New Globoseries Glycosphingolipids in Human Teratocarcinoma Reactive with the Monoclonal Antibody Directed to a Developmentally Regulated Antigen, Stage-specific Embryonic Antigen 3*

(Received for publication, February 8, 1983)

Reiji Kannagi†, Steven B. Levery‡, Funatsuugo Ishigami§, Sen-itiroh Hakomori¶, Lynne H. Shevinsky††, Barbara B. Knowles†††, and Davor Solter†††

From the Division of Biochemical Oncology, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104 and the Wistar Institute for Anatomy and Biology, Philadelphia, Pennsylvania 19104

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

- **Galα1-3Galβ1-4Glcα1-Cer** (α, Glcα1-4-antigen)
- **Galβ1-Nacα1-3Galβ1-4Glcα1-Cer** (β, Glcα1-3-antigen)
- **Galβ1-4Glcα1-4Galβ1-4Glcα1-Cer** (γ)
- **Galβ1-3Galβ1-4Galβ1-4Glcα1-Cer** (δ)
- **Galβ1-4Glcα1-4Galβ1-3Galβ1-4Glcα1-Cer** (ε)
- **Galβ1-4Glcα1-4Glcα1-4Glcα1-Cer** (ζ)

Synthesis of these structures by serial addition of galactose, fucose, and N-acetylneuraminic acid to globo-side (Gbα) in this teratocarcinoma is obvious, although further elongation of Gbα 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 (Fe't group) and in some human cancers. The latter four glycolipids (b–e), with the common internal structure $R \rightarrow 3$GalαNac$O\rightarrow 3Galα1-4\rightarrow 4R'$, 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) (Shevinsky, L. H., Knowles, B. B., Damjanov, I., and Solter, D. (1982) Cell 30, 697–705); structure (ε) 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, fucoside, 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 splenocyte fused with mouse myeloma cells (rat IgM) was prepared as described previously (10). A monoclonal antibody to Pα was a gift from J. Weis, M. Lipinski, and T. Turz, Institut Gustave-Roussy, Villejuif, France (13).

*This work was supported by National Institutes of Health Grants CA19224, CA10815, and HD12847. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

††† Recipient of National Science Foundation Grant PCM 81-18801 in support of this work.

** The abbreviations used are: SSEA, stage-specific embryonic antigen; TLC, thin layer chromatography; HPTLC, high performance thin layer chromatography; HPLC, high performance liquid chromatography; GC-MS, gas chromatography-mass spectrometry; PBS, phosphate-buffered saline, pH 7.4; HexCer, hexosylceramide Glcβ1→1Cer; LacCer, lactosylceramide, Galβ1-4Glcβ1→1Cer; Gβ, globotriaosylceramide (ceramide trihexoside, CTH), Galα1-4Galβ1-4Glcβ1→1Cer; Gα, globotetraosylceramide (paragloboside), Ga1β1-4Glcβ1-4Galβ1-4Glcβ1→1Cer; Gt, globotriaosylceramide (cytolipin R), Galα1-4Glcβ1-4Galβ1-4Glcβ1→1Cer; Gb, globotetraosylceramide (paragloboside), Ga1β1-4Glcβ1-4Galβ1-4Glcβ1→1Cer; Gb, globotriaosylceramide (asialo GMα), Ga1β1-4Glcβ1-4Galβ1-4Glcβ1→1Cer; Gβ, globotetraosylceramide (asialo GMβ), Galβ1-4Glcβ1-4Galβ1-4Glcβ1→1Cer; Gt, globotriaosylceramide (asialo GMt), Galβ1-4Glcβ1-4Galβ1-4Glcβ1→1Cer; Gb, globotetraosylceramide (asialo GMβ), Galβ1-4Glcβ1-4Galβ1-4Glcβ1→1Cer; IVα, NeuAcα2→3Galβ1-4Glcβ1-4Galβ1-4Glcβ1→1Cer; GMα, NeuAcα2→3Galβ1-4Glcβ1-4Galβ1-4Glcβ1→1Cer; GL, glycolipid; PNA, peanut agglutinin.

8934
Extraction and Purification of Glycolipids—Packed cells were homogenized and extracted with 20 volumes of chloroform/methanol (2:1, 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 X 50 cm) of Iatrobeads (IRS 8010, 10-μm diameter, Iatron, Tokyo) and eluted with a gradient of isopropanol/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 chloroform-methanol (1:1). Permethylated neutral glycolipids were hydrolyzed in 90% acetic acid containing 0.5 M sulfuric acid at 80°C for 6-8 h (19). Methanolysis was performed to obtain derivatives of sialic acid of the acidic glycolipids (20). Partially methylated alditol acetates were analyzed by chemical ionization gas chromatography—mass spectrometry using a Finnigan 3300 mass spectrometer with a 6110 data system. A DB-5 column (J and W Scientific Co., Rancho Cordova, CA) was used for gas chromatographic analysis. Alternatively, an OV-225 column (Supelco Inc., Bellefonte, PA) was used for separation of 2,4,6-Me3-O from 3,4,6-Me3-Gal.

H-NMR was performed with deuterium-exchanged 100-μg purified glycolipids dissolved in 0.4 ml of dimethyl sulfoxide-d6 containing 2% D2O and 1% trimethylsilyl (25) and recorded with a 500-MHz NMR spectrometer (model WM-500, Bruker, W. Germany) in the Fourier-transform mode using quadrature detection, an excitation pulse angle of 90°, and collecting 16,000 data points for a 4-KHz spectral width.

Immunological Reactivities of Glycolipids—The reactivity of the glycolipid with antibodies was ascertained by three different methods. TLC immunostaining was performed as described previously (8, 21). Briefly, glycolipid was chromatographed on HPTLC plates (Si-HPF plates, J. T. Baker Chemical Co.) and reacted successively with 1:1000 diluted monoclonal SSEA-3 antibody (both rat IgM, 1:1000 rabbit anti-rat IgM (μ-chain specific), and 125I-labeled protein A solution. After washing, TLC plates were subjected to autoradiography. Solid phase radioimmunoassay was performed with vinyl assay strips (Costar, Cambridge, MA). Glycolipids (~50 ng/well) were dissolved in ethanol with phosphatidylycerine and cholesterol (200 μg mg/well), dried at 37°C to remove adsorption to the bottom of each well. After treatment with PBS containing 5% bovine serum albumin, the well was reagent successively with 1:500 diluted monoclonal SSEA-3 antibody (IgM), 1:1000 diluted rabbit anti-rat IgM, and 125I-protein A solution. After washing, the radioactivity of each well was measured by a γ-scintillation counter. The cell-binding inhibition test was performed as follows: 5 X 10⁶ 2102Ep cells were incubated for 1 h at 37°C with a 1:1000 dilution of monoclonal SSEA-3 antibody (50 μl/tube) in the presence or absence of liposome suspensions (50 μl/tube) containing various amounts of glycolipids. Liposomes were made from 50 μg of glycolipid, 200 μg of phosphatidylycerine, 150 μg of cholesterol, and 7.5 μg of dicetylphosphate, suspended by sonication in 0.25 ml of PBS. Serial dilution was made by diluting this liposome suspension with PBS. After incubation at room temperature for 1 h, liposomes and unreacted antibody were removed by washing the cells three times with PBS. The cells were then incubated with 50 μl/tube of a 1:1000 dilution of second antibody (rabbit anti-rat IgM) at room temperature for 1 h, washed, and reacted with 125I-labeled protein A solution. Radioactivity absorbed to the cells was determined with a γ-scintillation counter.

RESULTS

Purification of 2102Ep Teratocarcinoma Glycolipids on HPLC—A typical elution pattern on HPLC, of neutral and acidic glycolipids prepared from the teratocarcinoma cells is shown in Fig. 1. The neutral glycolipid fraction apparently 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/water, 60:40:0.1, v/v) and tentatively termed GL-5a, 5b, and 5c. GL-5b was a

FIG. 1. Purification of human teratocarcinoma 2102Ep glycolipids on HPLC. a, neutral glycolipids; b, acidic glycolipids (gangliosides). Gradient system for HPLC was from isopropanol/hexane/water (55:43:2, v/v/v) to 55:30:15 for 200 min at a flow rate of 3 ml/min. The pressure was maintained between 44 to 85 atm throughout the chromatography. Elute was collected at every 2 min (6 ml). Glycolipids in each fraction were detected by TLC stained with orcinol/H2SO4 reagent. Solvent system for TLC was chloroform/methanol/water (60:35:8, v/v/v). C, total neutral glycolipids; C', total acidic glycolipids prepared from the teratocarcinoma cells, serving as mobility controls.

FIG. 2. HPTLC pattern of purified glycolipids of human teratocarcinoma 2102Ep cells. Lane 1, total neutral glycolipids; lane 2, total acidic glycolipids (gangliosides); lane 3, purified GL-3; lane 4, GL-4; lane 5, GL-5; lanes 6 and 7, GL-5a and 5c after acetylation purification; lane 8, GL-6; lane 9, GL-7. Solvent system, chloroform/methanol/water (60:35:8, v/v/v); stained with orcinol/H2SO4 reagent.

Human Teratocarcinoma SSEA-3 Glycolipids

8935

Downloaded from http://www.jbc.org/ by guest on March 24, 2020
Human Teratocarcinoma SSEA-3 Glycolipids

1. d-2

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 5c (Fig. 2, lanes 6 and 7) were further analyzed.

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 Gb1 (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-Me3-Gal, and 2,3,6-O-Me3-Glc (m/z 421) 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 Pκ antibody (Fig. 4b). From these findings, the structure of GL-3 is identified as Galβ1→4Glcβ1→1Cer (Gb1).

GL-4 comigrated with a Gb1 standard prepared from human erythrocytes on TLC. Direct probe mass spectrometry showed the characteristic ions for HexNAc- (m/z 260, 228), HexNAc-0-Hex- (m/z 464, 432), and HexNAc-0-Hex-0-Hex- (m/z 668, 636, data not shown). Methylation analysis (Fig. 3) showed

glycolipids. a, GL-3; b, GL-4; c, GL-5c; d, GL-6 (d-1, DB-5 column; d-2, OV-225 column); e, GL-7 (e-1, after acetylation; e-2, after methanolation). A mixture of acetic acid and sulfuric acid was used for the hydrolysis of GL-3 to GL-7 (19). Methanolation was performed with an aliquot of permethylated GL-7 to obtain derivatives of sialic acid residues. Gas chromatography was performed on a DB-5 bonded phase fused silica capillary column with oven temperature programmed from 140–240 °C at 4 °C/min except in d-2, where the OV-225 column was used for the separation of 2,4,6-O-Me3-Gal, and in e-2 where the temperature was programmed at 230 °C isothermal (DB-5 column). Abscissa, scan number; ordinate, the sum of the intensities of the ions at m/z [MH+60]+ and [MH-32]+ for O-methylhexitol acetates, and at m/z [MH-60]+ and MH+ for O-methylhexosaminitol acetates.

Fig. 4. Immunostaining of human teratocarcinoma glycolipids with monoclonal (a) SSEA-3 and (b) Pκ antibodies. Total neutral glycolipids from teratocarcinoma 2102Ep cells were chromatographed on a HPTLC plate with solvent system of chloroform/methanol/water (60:35:2, 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-Me₃-GalNac (terminal GalNac), 2,4,6-O-Me₃-Gal (→3Gal→), 2,3,6-O-Me₃-Gal (→4Gal→), and 2,3,6-O-Me₃-Glc (→3Glc→). GL-4 was completely cleaved by β-N-acetylgalactosaminidase 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→3Galα1→4Glcβ1→1Cer (Gb₃).

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, -0-HexNac-O-Hex- (m/z 450, 418), were also observed. The strong m/z 228 ion is the characteristic product from R-→HexNac1→ 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-Me₃-Gal (terminal Gal), 4,6-O-Me₃-GalNac (→3GalNac→), 2,4,6-O-Me₃-Gal (→3Gal→), 2,3,6-O-Me₃-Gal (→4Gal→), and 2,3,6-O-Me₃-Glc (→3Glc→) as shown in Fig. 3c. The same neutral sugar derivatives were detected by GC-MS using an OV-225 capillary column (amino sugar side-chain). The strong m/z 228 ion is the characteristic product from R-→HexNac1→ 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-Me₃-Gal (terminal Gal), 4,6-O-Me₃-GalNac (→3GalNac→), 2,4,6-O-Me₃-Gal (→3Gal→), 2,3,6-O-Me₃-Gal (→4Gal→), and 2,3,6-O-Me₃-Glc (→3Glc→) as shown in Fig. 3c. The same neutral sugar derivatives were detected by GC-MS using an OV-225 capillary column (amino sugar side-chain). The strong m/z 228 ion is the characteristic product from R-→HexNac1→ 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-Me₃-Gal (terminal Gal), 4,6-O-Me₃-GalNac (→3GalNac→), 2,4,6-O-Me₃-Gal (→3Gal→), 2,3,6-O-Me₃-Gal (→4Gal→), and 2,3,6-O-Me₃-Glc (→3Glc→) as shown in Fig. 3c. The same neutral sugar derivatives were detected by GC-MS using an OV-225 capillary column (amino sugar side-chain). The strong m/z 228 ion is the characteristic product from R-→HexNac1→ 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-Me₃-Gal (terminal Gal), 4,6-O-Me₃-GalNac (→3GalNac→), 2,4,6-O-Me₃-Gal (→3Gal→), 2,3,6-O-Me₃-Gal (→4Gal→), and 2,3,6-O-Me₃-Glc (→3Glc→) as shown in Fig. 3c. The same neutral sugar derivatives were detected by GC-MS using an OV-225 capillary column (amino sugar side-chain). The strong m/z 228 ion is the characteristic product from R-→HexNac1→ 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.

FIG. 6. HPTLC pattern of GL-5 after hydrolysis with C. lamrus β-galactosidase. Lane 1, control GL-5a; lane 2, GL-5a 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 1 mg/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; Gb₃, globoside.

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.
Glycosyl H-1 chemical shifts (ppm from trimethylsilyl) and $^3J_{1,2}$ coupling constants (Hz) of glycolipids from human teratocarcinoma cells

| Glycolipid  | Fucal-1→2 | Galβ1→3 | Galαβ1→3 | Galα1→4 | Galβ1→4 | Glcβ1→1Cer |
|------------|-----------|---------|---------|---------|---------|-------------|
| Glycolipid  | $\delta$ (J) | $\delta$ (J) | $\delta$ (J) | $\delta$ (J) | $\delta$ (J) | $\delta$ (J) |
| Gbα*      |           |         |         |         |         |             |
| GL-5       | 4.20 (7.3) | 4.61 (8.3) | 4.80 (3.5) | 4.26 (6.3) | 4.17 (7.8) |             |
| GL-6       | 4.05 (2.4) | 4.46 (7.3) | 4.61 (8.3) | 4.26 (6.3) | 4.17 (7.8) |             |
| GL-7 (NeuAc2→3) | 4.24 (7.8) | 4.57 (8.3) | 4.81 (2.9) | 4.26 (7.8) | 4.19 (6.3) |             |

*Data on Gbα (globoside) was taken from Dabrowski et al. (25).

The structure was further confirmed with the $^1$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 ($J_{1,2} = 2.4$ Hz), which is compatible with a terminal Fucal-1→2 residue. The proton signals at 4.46 ppm, therefore, correspond to the Galαβ1→3 H-1 which has shifted upfield ($\Delta\delta = -0.15$ ppm) and the Galβ1→3 H-1 which has shifted downfield ($\Delta\delta = 0.26$ 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 stereochemistry and vicinal 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 Fucal-1→2 of the type 1 chain H-terminal. For the type 1 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 ($\Delta\delta = -0.06$) 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.
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→GalNAcβ1→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→GalNAcβ1→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 Gg₃ which has the GalNAcβ1→4Galβ→R terminal or IVβ2GalNAcLe₄ (X₀ glycolipid, Ref. 22) which has a GalNAcβ1→3Galβ→R terminal indicates the importance of the 1→3 linkage between IVβ2GalNAc 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 Gb₄.

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 glycolipid which were visible on TLC by orcinol reaction.

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 raises 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.²

³Previously 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 IVβ2NeuAcGg₄, 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 IVβ2NeuAcGg₄, since the desialylated G5 reacted with a conventional anti-Gg₄ (asialo GM₃) antibody. The only difference between erythrocyte G5 and IVβ2NeuAcGg₄ (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 disialosyl 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 anomic structure remain to be elucidated. Other examples of the presence of "extended globoseries" glycolipids in human tissue are Forsmann antigen in tissues of a small Forsmann positive population (27) and "para-Forsmann antigen" as a very minor component of human erythrocytes (28).

GL-5 carries the terminal sugar sequence, Galα1→3GalNAcβ1→3Galα1→4Glcβ1→1Cer, which is identical with the terminal sequence of Gaβ, (asialo GM1). Some of the asialo GM1-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 ganglioseries 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 IVαNeuAcα1→3Galβ1→4Glcβ1→1Cer. 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 ganglio- or lactoseries glycolipids 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 kidney cells (31). However, the anomic structure and/or linkage of sugar residues have not been fully elucidated. The anomic structure of the terminal Gal 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 anomic 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→3Galβ1→3Galα1→4Glcβ1→1Cer, 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 to an internal sequence are known; a monoclonal IgM antibody reactive with both globoside and Forssmann (34) and various types of Ig-reactive antibodies (29) are good examples. Even though the antibody is directed to the internal structure, its reactivity is indirectly affected by the terminal

| Glycolipid | Structure | SSEA-3 activity |
|-----------|-----------|-----------------|
| GL-3      | Galα1→4Galβ1→4Glcβ1→1Cer | (+)             |
| GL-4      | Galβ1→3GalNAcβ1→3Galα1→4Glcβ1→1Cer | (+)             |
| GL-5      | Galα1→3GalNAcβ1→3Galα1→4Glcβ1→1Cer | (+)             |
| GL-6      | Fuco1→2Galβ1→3GalNAcβ1→3Galα1→4Glcβ1→1Cer | (+)             |
| GL-7      | NeuAcα2→3Galβ1→3GalNAcβ1→3Galα1→4Glcβ1→1Cer | (+)             |

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, it is well known that individuals of rare pphenotype have a high incidence of abortion, and it is suggested to be due to the reaction of anti-PP,P 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 been also 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
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 globoseries glycolipids. Thus an extensive change in the synthesis of lacto- and globoseries glycolipids was reported to occur during the course of differentiation of a mouse leukemia cell line (38).

Acknowledgments—We thank Drs. Gary Drobny, Dennis Hare, and Eric Shankland of the University of Washington for assistance in NMR spectrometry.

REFERENCES

1. Jacob, F. (1977) Immunol. Rev. 33, 3–32
2. Solter, D., and Knowles, B. B. (1979) Curr. Top. Dev. Biol. 13, 139–165
3. Saulman, A. E. (1980) Curr. Top. Dev. Biol. 14, 127–145
4. Stern, P. L., Willison, K. R., Lennox, E., Galfrè, G., Milstein, C., Secher, D., Ziegler, A., and Springer, T. (1978) Cell 14, 775–783
5. Kapadia, A., Feizi, T., and Evans, M. J. (1981) Exp. Cell. Res. 131, 185–195
6. Solter, D., and Knowles, B. B. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 5565–5569
7. Gooi, H. C., Feizi, T., Kapadia, A., Knowles, B. B., Solter, D., and Evans, J. M. (1981) Nature (Lond.) 292, 156–158
8. Kannagi, R., Nudelman, E., Levery, S. B., and Hakomori, S. (1982) J. Biol. Chem. 257, 14865–14874
9. Willison, K. R., Karol, R. A., Suzuki, A., Kundu, S. K., and Marcus, D. M. (1982) J. Immunol. 129, 603–609
10. Shevinsky, L. H., Knowles, B. B., Damjanov, I., and Solter, D. (1982) Cell 30, 697–705
11. Andrews, P. W., Goodfellow, P. N., Shevinsky, L. H., Bronson, D. L., and Knowles, B. B. (1982) Int. J. Cancer 29, 523–531
12. Wang, N., Trend, B., Bronson, D. L., and Fraley, E. E. (1980) Cancer Res. 40, 796–802
13. Nudelman, E., Kannagi, R., Hakomori, S., Parsons, M., Lipinski, M., Wiels, J., Fellows, M., and Tursz, T. (1983) Science 220, 509–511
14. Folch, J., Arsov, S., and Meath, J. A. (1951) J. Biol. Chem. 191, 819–831
15. Saito, T., and Hakomori, S. (1971) J. Lipid Res. 12, 257–259
16. Yu, R. K., and Ledeen, R. W. (1972) J. Lipid Res. 13, 689–686
17. Watanabe, K., and Arah, Y. (1981) J. Lipid Res. 22, 1020–1024
18. Hakomori, S. (1964) J. Biochem. (Tokyo) 55, 259–268
19. Stellner, K., Saito, H., and Hakomori, S. (1973) Arch. Biochem. Biophys. 155, 464–472
20. Rauvala, H., and Kärkkäinen, J. (1977) Carbohydr. Res. 56, 1–9
21. Magnani, J. L., Smith, D. F., and Ginsburg, V. (1980) Anal. Biochem. 109, 399–402
22. Kannagi, R., Fukuda, M. N., and Hakomori, S. (1982) J. Biol. Chem. 257, 4438–4442
23. Egge, H., and Hanfland, P. (1981) Arch. Biochem. Biophys. 210, 396–404
24. Hanfland, P., and Egge, H. (1975) Chem. Phys. Lipids 15, 243–247
25. Dabrowski, J., Hanfland, P., and Egge, H. (1980) Biochemistry 19, 5652–5658
26. Dabrowski, J., Hanfland, P., Egge, H., and Dabrowski, U. (1981) Arch. Biochem. Biophys. 210, 405–411
27. Hakomori, S., Wang, S. M., and Young, W. W., Jr. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 3023–3027
28. Ando, S., Kon, K., Nagai, Y., and Yamakawa, T. (1982) in New Vistas in Glycolipid Research (Makita, A., Handa, S., Taketomi, T., and Nagai, Y., eds) pp. 71–81, Plenum Publishing Corp., New York
29. Hakomori, S. (1981) Semin. Hematol. 18, 39–62
30. Wiegandt, H. (1973) Z. Physiol. Chem. 354, 1049–1056
31. Bloemberg, J., Breimer, M. E., and Karlsson, K-A. (1982) Biochim. Biophys. Acta 711, 469–477
32. Chien, J-L., and Hogan, E. L. (1980) Fed. Proc. 39, 2183 (Abstr. 3040)
33. Hogan, E., Happel, R. D., and Chien, J-L. (1982) in New Vistas in Glycolipid Research (Makita, A., Handa, S., Taketomi, T., and Nagai, Y., eds) pp. 273–278, Plenum Publishing Corp., New York
34. Naiki, M., and Marcus, D. M. (1977) J. Immunol. 119, 537–539
35. Marcus, D. M., Naiki, M., and Kundu, S. K. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 3263–3267
36. Iseki, S., Masaki, S., and Levine, P. (1954) Nature (Lond.) 173, 1193–1194
37. Levine, P., and Koch-Elizabeth, A. (1964) Science 120, 239–241
38. Kannagi, R., Levery, S. B., and Hakomori, S. (1983) Proc. Natl. Acad. Sci. U. S. A., in press
39. Watanabe, K., Powell, M. E., and Hakomori, S. (1979) J. Biol. Chem. 254, 8223–8229
40. Kundu, S. K., Marcus, D. M., Pascher, I., and Samuelsson, B. E. (1981) Fed. Proc. 40, 1545 (Abstr. 37)
41. Vliegenthart, J. F. G., Van Halbeek, H., and Dorland, L. (1981) Pure Appl. Chem. 53, 45–77
New globoseries glycosphingolipids in human teratocarcinoma reactive with the monoclonal antibody directed to a developmentally regulated antigen, stage-specific embryonic antigen 3.

R Kannagi, S B Levery, F Ishigami, S Hakomori, L H Shevinsky, B B Knowles and D Solter

J. Biol. Chem. 1983, 258:8934-8942.