Human Tumor-Associated Le^a-Le^x Hybrid Carbohydrate Antigen IV^3(Galβ1→3[Fucα1→4]GlcNAc)III^{3}FucnLc$_4$ Defined by Monoclonal Antibody 43-9F: Enzymatic Synthesis, Structural Characterization, and Comparative Reactivity with Various Antibodies†

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ABSTRACT: Two Le^a-cross-reacting monoclonal antibodies (mAbs) were previously established which define complex tumor-associated carbohydrate antigens. The first mAb, 43-9F, was raised against human squamous cell lung carcinoma and shows preferential reactivity with various human cancers over normal cells. Its tumor cell binding activity is best inhibited by a milk oligosaccharide characterized as Le^a-Le^x [Mårtensson, S., et al. (1988) *Cancer Res. 48*, 2125], Galβ1→3[Fucα1→4]GlcNAcβ1→3Galβ1→4[Fucα1→3]-GlcNAcβ1→3Galβ1→4Glc (**1**). The second mAb, ST-421, was raised against human gastric cancer xenograft in nude mice and found to have strong tumor growth-suppressing activity in nude mice. The epitope recognized by ST-421 was chemically identified as Le^a-Le^a, Galβ1→3[Fucα1→4]GlcNAcβ1→3Galβ1→3-[Fucα1→4]GlcNAcβ1→3Galβ1→4Glc (**2**) [Stroud, M. R., et al. (1991) *J. Biol. Chem. 266*, 8439]. Both 43-9F and ST-421 cross-react with Le^a. Identification of the 43-9F antigen as structure **1** (Le^a-Le^x) is tentative since it was not based on isolation and chemical characterization of antigen from tumor cells or tissues. We therefore synthesized structure **1** starting from sialyl-*nor*-hexaosylceramide (VI^{3}NeuAcnLc$_6$), with sequential enzymatic hydrolysis by sialidase and β-galactosidase followed by addition of β1→3Gal with β1→3 galactosyltransferase. This yielded the hybrid type 1/type 2 chain core structure IV^3-(Galβ1→3GlcNAc)nLc$_4$, which was fucosylated with α1→3/4 fucosyltransferase. The structure of the final product was characterized as Le^a-Le^x by ^{1}H-NMR, $^+$FAB-MS, and methylation analysis. We compared reactivities of glycosphingolipids (GSLs) bearing structure **1** or **2** with various mAbs. GSLs with structure **1** showed preferential reactivity with mAb 43-9F. In contrast, GSLs with structure **1** or **2** showed nearly identical reactivity with mAb ST-421 and only weak reactivity with anti-Le^a mAb CA3-F4. We conclude that structure **1** is the epitope of tumor-associated antigens recognized by mAb 43-9F.

During the past decade, the structures of a number of tumor-associated carbohydrate antigens (TACAs[1]) originally defined by mAbs have been characterized by chemical, spectroscopic, and enzymatic methods, e.g., methylation analysis, EI-MS, FAB-MS, ^{1}H-NMR, and enzymatic degradation. The best-characterized TACAs are (i) lacto series type 1 and type 2 chain GSLs with fucosyl and/or sialosyl substitution (e.g., Le^x, Le^y, Le^a, Le^b, sialosyl-Le^a, sialosyl-Le^x); (ii) the core structure of *O*-linked carbohydrate chains (e.g., Tn, sialosyl-Tn); and (iii) precursors of ganglio or globoseries antigens (e.g., GD2 and GD3 gangliosides in melanoma and neuroblastoma, Gb3 in Burkitt lymphoma) (Hakomori, 1984, 1989). In many cases, new TACAs have been discovered and identified using mAbs which exhibit preferential or specific reactivity with human cancer relative to normal cells or tissues followed by characterization of GSLs isolated from tumor cells expressing the mAb epitope. Conversely, the fine specificities of these mAbs have often been elucidated on the basis of their reactivity with well-characterized GSLs.

mAb 43-9F was established by standard procedures after immunization of mice with biopsied human squamous cell carcinoma. The mAb was claimed to show high specificity for malignant cells and tissues, and its antigen level in sera of patients was closely correlated with tumorigenesis and degree of malignancy (Olsson et al., 1984; Pettijohn et al., 1988). 43-9F showed significant cross-reactivity with Le^a antigen (Pettijohn et al., 1987). In an inhibition assay, however, the oligosaccharide difucosylated *p*-lacto-*N*-hexaose ("Le^a-X"; now called Le^a-Le^x; structure **10** in Table 1), originally found in feces from preterm infants fed on breast

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[1] Abbreviations: Cer, ceramide; CMW, chloroform–methanol–water; $^+$FAB-MS, positive ion fast atom bombardment mass spectrometry; FT, fucosyltransferase; Fuc, L-fucose; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; GSL, glycosphingolipid; ^{1}H-NMR, proton nuclear magnetic resonance; HPTLC, high-performance thin-layer chromatography, IHW, isopropyl alcohol-hexane–water; mAb, monoclonal antibody; TACA, tumor-associated carbohydrate antigen. Glycolipids are abbreviated according to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [*Lipids* (1977) *12*, 455–463]; however, the suffix -OseCer is omitted.

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Table 1: Structures of GSLs Used in This Study

1	III^{4}FucLc$_4$ (monomeric Le^a)	Galβ1→3GlcNacβ1→3Galβ1→4Glcβ1→1Cer 4 ↑ Fucα1
2	IV^{3}NeuAcIII^{4}FucLc$_4$ (sialyl-Le^a)	NeuAcα2→3Galβ1→3GlcNacβ1→3Galβ1→4Glcβ1→1Cer 4 ↑ Fucα1
3	IV^{3}GlcNAcnLc$_4$	GlcNAcβ1→3Galβ1→4GlcNacβ1→3Galβ1→4Glcβ1→1Cer
4	IV^3(Galβ1→3GlcNAc)nLc$_4$	Galβ1→3GlcNAcβ1→3Galβ1→4GlcNacβ1→3Galβ1→4Glcβ1→1Cer
5	nLc$_6$	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNacβ1→3Galβ1→4Glcβ1→1Cer
6	VI^{3}NeuAcnLc$_6$	NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNacβ1→3Galβ1→4Glcβ1→1Cer
7	III^{3}V^{3}Fuc$_2$nLc$_6$ (Le^x-Le^x)	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNacβ1→3Galβ1→4Glcβ1→1Cer 3 3 ↑ ↑ Fucα1 Fucα1
8	IV^3(Galβ1→3[Fucα1→4]GlcNAc)nLc$_4$	Galβ1→3GlcNAcβ1→3Galβ1→4GlcNacβ1→3Galβ1→4Glcβ1→1Cer 4 ↑ Fucα1
9	IV^3(Galβ1→3GlcNAc)III^{3}FucnLc$_4$	Galβ1→3GlcNAcβ1→3Galβ1→4GlcNacβ1→3Galβ1→4Glcβ1→1Cer 3 ↑ Fucα1
10	IV^3(Galβ1→3[Fucα1→4]GlcNAc)III^{3}FucnLc$_4$ (Le^a-Le^x)	Galβ1→3GlcNAcβ1→3Galβ1→4GlcNacβ1→3Galβ1→4Glcβ1→1Cer 4 3 ↑ ↑ Fucα1 Fucα1
11	V^{4}III^{4}Fuc$_2$nLc$_4$ (Le^a-Le^a)	Galβ1→3GlcNAcβ1→3Galβ1→3GlcNacβ1→3Galβ1→4Glcβ1→1Cer 4 4 ↑ ↑ Fucα1 Fucα1

milk (Sabharwal et al., 1988), was by far the most active inhibitor (Mårtensson et al., 1988). This compound was at least 50-fold more active than the Le^a-active lacto-*N*-fucopentaose II. The inhibitory oligosaccharide was characterized by mass spectrometry and ^{1}H-NMR spectroscopy (Sabharwal et al., 1988). In affinity chromatography, when a mixture of milk oligosaccharides was passed over a 43-9F column, Le^a-Le^x oligosaccharide was specifically retarded. Isolation and characterization from tumor tissue of the TACA defined by mAb 43-9F were not achieved.

mAb ST-421 was established by Watanabe et al. (1985) using a novel procedure, i.e., nude mice with human gastric cancer xenograft were transfused with immunocompetent mouse spleen lymphocytes, and hybridoma was prepared from host lymphocytes following regression of xenograft tumors. The mAb had a strong cytotoxic effect on ST-421 antigen-positive (but not ST-421-negative) tumors and strongly suppressed growth of ST-421-positive tumors in nude mice. ST-421, like 43-9F, showed cross-reactivity with Le^a antigen yet did not produce any cytotoxic effect on Le^a-positive normal cells *in vitro* or *in vivo* in nude mice (Watanabe et al., 1991). These findings suggested that 43-9F and ST-421 recognize the same or a similar antigen. However, the antigen present on Colo205, a typical ST-421-positive colon carcinoma cell line, was identified unequivocally as an extended, difucosylated type 1 chain GSL with Le^a-Le^a (dimeric Le^a) structure (Table 1, structure **11**) (Stroud et al., 1991). Not even a trace amount of GSL bearing Le^a-Le^x (structure **10**) was detectable in Colo205 cells or pooled human colon carcinoma extract (Stroud et al., 1991).

These previous results suggest that Le^a-Le^x epitope is present on a glycoprotein side chain rather than on GSL. In order to better characterize the epitope defined by mAb 43-9F, we synthesized GSL molecule with Le^a-Le^x epitope by sequential application of glycosylhydrolases and -transferases and unequivocally verified the structure of the final product on a chemical and spectroscopic basis. Comparative antibody binding studies with Le^a-Le^x, Le^a-Le^a, and monomeric Le^a revealed that the real epitope of mAb 43-9F is Le^a-Le^x.

MATERIALS AND METHODS

Enzyme Preparation. Colo205 cells (ATCC) were grown in RPMI 1640 medium containing 10% FCS. Cells were grown to confluency, trypsinized, centrifuged, washed twice with phosphate-buffered saline (pH 7.4), and counted using a hemocytometer. Cells (4×10^6) were injected subcutaneously into athymic (nude) mice, and tumors were excised after 2 weeks and stored frozen at −80 °C until needed.

Solubilization of FT was performed by homogenizing Colo205 tumors in two volumes of 50 mM Hepes (pH 7.0), 0.5 M sucrose, 1 mM EDTA, and 1% Triton CF-54. The homogenate was centrifuged at 100000*g* for 1 h. The supernatant was concentrated to the original volume of tumors

by dialysis, and the enzyme preparation was stored at −80 °C until needed.

Solubilization of β1→3 galactosyltransferase was performed by homogenizing Colo205 tumors in two volumes of 50 mM Hepes (pH 7.0), 25% glycerol, and 1 mM EDTA followed by centrifugation and rehomogenization of the pellet in the above buffer containing 0.2% Triton X-100 as described previously (Holmes & Levery, 1989b). Following centrifugation, the supernatant was concentrated by dialysis, and the β1→3 galactosyltransferase was separated from the β1→4 galactosyltransferase by chromatography on α-lactalbumin-Sepharose (Holmes & Levery, 1989b). All enzyme assays were performed as described previously (Holmes & Levery, 1989a,b; Holmes, 1989).

GSL Preparation. All GSL samples used in this study were either isolated or prepared enzymatically in this laboratory (see Table 1 for structures). VI^{3}NeuAcnLc$_6$ and IV^3-NeuAcIII^{4}FucLc$_4$ were isolated from human placenta and Colo205 cells, respectively, by sequential extraction with IHW (55:25:20), Folch partition (Folch-Pi et al., 1951), DEAE-Sephadex chromatography (Yu & Ledeen, 1972), and HPLC on an Iatrobeads 6RS 8010 column (Ando et al., 1976). nLc$_6$ and III^{4}FucLc$_4$ were prepared by desialylation of VI^3-NeuAcnLc$_6$ and IV^{3}NeuAcIII^{4}FucLc$_4$, respectively. IV^3-GlcNAcnLc$_4$, IV^3(Galβ1→3GlcNAc)nLc$_4$, IV^3(Galβ1→3-[Fucα1→4]GlcNAc)nLc$_4$, and IV^3(Galβ1→3[Fucα1→4]-GlcNAc)III^{3}FucnLc$_4$ (Le^a-Le^x) were prepared as described in the following section. IV^3(Galβ1→3GlcNAc)III^{3}FucnLc$_4$ was prepared by α-fucosidase treatment of IV^3(Galβ1→3[Fucα1→4]GlcNAc)III^{3}FucnLc$_4$ (Le^a-Le^x). Le^a-Le^a was purified as described previously (Stroud et al., 1991).

Enzymatic Synthesis of Le^a-Le^x. VI^{3}NeuAcnLc$_6$, obtained from human placenta, was hydrolyzed with sialidase and β-galactosidase, yielding the substrate IV^{3}GlcNAcnLc$_4$. Enzymatic synthesis of IV^3(Galβ1→3GlcNAc)nLc$_4$ was performed in a reaction mixture containing 4 mg of IV^3-GlcNAcnLc$_4$, 2 mg of Triton CF-54, 75 μmol of Hepes buffer (pH 7.0), 30 μmol of $MnCl_2$, 10 μmol of UDP-galactose, 5 μmol of D-galactono-1,5-lactone, and 1.6 mL of enzyme preparation in a total volume of 2.5 mL. The reaction mixture was incubated at 37 °C overnight. The reaction mixture was centrifuged, the pellet was washed twice with water, and the washes were pooled. These, along with the first supernatant, were passed over a C-18 column and eluted with methanol after extensive washing with water. The pellet was extracted with IHW (55:25:20) and centrifuged and the supernatant pooled with the C-18 elution. The products were subjected to HPLC on an Iatrobeads 6RS 8010 column using a gradient elution of IHW from 55:40:5 to 55:25:20 over 200 min. Two-milliliter fractions were collected and pooled according to HPTLC migration in CMW (50:40:10).

Biosynthesis of Le^a-Le^x GSL was performed in a reaction mixture containing 2 mg of IV^3 (Galβ1→3GlcNAc)nLc$_4$, 2 mg of deoxytaurocholate, 15 μmol of $MnCl_2$, 150 μmol of Tris buffer (pH 7.4), 15 μmol of CDP-choline, 6 μmol of GDP-fucose, and 1.5 mL of enzyme preparation in a total volume of 3 mL. The reaction mixture was incubated at 37 °C, and 5-μL aliquots were streaked onto an HPTLC plate at various times to monitor the fucosylation process. The HPTLC plate was washed extensively with water, dried, and developed in CMW (50:40:10). The final product was purified as described above for IV^3(Galβ1→3GlcNAc)nLc$_4$.

mAbs and Immunoassays. mAbs ST-421 and 43-9F were established as described previously (Watanabe et al., 1985; Pettijohn et al., 1987). mAb CA3-F4 was derived from spleen cells of mice immunized with neutral GSLs from human meconium (Young et al., 1983). The fine structural specificity of this mAb was previously characterized as requiring the entire Le^a tetrasaccharide epitope, i.e., Galβ1→3[Fucα1→4]-GlcNAcβ1→3Gal, as opposed to only the terminal trisaccharide. Enzyme-linked immunosorbant assay (ELISA) was performed by the method of Singhal et al. (1990), as follows. GSLs were mixed with phosphatidylcholine and cholesterol (1:5:3, w/w/w) in ethanol to a final GSL concentration of 5 μg/mL. The solution was serially diluted from 100 ng of GSL/well to 0.78 ng/well in a 96-well microtiter plate and incubated at 37 °C until all ethanol was evaporated. The plate was blocked with 1% BSA in PBS (pH 7.2) for 1 h followed by 2 h of incubation with mAb, washing with PBS, and incubation with peroxidase-conjugated goat anti-mouse Ig for 1 h. After washing with PBS, the reaction was visualized with *O*-phenylenediamine and read at A_{490}. mAbs were used as undiluted culture supernatants.

HPTLC immunostaining was performed by the method of Mårtensson et al. (1986). Briefly, 5 μg of each GSL was spotted onto an HPTLC plate and developed in CMW (50:40:10). The plate was coated with a solution of 0.05% poly(isobutyl methacrylate) in ether for 1 min, thoroughly dried using a hairdryer, and blocked for 2 h in 5% BSA in PBS (pH 7.2). The plate was then washed with PBS, incubated overnight at 4 °C with mAb from undiluted culture supernatant, washed thoroughly with PBS, and incubated for 1 h with a $^1/_{500}$ dilution of biotinylated horse anti-mouse Ig (Vector Labs, Burlingame, CA). The plate was washed, covered for 30 min with avidinbiotinylated horseradish peroxidase complex (Vector Labs) diluted $^1/_{250}$ in 1% BSA, washed again, and developed for 2 min in a solution consisting of 20 mg of 3-amino-9-ethylcarbazole, 2.5 mL of dimethylformamide, 50 mL of 0.15 M sodium acetate buffer (pH 5.5), and 25 μL of H_2O_2.

^{1}H-NMR Spectroscopy. Samples (1–2 mg) were deuterium exchanged by repeated lyophilization from DMSO-d_6/D_2O (98:2) and then dissolved in 0.4 mL of this solvent. One-dimensional spectra were recorded at 308 and 328 ± 2 K on a Bruker (Karlsruhe, West 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 (PD) period. The PD was 2.0 s. A Lorentzian to Gaussian transformation was used for resolution enhancement.

$^+$FAB-MS. A sample of the biosynthetic GSL (≈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 30 min, and MeI was removed by flushing with N_2 for 15 min at 37 °C prior to partitioning between $CHCl_3$ and H_2O. After the $CHCl_3$ was dried under N_2, 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 (Isobe et al., 1987; Holmes & Levery, 1989a;b). Scan range was 100-3000 amu; scan slope 2 min 45 s; acceleration voltage 10 kV; resolution 3000; xenon beam 8 kV. Three scans were accumulated for each spectrum. KI/CsI was used as calibration standard.

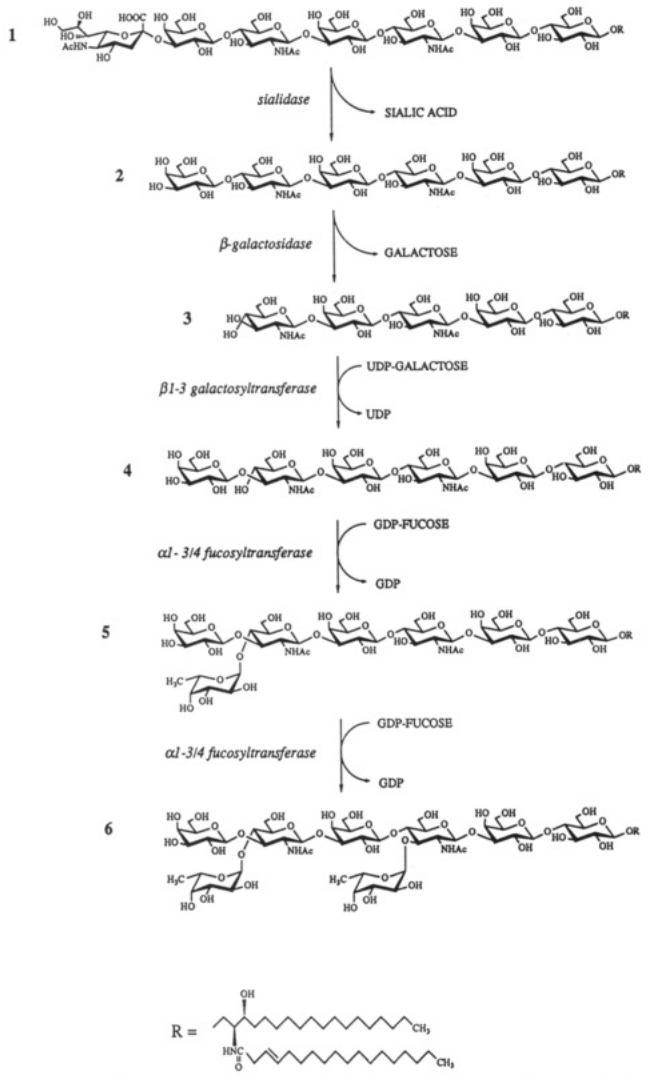

FIGURE 1: Enzymatic pathway used to synthesize Le^a-Le^x. 1, VI^3-NeuAcnLc$_6$. 2, nLc$_6$. 3, IV^{3}GlcNAcnLc$_4$. 4, IV^3(Galβ1→3GlcNAc)nLc$_4$. 5, IV^3(Galβ1→3[Fucα1→4]GlcNAc)nLc$_4$. 6, IV^3(Galβ1→3[Fucα1→4]GlcNAc)III^{3}FucnLc$_4$. R = Cer.

Methylation Analysis. The remainder of the permethylated sample was hydrolyzed, reduced, and acetylated as described previously (Levery & Hakomori, 1987). GC-MS analysis of partially methylated alditol acetates was performed using a 30-m DB-5 (0.25-mm i.d., 0.25-μm film thickness) bonded-phase fused silica capillary column as described previously (Clausen et al., 1987; Ostrander et al., 1988).

RESULTS

In order to further characterize the epitope structure of the tumor-associated antigen defined by mAb 43-9F, the antigen Le^a-Le^x was synthesized enzymatically and compared with previously isolated antigen Le^a-Le^a in side-by-side immunoassays using mAbs 43-9F and ST-421, and anti-Le^a mAb CA3-F4. The enzymatically synthesized Le^a-Le^x GSL was unequivocally identified by ^{1}H-NMR spectroscopy, $^+$FAB-MS, and methylation analysis as described in the following sections.

Enzymatic Synthesis of Le^a-Le^x. The enzymatic pathway employed in the biosynthesis of Le^a-Le^x is shown in Figure 1. IV^{3}NeuAcnLc$_6$, obtained from human placenta, was hydrolyzed with sialidase and β-galactosidase, yielding IV^3-GlcNAcnLc$_4$. The subsequent transfer of galactose from

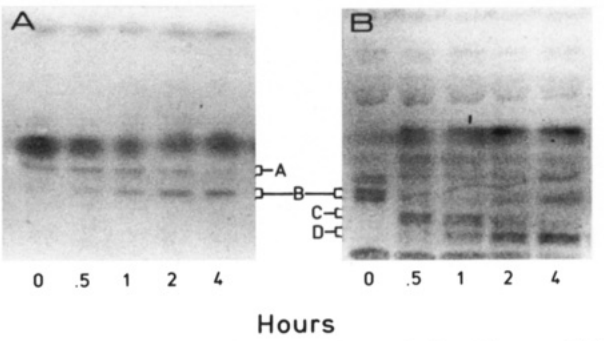

FIGURE 2: Time course of Le^a-Le^x biosynthesis. Aliquots of the reaction mixture were spotted at the indicated time intervals and the plates developed as described previously. Panel A, biosynthesis of IV^3(Galβ1→3GlcNAc)nLc$_4$. Panel B, biosynthesis of Le^a-Le^x. Band A, IV^{3}GlcNAcnLc$_4$. Band B, IV^3(Galβ1→3GlcNAc)nLc$_4$. Band C, IV^3(Galβ1→3[Fucα1→4]GlcNAc)nLc$_4$. Band D, IV^3(Gal-β1→3[Fucα1→4]GlcNAc)III^{3}FucnLc$_4$.

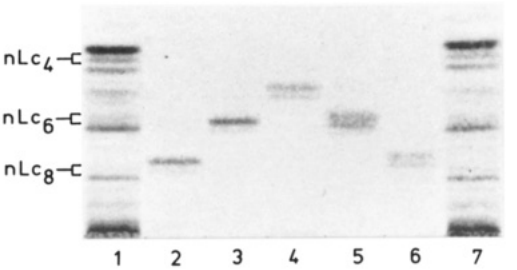

FIGURE 3: HPTLC pattern of the major GSLs formed in each step of Le^a-Le^x enzymatic synthesis. GSL pattern was detected by 0.5% orcinol in 2 N sulfuric acid. Lanes 1 and 7, standard upper neutral GSL fraction from human type O erythrocytes. Lane 2, VI^3-NeuAcnLc$_6$. Lane 3, nLc$_6$. Lane 4, IV^{3}GlcNAcnLc$_4$. Lane 5, IV^3-(Galβ1→3GlcNAc)nLc$_4$. Lane 6, IV^3(Galβ1→3[Fucα1→4]Glc-NAc)III^{3}FucnLc$_4$.

UDP-galactose to IV^{3}GlcNAcnLc$_4$ by β1→3 galactosyltransferase using Colo205 as an enzyme source yielded an hexaosyl-Cer after 4 h of incubation at 37 °C (Figure 2A) which comigrated with nLc$_6$ on HPTLC (Figure 3). Fucosyl substitution by α1→3/4 FT from Colo205 yielded a monofucosylated intermediate at 30 min (Figure 2B) and a difucosylated end product at 4 h of incubation at 37 °C (Figures 2B and 3). The HPTLC pattern of the major GSLs formed in each step of Le^a-Le^x enzymatic synthesis is shown in Figure 3.

Immunoassays. Results from ELISA using mAb 43-9F showed a much stronger reactivity with enzymatically synthesized Le^a-Le^x than with monomeric Le^a (Figure 4). No reactivity with 43-9F was observed for Le^a-Le^a. mAb ST-421 could not distinguish between Le^a-Le^x and Le^a-Le^a. However, this antibody showed a clear preference for these two antigens compared to monomeric Le^a. mAb CA3-F4 reacted strongly only with monomeric Le^a and showed little reactivity with either Le^a-Le^a or Le^a-Le^x.

Reactivity of three GSLs containing terminal Le^a was compared by TLC immunostaining with mAb 43-9F. Reactivity of GSL containing Le^a-Le^x was much stronger than that of GSLs containing Le^a-Le^a or monomeric Le^a (Figure 5). Although a minor amount of monofucosylated intermediate is present in the Le^a-Le^x sample (<0.5 μg), its presence does not influence the results of the ELISA, since no band was detected migrating higher than the Le^a-Le^x doublet.

Since two GlcNAc residues are present on the Le^a-Le^x precursor IV^3(Galβ1→3GlcNAc)nLc$_4$ (Table 1, structure 4), one of two possible monofucosylated intermediates could be formed by the action of α1→3/4 FT on this substrate. If the fucose were first transferred to the terminal GlcNAc, an Le^a-

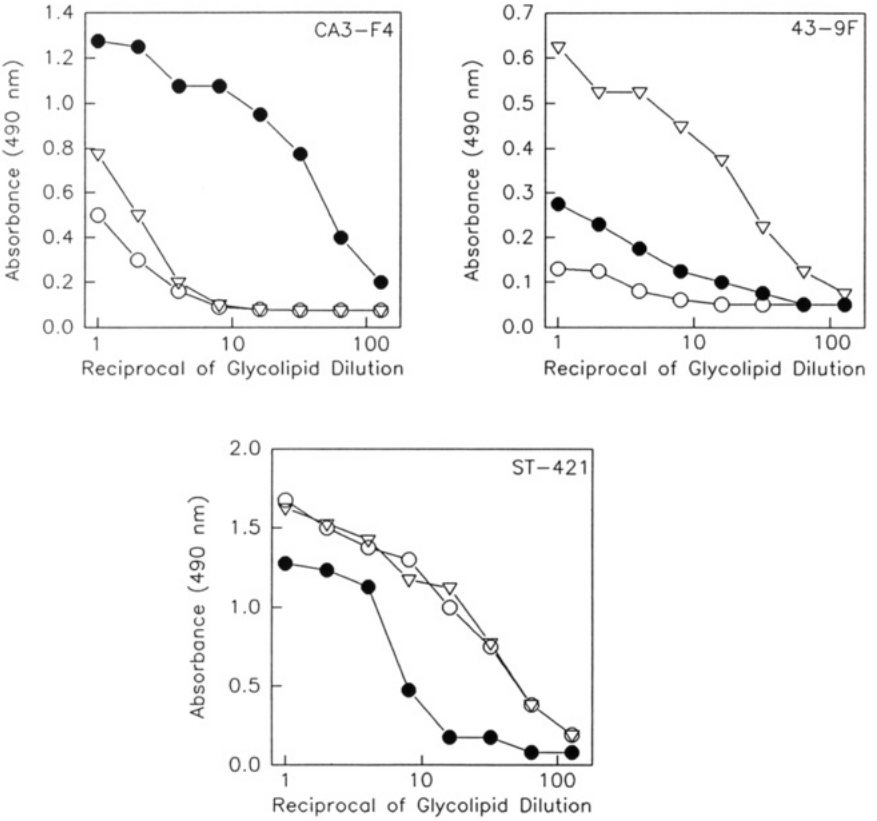

FIGURE 4: Immunoreactivity of mAbs with biosynthetic Le^a-Le^x and related GSLs by ELISA. Liposomes were prepared by sonicating a mixture of phosphatidylcholine, cholesterol, and GSL (5:3:1, w/w/w) in ethanol so that a final GSL concentration of 5 μg/mL was obtained. Serial double dilutions of GSL-containing liposomes were prepared in ethanol, added to 96-well flat-bottom assay plates (Probind, Falcon), and dried at 37 °C. Amount of GSL added to the first well was 100 ng. mAb binding assay was performed by ELISA as described in the text. mAbs were used as undiluted culture supernatants. ●, monomeric Le^a. ▽, Le^a-Le^x. ○, Le^a-Le^a.

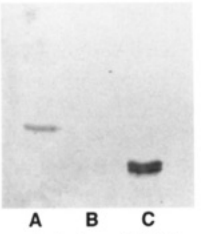

FIGURE 5: TLC immunostaining of GSLs with mAb 43-9F. A, monomeric Le^a. B, Le^a-Le^a. C, Le^a-Le^x. See Table 1 for structures. Each lane contained the same amount (5 μg) of GSL. Immunostaining was performed by ELISA with peroxidase-conjugated secondary antibody.

active epitope would be generated. If the fucose were transferred to the internal GlcNAc first, no Le^a activity would be detected. The simplest carbohydrate structure required for binding of mAb CA3-F4 is the entire Le^a-active tetrasaccharide Galβ1→3[Fucα1→4]GlcNAcβ1→3Gal. The observed strong binding of this mAb to the monofucosylated intermediate suggests that the order of fucosylation begins with the terminal GlcNAcV, forming an extended Le^a structure (IV^3(Galβ1→3[Fucα1→4]GlcNAc)nLc$_4$). In contrast, when the terminal fucose is removed by limited digestion of Le^a-Le^x with bovine kidney α-fucosidase, forming an internally fucosylated derivative (IV^3(Galβ1→3GlcNAc)III^{3}FucnLc$_4$), no reactivity is observed (Figure 6).

^{1}H-NMR Spectroscopy. Proton spectra were acquired at two temperatures, both to check for hidden or overlapped resonances and to compare with existing NMR databases on related GSLs (Dabrowski et al., 1981; Levery et al., 1986; Nudelman et al., 1988; Stroud et al., 1991, 1992). Eight anomeric resonances were found in the downfield region (Figure 7), two with $^3J_{1,2} \approx 4$ Hz (indicating α-configuration)

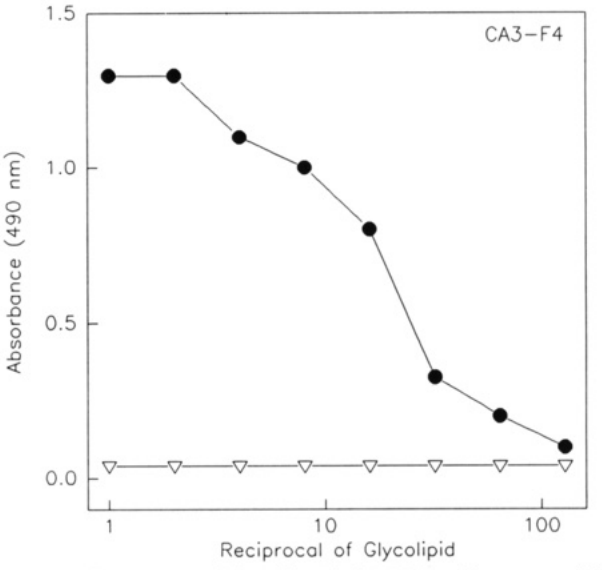

FIGURE 6: Immunoreactivity of mAb CA3-F4 with two possible monofucosylated intermediates. Procedures were the same as those in Figure 4. ●, IV^3(Galβ1→3[Fucα1→4]GlcNAc)nLc$_4$. ▽, IV^3(Galβ1→3GlcNAc)III^{3}FucnLc$_4$.

and six with $^3J_{1,2} \approx 7$–8 Hz (indicating β-configuration). Of the latter group, one was found in the usual position for H-1 of β-Glc attached to Cer with nonhydroxylated fatty acids and D18:1 or D20:1 sphingosines. Three were found in the region for H-1 of β-Gal and two in the region for β-GlcNAc H-1 (Dabrowski et al., 1980). That the two α-anomeric resonances belonged to Fuc residues was confirmed by the finding of two coincident H-5 broadened quartets coupled to CH_3 doublets. One group of signals corresponded well (within

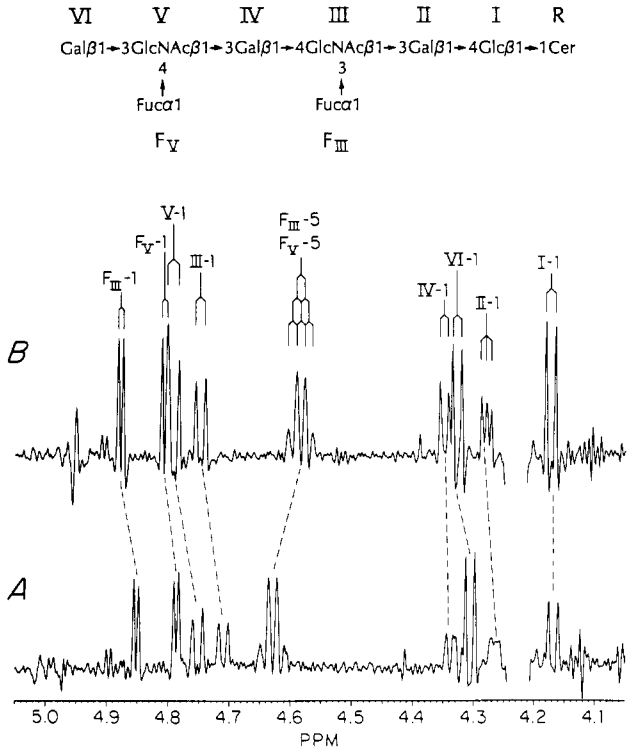

FIGURE 7: Anomeric region of resolution-enhanced 500-MHz ^{1}H-NMR spectra of biosynthetic difucosylated GSL in dimethyl sulfoxide-d_6–2% D_2O + 1% tetramethylsilane as chemical shift reference. A, acquired at 308 ± 2 K. B, acquired at 328 ± 2 K. Arabic numerals refer to ring protons of residues designated by roman numerals in the structure shown at the top.

±0.01 ppm) to diagnostic resonances found at 328 K for an internal Le^x trisaccharide (type 2 chain) structure (Levery et al., 1986; Nudelman et al., 1988): α-Fuc H-1 at 4.878 ppm, H-5 at 4.584 ppm, and H-6 at 1.019 ppm, β-GlcNAc H-1 at 4.748 ppm, and β-Gal H-1 at 4.349 ppm. Another group correlated fairly well (within ±0.03 ppm) to resonances found previously at 338 K for Le^a-terminated (type 1 chain) pentaglycosyl-Cer (Dabrowski et al., 1981): α-Fuc H-1 at 4.807 ppm and H-5 at 4.584 ppm, β-GlcNAc H-1 at 4.792 ppm, and β-Gal H-1 at 4.328 ppm. These assignments are consistent with the expected structure, particularly in the relative chemical shifts of the α-Fuc and β-GlcNAc H-1 resonances, in the near coincidence of the α-Fuc H-5 resonances, and in the assignment of internal vs terminal β-Gal H-1 resonances. The remaining β-Gal H-1 was found at a position (4.279 ppm) nearly always found for β-Gal II in both type 1 and type 2 chain GSLs (Dabrowski et al., 1980, 1981; Levery et al., 1986; 1988; Nudelman et al., 1988; Stroud et al., 1991, 1992). ^{1}H-NMR data are summarized in Table 2.

$^+$FAB-MS. Confirmation of the glycosyl sequence of the difucosylated biosynthetic GSL was provided by $^+$FAB-MS of the intact permethylated compound (Figure 8). In addition to clear pseudomolecular ion clusters, which showed it to be composed of deoxyHex$_2$·Hex$_4$·HexNAc$_2$ attached to Cer moieties composed primarily of d18:1 sphingosine in combination with 16:0, 22:0, and 24:0 fatty acids (see scheme, Figure 8), familiar B_n fragments [nomenclature of Domon and Costello (1988)] were observed with preferential cleavage at HexNAc residues (Dell, 1987; Egge & Peter-Katalinic, 1987). Key fragments showing the location of Fuc residues could be observed at m/z 638 (deoxyHex·Hex·HexNAc) and 1261 (deoxyHex$_2$·Hex$_2$·HexNAc$_2$). In addition, the abundant ion at m/z 402 is consistent with the 1→3 linkage of the terminal Hex residue, as this fragment is expected to be formed from that at m/z 638 via the frequently observed preferential neutral loss of 3-linked substituents from reducing-end-HexNAc-containing primary fragments (Dell, 1987; Egge & Peter-Katalinic, 1987). In this case, the lost fragment corresponds to HexOH. On the other hand, the same mechanism leads to formation of the ion at m/z 1055 from that at m/z 1261, indicating in this case the 1→3 linkage of the internal deoxyHex residue. These results clearly confirm the Le^x-internal/Le^a-terminal arrangement of the product and can be contrasted with results obtained under identical conditions from a permethylated biosynthetic GSL containing only type 2 chain linkages (Le^x-Le^x), which produces negligible amounts of the fragment m/z 402 (Holmes & Levery, 1989a), and from a permethylated tumor GSL containing only type 1 chain linkages (Le^a-Le^a), in whose mass spectrum the fragment m/z 1055 is of extremely low abundance (Stroud et al., 1991).

Fragments at m/z 1291 (deoxyHex·Hex$_3$·HexNAc$_2$), 1087 (deoxyHex·Hex$_2$·HexNAc$_2$), and 881 (neutral loss of deoxyHexOH from m/z 1087) indicate the presence of some monofucosylated product. The absence of a fragment corresponding to Hex·HexNAc (m/z 464) indicated that in this product the Fuc residue must be missing from the internal GlcNAcIII rather than from the penultimate GlcNAcV. The production of the fragment m/z 881 from the primary fragment m/z 1087 implies that some of this Fuc residue is linked α1→3 to a type 2 chain β-GlcNAc, since the isomeric type 1 chain case would be expected to yield a fragment, m/z 851, from loss of 3-linked HexOH. The m/z 881 fragment is produced in greater abundance than that at m/z 851. The existence of some monofucosylated product in a type 2 chain form is also consistent with the appearance of a fragment at m/z 432 which, in the absence of its usual parent at m/z 464, must be produced by loss of 3-linked deoxyHexOH from the terminal trisaccharide fragment m/z 638.

A comparison of the relative abundance of the m/z 432 and 402 ions, assuming both to be products of an isomeric parent at m/z 638, yielded an estimate of 10% for the type 2 chain terminal responsible for the former ion. However, there is no evidence that such estimates based on abundances of ions produced in these FAB spectra are reliable, nor is there any basis for determining error limits for such estimates.

Methylation Analysis. Confirmation of linkage position was further provided by methylation analysis (Figure 9). Partially methylated alditol acetates (PMAAs) were detected corresponding to →3(→4)-linked GlcNAc, →4Glc, →3Gal, terminal Fuc, and terminal Gal residues. A small contamination of →4GlcNAc again indicated some monofucosylated product, in which the Fuc residue is missing from the type 2 chain (internal) GlcNAcIII. From the ratios of the →4- and →3(→4)GlcNAc derivatives, it was again estimated that the monofucosyl product constituted about 10% of the total, but, as with the FAB-MS data, there is little basis for discussing the reliability of this estimate, since it would depend strongly on an assumption that the kinetics of production and the relative stabilities of the two derivatives are similar, an assumption that we are not prepared to support. However, since past experience has given us the impression that the →3(→4)GlcNAc derivative is somewhat underproduced compared to the →3GlcNAc derivative, 10% may constitute an upper, rather than a lower, limit on the amount of monofucosyl derivative present.

DISCUSSION

Lacto series type 1 or type 2 chain structures constitute the majority of human TACAs (Hakomori, 1984, 1989; Stroud

Table 2: Chemical Shifts (ppm from Tetramethylsilane) and $^3J_{1,2}$ Coupling Constants (Hz) of Glycosyl H-1 Resonances[a] for Biosynthetic GSL in Dimethyl Sulfoxide-d_6

	Galβ1——→3	GlcNAcβ1—→3Galβ1——→4		GlcNAcβ1—→3Galβ1——→4Glcβ1→1Cer				
T(°K)		Fucα1——→4			Fucα1——→3			
308:	4.305 (7.3)	4.786 (3.7)	4.751 (7.9)	4.338 (7.3)	4.851 (4.3)	4.709 (7.9)	4.264 (6.7)	4.168 (7.3)
328:	4.328 (7.3)	4.807 (4.3)	4.792 (7.9)	4.349 (6.7)	4.878 (4.3)	4.748 (7.9)	4.279 (7.9)	4.173 (7.9)

[a] Additional resonances were found as follows: (A) at 308 K, α-Fuc H-5, both at 4.629 ppm ($^3J_{5,6}$ = 6.7 Hz); α-Fuc H-6 (CH_3), at 1.001 and 1.006 ppm; β-GlcNAc NAc, at 1.815 and 1.818 ppm; (B) at 328 K, α-Fuc H-5, both at 4.584 ppm ($^3J_{5,6}$ = 6.7 Hz); α-Fuc H-6 (CH_3), at 1.019 and 1.024 ppm; β-GlcNAc NAc, at 1.822 and 1.828 ppm.

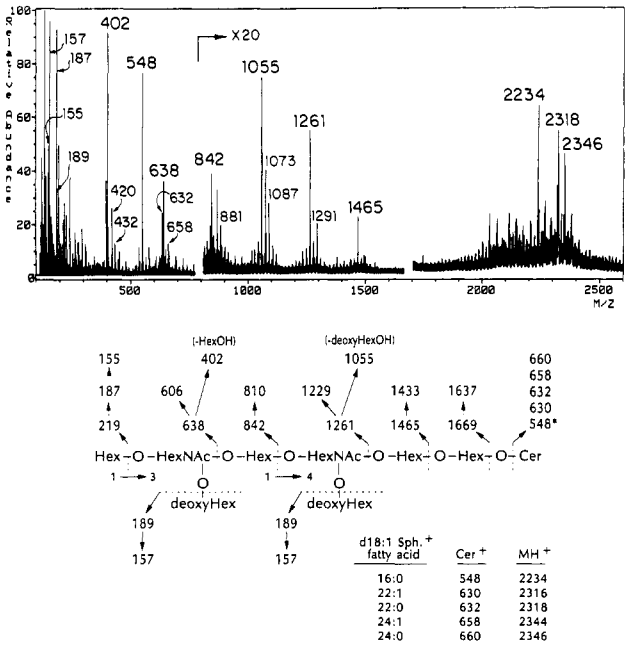

FIGURE 8: $^+$FAB mass spectrum of permethylated biosynthetic difucosylated GSL in NBA matrix. Spectrum shown at the top is a composite of three acquisitions optimized for sensitivity in different mass ranges. Segments from 800 to 2600 amu were acquired with 15-crown-5 added to the matrix. The segment below 800 amu was acquired without 15-crown-5. A proposed fragmentation scheme is shown below. All assignments are nominal monoisotopic masses.

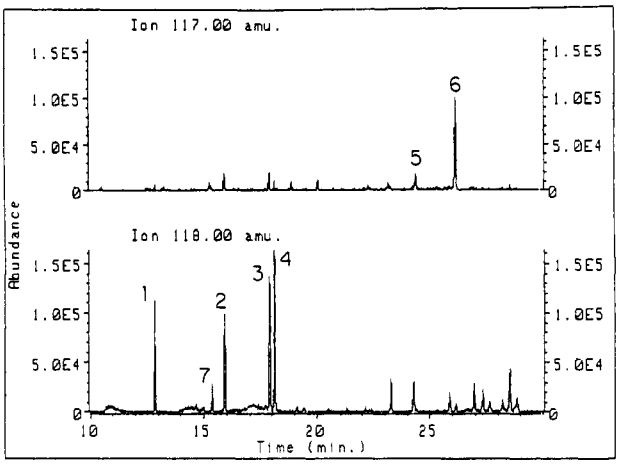

FIGURE 9: Selected electron impact mass chromatograms of monodeuterated partially *O*-methylated deoxyhexitol, hexitol, and 2-*N*-methyl-*N*-acetylhexosaminitol acetates obtained from the hydrolysis of permethylated biosynthetic fucolipids. Separation was performed on a 30-m DB-5 bonded-phase fused silica capillary column (0.25-mm i.d., 0.25-μm film thickness) using splitless injection. Electron impact mass spectra were acquired from 50 to 500 amu at 0.95 scans/s. The mass chromatograms for ions at m/z 117 (characteristic for 1-*O*-acetyl-2-*N*-(methylacetamido)-2-deoxyalditols) and m/z 118 (characteristic for 1-*O*-acetyl-2-*O*-methylalditols) are reproduced for the biosynthetic difucosyl GSL. Peaks identified were (1) 2,3,4-tri-*O*-Me-Fuc; (2) 2,3,4,6-tetra-*O*-Me-Gal; (3) 2,3,6-tri-*O*-Me-Glc; (4) 2,4,6-tri-*O*-Me-Gal; (5) 3,6-di-*O*-Me-GlcNAcMe; (6) 6-mono-*O*-Me-GlcNAcMe; (7) 2,3,4,6-tetra-*O*-Me-Glc (impurity).

et al., 1993). Type 1 antigens, constituting histo blood group Lewis (i.e., Le^a, Le^b, Le^c, Le^d) and bearing A and B determinants, are highly expressed in normal endodermal and secretory epithelial tissues and meconium. They have been found to be expressed on glycoprotein side chains and as GSLs with a short tetrasaccharide backbone. These type 1 antigens are relatively minor components in human tumors, as compared to the predominant type 2 chain structures, which include unbranched repeating poly(lactosamine) structures having sialosyl/fucosyl substitution. Epitopes based on type 2 chain (e.g., Le^x, Le^y, sialosyl-Le^x) are also highly expressed on granulocytes and monocytes, whereas type 1 chain epitopes are completely absent from blood cells. Because of the predominance of type 2 chain GSLs in tumors, our knowledge of the chemical nature of type 1 chain antigens found in human tumors is limited. In fact, type 1 chain paragloboside and its sialylated derivative were only clearly identified in meconium (Karlsson & Larson, 1979). The accumulation and enhanced synthesis of Le^a and Le^b in various human cancers were recognized 24 years ago (Hakomori & Andrews, 1970). In fact, recent studies indicate that the Le^a antigen can be synthesized in tumors from "genuine Le^{a-b-} individuals" (Ørntoft et al., 1991). Sialosyl-Le^a epitope ($IV^3NeuAcIII^4$-$FucLc_4$), defined by mAb N19-9 (Magnani et al., 1982) and many other mAbs, was also shown to be tumor associated.

Two types of Le^a-cross-reacting TACAs have been found, those defined by mAb ST-421 (Stroud et al., 1991) and those defined by mAb 43-9F. A GSL extracted from Colo205 cells and from pooled ST-421-reactive human colonic tumors was unequivocally identified as extended difucosylated type 1 chain (Le^a-Le^a; structure **11** in Table 1), whereas no GSL having Le^a-Le^x (structure **10**) was obtained from these sources (Stroud et al., 1991). In affinity chromatography, mAb 43-9F was shown to retard an Le^a-Le^x-active oligosaccharide from a mixture of milk oligosaccharides (Mårtensson et al., 1988). The compound was partially characterized by mass spectrometry and showed the same spectrum as difucosylated *p*-lacto-*N*-hexaose ("Le^a-X"), previously isolated from feces of preterm infants fed on breast milk (Sabharwal et al., 1988). The Le^a-Le^x antigen was not isolated from any tumor tissue. A sialylated GSL derivative of Le^a-Le^x, however, has recently been isolated from human rectal adenocarcinoma (Kitagawa

et al., 1993). The failure to detect any GSL having Le^a-Le^x suggests that this epitope may be expressed exclusively on glycoproteins. There are many well-documented cases in which a specific epitope is expressed only on glycoproteins but not on GSLs; e.g., the Le^y epitope, when expressed in T cell lymphoma Hat78 or activated T cells, is found exclusively on glycoprotein. In contrast, Le^y in K562 cells is expressed on both glycoprotein and GSL (K. Handa, K. Tashiro and S. Hakomori, unpublished data).

Compared with glycoproteins, GSLs are advantageous for both structure elucidation and epitope characterization for several reasons. (1) They carry only one carbohydrate chain per lipid molecule, and their relative abundance in most cell membranes facilitates the purification and subsequent structural characterization of the immunoreactive components. (2) The Cer moiety of GSLs adsorbs strongly to solid-phase supports, allowing direct immunostaining with mAbs directed to their carbohydrate epitopes. (3) With the exception of mannose-containing structures found on *N*-linked glycoproteins, most known carbohydrate epitopes on proteins can be found on GSLs and to a large degree show the same glycosylation patterns as those found on GSLs. However, due to the hydrophilic properties and heterogeneity of oligosaccharides associated with proteins, the immunological methods normally used with GSLs cannot be applied to determine the specificities of mAbs recognizing glycoprotein antigens. Possible approaches are to isolate oligosaccharide from either *O*-linked or *N*-linked structure and show its inhibition of mAb binding to antigen or convert it into "neo-GSL" and demonstrate binding of mAb to this neo-GSL (Tang et al., 1985). Because of the limited yield of oligosaccharides or neo-GSLs, the success of these approaches has been limited. This difficulty led us to design a procedure employing a combination of specific glycosidases and glycosyltransferases to synthesize Le^a-Le^x from a readily obtainable GSL precursor.

Two possible monofucosylated intermediates can be formed by the action of α1→3/4 FT on IV^3(Galβ1→3GlcNAc)nLc$_4$ during the synthesis of Le^a-Le^x. If the penultimate GlcNAcV is fucosylated first, a GSL intermediate having Le^a activity would be generated. If the order of fucosylation is reversed, the internal GlcNAcIII would be fucosylated prior to GlcNAcV and the resulting intermediate would have an internal Le^x structure with an unsubstituted type 1 chain terminus. A monofucosylated intermediate, reacting strongly with mAb CA3-F4 (Figure 6), did not react with IgM mAb MNH-1, which was prepared by immunization of mice with the GSL IV^3(Galβ1→3GlcNAc)nLc$_4$ (Table 1, structure **4**), and is directed to unsubstituted type 1 chain (Galβ1→3GlcNAcβ1-R) (M. R. Stroud and S. Hakomori, unpublished data). In contrast, when only the terminal fucose was removed from Le^a-Le^x after limited α-fucosidase treatment, the resulting internally fucosylated structure (IV^3-(Galβ1→3GlcNAc)III^{3}FucnLc$_4$) reacted with mAb MNH-1 (data not shown) but did not react with mAb CA3-F4 (Figure 6). These results suggest that enzymatic synthesis of Le^a-Le^x from unsubstituted type 1/type 2 chain precursor proceeds by fucosylation of the penultimate GlcNAc followed by the internal GlcNAc residue. This contrasts with the order of GlcNAc fucosylation with the same enzyme source when nLc$_6$ (Table 1, structure **5**) is used as a substrate (Holmes & Levery, 1989a). Although a number of FTs are known (Macher et al., 1991), the Lewis FT is the only enzyme known that fucosylates the GlcNAc residue of type 1 chain structures, i.e., Galβ1→3GlcNAcβ1→R. Since this enzyme has also been purified from human milk, the same source from which the Le^a-Le^x oligosaccharide was first isolated, the pathway leading to the biosynthesis of the Le^a-Le^x oligosaccharide *in vivo* may parallel the pathway we utilized to synthesize enzymatically the GSL derivative of Le^a-Le^x.

The enzymatic synthesis of Le^a-Le^x GSL allowed direct comparison of the specificities of mAbs 43-9F, CA3-F4, and ST-421. mAb ST-421 prefers the extended Le^a epitopes Le^a-Le^a and Le^a-Le^x over monomeric Le^a (pentaglycosyl-Cer) regardless of internal core structure (Stroud et al., 1991). mAb 43-9F discriminates between the two difucosylated isomers, recognizing only Le^a-Le^x (present study), whereas mAb CA3-F4 reacts only with monomeric Le^a. This is consistent with earlier studies which describe the necessity of the internal galactose residue of the Le^a-tetrasaccharide epitope for CA3-F4 binding (Young et al., 1983). When the Le^a-trisaccharide was attached to the C6 position of galactose in a β-anomeric configuration, the ability of CA3-F4 to bind to the Le^a-trisaccharide portion was lost. The lack of binding to Le^a-Le^a and Le^a-Le^x is most likely due to interference from the internal fucose residue, preventing recognition of the internal galactose. As mentioned above, mAb 43-9F is strongly inhibited by a milk octasaccharide described as "Le^a-X." Our data confirm both the structure and the specificity of this antibody as previously proposed and rule out Le^a-Le^a as a potential epitope.

With the continuing discovery and availability of new glycosyltransferases, the use of biosynthetic methods to prepare relatively large quantities of desired GSLs will facilitate both their structural characterization and the study of the immunochemical properties associated with their complex carbohydrate epitopes.

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