antibodies (29) are good examples. Even though the antibody is directed to the internal structure, its reactivity is indirectly affected by the terminal structure, probably due to changes in the tertiary structure of the internal sugar chain, as suggested by the NMR study.

The presence of SSEA-3 antigens in human teratocarcinoma cells raises the possibility that the antigen is also present in human embryos and plays a role as a stage-specific antigen. The presence of P and Pk antigens on the mouse embryo has been detected using polyclonal antisera (9). It is noteworthy that the structure of SSEA-3 active human glycolipids described in this paper includes the P-blood group antigen and its further metabolites. Since all the globoseries glycolipids so far characterized play a role as alloantigens in the P-blood group system (35), it could be predicted that some of the new structures found in the human teratocarcinoma cells may display previously uncharacterized antigens in P-blood group system. It is well known that individuals of rare phenotype have a high incidence of abortion and it is suggested to be due to the reaction of anti-PP, Pα antibody in the maternal serum with corresponding antigens in the fetus (36, 37). Frequency of the abortions is particularly high at the early stages of pregnancy. It is possible that P-antigen and/or other antigens in P-blood group system play a role as stage-specific developmental antigens not only in mouse but also in human embryogenesis, and the frequency of abortion depends upon the variable degree of surface expression of these antigens on the fetal cells and tissues during the course of embryogenesis.

Recently we have also elucidated the complete structures of the SSEA-1-containing glycolipids (8). This antigenic determinant, like SSEA-3, is found on the surface of murine embryo, but it is expressed at a later stage of preimplantation development (7, 10). The finding that both of these antigenic determinants are carbohydrates which can be borne by glycolipid molecules indicates the importance of changes in the cell surface glycolipids in the developing embryo.

The antigenic transformation from SSEA-3"SSEA-1" to SSEA-3"SSEA-1" status has also been detected during the course of in vitro differentiation system of human teratocarcinoma cells (11). SSEA-1 antigens are carried by a set of lactoseries glycolipids (8), and SSEA-3 antigens are carried

<table>
<thead>
<tr>
<th>Glycolipid</th>
<th>Structure</th>
<th>SSEA-3 activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>GL-3</td>
<td>Galα1→Galβ1→Glcβ1→1Cer</td>
<td>(+)</td>
</tr>
<tr>
<td>GL-4</td>
<td>Galβ1→3GalNAcβ1→Galβ1→3Gal1→4Glc1→1Cer</td>
<td>(+)</td>
</tr>
<tr>
<td>GL-5</td>
<td>Galβ1→3GalNAcβ1→Galβ1→3Gal1→4Glc1→1Cer</td>
<td>(+)</td>
</tr>
<tr>
<td>GL-6</td>
<td>Fucα1→2Galβ1→3GalNAcβ1→Galβ1→3Gal1→4Glc1→1Cer</td>
<td>(+)</td>
</tr>
<tr>
<td>GL-7</td>
<td>NeuAca2→3Galβ1→3GalNAcβ1→Galβ1→3Gal1→4Glc1→1Cer</td>
<td>(+)</td>
</tr>
</tbody>
</table>

Fig. 9. Synthetic pathways of SSEA-1 and SSEA-3 active glycoliposidolipids. a, the synthetic pathway of glycoliposidolipids which leads to the synthesis of a set of SSEA-3 active glycolipids; b, the synthetic pathway of neolactoseries glycolipids, fucosylation at internal GalNAc residues which leads to the synthesis of a set of SSEA-1 active glycolipids (8). A switching or shift of glycolipid synthesis from pathway a to pathway b is suggested to occur in mouse early embryogenesis.
by a set of globoseries glycolipids. Thus, in terms of glycolipid antigens, SSEA-1 and SSEA-3 antigens belong to entirely different species of glycolipids, and the synthetic pathways for the two antigens are also entirely different (Fig. 9). Therefore, the transition in expression of these antigens observed in mouse embryogenesis and differentiation of human teratocarcinoma cells is not due to the simple addition of one or a few sugar residues to pre-existing carbohydrate chains, but involves dynamic changes covering multiple synthetic pathways of cellular glycolipids, i.e. synthesis of lactoseries and globoseries glycolipids. Thus an extensive change in the synthesis of cell surface carbohydrates might occur between the 4–8 cell and morula stages. This type of drastic change in glycolipid synthesis involving more than one synthetic pathway is not necessarily unusual; a similar type of alteration of cellular antigenicity carried by lacto- and globoseries glycolipids was reported to occur during the course of differentiation of a mouse leukemia cell line (38).

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