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MONOCLONAL ANTIBODY DIRECTED TO THE STAGE-SPECIFIC EMBRYONIC ANTIGEN (SSEA-1) REACTS WITH A BRANCHED GLYCOSPHINGOLIPID SIMILAR IN STRUCTURE TO Ii ANTIGEN*

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Summary - A monoclonal antibody reacting with early mouse embryos and murine embryonal carcinoma cells (F9) defines the stage-specific embryonic antigen (SSEA-1). We now report that the antigen (SSEA-1) is a complex glycolipid with the branched lacto-N-glycosyl series. Antibody to SSEA-1 reacts strongly with the branched H_4-glycosphingolipid but not with other various glycolipids so far tested. This reactivity was abolished by endo-β-galactosidase treatment. The homogeneous H_4-glycolipid not only reacted with the monoclonal antibody to SSEA-1 but also with antibody to I-(Ma), i-(Dench) and with anti-H specific lectin. Chemical analysis, including methylation, also indicates that the glycolipid antigen had a close resemblance to I-antigen.

Cellular interactions during embryonic development and differentiation are probably mediated through cell surface molecules. Such molecules have been detected by immunological methods and have been described as developmentally regulated antigens. These include ABH (1), Forssman (2,3), Ii (4,5,6) and SSEA-1 (7) as well as complex antigenic mixtures detected by antisera raised against embryonal carcinoma cells like F9 (8,9) and TerC (10). In each of these cases the antigenic molecules were found to be glycolipid or glycoprotein. A sequential change in the branching process of a certain carbohydrate chain in the early embryo (5) and in the later stage of erythrocyte differentiation (6) has been described. The importance of carbohydrate-containing molecules in

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Abbreviations: TLC: thin-layer chromatography, HPLC: high performance liquid chromatography, Pi/salt: 140 mM NaCl, 10 mM sodium phosphate, pH adjusted to 7.0. Gangliosides are abbreviated according to the assignment of Svennerholm (38), and other glycolipids are named according to the recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature (39).

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development is further emphasized by results indicating that inhibition of glycosylation by tunicamycin prevents development of mouse embryos (11) and that antibodies raised against F9 cells prevent compaction of mouse morulae (12,13).

We have previously described a monoclonal antibody, prepared by immunization of mice with the F9 embryonal carcinoma cell line (7). This antibody reacts with teratocarcinoma stem cells and not with their differentiated derivatives (14,15, 16) and with preimplantation mouse embryos beginning at the eight cell stage. During subsequent post-implantation development, SSEA-1 is selectively expressed on only some germ layers and organ anlage (15).

Our preliminary data (7) suggested that SSEA-1 is a glycolipid. We now report the specific reactivity of the monoclonal antibody directed to SSEA-1 antigen with the complex branched glycosphingolipid of human erythrocyte membranes. The glycolipid was also characterized by the presence of blood group activities H, I-(Ma) and i-(Dench). The activity with antibody to the differentiation antigen SSEA-1, as well as to H and Ii were abolished by endo-β-galactosidase of *Escherichia freundii*. Methylation analysis also indicates that the glycolipid antigen had a close resemblance to I-antigen.

MATERIALS AND METHODS

Antibodies and Immunoassay: The monoclonal antibody directed to the stage-specific embryonic antigen SSEA-1 of mouse teratocarcinoma F9 was obtained as previously described (7). The reactivity of the antibody with various glycolipids was determined by absorption assay on plastic plate using [^{125}I]-Protein A of *Staphylococcus aureus* (IPA) (17,18). Briefly, the glycolipid solution in water (not in buffer solution) was incubated in Microtest II wells (Falcon, Oxnard, CA) at 37°C overnight. Each well was washed with Pi/saline and blocked with 5% bovine serum albumin for 2 hours at 37°C. The antibody binding to glycolipid was determined with diluted monoclonal anti-SSEA-1 antibody (1:4000) followed by rabbit antibody to mouse IgM (heavy chain specific) followed by [^{125}I]-Protein A. The purified glycolipid showing the activity was further confirmed by immunoprecipitation on double diffusion agar plates using glycolipid-liposomes prepared from one part of glycolipid, 5 parts of egg lecithin, and 3 parts of cholesterol. Anti-I(Ma), anti-I(Step) and anti-i(Dench) were kindly donated through Dr. Eloise R. Giblett (Puget Sound Blood Bank) and Mrs. Marie Crookston (Toronto General Hospital). Various glycolipids, such as lactosylceramide, ceramide trihexoside, globoside,paragloboside (lacto-*neo*-tetraosylceramide), H_1, H_2, and H_3-glycolipid, sialylparagloboside, GM_1 ganglioside, GM_3 ganglioside, were prepared according to the procedure established in this laboratory (19).

Fractionation of Glycolipids and Preparation of Active Fraction, H_4-glycolipid from Human Erythrocyte Membranes: Preparation of total lipid extract and subsequent fractionation to obtain the Folch's upper phase glycolipid was carried

out according to the method described previously (20) and the neutral fraction was separated from gangliosides, the total upper phase glycolipid, by chromatography through DEAE-Sephadex (21). The upper phase neutral glycolipid thus prepared was further fractionated by the following 2 step purification. a) Low pressure chromatography in Varian HPLC apparatus with the column Iatrobeads 100 cm x 1 cm (6RS-860; 60 micron, Iatron Laboratory, Tokyo, see 22), gradient from chloroform-methanol-water 75:25:3 to chloroform-methanol-water 20:80:15, applying a low pressure in order to obtain the flow rate, 2 ml/min. Four ml fractions were collected and each fraction was analyzed by TLC. The fractions that contain glycolipids were collected and analyzed for the reaction with antibody to SSEA-1. b) The active fractions which were eluted as associated with "H_4"-fraction (see Fig. 1) were further fractionated by high-pressure HPLC on Iatrobeads 10 micron column, 10 x 500 mm (6RS-810) with the solvent system devised by Watanabe (personal communication with Dr. Kiyohiro Watanabe, Shigei Medical Research Institute, Okayama, Japan). Namely, gradient elution from hexane-isopropanol-water (40:55:5) to hexane-isopropanol-water (30:55:15), followed by elution with hexane-isopropanol-water (25:55:20). A pressure (150 PSI) was applied in order to obtain 1 ml/min in Varian HPLC apparatus. Two ml fractions were collected and each fraction was analyzed by TLC and further analyzed for the activity with anti-SSEA-1 antibody.

Enzymatic Degradation with Endo-β-Galactosidase: Endo-β-galactosidase of *Escherichia freundii* was prepared as previously described (23) and was donated by Dr. Michiko N. Fukuda of this laboratory. The conditions for enzymatic degradation (conditions 1 and 2) and examination of degradation products, glycolipids and oligosaccharides, were carried out according to the method as previously described (24).

Methylation Analysis: Glycolipids were permethylated (25), and analyzed by direct probe mass spectrometry (26) or by gas chromatography-mass spectrometry for the partially O-methylated sugars after hydrolysis (27,28).

RESULTS

A strong reactivity with the monoclonal antibody against SSEA-1 antigen was found only in the fraction previously assigned as H_4-glycolipid, the fourth blood group H-active component of blood group O erythrocyte membranes (29). No other glycolipids tested, including H_1, H_2, H_3, showed this activity (see Table 1). The H_4 fraction, eluted from low-pressure HPLC (see Fig. 1) was further separated by high-pressure HPLC into two homogeneous components, H_4-a and H_4-b (see Fig. 2). Both fractions were equally active on the plastic plate assay (Table I), and in double diffusion precipitin reactions with antibody to SSEA-1 (see Fig. 3).

The fraction H_4-a was also characterized by a reactivity with anti-I(Ma) and a weak reaction with anti-i(Dench) and with anti-H *Ulex europeus* lectin (see Fig. 3). Interestingly, the fraction did not give a precipitin line with anti-I(Step).

TABLE I.

Reactivity of Various Glycolipids to the Monoclonal Antibody Directed to the Stage-Specific Embryonic Antigen (SSEA-1) (by the plate-binding assay)

Lac-Cer, globotriosylcer, globoside, Forssman, lacto-N-*neo*-tetraosylcer (paragloboside), lacto-N-triosylcer, lacto-N-*nor*-hexaosylcer, H_1, H_2, H_3, GM_3, GM_1, sialylparagloboside, ganglio-N-triosylcer, ganglio-N-tetraosylcer	-*
H_4-glycolipid	+++†
H_4-a, H_4-b	+++†
H_4-glycolipid incubated with endo-β-galactosidase	-*

*Negative at the first well that was incubated with a glycolipid solution with 20 μg/100 μl concentration.
†Positive at the fourth to fifth well that was incubated with a glycolipid solution with 1-2 μg/100 μl concentration.

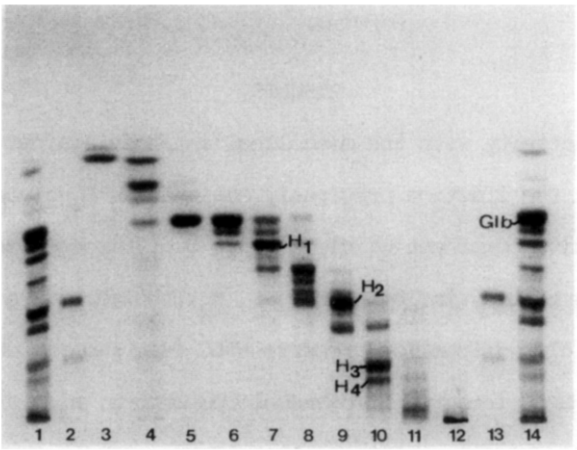

Fig. 1. TLC pattern of neutral glycolipids of human erythrocyte membranes separated through low pressure HPLC on a column of Iatro beads, 6RS-860 (60 micron) through Varian HPLC apparatus. Conditions, see the text. 4 ml fractions were collected and analyzed on TLC. Fractions showing a similar TLC pattern were combined and analyzed again on TLC, which is shown above. Lane numbers are identified as follows: 1 and 14, unfractionated total upper neutral glycolipid. 2 and 13, reference H_2 (upper spot) and H_3 (lower spot). Fraction numbers pooled as: 3: 43-57, 4: 58-77, 5: 78-91, 6: 92-103, 7: 104-115, 8: 116-127,9: 128-143, 10: 144-165, 11: 165-185, 12: methanol-water 80:20 wash, 100 ml. Spots for H_1, H_2, H_3 and H_4 are marked. The activity with the anti-SSEA-1 antibody was only demonstrated with the fraction which contains H_4-glycolipid, *i.e.*, fractions 10 and 11.

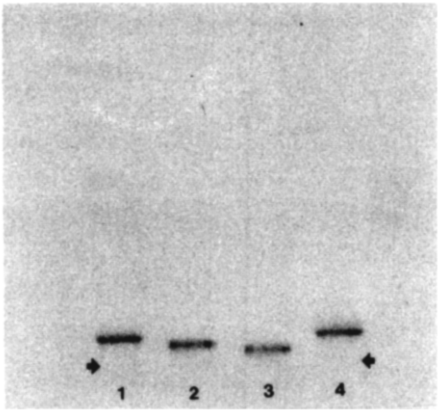

Fig. 2. High performance TLC of purified SSEA-1 glycolipid antigen (H_4-a and H_4-b). Lanes 1 and 4: A standard H_3-glycolipid (20,29). Lane 2: H_4-a glycolipid and Lane 3: H_4-b glycolipid separated from fraction 10 of Fig. 1 by Varian HPLC apparatus as described in the text. Arrows indicate the origin.

Reactivity with anti-SSEA-1, anti-H, and anti-Ii antibodies was abolished by endo-β-galactosidase treatment of the glycolipid fraction . The degradation with endo-β-galactosidase was clearly seen on TLC (see Fig. 4). A ceramide trisaccharide, but not a ceramide monosaccharide, was the major glycolipid liberated under the "condition 1" (24). This is in a striking contrast to H_3-glycolipid

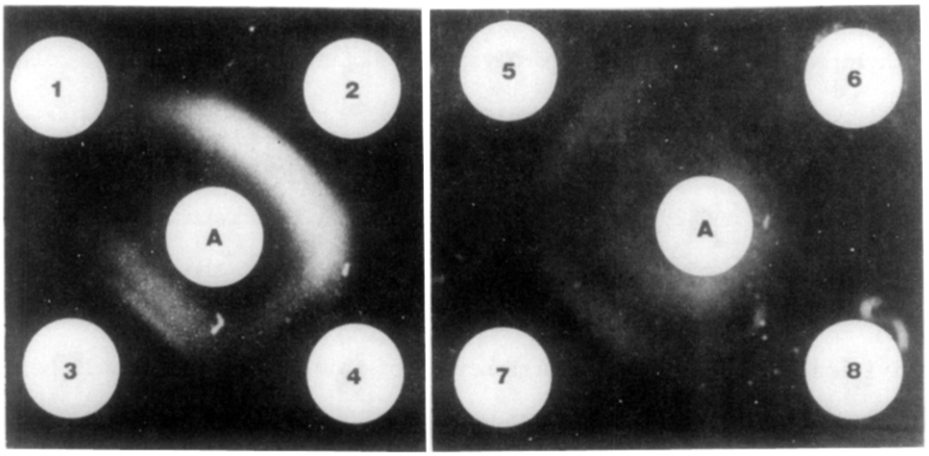

Fig. 3. Ouchterlony double diffusion precipitation of the SSEA-1 active antigen (fraction H_4-a). Center well (A) contains liposome with SSEA-1 active (H_4-a) glycolipid. The following reagents were added in each well. 1. *Rana catesbiana* egg lectin reactive to α-GalNAc residue (37). 2. Anti-I(Ma). 3. Anti-SSEA-1 monoclonal antibody, 1:4 dilution. 4. Anti-SSEA-1 monoclonal antibody, 1:40 dilution. 5. Anti-i(Dench). 6. Anti-i(Dench) 1:4 dilution. 7. Anti-H *Ulex europeus*. 8. None.

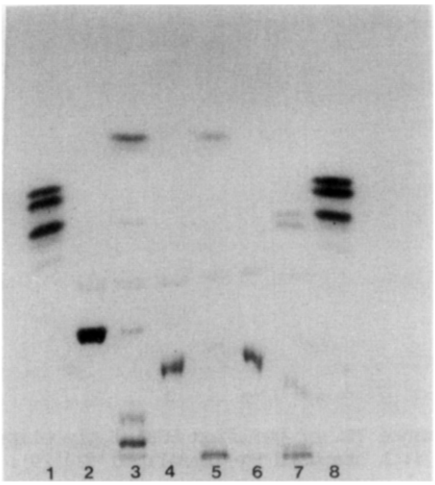

Fig. 4. The cleavage pattern of H_2, H_3, and H_4-a glycolipid by endo-β-galactosidase of *Escherichia freundii* under the condition 1 (24). Developed with chloroform-methanol-water 56:38:10 and spots revealed by orcinol-H_2SO_4. 1 and 8. Lactosylceramide and globotriosylceramide. 2. H_2 control. 3. H_2 degraded by the enzyme. 4. H_3 control. 5. H_3 degraded by the enzyme. 6. H_4-a control. 7. H_4-a degraded by the enzyme.

which yields only a ceramide monosaccharide on enzyme degradation (see Fig. 4), in agreement with previous result (24).

Direct probe mass spectrometry of the permethylated H_4-a glycolipid indicated the presence of two termini; Hex-O→HexN (m/e 219→187; m/e 464→432), and Fuc-O→Hex-O→HexN (m/e 189→157; m/e 393→361; m/e 638 +1). Hydrolysis of permethylated H_4-a yielded, on hydrolysis, reduction and acetylation, following partially O-methylated alditol acetates: 2,3,4-Me_3-Fuc, 2,3,4,6-Me_4-Gal, 2,4,6-Me_3-Gal, 3,4,6-Me_3-Gal, 2,4,6-Me_3-Glc, 2,4-Me_2-Gal and 3,6-Me_2-GlcNAcMe. Since 2,4-Me_2-Gal and 2,3,4,6-Me_4-Gal were present, the branched structure should be at the 3 and 6 position of galactose and one of the carbohydrate chains has the terminal galactose. These two structural points are the requirement for I-activities (30,31). Details in structure of this H_4-glycolipid will be published elsewhere.

DISCUSSION

The presence of stage-specific cell surface molecules and their orderly changes during ontogenesis and differentiation have been suggested by immunological approaches, *i.e.*, that stage-specific antibodies can be raised by immunization with

embryonal carcinoma cells (32,33) or with mouse embryos (34,34). The results of the present study clearly indicate that one such cell surface molecule, SSEA-1, is a specific glycosphingolipid with a branched lacto-N-glycosyl series. The activity was associated with H_4-glycolipid fraction which was further separated into two active components through a high-pressure HPLC on 10 micron Iatro beads column. They are homogeneous on TLC and assigned, respectively, H_4-a and H_4-b. A preliminary study on H_4-a indicates that the active component has a close structural similarity with I-antigen having also an additional H-active determinant. A weak i(Dench) activity was also present which may be due to the internal structure with repeating N-acetyl-lactosamine. A tentative structural assignment, as seen in Figure 5, should be further confirmed by extensive chemical study.

The cell surface carbohydrate structures recognized by monoclonal anti-I(Ma), anti-I(Step), and anti-i(Dench) have been independently shown to be expressed on early post-implantation mouse embryos and embryonal carcinoma cells. Undifferentiated teratocarcinoma stem cells were rich in surface-associated and cytoplasmic I-antigen; whereas i-antigen appeared when cells were differentiated into primary endoderm (5). Ii-determinants have been chemically well established (30,31) and are known to be highly susceptible to endo-β-galactosidase treatment (36). The glycopeptides of embryonal carcinoma cells, showing a clear differen-

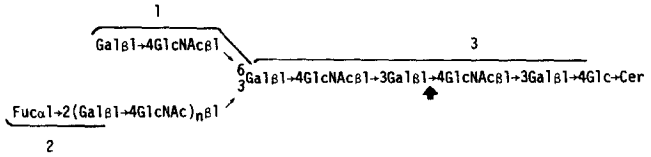

Fig. 5. A tentative structure, assigned for H_4-a glycolipid that reacts to the monoclonal antibody directed to the stage-specific embryonic antigen (SSEA-1). Two termini, Galβ1→4GlcNAc, and Fucα1→2Galβ1→4GlcNAc are linked by branching to the core carbohydrate chain which is highly susceptible to endo-β-galacto sidase under the "condition 1" (24). The degradation product was GlcNAc→Gal→ Glc→Cer rather than Glc→Cer. Therefore, a repeating N-acetyllactosamine must be present at the internal region (region 3) and the endo-β-galactosidase-susceptible linkage is indicated by an arrow. Results of methylation analysis and direct probe mass spectrometry support this structure. The number of N-acetyllactosamine residues (shown in parenthesis) at the side chain is unknown, but probably 1 (n=1). The region 1 for I(Ma) (30,31), the region 2 for H activities, and probably region 3 for a weak i activity. The structure responsible for SSEA-1 activity will be described elsewhere.

tiation-dependent change, were also found to be susceptible to endo-β-galactosidase. These results are comparable to our finding that the stage-specific embryo antigen SSEA-1 is related to Ii-antigen. Further chemical characterization of two components, H_4-a and H_4-b, will establish the molecular mechanism which may define the cell social behavior of early embryo.

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