Structural Study of the Sugar Chains of Human Leukocyte Common Antigen CD45†

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ABSTRACT: The leukocyte cell surface glycoprotein CD45 is a protein tyrosine phosphatase and is involved in signal transduction mediated by the T cell antigen receptor. The asparagine-linked sugar chains were released as oligosaccharides from purified CD45 by hydrazinolysis. Approximately 6 mol of sugar chains was released from 1 mol of CD45. These sugar chains were converted to radioactive oligosaccharides by reduction with NaB^3H_4 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. Binding of the sialylated oligosaccharides to an SNA-agarose column as well as methylation analysis revealed that the oligosaccharides have only α-2,6-linked sialic acid residues. The neutral and sialidase-treated acidic oligosaccharides were fractionated by serial lectin column chromatography 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 CD45 contains mainly bi-, tri-, and tetraantennary complex-type sugar chains. About 46% of the tetraantennary complex-type sugar chains had the poly(*N*-acetyllactosamine) groups and 18% of the 2,4-branched triantennary complex-type sugar chains had the fucosyl *N*-acetyllactosamine group. A portion of the bi- and 2,4-branched triantennary complex-type sugar chains were bisected. In addition to these sugar chains, a small amount of high mannose-type and hybrid-type sugar chains were detected.

CD45 is a leukocyte common antigen previously termed as L-CA or T200 glycoprotein expressed on all hematopoietic cells except for mature erythrocytes. Alternative splicing of the three exons can generate eight isotypes of CD45 with different extracellular domains [reviewed by Trowbridge (1991)]. These isotypes consist of glycoproteins with molecular weights from 180K to 220K and are present in a cell-type specific manner on different classes of leukocytes. CD45 has been shown to be a protein tyrosine phosphatase (Charbonneau et al., 1988; Tonks et al., 1988; Tan et al., 1993) which can activate the tyrosine protein kinases $p56^{lck}$ and $p59^{fyn}$ by dephosphorylating tyrosine-505 and tyrosine-528, respectively (Ostergaard et al., 1989; Mustelin et al., 1989, 1992). Since $p56^{lck}$ is associated noncovalently with CD4 and CD8 (Veillette et al., 1988; Turner et al., 1990), the former of which is engaged in T cell antigen receptor complex (Ledbetter et al., 1988), and since $p59^{fyn}$ is physically associated with the T cell antigen receptor (Samelson et al., 1990; Cooke et al., 1991), CD45 is considered to play a critical role in signal transduction mediated by T cell antigen receptor (Pingel & Thomas, 1989; Koretzky et al., 1990, 1991; Justement et al., 1991; Weaver et al., 1991).

Recently, the B cell surface adhesion molecule CD22 has been shown to bind CD45, thus mediating interaction of B cells with T cells (Stamenkovic et al., 1991; Aruffo et al., 1992). Accumulating evidence implies that CD22 binds to the carbohydrate residues of CD45 (Stamenkovic et al., 1991; Aruffo et al., 1992). Although CD22 does not have a C-type lectin domain, it has been shown to be a sialic acid binding lectin (Sgroi et al., 1993), recognizing asparagine-linked sugar chains containing α-2,6-linked sialic acid residues (Powell et al., 1993). Because CD45 is one of the heavily glycosylated proteins in leukocytes (Gahmberg et al., 1976; Jackson & Barclay, 1989), it is possible that the sugar chains of CD45 are involved in CD22-mediated cell adhesion. Therefore, an extensive structural study of the sugar chains of human CD45 has now been performed. The data indicated that CD45 contains mainly tetra- and triantennary complex-type sugar chains with the poly(*N*-acetyllactosamine) groups and exclusively α-2,6-linked sialic acid residues. This suggests that the sialylated oligosaccharides of CD45 are involved in binding to CD22.

EXPERIMENTAL PROCEDURES

Purification of CD45. Packed human buffy coat cells, which are enriched in T cells, were prepared by Ficoll-Isopaque gradient centrifugation from pooled human blood supplied by Finish 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 centrifuged at 100000*g* for 45 min. CD45 was purified from the final supernatants, in which solubilized membrane-bound glycoproteins were recovered, by affinity chromatography using a column containing the monoclonal antibody MEM-28 bound to Sepharose 4B. The MEM-28 antibody previously characterized (Cobbold et al., 1986) was kindly provided by Dr. V. Horejsi in the Czechoslovakian Academy of Sciences. The

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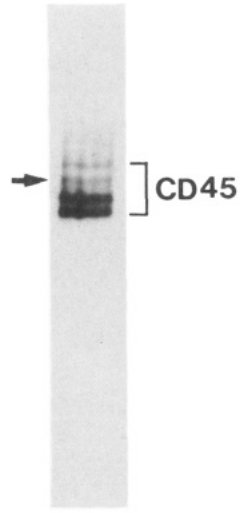


FIGURE 1: SDS–polyacrylamide gel electrophoretogram of purified CD45. The gel was stained with Coomassie Brilliant Blue. An arrow indicates the position where a 200K molecular weight marker, myosin, migrated.

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. CD45 thus prepared contained four bands migrating with apparent molecular weights of 180K, 190K, 205K, and 220K as determined by SDS–PAGE,[1] which is shown in Figure 1. Western blot analysis with use of the monoclonal antibody MEM-28 revealed that the antibody bound to all four bands (data not shown).

Liberation of Asn-Linked Sugar Chains from CD45. CD45 (4 mg), which was dried thoroughly over P_2O_5 *in vacuo*, was subjected to hydrazinolysis for 10 h as described previously (Takasaki et al., 1982). After *N*-acetylation, two-thirds of the liberated oligosaccharide mixture from CD45 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-tenth of the tritium-labeled oligosaccharides was added. Since each oligosaccharide contains only one reducing terminal, each oligosaccharide has the same specific radioactivity after reduction with NaB^3H_4.

In order to determine the number of asparagine-linked sugar chains included in 1 mol of glycoprotein sample, a time-course study of the liberation of sugar chains by hydrazinolysis was performed with 50 μg of CD45 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 (53 000 cpm/nmol) and the oligosaccharide fraction (for example, 79 000 cpm obtained at incubation time of 10 h) and the molecular weight of CD45, the approximate number of asparagine-linked sugar chains liberated from 1 mol of CD45 can be calculated. Because the present CD45 preparation contained four isotypes with different molecular weights, as shown in Figure 1, and the molar ratio of them in the CD45 sample was not known, it was impossible to calculate the exact number of the sugar chains attached to CD45. However, by taking an average molecular weight of CD45 as 200K (50 μg is equivalent to 0.25 nmol), approximately 6 mol of asparagine-linked sugar chains is bound to 1 mol of CD45 was obtained (data not shown).

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 neutral oligosaccharides by Bio-Gel P-4 column chromatography was performed as reported by Yamashita et al. (1982). Methylation analysis and periodate oxidation of oligosaccharides were conducted as described in previous papers (Furukawa et al., 1989; Yamashita et al., 1981).

Affinity Chromatography of Oligosaccharides on Immobilized Lectin Columns. Lectin column chromatography using immobilized E_4-PHA-agarose, Con A-Sepharose, DSA-Sepharose, AAL-Sepharose and SNA-agarose was performed as described previously (Yamashita et al., 1983, 1985, 1987; Ogata et al., 1975; Shibuya et al., 1987). In brief, samples on an E_4-PHA-agarose column were eluted with 10 mM Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl and 1 mM each of $MgCl_2$, $MnCl_2$, and $CaCl_2$. Samples on a Con A-Sepharose column were eluted with 10 mM Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl and 1 mM each of $MgCl_2$, $MnCl_2$, and $CaCl_2$, followed by the same buffer containing 5 mM α-methyl D-glucopyranoside and with the same buffer containing 100 mM α-methyl D-mannopyranoside. Samples on a DSA-Sepharose column were eluted with 10 mM Tris-HCl buffer, pH 7.4, followed by the same buffer containing 1% *N*-acetylglucosamine oligomers. Samples on an AAL-Sepharose column were eluted with 10 mM Tris-HCl buffer, pH 7.4, followed by the same buffer containing 1 mM L-fucose. Samples on an SNA-agarose column were eluted with phosphate-buffered saline, pH 7.4, followed by the same buffer containing 100 mM lactose.

Chemicals, Enzymes, and Lectins. The following lectins were purchased: E_4-PHA-agarose from Hohnen Oil Co., Tokyo; Con A-Sepharose from Pharmacia Fine Chemical Co., Tokyo; DSA-Sepharose from Wako Pure Chemical Co., Tokyo; AAL-Sepharose from Nichirei Co., Tokyo; SNA-agarose from E. Y. Labs, San Mateo, CA. NaB^3H_4 (470 mCi/mmol) was purchased from New England Nuclear, Boston, MA. NaB^2H_4 and sialidase from *Arthrobacter ureafaciens* were obtained from Nacalai Tesque Co., Kyoto. Diplococcal β-galactosidase and β-*N*-acetylhexosaminidase were purified from culture fluid of *Diplococcus pneumoniae* according to the method of Glasgow et al. (1977). Jack bean β-*N*-acetylhexosaminidase and α-mannosidase were purified from jack bean meal (Sigma Chemical Co., St. Louis, MO) by the method of Li and Li (1972). *Escherichia freundii* endo-β-galactosidase (Fukuda & Matsumura, 1976) and snail β-mannosidase (Sugahara et al., 1972) were supplied from Seikagaku Kogyo Co., Tokyo. Bovine epididymal α-L-fucosidase was obtained from Sigma Chemical Co., St. Louis, MO. *Aspergillus saitoi* α-mannosidase I was purified according to the method of Kobata and Amano (1987).

[1] Abbreviations: AAL, *Aleuria aurantia* lectin; Con A, concanavalin A; DSA, *Datura stramonium* agglutinin; E_4-PHA, erythroagglutinating *Paseorus vulgaris* agglutinin; SNA, *Sambucus nigra* agglutinin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis. A subscript OT is used to indicate an NaB^3H_4-reduced oligosaccharide. All sugars mentioned in this paper have the D-configuration except for fucose which has the L-configuration.

Almond emulsin α-L-fucosidase I was purified by the method of Kobata (1982).

Exo- and Endoglycosidase Digestion. Unless otherwise mentioned, oligosaccharides (1–5 × 10^4 cpm) were incubated with one of the following mixtures at 37 °C for 18 h: (1) *A. ureafaciens* sialidase (50 milliunits) in 50 μL of 0.5 M acetate buffer, pH 5.0; (2) diplococcal β-galactosidase (2 milliunits) in 50 μL of 0.3 M citrate phosphate buffer, pH 6.0; (3) diplococcal β-*N*-acetylhexosaminidase (4 milliunits) in 50 μL of 0.3 M citrate phosphate buffer, pH 6.0; (4) jack bean β-*N*-acetylhexosaminidase (0.5 unit) in 50 μL of 0.3 M citrate phosphate buffer, pH 5.0, containing 100 μg of γ-galactonolactone; (5) *A. saitoi* α-mannosidase I (0.15 μg) in 30 μL of 0.5 M acetate buffer, pH 5.0; (6) jack bean α-mannosidase (0.8 unit) in 50 μL of 0.5 M acetate buffer, pH 4.5, containing 1 mM $ZnCl_2$; (7) snail β-mannosidase (10 milliunits) in 50 μL of 0.05 M sodium citrate buffer, pH 4.0; (8) bovine epididymal α-fucosidase (35 milliunits) in 50 μL of 0.2 M sodium citrate buffer, pH 6.0; (9) *E. freundii* endo-β-galactosidase (20 milliunits) in 50 μL of 0.3 M citrate phosphate buffer, pH 6.0; (10) almond emulsin α-fucosidase I (40 milliunits) in 50 μL of 0.1 M acetate buffer, pH 5.0. One drop of toluene was added to all reaction mixtures to inhibit bacterial growth during incubation. Digestions were 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$_3$GlcNAcGlcNAc$_{OT}$) and Neu5Acα2→6Galβ1→4GlcNAcβ1→2Manα1→6 or 3(Galβ1→4GlcNAcβ1→2Manα1→3 or 6)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Neu5AcGal$_2$GlcNAc$_2$Man$_3$GlcNAcGlcNAc$_{OT}$) were prepared from human transferrin (Spik et al., 1975) by hydrazinolysis, and their desialylated oligosaccharide (Gal$_2$GlcNAc$_2$Man$_3$GlcNAcGlcNAc$_{OT}$) was prepared by digestion with *A. ureafaciens* sialidase. Galβ1→4GlcNAcβ1→2Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (Gal$_2$GlcNAc$_2$Man$_3$GlcNAcFucGlcNAc$_{OT}$) was prepared from human platelet thrombospondin (Furukawa et al., 1989). Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Man$_3$GlcNAcGlcNAc$_{OT}$) and Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (Man$_3$GlcNAcFucGlcNAc$_{OT}$) were obtained from Gal$_2$GlcNAc$_2$Man$_3$GlcNAcGlcNAc$_{OT}$ and Gal$_2$GlcNAc$_2$Man$_3$GlcNAcFucGlcNAc$_{OT}$, respectively, by digestion with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase. Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (ManGlcNAcGlcNAc$_{OT}$) and Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (ManGlcNAcFucGlcNAc$_{OT}$) were prepared from Man$_3$GlcNAcGlcNAc$_{OT}$ and Man$_3$GlcNAcFucGlcNAc$_{OT}$, respectively, by digestion with jack bean α-mannosidase. Manα1→6(Manα1→3)Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Man$_5$GlcNAcGlcNAc$_{OT}$) was obtained from bovine pancreatic ribonuclease B (Liang et al., 1980).

RESULTS

Fractionation of Oligosaccharides by Paper Electrophoresis. The radioactive oligosaccharides derived from CD45 were subjected to paper electrophoresis and separated into neutral (N) and acidic (A) fractions (Figure 2a). The percent molar ratios of fractions N and A calculated from their radioactivities

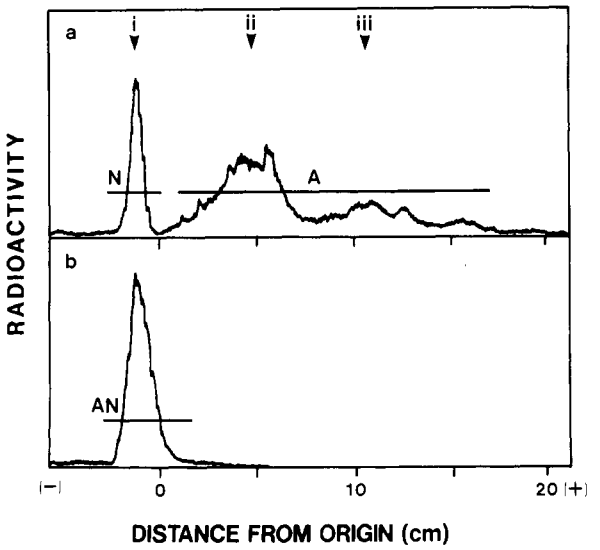


FIGURE 2: Paper electrophoresis of oligosaccharides released from CD45 (a) and of sialidase digests of the acidic oligosaccharides (indicated by a bar A) (b). The arrow heads at the top of the figure indicate the positions to which authentic oligosaccharides migrated: (i) lactitol; (ii) Neu5AcGal$_2$GlcNAc$_2$Man$_3$GlcNAcGlcNAc$_{OT}$; (iii) Neu5Ac$_2$Gal$_2$GlcNAc$_2$Man$_3$GlcNAcGlcNAc$_{OT}$.

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

	molar ratio (%)			
	N		AN	
fractions	AAL$^-$	AAL$^+$	AAL$^-$	AAL$^+$
I. E$_4$-PHA$^-$·Con A$^-$·DSA$^+$	2.8	8.0	3.8	26.3
II. E$_4$-PHA$^-$·Con A$^-$·DSA^r	0.5	2.0	4.7	14.4
III. E$_4$-PHA$^-$·Con A$^-$·DSA$^-$	1.0	1.1	5.3	2.9
IV. E$_4$-PHA$^-$·Con A$^+$	1.0	3.1	8.4	8.0
V. E$_4$-PHA$^-$·Con A^{++}	1.1	tr[a]	1.6	tr
VI. E$_4$-PHA^r	0.5	0.6	0.9	2.0

[a] Trace, less than 0.4%.

were 22% and 78%, respectively. When fraction A was digested exhaustively with *A. ureafaciens* sialidase, all of the acidic oligosaccharides were converted to neutral ones (AN) (Figure 2b). 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 Serial Lectin Column Chromatography. Oligosaccharides in fractions N and AN were initially subjected to E$_4$-PHA-agarose column chromatography in order to separate oligosaccharides with and without the bisecting *N*-acetylglucosamine residue. The fraction that passed through the column was designated as E$_4$-PHA$^-$ and that retarded in the column as E$_4$-PHA^r. As shown in Table I, 4% of the total oligosaccharides was retarded in the column. Oligosaccharides in the E$_4$-PHA fraction were applied to a Con A-Sepharose column, and the pass-through fraction (E$_4$-PHA$^-$·Con A$^-$), the bound fraction eluted with 5 mM α-methyl D-glucopyranoside (E$_4$-PHA$^-$·Con A$^+$) and the bound fraction eluted with 100 mM α-methyl D-mannopyranoside (E$_4$-PHA$^-$·Con A^{++}) were obtained. Oligosaccharides in fraction E$_4$-PHA$^-$·Con A$^-$ were applied to a DSA-Sepharose column, and the pass-through fraction (E$_4$-PHA$^-$·Con A$^-$·DSA$^-$), the fraction that was retarded in the column (E$_4$-PHA$^-$·Con A$^-$·DSA^r), and the bound fraction eluted with 1% *N*-acetylglucosamine oligomers (E$_4$-PHA$^-$·Con A$^-$·DSA$^+$) were obtained. The six fractions thus obtained were named fractions I–VI as listed in Table I. Finally, oligosaccharides in each of the six fractions were

applied to an AAL-Sepharose column. The pass-through fraction (AAL^-) and the fraction bound to the column and eluted with 1 mM L-fucose (AAL^+) were obtained. The molar ratios of oligosaccharides in each fraction 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 Fraction I. Oligosaccharides in fraction I contained 40.9% of the total oligosaccharides. Oligosaccharides in fraction AAL^+ of fraction I from AN (named AN-I) were separated into four components a, b, c and d with effective sizes of 16.5, 19.0, 22.0, and 25.0 glucose units by Bio-Gel P-4 column chromatography (Figure 3A). When component a was digested with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, three galactose residues (solid line peak in Figure 3B) and one *N*-acetylglucosamine residue (solid line peak in Figure 3C) were released. After digestion of the solid line peak in Figure 3C with jack bean β-*N*-acetylhexosaminidase, two *N*-acetylglucosamine residues were released, and the product was eluted at 8.2 glucose units (solid line peak in Figure 3D). By digestion with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, four galactose residues (dotted line peak in Figure 3B) and one *N*-acetylglucosamine residue (dotted line peak in Figure 3C) were released from component b in Figure 3A. When dotted line peak in Figure 3C was digested with jack bean β-*N*-acetylhexosaminidase, three *N*-acetylglucosamine residues were released and the product was also eluted at 8.2 glucose units (dotted line peak in Figure 3D).

When component c was digested with *E. freundii* endo-β-galactosidase, the product was eluted at 18.0 glucose units (solid line peak in Figure 3E). Upon digestion with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, three galactose residues (solid line peak in Figure 3F) and one *N*-acetylglucosamine residue (solid line peak in Figure 3G) were released from the solid line peak in Figure 3E. When the solid line peak in Figure 3G was digested with jack bean β-*N*-acetylhexosaminidase, three *N*-acetylglucosamine residues were released and the product was eluted at 8.2 glucose units (solid line peak in Figure 3H). When component d was digested with *E. freundii* endo-β-galactosidase, it was separated into two products e and f with effective sizes of 17.0 and 18.0 glucose units (dotted line peaks in Figure 3E). By digestion with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, two galactose residues (dotted line peak in Figure 3F) and one *N*-acetylglucosamine residue (dotted line peak in Figure 3G) were released, from peak e in Figure 3E. When the dotted line peak in Figure 3G was digested with jack bean β-*N*-acetylhexosaminidase, three *N*-acetylglucosamine residues were released and the product was eluted at 8.2 glucose units (dotted line peak in Figure 3H). When peak f in Figure 3E was digested with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, three galactose residues (dot-dashed line peak in Figure 3F) and one *N*-acetylglucosamine residue (dot-dashed line peak in Figure 3G) were released. When the dot-dashed line peak in Figure 3G was digested with jack bean β-*N*-acetylhexosaminidase, three *N*-acetylglucosamine residues were released, and the product was eluted at 8.2 glucose units (dot-dashed line peak in Figure 3H). That the solid and dotted line products in Figure 3D and the solid, dotted, and dot-dashed line products in Figure 3H have the structure Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)-$GlcNAc_{OT}$ was confirmed by sequential exoglycosidase digestion with jack bean α-mannosidase, snail β-mannosidase, jack bean β-*N*-acetylhexosaminidase, and bovine epididymal α-fucosidase and by methylation analysis (data not shown).

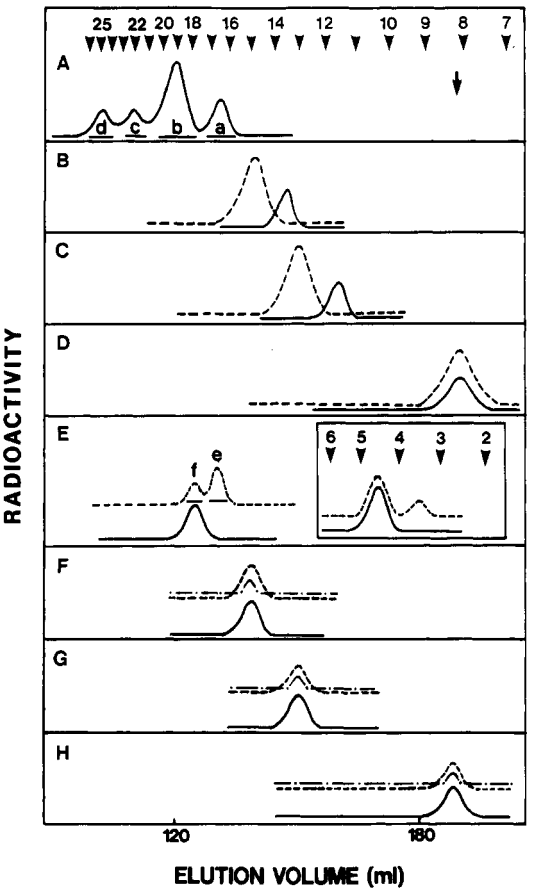


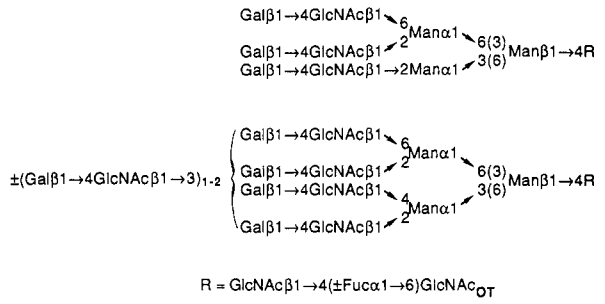
FIGURE 3: Sequential exoglycosidase digestion of fraction AN-I. The fraction and its exoglycosidase digestion products were analyzed by Bio-Gel P-4 column chromatography: (A) fraction AAL^+ of fraction AN-I; (B) solid and dotted line peaks indicate components a and b in panel A digested with diplococcal β-galactosidase, respectively; (C) solid and dotted line peaks indicate solid and dotted line peaks in panel B digested with diplococcal β-*N*-acetylhexosaminidase, respectively; (D) solid and dotted line peaks indicate solid and dotted line peaks in panel C digested with jack bean β-*N*-acetylhexosaminidase, respectively; (E) solid and dotted line peaks indicate components c and d in panel A digested with *E. freundii* endo-β-galactosidase, respectively; (inset in panel E) the oligosaccharides released by the enzyme digestion were reduced with NaB^3H_4 and subjected to Bio-Gel P-4 column chromatography; (F) solid, dotted, and dot-dashed line peaks indicate solid line peak and dotted line peaks e and f in panel E digested with diplococcal β-galactosidase, respectively; (G) solid, dotted, and dot-dashed line peaks indicate solid, dotted and dot-dashed line peaks in panel F digested with diplococcal β-*N*-acetylhexosaminidase, respectively; (H) solid, dotted, and dot-dashed line peaks indicate solid, dotted, and dot-dashed line peaks in panel G digested with jack bean β-*N*-acetylhexosaminidase, respectively. The 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. A black arrow indicates the elution position of authentic oligosaccharide $Man_3GlcNAcFucGlcNAc_{OT}$.

The oligosaccharides released by digestion of components c and d 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 eluted at 4.5 and/or 3.5 glucose units (solid and dotted line peaks in Figure 3E inset, derived from components c and d, respectively). By digestion with diplococcal β-galactosidase, the products eluted at 4.5 glucose units were converted to oligosaccharides with mobility of 3.5 glucose units. These products and the product

eluted at 3.5 glucose units in Figure 3E (inset) were converted to radioactive galactitol by digestion with jack bean β-*N*-acetylhexosaminidase (data not shown). These results indicated that components c and d in Figure 3A are tetraantennary complex-type sugar chains with one *N*-acetyllactosamine repeating unit and two *N*-acetyllactosamine repeating units or one Galβ1→4GlcNAcβ1→Galβ1→4GlcNAcβ1→Gal group, respectively.

Sequential exoglycosidase digestion of oligosaccharides in fraction AAL$^+$ of fraction N-I gave exactly the same series of results as described for fraction AAL$^+$ of fraction AN-I. The elution profiles of oligosaccharides in fractions AAL$^-$ of fractions AN-I and N-I and their sequential exoglycosidase digestion products from a Bio-Gel P-4 column were very similar to those of fraction AAL$^+$ of fraction AN-I, except that the elution position of each peak was smaller than the corresponding peak in fraction AAL$^+$ by one glucose unit (data not shown).

These results indicated that oligosaccharides in fraction I have the following structures.



Structures of Oligosaccharides in Fraction II. Fraction II contained 21.6% of the total oligosaccharides. Oligosaccharides in fraction AAL$^+$ of fraction AN-II were eluted from a Bio-Gel P-4 column as a single peak with an effective size of 17.0 glucose units (Figure 4A). When the oligosaccharide in Figure 4A was digested with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, three galactose residues (Figure 4B) and two *N*-acetylglucosamine residues (Figure 4C) were released. The peak in Figure 4C was further digested with jack bean β-*N*-acetylhexosaminidase, and the product was eluted at the same position as authentic $Man_3GlcNAcFucGlcNAc_{OT}$, releasing one *N*-acetylglucosamine residue (Figure 4D). That the product in Figure 4D has the structure Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)$GlcNAc_{OT}$ was confirmed by the methods described earlier.

Sequential exoglycosidase digestion of the oligosaccharide in fraction AAL$^+$ of fraction N-II gave the same series of results as described earlier for the oligosaccharide in Figure 4A. The elution profiles of oligosaccharides in fractions AAL$^-$ of fractions AN-II and N-II and their sequential exoglycosidase digestion products from a Bio-Gel P-4 column were very similar to those of fraction AAL$^+$ of fraction AN-II, except that the elution position of each peak was smaller than the corresponding peak in the AAL$^+$ fraction by one glucose unit (data not shown).

These results as well as the substrate specificity of diplococcal 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, indicated that the oligosaccharides in fraction II are triantennary complex-type sugar chains with the GlcNAcβ1→4(GlcNAcβ1→2)Man group as shown in Table III.

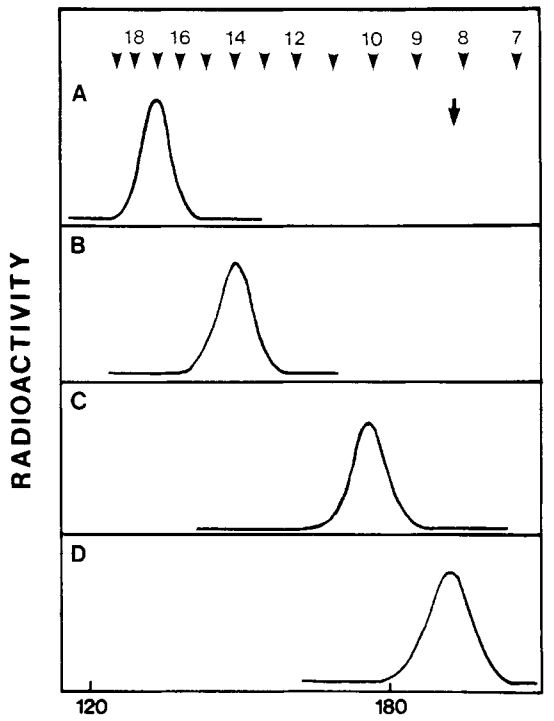


FIGURE 4: Sequential exoglycosidase digestion of fraction AN-II. The fraction and its digestion products were analyzed by Bio-Gel P-4 column chromatography: (A) fraction AAL$^+$ of fraction AN-II; (B) the oligosaccharide in panel A digested with diplococcal β-galactosidase; (C) the peak in panel B digested with diplococcal β-*N*-acetylhexosaminidase; (D) the peak in panel C digested with jack bean β-*N*-acetylhexosaminidase. Arrowheads at the top of the figure and the black arrow are the same as in Figure 3.

Structures of Oligosaccharides in Fraction III. Oligosaccharides in fraction AAL$^+$ of fraction AN-III, which contained 2.9% of the total oligosaccharides, were eluted from a Bio-Gel P-4 column as a single peak with an effective size of 17.5 glucose units (Figure 5A). When the oligosaccharide in Figure 5A was digested with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, two galactose residues (Figure 5B) and two *N*-acetylglucosamine residues (Figure 5C) were released. The peak in Figure 5C was digested with almond emulsin α-fucosidase I, which cleaves the Fucα1→3GlcNAc and Fucα1→4GlcNAc linkages, and the product was eluted at 11.2 glucose units, releasing one fucose residue (Figure 5D). When the peak in Figure 5D was digested with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, one galactose residue was released (Figure 5E), but no *N*-acetylglucosamine residue was released (Figure 5F). The peak in Figure 5F was digested with jack bean β-*N*-acetylhexosaminidase, and the product was eluted at 8.2 glucose units (Figure 5G). That the product in Figure 5G has the structure Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)$GlcNAc_{OT}$ was confirmed by the methods described earlier.

The elution profiles of the oligosaccharide in fraction AAL$^-$ of fraction AN-III and their sequential exoglycosidase digestion products from a Bio-Gel P-4 column were very similar to those of fraction AAL$^+$ of fraction AN-III, except that the elution position of each peak was smaller than the corresponding peak in fraction AAL$^+$ by one glucose unit (data not shown). When oligosaccharides in fraction AAL$^-$ of fraction N-III were subjected to Bio-Gel P-4 column chromatography, they were separated into several peaks, each of which amounted to less than 0.2% of the total oligosaccharides. Therefore,

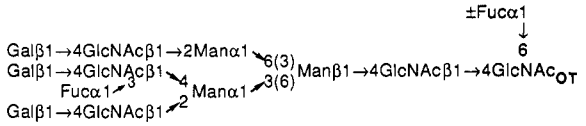
Table II: Methylation Analysis of Fraction A, Fraction AN, and Each Lectin Fraction Obtained from a Mixture of Fractions N and AN

partially *O*-methylated sugars	molar ratio[a]					
	fraction A	fraction AN	Con A$^-$	(Con A$^-$)[b]	Con A$^+$	Con A^{++}
fucitol						
2,3,4-tri-*O*-methyl (1,5-di-*O*-acetyl)	0.8	0.7	0.8	0.7	0.5	–[c]
galactitol						
2,3,4,6-tetra-*O*-methyl (1,5-di-*O*-acetyl)	2.4	3.6	3.4	3.5	1.9	0.3
2,4,6-tri-*O*-methyl (1,3,5-tri-*O*-acetyl)	0.2	0.2	0.2	0.2	0.1	–
2,3,4-tri-*O*-methyl (1,5,6-tri-*O*-acetyl)	1.3	–	–	–	–	–
mannitol						
2,3,4,6-tetra-*O*-methyl (1,5-di-*O*-acetyl)	tr[d]	tr	–	–	–	1.1
3,4,6-tri-*O*-methyl (1,2,5-tri-*O*-acetyl)	0.9	0.8	0.5	0.5	1.8	0.8
3,6-di-*O*-methyl (1,2,4,5-tetra-*O*-acetyl)	0.7	0.7	0.8	0.7	–	–
3,4-di-*O*-methyl (1,2,5,6-tetra-*O*-acetyl)	0.4	0.4	0.5	0.5	–	–
2,4-di-*O*-methyl (1,3,5,6-tetra-*O*-acetyl)	1.0	1.0	0.9	0.9	1.0	1.0
2-mono-*O*-methyl (1,3,4,5,6-penta-*O*-acetyl)	tr	tr	0.1	0.1	–	–
2-*N*-methylacetamido-2-deoxyglucitol						
6-mono-*O*-methyl (1,3,4,5-tetra-*O*-acetyl)	0.1	0.1	0.1	–	–	–
3,6-di-*O*-methyl (1,4,5-tri-*O*-acetyl)	4.2	4.3	4.5	4.6	3.1	0.8
1,3,5,6-tetra-*O*-methyl (4-mono-*O*-acetyl)	0.2	0.2	0.3	0.2	0.4	0.6
1,3,5-tri-*O*-methyl (4,6-di-*O*-acetyl)	0.6	0.7	0.7	0.7	0.5	–
3,4,6-tri-*O*-methyl (1,5-di-*O*-acetyl)	tr	tr	0.1	0.1	–	–

[a] Numbers in the table were calculated by taking the value of 2,4-di-*O*-methylmannitol and/or 2-mono-*O*-methylmannitol as 1.0. [b] After almond emulsin α-fucosidase I digestion. [c] Not detectable. [d] Trace, less than 0.1.

their structural analysis could not be performed and are included in "not determined" in Table III.

These results indicated that oligosaccharides in fraction AN-III have the following structures.



Structures of Oligosaccharides in Fraction IV. Oligosaccharides in fraction IV contained 20.5% of the total oligosaccharides. When oligosaccharides in fraction AAL$^+$ of fraction AN-IV were subjected to Bio-Gel P-4 column chromatography, two components g and h with effective sizes of 14.2 and 17.2 glucose units were eluted (Figure 6A). By digestion with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, two galactose residues (solid line peak in Figure 6B) and two *N*-acetylglucosamine residues were released from component g in Figure 6A, and the radioactive product was eluted at the same position as authentic Man_3-$GlcNAcFucGlcNAc_{OT}$ (solid line peak in Figure 6C). When component h in Figure 6A was digested with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, two galactose residues (dotted line peak in Figure 6B) and two *N*-acetylglucosamine residues were released, and the product was eluted at the position of 11.2 glucose units (dotted line peak in Figure 6C). By further digestion with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, one galactose residue (Figure 6D) and one *N*-acetylglucosamine residue were released from the dotted line peak in Figure 6C, and the product was eluted at the same position as authentic $Man_3GlcNAcFucGlcNAc_{OT}$ (Figure 6E). These results indicated that oligosaccharide h is 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 dotted line peak in Figure 6C was applied to a Con A-Sepharose column. All oligosaccharides bound to the column, and 40% of the bound oligosaccharides was eluted from the column with 5 mM α-methyl D-glucopyranoside, and the remaining 60% was eluted with 100 mM α-methyl D-mannopyranoside (data not shown). Since Galβ1→4GlcNAcβ1→2Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(±Fucα1→6)GlcNAc$_{OT}$ and Galβ1→4GlcNAcβ1→2Manα1→3(Manα1→6)-Manβ1→4GlcNAcβ1→4(±Fucα1→6)$GlcNAc_{OT}$ bind to a Con A-Sepharose column and are eluted with 5 mM α-methyl D-glucopyranoside and 100 mM α-methyl D-mannopyranoside, respectively (Yamashita et al., 1981; Merkle & Cummings, 1987), the tetrasaccharide outer chain of the peak h in Figure 6A should be linked almost evenly to both α-mannosyl arms of trimannosyl core. That the solid line product in Figure 6C and the dotted line product in Figure 6E have the structure Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)-$GlcNAc_{OT}$ was confirmed by the methods described earlier.

Sequential exoglycosidase digestion of oligosaccharides in fraction AAL$^+$ of fraction N-IV gave the same series of results as described earlier for components g and h in Figure 6A. The elution profiles of oligosaccharides in fractions AAL$^-$ of fractions AN-IV and N-IV and their sequential exoglycosidase digestion products from a Bio-Gel P-4 column were very similar to those of fraction AAL$^+$ of fraction AN-IV, except that the elution position of each peak was smaller than the corresponding peak in AAL$^+$ fraction by one glucose unit (data not shown).

These results indicated that the oligosaccharides in this fraction are biantennary complex-type with and without one poly(*N*-acetyllactosamine) group as shown in Table III.

Structures of Oligosaccharides in Fraction V. Oligosaccharides in fraction V contained 2.7% of the total oligosaccharides and passed through an AAL-Sepharose column, indicating that they lack the L-fucose residue attached to their proximal *N*-acetylglucosamine residue. When oligosaccharides in fraction AAL$^-$ of fraction N-V was subjected to Bio-Gel P-4 column chromatography, they were eluted from the column as multiple peaks larger than eight glucose units (Figure 7A). After digestion with *A. saitoi* α-mannosidase I, which cleaves only the Manα1→2Man linkage, the oligosaccharides in Figure 7A were separated into two peaks i and j, the former of which was eluted at the same position as authentic $Man_5GlcNAcGlcNAc_{OT}$ (Figure 7B). Peak i in

Table III: Proposed Structures of the Asn-Linked Sugar Chains of Human CD45

fractions[a]	structures[b]	molar ratio (%)
I.	$(Neu5Ac\alpha2\rightarrow6)_{0\sim2}$ {Galβ1→4GlcNAcβ1→6(2)Manα1→6(3)Manβ1→4R; Galβ1→4GlcNAcβ1→3(6)Manβ1→4R; Galβ1→4GlcNAcβ1→2Manα1→3(6)Manβ1→4R}	7.2
I.	$(Neu5Ac\alpha2\rightarrow6)_{0\sim2}$ {Galβ1→4GlcNAcβ1→6(2)Manα1→6(3)Manβ1→4R; Galβ1→4GlcNAcβ1→3(6)Manβ1→4R; Galβ1→4GlcNAcβ1→4(2)Manα1→3(6)Manβ1→4R; Galβ1→4GlcNAcβ1→2Manα1→3(6)Manβ1→4R}	17.8
I.	$(Gal\beta1\rightarrow4GlcNAc\beta1\rightarrow3)_{1\sim2}$ $(Neu5Ac\alpha2\rightarrow6)_{0\sim2}$ {Galβ1→4GlcNAcβ1→6(2)Manα1→6(3)Manβ1→4R; Galβ1→4GlcNAcβ1→3(6)Manβ1→4R; Galβ1→4GlcNAcβ1→4(2)Manα1→3(6)Manβ1→4R; Galβ1→4GlcNAcβ1→2Manα1→3(6)Manβ1→4R}	15.1
II.	$(Neu5Ac\alpha2\rightarrow6)_{0\sim2}$ {Galβ1→4GlcNAcβ1→2Manα1→6(3)Manβ1→4R; Galβ1→4GlcNAcβ1→4(2)Manα1→3(6)Manβ1→4R; Galβ1→4GlcNAcβ1→2Manα1→3(6)Manβ1→4R}	21.6
III.	$(Neu5Ac\alpha2\rightarrow6)_{0\sim2}$ {Galβ1→4GlcNAcβ1→2Manα1→6(3)Manβ1→4R; Galβ1→4GlcNAcβ1→4(2)Manα1→3(6)Manβ1→4R; Fucα1→3; Galβ1→4GlcNAcβ1→2Manα1→3(6)Manβ1→4R}	4.7
IV.	$(Neu5Ac\alpha2\rightarrow6)_{0\sim2}$ {Galβ1→4GlcNAcβ1→2Manα1→6(3)Manβ1→4R; Galβ1→4GlcNAcβ1→2Manα1→3(6)Manβ1→4R}	18.6
IV.	$(Gal\beta1\rightarrow4GlcNAc\beta1\rightarrow3)$ $(Neu5Ac\alpha2\rightarrow6)_{0\sim2}$ {Galβ1→4GlcNAcβ1→2Manα1→6(3)Manβ1→4R; Galβ1→4GlcNAcβ1→2Manα1→3(6)Manβ1→4R}	1.5
V.	$(Man\alpha1\rightarrow2)_{0\sim4}$ {Manα1→6(3)Manα1→6(3)Manβ1→4R[c]; Manα1→3(6)Manβ1→4R[c]}	0.9
V.	Manα1→6(3)Manα1→6(3)Manβ1→4R[c]; Manα1→3(6)Manβ1→4R[c]; $(Neu5Ac\alpha2\rightarrow6)_{0\sim1}$ Galβ1→4GlcNAcβ1→2Manα1→3(6)Manβ1→4R[c]	1.4
VI.	$(Neu5Ac\alpha2\rightarrow6)_{0\sim2}$ {Galβ1→4GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)3Manβ1→4R; Galβ1→4GlcNAcβ1→2Manα1→3(6)Manβ1→4R}	2.6
VI.	$(Neu5Ac\alpha2\rightarrow6)_{0\sim2}$ {Galβ1→4GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)3Manβ1→4R; Galβ1→4GlcNAcβ1→4(2)Manα1→3(6)Manβ1→4R; Galβ1→4GlcNAcβ1→2Manα1→3(6)Manβ1→4R}	1.1
	Not Determined	7.5

[a] Fractions I–VI shown in Table I. [b] R = GlcNAcβ1→4(±Fucα1→6)GlcNAc$_{OT}$. [c] R = GlcNAcβ1→4GlcNAc$_{OT}$.

Figure 7B was further digested with jack bean α-mannosidase and converted to the product with the same elution position as authentic ManGlcNAcGlcNAc$_{OT}$ (Figure 7C). That the product in Figure 7C has the structure Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ was confirmed by sequential exoglycosidase digestion with snail β-mannosidase and jack bean β-*N*-acetylhexosaminidase and by methylation analysis (data not shown). These results indicated that 90% of the oligosaccharides in fraction AAL$^-$ of fraction N-IV are a series of high mannose-type: (Manα1→2)$_{0-4}$Man$_5$GlcNAcGlcNAc$_{OT}$.

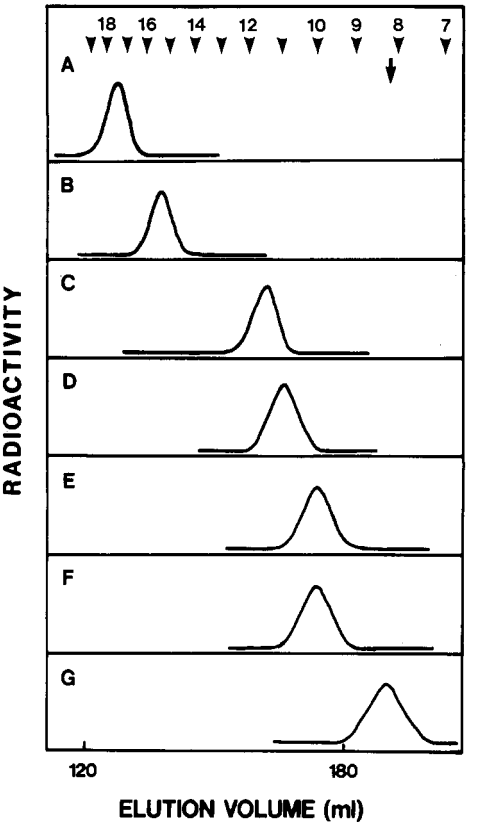


FIGURE 5: Sequential exoglycosidase digestion of fraction AN-III. The fraction and its digestion products were analyzed by Bio-Gel P-4 column chromatography: (A) fraction AAL$^+$ of fraction AN-III; (B) the oligosaccharide in panel A digested with diplococcal β-galactosidase; (C) the peak in panel B digested with diplococcal β-*N*-acetylhexosaminidase; (D) the peak in panel C digested with almond emulsin α-fucosidase I; (E) the peak in panel D digested with diplococcal β-galactosidase; (F) the peak in panel E digested with diplococcal β-*N*-acetylhexosaminidase; (G) the peak in panel F digested with jack bean β-*N*-acetylhexosaminidase. The arrowheads at the top of the figure and the black arrow are the same as in Figure 3.

When peak j in Figure 7B was digested with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, one galactose residue (Figure 7D) and one *N*-acetylglucosamine residue (Figure 7E) were released. Upon digestion with jack bean α-mannosidase, four mannose residues were released from the peak in Figure 7E, and the product was eluted at the same position as authentic ManGlcNAcGlcNAc$_{OT}$ (Figure 7F). That the radioactive product in Figure 7F has the structure Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ was confirmed by the methods described earlier. Since methylation analysis showed that oligosaccharide j after digestion with a mixture of diplococcal β-galactosidase and diplococcal β-*N*-acetylhexosaminidase contains only 2,3,4,6-tetra-*O*-methyl and 2,4-di-*O*-methyl mannitols as the methylated mannitols in the molar ratio of 3:2 (data not shown), the structure of the peak in Figure 7E can be written either as Manα1→6(Manα1→3)Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ or as Manα1→6(Manα1→3)-Manα1→3(Manα1→6)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$. In order to determine which of the structures

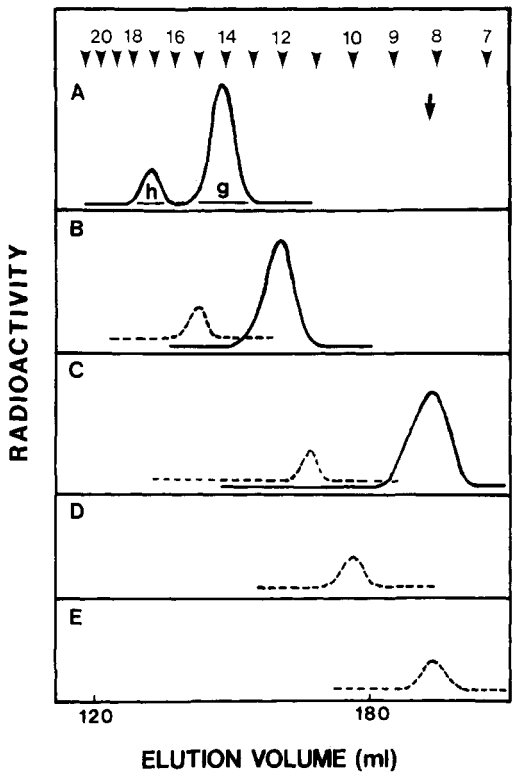


FIGURE 6: Sequential exoglycosidase digestion of fraction AN-IV. The fraction and its digestion products were analyzed by Bio-Gel P-4 column chromatography: (A) fraction AAL^+ of fraction AN-IV; (B) solid and dotted line peaks indicate components g and h in panel A digested with diplococcal β-galactosidase, respectively; (C) solid and dotted line peaks indicate solid and dotted line peaks in panel B digested with diplococcal β-*N*-acetylhexosaminidase, respectively; (D) dotted line peak in panel C digested with diplococcal β-galactosidase; (E) dotted line peak in panel D digested with diplococcal β-*N*-acetylhexosaminidase. Arrowheads at the top of the figure and the black arrow are the same as in Figure 3.

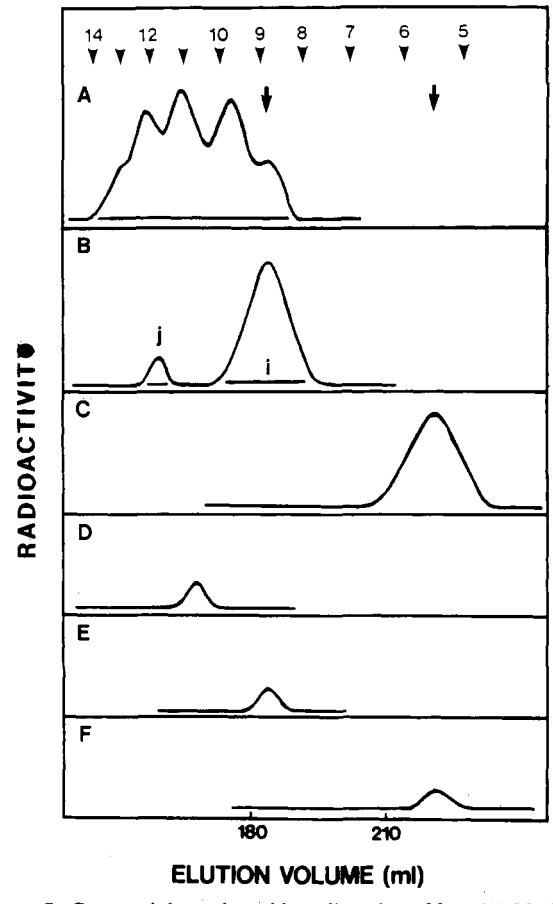


FIGURE 7: Sequential exoglycosidase digestion of fraction N-V. The fraction and its digestion products were analyzed by Bio-Gel P-4 column chromatography: (A) fraction AAL^- of fraction N-V; (B) oligosaccharides indicated by a bar in panel A digested with *A. saitoi* α-mannosidase I; (C) peak i in panel B digested with jack bean α-mannosidase; (D) peak j in panel B digested with diplococcal β-galactosidase; (E) the peak in panel D digested with diplococcal β-*N*-acetylhexosaminidase; (F) the peak in panel E digested with jack bean α-mannosidase. Arrowheads at the top of the figure are the same as in Figure 3. Black arrows indicate the elution positions of authentic oligosaccharides: (I) $Man_5GlcNAcGlcNAc_{OT}$; (II) $ManGlcNAcGlcNAc_{OT}$.

is the correct one, the radioactive product in Figure 7E was subjected to periodate oxidation, and the product was analyzed by Bio-Gel P-4 column chromatography. A radioactive peak with the same mobility as authentic Manα1→6Manβ1→4GlcNAcβ1→4XylNAc$_{OT}$ was obtained (data not shown). This radioactive tetrasaccharide was then subjected to a second periodate oxidation. Analysis of the radioactive product by Bio-Gel P-4 column chromatography revealed that a single radioactive peak corresponding to GlcNAcβ1→4XylNAc$_{OT}$ was obtained (data not shown). These results indicated that the radioactive tetrasaccharide obtained by the first periodate oxidation is not Manα1→3Manβ1→4GlcNAcβ1→4XylNAc$_{OT}$ but Manα1→6Manβ1→4GlcNAcβ1→4XylNAc$_{OT}$. Therefore, the structure of the peak in Figure 7E should be Manα1→6(Manα1→3)Manα1→6(Manα1→3)-Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$, because Manα1→6-(Manα1→3)Manα1→3(Manα1→6)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ must be converted to Manα1→3Manβ1→4GlcNAcβ1→4XylNAc$_{OT}$ by the first periodate oxidation. These results indicated that the component j in Figure 7B is a typical hybrid-type sugar chain.

When oligosaccharides in fraction AAL^- of fraction AN-V were subjected to Bio-Gel P-4 column chromatography, a single peak with an effective size of 11.9 glucose units was eluted (data not shown). The elution profiles of its sequential exoglycosidase digestion products as well as the periodate oxidation products from a Bio-Gel P-4 column were the same as those of oligosaccharide j in Figure 7B (data not shown). Therefore, these results indicated that the oligosaccharide in fraction AAL^- of fraction AN-V is also a typical hybrid-type sugar chain as shown in Table III.

Structures of Oligosaccharides in Fraction VI. Since all of oligosaccharides in fraction VI were retarded in an E_4-PHA-agarose column, they should have bisecting *N*-acetylglucosamine. Oligosaccharides in fraction AAL^+ of fraction AN-VI, which contained 2.0% of the total oligosaccharides, were separated into two components k and l with effective sizes of 15.0 and 17.8 glucose units by Bio-Gel P-4 column chromatography (Figure 8A). When component k in Figure 8A was digested with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, two galactose residues (solid line peak in Figure 8B) and one *N*-acetylglucosamine residue (solid line peak in Figure 8C) were released. After digestion of the solid line peak in Figure 8C with jack bean β-*N*-acetylhexosaminidase, the radioactive product was eluted at 8.2 glucose units (Figure 8D). When component l in Figure 8A was digested with diplococcal β-galactosidase followed by diplococcal β-*N*-acetylhexosaminidase, three galactose residues (dotted line peak in Figure 8B) and one *N*-acetylglucosamine residue (dotted line peak in Figure 8C) were released. After digestion of the dotted line peak in Figure 8C with jack bean β-*N*-acetylhexosaminidase, the radioactive product was eluted at 8.2 glucose units (dotted line peak in Figure 8D).

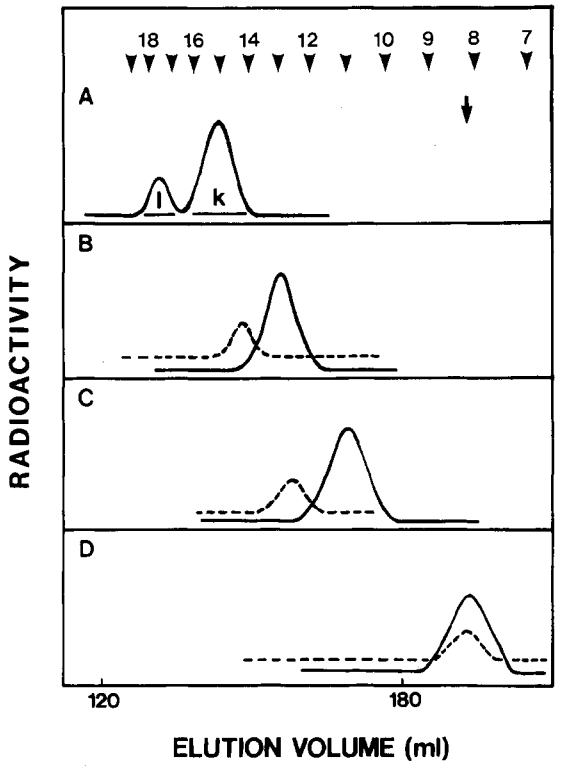
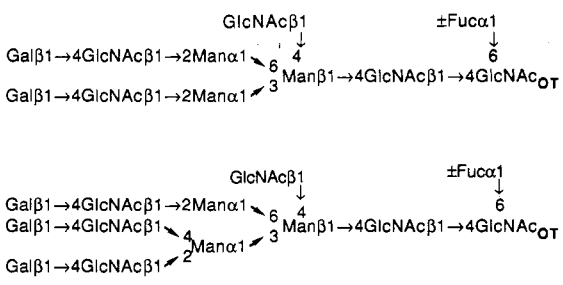


FIGURE 8: Sequential exoglycosidase digestion of fraction AN-VI. The fraction and its digestion products were analyzed by Bio-Gel P-4 column chromatography: (A) fraction AAL$^+$ of fraction AN-VI; (B) solid and dotted line peaks indicate components k and l in panel A digested with diplococcal β-galactosidase, respectively; (C) solid and dotted line peaks indicate solid and dotted line peaks in panel B digested with diplococcal β-*N*-acetylhexosaminidase, respectively; (D) solid and dotted line peaks indicate solid and dotted line peaks in panel C digested with jack bean β-*N*-acetylhexosaminidase, respectively. Arrowheads at the top of the figure and the black arrow are the same as in Figure 3.

That the solid and dotted line peaks in Figure 8D have the structure Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4-(Fucα1→6)GlcNAc$_{OT}$ was confirmed by the methods described earlier.

Sequential exoglycosidase digestion of oligosaccharides in fraction AAL$^+$ of fraction N-VI gave the same series of results as described earlier for the components k and l in Figure 8A (data not shown). The elution profiles of oligosaccharides in fractions AAL$^-$ of fractions AN-VI and N-VI and their sequential exoglycosidase digestion products from a Bio-Gel P-4 column were very similar to those of oligosaccharides k and l in fraction AAL$^+$ of fraction AN-VI, except that the elution positions of each peak were smaller than the corresponding peaks in fraction AAL$^+$ by one glucose unit (data not shown). These results indicated that the structures of oligosaccharides in fraction AAL$^-$ contain the non-fucosylated trimannosyl core. These results and the binding specificity of E_4-PHA indicated that oligosaccharides in fraction VI have the following structures:



Structure of the Sialylated Oligosaccharides. Since approximately 80% of the oligosaccharides released from CD45 were sialylated, they were subjected to SNA-agarose column chromatography. SNA is known to bind the sialylated oligosaccharides with the Neu5Acα2→6Gal/GalNAc group but not with the Neu5Acα2→3Gal/GalNAc group or the Galβ1→3(Neu5Acα2→6)GlcNAc group (Shibuya et al., 1987). Consistent with the binding specificity of this lectin, a biantennary complex-type sugar chain with the Neu5Acα2→6Gal group prepared from human transferrin (Spik et al., 1975) bound and was eluted from the column with 100 mM lactose, but the complex-type sugar chains with the Neu5Acα2→3Gal group prepared from human placental alkaline phosphatase (Endo et al., 1988) and the desialylated human transferrin sugar chain did not bind (data not shown). Under the same condition as above, all of the sialylated sugar chains bound and were eluted from the column with 100 mM lactose (data not shown), indicating that the sialylated sugar chains contain the Neu5Acα2→6Gal groups.

Comparison of the methylation data for oligosaccharides in fraction A and fraction AN indicated that all of the sialic acid residues are linked at the C-6 position of the galactose residues, since only 2,3,4-tri-*O*-methyl galactitol disappeared after digestion with *A. ureafaciens* sialidase (Table II). These results suggest that the sialylated oligosaccharides contain only the Neu5Acα2→6Gal groups.

Methylation Analysis of Oligosaccharides. In order to confirm each glycosidic linkage in the oligosaccharides of CD45 as determined mainly by sequential exoglycosidase digestion, deuterium-labeled oligosaccharides in fractions N and AN 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 from the columns 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 mannitol and/or 2-mono-*O*-methyl mannitol as 1.0. Comparison of the data for oligosaccharides in fraction Con A$^-$ before and after the α-fucosidase I digestion indicated that 12.5% of the fucose residues is linked at the C-3 position of the *N*-acetylglucosamine residue, since 0.1 mol each of 2,3,4-tri-*O*-methyl fucitol and 6-mono-*O*-methyl 2-(*N*-methylacetamido)-2-deoxyglucitol disappeared after the α-fucosidase I digestion. Detection of 0.1 mol of 2-mono-*O*-methyl mannitol in fraction Con A$^-$ indicated that approximately 10% of the oligosaccharides in this fraction are bisected. Comparison of the data for oligosaccharides in fraction Con A$^-$ before and after the endo-β-galactosidase digestion indicated that about 20% of the oligosaccharides in this fraction contain the poly-(*N*-acetyllactosamine) groups, since 0.2 mol of 2,4,6-tri-*O*-methyl galactitol disappeared after the enzyme digestion (data not shown). The other methylation data indicated that fraction Con A$^-$ contain bi-, tri-, and tetraantennary complex-type sugar chains.

The methylation data of fractions Con A$^+$ and Con A^{++} indicated that these fractions contain biantennary complex-type and high mannose-type and hybrid-type sugar chains, respectively.

The proposed oligosaccharide structures of human CD45, as determined by sequential exoglycosidase digestion and methylation analysis, are summarized in Table III.

DISCUSSION

On the basis of the results presented here, CD45 was shown to contain approximately six asparagine-linked sugar chains per molecule, consisting of bi-, tri-, and tetraantennary complex-type sugar chains. Since the 220K isotype of human CD45 contains 17 potential *N*-glycosylation sites as calculated from the primary structure deduced from the cDNA sequence (Ralph et al., 1987; Streuli et al., 1987), the result showed that only one-third of the glycosylation sites are occupied with the sugar chains described above. This is partly due to changes in the number of potential *N*-glycosylation sites present in each isotype of CD45 generated by the variable use of three exons which encode peptides including the potential *N*-glycosylation sites [reviewed by Thomas (1989)]. Quite interestingly, about half of the tetraantennary complex-type sugar chains, which amounted to 32.9% of the total oligosaccharides obtained from CD45, was shown to contain one and two Galβ1→4GlcNAcβ1→3Gal groups or one Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Gal group. As shown in Table III, these poly(*N*-acetyllactosamine) groups were not fucosylated. However, the Galβ1→4GlcNAcβ1→4Man group of triantennary complex-type sugar chains was fucosylated thus forming the X group, Galβ1→4-(Fucα1→3)GlcNAc. Because all of the sialic acid residues are shown to link at the C-6 position of the galactose residues, the CD45 sugar chains may not have the sialyl X group, Neu5Acα2→3Galβ1→4(Fucα1→3)GlcNAc, which is one of the ligands for cell adhesion molecules grouped in the selectin family (Bevilacqua et al., 1991).

In a previous study, we elucidated the structures of the asparagine-linked sugar chains of the human leukocyte integrins CD11/CD18 (Asada et al., 1991). The major sugar chains of CD11/CD18 were high mannose-type and tri- and tetraantennary complex-type sugar chains with and without the X and/or sialyl X groups, the latter of which amounted to 38% of the total oligosaccharides. Recently, the sialyl X groups expressed on the CD11/CD18 sugar chains have been shown to bind E-selectin, indicating the possibility that this binding induces the interaction of leukocytes with activated vascular endothelial cells (Kotovuori et al., 1993). Because CD11/CD18 itself is a cell adhesion molecule which is considered to be involved in the second step of the adhesion process (Butcher et al., 1991) by binding to the intercellular adhesion molecules 1 and 2 (ICAM-1, ICAM-2) expressed on the activated leukocytes and endothelial cells, the expression of the sialyl X groups, which are involved in the first step of the adhesion, on CD11/CD18 molecules may be of particular importance in leukocyte adhesion. However, the present study showed that the CD45 sugar chains do not have the sialyl X group, even though CD45 and CD11/CD18 are synthesized in the same cells. This suggests that the glycosylation of individual glycoproteins is regulated by different mechanisms.

Since most of the acidic oligosaccharides obtained from CD45 are mono- and disialylated sugar chains, the tetra- and triantennary complex-type sugar chains are not fully sialylated but have one or two Neu5Acα2→6Gal groups. This sialyl group may also be present on the poly(*N*-acetyllactosamine) groups expressed on the highly branched sugar chains. These results support the recent finding (Powell et al., 1993) demonstrating that the B cell adhesion molecule CD22β recognizes the α-2,6-linked sialic acid residue of asparagine-linked sugar chains of CD45RO, the 180K isotype, which is present in T cells (Thomas, 1989). Because the present CD45 preparation contained CD45RO as one of the major isotypes (Figure 1), the mono- and disialylated sugar chains described here could be ligands for CD22β.

In addition to these asparagine-linked sugar chains, CD45 has been shown to contain mucin-type sugar chains, some of which carried I and i antigens (Childs et al., 1983) or the isotype specific carbohydrate antigens (Pulido & Sanchez-Madrid, 1990). Because treatment of CD45 with *O*-glycanase or sialidase resulted in the loss of CD45RO and CD45RB determinants (Pulido & Sanchez-Madrid, 1990), the latter of which is shared by the 220K, 205K, and 190K isotypes present in T and B cells (Thomas, 1989), the mucin-type sugar chains of CD45 may be involved in the isotype-specific functions.

Leukocyte adhesion is mediated by several classes of adhesion molecules (Springer, 1990; Patarroyo et al., 1990) including CD22 and its ligand CD45 (Stamenkovic et al., 1991). Cross-linking of CD3 and CD22 ligands with anti-CD3 antibody and soluble CD22, and co-ligation of CD3 and CD45 by antibodies, resulted in the suppression of intracellular calcium increase and of tyrosine phosphorylation of phospholipase Cγ1 (Aruffo et al., 1992). This induces an increase of phosphatidylinositol turnover leading to activation of protein kinase C and of intracellular calcium levels. Therefore, CD45 must play a functional role in CD22-induced T cell stimulation, and the asparagine-linked sugar chains containing α-2,6-linked sialic acid residues of CD45 should be particularly important for the CD22-mediated signal transduction in T cells. Although it is unknown whether or not the *N*-glycosylation of each CD45 isotype is the same, the isotypes expressed in different classes of leukocytes may have cell-type specific glycosylation, which may alter the CD22 binding affinity.

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