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Structural Characterization of x_2 Glycosphingolipid, Its Extended Form, and Its Sialosyl Derivatives: Accumulation Associated with the Rare Blood Group p Phenotype†

Jens J. Thorn,‡,§,‖ Steven B. Levery,‡ Mary Ellen K. Salyan,‡ Mark R. Stroud,‡ Bertil Cedergren,⊥ Birgitta Nilsson,⊥ Sen-itiroh Hakomori,*,‡ and Henrik Clausen‡,#

The Biomembrane Institute, 201 Elliott Avenue West, Seattle, Washington 98199, Department of Pathobiology, University of Washington, Seattle, Washington, Department of Oral Medicine and Oral Surgery, University Hospital, Copenhagen, Denmark, Department of Oral Pathology and Oral Medicine and Department of Oral Diagnosis, School of Dentistry, Faculty of Health Sciences, University of Copenhagen, Denmark, and Bloodcentral, Umeå, Sweden

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ABSTRACT: It has been suggested that the x_2 glycosphingolipid (GSL) could offer a structural basis for a P-like antigen activity found in blood group p individuals [Kannagi R., Fukuda, M. N., Hakomori, S. (1982) *J. Biol. Chem. 257*, 4438]. The structures of the x_2 and sialosyl-x_2 GSLs have been confirmed unequivocally as shown below by $^+$FAB-MS, methylation analysis by GC-MS, and ^{1}H-NMR. We have established a

x_2 GalNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer

sialosyl-x_2 NeuAcα2→3GalNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer

monoclonal antibody (TH2) specific for the GalNAcβ1→3Galβ1→4GlcNAc epitope, the terminal trisaccharide of x_2 GSL. Application of MAb TH2 on TLC immunoblotting together with chemical analysis indicates the following points of interest: (i) the existence of extended type GSLs having the same x_2 terminal structure; (ii) the chemical quantities of x_2, sialosyl-x_2, and extended x_2 found in blood cells and in various tissues including carcinomas being nearly the same; (iii) considerably larger quantities of x_2 and x_2-derived structures found in blood samples of rare blood group p individuals. The accumulation of x_2 and its derivatives in blood cells of p individuals is in contrast to the occurrence of these GSLs as extreme minor components in normal human red blood cells and tissues, and they may be responsible for the reported P-like activity in blood group p individuals [Naiki, M., & Marcus, D. M. (1977) *J. Immunol. 119*, 537].

First described by Landsteiner and Levine (1927), the P blood group system consists of three antigens, P, P_1, and P^k, and five phenotypes, the common P_1 and P_2 and the rare p, P_1^k, and P_2^k [for reviews, see Prokop and Uhlenbruck (1969) and Race and Sanger (1975)]. The P_1 and P^k antigens, while structurally related by virtue of having the identical immunodominant terminal sugar, are products of divergent pathways and possess distinct serological activities. The P^k and P antigens, on the other hand, are directly biosynthetically related (see Figure 1) [for reviews on immunochemistry of the P system, see Watkins (1980) and Marcus et al. (1981)].

Although blood group p individuals do not express the P antigen identified as Gb_4, p RBCs[1] show reactivity with anti-P serum (Naiki & Marcus, 1977). One candidate for this P-like

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* Address correspondence to this author.
‡ The Biomembrane Institute and Department of Pathobiology, University of Washington.
§ Department of Oral Medicine and Oral Surgery, University Hospital, Copenhagen.
‖ Department of Oral Pathology and Oral Medicine, School of Dentistry, Faculty of Health Sciences, University of Copenhagen.
⊥ Bloodcentral.
Department of Oral Diagnosis, School of Dentistry, Faculty of Health Sciences, University of Copenhagen.

[1] Abbreviations: BSA, bovine serum albumin; CM, chloroform–methanol; CMW, chloroform–methanol–water; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunostaining assay; $^+$FAB-MS, positive ion fast atom bombardment mass spectrometry; FITC, fluorescein isothiocyanate; GC–MS, gas chromatography–mass spectrometry; GSL, glycosphingolipid; ^{1}H-NMR, proton nuclear magnetic resonance spectroscopy; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; Ig, immunoglobulin; IHW, isopropanol–hexane–water; MAb, monoclonal antibody; PBS, phosphate-buffered saline; PG, paragloboside; PMAA, partially methylated alditol acetate; RBC, red blood cell; RT, room temperature; SPG, sialosylparagloboside; WBC, white blood cell.

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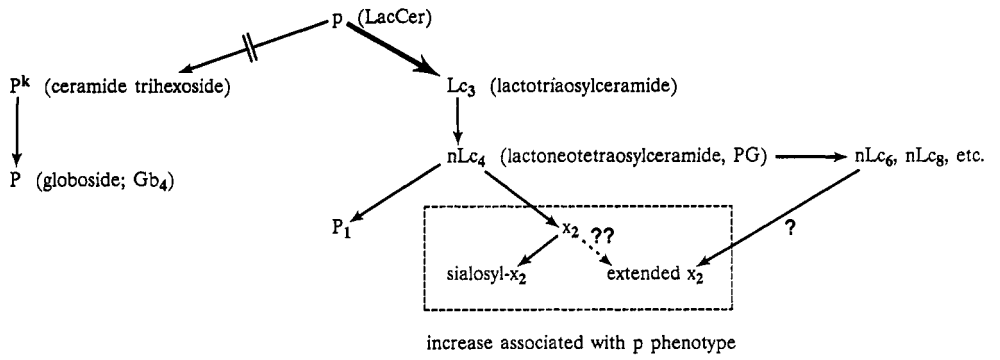


p	(LacCer)	Galß1→4Glcß1→1Cer
Pk	(CTH)	Galα1→4Galß1→4Glcß1→1Cer
P	(Gb_4)	GalNAcß1→3Galα1→4Galß1→4Glcß1→1Cer
P_1		Galα1→4Galß1→4GlcNAcß1→3Galß1→4Glcß1→1Cer
x_2		GalNAcß1→3Galß1→4GlcNAcß1→3Galß1→4Glcß1→1Cer

FIGURE 1: Synthetic scheme and structures of GSLs.

activity was proposed by Kannagi et al. (1982a) to be the so-called "x_2" GSL, on the basis of cross-reactivity with anti-Gb_4 MAbs. Both the x_2 GSL and its sialosylated form were separated from human RBC membranes and tentatively characterized by direct probe mass spectrometry and degradation with exo- and endoglycosidases (Watanabe & Hakomori, 1979; Kannagi et al., 1982a). For various reasons (see Discussion), structural assignment of both x_2 and sialosyl-x_2 was tentative, and it awaited verification by more modern methods. These GSLs are known to be present in very small quantities in normal RBCs, but their general tissue distribution is unknown, as is their expression in blood group p individuals.

Here we report further characterization of x_2 and sialosyl-x_2 and, with the aid of a newly-established MAb specific for the x_2 epitope, demonstrate the existence of more complex GSLs with these epitopes. We also report the application of this MAb in studies of tissue and cell distribution of x_2 and sialosyl-x_2. The epitope was found to be highly expressed in blood group p individuals lacking both P (Gb_4) and A and H variants (H/A-Gb_4) thereof.

Experimental Procedures

Isolation of GSLs. Pooled outdated human whole blood was lysed in ice-cold tap water containing 0.25% acetic acid, and membranes were prepared by continuous centrifugation on a Sharpless centrifuge and extracted with IHW (55:25:20 v/v/v). The Folch "upper phase" GSL fraction was prepared as previously described (Kannagi et al., 1982c). The neutral GSL and the monosialosyl fractions were separated by DEAE-Sephadex (A-25) chromatography (Yu & Ledeen, 1972). x_2 and sialosyl-x_2 were obtained from the neutral and the monosialosyl fractions respectively. Fractionation was accomplished by low-pressure HPLC on a 1 × 50 cm column of porous silica gel, Iatrobeads 6RS-8060 (60-μm particles; Iatron Chemical Co., Tokyo, Japan) (Ando et al., 1976). The elution was carried out with a programmed gradient of IHW (55:42:3 to 55:20:25) during 200 min with a flow rate of 1 mL/min. Each 2-mL fraction was collected on a fraction collector. Fractions containing x_2 were detected by HPTLC in CMW (50:40:10). The procedure was repeated until only one major band in the x_2 area was distinguishable. This was further separated into seven bands as acetates by HPTLC in dichloroethane–acetone–water (40:60:2.5). Acetylation was performed in pyridine–acetic acid anhydride (2:1) at RT for 18 h. Preparative HPTLC was performed on Merck plates (Darmstadt, West Germany). GSLs on HPTLC plates were detected by primulin spray (Aldrich) followed by observation under UV light (Skipski, 1975). GSL acetates were extracted from HPTLC silica by sonication in CM (2:1) and deacetylated in CM (2:1) containing 1% sodium methoxide at RT for 1 h (Clausen et al., 1987). The fraction containing the x_2 GSL was determined by enzymatic degradation of native compounds. The reaction mixture contained 5 μg of GSL, 30 μg of taurocholate, 40 μL of sodium acetate (0.2 M, pH 4.6), and 0.2 unit of β-*N*-acetylhexosaminidase (Jack Bean, Sigma). β-Galactosidase (Jack Bean, Sigma) was used as a control. The reaction was performed at 37 °C overnight and stopped by N_2 evaporation. Only one fraction showed a change in mobility on HPTLC to that of nLc_4 (PG), after treatment with β-*N*-acetylhexosaminidase.

HPLC fractions containing sialosyl-x_2 were found by positive reactivity with TH2 on HPTLC immunostaining following treatment of GSLs with 1% acetic acid for 60 min at 90 °C. Final separation and preparative HPTLC were carried out using a solvent system of 2-propanol–NH_4OH–water (6:1.3:1).

GSLs from various human tissues were extracted with IHW (55:25:20), followed by a Folch partition. Neutral fractions, monosialosyl fractions, and disialosyl fractions were obtained by DEAE-Sephadex chromatography (Yu & Ledeen, 1972). Reactivity of MAb TH2 with monosialosyl and disialosyl fractions was tested after desialosylation with 1% acidic acid at 98 °C for 1 h.

Generation of anti-x_2 MAb TH2. The x_2 GSL was inserted into liposomes with lipid A according to a method previously described (Brodin et al., 1986). A mouse MAb (TH2) was obtained by immunization iv with 2 μg of x_2 per injection, and spleen cells were fused with NS-1 myeloma cells (Köhler & Milstein, 1975), three injections followed by eyebleed screening, and a fourth injection three days prior to fusion. Hybridomas were cloned by limited dilution at least three times. The reactivity of hybridoma MAbs was tested by ELISA and HPTLC immunostaining using various GSL

standards (Kannagi et al., 1982c). Particle-concentrated fluorescence immunoassay with isotype-specific goat anti-mouse FITC-conjugated MAbs was used to determine isotypes of the secreted MAbs as described previously (Clausen et al., 1988).

ELISA Assays. ELISA was performed by coating 96-well plates (Falcon, Pro-bind assay plate) with x_2 (25 ng/well) (Gb_4 as a control) and blocking with 5% BSA in PBS. The GSLs were incubated with supernatant for 4 h, coupled with (1:1000) goat anti-mouse Ig(M+G) conjugated to peroxidase (Boehringer-Manheim, Indianapolis, IN) and visualized by 0.1 M citric acid, pH 5, with 0.2 μg/mL *o*-phenylenediamine dihydrochloride and 5 μg/mL H_2O_2.

HPTLC Immunostaining of GSLs. GSLs were streaked onto HPTLC plates (Whatman, England) and subjected to ascending chromatography using solvent systems described previously. After being dried, the plates were blocked with 5% BSA in PBS for 1 h and incubated overnight at 4 °C in undiluted MAb TH2 supernatant. After being washed, plates were incubated with rabbit anti-mouse IgG (ICN Immuno-Biologicals) diluted 1:1000 for 1 h, followed by incubation with ^{125}I-labeled protein A (2×10^6 cpm/microliter) for 45 min. The plates were examined by autoradiography on Kodak X-OMAT AR5 film.

Biosynthesis of Fucosyl-x_2. Reaction mixtures contained 5 μmol of Tris buffer, pH 7.4, 2 μg of acceptor GSL (x_2 or nLc_4), 2 μg of taurodeoxycholate, 0.5 μmol of $MnCl_2$, 5 μmol of CDP-choline, 5 μg of GDP-fucose, or 10 nmol of GDP-[^{14}C]fucose (20 000 cpm/nmol), and 50 μL of a Triton-X100 extraction of Colo205 cells (Holmes & Levery, 1989a), all in a total volume of 100 μL. Assays lacking acceptor GSL were performed to control for endogenous GSL substrates. The reaction mixture was incubated for 2 h at 37 °C and stopped by addition of 100 μL of CM (2:1), followed by N_2 evaporation. Radioactive GSLs were located by autoradiography, and HPTLC immunostaining was performed on both nonradioactive and radioactive GSLs (data not shown).

Immunofluorescence Staining Procedure. Cell lines were grown according to ATCC recommendations, air-dried on slides in 5% solution of PBS at RT for 2 h and fixed with ice-cold acetone for 5 min, incubated with primary MAb overnight at 4 °C, washed, and incubated with fluorescein-conjugated rabbit anti-mouse Ig (Dakopatts, Denmark) diluted 1:80 for 40 min, 20 °C. Control reactions included staining with the conjugate alone and substitution of the primary MAb with other mouse IgM MAbs. Human carcinoma cell lines studies were A431, MCF-7, T-47D, Calu-3, Sk-Lu-1, H2981, Sk-Mes-1, Lu-65, MKN-45, Kato-III, SW-48, SW-480, Colo205, LS-174-T, HT-29, SW-1116, HRT-18, H-1733, Ramos, U-87-MG, SK-N-SH, KO, KANR, and Y79.

Flow Cytometry (FACS) Analysis. A 100 μL-cell suspension of 2×10^6 WBCs or 2×10^8 RBC/mL in 1% BSA–PBS was incubated with undiluted TH2 supernatant for 30 min at RT, followed by wash and incubation for 30 min with FITC-conjugated goat anti-mouse IgG (Fluoricon, Baxter) before analysis in a FACSCAN flow cytometer (EPICS, Coulter, Hialeah, FL). Isotype controls were performed, as well as negative controls with only secondary MAb. A total of 5×10^3 WBCs and 10^5 RBCs were analyzed for each sample.

^{1}H-NMR. Approximately 0.5 mg of sample was deuterium exchanged by repeated lyophilization from DMSO-d_6/D_2O (98:2) and then dissolved in 0.4 mL of this solvent for ^{1}H-NMR analysis. One-dimensional spectra were recorded at 308 ± 2 K on a Bruker (Karlsruhe, Germany) AM-500 Fourier transform spectrometer/Aspect 3000 data system, using quadrature detection. The sweep width was 5000 Hz, collected over 16K data points. The residual HOD resonance was suppressed using a presaturation pulse during the preparatory delay period. The preparatory delay was 2.0 s. A Lorentzian to Gaussian transformation was used for resolution enhancement.

$^+$FAB-MS. A sample of GSL (100–150 μg) was permethylated by the method of Ciucanu and Kerek (1984), as modified by Larson et al. (1987), except that equal volumes of MeI and DMSO were used (200 μL each). The reaction time was 60 min, and MeI was removed by flushing with N_2 for 25 min at 37 °C prior to partitioning between $CHCl_3$ and H_2O. After being washed three times with H_2O, the $CHCl_3$ was dried under N_2, and a portion of the permethylated sample was subjected to $^+$FAB-MS, performed on a JEOL (Tokyo, Japan) HX-110/DA-5000 mass spectrometer/data system. Aliquots of permethylated sample (≈20 μg) in MeOH were transferred to a FAB target and suspended in 3-nitrobenzyl alcohol matrix (Meili & Seibl, 1984; Barber et al., 1988) with and without 15-Crown-5 (Holmes & Levery, 1989a,b; Isobe et al., 1987). Additional experiments were performed with the addition of sodium acetate to matrix (Dell, 1987; Egge & Peter-Katalinic, 1987). The scan range was 100–3000 atomic mass units; scan slope was 1 min, 30 s; acceleration voltage was 10 kV; resolution was 3000; xenon beam was 6 kV. Three scans were accumulated for each spectrum. KI/CsI was used as calibration standard.

Methylation Analysis. The remainder of the permethylated sample was hydrolyzed, reduced, and acetylated according to published procedures (Levery & Hakomori, 1987). Partially O-methylated, N-methylated deoxyhexitol, hexitol, and hexosaminitol acetates were analyzed by GC–MS under conditions described in the legend to Figure 6. Derivatives were identified by characteristic ions (Levery & Hakomori, 1987; McNeil & Albersheim, 1977; Laine, 1981; Björndal et al., 1970; Jansson et al., 1976; Stellner et al., 1973; Stoffel & Hanfland, 1973; Tai et al., 1975) and retention times, verified by coinjection with standards when necessary.

Results

Isolation of x_2 and Sialosyl-x_2 GSLs. An HPLC fraction migrating as one major band in the solvent system CMW (50:40:10) was separated into seven bands as acetates by preparative HPTLC. The band containing x_2 GSL was detected by exoglycosidase treatment, i.e., cleaving off β-GalNAc to produce a GSL co-migrating with nLc_4.

HPLC fractions containing sialosyl-x_2 GSL were selected by reactivity to MAb TH2 (see below) after desialosylation by 1% acetic acid. Purification was accomplished by preparative HPTLC using a solvent system of 2-propanol–NH_4OH–water (6:1.3:1).

Production and Characterization of MAb TH2. A mouse hybridoma secreting IgG_3 MAb (TH2) was isolated after immunization with x_2 GSL and selected by reactivity with x_2. Specificity of TH2 was further tested using HPTLC immunostaining and ELISA against a variety of purified GSLs. It reacted strongly with x_2 but not with Gb_4 which has the same terminal GalNAcβ1→3Gal (Figure 2). It did not react with Forssman-GM_1 (GalNAcα1→3GalNAc), with asialo-GM_2 (GalNAcβ1→4Gal), or with nLc_6, asialo-GM_1, GM_1, or GM_2.

Extended Structure with TH2 Reactivity. In the neutral fraction, among the upper phase GSLs in human blood group O, three bands reacting with TH2 were distinguishable (Figure 3, lane 6). The second (first extended form), having a slower mobility than x_2, was seen in the area between H_2 and H_3. The

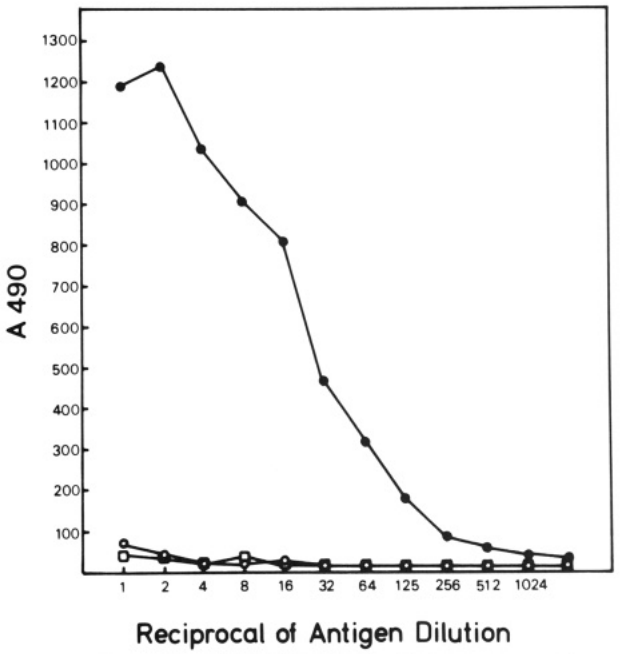


FIGURE 2: Reactivity of MAb TH2. Symbols: ●, x_2; □, Gb_4; ○, background. ELISA was as described in text.

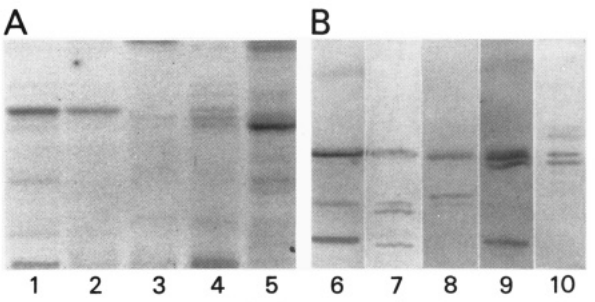


FIGURE 3: HPTLC patterns of GSL extracts of various tissues. Panel A: visualized with orcinol/H_2SO_4 reagent. Panel B: immunostain with MAb TH2. Lanes 1 and 6: upper neutral fraction of O RBCs. Lanes 2 and 7: upper neutral fraction of A type RBCs from umbilical cord. Lanes 3 and 8: desialosylated, disialosyl fraction of normal lung tissue. Lanes 4 and 9: prostate tumor, upper neutral fraction. Lanes 5 and 10: desialosylated, monosialosyl fraction of lung tumor.

third band (second extended form) was found to migrate below H_3. All three forms were found in RBCs from the umbilical cord of a blood type A person (Figure 3, lane 7).

After acetic acid treatment of the monosialosyl fraction of the "upper phase" GSLs of pooled human blood, a series of sialosylated GSLs was found to be reactive with MAb TH2. Following desialosylation, the uppermost sialosyl-x_2 GSL band migrated at the x_2 position (Figure 4, band A), while two extended forms co-migrated with the first and second extended forms of x_2 found in the nonsialoylated fractions (Figure 4, bands B and C).

Some monosialosylated GSL extracts of RBCs of type A and B distinctly showed more extended forms (Figure 4, bands D, E, and F). Whether these additional bands are due to differences in the ceramide moiety or represent three more structurally distinct extended forms is unclear at this time.

A minor fraction of the second extended sialosylated form was partially purified by HPLC and preparative HPTLC. Preliminary studies of the inner core of the extended structure were performed using sequential exoglycosidase treatment and subsequent HPTLC immunostaining with MAbs to *N*-acetyllactosamine and asialo-GM_1 (data not shown). By these criteria, however, the presence of neolacto-series or ganglio-series core structures was not confirmed. To determine if the

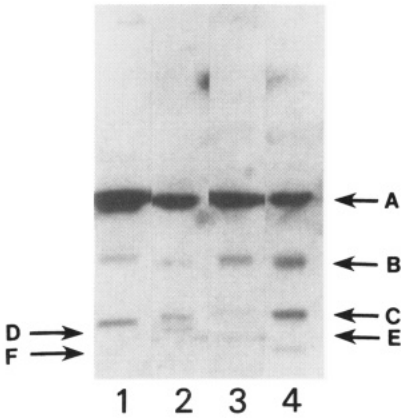


FIGURE 4: HPTLC immunostaining with MAb TH2. Lane 1: upper neutral GSL fraction of blood group O RBCs. Lane 2: monosialosyl fraction of A_1 RBCs desialosylated with 1% acidic acid. Lane 3: desialosylated monosialosyl fraction of A_2 RBCs. Lane 4: desialosylated monosialosyl fraction of B RBCs. Band A: x_2. Band B: first extended form of x_2. Band C: second extended form of x_2. Bands D, E, and F: see text.

first extension of x_2 is due to fucosylation, biosynthesis of fucosyl-x_2 was performed. From this experiment, it was concluded that Colo205 fucosyltransferase can utilize x_2 as an acceptor substrate. However, fucosyl-x_2 did not co-migrate with the first extended form of x_2 and was not recognized by MAb TH2 (data not shown).

Chemical Characterization of x_2 GSL. The purified x_2 GSL fraction was permethylated, and an aliquot of the intact product was subjected to $^+$FAB-MS analysis (Figure 5). Clear pseudomolecular ion clusters were observed, consistent with a sugar composition of $Hex_3{\cdot}HexNAc_2$, in combination with ceramides consisting predominantly of d18:1 sphingosine plus 22:0, 24:1, and 24:0 fatty acids (see scheme presented in Figure 5). In addition, familiar A^1-type fragments (Kochetkov & Chizhov, 1966; Dell, 1987) were observed, with preferential cleavage at HexNAc residues, and charge retention on the nonreducing portions (Dell, 1987; Egge & Peter-Katalinic, 1987). Thus, the predominant fragment pairs at m/z 260→228 (terminal HexNAc) and 709→677 ($Hex{\cdot}HexNAc_2$), along with the less abundant fragments at 464→432 (Hex·HexNAc) and 913→881 ($Hex_2{\cdot}HexNAc_2$), clearly demonstrate the linear nonreducing saccharide sequence HexNAc-O-Hex-O-HexNAc-O-Hex-. Further confirmation of structure was provided by the ion group at m/z 1285, 1311, and 1313, representing the reducing-end $Hex_2{\cdot}HexNAc{\cdot}Cer$ fragment.

The monosaccharide composition and linkage structure were provided by methylation analysis with GC–MS of PMAAs produced following hydrolysis of the permethylated compound (Figure 6A). Taken with the $^+$FAB-MS data, the detection of 3,4,6-tri-*O*-Me-GalNAcMe (T-GalNAc), 3,6-di-*O*-Me-GlcNAcMe (4-linked GlcNAc), 2,4,6-tri-*O*-Me-Gal (3-linked Gal), and 2,3,6-tri-*O*-Me-Glc (4-linked Glc), in an approximate ratio of 1:1:2:1, indicates that the structure must be GalNAcl→3Gal1→4GlcNAc1→3Gal1→4Glc1→1Cer, provided one assumes the Glc residue is attached to ceramide.

The anomeric configuration of each saccharide residue, as well as confirmation of other structural features, was provided by 1-D ^{1}H-NMR of the native GSL in DMSO-d_6/2% D_2O at 308 K (Figure 7). The downfield region clearly displayed five β-anomeric resonances ($^3J_{1,2}$ = 7–9 Hz), which were assigned by comparison with spectra of other GSLs obtained under similar conditions (Levery et al., 1988; Ostrander et al., 1988). The chemical shifts of three resonances at 4.168 ppm

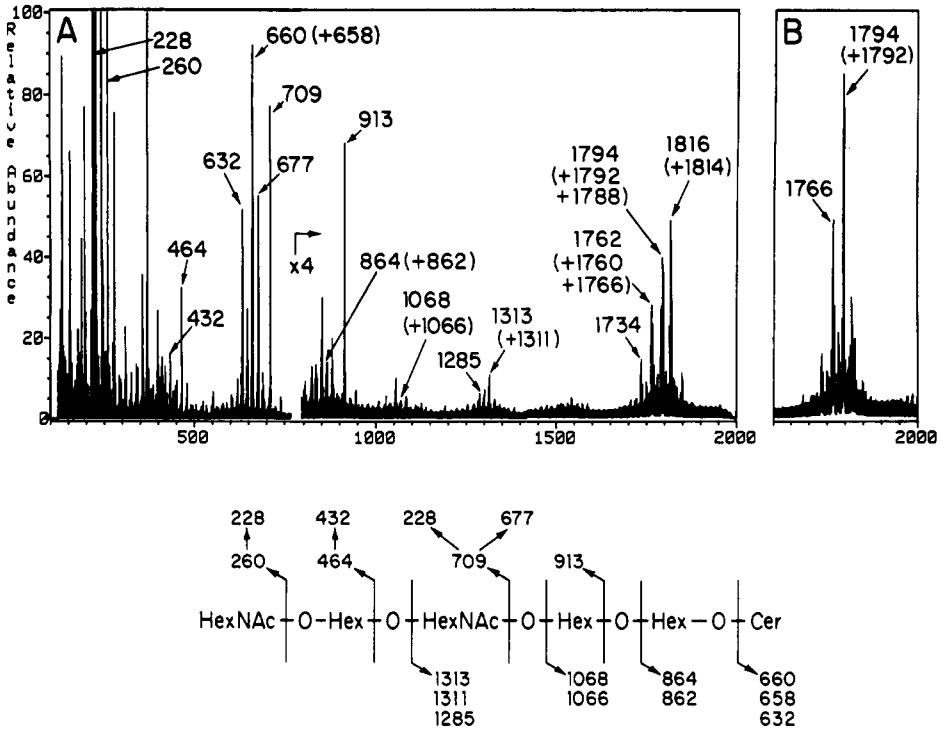


FIGURE 5: $^{+}$FAB mass spectra of permethylated x_2 GSL in NBA matrix. Panel A is a composite of two separate determinations with conditions optimized for acquisition of different mass ranges. In panel B, 15-crown-5 was added to the matrix to suppress formation of sodium-complexed pseudomolecular ions. Drawn below the spectra is a proposed fragmentation scheme. All assignments are nominal, monoisotopic masses. Axes: ordinate, relative ion abundance; abscissa, mass to charge ratio (m/z).

($^3J_{1,2}$ = 7.3 Hz), 4.260 ppm ($^3J_{1,2}$ = 6.7 Hz), and 4.659 ppm ($^3J_{1,2}$ = 7.9 Hz), were virtually identical to those found for H-1 of Glc I, Gal II, and GlcNAc III, respectively, of nLc$_4$Cer (Levery et al., 1988). A fourth resonance, at 4.275 ppm ($^3J_{1,2}$ = 7.3 Hz), could be assigned to Gal IV of an internal nLc$_4$Cer sequence, on the basis of an expected glycosylation-induced downfield shift resulting from the additional terminal GalNAcβ1→3 residue. The resonance found at 4.583 ppm ($^3J_{1,2}$ = 8.5 Hz) could then be assigned to this terminal residue. A similar H-1 chemical shift (4.589 ppm) was found for the terminal residue of the sequence GalNAcβ1→3Galα1→3, derived from a GSL found in English sole liver (Ostrander et al., 1988), while that found in the GalNAcβ1→4Galβ1→4 sequence resonated considerably upfield (4.462 ppm) (Koerner et al., 1983). Three Gal H-4 resonances were tentatively identified and assigned as shown in Figure 7. Two HexNAc *N*-acetyl methyl signals (not shown) were found at 1.820 ppm (identical to β-GlcNAc NAc of type 2 *N*-acetyllactosamine sequences) and at 1.855 ppm (assigned to the β-GalNAc residue). Thus, all data were consistent with the structure GalNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer for x_2, as proposed previously by Kannagi et al. (1982a).

Chemical Characterization of Sialosyl-x_2 GSL. Considerably less material was available to work with in this fraction, precluding acquisition of a useful NMR spectrum. Therefore, structural elucidation depended on chemical data consisting solely of $^{+}$FAB-MS and methylation analysis. Nevertheless, combination of these results with HPTLC, enzymatic degradation, and immunostaining data allowed structural identification of this fraction with a high degree of confidence. In the $^{+}$FAB mass spectrum of the permethylated GSL fraction (Figure 8), clear pseudomolecular ion clusters were observed, consistent with a sugar composition of NeuAc·Hex$_3$·HexNAc$_2$, in combination with ceramides consisting predominantly of d18:1 sphingosine plus 22:0, 24:1, and 24:0 fatty acids (see scheme presented in Figure 8). Predominant A^1-type fragments were observed at m/z 376→344 (terminal NeuAc), 621 (NeuAc·HexNAc), and 1070→1038 (NeuAc·Hex·HexNAc$_2$), along with less abundant fragments at 825 (NeuAc·Hex·

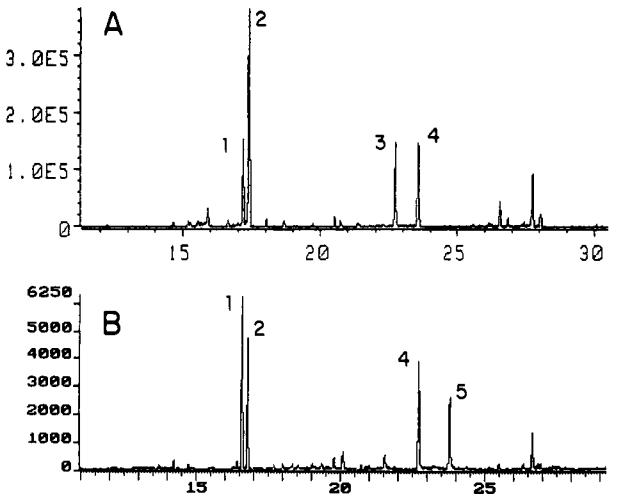


FIGURE 6: GC–MS of monodeuterated partially O-methylated hexitol and 2-*N*-methyl-*N*-acetylhexosaminitol acetates obtained from the hydrolyses of permethylated GSLs. Panel A: x_2. Panel B: sialosyl-x_2. Separations were performed on a 30-m DB-5 bonded phase fused silica capillary column (0.25 mm i.d.) using splitless injection. The temperature was programmed from 140 to 250 °C at 4 deg/min. In panel A, the mass spectrometer was a Hewlett-Packard 5970B mass selective detector (electron impact ionization mode). Axes: ordinate, summation of selected ion intensities (m/z 117, 118); abscissa, retention time in minutes. In panel B, the mass spectrometer was an Extrel ELQ 400 (chemical ionization, methane reagent gas). Axes: ordinate, summation of intensities of all relevant MH$^+$, (MH-32)$^+$, and (MH-60)$^+$ ions; abscissa, retention time in minutes. Peaks identified: (1) 2,3,6-tri-O-Me-Glc; (2) 2,4,6-tri-O-Me-Gal; (3) 3,4,6-tri-O-Me-GalNAcMe; (4) 3,6-di-O-Me-GlcNAcMe; (5) 4,6-di-O-Me-GalNAcMe. Note that since the retention times of components were affected by the different instruments and conditions used, the abscissas have been adjusted so that identical compounds appear, for the sake of clarity, at the same position.

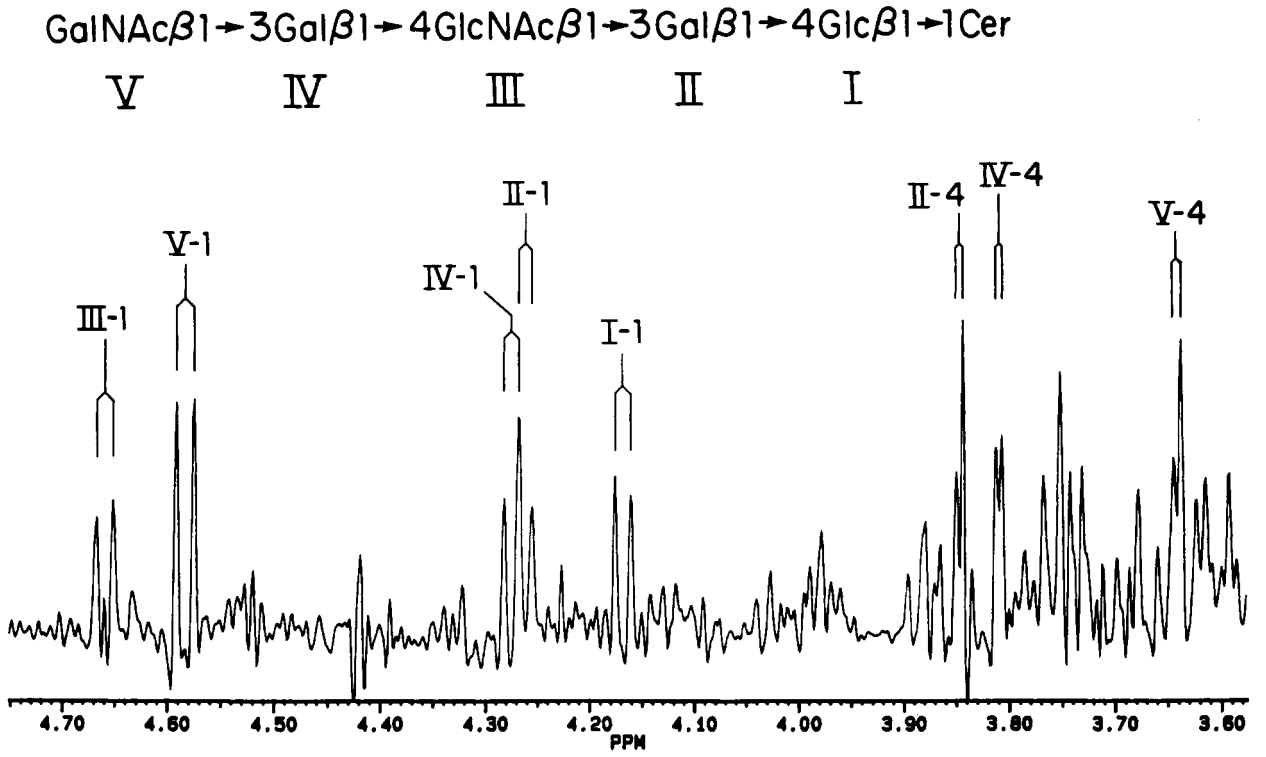
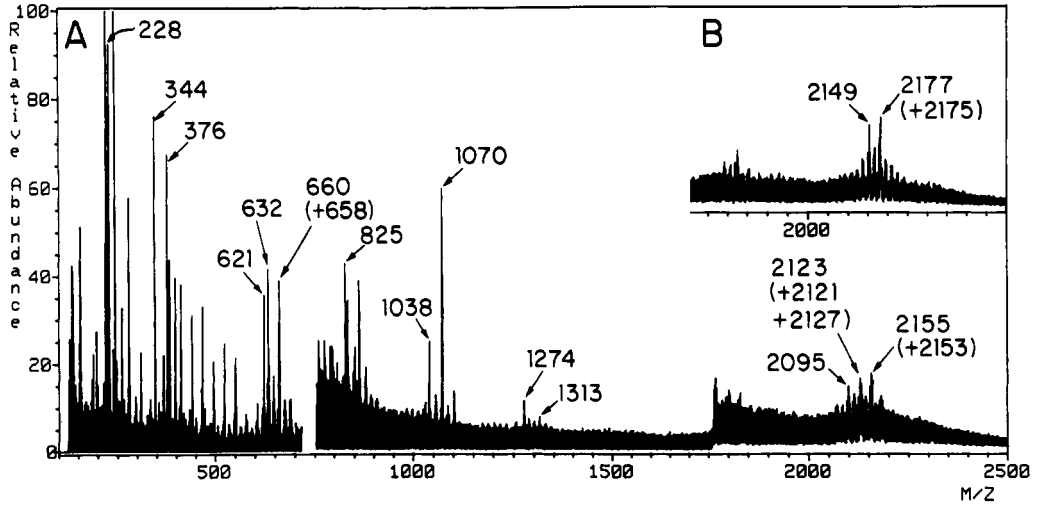
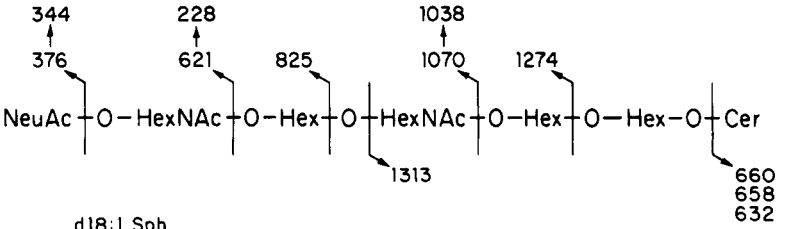


FIGURE 7: Downfield region of resolution-enhanced 500-MHz ^{1}H-NMR spectrum of x_2 GSL in DMSO-d_6/2% D_2O at 308 ± 2 K. Arabic numerals refer to ring protons of residues designated by Roman numerals in the corresponding structure.





d18:1 Sph + fa	MH^+	$[MH-32]^+$	MNa^+
24 : 0	2155	2123	2177
24 : 1	2153	2121	2175
22 : 0	2127	2095	2149

FIGURE 8: $^+$FAB mass spectra of permethylated sialosyl-x_2 GSL in NBA matrix. In spectrum A, 15-crown-5 was added to matrix; for inset B, sodium acetate was added to matrix. Above 750 amu, ion intensities multiplied 5 times. The discontinuity at m/z 1760 in spectrum A is due to the xenon FAB gun being turned on during the first accumulated scan. Other comments are as in Figure 5.

HexNAc) and 1274 (NeuAc·Hex_2·$HexNAc_2$), clearly demonstrating the linear nonreducing saccharide sequence NeuAc-O-HexNAc-O-Hex-O-HexNAc-O-Hex. A ceramide-containing ion found in the spectrum of x_2, providing further confirmation of structure, was observed at m/z 1313, again representing the reducing-end Hex_2·HexNAc·Cer fragment.

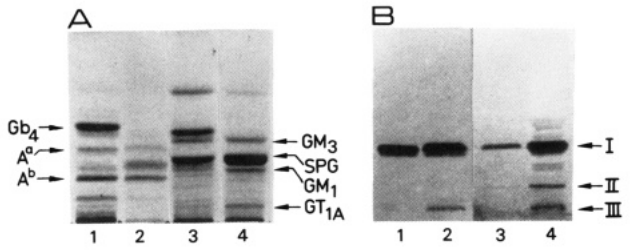


FIGURE 9: HPTLC pattern of Folch upper neutral and monosialosyl GSL extracts from blood group A_1 and A_1,p RBCs. Panel A: orcinol/H_2SO_4 reagent. Panel B: immunostain with MAb TH2. Lane 1: RBC A, upper neutral fraction. Lane 2: RBC $A_{1,p}$, upper neutral fraction. Lane 3: RBC A_1, monosialosyl fraction. Lane 4: RBC A_1,p, monosialosyl fraction. Lanes 3 and 4 in panel B: fraction desialosylated prior to HPTLC analysis. Band I: x_2. Band II: first extended x_2 form. Band III: second extended x_2 form. Note the lack of band in the orcinol stain of p upper neutral sample co-migrating with Gb_4 of A sample.

A number of ions of unknown origin were observed, indicating some increased levels of contamination, as might be expected when working with much smaller amounts of sample.

The monosaccharide composition and linkage structure were again provided by methylation analysis (Figure 6B), although the relative amounts of derivatives detected would not allow a quantitative assessment to be made. In this analysis, no PMAAs arising from terminal Hex or HexNAc residues were detected, consistent with the sialosylated structure indicated by $^+$FAB-MS data. The detection of 4,6-di-*O*-Me-GalNAcMe (3-linked GalNAc), 3,6-di-*O*-Me-GlcNAcMe (4-linked GlcNAc), 2,4,6-tri-*O*-Me-Gal (3-linked Gal), and 2,3,6-tri-*O*-Me-Glc (4-linked Glc) is consistent with the structure NeuAc2→3GalNAc1→3Gal1→4GlcNAc1→3Gal1→4Glc1→1Cer, provided one assumes that (a) the Glc residue is attached to ceramide and (b) the position of HexNAc residues is similar to that in x_2 and other known structures with nLc_4Cer cores. These assumptions are consistent with all other data obtained for this fraction; thus, it is reasonably clear that this GSL is the NeuAcα2→3 derivative of x_2 proposed previously by Watanabe and Hakomori (1979).

Cell and Tissue Distribution of x_2 and Sialosyl-x_2. HPTLC immunostaining showed that x_2 and sialosyl-x_2 were expressed in RBCs regardless of ABO status, and in WBCs as well, whereas fresh plasma was devoid of these antigens (data not shown). These antigens could not, however, be detected on the surface of RBCs or WBCs by flow cytometry with or without neuraminidase treatment, and RBCs were not agglutinated even after protease, trypsin, or neuraminidase pretreatment.

HPTLC immunostaining of neutral fractions of various human tissues showed a doublet band in the x_2 area of liver, spleen, lung, kidney, colon, pancreas, brain, and placenta, but not in bone marrow. Doublets were visible in the area of the first extended form of x_2 in liver, lung, kidney, and placenta. Of 14 samples of mono-, di-, and trisialosyl fractions of normal human tissues, 12 showed reactivity with MAb TH2 in the x_2 region, and 4 showed reactivity in the first extended x_2 region. Of the neutral fractions of 20 different human carcinomas, the x_2 structure was detected in 13; four of these showed extended structures. Monosialosyl fractions of 24 human tumors were treated with acetic acid, and definite reactivity in the x_2 area was found in 23, while traces of extended structures were found in several (data not shown). Examples are presented in Figure 3.

Immunochemical staining with MAb TH2 was negative for all human carcinoma cell lines tested.

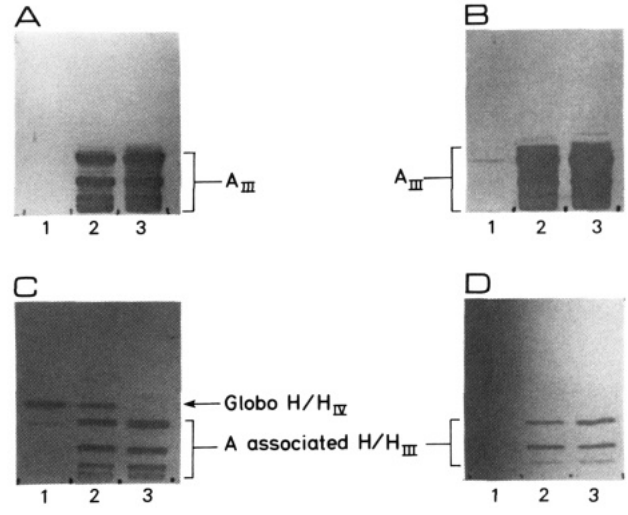


FIGURE 10: HPTLC immunostains of type O, A and A_1,p RBC GSL extracts. Panel A: MAb TH1 (A, type III). Panel B: MAb HH5 (A, type III,IV). Panel C: MAb MBr1 (globo-H; H type III). Panel D: MAb HH14 (H type III). Lane 1: O upper neutral fraction. Lane 2: A upper neutral fraction. Lane 3: A_1,p upper neutral fraction.

GSLs of Blood Group p RBCs. From an extract of RBCs of a single A_1,p individual, MAb TH2 identified x_2 and sialosyl-x_2 in upper neutral and monosialosyl fractions (Figure 9). The blood group p status of this individual was verified by the absence of Gb_4 (Figure 9A) and CTH and accumulation of CDH in the lower phase fraction. Compared with the GSL extract from A RBCs, the accumulation of x_2 in the A_2,p neutral fraction was not exceptionally greater (Figure 9B, lanes 1–2). However, comparison of the monosialosyl fractions showed considerable accumulation of sialosyl-x_2 in A_1,p relative to A RBCs (lanes 3–4). This was also manifested in the enhanced detection of extended forms of sialosyl-x_2 in the A_1,p RBC extract (lane 4). SPG was also found in greater abundance in the A_1,p RBC extract.

Two prominent bands with slower mobility than SPG were found in the ganglioside fraction (Figure 9, lane 4). These had the same mobilities as GM_1 and GT_{1a}, respectively. Traces of fucosyl-GM_1 were detected only in the A_1,p UN by MAb TKH5 (data not shown) (Vangsted et al., 1991).

MAbs HH5 and MBr-1, defining type 3/4 chain A and H antigen, respectively, clearly identified globo-A and globo-H (type 4 antigens) in normal RBC, as shown in Figure 10. As expected, the p upper neutral fraction was found to be completely devoid of these antigens. In contrast, the A-associated type 3 chain A/H variants were found to be equally expressed in normal and p RBCs (Figure 10).

Discussion

Structure and Distribution of x_2 and Sialosyl-x_2 Epitopes. The x_2 and sialosyl-x_2 GSLs previously isolated as minor GSL components from blood group O RBCs were tentatively characterized (Kannagi et al., 1982a; Watanabe & Hakomori, 1979). The previous structure determinations relied heavily on methylation analysis, electron impact mass spectrometry of permethylated materials, and glycosidase digestion monitored by changes in TLC mobility. Because the last method may sometimes yield errors due to contamination with enzymes having different specificities, and because the mass spectrometer used in that work had an attenuated mass range (1000 amu), we considered it worthwhile to confirm the structures using modern methods. Here we have isolated both components, characterized x_2 by 1-D ^{1}H-NMR (Figure 7), and both x_2 and sialosyl-x_2 by $^+$FAB-MS (Figures 5 and 8) and GC–MS

analysis (Figure 6), and by the use of a new MAb TH2, we demonstrated that the x_2 epitope exists on several extended structures (Figures 3 and 4). Our results essentially confirmed the previous structural proposals (Kannagi et al., 1982a; Watanabe & Hakomori, 1979) except for one key difference. Whereas the sialosyl-x_2 isolated by Watanabe and Hakomori (1979) was found to have a ceramide moiety consisting predominantly of 14:0 fatty acid in combination with d18:0 sphingosine, the fraction described herein appeared to have a ceramide consisting mainly of 22:0, 24:1, and 24:0 fatty acids with d18:1 sphingosine. The reasons for this discrepancy are not obvious, but it is possible that the material previously analyzed was contaminated with a nonglycosylated lipid component. This may explain the appearance of several unusual peaks in the electron impact mass spectrum of permethylated sialosyl-x_2 [Figure 1A of Watanabe and Hakomori (1979)]. Interestingly, these peaks are not seen in the mass spectrum of the desialosylated and permethylated material [Figure 1B of Watanabe and Hakomori (1979)]; instead, ceramide ions at m/z 632, 658, and 660, although unmarked, are plainly observed. Since GSLs with short fatty acid aliphatic chains have been proposed to be serum derived (Koscielak et al., 1978), and because sialosyl-x_2 was previously proposed to have a large amount of C14 fatty acid, it was speculated whether this ganglioside was present in serum in larger amounts (Watanabe & Hakomori, 1979). Our findings indicate instead a strict cellular origin of this structure.

The presence of two neutral and at least three sialosylated extended forms of x2, verified by immunoreactivity with MAb TH2 (Figures 3 and 4), is reported here for the first time. They were found in RBCs, WBCs, umbilical cord blood, and a variety of human normal tissues and carcinomas (Figure 3). We anticipated a polylactosamine inner core structure in the more complex GSLs with the x_2 epitope. We were, however, unable to verify this by exoglycosidase treatment or, by the same means, to demonstrate a ganglio-series core. We did not test for globo-series core, but this possibility seemed highly unlikely. On the basis of studies of Colo205 fucosyltransferase (Holmes & Levery, 1989a), it was thought that fucose might be transferred using this enzyme to the internal GlcNAc residue of x_2 in an α1→3 linkage. MAb TH2 did not recognize the product of this reaction, however, nor did the product co-migrate with any extended x_2 band. Therefore, the extension of x_2 recognized by TH2 may not be due to α1→3 fucosylation of GlcNAc. The structure of the inner core on extended x_2 remains unresolved at present.

The neutral and the sialosylated x_2 epitope were both found on GSLs isolated from RBCs and WBCs regardless of ABO blood group. However, the epitopes could not be detected on the membrane of RBCs by hemagglutination or by FACS analysis. The x_2 epitope seems to be widely distributed in other normal tissues in the form of GSLs. Most normal tissues contained both x_2 and sialosyl-x_2. These compounds were not restricted to normal tissues, as they were found in most of the tested tumors as well.

The x_2 GSL has been reported to be present in a high concentration in gastric cancer tissue from a p blood group individual (Kannagi et al., 1982b). A low level of PG in the tumor as compared to in the RBCs suggested a high conversion rate to x_2, and it was anticipated that normal tissues of p individuals would contain the structure. It has not yet been possible for us to test other tissues from such persons.

Relationship to p Blood Group. The neutral GSL extract of RBCs from a blood group A_1,p individual showed no Gb_4 (Figure 9A) or CTH, but it did show accumulation of CDH, confirming the results of Marcus et al. (1976, 1981). The x_2 glycolipid was present in the neutral fraction of A_1,p RBCs in an amount similar to that of A RBCs (Figure 9). Except for SPG, the ganglioside profile of blood group p RBCs was reported to be normal (Marcus et al., 1976, 1981). We found, however, that in addition to accumulation of SPG (Figure 9A), sialosyl-x_2 could also be detected in excess amount, and the ganglio-series GM_3, GM_1, and GT_{1a} could be detected as major bands as well (Figure 9A). Interestingly, trace amounts of fucosyl-GM_1 were detected. This ganglioside has not been detected in normal RBCs (Nilsson et al., 1986) and is therefore further evidence of the abnormal biosynthetic pathways in p RBCs outlined above. The lack of blood group A and O epitopes on globo-core structures (type 4) in these RBCs, along with the normal appearance of A-associated type 3 chain A/H variants, underlines this notion. It has recently been reported that normal tissues from blood group p individuals lack the globo-series GSLs as well (Lindstrom et al., 1991).

It is assumed that the P^k genes code for the α-galactosyltransferase that synthesizes CTH from CDH and P genes code for the transferase that adds GalNAc to CTH to form Gb_4 (Marcus et al., 1981; Watkins & Morgan, 1976). It has been elegantly shown, by in vitro fusion of fibroblasts from a p and a P^k individual, that p blood group persons express the *N*-acetylgalactosamine transferase that converts CTH to Gb_4 (Fellous et al., 1977). Therefore, only the P^k gene is nonfunctioning in p individuals. The lack of the UDP–Gal:CDH α1→4galactosyltransferase in blood group p individuals could be expected to lead to a relatively higher substrate concentration for neolacto/lacto- and ganglio-series GSLs, thus leading to accumulation of other GSLs. The globo-series GSLs account for the major proportion of GSL biosynthesis in RBCs. Deletion of these at the CDH/CTH stage obviously favors the biosynthesis of other GSL series because the CDH structure is the common precursor structure for the various GSL core classes (Clausen & Hakomori, 1989). We have shown that x_2, with the same terminal GalNAcβ1→3Gal epitope as Gb_4, is present on p RBCs. Accordingly, it could be the same enzyme that acts on CTH and PG, and the conversion of PG to x_2 therefore is regulated by the P gene. The acceptor specificity for this enzyme could be unexpectedly low since the terminal galactose is in α-anomeric configuration in CTH and in β-anomeric configuration in PG.

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