Novel Oligosaccharides with the Sialyl-Le^a Structure in Human Milk†

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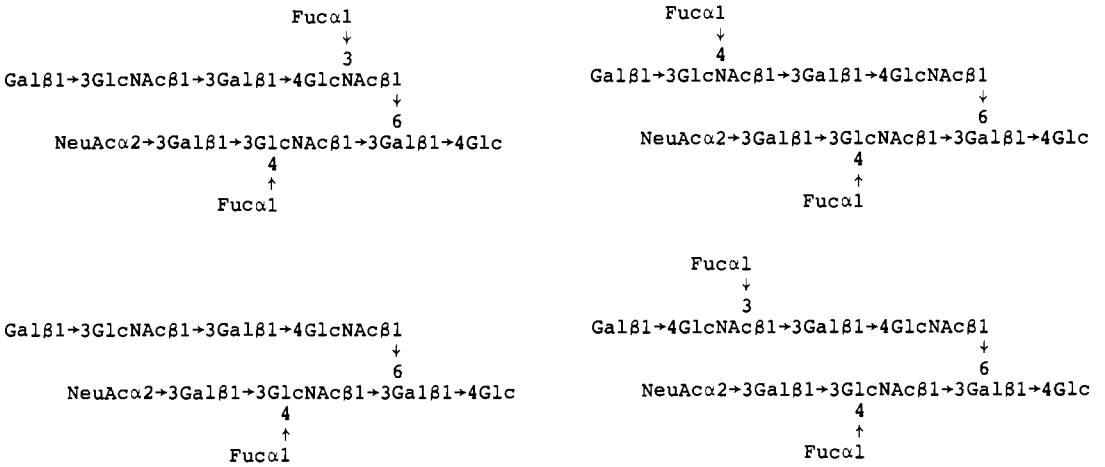
ABSTRACT: We have isolated four novel oligosaccharides with the sialyl-Le^a structure from human milk using a monoclonal antibody, MSW 113. These oligosaccharides were purified by affinity chromatography on a column of the immobilized monoclonal antibody and by high-performance liquid chromatography. The results of structural analyses, i.e., 500-MHz ^{1}H NMR spectroscopy, fast atom bombardment mass spectrometry, and binding to specific anticarbohydrate antibodies, are consistent with the following structures.



Structural homology has been recognized between oligosaccharides in human milk and carbohydrates carried by glycoproteins and glycolipids on cell surfaces. Thus, such oligosaccharides have been widely used as substrates for studies on glycosyltransferases and glycosidases and as model compounds for determining the structural requirements of lectins and monoclonal antibodies (Hearn et al., 1968; Wiederschain & Rosenfeld, 1971; Hansson & Zopf, 1985; Greenwell et al., 1986; Dakour et al., 1986; Kitagawa et al., 1988a).

During the past few years, several laboratories have produced monoclonal antibodies that specifically bind to human cancer cells. Many of them recognize complex carbohydrate antigens (Ginsburg et al., 1985). Such antigens are often present in the bloodstream of cancer patients, and most of them have been identified as mucin-type glycoproteins. We have recently established several monoclonal antibodies directed toward mucin carbohydrates (Fukui et al., 1988; Kurosaka et al., 1987, 1988; Kitagawa et al., 1988a; Numata et al., 1990). During studies to determine the epitopic structure for one of these antibodies, MSW 113, we found that oligosaccharides in human milk significantly inhibited the antigen–antibody reaction. By means of immunoaffinity chromatography with MSW 113, we were able to isolate these inhibitory oligosaccharides (Kitagawa et al., 1988b, 1989, 1990). In this paper, we describe the isolation and structural determination of four of these oligosaccharides that had not been identified previously, either as free oligosaccharides in milk or as the carbohydrate moieties of glycolipids or glycoproteins.

Materials and Methods

Materials. The human milk samples were gifts from the Department of Gynecology and Obstetrics, Faculty of Medicine, Kyoto University, Kyoto, and Hikone City Hospital, Hikone. The murine monoclonal antibodies MSW 113 (IgG3) and MSW 124 (IgG3), which recognize the sialyl-Le^a and Le^a structures, respectively, were established by Kitagawa et al. (1988a and unpublished, respectively). The anti-Le^x monoclonal antibody X001 (IgM) was purchased from BioCarb AB, Lund. MSW 113–protein A–Sepharose CL-4B (6 mg of antibody/mL of resin) was prepared according to Schneider et al. (1982). Le^a pentasaccharide (lacto-*N*-fucopentaose II), Le^x pentasaccharide (lacto-*N*-fucopentaose III), lacto-*N*-tetraose, lacto-*N*-neotetraose, and the Le^a– and Le^x–HSA

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(human serum albumin) conjugates were purchased from BioCarb AB, Lund. Bio-Gel P-4 was purchased from Bio-Rad Laboratories, Richmond. The column used for high-performance liquid chromatography (HPLC)[1] was TSK-GEL NH_2-60, which was obtained from TOSOH Ltd., Tokyo. Sephadex G-25 was purchased from Pharmacia, Uppsala. Sialyl-Le^a hexasaccharide (sialyllacto-*N*-fucopentaose II) was prepared from human milk by affinity chromatography on a column of MSW 113–protein A–Sepharose CL-4B, as described previously (Kitagawa et al., 1988b).

Fractionation of Oligosaccharides. Human milk oligosaccharides were prepared according to Smith et al. (1978). The mixed sialyl oligosaccharides obtained from 3 L of milk were dissolved in 50 mM pyridine–acetic acid buffer, pH 5.0, and then the solution was applied to a column (4.0 × 60 cm) of Bio-Gel P-4 (100–200 mesh) previously equilibrated with the same buffer. The column was washed with the same buffer, and sialic acid containing fractions detected by the resorcinol method of Jourdian et al. (1971) were pooled and lyophilized.

Affinity Chromatography. The dried material was dissolved in 50 mM phosphate buffer, pH 7.2, and the solution was applied to a column of MSW 113–protein A–Sepharose CL-4B, which had been equilibrated with the same buffer. The column was washed with the same buffer, and then antigens retained on the column were eluted with 50 mM diethylamine, pH 11.5. The eluates were neutralized with acetic acid, pooled, and then lyophilized. All procedures were carried out at 4 °C.

High-Performance Liquid Chromatography (HPLC). The oligosaccharides obtained on affinity chromatography were dissolved in 50 mM pyridine–acetic acid buffer, pH 5.0, and the solution was passed through a column of Sephadex G-25 equilibrated with the same buffer. The effluent was lyophilized. The oligosaccharides were further fractionated by HPLC, which was carried out with a Shimadzu Model LC-6A System on a TSK-GEL NH_2-60 column (4.6 × 25 cm), with elution with a gradient of acetonitrile/15 mM potassium phosphate, pH 5.2, as described previously (Kitagawa et al., 1989). Fractionation of asialooligosaccharides was also carried out by HPLC with the same column and system, except that elution was performed isocratically for the initial 3 min with acetonitrile/water (80/20 v/v) and then with a linear gradient of 80/20 to 20/80 (v/v) acetonitrile/water over 60 min (Figure 2).

500-MHz ^1H NMR Spectroscopy. NMR spectra of the oligosaccharides were measured with a JEOL JNM-GX500 500-MHz ^1H NMR spectrometer at 40 °C. NMR samples were dissolved in 150 μL of D_2O (99.96% CEA) in microcells, the pH of the sample solutions being ca. 7.0. Homogated decoupling was performed to suppress residual HDO resonance. The Gaussian function was applied for resolution enhancement of 1D spectra. For measurement of 2D homonuclear Hartmann–Hahn (2D HOHAHA) spectra (Bax & Davis, 1985), 64 scans were accumulated for each t_1, with a mixing time of 100 ms, and 512 × 2048 data points were used, with a spectral width of 3000 Hz and a relaxation delay of 1 s. The digital resolution was ca. 3.0 Hz/point in both dimensions after zero filling in the t_1 dimension. 2,2-Dimethyl-2-silapentane-5-sulfonate (DSS) was used as an internal standard for chemical shifts.

Fast Atom Bombardment Mass Spectrometry (FAB-MS). FAB-MS analysis was carried out as described previously (Kitagawa et al., 1989).

Solid-Phase Radioimmunoassay. Antibodies were reacted with oligosaccharide–HSA conjugates, coated on poly(vinyl chloride) plates (Costar, Cambridge) according to the method of Mårtensson et al. (1988), and then anti-mouse IgG rabbit serum and ^{125}I-labeled protein A (about 100000 dpm/12.5 ng) were added to the immune complex. The amount of antibodies bound was expressed as the radioactivity of bound ^{125}I-labeled protein A, as determined with a γ spectrophotometer (Gamma 5000; Beckman).

Other Materials. [^{125}I]NaI, carrier free, 16 mCi/mmol, was purchased from Amersham. Protein A (from *Staphylococcus aureus*) was from nacarai tesque, Kyoto, and anti-mouse IgG rabbit serum was from Nordic Immunological Laboratories, Tilburg. Preparation of ^{125}I-labeled protein A was carried out by the chloramine T method of Langone (1980), except that termination of the reaction was performed with saturated tyrosine.

Results

Isolation of Immunoreactive Oligosaccharides from Human Milk. Immunoreactive oligosaccharides were isolated from human milk by affinity chromatography on an MSW113–protein A–Sepharose CL-4B column, and then the eluate was further fractionated by HPLC, as described previously (Kitagawa et al., 1989). The eluate gave several peaks, and the structures of the materials in six of them were determined (Kitagawa et al., 1988b, 1989, 1990), as reported previously. In the present study, the fractions corresponding to the two peaks at elution times of 47 min (peak A) and 48.5 min (peak B) were pooled and subjected to structural determination, as described below.

Structural Determination of the Oligosaccharides. (A) Structure of Peak A. Negative ion FAB-MS (Figure 1a) of the oligosaccharides in the HPLC fraction at the elution time of 47 min (peak A) gave molecular ions $(M - H)^-$ of 1873 and 2019. This suggests that this fraction comprises a mixture of oligosaccharides with two different molecular weights and that the difference corresponds to the molecular weight of one fucose residue. Complete separation of these oligosaccharides by HPLC under different conditions was attempted without success. Thus, these oligosaccharides were desialyzed with sialidase from *Arthrobacter ureafaciens*, and then the asialooligosaccharides were fractionated by HPLC. As shown in Figure 2, the asialo compounds gave two peaks (peaks A-1′ and 2′). Negative ion FAB-MS of the two asialo compounds gave molecular ions $(M - H)^-$ of 1728 (peak A-1′) and 1582 (peak A-2′) (Figure 1, panels b and c, respectively). To determine their complete structures, the two asialo compounds and the native oligosaccharides were subjected to 500-MHz ^1H NMR spectroscopy.

(B) Structural Characterization of Peak A by 500-MHz ^1H NMR Spectroscopy. Figure 3a shows the 500-MHz ^1H NMR spectrum of peak A. The spectrum is closely similar to those of sialyl-Le^a oligosaccharides (Kitagawa et al., 1988b, 1989), showing that the oligosaccharides have a common sialyl-Le^a structure, which was further supported by analysis of the 2D HOHAHA spectrum (Figure 4). From the chemical shifts and the splitting patterns of cross peaks, the constituent monosaccharides of peak A were identified (Figure 4). Considering the intensities of the proton resonances in the 1D spectrum and those of cross peaks in the 2D spectrum, peak

[1] Abbreviations: HPLC, high-performance liquid chromatography; 2D HOHAHA, two-dimensional homonuclear Hartmann–Hahn spectroscopy; FAB-MS, fast atom bombardment mass spectrometry; Fuc, L-fucose; Gal, D-galactose; Glc, D-glucose; GlcNAc, *N*-acetyl-D-glucosamine; peaks A and B, the fractions corresponding to two peaks at elution times of 47 and 48.5 min on HPLC (Kitagawa et al., 1989), respectively.

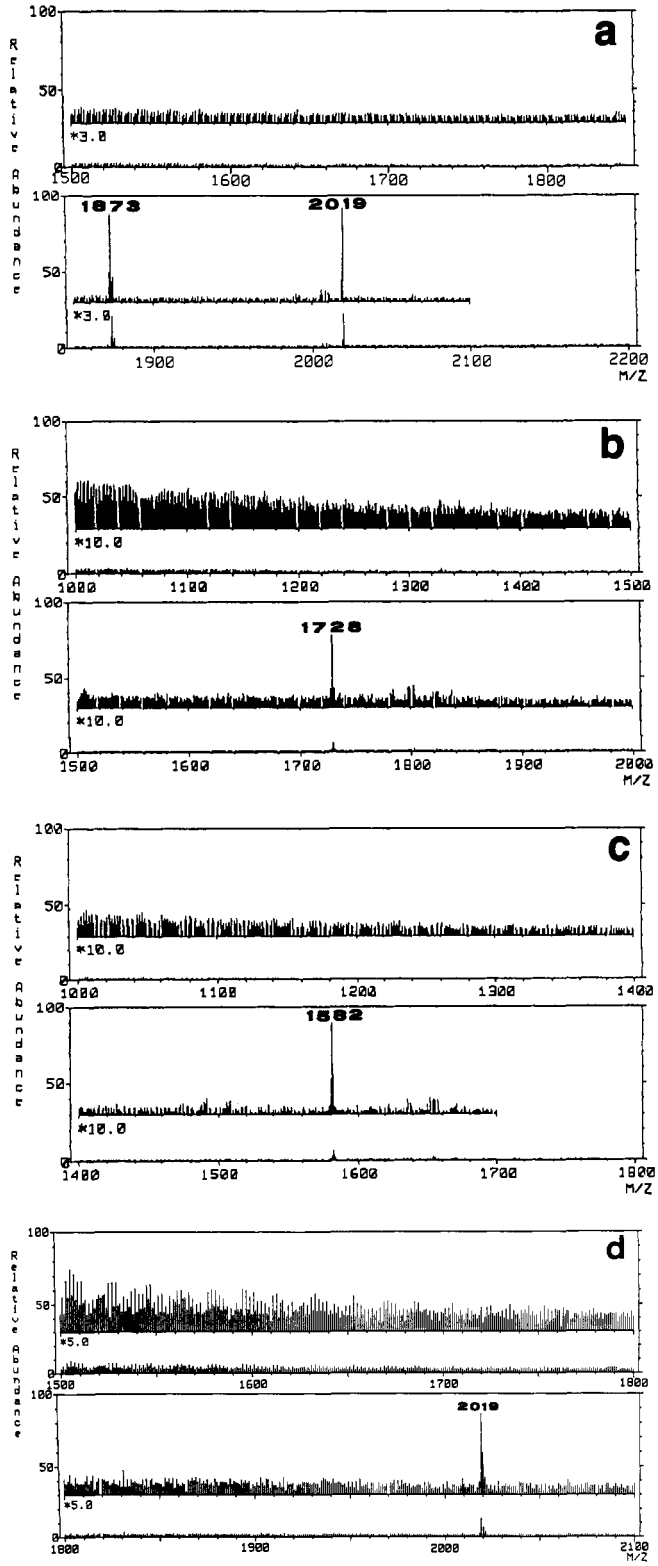
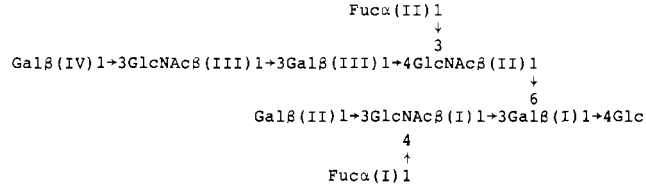
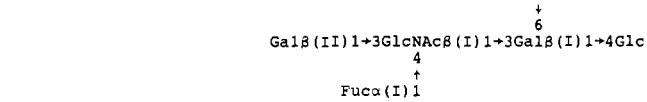


FIGURE 1: Negative ion fast atom bombardment mass spectra of the oligosaccharides. (a) Peak A oligosaccharides (see Results); (b) A-1′ oligosaccharides; (c) A-2′ oligosaccharide (see Figure 2); (d) peak B oligosaccharides (see Results).

A contains 1 mol each of Glc, Galβ(I), Galβ(II), Galβ(IV), Fucα(I), GlcNAcβ(I), and GlcNAcβ(III), with 1–3 linkages, and NeuAcα. In addition, peak A contains approximately 0.5 mol each of Galβ(III), Galβ(III′), Fucα(II), GlcNAcβ(II), and GlcNAcβ(II′), with 1–6 linkages. Pattern matching of the constituent monosaccharides of fraction A with known sialyl-Le^a oligosaccharides led to the definite conclusion that peak A contains the sialyl-Le^a structure (Kitagawa et al., 1988, 1989). However, as shown by the FAB-MS spectrum (Figure 1a), peak A is a mixture of two oligosaccharides, A-1 and A-2. This is consistent with the observation of the occurrence of 0.5 mol of Fucα(II), 0.5 mol each of two types of GlcNAcβ [GlcNAcβ(II) and GlcNAcβ(II′)], and 0.5 mol each of two types of Galβ [Galβ(III) and Galβ(III′)] (Figure 4). After sialidase treatment, the resulting two asialo compounds, A-1′ and A-2′, were separated, and each was subjected to NMR analysis. Panels b and c of Figure 3 show the 500-MHz ^{1}H NMR spectra of A-1′ and A-2′, respectively, and Figure 5 shows the 2D HOHAHA spectrum of A-1′, with the assignments of the constituent monosaccharides. Compared with the 2D HOHAHA spectrum of peak A, peak A-1′ contains Fucα(II), GlcNAcβ(II), and Galβ(III), and, subsequently, peak A-2′ contains GlcNAc(II′) and Galβ(III′) in addition to common constituent monosaccharides, i.e., Fucα(I), GlcNAcβ(III), GlcNAcβ(I), Glc, Galβ(II), Galβ(IV), and Galβ(I). Judging from the cross peak patterns of GlcNAcβ(II) in fractions 2 and 3 reported in a previous paper (Kitagawa et al., 1989), we concluded that A-1′ contains GlcNAcβ(II), which has Fucα(II) at the C3 position, whereas A-2′ does not contain this fucose. In addition, peaks A-1′ and A-2′ contain one more β-linked *N*-acetylglucosamine [GlcNAcβ(III)] and one more β-linked galactose [Galβ(IV)], compared with fractions 2 and 3. The low-field shift of Galβ(III) H4 is consistent with the linkage, GlcNAcβ(III)1→3Galβ(III). Furthermore, the low-field shifts of GlcNAcβ(III) H1 and H3 support the linkage, Galβ(IV)1→3GlcNAcβ(III) (Sabharwal et al., 1988). The structure of A-1′ was deduced to be



Since Fucα(II) is absent in A-2′, the structure of A-2′ was deduced to be



Thus, peak A is concluded to be a mixture of sialyl derivatives of A-1′ and A-2′, with the sialic acid residue being attached to the terminal galactose, Galβ(II), of the stem oligosaccharide chain. They are designated as oligosaccharides A-1 and A-2, and their structures are

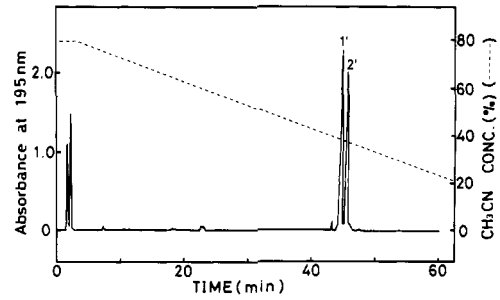
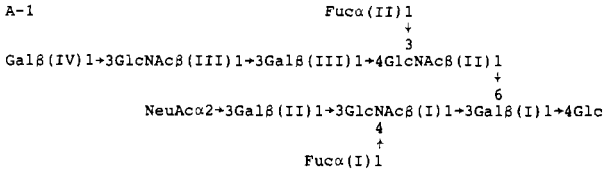
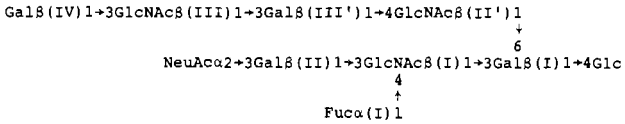


FIGURE 2: High-performance liquid chromatography (HPLC) of the asialo compounds derived from peak A oligosaccharides. The oligosaccharides were detected as to the absorbance at 195 nm. The flow rate was 1.0 mL/min.

A-1



A-2



In Table I, we show the chemical shifts of the structural reporter groups of A-1 and A-2 as well as those of A-1′ and A-2′.

(*C*) *Structure of Peak B.* Negative ion FAB-MS of the peak B oligosaccharide gave the molecular ion $(M - H)^-$ of 2019 (Figure 1d). The oligosaccharide was then subjected to 500-MHz ^{1}H NMR spectroscopy.

(*D*) *Structural Characterization of Peak B by 500-MHz ^{1}H NMR Spectroscopy.* The 500-MHz ^{1}H NMR spectrum of peak B is shown in Figure 3d. The spectrum is quite similar to that of peak A, suggesting that peak B also contains the sialyl-Le^a structure, similar to that in peak A. From the integral, it was found that peak B contains 1.5 mol of fucose with the α1→4 linkage and 0.5 mol of fucose with the α1→3 linkage, suggesting that peak B is an equimolar mixture of two structurally related sialyl oligosaccharides, one with two α1→4-linked fucose residues, and the other with one α1→4-linked residue and one α1→3-linked fucose residue.

Further fractionation of peak B (intact or after sialidase treatment) into individual oligosaccharides was attempted by changing the HPLC conditions without success. The 2D HOHAHA spectrum of peak B showed that H1 of GlcNAc with the β1-3 linkage in GlcNAcβ1→3Galβ was split into two peaks [GlcNAcβ(III) and GlcNAcβ(III′)] (data not shown). The pattern of cross peaks at higher field (4.711 ppm) was characteristic of Fucα1→4GlcNAcβ, whereas the pattern of cross peaks at lower field (4.736 ppm) was characteristic of Fucα1→3GlcNAcβ, as reported previously (Kitagawa et al., 1989). Galβ(IV) H1 was also split into two peaks [Galβ(IV) and Galβ(IV′)] with an equimolar ratio, as shown in Figure 3d. The lower field resonance [Galβ(IV)] was deduced to be due to the galactose residue in the Le^a moiety and the higher field resonance [Galβ(IV′)] due to the Le^x moiety (Sabharwal et al., 1988). The cross peak patterns of GlcNAcβ(II) could not be identified due to bleaching caused by water suppression. However, fucosylation of GlcNAcβ in the Galβ1→4GlcNAcβ unit generally induces a high-field shift of H4 of the Galβ residue from 4.129 ppm to 4.090 ppm (Table I) (Sabharwal et al., 1988). Thus, it was concluded, on the basis of the chemical shift of Galβ(III) H4 at 4.123 ppm, that GlcNAcβ(II) was not fucosylated. Since the chemical shifts of Fucα H1 have been shown to be 5.10 and 5.02 ppm for Le^x and Le^a (Sabharwal et al., 1988), respectively, the chemical shift of Fucα(II′) H1 at 5.088 ppm was assigned to the Le^x moiety and that of Fucα(II) H1 at 5.020 ppm to the Le^a moiety. In addition, the chemical shift of Fucα(I) H1 at 5.002 ppm was assigned to the sialyl-Le^a moiety (Kitagawa et al., 1989).

Thus the structures of the nonreducing terminals of the side chains arising from position 6 of 3,6-linked galactose of these oligosaccharides correspond to the Le^a and Le^x antigens, respectively. This was confirmed by the next experiment. The reactivities of peak B oligosaccharides with an anti-Le^a antibody (MSW 124) and an anti-Le^x antibody (X001) were about half on a molar basis, compared to the reactivities of lacto-*N*-fucopentaoses II and III, respectively (Figure 6). It should be noted that the peak A oligosaccharides showed no reactivity with the antibodies. These results suggest that peak B is an about equimolar mixture of two oligosaccharides isomers. One of them possesses a terminal galactose on C4 of

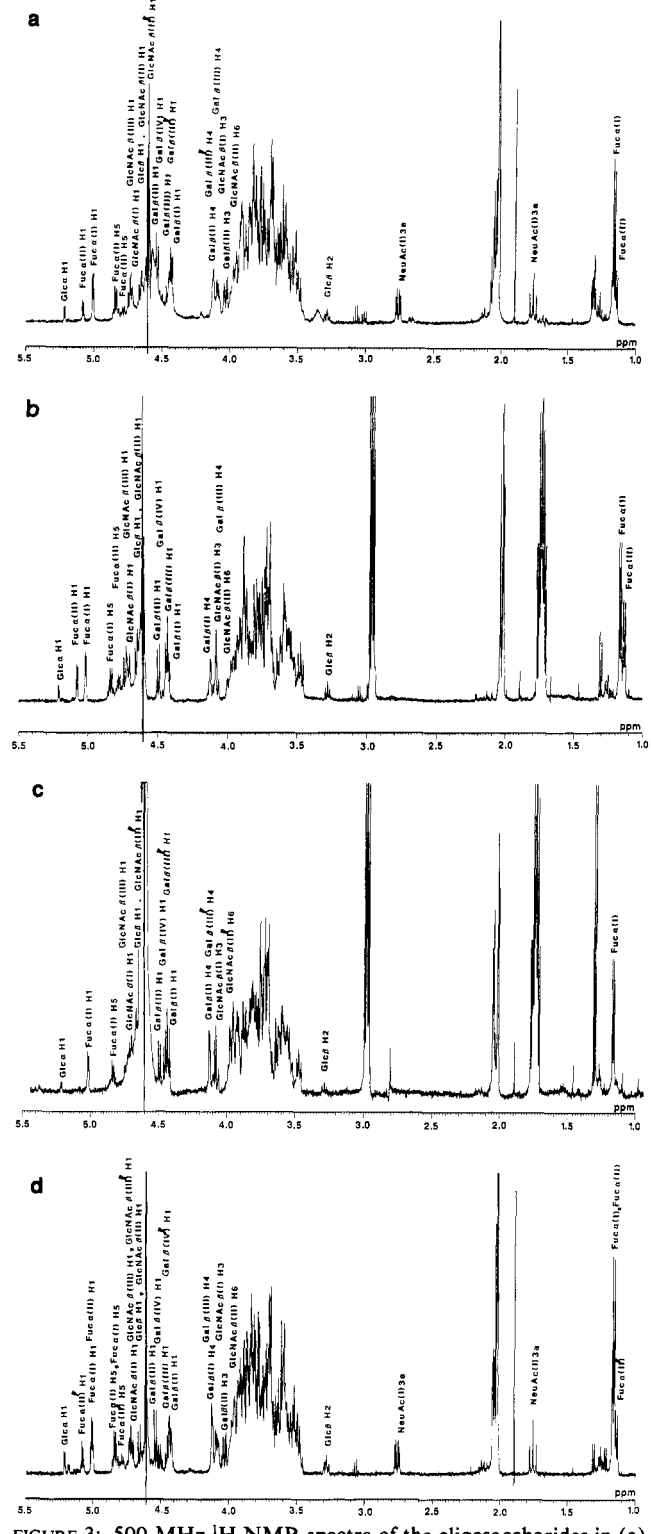


FIGURE 3: 500-MHz ^{1}H NMR spectra of the oligosaccharides in (a) peak A, (b) peak A-1′, (c) peak A-2′, and (d) peak B, with the assignments of the structural reporter groups. Base-line correction was performed to remove the background at around 4.7 ppm. Due to water suppression caused by homogenated decoupling, the resonances around HDO were suppressed.

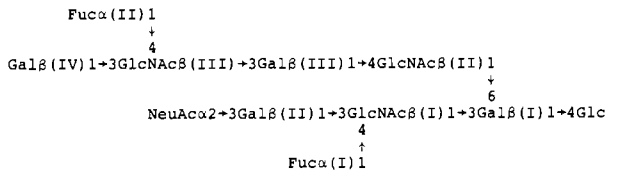
Table I: Chemical Shifts (in ppm) of Structural Reporter Group Protons of the Constituent Monosaccharides of Oligosaccharides

residue	^{1}H	A-1	A-2	A-1′	A-2′	B-1	B-2
Glcβ	H1	4.658	4.658	4.656	4.656	4.656	4.656
Galβ(I)	H1	4.427	4.427	4.428	4.430	4.429	4.429
	H4	4.126	4.126	4.123	4.125	4.125	4.125
GlcNAcβ(I)	H1	4.720	4.720	4.718	4.718	4.720	4.720
	H3	4.086	4.086	4.084	4.079	4.081	4.081
Galβ(II)	H1	4.538	4.538	4.496	4.495	4.538	4.538
	H3	4.032	4.032				
Fucα(I)	H1	5.003	5.003	5.019	5.021	5.002	5.002
	H5	4.838	4.838	4.838	4.838	4.839	4.839
	H6	1.174	1.174	1.174	1.174	1.170	1.170
NeuAcα	H3a	1.760	1.760			1.760	1.760
	H3e	2.762	2.762			2.764	2.764
GlcNAcβ(II)	H1	4.644		4.645		*[a]	*
GlcNAcβ(II′)	H1		4.632		*		
Galβ(III)	H1	4.430		4.432		4.430	4.430
	H4	4.087		4.090		4.123	4.123
Galβ(III′)	H1		4.428		4.430		
	H4		4.124		4.129		
Fucα(II)	H1	5.080		5.080		5.020	
	H5	4.776		4.779		4.839	
	H6	1.141		1.143		1.174	
Fucα(II′)	H1						5.088
	H5						4.789
	H6						1.151
GlcNAcβ(III)	H1	4.740	4.740	4.740	4.740	4.711	
GlcNAcβ(III′)	H1						4.736
Galβ(IV)	H1	4.440	4.440	4.439	4.439	4.509	
Galβ(IV′)	H1						4.455

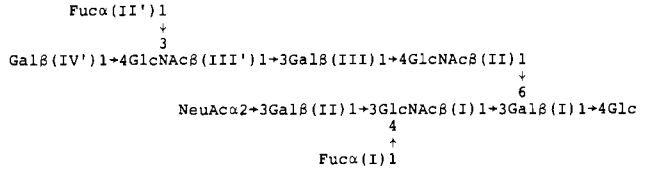
[a] An asterisk indicates not observed due to overlapping with HDO resonance.

N-acetylglucosamine (type 2 chain) to form the Le^x structure and the other one on C3 (type 1 chain) to form the Le^a structure. The structures of the peak B oligosaccharides are

B-1



B-2



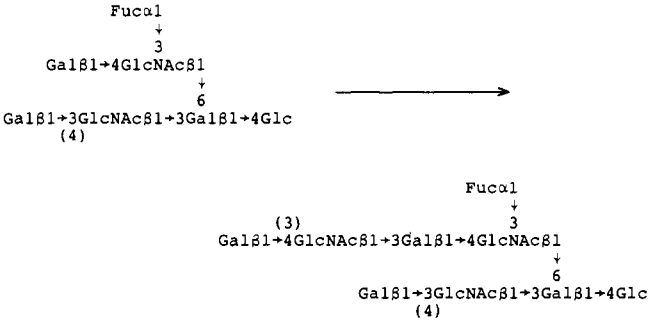
In Table I, the chemical shifts of the structural reporter groups of B-1 and B-2 are shown.

Discussion

Many of the oligosaccharides that occur in human milk constitute the carbohydrate moieties of cell surface glycolipids and glycoproteins. Some of them are structural determinants of oncofetal antigens such as sialyl-Le^a oligosaccharides (Montreuil, 1960; Wieruszeski et al., 1985; Smith et al., 1987; Kitagawa et al., 1988b, 1989, 1990). This similarity in structure of the oligosaccharides of human milk to the oncofetal antigens led to our studies to determine the epitopic structure recognized by the monoclonal antibody MSW 113, which recognizes tumor-associated carbohydrate antigens.

In general, isolation of such oligosaccharides is troublesome because of the need of laborious, multistep separation procedures. By means of immunoaffinity purification with MSW 113, however, we succeeded in isolating several novel oligosaccharides from human milk. All of them had the sialyl-Le^a structure (Kitagawa et al., 1988b, 1989, 1990). This work was further extended to the isolation of four novel oligosaccharides, as reported in the present paper.

So far, the structures of more than 50 different milk oligosaccharides have been determined (Yamashita et al., 1982). These oligosaccharides may be classified into nine types on the basis of the core structure. The core structures are lactose, lacto-*N*-tetraose, lacto-*N*-neotetraose, two lacto-*N*-hexaoses, two lacto-*N*-parahexaoses, and two lacto-*N*-octaoses, with an exception being the unusual oligosaccharide we recently isolated, which lacks an internal lactose structure (Kitagawa et al., 1990). These core structures and the additional unusual structure can be further modified through the actions of sialyl-, galactosyl-, glucosaminyl-, and fucosyltransferases, with a multiple of oligosaccharide structures being produced (Egge et al., 1983; Bruntz et al., 1988). According to Tachibana et al. (1978), fucosyllacto-*N*-hexaose IV and fucosyllacto-*N*-neohexaose II are precursors of monofucosyllacto-*N*-octaose and monofucosyllacto-*N*-neooctaose, respectively. Thus, the fucosyl linkage Fucα1→3GlcNAc should be included in the core structure of these oligosaccharides. The biosynthesis would proceed as follows.



However, three of the lacto-*N*-octaose series oligosaccharides (oligosaccharides A-2, B-1, and B-2, see Results), which we

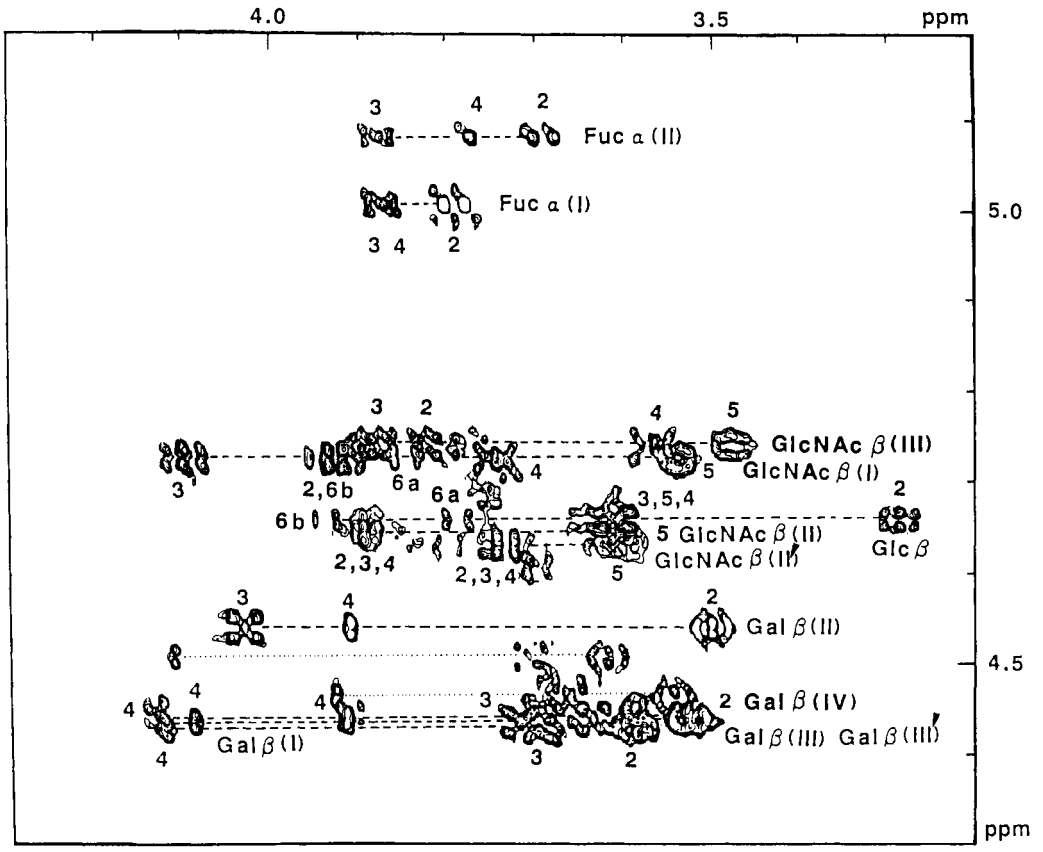


FIGURE 4: 2D HOHAHA spectrum of fraction A in the cross peak region of the anomeric proton and other sugar proton resonances. The type and anomeric configuration of each sugar component are shown as well as the assignments of the proton resonances. Impurity cross peaks due to Galβ are connected by dotted lines.

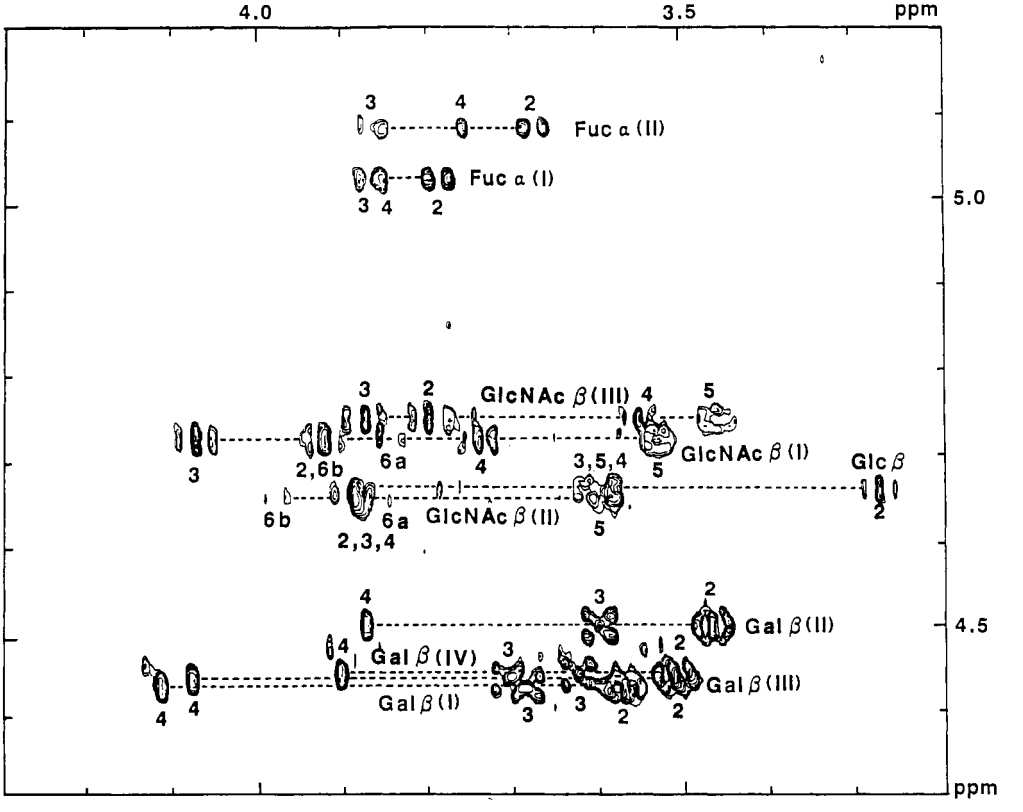


FIGURE 5: 2D HOHAHA spectrum of fraction A-1′ in the cross peak region of the anomeric proton and other sugar proton resonances. The type and anomeric configuration of each sugar component are shown as well as the assignments of the proton resonances.

isolated and determined the structures of in this study, lack this fucosyl linkage. This would suggest that this fucosyl linkage is not essential for extension of the lactosamine unit.

Two of the four newly isolated oligosaccharides (oligosaccharides B-1 and B-2, see Results) have both the sialyl-Le^a and Le^a or Le^x-i structures, respectively. These structures have

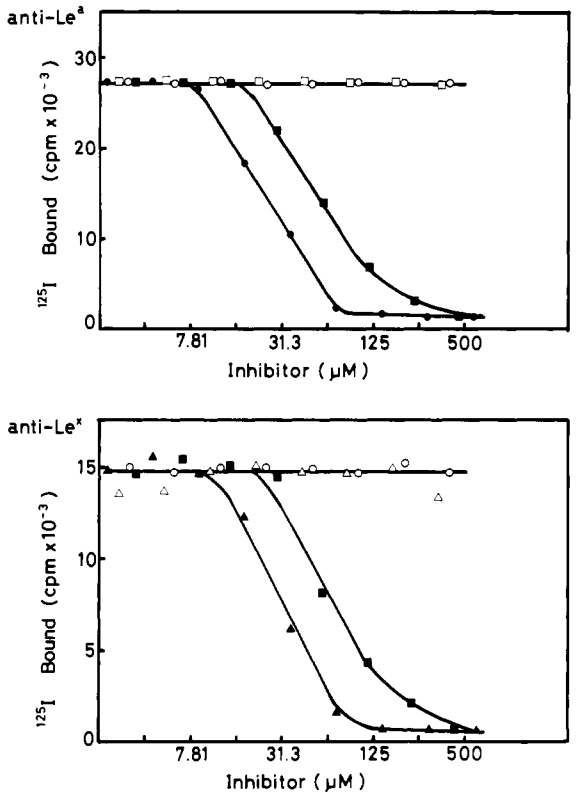


FIGURE 6: Inhibition of binding of monoclonal antibodies (a) anti-Le^a (MSW 124) and (b) anti-Le^x (X001) to the respective antigens by peak B oligosaccharides from human milk and by various other oligosaccharides. Poly(vinyl chloride) plates were coated with oligosaccharide–HSA; for anti-Le^a (MSW 124) and anti-Le^x (X001) monoclonal antibodies, lacto-*N*-fucopentaose II–HSA and lacto-*N*-fucopentaose III–HSA were used, respectively. (○) Sialyl-Le^a hexasaccharide (NeuAcα2→3Galβ1→3(Fucα1→4)GlcNAcβ1→3Galβ1→4Glc) or peak A oligosaccharides (see the structures under Results); (●) lacto-*N*-fucopentaose II (Le^a: Galβ1→3(Fucα1→4)GlcNAcβ1→3Galβ1→4Glc); (□) lacto-*N*-tetraose (Galβ1→3GlcNAcβ1→3Galβ1→4Glc); (■) peak B oligosaccharides (see the structures under Results); (△) lacto-*N*-neotetraose (Galβ1→4GlcNAcβ1→3Galβ1→4Glc); (▲) lacto-*N*-fucopentaose III (Le^x: Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4Glc).

separately been found in mucin-type glycoproteins and glycolipids in a variety of human cancers, as described in a previous paper (Kitagawa et al., 1989). Similar oligosaccharides with both the sialyl-Le^a and blood-group H structures or with both the sialyl-Le^a and difucosyl Le^a-Le^x structures would also occur in human milk, and the latter, the Le^a-Le^x structure, exhibits high affinity to an antibody directed to a human squamous lung carcinoma (Yamashita et al., 1982; Mårtensson et al., 1988).

The amounts of the oligosaccharides in peaks A-1′, A-2′, and B were roughly 20–50, 20–50, and 10–30 μg/L, respectively, but the values varied widely among individuals.

In previous papers (Kitagawa et al., 1988a, 1989, 1990), we reported that MSW 113 was distinct from NS 19-9 (Koprowski et al., 1979), which is also sialyl-Le^a structure directed (Magnani et al., 1982), in epitope recognition, as judged from the results of immunoreactivity with the milk oligosaccharides. The same experiment was carried out on the newly isolated oligosaccharides (peaks A and B). The results showed that MSW 113 reacted with the A and B oligosaccharides about 30-fold more strongly than NS 19-9. Accordingly, oligosaccharides isolated from human milk have been regarded as being very useful as hapten inhibitors to determine the binding specificities of anti-carbohydrate antibodies and as models for studies on the acceptor specificities of glycosyltransferases and on the biosynthesis of glycoprotein and glycolipid sugar chains.

It is also worthy of mentioning that MSW 113 is useful in detecting cancer-associated antigens in sera of patients with colorectal, gastric, gall bladder, and pancreatic cancers, probably due to the broader reactivity towards various oligosaccharides with the sialyl-Le^a structure compared to that of NS 19-9. This would suggest that these oligosaccharides occur on mucins produced by cancer cells.

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Registry No. [(Galβ1→3GlcNAcβ1→3Galβ1→4)[Fucα1→3]-GlcNAcβ1→6](NeuAcα2→3Galβ1→3[Fucα1→4]GlcNAcβ1→3)-Galβ1→4Glc, 132020-01-0; (Galβ1→3GlcNAcβ1→3Galβ1→-4GlcNAcβ1→6)[(NeuAcα2→3Galβ1→3)[Fucα1→4]GlcNAcβ1→-3]Galβ1→4Glc, 132020-02-1; [(Fucα1→4)(Galβ1→3)GlcNAcβ1→-3Galβ1→4GlcNAcβ1→6][(NeuAcα2→3Galβ1→3)[Fucα1→4]-GlcNAcβ1→3]Galβ1→4Glc, 132020-03-2; [(Galβ1→4)(Fucα1→-3)GlcNAcβ1→3Galβ1→4GlcNAcβ1→6][(NeuAcα2→3Galβ1→-3)[Fucα1→4]GlcNAcβ1→3]Galβ1→4Glc, 132020-04-3.

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Exchange of Phosphoryl Groups between HPr Molecules of the Phosphoenolpyruvate-Dependent Phosphotransferase System Is an Autocatalytic Process†

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ABSTRACT: HPr, a central component of the phosphoenolpyruvate-dependent phosphotransferase system, can exist in *Escherichia coli* in a phosphorylated (PHPr) and a nonphosphorylated form. We show that, beside the normal transfer of the phosphoryl group from PHPr to enzymes II and III, PHPr can phosphorylate other HPr molecules in an autocatalytic exchange reaction. The reaction is very fast but is inhibited by labeling the protein with Bolton–Hunter reagent. We demonstrate that the exchange reaction can be used to determine the $\Delta G°$ of the phosphoryl group of mutant forms of PHPr relative to wild-type PHPr. Two HPr mutants were constructed by site-directed mutagenesis, HPr P11E and HPr E68A. Both show altered phosphoryl group potentials but show no significantly altered K_M or V_{max} values compared to wild-type HPr, illustrating the sensitivity of the exchange process. The exchange reaction does not occur between HPr from *E. coli* and HPr from *Staphylococcus carnosus*.

The phosphocarrier protein HPr is a central component of almost all bacterial phosphoenolpyruvate (PEP)[1]-dependent sugar phosphotransferase systems (PTS). Its primary function is the catalysis of the transfer of a phosphoryl group from EI, the first general component of the PTS, to the next PTS component, which is sugar-specific (e.g., $EIII^{glc}$ or EII^{mtl}; see Figure 1). In *Escherichia coli*, HPr can exist in a phosphorylated form (PHPr) and a nonphosphorylated form. During phosphorylation, the phosphoryl group is carried on the Nδ1 position of the His15 imidazole ring, as has been reported by several investigators (Weigel et al., 1982a,b; Waygood et al., 1985; van Dijk et al., 1990); the hydrolysis characteristics of this active-site residue have also been established (Waygood et al., 1985). In a study on the reversibility of the phosphotransfer reactions catalyzed by HPr, Sutrina et al. (1987) showed that EI, EII^{mtl}, and $EIII^{glc}$ can catalyze the exchange of a phosphoryl group between HPr and PHPr.

In the present study, we show that the phosphoryl group exchange between HPr molecules is a highly efficient, autocatalytic process which does not require the presence of EI, EII, or EIII. This self-exchange process enables us to characterize HPr mutants with respect to their phosphoryl group potential.

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[1] Abbreviations: decyl PEG, decyl poly(ethylene glycol) 300; DTT, dithiothreitol; (P)EI, II, III, (phosphorylated) enzyme I, II, III; glc, glucose; mtl, mannitol; PEP, phosphoenolpyruvate; PTS, phosphoenolpyruvate-dependent phosphotransferase system. Note HPr mutants are denoted as follows: HPr A*xx*B in which A is the wild-type amino acid at residue number *xx*, which in the mutated HPr is replaced by amino acid B.

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