Structural Study of the Sugar Chains of Human Leukocyte Cell Adhesion Molecules CD11/CD18†

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Received June 8, 1990; Revised Manuscript Received September 4, 1990

ABSTRACT: Leu-CAMs (CD11/CD18) consisting of LFA-1, Mac-1, and p150/95 are leukocyte cell surface glycoproteins that are involved in various leukocyte functions. The asparagine-linked sugar chains were released as oligosaccharides from Leu-CAMs by hydrazinolysis. About 12 mol of sugar chains was released from 1 mol of Leu-CAMs. These sugar chains were converted to radioactive oligosaccharides by reduction with sodium borotritide and separated into neutral and acidic fractions by paper electrophoresis. All of the acidic oligosaccharides were converted to neutral ones by digestion with sialidase, indicating that they are sialyl derivatives. The neutral and sialidase-treated acidic oligosaccharides were fractionated by chromatography on lectin columns followed by Bio-Gel P-4 column chromatography. Structural studies of each oligosaccharide by sequential exo- and endoglycosidase digestion and by methylation analysis revealed that Leu-CAMs contain mainly high mannose type and high molecular weight complex type sugar chains. The latter sugar chains were of bi-, tri-, and tetraantennary complex types with the Galβ1→4(Fucα1→3)GlcNAcβ1→ and/or the Galβ1→3GlcNAcβ1→ groups together with the Galβ1→4GlcNAc group in their outer-chain moieties. In addition to these sugar chains, a small amount of monoantennary complex type and hybrid type sugar chains was found in Leu-CAMs. Furthermore, analysis of the asparagine-linked sugar chains released from the β-subunit of Leu-CAMs by a series of lectin chromatography showed that subunit-specific glycosylation is not observed between the α- and β-subunits of Leu-CAMs.

The leukocyte cell adhesion molecules (Leu-CAMs or CD11/CD18)[1] are members of the integrin superfamily (Hynes, 1987; Kishimoto et al., 1987a; Ruoslahti & Pierschbacher, 1987; Arnaout, 1990). They consist of the lymphocyte function-associated antigen 1 (LFA-1, CD11a/CD18), which is distributed in most leukocytes, the myeloid antigen 1 (Mac-1, CD11b/CD18), and the glycoproteins p150/95 (CD11c/CD18) expressed mainly on the surfaces of macrophages and hairy leukemia cells (Patarroyo et al., 1985a,b; Schwarting et al., 1985; Hogg et al., 1986; Miller et al., 1986). All of these three molecules are composed of α- and β-subunits in which the common β-subunit (CD18) (M_r 95000) is associated noncovalently with a unique α-subunit having a different molecular weights (M_r 180000 for CD11a, M_r 170000 for CD11b, and M_r 150000 for CD11c) (Kurzinger & Springer, 1982; Springer et al., 1987).

Extensive studies on the functions of the CD11a/CD18 molecule revealed that it is involved in numerous adhesion-dependent phenomena, including T cell mediated immune responses. Monoclonal antibodies directed against CD11a/CD18 block the proliferation of antigen-specific helper T cells and cytotoxic T cell mediated cytolysis, antibody-dependent cytotoxity by granulocytes, and natural killer cell activity (Davignon et al., 1981; Krensky et al., 1983; Miedema et al., 1984; Timonen et al., 1988, 1990). In granulocytes and monocytes, a large proportion of CD11b/CD18 and CD11c/CD18 is stored in intracellular compartments and translocated to the cell surfaces upon stimulation of inflammatory mediators, resulting in increased adhesiveness of these cells to endothelial cells (Berger et al., 1984; Todd et al., 1984; Miller et al., 1987).

The oligosaccharide moieties of cell surface glycoproteins appear to be involved in specific cell to cell interactions in many biological systems (Heifetz & Lennarz, 1979; Geltosky et al., 1980; Rutishauser, 1984). Furthermore, carbohydrate groups have been implicated in the recognition processes of lymphocytes (Hart, 1982; Cowing & Chapdelaine, 1983). It has been demonstrated that the structures of the asparagine-(Asn-) linked sugar chains on lymphocyte cell surfaces influence the recognition of Ia antigens by responding T cells in the mixed lymphocyte reaction (Pimlott & Miller, 1986; Powell et al., 1985).

Although Leu-CAMs have been studied extensively because of their involvement in various recognition and/or adhesion phenomena, little is known about their carbohydrate structures. Recently, the genes encoding CD11a/CD18, CD11b/CD18, and CD11c/CD18 molecules have been cloned, and their deduced amino acid sequences showed that there are 12 potential glycosylation sites in CD11a (Larson et al., 1989), 19 in CD11b (Stastre et al., 1986; Arnaout et al., 1988; Corbi et al., 1988), 10 in CD11c, and 6 in CD18 (Corbi et al., 1987; Kishimoto et al., 1987b; Law et al., 1987). However, these consensus sequences in Leu-CAMs are apparently not all glycosylated, as estimated by the decrease in their molecular

† This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan and by research grants from the Academy of Finland and the Sigrid Juselins Foundation.

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[1] Abbreviations: CD11/CD18, Leu-CAM, heterocomplex of leukocyte adhesion molecules consisting of specific α-chains and the common β-chain; CD11a/CD18, Leu-CAMa, LFA-1; CD11b/CD18, Leu-CAMb, Mac-1; CD11c/CD18, Leu-CAMc, p150/95; Con A, concanavalin A; AAL, *Aleuria aurantia* lectin; RCA, *Ricinus communis* agglutinin 120. Subscript OT is used to indicate NaB^3H_4-reduced oligosaccharides. All sugars mentioned in this paper have the D-configuration except for fucose, which has the L-configuration.

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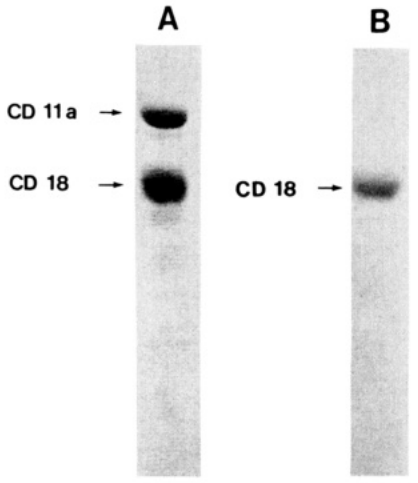


FIGURE 1: SDS–polyacrylamide gel electrophoretogram of purified CD11/CD18 and CD18: (A) pattern of the Leu-CAM complex purified by affinity chromatography; (B) pattern of isolated CD18. The gels were stained with Coomassie Brilliant Blue.

weights on SDS–polyacrylamide gel electrophoresis (SDS–PAGE) after digestion with endoglycosidases (Miller & Springer, 1987; Kantor et al., 1988). In agreement with this, only half of the potential sites in a fibronectin receptor, which is also a member of an integrin family, are reported to be glycosylated (Akiyama & Yamada, 1987). As a first step to elucidate the functional roles of the integrin, an extensive structural study of the sugar chains of Leu-CAMs has been performed in this study.

Experimental Procedures

Purification of Leu-CAMs. Packed human buffy coat cells, which are enriched in T cells, were prepared by Ficoll-Isopaque gradient centrifugation from pooled human blood supplied by Finnish Red Cross Blood Transfusion Service, Helsinki. The cells were homogenized by a Potter-Elvehjem homogenizer in 10 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl, 1% Triton X-100, and 1 mM phenylmethanesulfonyl fluoride. The cell homogenates were centrifuged at 20000*g* for 15 min, and the resultant supernatants were further spun down at 100000*g* for 45 min. Leu-CAMs were purified from the final supernatants, in which solubilized membrane-bound glycoproteins were recovered, by affinity chromatography using a column containing the monoclonal antibody mAb-59 bound to Sepharose 4B. This antibody recognizes the β-subunit common to the three Leu-CAMs. The column was washed with 20 mM glycine–NaOH buffer, pH 9.0, containing 0.1% sodium deoxycholate to remove nonspecifically adsorbed proteins. The bound materials were eluted with 50 mM diethylamine solution, pH 11.5. The eluates were neutralized, dialyzed against distilled water, and lyophilized (Kantor et al., 1988). Leu-CAMs thus prepared contained two major bands migrating with apparent molecular weights of 180000 and 95000 as determined by SDS–PAGE, which is shown in Figure 1. For separation of the β-subunit, preparative SDS–PAGE was used. The isolated protein showed a single band by SDS–PAGE (Figure 1).

Liberation of Asn-Linked Sugar Chains from Leu-CAMs. Leu-CAMs (5 mg) and the isolated β-subunit (approximately 0.1 mg), which were dried thoroughly over P_2O_5 in vacuo, were subjected to hydrazinolysis for 10 h as described previously (Takasaki et al., 1982). After N-acetylation, two-thirds of the liberated oligosaccharide mixture from Leu-CAMs was reduced with NaB^3H_4 to obtain tritium-labeled oligosaccharides for structural analysis, and the remainder was reduced with NaB^2H_4 to obtain deuterium-labeled oligosaccharides for methylation analysis. To facilitate the detection of the deuterium-labeled oligosaccharides, one-ninth of the tritium-labeled oligosaccharides was added. Due to the limited amount of the β-subunit available, the oligosaccharide mixture obtained from this glycoprotein was solely reduced with NaB^3H_4 for structural analysis.

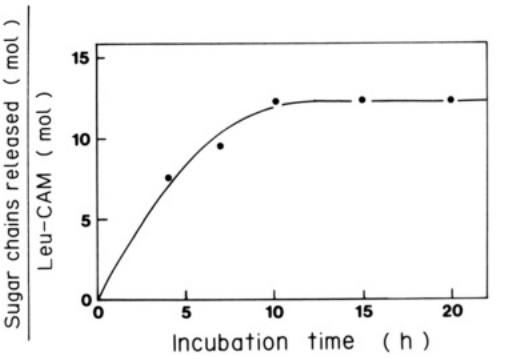


FIGURE 2: Time course of the release of oligosaccharides from Leu-CAMs by hydrazinolysis. The number of sugar chains released from Leu-CAMs was calculated on the basis of the radioactivities incorporated into the oligosaccharides and xylitol and a molecular weight for Leu-CAMs of 275000 as described in the text.

In order to determine the number of Asn-linked sugar chains included in one molecule of glycoprotein sample, a time-course study of the liberation of sugar chains by hydrazinolysis was performed with 100 μg of Leu-CAMs for each incubation time. Xylose was added as an internal standard according to the previously published method (Takasaki et al., 1982). For instance, xylose (25 nmol) was mixed with the released oligosaccharides prior to reduction with NaB^3H_4. The radioactive oligosaccharides and [^{3}H]xylitol were separated by paper chromatography using 1-butanol–ethanol–water (4:1:1 v/v) as a solvent. On the basis of the radioactivities incorporated into xylitol and the oligosaccharide fraction and the molecular weight of Leu-CAMs as 275000, the approximate number of Asn-linked sugar chains liberated from 1 mol of Leu-CAMs was calculated to be 12 mol (Figure 2). This value is consistent with those estimated from the decrease in the molecular weight of CD11a/CD18 and CD11c/CD18 on SDS–PAGE after endoglycosidase treatment (Miller & Springer, 1987; Kantor et al., 1988). The data indicated that about 5–6 Asn-linked sugar chains are included in each subunit.

Analytical Methods. The radioactive oligosaccharides were subjected to high-voltage paper electrophoresis in pyridine–acetate buffer, (3:1:387 pyridine:acetic acid:water), pH 5.4, at 70 V/cm for 90 min. Fractionation of the radioactive oligosaccharides by Bio-Gel P-4 column chromatography was performed as reported by Yamashita et al. (1982). Methylation analysis of oligosaccharides was conducted as described in a previous paper (Furukawa et al., 1989).

Affinity Chromatography of Oligosaccharides on Immobilized Lectin Columns. Lectin column chromatography using immobilized concanavalin A (Con A), *Aleuria aurantia* lectin (AAL), and *Ricinus communis* agglutinin 120 (RCA) was performed as described previously (Ogata et al., 1975; Yamashita et al., 1985; Harada et al., 1987). In brief, the mixture of radioactive oligosaccharides was applied to a Con A–Sepharose column that was equilibrated with 10 mM Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. The column was washed with 10 bed volumes of the buffer, and the bound oligosaccharides were eluted with the buffer containing 5 mM methyl α-D-glucopyranoside and then with the buffer containing 100 mM

methyl α-D-mannopyranoside. In the case of an AAL–Sepharose column that was equilibrated with 10 mM Tris-HCl buffer, pH 7.4, the column was washed with 10 bed volumes of the buffer, and the bound oligosaccharides were eluted with the buffer containing 1 mM L-fucose. In the case of an RCA–agarose column that was equilibrated with phosphate-buffered saline, pH 7.4, the column was washed with 10 bed volumes of the buffer, and the bound oligosaccharides were eluted with the buffer containing 10 mM lactose.

Chemicals, Enzymes, and Lectins. Con A–Sepharose and RCA–agarose were purchased from Pharmacia Fine Chemical Co., Tokyo, and Hohnen Oil Co., Tokyo, respectively. AAL–Sepharose was kindly supplied by Dr. N. Kochibe, Gunma University. NaB^3H_4 (600 mCi/mmol) was purchased from New England Nuclear (Boston, MA), and NaB^2H_4 was obtained from Nacalai Tesque Co., Kyoto. Streptococcal β-galactosidase and β-*N*-acetylhexosaminidase were purified from culture fluid of *Streptococcus pneumoniae* according to the method of Glasgow et al. (1977). Jack bean β-*N*-acetylhexosaminidase was purified from jack bean meal (Sigma Chemical Co., St. Louis, MO) by the method of Li and Li (1972). β-Galactosidase from *Streptococcus* 6646K, which will be referred to as 6646K β-galactosidase in this paper, and β-mannosidase from snail were kindly supplied by Seikagaku Kogyo Co., Tokyo. *Aspergillus saitoi* α-mannosidases I and II were purified according to the method of Kobata and Amano (1987). Almond emulsin α-fucosidase I was purified according to the method of Kobata (1982). Sialidase from *Arthrobacter ureafaciens* and endo-β-galactosidase from *Escherichia freundii* were purchased from Nacalai Tesque Co., Kyoto, and Seikagaku Kogyo Co., Tokyo, respectively.

Exo- and Endoglycosidase Digestion. Unless otherwise mentioned, oligosaccharides [(3–5) × 10^3 cpm] were incubated with one of the following mixtures at 37 °C for 18h: (1) *Ar. ureafaciens* sialidase (50 milliunits) in 50 μL of 0.5 M acetate buffer (pH 5.0); (2) a mixture of streptococcal β-galactosidase (1 milliunit) and β-*N*-acetylhexosaminidase (4 milliunits) in 80 μL of 0.3 M citrate phosphate buffer (pH 6.0) containing 7.5 μmol of mannose; (3) a mixture of *Streptococcus* 6646K β-galactosidase (5 milliunits) and jack bean β-*N*-acetylhexosaminidase (0.5 units) in 50 μL of 0.3 M citrate phosphate buffer (pH 5.5); (4) *Streptococcus* 6646K β-galactosidase (5 milliunits) in 50 μL of 0.3 M citrate phosphate buffer (pH 6.0); (5) almond emulsin α-fucosidase I (40 microunits) in 50 μL of 0.1 M acetate buffer (pH 5.0); (6) *As. saitoi* α-mannosidase I (0.15 μg) in 30 μL of 0.5 M acetate buffer (pH 5.0); (7) *As. saitoi* α-mannosidase II (20 milliunits) in 50 μL of 0.5 M acetate buffer (pH 5.0); (8) snail β-mannosidase (10 milliunits) in 50 μL of 0.05 M sodium citrate buffer (pH 4.0); (9) jack bean β-*N*-acetylhexosaminidase (0.5 units) in 50 μL of 0.3 M citrate phosphate buffer containing 100 μg of γ-galactonolactone; (10) *E. freundii* endo-β-galactosidase (20 milliunits) in 50 μL of 0.1 M acetate buffer (pH 6.0). One drop of toluene was added to all reaction mixtures to inhibit bacterial growth during incubation. The digestion was terminated by heating the reaction mixture in a boiling water bath for 3 min, and the product was desalted and analyzed by Bio-Gel P-4 column chromatography.

Oligosaccharides. Neu5Acα2→6Galβ1→4GlcNAcβ1→2Manα1→6(Neu5Acα2→6Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Neu5Ac$_2$·Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·GlcNAc$_{OT}$) and Neu5Acα2→6Galβ1→4GlcNAcβ1→2Manα1→6 or 3-(Galβ1→4GlcNAcβ1→2Manα1→3 or 6)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Neu5Ac·Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·GlcNAc$_{OT}$) were prepared from human transferrin (Spik et al., 1975) by hydrazinolysis, and their desialylated oligosaccharide (Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·GlcNAc$_{OT}$) was prepared by digestion with *Ar. ureafaciens* sialidase. Neu5Acα2→6Galβ1→4GlcNAcβ1→4(Neu5Acα2→6Galβ1→4GlcNacβ1→2)Manα1→3(Neu5Acα2→6Galβ1→4GlcNAcβ1→2Manα1→6)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Neu5Ac$_3$·Gal$_3$·GlcNAc$_3$·Man$_3$·GlcNAc·GlcNAc$_{OT}$) and its di- and monosialylated oligosaccharide (Neu5Ac$_2$·Gal$_3$·GlcNAc$_3$·Man$_3$·GlcNAc·GlcNAc$_{OT}$ and Neu5Ac·Gal$_3$·GlcNAc$_3$·Man$_3$·GlcNAc·GlcNAc$_{OT}$) were prepared from fetuin (Takasaki & Kobata, 1986) by hydrazinolysis. Galβ1→4GlcNAcβ1→2Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$(Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) was prepared from human platelet thrombospondin (Furukawa et al., 1989). Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Man$_3$·GlcNAc·GlcNAc$_{OT}$) and Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) were obtained from Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·GlcNAc$_{OT}$ and Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$, respectively, by digestion with a mixture of streptococcal β-galactosidase and β-*N*-acetylhexosaminidase. Manα1→6(Manα1→3)Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Man$_5$·GlcNAc·GlcNAc$_{OT}$) was obtained from bovine pancreatic ribonuclease B (Liang et al., 1980), and Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$(Man·GlcNAc·GlcNAc$_{OT}$) was prepared from its digest with jack bean α-mannosidase.

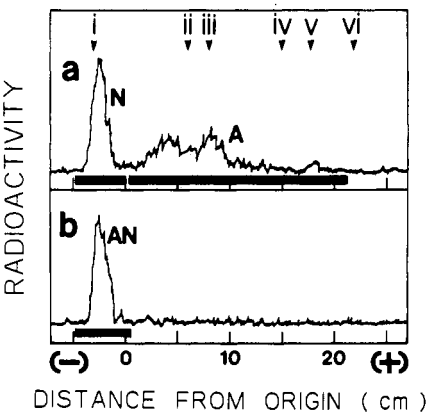


FIGURE 3: Paper electrophoretogram of oligosaccharides released from Leu-CAMs. The radioactive oligosaccharides released from Leu-CAMs by hydrazinolysis followed by reduction with NaB^3H_4 were subjected to paper electrophoresis (a). Fractions N and A were recovered from the paper by elution with water. A part of fraction A was digested with *Ar. ureafaciens* sialidase and analyzed by paper electrophoresis (b). Arrowheads at the top of the figure indicate the migrating positions of authentic oligosaccharides: (i) lactitol; (ii) Neu5Ac·Gal$_3$·GlcNAc$_3$·Man$_3$·GlcNAc·GlcNAc$_{OT}$; (iii) Neu5Ac·Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·GlcNAc$_{OT}$; (iv) Neu5Ac$_2$·Gal$_3$·GlcNAc$_3$·Man$_3$·GlcNAc·GlcNAc$_{OT}$; (v) Neu5Ac$_2$·Gal$_2$·GlcNAc$_2$·Man$_3$·GlcNAc·GlcNAc$_{OT}$; (vi) Neu5Ac$_3$·Gal$_3$·GlcNAc$_3$·Man$_3$·GlcNAc·GlcNAc$_{OT}$.

RESULTS

Fractionation of Oligosaccharides by Paper Electrophoresis. The radioactive oligosaccharides released from Leu-CAMs by hydrazinolysis were subjected to paper electrophoresis and separated into neutral (N) and acidic (A) fractions (Figure 3a). The percent molar ratios of the fractions N and A calculated from their radioactivities were 56% and 44%, respectively. When fraction A was digested exhaustively with

Table I: Percent Molar Ratio of Oligosaccharides Fractionated by Serial Immobilized Lectin Column Chromatography

		% molar ratio			
		αβ complex		β-subunit	
	fractions	N	AN	N	AN
I	Con A^-, AAL^-	5	3	8	9
II	Con A^-, AAL^+	7	26	5	23
III	Con A^+, AAL^-	1	3	2	4
IV	Con A^+, AAL^+	3	10	2	7
V	Con A^{++}, AAL^-	40	2	38	2
VI	Con A^{++}, AAL^+	0	tr[a]	0	tr

[a] Trace, less than 1% of the total oligosaccharides.

Ar. ureafaciens sialidase, all of the acidic oligosaccharides were converted to neutral ones (AN) as shown in Figure 3b. Therefore, most of the acidic nature of the oligosaccharides could be ascribed to their sialic acid residues.

Fractionation of Oligosaccharides in Fractions N and AN by Lectin Chromatography. Oligosaccharides in fractions N and AN were subjected to Con A–Sepharose column chromatography, since Leu-CAMs are reported to contain high mannose type sugar chains (Miller & Springer, 1987). The pass-through fraction (Con A^-), the bound fraction eluted with 5 mM methyl α-D-glucoside (Con A^+), and the fraction eluted with 100 mM methyl α-D-mannoside (Con A^{++}) were obtained. These fractions were applied to an AAL–Sepharose column in order to separate oligosaccharides with and without the Fucα1→6GlcNAc group in their trimannosyl core. The fraction that passed through the column is designated as AAL^- and that bound to the column and eluted with 1 mM L-fucose as AAL^+. The molar ratios of oligosaccharides in each fraction thus separated by the serial lectin column chromatography were calculated on the basis of the radioactivities of the fractions and are summarized in Table I.

Structures of Oligosaccharides in Fractions V and VI. When fraction N-V (Con A^{++}, AAL^-), which contained 40% of the total oligosaccharides, was subjected to Bio-Gel P-4 column chromatography, it was separated into five peaks (Figure 4A). After digestion with *As. saitoi* α-mannosidase I, which cleaves only the Manα1→2Man linkage, about 95% of the oligosaccharides was converted to a radioactive component, the effective size of which was the same as authentic Man_5·GlcNAc·$GlcNAc_{OT}$ (component a in Figure 4B). When component a in Figure 4B was further digested with jack bean α-mannosidase, the product was eluted at the same position as authentic Man·GlcNAc·$GlcNAc_{OT}$ (Figure 4C). That the radioactive product in Figure 4C has the structure Manβ1→GlcNAcβ1→$GlcNAc_{OT}$ was confirmed by sequential digestion with snail β-mannosidase and jack bean β-*N*-acetylhexoaminidase (data not shown). These results indicated that approximately 95% of the oligosaccharides in fraction N-V were a series of high mannose type: $(Man\alpha1\rightarrow2)_{0\sim4}$·$Man_5$·GlcNAc·$GlcNAc_{OT}$.

The rest of the oligosaccharides in fraction N-V were resistant to α-mannosidase I digestion and were separated into two radioactive components (b and c in Figure 4B). When component c was digested with streptococcal β-galactosidase, one galactose residue was released (solid line in Figure 4D). When it was further digested with streptococcal β-*N*-acetylhexosaminidase, one *N*-acetylglucosamine residue was released and eluted at the same position as authentic Man_5·GlcNAc·$GlcNAc_{OT}$ (solid line in Figure 4E). That the radioactive solid-line product in Figure 4E has the same structure as Man_5·GlcNAc·$GlcNAc_{OT}$ was further confirmed by the fact that it was converted to Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ by jack bean α-mannosidase digestion (solid line in Figure 4F).

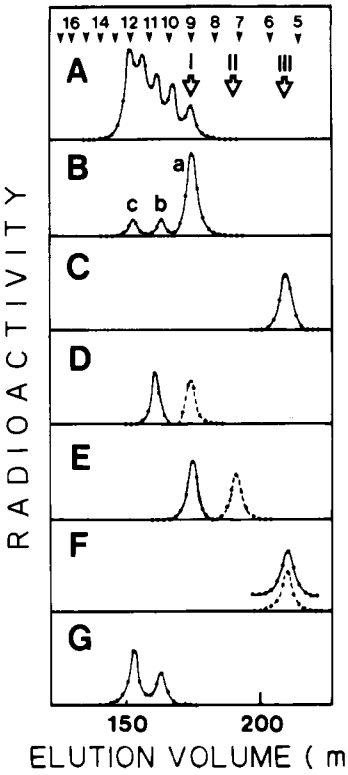
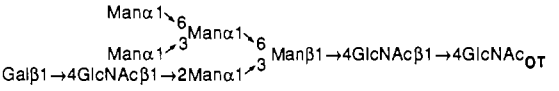


FIGURE 4: Sequential exoglycosidase digestion of fractions N-V and AN-V. Fractions and their digestion products were analyzed by Bio-Gel P-4 column chromatography: (A) fraction N-V; (B) oligosaccharides in panel A digested with *As. saitoi* α-mannosidase I; (C) component a in panel B digested with jack bean α-mannosidase; (D) components b (dotted line) and c (solid line) digested with streptococcal β-galactosidase; (E) solid and dotted lines representing the digestion products of solid- and dotted-line peaks in panel D with streptococcal β-*N*-acetylhexosaminidase, respectively; (F) solid and dotted lines representing the digestion products of solid- and dotted-line peaks in panel E with jack bean α-mannosidase, respectively; (G) fraction AN-V. Arrowheads at the top of the figure indicate the elution positions of glucose oligomers used as internal standards, and the numbers indicate the glucose units. White arrows indicate the elution positions of authentic oligosaccharides: (I) Man_9·GlcNAc·$GlcNAc_{OT}$; (II) Man_5·GlcNAc·$GlcNAc_{OT}$; (III) Man·GlcNAc·$GlcNAc_{OT}$.

These results and Con A binding specificity of the oligosaccharide indicated that component c in Figure 4B has the structure



Component b also released one galactose residue by digestion with streptococcal β-galactosidase (dotted line in Figure 4D) and one *N*-acetylglucosamine residue with streptococcal β-*N*-acetylhexosaminidase (dotted line in Figure 4E). The resulting radioactive product was eluted at the same position as authentic Man_3·GlcNAc·$GlcNAc_{OT}$. The dotted-line product in Figure 4E was converted to Man·GlcNAc·$GlcNAc_{OT}$ by digestion with jack bean α-mannosidase (dotted line in Figure 4F). That the dotted line products in Figure 4F have the structure Manβ1→GlcNAcβ1→$GlcNAc_{OT}$ was confirmed by sequential exoglycosidase digestion as described above. The results indicated that component b in Figure 4B is a monoantennary complex type sugar chain with the Galβ1→4GlcNAcβ1→2 group at its outer chain. Since component b was totally resistant to digestion with *As. saitoi* α-mannosidase II, which releases a mannose residue from the Galβ1→4GlcNAcβ1→2Manα1→6(Manα1→3)Man group but not from the Galβ1→4GlcNAcβ1→2Manα1→3-

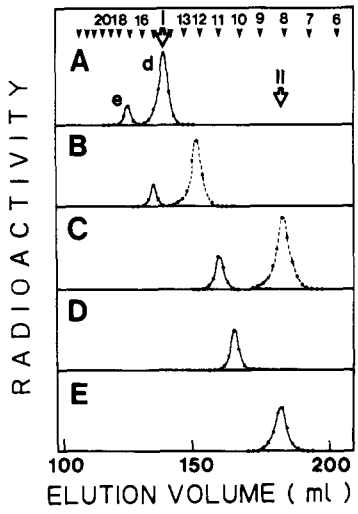


FIGURE 5: Sequential exoglycosidase digestion of fraction AN-IV. The fraction and its digestion products were analyzed by Bio-Gel P-4 column chromatography: (A) fraction AN-IV; (B) dotted and solid lines representing the digestion products of components d and e in panel A with streptococcal β-galactosidase, respectively; (C) dotted and solid lines representing the digestion products of dotted- and solid-line peaks in panel B with streptococcal β-*N*-acetylhexosaminidase; (D) the solid-line peak in panel C digested with streptococcal β-galactosidase; (E) the peak in panel D digested with streptococcal β-*N*-acetylhexosaminidase. Arrowheads at the top of the figure are the same as in Figure 4. White arrows indicate the elution positions of authentic oligosaccharides: (I) Gal_2·$GlcNAc_2$·Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$; (II) Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$.

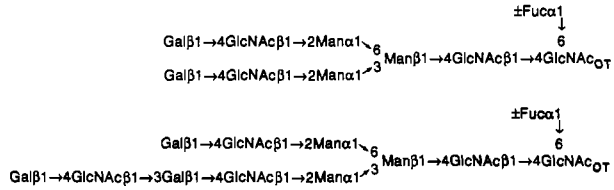
(Manα1→6)Man group (data not shown), the Galβ1→4GlcNAc group should be attached to the Manα1→3 side. In order to further confirm the structures of components b and c, oligosaccharides in fraction N-V were applied to an RCA–agarose column, which recognizes the terminal galactose residues. About 5% of the oligosaccharides in this fraction was retarded on the column and was separated into two components that were eluted on a Bio-Gel P-4 column at the same positions as those of components b and c in Figure 4B, respectively. Structural studies of these oligosaccharides by sequential glycosidase digestion gave the same series of results as described for components b and c (Figure 4D–F) (data not shown).

The oligosaccharides in fraction AN-V (Con A^{++}, AAL^-) were separated on a Bio-Gel P-4 column into two components whose elution positions were the same as those of components b and c in Figure 4B (Figure 4G). Sequential exoglycosidase digestion of these components gave the same series of results as described already for components b and c in Figure 4B, respectively (data not shown). Therefore, these two components should have the same structures as components b and c. Since fraction AN-VI (Con A^{++}, AAL^+) contained oligosaccharides amounting to less than 1% of the whole oligosaccharide mixture, their structural study was not performed.

Structures of Oligosaccharides in Fractions III and IV. Fraction AN-IV (Con A^+, AAL^+), which included 10% of the total oligosaccharides, was separated into two components with effective sizes of 14.5 and 17.5 glucose units by Bio-Gel P-4 column chromatography (components d and e in Figure 5A). When radioactive components d and e were digested with streptococcal β-galactosidase followed by streptococcal β-*N*-acetylhexosaminidase, two galactose residues (dotted line and solid line in Figure 5B, respectively) and two β-*N*-acetylglucosamine residues (dotted line and solid line in Figure 5C, respectively) were released. The effective size of the dotted-line product in Figure 5C was the same as authentic Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$. That the product has the structure Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)$GlcNAc_{OT}$ was confirmed by the method reported previously (Furukawa et al., 1989) (data not shown). These results indicated that component d has the structure Galβ1→4GlcNAcβ1→2Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)$GlcNAc_{OT}$.

When the solid-line component in Figure 5C was digested with streptococcal β-galactosidase followed by streptococcal β-*N*-acetylhexosaminidase, one galactose residue (Figure 5D) and one *N*-acetylglucosamine residue (Figure 5E) were released. The radioactive product at this stage was eluted at the same position as authentic Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$. On the basis of the specifities of the enzymes and the lectins, component e was considered to be a biantennary complex type sugar chain with one each of the Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAc and the Galβ1→4GlcNAc outer chains. In order to determine the location of the tetrasaccharide outer chain, the solid-line peak in Figure 5C was digested with *As. saitoi* α-mannosidase II, which releases a mannose residue from the Galβ1→4GlcNAcβ1→2Manα1→6(Manα1→3)Man group but not from the Galβ1→4GlcNAcβ1→2Manα1→3-(Manα1→6)Man group. Since it was totally resistant to the enzyme digestion, the tetrasaccharide outer chain in component e was considered to be attached to the Manα1→3Man arm of the trimannosyl core (data not shown). Oligosaccharides in fraction N-IV (Con A^+, AAL^+) showed the same elution patterns as those of oligosaccharides in fraction AN-IV before and after each digestion with glycosidases.

The elution profiles of oligosaccharides in fraction N-III and AN-III (Con A^+, AAL^-) were also the same as those of oligosaccharides in fraction AN-IV before and after enzyme digestions except that the elution position of each peak was smaller by one glucose unit than the respective peak from fraction AN-IV, reflecting the absence of a fucose residue linked to the trimannosyl core. Therefore, fraction III contains the nonfucosylated forms and fraction IV the fucosylated forms of the oligosaccharides



Structures of Oligosaccharides in Fractions I and II. Oligosaccharides in fraction AN-II (Con A^-, AAL^+), which amounted to 26% of the total oligosaccharides, were eluted from a Bio-Gel P-4 column as multiple peaks larger than 15 glucose units (Figure 6A). After digestion with *E. freundii* endo-β-galactosidase, almost all the oligosaccharides were converted to smaller ones, the major peak of which was eluted at 17 glucose units (Figure 6B). The oligosaccharides released by digestion with the endo-β-galactosidase and eluted at smaller than 10 glucose units were recovered and reduced with NaB^3H_4. When these oligosaccharides were subjected to Bio-Gel P-4 column chromatography, they were eluted mainly at 4.5 glucose units with an accompanying small peak at 3.5 glucose units (Figure 6B inset). By digestion with streptococcal β-galactosidase, most of the oligosaccharides eluted at 4.5 glucose units were converted to a radioactive oligosaccharide with mobility of 3.5 glucose units. The oligosaccharide eluted at 3.5 glucose units was further digested with jack bean β-*N*-acetylhexosaminidase, and the radioactive oligosaccharide was eluted at 1.5 glucose units. Analysis of the radioactive product

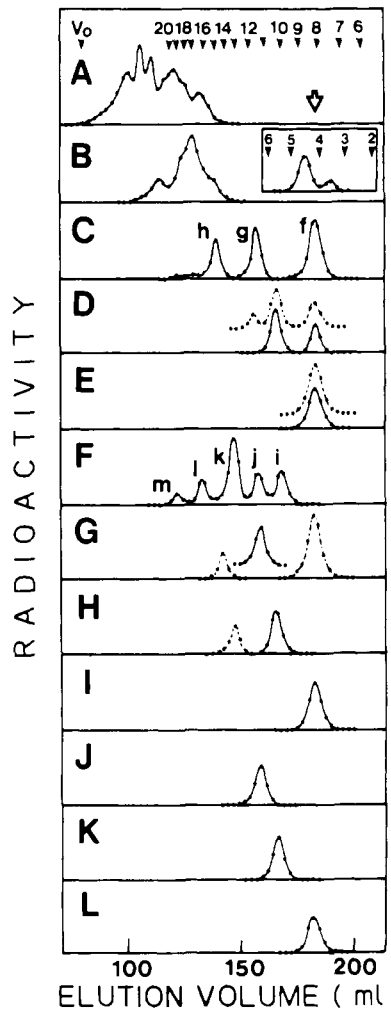


FIGURE 6: Sequential exo- and endoglycosidase digestion of fraction AN-II. The fraction and its digestion products were analyzed by Bio-Gel P-4 column chromatography: (A) fraction AN-II; (B) oligosaccharides in panel A digested with *E. freundii* endo-β-galactosidase; (inset in panel B) the oligosaccharides released by the enzyme digestion reduced with NaB^3H_4 and subjected to Bio-Gel P-4 column chromatography; (C) oligosaccharides in panel A digested with a mixture of 6646K β-galactosidase and jack bean β-*N*-acetylhexosaminidase; (D) solid and dotted lines representing the digestion products of components g and h in panel C treated by one and two cycles of enzyme digestion with almond emulsin α-fucosidase I followed by a mixture of streptococcal β-galactosidase and β-*N*-acetylhexosaminidase, respectively; (E) solid and dotted lines representing the digestion products of the solid-line peak eluted at 10.0 glucose units and the dotted-line peaks eluted at 10.0 and 12.2 glucose units in panel D with jack bean β-*N*-acetylhexosaminidase, respectively; (F) oligosaccharides in panel A treated with two cycles of enzyme digestion first with almond emulsin α-fucosidase I and subsequently with a mixture of streptococcal β-galactosidase and β-*N*-acetylhexosaminidase; (G) dot-dashed line representing each digestion product of components i, j, and k in panel F with jack bean β-*N*-acetylhexosaminidase and solid and dotted lines representing the digestion products of components l and m in panel F with jack bean β-*N*-acetylhexosaminidase, respectively; (H) solid and dotted lines representing the digestion products of solid- and dotted-line peaks in panel G with 6646K β-galactosidase, respectively; (I) solid-line peak in panel H digested with streptococcal β-*N*-acetylhexosaminidase; (J) dotted-line peak in panel H digested with streptococcal β-*N*-acetylhexosaminidase; (K) the peak in panel J digested with streptococcal β-galactosidase; (L) the peak in panel K digested with streptococcal β-*N*-acetylhexosaminidase. Component f in panel C, the solid- and dotted-line peaks eluted at 8.2 glucose units in panel D, the solid- and dotted-line peaks in panel E, the dot-dashed-line peak in panel G, the peak in panel I, and the peak in panel L were shown to have the structure Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ by sequential enzyme digestion as described in the text. Arrowheads at the top of the figure are the same as in Figure 4. The white arrow indicates the elution position of authentic oligosaccharide Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$.

that is a reducing terminal sugar revealed that it is a galactitol (data not shown). These results indicated that the major and minor oligosaccharides in the Figure 6B inset are Galβ1→4GlcNAcβ1→Gal_{OT} and GlcNAcβ1→Gal_{OT}. Therefore, the majority of oligosaccharides in the Con A^- fraction contain *N*-acetyllactosamine groups in their outer chain moieties.

When oligosaccharides in Figure 6A were digested with a mixture of 6646K β-galactosidase and jack bean β-*N*-acetylhexosaminidase, which removes all Galβ1→GlcNAcβ1→ groups, they were converted to three components (components f, g, and h in Figure 6C). When digested with almond emulsin α-fucosidase I, which cleaves the Fucα1→3GlcNAc and the Fucα1→4GlcNAc linkages, and then digested with a mixture of streptococcal β-galactosidase and β-*N*-acetylhexosaminidase, component g was converted to a mixture of two radioactive oligosaccharides with mobilities of 10.0 and 8.2 glucose units (solid line in Figure 6D). Component h was converted to a mixture of three radioactive oligosaccharides with mobilities of 12.0, 10.0, and 8.2 glucose units when treated with two cycles of the same treatment as above (dotted line in Figure 6D). By digestion with jack bean β-*N*-acetylhexosaminidase, all these oligosaccharides were converted to a single radioactive oligosaccharide eluted at the same position as authentic Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ (Figure 6E). The results indicated that component g in Figure 6C contains Galβ1→4(Fucα1→3)GlcNAcβ1→2, 4, or 6Manα1→3 or 6Manβ1→4GlcNAcβ1→4(Fucα1→6)-$GlcNAc_{OT}$ and that component h in Figure 5C contains Galβ1→4(Fucα1→3)GlcNAcβ1→2Manα1→6[Galβ1→4-(Fucα1→3)GlcNAcβ1→2Manα1→3]Manβ1→4GlcNAc-β1→4(Fucα1→6)$GlcNAc_{OT}$, Galβ1→4(Fucα1→3)-GlcNAcβ1→2[Galβ1→4(Fucα1→3)GlcNAcβ1→4 or 6]-Manα1→3 or 6(Manα1→6 or 3)Manβ1→4GlcNAcβ1→4-(Fucα1→6)$GlcNAc_{OT}$, Galβ1→4(Fucα1→3)GlcNAcβ1→-4Manα1→3[Galβ1→4(Fucα1→3)GlcNAcβ1→6Manα1→-6]Manβ1→4GlcNAcβ1→4(Fucα1→6)$GlcNAc_{OT}$, or Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4(Fucα1→3)-GlcNAcβ1→2, 4, or 6Manα1→3 or 6Manβ1→-4GlcNAcβ1→4(Fucα1→6)$GlcNAc_{OT}$. Since the oligosaccharides released by digestion with endo-β-galactosidase were not fucosylated at all, the fucose residue should be attached to the *N*-acetylglucosamine residues that form the branches of the sugar chains, and when the *N*-acetyllactosamine repeat was fucosylated, the repeat should be attached to the fucosylated branch of the sugar chains. In fraction AN-II, Galβ1→GlcNAcβ1→ and its repeating outer chains should be added to these oligosaccharides forming bi-, tri-, and tetraantennary complex type sugar chains.

When fraction AN-II was treated with two cycles of enzyme digestion with almond emulsin α-fucosidase I and then with a mixture of streptococcal β-galactosidase and β-*N*-acetylhexosaminidase, it was converted to a mixture of five oligosaccharides (i–m in Figure 6F). Components, i, j, and k were all converted to a single radioactive product with the same mobility as authentic Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ by digestion with jack bean β-*N*-acetylhexosaminidase (dot-dashed line in Figure 6G). Therefore, the differences in size of components i, j, and k were ascribed to the different numbers of *N*-acetylglucosamine residues. On the basis of the specificity of streptococcal β-*N*-acetylhexosaminidase, which cleaves the GlcNAcβ1→2Man linkage of the GlcNAcβ1→4-(GlcNAcβ1→2)Man group but not that of the GlcNAcβ1→-6(GlcNAcβ1→2)Man group, components i and j were considered to be derived from triantennary complex type oligosaccharides having the GlcNAcβ1→4(GlcNAcβ1→2)Man group and the GlcNAcβ1→6(GlcNAcβ1→2)Man group, re-

Table II: Methylation Analysis of Each Con A Fraction Obtained from a Mixture of Fractions N and AN

partially methylated sugars	molar ratio[a]			
	Con A$^-$	Con A$^-$[b]	Con A$^+$	Con A^{++}
fucitol				
2,3,4-tri-*O*-methyl-1,5-di-*O*-acetyl	1.8	0.8	0.8	tr[c]
galactitol				
2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl	3.6	3.6	2.0	tr
2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetyl	0.5	0.5	tr	
mannitol				
2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl				1.4
3,4,6-tri-*O*-methyl-1,2,5-tri-*O*-acetyl	0.3	0.3	2.0	1.2
3,6-di-*O*-methyl-1,2,4,5-tetra-*O*-acetyl	0.9	0.9		
3,4-di-*O*-methyl-1,2,5,6-tetra-*O*-acetyl	0.8	0.8		
2,4-di-*O*-methyl-1,3,5,6-tetra-*O*-acetyl	1.0	1.0	1.0	1.0
2-(*N*-methylacetamido)-2-deoxyglucitol				
6-mono-*O*-methyl-1,3,4,5-tetra-*O*-acetyl	1.0			
3,6-di-*O*-methyl-1,4,5-tri-*O*-acetyl	4.0	4.9	3.1	0.5
4,6-di-*O*-methyl-1,3,5-tri-*O*-acetyl	0.2	0.2		
1,3,5,6-tetra-*O*-methyl-4-mono-*O*-acetyl	0.2	0.2	0.2	0.5
1,3,5-tri-*O*-methyl-4,6-di-*O*-acetyl	0.8	0.8	0.8	tr

[a] Numbers in the table were calculated by taking the value of 2,4-di-*O*-methyl-1,3,5,6-tetra-*O*-acetylmannitol as 1.0. [b] After almond emulsin fucosidase I digestion. [c] Trace, less than 0.1.

spectively, and component k was considered to be derived from a tetraantennary complex type oligosaccharide.

When peaks l and m in Figure 6F were digested with jack bean β-*N*-acetylhexosaminidase, three *N*-acetylglucosamine residues were removed and the products were eluted at 11.2 and 14.0 glucose units (solid and dotted lines in Figure 6G, respectively). When the solid- and dotted-line components in Figure 6G were digested with 6646K β-galactosidase, which cleaves the Galβ1→3GlcNAc linkage as well as the Galβ1→4GlcNAc linkage, both components released one galactose residue (solid and dotted lines in Figure 6H, respectively). The resulting solid-line product was converted to the fucosylated trimannosyl core by digestion with sterptococcal β-*N*-acetylhexosaminidase (Figure 6I), and the dotted-line product was also converted to the core by sequential digestion with streptococcal β-*N*-acetylhexosaminidase (Figure 6J), streptococcal β-galactosidase (Figure 6K), and streptococcal β-*N*-acetylhexosaminidase (Figure 6L). The results indicated that component l contains the Galβ1→3GlcNAcβ1→2Manα1→3 or 6Man group and component m contains the Galβ1→3GlcNAcβ1→3Galβ1→4GlcNAcβ1→2Manα1→3 or 6Man group. Since the fucosylated components g and h in Figure 6C were converted to the afucosylated and agalactosylated derivatives (Figure 6D) by one and two cycles of enzyme digestion with almond emulsin α-fucosidase I followed by a mixture of streptococcal β-galactosidase and β-*N*-acetylhexosaminidase, respectively, the fucosylated components were shown to consist of the Galβ1→4GlcNAc group but not the Galβ1→3GlcNAc group. Therefore, the type I group in components l and m in Figure 6F were not fucosylated. Although this type I group in component m should be released upon digestion with endo-β-galactosidase, it was not detected in the oligosaccharides released by the above enzyme treatment, probably due to the lower limitation for detection.

The elution profiles of the oligosaccharides in fraction N-II (Con A$^-$, AAL$^+$) that comprised 7% of the total oligosaccharides were almost identical with those in fraction AN-II. Structural studies of oligosaccharides in this fraction by sequential glycosidase digestion also gave a series of results similar to that described for oligosaccharides in fraction AN-II (data not shown). The elution profiles of oligosaccharides in fraction I (Con A$^-$, AAL$^-$) of fractions N (5%) and AN (3%) were also similar to those of oligosaccharides in fraction II before and after enzyme digestions except that the elution position of each peak was smaller than the respective peak from fraction II by one glucose unit, reflecting the absence of a fucose residue linked to the trimannosyl core. However, in these fractions, the Galβ1→3GlcNAc group could not be detected in a tetraantennary complex type sugar chain, probably due to the lower limitation for detection.

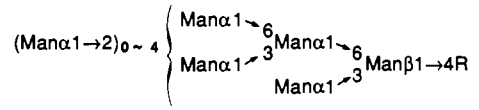
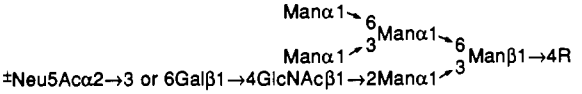
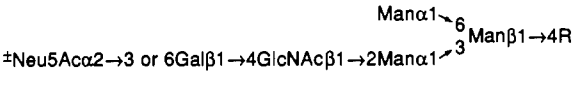
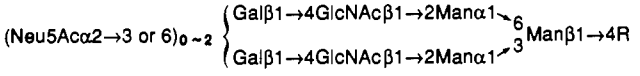
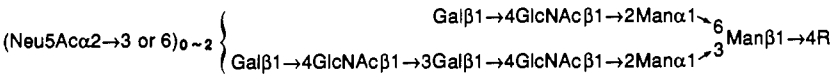
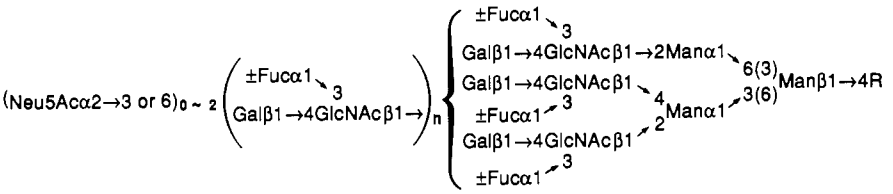
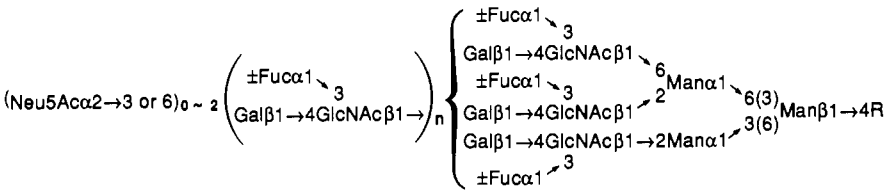
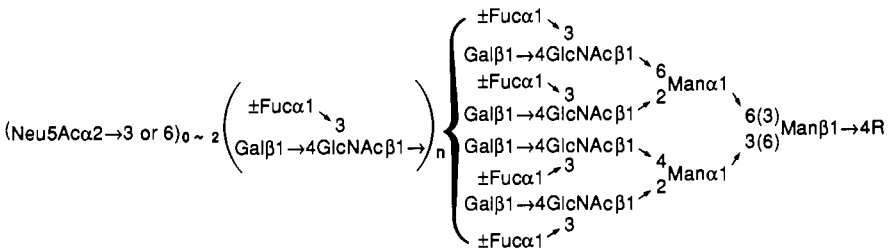
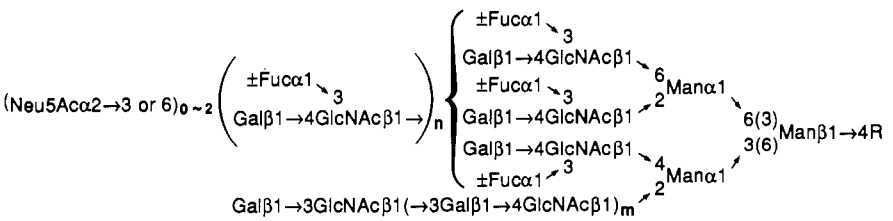
Analysis of the Carbohydrate Structures of the β-Subunit of Leu-CAMs. Radioactive oligosaccharides released from the β-subunit of Leu-CAMs were also analyzed mainly by lectin column chromatography. Due to the limited amount of the glycoprotein sample available, the number of sugar chains attached to the β-subunit was not determined. The percent molar ratio of the oligosaccharides in each fraction obtained by Con A–Sepharose and AAL–Sepharose column chromatography is summarized in Table I.

It became apparent that the percent molar ratio of oligosaccharides in each fraction is almost identical with that of the corresponding fraction obtained from αβ complexes. In addition, the elution profile of the oligosaccharides in each fraction from a Bio-Gel P-4 column was almost identical with that of the oligosaccharides in the corresponding fraction from αβ complexes (data not shown). Because the amount of sample available was limited, methylation analysis of oligosaccharides from the β-subunit could not be performed.

Methylation Analysis of Oligosaccharides. In order to confirm each glycosidic linkage of the carbohydrate structures of Leu-CAMs determined mainly by sequential exoglycosidase digestion, deuterium-labeled fractions N and AN were prepared for methylation analysis. These two fractions were combined and subjected to Con A–Sepharose column chromatography to separate the Con A$^-$, Con A$^+$, and Con A^{++} fractions. After the haptenic monosaccharides used for eluting the bound materials were removed by Bio-Gel P-4 column chromatography, each fraction was subjected to methylation analysis. In the case of the Con A$^-$ fraction, the oligosaccharides before and after almond emulsin α-fucosidase I digestion were also subjected to methylation analysis in order to confirm the structure of the fucosyl-*N*-acetyllactosamine group.

As shown in Table II, the molar ratio of each methylated sugar was calculated by taking the value of 2,4-di-*O*-methyl-1,3,5,6-tetra-*O*-acetylmannitol as 1.0. Comparison of the data for oligosaccharides in fraction Con A$^-$ before and after α-fucosidase I digestion indicated that 40% of the fucose residues is linked at the C-3 position of the *N*-acetylglucosamine residue of the *N*-acetyllactosamine groups, since 6-

Table III: Proposed Structures of the Asn-Linked Sugar Chains of Leu-CAMs

proposed structures[a]	molar ratio (%)
	38
	3
	2
	17
	1
	7
	4
	18
	9

[a] R = GlcNAcβ1→4(±Fucα1→6)GlcNAc$_{OT}$ n = 0–4; m = 0–1. The number of fucose residues attached via α1→3 to *N*-acetylglucosamine in the complex-type sugar chains is not more than two.

mono-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyglucitol, which was detected in fraction Con A$^-$, disappeared after the α-fucosidase I digestion. Since 2-(*N*-methylacetamido)-2-deoxyglucitol was detected in a trace amount as a 4,6-di-*O*-methyl derivative in oligosaccharides in fraction Con A$^-$ before and after the enzyme digestion, the presence of the Galβ1→3GlcNAc group in fraction Con A$^-$ is also confirmed by methylation analysis.

Detection of 2,4,6-tri-*O*-methylgalactitol in fraction Con A$^+$ indicated that 3% of the galactose residues of biantennary complex type sugar chains is substituted with an *N*-acetyllactosamine group. The methylation data of fraction Con A^{++} also indicated that oligosaccharides in the fraction are mainly of high mannose type. However, the presence of 2,3,4,6-tetra-*O*-methylgalactitol and 3,6-di-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyglucitol in the same fraction supported the

occurrence of monoantennary complex type and hybrid type sugar chains as determined by sequential exoglycosidase digestion (Figure 4).

The proposed oligosaccharide structures of human Leu-CAMs, as determined by sequential exoglycosidase digestion and methylation analysis, are shown in Table III.

Discussion

Since human buffy coat cells are enriched in T cells, the predominant class of molecules present in the Leu-CAMs should be CD11a/CD18. This estimation is also supported by the fact that the α-subunit band corresponding to M_r 180 000 was detected by SDS–PAGE analysis of the sample (Figure 1). The CD11a/CD18 molecule is expressed on the surface of virtually all leukocytes and is involved in various functions such as cell adhesions. Monoclonal antibodies against CD11a/CD18 inhibited proliferation of antigen-specific helper T cells and cytolytic functions mediated by cytotoxic T cells and by natural killer cells (Davignon et al., 1981; Krensky et al., 1983; Miedema et al., 1984). Adhesion of leukocytes to endothelial cells, fibroblasts, and keratinocytes at the sites of inflammation is also mediated by the CD11a/CD18 molecule (Dustin & Springer, 1988; Patarroyo et al., 1990). The oligosaccharide moieties of leukocyte cell surface glycoproteins have been implicated in the recognition processes of lymphocytes, including the recognition of Ia molecules by responding T cells in the mixed lymphocyte reaction. Also, homing of lymphocytes to various organs may be due to sugar-binding proteins (Gallatin et al., 1983; Stoolman & Rosen, 1983; Lasky et al., 1989). Little is known, however, about their carbohydrate structures involved in these phenomena.

By use of established methods for structural analysis of oligosaccharides released by hydrazinolysis, structures of the Asn-linked sugar chains of Leu-CAMs have been determined. On the basis of the results presented here, Leu-CAMs were shown to have 12 Asn-linked sugar chains per molecule and their structures are proposed as shown in Table III. The major Asn-linked sugar chains of Leu-CAMs were of high mannose type and bi-, tri-, and tetraantennary complex types. Although the outer chains of biantennary complex type sugar chains contain only the Galβ1→4GlcNAcβ1→ group, those of tri- and tetraantennary sugar chains are enriched with the Galβ1→4(Fucα1→3)GlcNAc group. Small amounts of the Galβ1→3GlcNAcβ1→ and the Galβ1→3GlcNAcβ1→3Galβ1→4GlcNAc groups are also found in the outer-chain moieties of the tetraantennary sugar chains. These type I structures were not fucosylated as in the case of the Galβ1→4GlcNAc outer chain from which the type I structure extends. This structural characteristic is quite unique to Leu-CAMs. The fucosylation of the *N*-acetyllactosamine group of tri- and tetraantennary complex type sugar chains is also characteristic to these molecules. Recently, fucosyl-*N*-acetyllactosamine, which is expressed on cell surface glycoconjugates in a stage-specific manner during the development of mouse embryos (Gooi et al., 1985), has been shown to interact homotypically, thus inducing compaction, a tight cellular adhesion (Eggens et al., 1989). Therefore, the fucosyl-*N*-acetyllactosamine residues expressed on Leu-CAMs might be important for initial interaction with ligands such as ICAM-1 and ICAM-2 (Rothlein et al., 1986; Patarroyo et al., 1987; Staunton et al., 1989). The presence of fucosyl-*N*-acetyllactosamine on CD11a/CD18 has previously been demonstrated by using a monoclonal antibody (Spitalnik et al., 1989).

In addition, Leu-CAMs contain a small amount of hybrid type and monoantennary complex type sugar chains. These sugar chains are not widely distributed in mammalian glycoproteins. The latter structure has been found limitedly in chorionic gonadotropin (Endo et al., 1979), urinary ribonuclease (Hitoi et al., 1987), placental β-glucocerebrosidase (Takasaki et al., 1984), and platelet thrombospondin (Furukawa et al., 1989) of human origin.

Comparative study of the oligosaccharides released from $\alpha\beta$ complexes and the β-subunit by lectin chromatography showed that similar series of oligosaccharides were distributed evenly in both α- and β-subunits. The results are consistent with previous reports that the sugar chains of Leu-CAMs were susceptible to endo-β-*N*-acetylglucosaminidases H and F (endo F) and that the reduction of molecular sizes of α- and β-subunits determined by SDS–PAGE after treatment with endo F suggests the presence of 5–6 sugar chains in each subunit (Miller & Springer, 1987; Kantor et al., 1988). The presence of sulfate groups in the sugar chains of the CD11a/CD18 molecule, which was detected by metabolic labeling of mouse T lymphoma EL-4 cells with $^{35}SO_4^{2-}$ (Dahms & Hart, 1985), was not confirmed by the present study. Therefore, the amount of sulfated sugar chains in the Leu-CAMs from peripheral T cells might be very small, even if they occur in CD11a/CD18. Whether hydrazinolysis does release sulfate groups from sugar chains or not was investigated by treatment of radioactive keratan sulfate fragment GlcNAc(6-SO_4)β1→3Gal_{OT} with hydrazine at 100 °C for 10 h. Since the electrophoretic mobility of the sulfated disaccharide was not affected by the treatment, hydrazinolysis does not release sulfate groups from sugar chains. In addition, the carbohydrate structures of the sulfated sugar chains were determined by use of the hydrazine-released oligosaccharides (Yamashita et al., 1983; Edge & Spiro, 1984).

Burkitt lymphoma cells lack or have a low level of the CD11a/CD18 molecule and are not metastatic (Patarroyo et al., 1988; Roossien et al., 1989). Metastatic lymphoma cells were shown to invade monolayers of lymphocytes and fibroblasts. The invasion was totally blocked by anti-CD11a/CD18 antibody. In addition, mutant lymphoma cells, which are deficient in the expression of the CD11a/CD18 molecule on cell surfaces due to the impaired synthesis of either the α- or β-subunit precursor, failed considerably to invade the cell layers. These results strongly suggested the involvement of CD11a/CD18 in the efficient metastasis of certain lymphoma cells. Since the carbohydrate structure of metastasis-associated glycoprotein was determined by the analysis of lectin mutant cell lines, which have lost the metastatic potential but not the tumorigenicity (Stanley, 1984; Dennis et al., 1987), it would be of interest to determine the carbohydrate structures of the CD11a/CD18 molecule isolated from the invasive lymphoma cells.

Acknowledgments

We express our gratitude to Miss Yumiko Kimizuka and Ms. Tamae Takahashi for their excellent secretarial assistance.

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Intron–Exon Organization of the Human Gene Coding for the Lipoprotein-Associated Coagulation Inhibitor: The Factor Xa Dependent Inhibitor of the Extrinsic Pathway of Coagulation†

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Received July 25, 1990; Revised Manuscript Received October 29, 1990

ABSTRACT: Blood coagulation can be initiated when factor VII(a) binds to its cofactor tissue factor. This factor VIIa/tissue factor complex proteolytically activates factors IX and X, which eventually leads to the formation of a fibrin clot. Plasma contains a lipoprotein-associated coagulation inhibitor (LACI) which inhibits factor Xa directly and, in a Xa-dependent manner, also inhibits the factor VIIa/tissue factor complex. Here we report the cloning of the human LACI gene and the elucidation of its intron–exon organization. The LACI gene, which spans about 70 kb, consists of nine exons separated by eight introns. As has been found for other Kunitz-type protease inhibitors, the domain structure of human LACI is reflected in the intron–exon organization of the gene. The 5′ terminus of the LACI mRNA has been determined by primer extension and S1 nuclease mapping. The putative promoter was examined and found to contain two consensus sequences for AP-1 binding and one for NF-1 binding, but no TATA consensus promoter element.

Blood coagulation is a host defense system that is involved in maintaining the integrity of the vascular circulatory system after blood vessel injury. The coagulation system consists of several plasma glycoproteins, including factor VII, factor IX, and factor X, which are zymogens of serine proteases. They are converted from an inactive form to an active enzyme by limited proteolysis. Coagulation is initiated when factor VII(a) binds to the transmembrane glycoprotein tissue factor [reviewed in Furie and Furie (1988) and Bach (1988)]. This factor VIIa/tissue factor complex proteolytically activates factors IX and X, triggering a cascade of events which eventually leads to the formation of insoluble fibrin.

Early studies regarding the regulation of the tissue factor initiated coagulation showed that incubation of tissue factor (in crude extracts) with serum inhibited its procoagulant activity (Schneider, 1947; Thomas, 1947; Lanchantin & Ware, 1953). Hjort (1957) confirmed and extended these observations and concluded that serum contains a component that inactivates the factor VIIa/tissue factor complex. Recent studies (Sanders et al., 1985; Hubbard & Jennings, 1987; Broze & Miletich, 1987a) have shown that this inhibitor, that is variously called the tissue factor inhibitor (Broze & Miletich, 1987b), the extrinsic pathway inhibitor (EPI) (Rao & Rapoport, 1987), or the lipoprotein-associated coagulation inhibitor (LACI) (Broze et al., 1988), binds to factor Xa and inhibits the formation of factors IXa and Xa by the factor VIIa/tissue factor complex in a factor Xa dependent manner. The inhibition of the factor VIIa/tissue factor complex is thought to involve the formation of an LACI/factor Xa complex which binds noncovalently to the factor VIIa/tissue factor complex, producing a quaternary factor VIIa/tissue factor/factor Xa/LACI complex (Broze et al., 1988).

The complete cDNA of LACI has recently been cloned (Girard et al., 1989a; Wun et al., 1988). The predicted amino acid sequence reveals that LACI contains several discernible domains, including a negatively charged NH_2 terminus and a positively charged COOH terminus. The center portion of the 32-kilodalton protein consists of three tandemly arranged homologous domains which have the typical cysteine backbone of the Kunitz-type inhibitor domain, a structure very common in basic protease inhibitors (Wun et al., 1988). Mutation experiments indicated that the first Kunitz domain binds to the active site of the factor VIIa/tissue factor complex (Girard et al., 1989b, 1990) and that the second Kunitz domain binds to the active site of factor Xa. The function of the third Kunitz domain is not known (Girard et al., 1989b).

The tissue distribution of LACI expression has not been studied in detail, but LACI transcripts have been identified in liver-derived cell lines (Wun et al., 1988) and platelets (Novotny et al., 1988). Furthermore, LACI activity has been demonstrated in conditioned media from endothelial cell cultures (Warn-Cramer et al., 1989) and in the media from

† The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J05312.

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0006-2960/91/0430-1571$02.50/0