Volume 228, number 2, 321-326 FEB 05587 February 1988

Identification of a tetrasialylated monofucosylated tetraantennary *N*-linked carbohydrate chain in human platelet glycocalicin

Sophia A.M. Korrel, Kenneth J. Clemetson*, Herman van Halbeek+, Johannis P. Kamerling, Jan J. Sixma° and Johannes F.G. Vliegenthart

*Department of Bio-Organic Chemistry, Utrecht University, Transitorium III, PO Box 80.075, 3508 TB Utrecht, The Netherlands, *Theodor Kocher Institute, Bern, Switzerland* and °*Department of Haematology, Utrecht University, Utrecht, The Netherlands*

Received 24 December 1987

Glycocalicin (140 kDa), the main constituent of the glycoprotein Ib α-chain (150 kDa) of the human platelet membrane, contains 4 putative *N*-glycosylation sites. For the structural analysis of the *N*-glycosidic carbohydrate chains of glycocalicin, the glycoprotein has been subjected to the hydrazinolysis procedure. The acidic carbohydrate chains obtained were fractionated by ion-exchange chromatography on DEAE-Sephadex A-25, and subsequently analyzed by sugar analysis, anion-exchange chromatography on Mono Q HR 5/5 and 500 MHz ^{1}H-NMR spectroscopy. A novel tetrasialylated monofucosylated tetraantennary chain was identified in the glycoprotein. It could also be deduced that in all structures the α2→6-linked NeuAc is attached exclusively at the Galβ1→4GlcNAcβ1→2Manα1→3 antenna, whereas the other antennae can be terminated with α2→3-linked NeuAc. As minor constituents sialylated *N*-linked carbohydrate chains with a terminal Fucα1→2Galβ1→. sequence were detected.

Glycoprotein Ib, Glycocalicin; *N*-linked carbohydrate

1. INTRODUCTION

Platelet membrane glycoproteins are important in vital biological processes such as platelet aggregation and adhesion [1,2]. One of the main sialoglycoproteins of the platelet plasma membrane is glycoprotein Ib. Release of glycocalicin, the main part of the α-chain of glycoprotein Ib, leads to loss of the platelet aggregation response to von Willebrand factor [3]. As was shown earlier, glycocalicin contains 40% (w/w) carbohydrate comprising *O*- as well as *N*-linked carbohydrate chains [4]. The 4 putative *N*-glycosylation sites on the α-chain of glycoprotein Ib lie within the glycocalicin part [5]. These carbohydrates may influence the course of the interaction mentioned above. Therefore, it is important to determine their structures.

Recently, structural studies on the *O*-linked carbohydrate chains of human platelet glycocalicin have been reported by us [4,6] and by others [7,8]. Sialylated di- and triantennary *N*-acetyllactosamine-type oligosaccharides with an α1→6-linked Fuc residue at the Asn-bound GlcNAc unit have been found in the *N*-linked chains [9,10]. Here we report on the additional presence of a tetrasialylated monofucosylated tetraantennary type of structure.

Abbreviations: Man, D-mannose; Gal, D-galactose; Fuc, L-fucose; GlcNAc, 2-acetamido-2-deoxy-D-glucose; GlcNAc-ol, 2-acetamido-2-deoxy-D-glucitol, NeuAc, *N*-acetylneuraminic acid

Correspondence address: S.M. Korrel, Department of Bio-Organic Chemistry, Utrecht University, Transitorium III, PO Box 80.075, 3508 TB Utrecht, The Netherlands

+*Present address:* Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30613, USA

2. MATERIALS AND METHODS

2.1. *Hydrazinolysis procedure and fractionation of carbohydrate chains*

Glycocalicin (40 mg), isolated from human platelet membranes [4], was subjected to the hydrazinolysis procedure

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

[6,11]. High-voltage paper electrophoresis of the mixture of tritium-labelled carbohydrate chains was carried out on Whatman 3MM paper (70 V/cm; 90 min) using a pyridine/acetic acid/water buffer (3:1:387, v/v), pH 5.4. The neutral and acidic fractions were recovered from the electropherogram by elution with water. The acidic fractions were combined and lyophilized. The residue was dissolved in 5 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 6.5, and subsequently applied to a column (60×1.5 cm) of DEAE-Sephadex A-25 (Pharmacia) equilibrated with the same buffer. The column was eluted with 400 ml of a linear concentration gradient from 5 to 30 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 6.5, followed by stepwise elution with 100 ml of 100 mM and 100 ml of 500 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 6.5. Fractions of 3.7 ml were collected at a flow rate of 18.5 ml/h. Further fractionation was performed on a Bio-Gel P-6 (Bio-Rad) column (100×2.5 cm) using 100 mM ammonium acetate buffer, pH 5.2. In the latter case fractions of 4 ml were collected at a flow rate of 20 ml/h.

Fractionation patterns were monitored by scintillation counting. Tritium-labelled peaks were pooled, lyophilized and desalted by filtration over a column (20×1 cm) of Bio-Gel P-2 (Bio-Rad) using water as eluent at a flow rate of 20 ml/h.

Analytical fractionations were carried out by medium-pressure ion-exchange chromatography on a Mono Q HR 5/5 column (Pharmacia) eluted with 3 ml of 10 mM 2,2-bis(hydroxymethyl)-2,2′,2″-nitriloethanol, pH 6.4, followed by linear concentration gradients of 3 ml of 0–10 mM NaCl, 13 ml of 10–50 mM NaCl and 10 ml of 50–120 mM NaCl in 10 mM 2,2-bis(hydroxymethyl)-2,2′,2″-nitriloethanol, pH 6.4. Fractions of 0.33 ml were collected at a flow rate of 1 ml/min. For calibration, sialyloligosaccharide-alditols prepared from fetuin were used as reference compounds (Bolscher, J.G.M. et al. unpublished).

2.2. *Sugar analysis*

Sugar analysis was carried out by gas-liquid chromatography on a CPsil5 WCOT fused silica capillary column (25 m×0.32 mm i.d., Chrompack) using a Varian Aerograph 3700 gas chromatograph. The trimethylsilylated methyl glycosides were prepared by methanolysis, *N*-(re)acetylation and trimethylsilylation [12].

2.3. *500 MHz ^{1}H-NMR spectroscopy*

Prior to ^{1}H-NMR spectroscopic analysis samples were repeatedly treated with ^2H_2O (99.96 atom% ^{2}H,Aldrich) at p^{2}H 7 and room temperature. 500 MHz ^{1}H-NMR spectra were recorded using a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands) operating in the pulsed Fourier-transform mode at a probe temperature of 27°C [13]. Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation [14]. Chemical shifts (δ) are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ = 2.225 ppm in ^2H_2O at 27°C).

3. RESULTS

For the release of the *N*-linked carbohydrate chains from glycocalicin the hydrazinolysis procedure was chosen [11], although the conditions applied also result in partial cleavage of GalNAcα-(1→*O*)Ser/Thr linkages [6]. After fractionation of the hydrazinolysate by paper electrophoresis, the acidic fractions were pooled and chromatographed on DEAE-Sephadex A-25 (see fig.1). The carbohydrate composition of the various fractions is summarized in table 1. Based on the GalNAc-ol, GalNAc and Man contents in table 1, it is clear that fractions I, II, V and VI contain only *N*-glycosidic type chains, III and IV only the *O*-glycosidic type and the remaining fractions both types of chains. Fractions I, II, VII, VIII and X did not contain sufficient carbohydrate material for structural analysis, whereas fractions III and IV contained alkaline degradation products of the *O*-glycosidic type of chains. In the following the fractions V, VI and IX will be discussed in more detail.

The major component of fraction VI turned out to be the disialylated diantennary *N*-acetyllactosamine type structure with an α1→6-linked Fuc residue at the Asn-bound GlcNAc unit, as reported

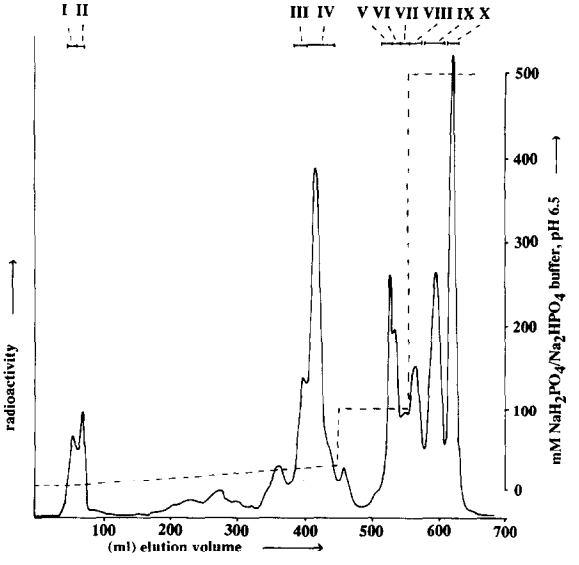


Fig.1. Fractionation pattern on a DEAE-Sephadex A-25 column (60 × 1.5 cm) of the ^{3}H-labelled acidic carbohydrate chains obtained after hydrazinolysis treatment of glycocalicin. The column is eluted with 400 ml of a linear concentration gradient from 5 to 30 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 6.5, followed by 100 ml of 100 mM and 100 ml of 500 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 6.5. Fractions of 3.7 ml are collected at a flow rate of 18.5 ml/h and assayed for ^{3}H radioactivity. Fractions are pooled as indicated by the bars.

in [9,10]. However, so far, the linkage type for the NeuAc residues at the specific antennae is not known. From the ^{1}H-NMR spectrum it could be deduced unambiguously that the Manα1→ branch bears specifically α2→3-linked NeuAc, and the Manα1→3 branch α2→6-linked NeuAc.

For fraction V the presence of a mixture of disialylated triantennary (2,4-disubstituted Man α1→3) and tetraantennary type of carbohydrate chains with an α1→6-linked Fuc unit at the Asn bound GlcNAc residue could be demonstrated by ^{1}H-NMR spectroscopy [9,10]. Concerning the assignments of the various sialic acid signals, it was found that α2→6-linked NeuAc is specifically attached at the Galβ1→4GlcNAcβ1→2Manα1→3 antenna and the other antennae can be terminated by α2→3-linked NeuAc. Each of the branches is partially asialo. The chromatographic behaviour of fraction V is in accordance with the occurrence of disialylated compounds. As minor constituents in fraction V compounds with the Fucα1→2Galβ1→4GlcNAcβ1→. sequence were detected.

Fraction IX contained a mixture of *N*- and *O*-glycosidic chains as was evident from the presence of GalNAc-ol and GalNAc besides GlcNAc-ol (see table 1). This fraction was subfractionated by Bio-Gel P-6 gel permeation chromatography. Sugar analyses of the subfractions indicated that IXa and IXb contain exclusively *N*-glycosidic and IXc and IXd *O*-glycosidic type carbohydrate chains. Because of the low amount of material and the complexity of the carbohydrate mixture present, fraction IXb will not be discussed. The ^{1}H-NMR spectrum of fraction IXa showed the typical ^{1}H-NMR structural-reporter-group signals of a fully sialylated tetraantennary structure, not demonstrated before for glycocalicin:

Table 1

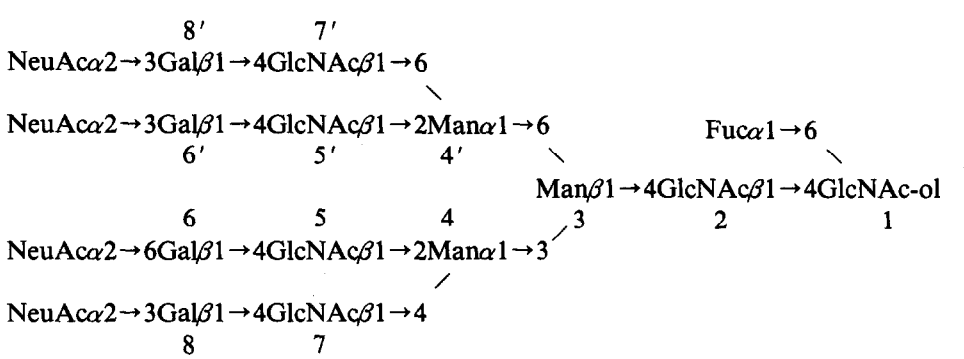
Molar carbohydrate composition of the desalted fractions I–X of the DEAE-Sephadex A-25 column

Mono-saccharides	Molar ratios in fractions									
	I	II	III	IV	V	VI	VII	VIII	IX	X
Fuc	1.4	1.5	–	–	0.9	0.9	1.1	0.9	0.9	–
Man	3.0	3.0	0.1	–	3.0	3.0	3.0	3.0	3.0	3.0
Gal	3.4	2.7	2.3	1.5	3.4	3.1	4.9	5.4	10.2	24.0
GlcNAc	3.0	2.8	1.0	1.0	3.8	3.8	4.8	5.4	6.9	15.6
GalNAc-ol	0.3	0.4	–	–	0.6	0.6	0.9	1.0	0.7	–
GalNAc	–	–	–	–	–	–	0.3	0.5	0.6	–
GalNAc-ol	–	–	0.3	0.1	–	–	–	–	1.3	1.2
NeuAc	+[a]	0.5	1.2	1.2	1.8	2.5	4.4	6.0	11.4	16.2

[a] Detectable, but <0.1

The molar ratios are calculated relative to 3 mol Man except fractions III and IV. These are calculated relative to 1 mol GlcNAc

Table 2 summarizes the relevant ^{1}H-NMR data of this fraction together with the data of two tetraantennary reference compounds [13,15]. The tetraantennary type of carbohydrate chain is reflected by the characteristic set of Man H-1 and H-2 structural-reporter-group signals for Man-3 (H-2, δ = 4.212 ppm), Man-4 (H-1, δ = 5.132 ppm; H-2, δ = 4.212 ppm), and Man-4′ (H-1, δ = 4.854 ppm; H-2, δ = 4.110 ppm). The α1→6 fucosylated *N,N′*-diacetylchitobiitol unit gives rise to the specific series of signals for GlcNAc-1-ol (NAc, δ = 2.055 ppm), GlcNAc-2 (NAc, δ = 2.085 ppm), and Fuc (H-1, δ = 4.900 ppm; CH_3, δ = 1.224 ppm) [16]. The NeuAc H-3 regions of the spectrum show multiplets indicating the presence of α2→3 (H-3e, δ = 2.757 ppm; H-3a, δ = 1.802 ppm) and α2→6 (H-3e, δ = 2.673 ppm; H-3a, δ = 1.717 ppm) linked NeuAc in the ratio 3:1. No asialo antennae are present since all Gal H-1 signals are typical for sialylated Gal residues. The Gal-6 residue is ex-



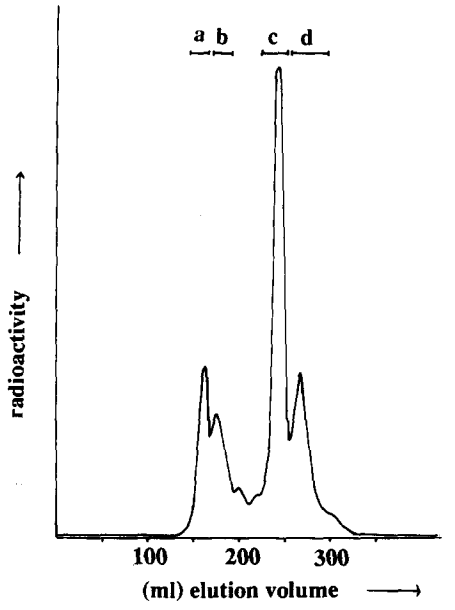


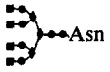
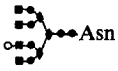
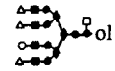
Fig.2. Fractionation pattern of fraction IX on a Bio-Gel P-6 column (110 × 2.5 cm) eluted with 100 mM ammonium acetate buffer, pH 5.2. Fractions of 4 ml are collected at a flow rate of 20 ml/h and assayed for ^3H radioactivity. Fractions are pooled as indicated by the bars.

clusively $\alpha 2\rightarrow 6$ sialylated, as is evident from the chemical shift of Man-4 H-1 ($\delta = 5.132$ ppm), in combination with the H-1 signal of Gal-6 ($\delta = 4.439$ ppm) and the NAc singlet of GlcNAc-5 ($\delta = 2.070$ ppm). The other Gal residues are $\alpha 2\rightarrow 3$ sialylated, which is concluded from the chemical shifts of the H-1 signals ($\delta = 4.55$ ppm). By comparison with the reference compounds, the NAc signal at $\delta = 2.076$ ppm can be assigned to GlcNAc-7 and that at $\delta = 2.040$ ppm to GlcNAc-5′ and GlcNAc-7′ [13,15].

^1H-NMR spectroscopic analysis of the fractions IXc and IXd, containing only *O*-glycosidic type chains, showed that the compound present in fraction IXc is identical with the main *O*-glycosidic oligosaccharide-alditol of glycocalicin reported earlier [4], i.e. NeuAc$\alpha 2\rightarrow$3Gal$\beta 1\rightarrow$4GlcNAc$\beta 1\rightarrow$6 (Neu Ac $\alpha 2\rightarrow$ 3Gal $\beta 1\rightarrow$ 3)Gal NAc- ol, whereas fraction IXd contained a mixture of IXc and the tetrasaccharide-alditol NeuAc$\alpha 2\rightarrow$3Gal$\beta 1\rightarrow$3(Neu Ac$\alpha 2\rightarrow$6)GalNAc-ol [4].

Table 2

^1H chemical shifts of structural-reporter-group protons of the constituent monosaccharides of fraction IXa obtained from glycocalicin, together with those of the reference compounds A [13] and B [15]

Reporter group	Residue[a]	Chemical shift[b] in[c]		
		A	B	IXa
				
H-1	GlcNAc-2	4.614	4.611	n.d.
	Man-3	4.757	4.76	n.d.
	Man-4	5.129	5.135	5.132
	Man-4′	4.868	4.870	4.854
	GlcNAc-5	4.573	4.593	n.d.
	GlcNAc-5′	4.596	4.593	n.d.
	GlcNAc-7	4.547	4.547	4.55
	GlcNAc-7′	4.553	4.554	4.55
	Gal-6	4.465	4.438	4.439
	Gal-6′	4.472	4.469	4.55
	Gal-8	4.470	4.469	4.55
	Gal-8′	4.481	4.480	4.55
	Fuc	–	–	4.900
H-2	GlcNAc-1-ol	–	–	4.22
	Man-3	4.210	4.218	4.212
	Man-4	4.224	4.229	4.212
	Man-4′	4.092	4.092	4.110
H-3	Gal-6′/8/8′	n.d.	n.d.	4.117
H-3a	NeuAc	–	1.717	1.717
	NeuAc′/*/″	–	–	1.802
H-3e	NeuAc	–	2.669	2.673
	NeuAc′/*/″	–	–	2.757
CH_3	Fuc	–	–	1.224
NAc	GlcNAc-1-ol	–	–	2.055
	GlcNAc-2	2.078	2.077	2.085
	GlcNAc-5	2.054	2.070	2.070
	GlcNAc-5′	2.042	2.042	2.040
	GlcNAc-7	2.079	2.078	2.076
	GlcNAc-7′	2.041	2.042	2.040
	NeuAc	–	2.030	2.031
	NeuAc′/*/″	–	–	2.031

[a] For numbering of monosaccharide residues, see text. NeuAc denotes the sialic acid $\alpha 2\rightarrow$6-linked to Gal-6, NeuAc′ the one $\alpha 2\rightarrow$3-linked to Gal-6′, NeuAc* the one $\alpha 2\rightarrow$3-linked to Gal-8, and NeuAc″ the one $\alpha 2\rightarrow$3-linked to Gal-8′

[b] Chemical shifts are given for neutral solutions at 27°C, in ppm downfield from internal 4,4-dimethyl-4-silapentane-1-sulfonate in ^2H_2O, acquired at 500 MHz (but were actually measured relative to internal acetone: $\delta = 2.225$ ppm)

[c] Structures are represented by short-hand notation [13]: ●, GlcNAc; ◆, Man; ■, Gal; △, NeuAc $\alpha 2\rightarrow$3; ○, NeuAc $\alpha 2\rightarrow$6; □, Fuc

4. DISCUSSION

In the past few years we [4,6,9] and other groups [7,8,10] have reported on the structural analysis of *N*- and *O*-glycosidic carbohydrate chains of glycocalicin. The high carbohydrate content (40%) comprising both types of chains gave rise to severe problems in selective release of the *N*- as well as *O*-linked chains. In fact, hydrazinolysis or alkaline borohydride degradation of the intact glycoprotein yielded mixtures of *N*- and *O*-linked oligosaccharide chains ([4,6] and this study). Our recently introduced approach for the analysis of the carbohydrate chains of *N,O*-glycoproteins using enzymatic release for the *N*-linked chains by peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase-F, followed by the alkaline borohydride method for the *O*-linked chains, has shown to be a promising alternative [17].

The *N*-linked carbohydrate chains of glycocalicin include sialylated di-, tri- (2,4-disubstituted Manα1→3 residue), and tetraantennary *N*-acetyllactosamine type structures with an α1→6-linked Fuc residue at the Asn-bound GlcNAc unit. Concerning the sialylation pattern of these three oligosaccharide chains, we have demonstrated that the Galβ1→ 4Glc NAcβ1→ 2Manα1→ 3 antenna bears exclusively an α→6-linked Neu-Ac residue. The α2→3 linkage of NeuAc occurs in the additional antennae. As mentioned, there are indications that α1→2-linked Fuc is also involved to some extent in terminating Galβ1→4GlcNAcβ1→. antennae. It should be noted that heterogeneity in branching pattern for a glycoprotein is often observed in serum glycoproteins, but so far not in membrane glycoproteins. The earlier reported structures for the *O*-linked carbohydrate chains are also terminated by α2→3-linked NeuAc and α1→2-linked Fuc at Galβ1→4GlcNAcβ1→. sequences [4,6,7,8].

Glycocalicin can be split into a 90 kDa fragment and a 45 kDa fragment [18]. Carbohydrate analysis of these fragments (not shown) shows that the 90 kDa fragment contains almost exclusively *O*-linked and the 45 kDa fragment *N*-linked carbohydrate chains. Glycocalicin contains 4 potential *N*-glycosylation sites [5] and the 45 kDa region two sites that are definitively *N*-glycosylated [19]. Experiments with different proteases indicate that the abundant *O*-glycosidic carbohydrate chains protect the glycoprotein against proteolysis [18]. The thrombin-binding site as well as the receptor site for von Willebrand factor are both located on the 45 kDa fragment [18]. Using a series of exoglycosidases, results were obtained suggesting that removal of sialic acid and galactose have no influence on the binding of von Willebrand factor to glycocalicin, but partial removal of *N*-acetylglucosamine reduces the binding of von Willebrand factor. It has still to be established if the carbohydrate residues indeed form part of the receptor site. The alternative explanation that removal of carbohydrate chains causes a conformational change cannot be excluded as yet.

Acknowledgements: The authors thank Mr W. Schnippering (Bern) for the preparation of glycocalicin and Mr G.J. Gerwig (Utrecht) for skilful assistance with the sugar analyses. Analyses on the Mono Q column were performed with the assistance of Drs J.G.M. Bolscher and W.P. van Beek at the Netherlands Cancer Institute (Amsterdam). This investigation was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO) and by the Netherlands Foundation for Cancer Research (KWF, grant UUKC-OC 83-13).

REFERENCES

[1] Berndt, M.C. and Phillips, D.R. (1981) in: Research Monographs in Cell and Tissue Physiology, vol. 5 (Dingle, J.T. and Gordon, J.L. eds) pp.43–75, Elsevier/North-Holland, Amsterdam.
[2] Nachman, R.L., Leung, L.L.K. and Polley, M.J. (1985) in: Platelet Membrane Glycoproteins (George, J.N. et al. eds) pp.245–257, Plenum, New York.
[3] Clemetson, K.J. (1983) Blood Cells 9, 319–329.
[4] Korrel, S.A.M., Clemetson, K.J., Van Halbeek, H., Kamerling, J.P., Sixma, J.J. and Vliegenthart, J.F.G. (1984) Eur. J. Biochem. 140, 571–576.
[5] Lopez, J.A., Chung, D.W., Fujikawa, K., Hagen, F.S., Papayannopoulou, T. and Roth, G.J. (1987) Proc. Natl. Acad. Sci. USA 84, 5615–5619.
[6] Korrel, S.A.M., Clemetson, K.J., Van Halbeek, H., Kamerling, J.P., Sixma, J.J. and Vliegenthart, J.F.G. (1985) Glycoconj. J. 2, 229–234.
[7] Judson, P.A., Anstee, D.J. and Clamp, J.R. (1982) Biochem. J. 205, 81–90.
[8] Tsuji, T., Tsunehisa, S., Watanabe, Y., Yamamoto, K., Tohyama, H. and Osawa, T. (1983) J. Biol. Chem. 258, 6335–6339.
[9] Korrel, S.A.M., Clemetson, K.J., Van Halbeek, H., Kamerling, J.P., Sixma, J.J. and Vliegenthart, J.F.G. (1984) Abstr. XIIth Int. Carbohydr. Symp. (Vliegenthart, J.F.G. et al. eds) p.423, Vonk, Zeist.
[10] Tsuji, T. and Osawa, T. (1987) J. Biochem. 101, 241–249.

[11] Takasaki, S., Mizuochi, T. and Kobata, A. (1982) Methods Enzymol. 83, 263–268.
[12] Kamerling, J.P. and Vliegenthart, J.F.G. (1982) Cell Biol. Monogr. 10, 95–125.
[13] Vliegenthart, J.F.G., Dorland, L. and Van Halbeek, H. (1983) Adv. Carbohydr. Chem. Biochem. 41, 209–374.
[14] Ernst, R.R. (1966) Adv. Magn. Reson. 2, 1–135.
[15] Joziasse, D.H., Schiphorst, E.C.M., Van den Eynden, D.H., Van Kuik, J.A., Van Halbeek, H. and Vliegenthart, J.F.G. (1987) J. Biol. Chem. 262, 2025–2033.
[16] Cahour, A., Debeire, P., Hartmann, L., Montreuil, J., Van Halbeek, H. and Vliegenthart, J.F.G. (1984) FEBS Lett. 170, 343–349.
[17] Damm, J.B.L., Kamerling, J.P., Van Dedem, G.W. and Vliegenthart, J.F.G. (1987) Glycoconj. J. 4, 129–144.
[18] Wicki, A.N. and Clemetson, K.J. (1987) Eur. J. Biochem. 163, 43–50.
[19] Titani, K., Takio, K., Handa, M. and Ruggeri, Z.M. (1987) Proc. Natl. Acad. Sci. USA 84, 5610–5614.
[20] Michelson, A.D., Loscalzo, J., Melnick, B., Coller, B.S. and Handin, R.I. (1986) Blood 67, 19–26.