A Novel Disialoganglioside ($IV^3NeuAcIII^6NeuAcLc_4$) of Human Adenocarcinoma and the Monoclonal Antibody (FH9) Defining This Disialosyl Structure†

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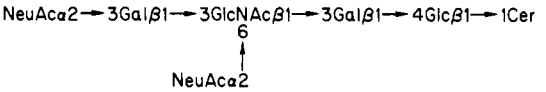
ABSTRACT: A novel disialoganglioside has been isolated from human colonic adenocarcinoma, and its structure has been determined by methylation analysis and electron-impact high mass range mass spectrometry:



This ganglioside is highly immunogenic, and immunization of mice with this disialoganglioside fraction coated on *Salmonella minnesota* followed by fusion of immunized spleen cells with mouse myeloma and selection of the hybridoma by positive reactivity with the purified disialoganglioside resulted in the establishment of a hybridoma secreting immunoglobulin G2a antibody FH9 that reacts specifically with the ganglioside antigen above but not with monosialosyllactotetraosylceramide I ($IV^3NeuAcLc_4$), monosialosyllactotetraosylceramide II ($III^6NeuAcLc_4$), or any other gangliosides tested.

The major change in carbohydrate chains associated with the most common human cancers, such as adenocarcinomas of colon, breast, and lung, is the accumulation of fucosylated type 2 chain with Le^x, di- or trimeric Le^x, Le^y, sialyl derivatives of Le^x, and multimeric Le^x structures (Yang & Hakomori, 1971; Hakomori et al., 1984; Fukushi et al., 1984a). Each structure has been defined by respective specific monoclonal antibodies (Fukushi et al., 1984b,c), some of which preferentially stain human adenocarcinoma over a large variety of normal adult tissues, with the exception of a few loci such as proximal tubuli of kidney, parietal cells of gastric epithelia, and Paneth cells of intestinal epithelia (Fukushi et al., 1984c). Each of these antibodies is reactive as well with embryonic and fetal tissues at a certain stage of development, and therefore, the structures they define are generally regarded as oncofetal antigens [see, for review, Hakomori & Kannagi (1983) and Hakomori (1984)]. On the other hand, various human cancer tissues also express modified type 1 chain antigens defined by specific monoclonal antibodies, although the chemical quantity of such antigens is much less than that of type 2 chain derivatives. Typical examples are sialyl Le^a antigen defined by monoclonal antibody N-19-9 (Magnani et al., 1982) and sialyllactotetraosylceramide antigen defined by antibody CA50 (Nilsson et al., 1985). This paper describes a new monoclonal antibody (termed FH9) defining a novel disialoganglioside with type 1 chain structure. The oligosaccharide moiety of this new antigen is identical with a milk oligosaccharide described previously (Smith et al., 1978).

Materials and Methods

Tumor Tissues, Tumor Cell Lines, and Glycolipid Samples. The antigen was extracted from metastatic deposits in liver from five cases of colonic cancer, which were obtained through the courtesy of Wilma Verrato of the Tumor Procurement Program of the National Cancer Institute, National Institutes of Health. The antigen disialosyllactotetraosylceramide was found as a common component in all tumor tissue examined. The extraction, preparation, and purification of glycolipids were performed as previously described (Hakomori et al., 1984) but will be detailed in a subsequent section. The following standard components were prepared from human erythrocyte membranes and human cancer tissue: sialosyl(2→3)lactoneotetraosylceramide ($IV^3NeuAcnLc_4$)[1] (Siddiqui & Hakomori, 1973; Hakomori et al., 1983a), sialosyl(2→6)lactoneotetraosylceramide ($IV^6NeuAcnLc_4$) (Hakomori et al., 1983a,b), and sialosyl(2→3)lactonorhexaosylceramide ($VI^3NeuAcnLc_6$) (Watanabe et al., 1979). The 6C ganglioside ($VI^6NeuAcIII^3FucnLc_6$) (Hakomori et al., 1983a) and 6B ganglioside ($VI^3NeuAcV^3FucIII^3FucnLc_6$) (Fukushi et al., 1984b) were prepared from the monosialoganglioside fraction of human colonic cancer as previously described. Sialosyl Le^a antigen ($IV^3NeuAcIII^4FucLc_4$) was prepared from the monosialoganglioside fraction of human adenocarcinoma as described by Magnani et al. (1982). Disialosyl Le^a and disialosyllactoneotetraosylceramide (disialosyl type 2 chain paragloboside, $IV^3[NeuAc\alpha2\rightarrow8NeuAc]nLc_4$) were prepared from human adenocarcinoma (E. Nudelman, Y. Fukushi, S. B. Levery, T. Higuchi, and S. Hakomori, submitted for publication), although the latter ganglioside was prepared previously from human kidney (Rauvala et al., 1978) and from human erythrocytes (Kundu et al., 1983). Disialosylgalactosylgloboside ($VI^3[NeuAc2\rightarrow3[NeuAc2\rightarrow6]Gal]Gb_4$) was prepared from the disialoganglioside fraction of human erythrocytes (Kundu et al., 1983) as well as from human

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[1] Glycolipids are designated according to the recommendation of the IUPAC Nomenclature Committee, but the suffix "OseCer" is omitted (IUPAC, 1977). Ganglio-series gangliosides are abbreviated according to Svennerholm (1964).

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adenocarcinoma (E. Nudelman, Y. Fukushi, S. B. Levery, T. Higuchi, and S. Hakomori, submitted for publication). GD_3 ganglioside was prepared from human melanoma cell line (Nudelman et al., 1982). Lactotetraosylceramide (Lc_4; type 1 chain paragloboside) was prepared from human meconium (Karlsson & Larsson, 1979). Monosialosyllactotetraosylceramide I ($IV^3NeuAcLc_4$; Table I) was prepared from meconium (E. Holmes, G. Ostrander, E. Nudelman, and S. Hakomori, unpublished observations), and monosialosyllactotetraosylceramide II ($III^6NeuAcLc_4$; Table I) was prepared from disialosyllactotetraosylceramide by hydrolysis with influenza virus type A_2 sialidase (Corfield et al., 1982).

Extraction, Subsequent Fractionation, and Purification of Glycolipids. Tissues or cells were extracted with 2-propanol–hexane–water (55:25:20) (Kannagi et al., 1982; Hakomori et al., 1984) followed by repeated Folch's partition (Folch-Pi et al., 1951). The total ganglioside fractions were adsorbed on DEAE-Sepharose and separated by stepwise elution into mono-, di-, and trisialoganglioside fractions by 0.05, 0.15, and 0.45 M ammonium acetate solution, respectively (Ledeen & Yu, 1982). Each ganglioside fraction was dialyzed against distilled water in a Spectrapor dialysis tube (Spectrum Medical Industries, Inc., Los Angeles, CA), followed by adsorption on an alkylated porous silica gel column (e.g., Whatman Partisphere C18, Kent, England), washed with 5 column volumes of water, and gangliosides were eluted with 3 column volumes of chloroform–methanol (2:1 v/v). If the quantity of ganglioside was small (less than 50 μg), a small column of Bond-Elut C18 (Analytichem International, Harbor City, CA) was used. The disialoganglioside fraction was exclusively used in this study.

Purification of Disialogangliosides. The fractions, first purified on a hydrophobic column as described above, were separated by high-performance liquid chromatography (HPLC)[2] in a 2-propanol–hexane–water system through an Iatrobeads 6RS-8010 column as previously described (Watanabe & Arao, 1980; Kannagi et al., 1982). Gradient elution was performed from 2-propanol–hexane–water (55:40:5 v/v/v) to 2-propanol–hexane–water (55:20:25 v/v/v) during 300 min followed by continuous elution with a constant solvent composition of 2-propanol–hexane–water (55:20:25 v/v/v) for an additional 100 min. The total volume of the solvent used for elution was 400 mL collected over 100 tubes during 400 min with a flow rate of 1.0 mL/min. Each fraction was analyzed by high-performance thin-layer chromatography (HPTLC) developed with chloroform–methanol–water (60:40:9 containing 0.02% $CaCl_2$). The major disialogangliosides, GD_3 and GD_{1a}, were eluted in tubes 1–30 (fractions 1–4). Fraction 5 (tubes 31–37) contained components with slower HPTLC mobility than that of GD_{1a}. In this fraction, three major slow-migrating components (bands 1–3) were separated on HPTLC. On HPTLC developed in chloroform–methanol–water (60:40:10 v/v/v) containing 0.02% $CaCl_2$, bands 1 and 2 were not well separated, but band 3 (the slowest-migrating band) was clearly separated from bands 1 and 2. Band 1 migrated clearly faster than bands 2 and 3 in propanol–water–28% ammonia (7:2:1 v/v/v) (see Figure 1A). These bands were separated, therefore, on preparative HPTLC (Merck HPTLC plates, Merck, Darmstadt, West Germany) on prolonged development along 20 cm in a chloroform–methanol solvent system, after which bands 1 and 2 were extracted and further separated in a propanol–water system. The bands separated on HPTLC were indicated by Primulin spray followed by UV detection (Skipski, 1975). The Primulin-positive bands were scraped, extracted with 2-propanol–hexane–water (55:40:45) under sonication for 5 min, and centrifuged, and the silica gel pellet was reextracted 3 times. The combined extracts were filtered through a Millipore filter (Millipore Corp., Bedford, MA) to eliminate any trace of silica gel. The recovery of glycolipids was nearly 100%. The purified band 1 (lane 6 in Figure 1A) was identified by methylation analysis as disialosyllactoneotetraosylceramide ($IV^3NeuAc2\rightarrow 8NeuAcnLc_4$), which was previously isolated from human kidney (Rauvala et al., 1978) and from human erythrocytes (Kundu et al., 1983). The purified band (lane 5 in Figure 1A) was a hitherto unknown ganglioside and is now identified as disialosyllactotetraosylceramide, the structure of which is described in this paper.

The slowest migrating component (band 3) was much enriched in HPLC eluates in tubes 38–50. This band was further separated on HPTLC into two components (bands 3 and 4) on prolonged development (20 cm for 3 h) in a propanol–water–ammonia system. Bands 3 and 4 were characterized as disialosyl Le^a and disialosylgalactosylgloboside, respectively (Nudelman et al., 1986).

Methylation Analysis and Direct-Probe Mass Spectrometry. Glycolipids were methylated (Hakomori, 1964), hydrolyzed, and reduced in sodium borodeuteride, and partially O-methylated hexitol and hexosaminitol acetates were prepared as previously described (Björndal et al., 1967; Stellner et al., 1973). They were analyzed by gas chromatography–chemical ionization mass spectrometry with selected ion monitoring under conditions described in the legend for Figure 5. The conditions are essentially a combination of various methods previously described (McNeil & Albersheim, 1977; Laine, 1980; Kannagi et al., 1983; Bremer et al., 1984), which have been recently compiled (S. B. Levery and S. Hakomori, submitted for publication).

Electron-Impact Mass Spectrometry of Permethylated Glycolipids. Permethylated glycolipids were analyzed by electron-impact mass spectrometry according to the principles described previously (Karlsson et al., 1974; Ledeen et al., 1974; Watanabe et al., 1975) employing a high mass range JEOL HX-110 mass spectrometer under the conditions described in the legend for Figure 3.

Partial and Complete Desialylation of Disialoganglioside. To effect desialylation of the α2→3 sialosyl–Gal linkage, 2–5 μg of the disialoganglioside were dissolved in 25 μL of 0.2 M acetate buffer (pH 4.5) and incubated with 25 μL of influenza virus A_2 sialidase (1 unit/mL activity) at 37 °C for 18 h. The influenza virus type 2 was kindly donated by Dr. Marion Cooney of the Department of Pathobiology, University of Washington. The enzyme derived from this virus preferentially or specifically hydrolyzes the α2→3 sialosyl residue linked to Gal but does not hydrolyze the α2→6 sialosyl residue linked to GlcNAc (Corfield et al., 1981; Schauer, 1982). The incubation mixture was passed through a column of C18 alkylated porous silica gel (Bond-Elut, Analytichem International, Harbor City, CA), washed with water, and the partially desialylated ganglioside was eluted with 5 column volumes of chloroform–methanol (1:1 v/v). For complete desialylation, 10 μg of the ganglioside and 50 μg of sodium deoxytaurocholate in chloroform–methanol were mixed and evaporated to dryness. The residue was dissolved in 25 μL of 100 mM acetate buffer (pH 4.5) and incubated with 25 μL of *Clostridium perfringens* sialidase (1 unit/mL activity) at 37 °C for 18 h. Degraded glycolipids in the incubation mixture were

[2] Abbreviations: HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography.

recovered through a Bond-Elut column as above and passed through a small column (0.3 × 1 cm) of DEAE-Sephadex in methanol–chloroform–water (60:30:8 v/v/v) to eliminate deoxytaurocholate.

Monoclonal Antibody FH9 Directed to Disialosyl Type 1 Chain Ganglioside and Other Monoclonal Antibodies Used for Characterization of Components. The disialoganglioside fraction containing disialosyllactoneotetraosylceramide (IV^3-[NeuAc2→8NeuAc]nLc$_4$) and disialosyllactotetraosylceramide (this ganglioside) was separated by HPLC (fraction 6; tubes 31–37), followed by preparative HPTLC developed in a solvent mixture of chloroform–methanol–water (60:40:9 v/v/v) containing 0.02% $CaCl_2$. About 5 μg of the ganglioside fraction (containing these two components in an approximate molar ratio of 1:2) was mixed with 20 μg of *Salmonella minnesota* and injected intravenously into BALB/c mice on day 0, and 2 μg of the ganglioside fraction mixed with 20 μg of the bacteria was injected every 4 days, with a total of seven intravenous injections. On the fourth day after the last injection, spleen cells were harvested and fused with SP-2 mouse myeloma cells. This procedure is essentially the same as previously described (Fukushi et al., 1984a,b). The hybridoma secreting Ig was selected by positive reaction with a purified disialosyl type 1 chain ganglioside (disialosyllactotetraosylceramide; $IV^3NeuAcIII^6NeuAcLc_4$) and negative reaction with disialosyllactoneotetraosylceramide (IV^3[NeuAc2→8NeuAc]nLc$_4$), disialosyl Le^a ($III^6NeuAcIII^4FucIV^3NeuAcLc_4$), disialosylgalactosylgloboside (IV[NeuAc2→6-[NeuAc2→3]Gal]Gb$_4$), and GD_3, GD_{1a}, and GD_{1b} gangliosides.

Other monoclonal antibodies, such as FH6 (Fukushi et al., 1984b), which defines sialosyl Le^x carried by a long type 2 chain structure, N-19-9 (Koprowski et al., 1979) or CSLEA-1 (Chia et al., 1985), both of which define sialosyl Le^a, and 1B9 (Hakomori et al., 1983b), which defines sialosyl 2→6Galβ1→4GlcNAcβ1→R structure, were established as previously described. 1B2 antibody, which defines type 2 chain N-acetyllactosamine (Young et al., 1981), and various antibodies (FH1, FH2, FH3) that define Le^x determinants were established as previously described (Fukushi et al., 1984a).

Determination of Specificity of Antibody FH9. The antibody specificity was determined by solid-phase radioimmunoassay with glycolipid/phospholipid/cholesterol coated on vinyl strip plates (Costar Laboratories, Cambridge, MA) as previously described (Kannagi et al., 1983b). The assay was performed with antibody dilution as well as antigen dilution. In addition, the reactivity was confirmed by immunostaining of various purified gangliosides separated on a mini-Baker HPTLC plate (5 × 6 cm) (J. T. Baker Chemical Co., Phillipsburg, NJ), performed as previously described (Fukushi et al., 1984b). The isotypes of the immunoglobulin were determined with subclass-specific antibodies purchased from Cappel Laboratories (Cochranville, PA).

Results

Isolation of Disialosyllactotetraosylceramide (Disialosyl Type 1 Chain Paragloboside). Eight to nine resorcinol-positive bands were separated on HPTLC of the disialoganglioside fraction derived from most cases of human colonic adenocarcinoma in a solvent of chloroform–methanol–water (60:40:9) containing 0.02% $CaCl_2$. The HPTLC patterns of the disialoganglioside fraction of colonic adenocarcinoma (TG126) and lung squamous carcinoma cell line QG56, and their purified components, are shown in Figure 1. A single band representing disialosyllactotetraosylceramide (disialosyl type 1 chain paragloboside) was isolated in homogeneous form

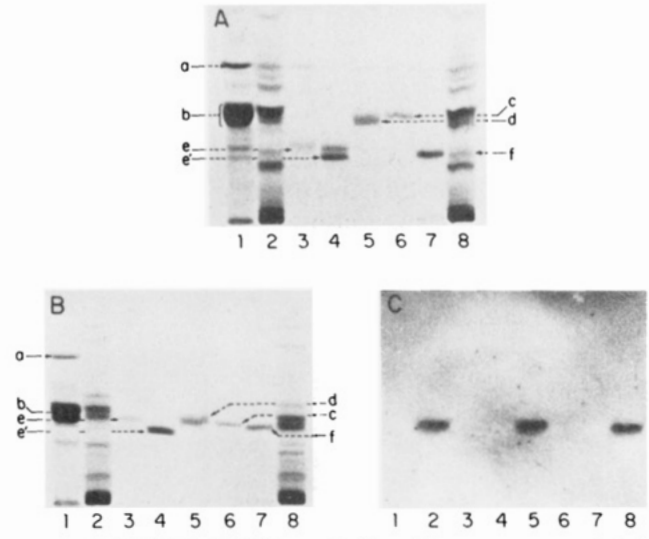
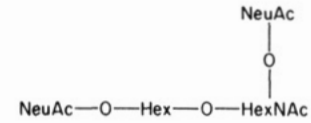


FIGURE 1: HPTLC of disialogangliosides of human cancer and their immunostaining pattern with FH9 antibody. (Panel A) HPTLC developed in propanol–water–28% ammonia (70:2.5:1 v/v/v). Spots were revealed after spraying with 0.2% orcinol in 2 M H_2SO_4 and heating at 120 °C for 7 min. (Lane 1) Disialoganglioside of human squamous lung cancer cell line QG56; (lanes 2 and 8) disialoganglioside fraction of human colonic adenocarcinoma TG126; (lane 3) fast-migrating band of disialosyl Le^a (spot e; about 1 μg); (lane 4) a mixture of slow-migrating bands of disialosyl Le^a (spot e') and disialosylgalactosylgloboside; (lane 5) disialosyllactotetraosylceramide (spot d; about 2 μg); (lane 6) disialosyllactoneotetraosylceramide (spot c; about 1 μg); (lane 7) disialosylgalactosylgloboside (spot f; about 2.5 μg). (Panel B) HPTLC developed in chloroform–methanol–water (60:40:9 v/v/v) containing 0.02% $CaCl_2$. Methods of spot detection and samples in each lane are identical with those in panel A. (Panel C) HPTLC of gangliosides separated as in panel A and immunostained with FH9 antibody.

(lane 5, Figure 1) as described under Materials and Methods. This component migrated on HPTLC close to GD_{1a} and disialosyllactoneotetraosylceramide (IV^3[NeuAcα2→8NeuAc]nLc$_4$), which was previously isolated from human kidney (Rauvala, 1978) and erythrocytes (Kundu et al., 1983). The compound was clearly distinguishable from disialosyl Le^a (Nudelman et al., 1985) (lane 3) and disialosylgalactosylgloboside (IV^3[NeuAc2→3[NeuAc2→6]Gal]Gb$_4$) (Kundu et al., 1983), particularly on HPTLC developed in propanol–water–28% ammonia (7:2:1 v/v/v) (Figure 1).

Characterization of Disialosyllactotetraosylceramide. (A) Homogeneity. This ganglioside, separated as described above, gave a single band in two solvent systems, chloroform–methanol–water (60:40:9 v/v/v) containing 0.02% $CaCl_2$ as well as 1-propanol–water–28% ammonia (7:2.5:1 v/v/v). After peracetylation, the ganglioside gave a single band on HPTLC developed in a solvent mixture of 1-chloroethane–methanol–water (55:40:5 v/v/v) or *n*-butylacetate–acetone–water (7:3:0.2 v/v/v).

(B) Electron-Impact Mass Spectrometry. Electron-impact mass spectrometry of the permethylated compound gave a spectrum with the following prominent fragment ions, which are of diagnostic value in defining the sugar sequence (see Figure 2). The prominent fragment ions m/z 1186, 811 (1186 − 376 + 1), and 436 [1186 − (2 × 376) + 2] are consistent with the sequence (see Figure 2C)



Another prominent ion, m/z 1015, could be derived from a

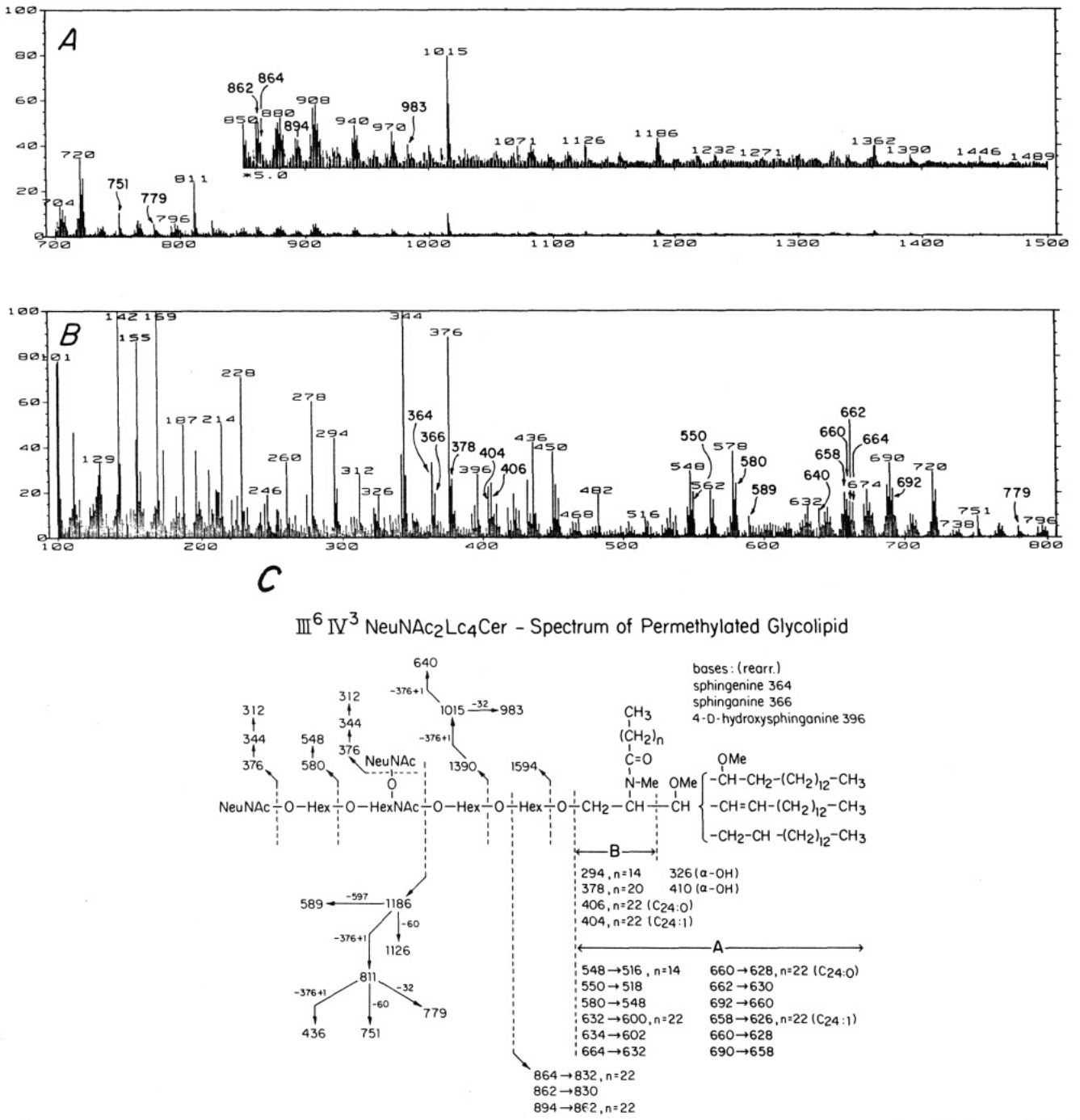
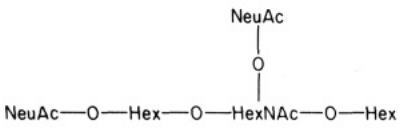


FIGURE 2: Electron-impact direct-probe mass spectrum of permethylated disialosyllactotetraosylceramide. About 5 μg of purified permethylated disialoganglioside was placed in a direct-probe cuvette, and the mass spectrum was obtained by electron-impact mode with an ionization potential of 27 eV and an ionizing current of 300 μA. The temperature of the probe was raised from 200 to 380 °C. Significant spectra appeared between 240 and 280 °C. (Panel A) Total spectrum. (Panel B) Detailed spectrum on expanded scale. (Panel C) Proposed fragmentation pattern of the structure. Ion mass numbers representing ceramide composition (B and A ions), which appeared in the spectrum of the disialoganglioside, are also shown.

clearly observable ion, m/z 1390 (1390 − 376 + 1), representing the sequence (see Figure 2C)



A series of ions, m/z 376 → 344 → 312, representing the terminal NeuAc, and m/z 580 → 548, representing NeuAc–O–Hex, are clearly seen. The electron-impact mass spectrum of disialosylgalactosylgloboside also showed a prominent ion, m/z 1015 (Kundu et al., 1983); however, disialosylgalactosylgloboside did not give prominent ion m/z 1186 nor m/z 580 → 548, indicating the terminal NeuAc–O–Hex. Rearrangement ions for sphingosine base (Egge & Hanfland, 1981; Karlsson et al., 1974) included m/z 364 (sphingenine), 366 (sphinganine), and 396 [4(D)-hydroxysphinganine]. A and B fragments for complete ceramide and fatty acyl portion, respectively (Egge & Hanfland, 1981; Karlsson et al., 1974), showed an extensive heterogeneity of the aglycon, as shown in the fragmentation scheme (Figure 2C).

(*C*) *Methylation Analysis.* On hydrolysis, reduction, and acetylation of the permethylated disialoganglioside, GC–MS of the resulting partially O-methylated alditol acetates yielded

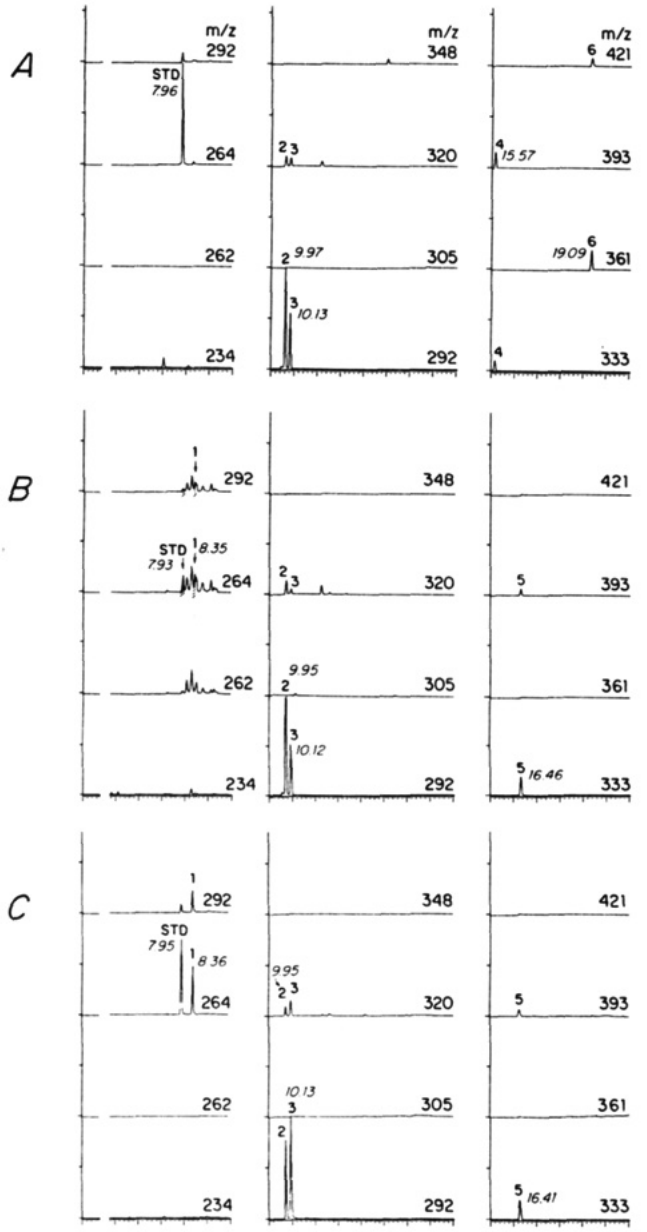


FIGURE 3: Selected ion chromatograms of partially O-methylated alditol and hexosaminitol acetates from the hydrolyzates of permethylated glycolipids. (A) Disialosyllactotetraosylceramide, intact. (B) The same ganglioside, after digestion with *Clostridium perfringens* sialidase. (C) Authentic lactotetraosylceramide (type 1 chain paragloboside) isolated from meconium. Derivatives were separated on a DB-5 capillary column, temperature programmed from 140 to 250 °C at 4 °C/min. Splitless injection was used, with splitter closed and oven temperature 50 °C for 40 s following injection, after which time the splitter was opened and the oven temperature was raised to the program starting temperature (taking 110 s more). Derivatives were identified by methane (300 μM) chemical ionization mass spectrometry with selected ion monitoring (cycle time 1 s), on a Finnigan 3300 gas chromatograph/mass spectrometer adapted for use with fused silica capillary columns and 6110 data system. (Ordinate) Intensity of each ion at mass number indicated. (Abscissa) Retention time. The italicized numbers are retention times in minutes, measured from point at which splitter is opened. The apparent shift of the peaks in (A) is due to a 6-s delay in turning on the data system. Peaks identified were (STD) 2,3,4,6-tetra-*O*-Me-Glc added as retention time standard, (1) 2,3,4,6-tetra-*O*-Me-Gal, (2) 2,3,6-tri-*O*-Me-Glc, (3) 2,4,6-tri-*O*-Me-Gal, (4) 3,6-di-*O*-Me-GlcNAcMe (from type 2 chain impurity), (5) 4,6-di-*O*-Me-GlcNAcMe, and (6) 4-mono-*O*-Me-GlcNAcMe. Identifications were made on the basis of appropriate MH^+, $(MH - 32)^+$, and $(MH - 60)^+$ ions and retention indices compared with authentic standards and were confirmed, particularly in the case of hexosaminitols, by coinjection.

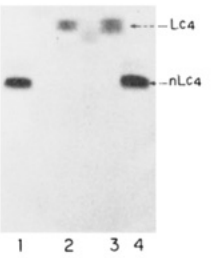


FIGURE 4: HPTLC of desialylated and acetylated core glycolipid derived from the disialoganglioside, acetylated lactotetraosylceramide (Lc_4), and acetylated lactoneotetraosylceramide (nLc_4). The disialoganglioside was desialylated, acetylated, and separated on HPTLC in 2-chloroethanol–methanol (95:5 v/v). (1 and 4) Acetylated nLc_4. (2) Acetylated Lc_4 from meconium. (3) Desialylated and acetylated glycolipid from the disialoganglioside. Spots were detected by orcinol–sulfuric acid.

2,4,6-tri-*O*-Me-Gal (peak 3), 4-mono-*O*-Me-GlcNAcMe, and 2,3,6-tri-*O*-Me-Glc (peak 2), as shown in Figure 3A. Following removal of two sialic acid residues by treatment with *C. perfringens* sialidase in the presence of detergent, methylation analysis of the desialylated product yielded 2,3,4,6-tetra-*O*-Me-Gal (peak 1), 2,4,6-tri-*O*-Me-Gal (peak 3), 4,6-di-*O*-Me-GlcNAcMe (peak 5), and 2,3,6-tri-*O*-Me-Glc (peak 2) (Figure 3B). Minor fast-migration peaks appearing after sialidase treatment (Figure 3B, ion plot m/z 292, 264, and 262) are due to unidentified impurities introduced by derivatization. Essentially the same partially O-methylated alditol and hexosaminitol acetates were produced from the permethylated authentic sample of lactotetraosylceramide (type 1 chain paragloboside) prepared from meconium (Figure 3C). These results clearly indicate that the intact glycolipid had a core type 1 chain lactotetraosylceramide substituted at the 3-position of the terminal Gal and at the 6-position of the GlcNAc residue.

(*D*) *HPTLC of Desialylated Core Structure*. The desialylated core structure of this ganglioside was further confirmed by HPTLC of peracetylated derivatives in 2-chloroethane–methanol (9:1 v/v) as described by Karlsson and Larsson (1979). The desialylated, peracetylated core glycolipid showed an HPTLC migration identical with that of peracetylated lactotetraosylceramide (Lc_4) (R_f 0.85) and had a much faster mobility than peracetylated lactoneotetraosylceramide (nLc_4) (R_f 0.55) (see Figure 4), although the HPTLC mobility of underivatized Lc_4 was identical with that of nLc_4 in chloroform–methanol–water or propanol–water systems.

(*E*) *Conversion of Disialoganglioside to Monosialoganglioside* ($III^6NeuAcLc_4$) *and to Lactotetraosylceramide* (Lc_4). The disialoganglioside was converted to lactotetraosylceramide (Lc_4) by complete desialylation with *C. perfringens* sialidase in the presence of sodium deoxytaurocholate. The product Lc_4 was characterized by methylation analysis and by HPTLC as an acetate as described above. On the other hand, the disialoganglioside was converted to a monosialoganglioside on incubation with influenza virus sialidase (Figure 5), which preferentially or specifically hydrolyzes the $\alpha 2 \rightarrow 3$ sialosyl residue linked to Gal (Corfield et al., 1982; Schauer, 1982). The product had approximately the same TLC mobility as other monosialogangliosides.

Preparation and Properties of Monoclonal Antibody (*FH9*) *Directed to This Ganglioside* (*Disialosyllactotetraosylceramide*). Hybridomas secreting antibodies showing positive reactivity with purified disialosyllactotetraosylceramide (a new ganglioside, $IV^3NeuAcIII^6NeuAcLc_4$) but negative reactivity with disialosyllactoneotetraosylceramide ($IV^3NeuAc\alpha 2 \rightarrow 8NeuAcnLc_4$) and other gangliosides were selected, and finally,

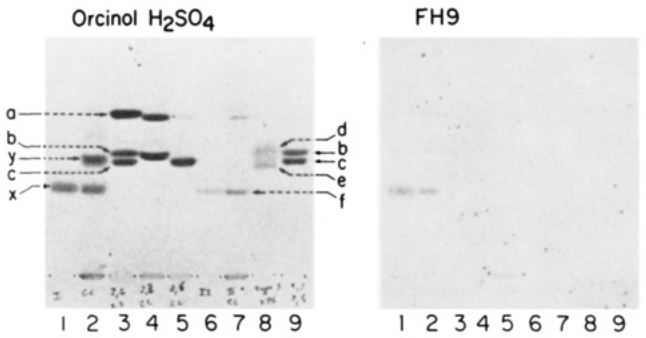


FIGURE 5: HPTLC patterns of various gangliosides, their cleavage products by influenza virus A_2 sialidase, and their immunostaining patterns with monoclonal antibody FH9. Left panel shows orcinol–sulfuric acid staining of various components. Right panel shows immunostaining pattern of the same HPTLC plate with monoclonal antibody FH9. (Lane 1) Intact disialoganglioside (disialosyllactotetraosylceramide) (spot x; about 1 μg). (Lane 2) Same as lane 1, incubated with influenza virus sialidase (spot y is a degradation product, which should be $III^6NeuAcLc_4$; undegraded spot can be eliminated by re-incubation). (Lane 3) Reference glycolipids: globoside (spot a; about 2 μg), sialosyl(2→3)lactoneotetraosylceramide (spot b; about 1 μg), and sialosyl(2→6)lactoneotetraosylceramide (spot c; about 1 μg). (Lane 4) Sialosyl(2→3)lactoneotetraosylceramide incubated with influenza virus sialidase (cleaved to lactoneotetraosylceramide). (Lane 5) Sialosyl(2→6)lactoneotetraosylceramide incubated with influenza virus sialidase (no cleavage). (Lane 6) Disialosyllactoneotetraosylceramide (from human erythrocytes). (Lane 7) Same as lane 6, incubated with influenza virus sialidase (cleaved partially). (Lane 8) Two monosialogangliosides of human meconium, the upper band (d) being sialosyl(2→3)lactotetraosylceramide ($IV^3NeuAcLc_4$) and the lower band (e) a mixture of sialosyl Le^a and sialosyl(2→6)lactoneotetraosylceramide ($IV^6NeuAcnLc_4$) of meconium containing α-hydroxy fatty acids. (Lane 9) Sialosyl(2→3)-lactoneotetraosylceramide (b) and sialosyl(2→6)lactoneotetraosylceramide (c).

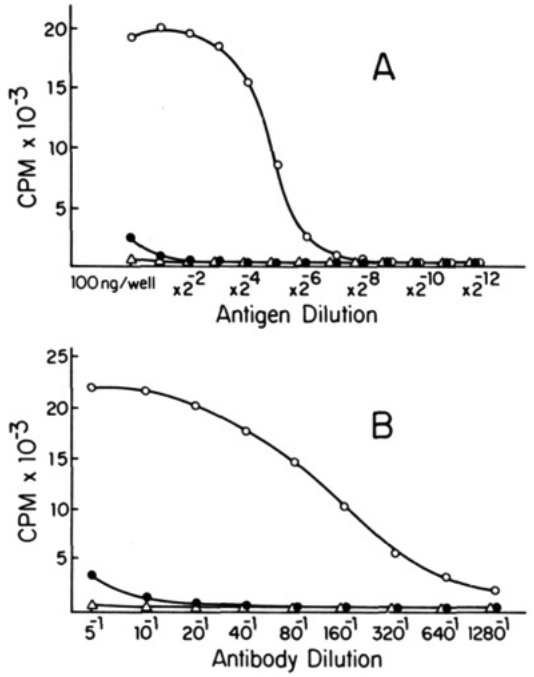


FIGURE 6: FH9 antibody binding to solid-phase glycolipids. (Panel A) Results of assay with antigen dilution. The first well contained 100 ng of glycolipid with 300 ng of cholesterol and 500 ng of lecithin, and a serial dilution is indicated in the abscissa. (Panel B) Results of assay with antibody dilution. The first well contained 5 times diluted culture supernatant, and the dilution is indicated in the abscissa. (○) Disialosyllactotetraosylceramide; (●) disialosyl Le^a; (△) monosialosyl Le^a ($IV^3NeuAcIII^4FucLc_4$), disialosyllactoneotetraosylceramide (IV^3[NeuAc2→8NeuAc]nLc_4), sialosyl(2→3)lactoneotetraosylceramide ($IV^3NeuAcnLc_4$), sialosyl(2→3)lactotetraosylceramide ($IV^3NeuAcLc_4$), and sialosyl(2→6)lactotetraosylceramide ($III^6NeuAcLc_4$; degradation product of disialosyllactotetraosylceramide by influenza virus).

one hybridoma antibody designated FH9 was established. The antibody was identified as IgG2a and showed a specific reactivity with disialosyllactotetraosylceramide but negative reactivity with disialosyl Le^a ($III^4FucIII^6NeuAcIV^3NeuAcLc_4$), disialosylgalactosylgloboside (IV^3[NeuAc2→6-[NeuAc2→3]Gal]Gb_4), disialosyllactoneotetraosylceramide, GD_3 ganglioside, and GD_{1a} ganglioside. The antibody was also not capable of reacting with monosialogangliosides with closely related structure, i.e., monosialosyllactotetraosylceramide I ($IV^3NeuAcLc_4$), isolated from meconium (Figure 5, lane 8), and monosialosyllactotetraosylceramide II ($III^6NeuAcLc_4$), which is the degradation product of the disialoganglioside by influenza virus sialidase (Figure 5, lane 2). Specificity of the antibody was further demonstrated by solid-phase radioimmunoassay with antigen dilution as well as antibody dilution, as shown in Figure 6.

Discussion

A number of tumor-associated carbohydrate antigens have been identified by the monoclonal antibody approach [see, for review, Hakomori & Kannagi (1983)]. Many of them have been established after immunization of mice by a specific type of human cancer cell line or by membrane fractions isolated from specific types of cancer, followed by selection of hybridomas secreting antibodies showing a positive reactivity with the tumor cells used as immunogen and showing negative or restricted reactivity with normal cells or tissues. We have been developing different approaches for establishing hybridoma antibodies that react preferentially with tumor tissue or cells. The procedures consist of (i) chemical analysis of a given type of tumor cells or tumor tissue and identification of a specific component or multiple components that accumulate in the tumor cells or tissues and are absent or present in small quantity in the normal counterpart, (ii) immunization of the specific component with or without adjuvant (e.g., coated on bacteria) followed by hybridization of immunized mice spleen cells with HAT-sensitive mouse myeloma cells, (iii) selection of hybridomas in the HAT media that react specifically with the immunized antigen and show no reactivity with other unrelated antigens, and (iv) determination of the specificity of antibodies, including immunohistological examination of various normal and tumor tissues. Through these procedures, we have established a large variety of monoclonal antibodies directed to fucosylated or fucosylated/sialylated type 2 chain antigens. Some of the antibodies defining di- or trimeric Le^x show preferential reactivity with human gastrointestinal and breast adenocarcinoma (Hakomori et al., 1984; Fukushi et al., 1984a,c), and some others, directed to sialosyl derivatives of di- or trimeric Le^x, show a specific reactivity with human lung, gastric, pancreas, and colonic cancer (Fukushi et al., 1984b). This approach has now been extended to antigens with type 1 chain fucosylated or sialylated structure. Previously, a well-known antigen, monosialyl Le^a, was identified by Magnani et al. (1984) as being defined by monoclonal antibody N-19-9 (Koprowski et al., 1979), which reacts preferentially with a large variety of human cancer tissues including gastric, colonic, and pancreatic cancer (Atkinson et al., 1982; Arends et al., 1983). The antigen has been detected in the form of mucin glycoprotein in higher quantities in a large variety of sera of patients with cancer than was detectable in sera of patients with nonmalignant diseases and normal subjects (Magnani et al., 1983).

This study clearly identified a new type of disialosyl type 1 chain glycolipid ($IV^3NeuAcIII^6NeuAcLc_4$; see Table I),

Table I: Structures and Reactivities of Various Type 1 Chain Gangliosides and Closely Related Structures with Monoclonal Antibody FH9

structure	reactivity with FH9
disialosyllactotetraosylceramide NeuAcα2→3Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1→1Cer 6 ↑ NeuAcα2	++
monosialosyllactotetraosylceramide (I) NeuAcα2→3Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1→1Cer	–
(II) Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1→1Cer 6 ↑ NeuAcα2	–
sialosyl Le^a NeuAcα2→3Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1→1Cer 4 ↑ Fucα1	–
monosialosyllactoneotetraosylceramide NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer	–
disialosyllactoneotetraosylceramide NeuAcα2→8NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer	–

although the same carbohydrate structure ("disialosyllacto-*N*-tetraose") present in this ganglioside was found previously as a milk oligosaccharide (Smith et al., 1978). The antibody FH9 was established, which is directed to this antigen and does not show any reactivity with other disialosyl or monosialosyl gangliosides. More recently, an antibody directed to a similar epitope. i.e., monosialosyl type 1 chain, has been suggested, although the exact structure of the antigen has not been clearly demonstrated (Nilsson et al., 1985). The antigen described in this paper is clearly defined as summarized in Table I, i.e., disialosyllactotetraosylceramide with one sialic acid substituted at the terminal Gal and the other sialic acid substituted at the 6-position of the subterminal GlcNAc. The antibody established is highly specific to this structure and seems to require both 2→3-linked and 2→6-linked sialic acid residues. The antibody does not cross-react with other monosialosyl and disialosyl gangliosides with either type 1 or type 2 chain structure (Table I; Figures 4 and 5). Smith and Ginsburg (1980) prepared polyclonal antibodies by immunizing rabbits with disialosyllacto-*N*-tetraose linked to keyhole limpet hemocyanin. The hapten inhibition study indicated that the antibodies were mainly directed to "LS-tetrasaccharide b" (Galβ1→3[NeuAcα2→6]GlcNAcβ1→3Galβ1→4Glc) but not to "LS-tetrasaccharide a" (NeuAcα2→3Galβ1→3GlcNAcβ1→3Galβ1→4Glc). Thus, the specificity of FH9, which requires two sialic acids, is clearly different from the majority of polyclonal antibodies that were described by Smith & Ginsburg (1980). A preliminary study indicates that the antibody FH9 also shows a strong reactivity with a number of human tumor cell lines but shows no reactivity with human erythrocytes, lymphocytes, and fibroblasts (E. Nudelman, Y. Fukushi, and S. Hakomori, unpublished results).

The chemical quantity of type 2 chain and its derivatives in human gastrointestinal tumors and perhaps in other types of human adenocarcinoma is much higher than that of type 1 chain derivatives. Nevertheless, type 1 chain antigens in either sialylated or sialylated/fucosylated form are absent or present in small quantities in normal gastrointestinal mucosae, since the majority of type 1 chains are converted to blood group H, Le^a, or Le^b as well as A and B determinants. In tumor tissue, however, sialylation of either the terminal Gal or the penultimate GlcNAc may take place preferentially. Subsequently, a terminally sialylated type 1 chain can be α1→4 fucosylated at the penultimate GlcNAc to form sialosyl Le^a (Hansson & Zopf, 1985). Therefore, monoclonal antibodies directed to type 1 chain derivatives are equally useful as those directed to type 2 chain derivatives in diagnosis of human cancer. As previously described by Magnani et al. (1983), those antigens chemically characterized as glycolipids of tumor cells represent only minor components; the majority of antigens may be present as glycoproteins.

A number of immunochemical and chemical investigations based on monoclonal antibodies directed to tumor-associated carbohydrate antigens clearly demonstrate that common changes occurring in the majority of human cancer are related to the carrier structures of blood group ABH and Lewis antigens; therefore, their biosynthesis and genetic basis are of great importance in understanding the phenotype of human malignancy.

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Registry No. NeuAcα2→3Galβ1→3GlcNAc(6←2NeuAcα2)-β1→3Galβ1→4Glcβ1-1Cer, 101374-99-6.

REFERENCES

Arends, J. W., Verstyne, C., Bosman, F. T., Hilgers, J., & Steplewski, Z. (1983) *Hybridoma 2*, 219–229.

Atkinson, B. F., Ernst, C. S., Herlyn, M., Steplewski, Z., Sears, H. F., & Koprowski, H. (1982) *Cancer Res. 42*, 4820–4823.

Björndal, H., Lindberg, B., & Svensson, S. (1967) *Carbohydr. Res. 5*, 433–440.

Bremer, E. G., Levery, S. B., Sonnino, S., Ghidoni, R., Canevari, S., Kannagi, R., & Hakomori, S. (1984) *J. Biol. Chem. 259*, 14773–14777.

Chia, D., Terasaki, P. I., Suyama, N., Galton, J., Hirota, M., & Katz, D. (1985) *Cancer Res. 45*, 435–437.

Corfield, A. P., Veh, R. W., Wember, M., Michaeski, J.-C., & Schauer, R. (1981) *Biochem. J. 197*, 293–299.

Folch-Pi, J., Arsov, S., & Meath, J. A. (1951) *J. Biol. Chem. 191*, 819–831.

Fukuda, M., Dell, A., & Fukuda, M. N. (1984) *J. Biol. Chem. 259*, 4782–4791.

Fukuda, M. N., Dell, A., Oates, J. E., & Fukuda, M. (1985) *J. Biol. Chem. 260*, 6623–6631.

Fukushi, Y., Hakomori, S., Nudelman, E., & Cochran, N. (1984a) *J. Biol. Chem. 259*, 4681–4685.

Fukushi, Y., Nudelman, E., Levery, S. B., Rauvala, H., & Hakomori, S. (1984b) *J. Biol. Chem. 259*, 10511–10517.

Fukushi, Y., Hakomori, S., & Shepard, T. (1984c) *J. Exp. Med. 159*, 506–520.

Hakomori, S. (1964) *J. Biochem. (Tokyo) 55*, 205–208.

Hakomori, S. (1984) *Annu. Rev. Immunol. 2*, 103–126.

Hakomori, S., & Kannagi, R. (1983) *JNCI, J. Natl. Cancer Inst. 71*, 231–251.

Hakomori, S., Nudelman, E., Levery, S. B., & Patterson, C. M. (1983a) *Biochem. Biophys. Res. Commun. 113*, 791–798.

Hakomori, S., Patterson, C. M., Nudelman, E., & Sekiguchi, K. (1983b) *J. Biol. Chem. 258*, 11819–11822.

Hakomori, S., Nudelman, E., Levery, S. B., & Kannagi, R. (1984) *J. Biol. Chem. 259*, 4672–4680.

Hansson, G. C., & Zopf, D. (1985) *J. Biol. Chem. 260*, 9388–9392.

IUPAC (1977) *Lipids 12*, 455–463.

Kannagi, R., Nudelman, E., Levery, S. B., & Hakomori, S. (1982) *J. Biol. Chem. 257*, 14865–14874.

Kannagi, R., Levery, S. B., Ishigami, F., Hakomori, S., Shevinsky, L. H., Knowles, B. B., & Solter, D. (1983a) *J. Biol. Chem. 258*, 8934–8942.

Kannagi, R., Stroup, R., Cochran, N. A., Urdal, D. L., Young, W. W., Jr., & Hakomori, S. (1983b) *Cancer Res. 43*, 4997–5005.

Karlsson, K.-A., & Larson, G. (1979) *J. Biol. Chem. 254*, 9311–9316.

Karlsson, K.-A., Pascher, I., Pimlott, W., & Samuelsson, B. E. (1974) *Biomed. Mass Spectrom. 1*, 49–56.

Koprowski, H., Steplewski, Z., Mitchell, K., Herlyn, M., Herlyn, D., & Fuhrer, P. (1979) *Somatic Cell Genet. 5*, 957–972.

Kundu, S. K., Samuelsson, B. E., Pascher, I., & Marcus, D. M. (1983) *J. Biol. Chem. 258*, 13857–13866.

Laine, R. A. (1980) in *Twenty-Seventh International Congress of Pure and Applied Chemistry* (Varmavuori, A., Ed.) pp 193–198, Pergamon, New York.

Ledeen, R., & Yu, R. K. (1982) *Methods Enzymol. 83*, 139–191.

Ledeen, R. W., Kundu, S. K., Price, H. C., & Fong, J. W. (1974) *Chem. Phys. Lipids 13*, 429–446.

Magnani, J. L., Nilsson, B., Brockhaus, M., Zopf, D., Steplewski, Z., Koprowski, H., & Ginsburg, V. (1982) *J. Biol. Chem. 257*, 14365–14369.

Magnani, J. L., Steplewski, Z., Koprowski, H., & Ginsburg, V. (1983) *Cancer Res. 43*, 5489–5492.

McNeil, M., & Albersheim, P. (1977) *Carbohydr. Res. 56*, 239–248.

Nilsson, O., Mansson, J. E., Lindholm, L., Holmgren, J., & Svennerholm, L. (1985) *FEBS Lett. 182*, 398–402.

Nudelman, E., Hakomori, S., Kannagi, R., Levery, S., Yeh, M.-Y., Hellström, K. E., & Hellström, I. (1982) *J. Biol. Chem. 257*, 12752–12756.

Nudelman, E., Fukushi, Y., Levery, S. B., Higuchi, T., & Hakomori, S. (1986) *J. Biol. Chem.* (in press).

Rauvala, H., Krusius, T., & Finne, J. (1978) *Biochim. Biophys. Acta 531*, 266–274.

Schauer, R. (1982) *Sialic Acids: Chemistry, Metabolism, and Function*, Springer-Verlag, Wien and New York.

Siddiqui, B., & Hakomori, S. (1973) *Biochim. Biophys. Acta 330*, 147–155.

Skipski, V. P. (1975) *Methods Enzymol. 35*, 396–425.

Smith, D., & Ginsburg, V. (1980) *J. Biol. Chem. 255*, 55–59.

Smith, D., Zopf, D., & Ginsburg, V. (1978) *Anal. Biochem. 85*, 602–608.

Stellner, K., Hakomori, S., & Warner, G. A. (1973) *Biochem. Biophys. Res. Commun. 55*, 439–445.

Svennerholm, L. (1964) *J. Lipid Res. 5*, 145–155.

Watanabe, K., & Arao, Y. (1981) *J. Lipid Res. 22*, 1020–1024.

Watanabe, K., Laine, R. A., & Hakomori, S. (1975) *Biochemistry 14*, 2725–2733.

Watanabe, K., Powell, M. E., & Hakomori, S. (1979) *J. Biol. Chem. 254*, 8223–8229.

Yang, H.-J., & Hakomori, S. (1971) *J. Biol. Chem. 246*, 1192–1200.

Young, W. W., Jr., Portoukalian, J., & Hakomori, S. (1981) *J. Biol. Chem. 256*, 10967–10972.