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Structures of Sialylated Fucosyl Polylactosaminoglycans Isolated from Chronic Myelogenous Leukemia Cells*

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Polylactosaminoglycans were isolated from human chronic myelogenous leukemia cells and their structures were elucidated. The lactosaminoglycan saccharides were isolated by hydrazinolysis and fractionated by QAE-Sephadex. The structures of fractionated oligosaccharides were analyzed by fast atom bombardment-mass spectrometry and methylation before and after treatment with specific exoglycosidases, such as α2→3 specific neuraminidase. Based on these experiments, the structures of sialyl polylactosaminoglycans of chronic myelogenous leukemia cells were found to contain the following unique structure which is absent in normal mature granulocytes:

NeuNAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→
3 3
↑α ↑α
1 1
Fuc Fuc

In addition to this, chronic myelogenous leukemia polylactosaminoglycans can be distinguished from normal granulocyte polylactosaminoglycans by the following characteristics. Leukemic polylactosaminoglycans are (*a*) shorter, (*b*) more highly sialylated and contain fully sialylated, tetrasialosyl polylactosaminoglycans, (*c*) are less fucosylated at C-3 of *N*-acetylglucosamine of polylactosaminyl side chains, and (*d*) contain a significant amount of sialyl Le^x, NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→3, structure. These results indicate that chronic myelogenous leukemia cells express unique polylactosaminoglycan structures which are distinct from normal mature granulocytes.

Chronic myelogenous leukemia (CML[1]) is a clonal myeloproliferative disorder and characterized by a marked overproduction of granulocytes (for review, see Ref. 1). The morphology and functions of granulocytes produced in CML are very similar to those of normal mature granulocytes (2), although most of the CML granulocytes can be distinctively characterized by the presence of a specific chromosome, the Philadelphia chromosome (3).

In order to detect cell surface structures specific to granulocytes of CML patients, several studies have been carried out. In particular, Baker *et al.* (4) found that CML granulocytes are highly sialylated and glycoproteins of those cells were not revealed by the galactose oxidase/NaB[^{3}H]$_4$ procedure without prior neuraminidase treatment, whereas normal granulocytes were labeled by the same procedure. This difference, however, was not detected when CML granulocytes and normal granulocytes were labeled by the galactose oxidase procedure after neuraminidase treatment (5). Westrick *et al.* (6) characterized gangliosides from CML cells and showed the absence of some glycolipids in CML cells. Consistent with Baker *et al.*'s findings, Van Beek *et al.* (7) described that CML granulocytes contain more sialylated glycopeptides than normal granulocytes. The binding of *Ricinus communis* agglutinin to CML granulocytes was significantly increased after neuraminidase treatment (8). These studies suggest that CML granulocytes are more sialylated than normal mature granulocytes; however, a detailed structural characterization of cell surface oligosaccharides on CML granulocytes has not been made.

Recently, we have reported that normal mature granulocytes contain a significant amount of polylactosaminoglycans with the terminal structure of Galβ1→4(Fucα1→3)GlcNAc (9). In addition, we have found that some of the polylactosaminoglycans are sialylated and contain the NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAc terminal structure (10). In the present study, we have extended our analysis to the characterization of sialylated polylactosaminoglycans isolated from CML granulocytes and have defined the major structures present.

EXPERIMENTAL PROCEDURES

The majority of the experimental procedures are the same as described in the previous papers (9, 10) except for the following.

Isolation of Glycopeptides from Granulocytic Cells of Chronic Myelogenous Leukemia Patients—Patients were admitted to the hospitals of the University of California at San Diego or The Medical Research Institute at San Francisco. All patients were in the chronic phase of CML and had blast counts of less than 10%. Leukocytes were obtained by therapeutic leukophoresis and further purified by

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[1] The abbreviations used are: CML, chronic myelogenous leukemia; FAB-MS, fast atom bombardment-mass spectrometry.

hypotonic lysis of contaminating erythrocytes as described (11). In order to minimize the damage of leukocytes, the lysing solution was immediately made isotonic by adding 10 times phosphate-buffered saline. The preparation obtained was found by their morphology to be more than 90% granulocytic cells with various degrees of maturation (segmented and banded neutrophils, metamyelocytes, and myelocytes).

Granulocytic cells were then extracted with 5 times the volume of hot ethanol and then with 10 times the volume of chloroform:methanol (2:1). The dried residue was subjected to extensive pronase digestion and digestion with deoxyribonuclease and the glycopeptide fractions were isolated by Sephadex G-25 gel filtration, as described previously (9). The glycopeptide fractions were then applied to DE52 column chromatography and the fractions which did not bind and which eluted with 100 mM phosphate buffer, pH 7.4, were combined and desalted as described (12). This preparation was called the glycopeptide fraction.

Glycosidase Treatment—Saccharides were incubated with various glycosidases as follows. Saccharides (50–100 μg) were digested with a mixture of β-galactosidase (0.3 units) and β-*N*-acetylglucosaminidase (0.17 units) in 50 μl of 0.02 M sodium citrate buffer, pH 4.1, at 37 °C for 48 h. Saccharides were also digested with these enzymes in the presence of almond emulsin α-L-fucosidase (0.05 units). Digestion with Newcastle disease virus neuraminidase (13) was with 4.5 milliunits of the enzyme and 20 μl of 0.1 M sodium cacodylate buffer, pH 6.5, at 37 °C for 48 h. In order to further the digestion of glycopeptides with β-galactosidase and β-*N*-acetylglucosaminidase, the sample was centrifuged to remove virus particles and the supernatant was adjusted to pH 4.1 with the addition of 0.1 M sodium citrate buffer, pH 3.0. The volume of the added citrate buffer was half of the sodium cacodylate buffer. After treatment, the digests were centrifuged and the supernatants were applied to a column of Sephadex G-25, equilibrated, and eluted with water. β-Galactosidase (jack bean) and β-*N*-acetylglucosaminidase (beef kidney) were obtained from Sigma and Boehringer Mannheim, respectively. α1→3/4 specific fucosidase was purified from almond emulsin as described (9, 14). Newcastle disease virus neuraminidase (13) was kindly donated by Dr. Paulson of the UCLA School of Medicine.

Methylation Analysis—The lactosaminoglycans were methylated by the method of Hakomori (15) with modifications as described (16, 17). Alditol and hexosaminol acetates of methylated sugars were analyzed as described (16) except for the following. During gas chromatography-mass spectrometry, the column temperature was programmed at 20 °C increments/min from 50 to 150 °C and then at 4 °C increments/min to 250 °C.

Fast Atom Bombardment-Mass Spectrometry—Fast atom bombardment-mass spectrometry (FAB-MS) were carried out as described previously (17). About 100–300 μg of saccharides were permethylated and about 10–30 μg of each derivative was loaded into the glycerol/thioglycerol matrix for each FAB-MS run. Complete coverage of the mass scale (100–3300 mass units) usually requires several runs.

RESULTS

Isolation and Fractionation of Saccharides from Granulocytic Cells of Chronic Myelogenous Leukemia—The glycopeptide fraction from each patient, prepared as described under "Experimental Procedures," was subjected to hydrazinolysis and the resultant saccharides were *N*-acetylated and reduced with $NaB[^3H]_4$ as described previously (17). The samples were then subjected to Sephadex G-50 gel filtration as shown in Fig. 1*A*. The saccharides which eluted at fractions of high molecular weight (see the *horizontal arrows* in Fig. 1*A*) were combined and reapplied to the same column (Fig. 1*C*). This saccharide fraction will be called CML polylactosaminoglycans. Saccharides were also prepared from normal, mature granulocytes in the same way as described for CML saccharides and subjected to the same gel filtration (Fig. 1*B*). It was found that the molecular weights of CML polylactosaminoglycans are smaller than those of granulocyte polylactosaminoglycans.

CML polylactosaminoglycans were then fractionated by QAE-Sephadex column chromatography (10, 18). As shown in Fig. 2*A*, the CML polylactosaminoglycans were separated into 5 fractions and A-b, A-c, A-d, and A-e were recovered as sialylated saccharides, whereas A-a was found to be a mixture

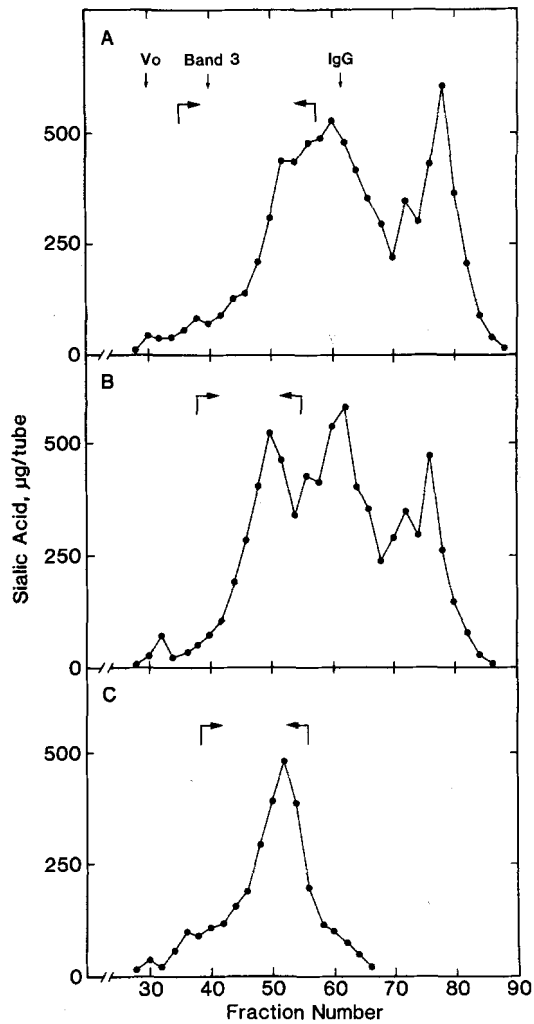

FIG. 1. **Sephadex G-50 gel filtration of saccharides obtained from chronic myelogenous leukemia cells and normal mature granulocytes.** Saccharides obtained after hydrazinolysis were applied to a column (1.0 × 94 cm) of Sephadex G-50 (superfine), equilibrated and eluted with 0.2 M NaCl. Each fraction contained 0.7 ml and aliquots (10–50 μl) were taken for determining sialic acid by the periodate/resorcinol procedure (34). *A*, saccharides obtained from CML cells. *B*, saccharides obtained from normal mature granulocytes. *C*, the rechromatography of fractions 34–56 of *A*. Fractions 38–56 of this chromatography were pooled for polylactosaminoglycans of chronic myelogenous leukemia cells. Elution positions of the standard saccharides, cord Band 3 polylactosaminoglycan (*Band 3*), and bovine IgG saccharides (IgG) are indicated by the *vertical arrows*.

of neutral saccharides (see below also). Sialylated CML polylactosaminoglycans eluted at later fractions than granulocyte polylactosaminoglycans under the same conditions (Fig. 2*B*). In the following sections, structural determinations of each fraction will be described separately.

Structure of A-a—Methylation analysis indicated that A-a is composed of 9 lactosaminyl units and is of tetraantennary structure (Table I). The same analysis indicates that A-a contains about 1 mol of fucose attached to polylactosaminyl side chains based on the amount of 6-*O*-methyl *N*-acetylglucosamine. Fucose is bound to C-3 of *N*-acetylglucosamine since A-a produced 3,6-di-*O*-methyl *N*-acetylglucosamine with the concomitant loss of 6-*O*-methyl *N*-acetylglucosamine after defucosylation with 0.1 N trichloroacetic acid at 100 °C for 1 h (19).

FAB-MS of permethylated A-a provided a series of signals

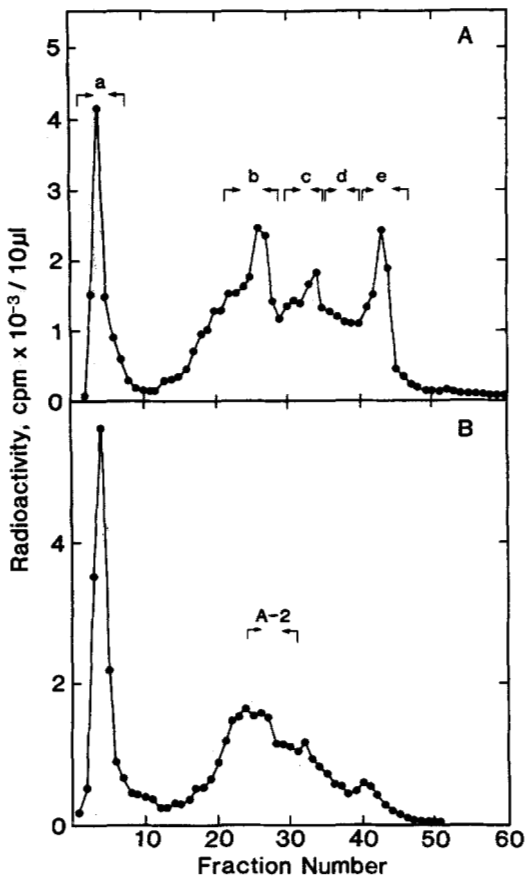

FIG. 2. **QAE-Sephadex A-25 column chromatography of saccharides obtained from CML cells (*A*) and normal mature granulocytes (*B*).** Saccharides were applied to a column (0.9 × 15 cm) of QAE-Sephadex equilibrated with 2 mM Tris-HCl, pH 8.0. After washing with 15 ml of the same buffer, the elution was carried out with a linear gradient of 2 mM Tris-HCl, pH 8.0, to 100 mM sodium phosphate buffer, pH 7.0 (50 ml of each solution). Fractions of 1.5 ml were collected and aliquots were taken for determination of radioactivity. Each fraction designated by *horizontal arrows* was desalted and rechromatographed under the same conditions.

TABLE I

Relative proportions of methylated sugars obtained from polylactosaminoglycan saccharides of chronic myelogenous leukemia cells

Methylated sugars	A-a	A-b	A-c	A-d	A-e
Fucitol					
2,3,4-tri-*O*-methyl	1.1	1.4	1.1	1.2	0.2
Galactitol					
2,3,4,6-tetra-*O*-methyl	3.6	2.8	1.4	0.9	0.2
2,4,6-tri-*O*-methyl	5.1	2.9	3.5	3.1	3.4
2,3,4-tri-*O*-methyl	0.3	1.1	1.2	1.1	1.1
Mannitol					
3,4,6-tri-*O*-methyl	0.1	0.3	0.1	0.5	0.3
3,6-di-*O*-methyl	0.8	0.8	0.9	0.7	0.7
3,4-di-*O*-methyl	1.1	0.9	1.0	0.8	1.0
2,4-di-*O*-methyl	1.0	1.0	1.0	1.0	1.0
2-*N*-methylacetamido-2-deoxy-glucitol[a]					
3,4,6-tri-*O*-methyl	Trace	0.1	0.2	0.3	0
3,6-di-*O*-methyl	7.7	5.5	4.6	4.0	4.6
6-*O*-methyl	0.7	1.1	0.9	0.4	0.1
3-*O*-methyl	0.3	Trace	0.2	0.1	Trace

[a] 1,3,5,6-Tetra-*O*-methyl GlcNAcMe was not detected. This indicates only a part of the molecules was reduced by $NaBH_4$. See also the previous papers (35, 36).

TABLE II

Compositional assignment for fragment ions derived from permethylated saccharides obtained from chronic myelogenous leukemia cells

m/z	Assignment	A-a	A-b	A-c	A-d	A-e
2346	NeuNAc·Fuc ·Hex_4·$HexNAc_4^+$			+		
2333	Fuc_3·Hex_4·$HexNAc_4^+$	+				
2172	NeuNAc ·Hex_4·$HexNAc_4^+$			+	+	
2159	Fuc_2·Hex_4·$HexNAc_4^+$	+				
2071	NeuNAc·Fuc_2·Hex_3·$HexNAc_3^+$			+	+	
1897	NeuNAc·Fuc ·Hex_3·$HexNAc_3^+$	±	+	+	+	
1884	Fuc_3·Hex_3·$HexNAc_3^+$	±				
1723	NeuNAc ·Hex_3·$HexNAc_3^+$		+	+	+	±
1710	Fuc_2·Hex_3·$HexNAc_3^+$	+	+	+	±	
1622	NeuNAc·Fuc_2·Hex_2·$HexNAc_2^+$			+	±	
1536	Fuc ·Hex_3·$HexNAc_3^+$	+	+	+		
1448	NeuNAc·Fuc ·Hex_2·$HexNAc_2^+$	±	+	+	+	+
1362	Hex_3·$HexNAc_3^+$	+	+	+		
1274	NeuNAc ·Hex_2·$HexNAc_2^+$	+	+	+	+	+
1261	Fuc_2·Hex_2·$HexNAc_2^+$	+	+			
1087	Fuc ·Hex_2·$HexNAc_2^+$	+	+	+	+	
999	NeuNAc·Fuc ·Hex ·$HexNAc^+$		+	+	+	±
913	Hex_2·$HexNAc_2^+$	+	+	+	+	
825	NeuNAc ·Hex ·$HexNAc^+$	+	+	+	+	+
638	Fuc ·Hex ·$HexNAc^+$	+	+	+	+	±
580	NeuNAc ·Hex^+		+	+	+	
464	Hex ·$HexNAc^+$	+	+	+	+	±
376	$NeuNAc^+$	+	+	+	+	+

which are derived from polylactosaminyl chains (Fig. 3*A*)[2] which are summarized in Table II. Since this polylactosaminoglycan did not reveal any significant difference from granulocyte neutral polylactosaminoglycans (see Ref. 9), further detailed studies were not carried out. It was, however, discovered that the neutral polylactosaminoglycan from CML cells contains less fucose attached to polylactosaminyl side chains than the neutral polylactosaminoglycan of normal granulocytes, which contains about 4 mol of fucose in polylactosaminyl side chains (9). Some of the sialylated saccharides apparently were not bound to QAE-Sephadex, judging from the presence of a sialylated fragment ion (*m/z* 825) in the A-a fraction (see Fig. 3*A*).

Structure of A-b—Rechromatography of fractions 21–28 on the QAE-Sephadex A-25 column yielded a symmetrical peak and this sample, A-b, was subjected to structural analysis. Methylation analysis indicated that A-b is composed of $\alpha 2\rightarrow$ 6 linked and $\alpha 2\rightarrow 3$ linked sialic acid (Table I). The same analysis indicates that A-b contains about 1 mol of fucose, which was shown to be attached to C-3 of *N*-acetylglucosamine since 6-*O*-methylglucosamine disappears after defucosylation (data not shown).

FAB-MS of permethylated A-b provided a series of signals which are derived from polylactosaminyl chains (Fig. 3*B*) and these are summarized in Table II. These signals are apparently derived from four different structures (Table III): first, 464 for Hex·$HexNAc^+$, 913 for Hex_2·$HexNAc_2^+$, and 1362 for Hex_3·$HexNAc_3^+$; second, 638 for Fuc·Hex·$HexNAc^+$, 1087 for Fuc·Hex_2·$HexNAc_2^+$, 1261 for Fuc_2·Hex_2·$HexNAc_2^+$, 1536 for Fuc·Hex_3·$HexNAc_3^+$, and 1710 for Fuc_2·Hex_3·$HexNAc_3^+$; third, 580 for NeuNAc·Hex^+, 825 for NeuNAc·Hex·$HexNAc^+$, 1274 for NeuNAc·Hex_2·$HexNAc_2^+$, 1723 for NeuNAc·Hex_3·$HexNAc_3^+$; and fourth, 999 for NeuNAc·Fuc·

[2] Portions of this paper (Figs. 3, 4, and 7 and Table III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-1092, cite the authors, and include a check or money order for $6.80 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

Hex·HexNAc$^+$, 1448 for NeuNAc·Fuc·Hex$_2$·HexNAc$_2^+$, and 1897 for NeuNAc·Fuc·Hex$_3$·HexNAc$_3^+$. It has been shown that cleavage in polylactosamine-containing compounds takes place preferentially at the hexosaminyl linkages (16, 20). These results, therefore, indicate that A-b contains structure 1, structure 2, one or more of structures 3–5 and one or more of structures 10–15 in Table III.

Combining the results obtained by FAB-MS with methylation studies, it was concluded that the structure of A-b has four different chains; one without any substitution, one terminated with α2→6 linked sialic acid, one terminated with α2→3 linked sialic acid, and one with various degrees of fucosylation. In order to confirm the above conclusion and elucidate the distribution of these side chains, A-b was digested extensively with a mixture of β-galactosidse and β-*N*-acetylglucosaminidase after desialylation with mild acid hydrolysis. Permethylation of the product yielded 0.7 mol of 2,3,4,6-tetra-*O*-methylmannose, 0.5 mol of 2,3,4-tri-*O*-methylmannose, and 0.2 mol of 2,3,6-tri-*O*-methylmannose with the concomitant loss of 0.8 mol each of 3,6-di-*O*-methylmannose and 3,4-di-*O*-methylmannose. The product also yielded 2.4 mol of 3,6-di-*O*-methyl *N*-acetylglucosamine, 0.9 mol of 6-mono-*O*-methyl *N*-acetylglucosamine, 0.4 mol of 2,3,4,6-tetra-*O*-methylgalactose and 1.4 mol of 2,4,6-tri-*O*-methylgalactose. These results indicate that the majority of fucose is attached to the side chains arising from position 6 of 2,6-substituted α-mannose and from position 4 of 2,4-substituted α-mannose.

Intact A-b was then digested extensively with a mixture of β-galactosidase and β-*N*-acetylglucosaminidase. Permethylation of the product yielded 0.8 mol of 2,3,4-tri-*O*-methylmannose, 0.7 mol of 3,4,6-tri-*O*-methylmannose with the concomitant decrease (0.8 mol) of 2,6- and 2,4-substituted mannose. Therefore, these results combined with the above results indicate that sialic acid is linked to the side chains arising from position 6 of 2,6-substituted α-mannose and position 2 of 2,4-substituted α-mannose. These results are summarized in Fig. 6*A*. It was presumed that α2→6 linked sialic acid is linked to the side chain arising from position 2 of 2,4-substituted mannose as shown in A-c (see below).

Structure of A-c—Methylation analysis of A-c indicates that A-c is composed of 6 lactosaminyl units and contains 2 mol of 2→3 linked and 1 mol of 2→6 linked sialic acid (Table I). The same analysis also indicates that about 1 mol of fucose is bound to *N*-acetylglucosamine in polylactosaminyl side chains judging from the amount of 6-*O*-methyl *N*-acetylglucosamine (Fig. 4*A*). FAB-MS of permethylated A-c afforded a series of signals of the same *m/z* values as those obtained from A-b (Fig. 5*A*). In addition, signals were present at *m/z* 1622 for NeuNAc·Fuc$_2$·Hex$_2$·HexNAc$_2^+$, 2071 for NeuNAc·Fuc$_2$·Hex$_3$·HexNAc$_3^+$, 2172 for NeuNAc·Hex$_4$·HexNAc$_4^+$, and 2346 for NeuNAc·Fuc·Hex$_4$·HexNAc$_4^+$.

This result indicates that structure 6 (and possibly 7 and 8) in Table III is present in A-c, whereas such a structure was undetectable in A-b. In order to determine the mode of sialyl linkage which forms NeuNAc→Gal→(Fuc→)GlcNAc→Gal(Fuc→)GlcNAc→, A-c was digested with α2→3 specific neuraminidase from Newcastle disease virus. This treatment removed α2→3 linked sialic acid while α2→6 linked sialic acid was not removed, as indicated by the fact that 2,4,6-tri-*O*-methylgalactose was decreased while 2,3,4-tri-*O*-methylgalactose remained in the methylation analysis (see Fig. 4*D*). As shown in Fig. 5*B*, this treatment diminished the signals for NeuNAc→Gal→(Fuc→)GlcNAc→Gal(Fuc→)GlcNAc (*m/z* 1622) upon FAB-MS analysis, indicating that sialic acid is linked by α2→3 linkage to form sialylated fucosyl or sialylated polyfucosyllactosamine. Combining these results with those obtained by various glycosidase digestions (see below), the presence of the following structure in intact A-c is indicated:

$$\begin{array}{l} \text{NeuNAc}\alpha2\rightarrow3\text{Gal}\beta1\rightarrow4\text{GlcNAc}\beta1\rightarrow3\text{Gal}\beta1\rightarrow4\text{GlcNAc}\rightarrow \\ \qquad\qquad\quad 3 \qquad\qquad\qquad\qquad 3 \\ \qquad\qquad\quad \uparrow\alpha \qquad\qquad\qquad\quad \uparrow\alpha \\ \qquad\qquad\quad 1 \qquad\qquad\qquad\qquad 1 \\ \qquad\qquad\quad \text{Fuc} \qquad\qquad\qquad\quad \text{Fuc} \end{array}$$

It is noteworthy that CML polylactosaminoglycans contain this structure since normal granulocyte polylactosaminoglycans were shown not to contain such a structure (see Ref. 10). In addition, it was noticed that fragment ions for sialylated fucosyl lactosaminyl structures (*m/z* 999 and *m/z* 1448, see Table II for their compositions) were much more prominent in CML polylactosaminoglycan A-c (Fig. 5*A*) than the corresponding counterpart of normal granulocyte polylactosaminoglycan A-2 (Fig. 3, *E* and *F*), when the spectra were taken under the same conditions.

The same analysis indicates that the side chain terminating with α2→6 linked sialic acid is shorter than those with α2→3 linked sialic acid, since NeuNAc·Hex$_3$·HexNAc$_3^+$ (*m/z* 1723) was not detected by FAB-MS after α2→3 specific neuraminidase treatment, although Fuc$_2$·Hex$_3$·HexNAc$_3^+$ (*m/z* 1710) was still detected (Fig. 5*B*).

In order to elucidate the distribution of sialic acid and fucose among the four side chains, A-c was digested with various glycosidases in the same way as A-b. First, A-c was digested with clostridial neuraminidase, β-galactosidase (jack bean), and β-*N*-acetylglucosaminidase (beef kidney) and the product was methylated. As shown in Fig. 4*B*, this treatment afforded approximately 1 mol of terminal mannose (2,3,4,6-tetra-*O*-methylmannose, peak 2), 0.3 mol of 4-substituted mannose (2,3,6-tri-*O*-methylmannose, peak 5), and 0.4 mol of 6-substituted mannose (2,3,4-tri-*O*-methylmannose, peak 7). In addition, 0.1 mol of 2-substituted mannose (3,4,6-tri-*O*-methylmannose, peak 4) was increased after this treatment. These results indicate that fucose is linked to the side chains arising from C-6 and possibly C-2 of 2,6-substituted α-mannose and from C-4 of 2,4-substituted α-mannose. The same treatment also afforded 2.2 mol of 3,6-di-*O*-methyl *N*-acetylglucosamine and 0.8 mol of 6-mono-*O*-methyl *N*-acetylglucosamine. This is consistent with the fact that A-c contains a total of 1 mol of polylactosaminyl side chain which contains fucose. For the second treatment, A-c was digested with α2→3 specific neuraminidase from Newcastle disease virus and then a mixture of β-galacosidase and β-*N*-acetylglucosaminidase and the product was permethylated. As shown in Fig. 4*C*, this product afforded 0.7 mol of 2-substituted mannose, 0.5 mol of 6-substituted mannose with the concomitant significant decrease of 2,4-substituted or 2,6-substituted mannose, but it did not produce 4-substituted mannose. This result indicates that α2→6 linked sialic acid terminates the side chain arising from position 2 of 2,4-substituted α-mannose; otherwise, the product should yield 4-substituted mannose (see also Fig. 6).

In a third treatment, A-c was extensively digested with a mixture of β-galactosidase and β-*N*-acetylglucosaminidase and the product was permethylated. The permethylated sample produced about 0.1 mol each of 2-, 4-, and 6-linked mannose with concomitant decrease of 2,6- and 2,4-linked mannose. These results combined with the above results indicate that α2→3 linked sialic acid terminates the side chains arising from position 6 and position 2 of 2,6-linked α-mannose and from position 4 of 2,4-linked α-mannose. It was not apparent which side chains are more heavily sialylated, although A-c contains only 2 mol of α2→3 linked sialic acid,

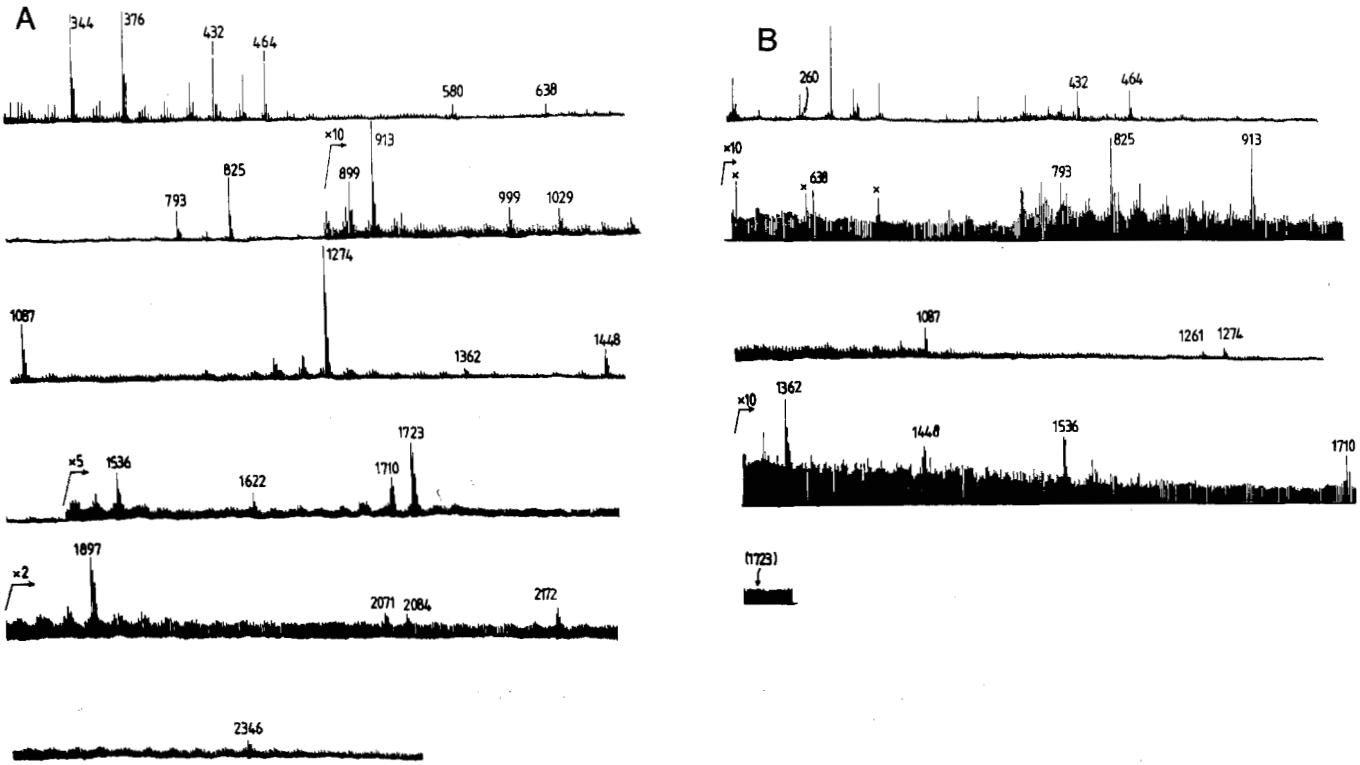

FIG. 5. **Fast atom bombardment-mass spectra of permethylated A-c saccharides.** A-c saccharides were permethylated before (*A*) and after treatment of 2→3 specific Newcastle disease virus neuraminidase (*B*). Permethylated samples were subjected to fast atom bombardment-mass spectrometry as described previously (10, 16) and the positive spectra were obtained. *Ordinate*, relative intensity; *abscissa*, mass number *m/z*. Signals are assigned as shown in Table II.

and one of the side chains should have no sialic acid (see Fig. 6*B*).

In a fourth treatment, A-c was desialyzed by mild acid treatment and digested with a mixture of α1→3/4 specific fucosidase (almond emulsin), β-galactosidase, and β-*N*-acetylglucosaminidase. The permethylation analysis of the product yielded approximately 1.6 mol of tetra-*O*-methylmannose, 0.1 mol of 3,4,6-tri-*O*-methylmannose, 0.1 mol of 2,3,6-tri-*O*-methylmannose, 0.2 mol of 2,3,4-tri-*O*-methylmannose, as well as 0.2 mol of 2,4,6-tri-*O*-methylgalactose, 0.3 mol of 6-*O*-methyl *N*-acetylglucosamine, and 1.7 mol of 3,6-di-*O*-methyl *N*-acetylglucosamine. The same digest was also applied to Bio-Gel P-4 gel filtration. As shown in Fig. 7, the majority of the resultant oligosaccharides eluted at the position of Manα1→6(Manα1→3)Manα1→4GlcNAcβ1→4GlcNAcOH and a minor component was at the position of Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAcOH. It is likely that oligosaccharides higher than the hexasaccharide were produced due to the incomplete removal of fucose, since the activity of α-L-fucosidase was low. The further identification of the hexasaccharide and pentasaccharide was carried out as described (21). These results confirmed the core structures as shown in Fig. 6.

Structure of A-d—Permethylation analysis of A-d indicates that it consists of 5 lactosaminyl units, 3 sialic acid residues (2 of 2→3 linked and 1 of 2→6 linked), and less than 1 fucosyl residue attached to *N*-acetylglucosamine in the polylactosaminyl side chains. FAB-MS of permethylated A-d afforded a similar series of signals to those observed in A-c (Fig. 3*C*) except for the following significant differences. A-d mainly yielded fragment ions derived from sialylated polylactosaminyl chains with or without fucose but only small amounts of fragment ions derived from nonsialylated fucosyl polylactosamines. In addition, polylactosaminyl side chains without fucose or sialic acid are very minor indeed and no fragment ions corresponding to $Hex_3 \cdot HexNAc_3^+$ or larger were detected. Another interesting observation was that a significant fraction of A-d must have triantennary structure since 0.5 mol of 2-substituted mannose was yielded (Table I, see Fig. 6).

Structure of A-e—Methylation analysis of A-e indicates that A-e consists of 5 lactosaminyl units and a negligible amount of fucose linked to polylactosaminyl side chains. The same analysis also indicates that the majority of A-e is fully sialylated with 3 mol of 2→3 linked sialic acid and 1 mol of 2→6 linked sialic acid (Table I). FAB-MS of permethylated A-e provided fragment ions, among which prominent signals are derived from sialylated polylactosamine without fucose (Fig. 3*D* and Table II). This result is consistent with the result obtained by methylation analysis. FAB-MS analysis also indicated that each polylactosaminyl side chain has a significant degree of heterogeneity and the number of lactosaminyl units in sialylated chains varies from 1 to 3, although the majority of them contain only the NeuNAc·Hex·HexNAc or $NeuNAc \cdot Hex_2 \cdot HexNAc_2$ lactosaminyl unit. This is because the signals (*m/z* 825 and 1274) are much higher than the signal (*m/z* 1723) for $NeuNAc \cdot Hex_3 \cdot HexNAc_3^+$ (see Fig. 3*D*).

Each α-linked mannose must have at least 1 lactosaminyl chain since no unsubstituted mannose was detected. Combining these results, the structure of A-e can be proposed as shown in Fig. 6*C*. It was assumed that 2→6 linked sialic acid resides on the side chain arising from position 2 of 2,4-substituted mannose.

Structures of CML Polylactosaminoglycans—Based on the results described above, the structures of CML sialyl polylac-

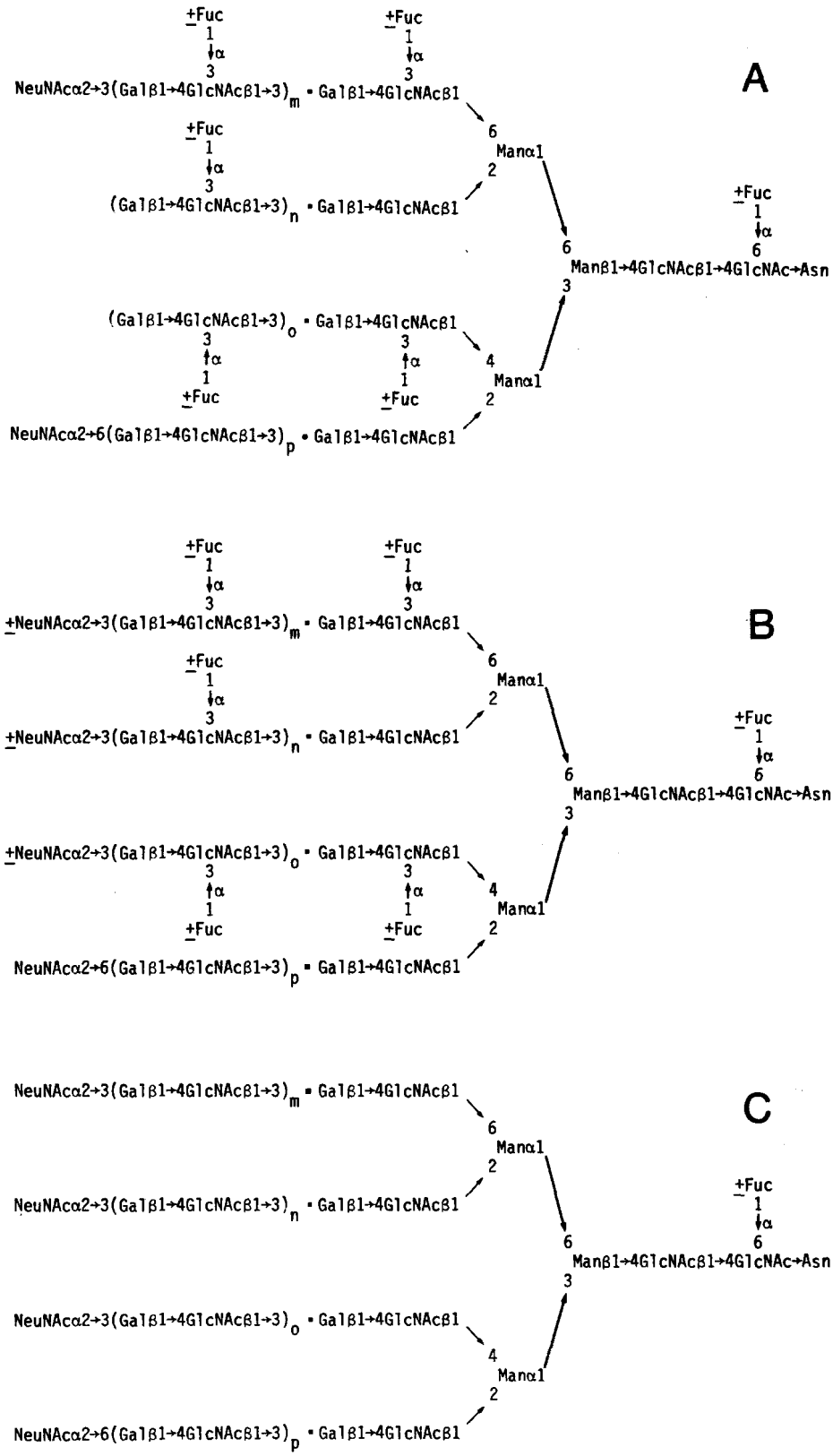

FIG. 6. **Proposed structures of sialyl polylactosaminoglycans isolated from chronic myelogenous leukemia cells.** Saccharide A-b is mainly composed of structure A and $m + n + o + p = 3$. Saccharide A-c and A-d is mainly composed of structure B and $m + n + o + p = 2$ (for A-c) and 1 (for A-d), respectively. Saccharide A-e is mainly composed of structure C where $m + n + o + p = 1$ on the average. About 50% of A-d contains triantennary structure, while less than 30% of the saccharides contain triantennary structure in A-b, A-c, and A-e. The side chain arising from C-4 of the α-mannose residue is absent in the triantennary structures.

tosaminoglycans are shown in Fig. 6. In order to determine which of the outer α-mannose residues is substituted with 2 and 4 positions, A-d was subjected to periodate oxidation followed by reduction and mild acid hydrolysis (Smith degradation) as described (21). The Smith degraded product produced 2,4,6-tri-*O*-methylmannose upon methylation analysis. This result indicates that periodate-resistant (2,4-substituted) mannose arises from position 3 of β-linked mannose as shown in Fig. 6.

CML sialyl polylactosaminoglycans can be grouped into three different saccharides: disialosyl-, trisialosyl-, and tetrasialosyl saccharides. It appears that there is greater sialylation

of the shorter polylactosaminyl side chains. Although we have shown data on one particular patient, similar results on methylation and FAB-MS were obtained on sialyl lactosaminoglycans prepared from the other CML patient. In addition, a similar QAE-Sephadex chromatographic profile of sialyl lactosaminyl saccharides was obtained from a third patient.

Polylactosaminoglycan Glycoproteins in the Normal and CML Granulocyte Plasma Membranes—In order to identify the carriers for polylactosaminoglycans, normal granulocytes and CML cells were labeled by the periodate/NaB[^{3}H]$_4$ technique, and the surface-labeled cells were subjected to endo-β-galactosidase treatment. As shown in Fig. 8, the carbohydrate moiety of the major cell surface glycoprotein(s) was significantly affected by endo-β-galactosidase, indicating the polylactosaminoglycan is carried by the major glycoprotein(s). The same experiment also revealed that the major glycoprotein in normal granulocytes exhibits a higher molecular weight ($M_r \sim 130{,}000$) than the major glycoprotein of CML granulocytes ($M_r \sim 120{,}000$).

DISCUSSION

This paper reveals the detailed structures of sialylated polylactosaminoglycans isolated from human chronic myelogenous leukemia cells. The side chains of these polylactosaminoglycans are terminated with the following structures:

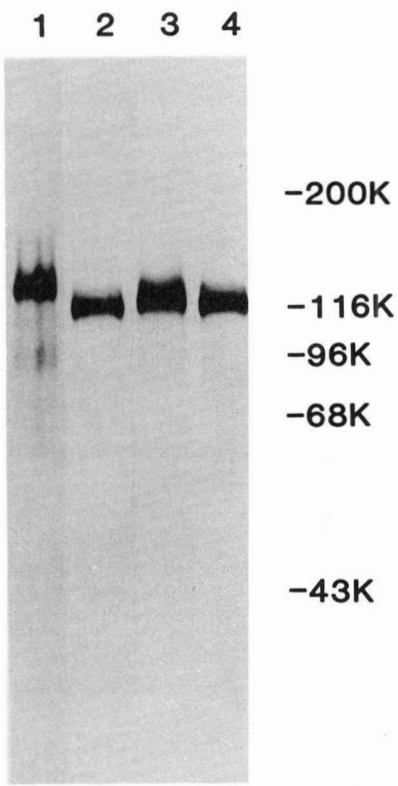

FIG. 8. **Fluorograms of sodium dodecyl sulfate-polyacrylamide gels of surface glycoproteins of normal and CML granulocytes before and after treatment with endo-β-galactosidase.** Normal granulocytes and CML cells were purified as described under "Experimental Procedures." Cells were labeled by periodate:NaB[^{3}H]$_4$, and surface-labeled cells were treated with endo-β-galactosidase as described previously (32). Cell surface glycoproteins were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by fluorography as described (32). *Lanes 1* and *2*, normal granulocytes treated with (*Lane 2*) and without endo-β-galactosidase (*Lane 1*); *Lanes 3* and *4*, CML granulocytes treated with (*Lane 4*) and without endo-β-galactosidase (*Lane 3*). The migration positions of molecular weight marker proteins are shown on the right side of the gel.

Galβ1→4GlcNAcβ1→3, Galβ1→4(Fucα1→3)GlcNAcβ1→3, NeuNAcα2→6Galβ1→4GlcNAcβ1→3,NeuNAcα2→3Galβ1→4GlcNAcβ1→3, NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→3, and NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4(Fucα1→3)GlcNAcβ1→. The average number of lactosaminyl units in each side chain is 1–3 and four or three of the polylactosaminyl side chains are bound to α-mannose residues of the core portion, Manα1→3(Manα1→6)Manβ1→4GlcNAcβ1→4(±Fucα1→6)GlcNAc→Asn.

It is significant that the CML polylactosaminoglycans contain NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4(Fucα1→3)GlcNAcβ1→3 (structure 6 in Table III). Previously we have shown that normal granulocyte polylactosaminoglycans contain a detectable amount of NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAc or NeuNAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4(Fucα1→3)GlcNAc→, but no sialylated lactosaminyl chain with 2 fucose residues was detected in normal granulocytes (10). To our knowledge, this is the first report of the presence of the NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4(Fucα1→3)GlcNAc→ structure in glycoproteins, although this structure was found in a glycolipid isolated from human colon carcinoma (22). In the present study, we have demonstrated this new structure by FAB-MS of permethylated polylactosaminoglycans before and after desialylation by α2→3 specific neuraminidase. Since polylactosaminoglycans contain more than one polylactosaminyl side chain, the analysis by FAB-MS before and after specific glycosidase treatment is essential to elucidate those structures. In addition, methylation analysis after digestions with various glycosidases, including linkage specific neuraminidase, revealed preferential distributions of different lactosaminyl chains among four possible sites attached to α-mannose. Mizoguchi *et al.* (23) recently analyzed glycopeptide structures of HL-60 promyelocytic cells. However, they did not detect such structures as sialyl Le^x or sialyl difucosyl polylactosamine, since the analysis was made only after desialylation.

The present study also revealed three interesting aspects of CML polylactosaminoglycan structures. First, considerable heterogeneity exists among different polylactosaminoglycans; whereas A-b, A-c, and A-d contain significant amounts of fucose and sialyl Le^x, NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→3 structure, A-e lacks this structure. Second, considerable differences exist among different lactosaminyl side chains. It is apparent that the side chains terminating with α2→6 linked sialic acid are shorter than those with α2→3 linked sialic acid (Fig. 5*B*). In addition, the NeuNAcα2→6Gal terminal is mainly present in the polylactosaminyl side chain arising from position 2 of 2,4-substituted α-mannose which is linked to C-3 of β-mannose, whereas the NeuNAcα2→3 terminals are present in the other polylactosaminyl side chains. These results are consistent with the previous finding of asymmetric distribution of side chains; that is, the side chain terminating with α2→6 linked sialic acid, which arises from C-3 of β-mannose, is shorter than that terminating with α2→3 linked sialic acid in erythrocyte Band 3 polylactosaminoglycans (17).

A similar selective localization of 2→6 linked sialic acid has also been reported in other glycoproteins of triantennary types (24–29). However, the sialic acid residue is linked through 2→6 to C-2 of α-mannose of the C-6 side in these triantennary glycopeptides. On the other hand, the sialic acid residue is linked through 2→3 to C-2 of α-mannose of the C-6 side in tetraantennary glycopeptides as shown in α_1-acid glycoprotein (13) and our studies on granulocyte polylactosaminoglycans. Joziasse *et al.* (30) recently showed that an α2→6 sialyl transferase adds sialic acid preferentially to the side chain of

the C-3 side only when nearly complete biantennary oligosaccharides were used as acceptors. It is likely that the addition of a fourth side chain to C-6 of α-mannose at the C-6 side affects the conformation of the side chain attached to C-2 of the same mannose.

Similarly, selective localization of fucose residues exists as shown previously in sialyl polylactosaminoglycan of normal granulocytes; fucose is preferentially linked to side chains arising from C-4 or C-6 of α-mannose. A small amount of fucosylation takes place at the side chain originating from C-2 of 2,6-linked α-mannose in contrast to the complete 2→6 sialylation of the other side chain originating from position 2 of 2,4-linked α-mannose.

The third aspect of heterogeneity concerns the individual polylactosaminyl side chains. This is particularly obvious in the degree of fucosylation. It is apparent that fucosylated chains have one or two fucoses in various positions regardless of whether they are terminated with sialic acid or not. It is also evident that each side chain must have various chain lengths. This is because the total number of maximum chain lengths detected by FAB-MS in each side chain always exceeds the total number of lactosaminyl units obtained by gas chromatography-mass spectrometry.

In addition to the presence of NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4(Fucα1→3)GlcNAc structure, CML polylactosaminoglycans have the following characteristic features. CML polylactosaminoglycans are more sialylated and contain much less neutral lactosaminoglycans than granulocyte polylactosaminoglycans (see Fig. 2). Consistent with this notion, CML polylactosaminoglycans A-c and A-d afforded a fragment ion for $NeuNAc_2 \cdot Hex_3 \cdot HexNAc_3^+$ (m/z 2084), whereas this fragment ion was not detected in normal granulocyte polylactosaminoglycans (see Figs. 3 and 5). At the same time, CML polylactosaminoglycans are shorter than granulocyte polylactosaminoglycans. Although the polylactosaminoglycans from the second patient are slightly larger than the first one, they are still smaller than normal granulocyte polylactosaminoglycans by 2–3 lactosaminyl units. Based on these results, it is tempting to speculate that CML cells contain a much higher activity of sialyl transferase so that chain elongation is terminated by sialic acid in relatively short polylactosamines. High sialylation may also be the reason that CML granulocytes have more sialylated fucosyl polylactosamine despite the fact that CML polylactosaminoglycans contain less fucose than normal ones. It is also possible that CML cells have a sialyl transferase which is different from normal mature cells or other cells which lack sialyl Le^x structure.

Chronic myelogenous leukemia usually has two phases. The relatively benign chronic phase gives way to either an accelerated period of disease or a phase known as blast crisis in the second phase. In a stage of blast crisis, patients now have a large amount of blastoid immature cells, which are distinctly different from relatively mature granulocytes observed in a chronic phase (31). It will, therefore, be interesting to see how the structure unique to CML cells is expressed during these different phases of the disease. In this connection, it is interesting to note that premyelocytic leukemia HL-60 cells express a significant amount of polylactosaminoglycans (23, 32), whereas our preliminary studies indicated that blastoid cells contain a negligible amount of polylactosaminoglycans (32). Another important point is the consideration of the possibility that cell surface carbohydrate structures of CML cells merely reflect those of immature cells in normal granulocyte development. Since immature cells of normal cell development can be obtained in a small quantity, it will be essential to examine those cells by immunochemical methods in order to see whether such immature cells express sialyl Le^x or sialyl difucosyl polylactosaminyl structure.

The present study also encourages us to look for unique structures which may be present in lymphocytes in chronic lymphocytic leukemia. In fact, Kornfeld (33) reported that lymphocytes in chronic lymphocytic leukemia express fewer phytohemagglutinin receptor sites than normal mature lymphocytes, although both lymphocytes show similar morphology and functions. It will be interesting to analyze cell surface carbohydrate structures of normal and chronic lymhocytic leukemia lymphocytes, hoping that recent advances in structural analysis might reveal unique structures in chronic lymphocytic leukemia cells.

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SUPPLEMENTAL MATERIAL
TO
STRUCTURES OF SIALYLATED FUCOSYL POLYLACTOSAMINOGLYCANS
ISOLATED FROM CHRONIC MYELOGENOUS LEUKEMIA CELLS

By

Minoru Fukuda, Brian Bothner, Priya Ramsamooj, Anne Dell,
Philip R. Tiller, Ajit Varki and John C. Klock

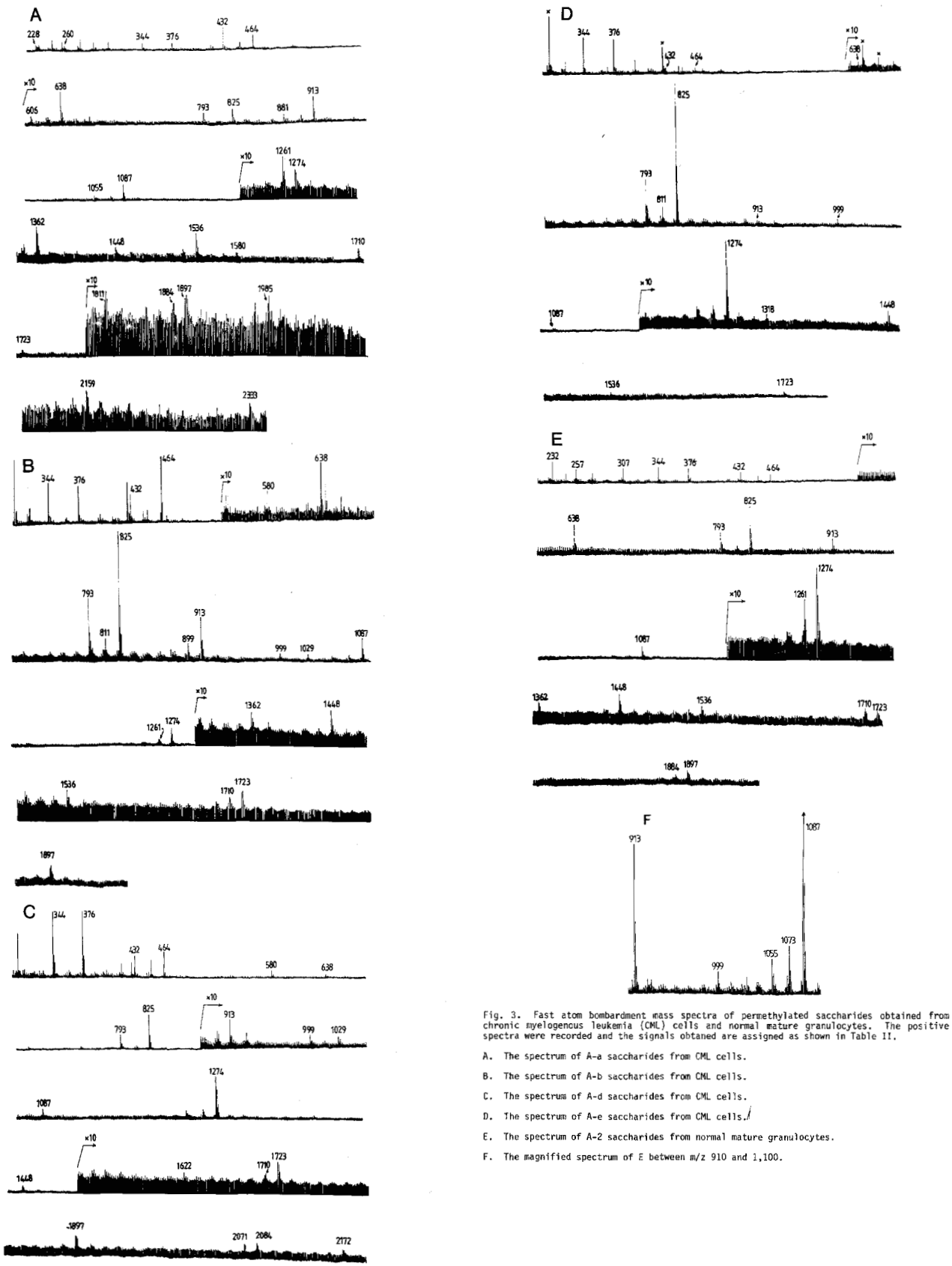


Fig. 3. Fast atom bombardment mass spectra of permethylated saccharides obtained from chronic myelogenous leukemia (CML) cells and normal mature granulocytes. The positive spectra were recorded and the signals obtaned are assigned as shown in Table II.

A. The spectrum of A-a saccharides from CML cells.

B. The spectrum of A-b saccharides from CML cells.

C. The spectrum of A-d saccharides from CML cells.

D. The spectrum of A-e saccharides from CML cells.

E. The spectrum of A-2 saccharides from normal mature granulocytes.

F. The magnified spectrum of E between m/z 910 and 1,100.

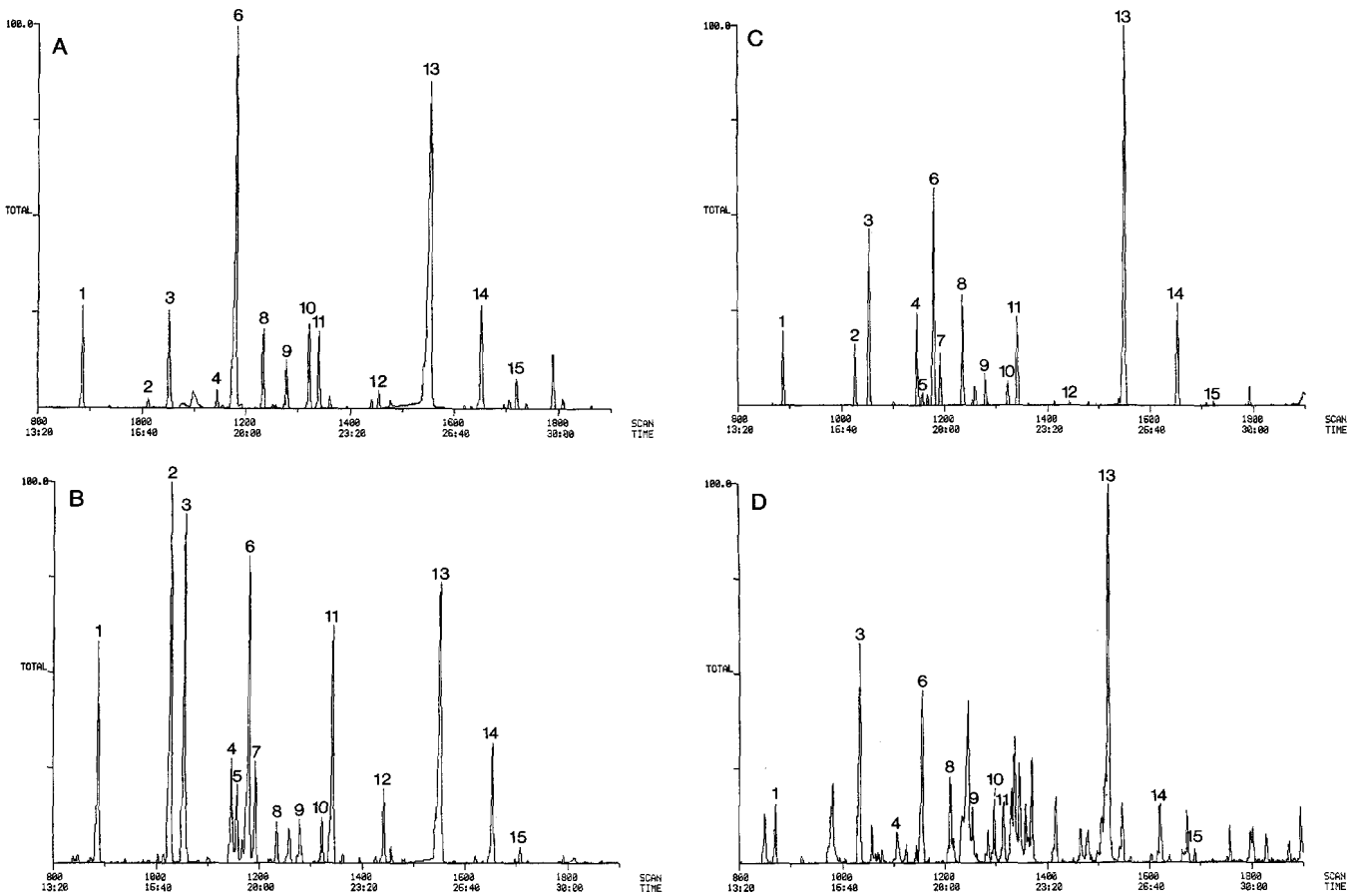


Fig. 4. Gas chromatograms of partially O-methylated sugars obtained from A-c saccharide fraction and its derivatives. The chromatogram was obtained by monitoring the total ion content.

The following peaks were identified as additol acetates of 1, 2,3,4-tri-O-methyl Fuc; 2, 2,3,4,6-tri-O-methyl Man; 3, 2,3,4,6-tetra-O-methyl Gal; 4, 3,4,6-tri-O-methyl Man; 5, 2,3,6-tri-O-methyl Man; 6, 2,4,6-tri-O-methyl Gal; 7, 2,3,4-tri-O-methyl Man; 8, 2,3,4-tri-O-methyl Gal; 9, 3,6-di-O-methyl Man; 10, 3,4-di-O-methyl Man; 11, 2,4-di-O-methyl Man; and hexosaminitol acetates of 12, 3,4,6-tri-O-methyl GlcNAcMe; 13, 3,6-di-O-methyl GlcNAcMe; 14, 6-mono-O-methyl GlcNAcMe; and 15, 3-mono-O-methyl GlcNAcMe. The other peaks were non-sugar contaminants. Ordinate, relative intensity; abscissa, retention time (13 min. 20 sec. to 31 min. 20 sec.).

A. The intact A-c saccharides.

B. The A-c saccharides after treatment with clostoidial neuraminidase and a mixture of β-galactosidase and β-N-acetylglucosaminidase.

C. The A-c saccharides after sequential treatment with α2→3 specific neuraminidase and then a mixture of β-galactosidase and β-N-acetylglucosaminidase.

D. The A-c saccharides after treatment with α2→3 specific neuraminidase.

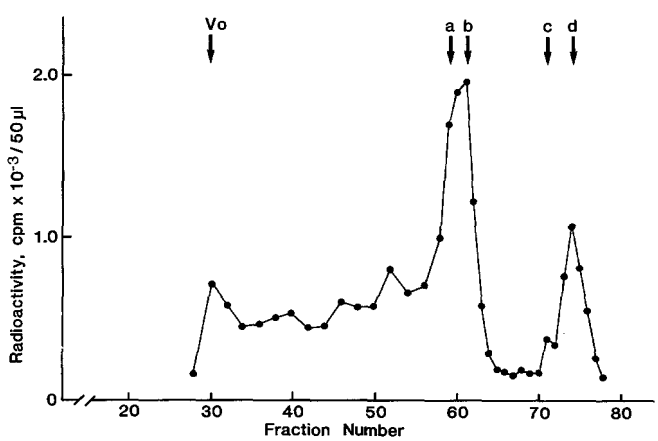
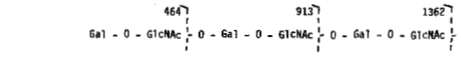
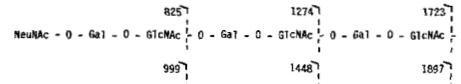
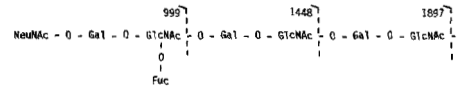
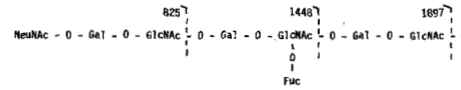
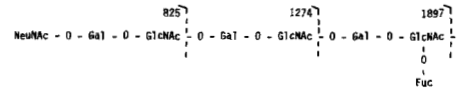
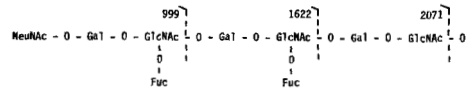
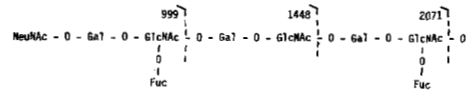
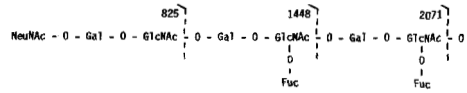
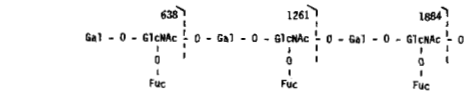
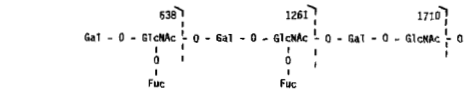
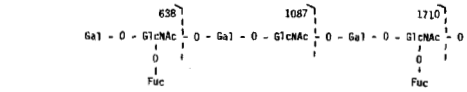
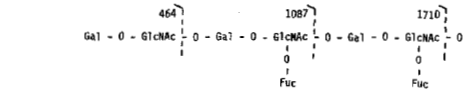
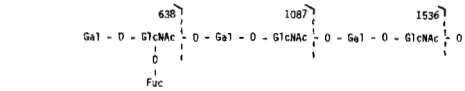
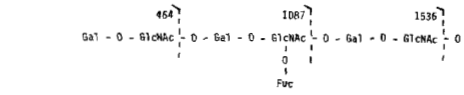


Fig. 7. Bio-Gel P-4 gel filtration of A-C saccharides after desialylation and extensive digestion with α1→3/4 fucosidase, β-galactosidase and β-N-acetylglucosaminidase.

The digest was applied to a column (1 x 160 cm) of Bio-Gel P-4 (200-400 mesh), equilibrated and eluted with water as described previously (17). Elution positions of standard oligosaccharides are: a, Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAcOH; b, Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAcOH; c, Fucα1→6GlcNAcOH; d, GlcNAcOH. Vo, void volume. It appeared that some of the oligosaccharides were digested further to produce Fucα1→6GlcNAcOH and GlcNAcOH, probably because of the contaminating glycosidases present in the α-L-fucosidase preparation.

Table III

Possible Structures of Side Chains Revealed by Fast Atom Bombardment Mass Spectrometry
Mass units were obtained for permethylated derivatives.

Number	Structure
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	
11	
12	
13	
14	
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