Primary structure of the major glycans of the *N*-acetyllactosamine type derived from the human immunoglobulins M from two patients with Waldenström's macroglobulinemia

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The carbohydrate chains of the pathological human immunoglobulins M from two patients with Waldenström's macroglobulinemia were released by hydrazinolysis. The *N*-acetyllactosamine-type glycans were obtained by affinity chromatography on concanavalin A and fractionated by high-voltage paper electrophoresis. The primary structure of the major compounds was elucidated on the basis of carbohydrate analysis, methylation analysis, including mass-spectrometry, and 500 MHz ^{1}H-NMR spectroscopy. For both patients, this appeared to be a monosialyl monofucosyl biantennary structure; the compounds differed by the presence of an intersecting *N*-acetylglucosamine residue.

Immunoglobulin M *Waldenström's macroglobulinemia* N-*Glycosidic glycan* *500 MHz ^{1}H-NMR spectroscopy*

1. INTRODUCTION

Immunoglobulins M (IgMs) are glycoproteins that have a relatively high (7–15%) carbohydrate content and show a large chemical heterogeneity of the oligosaccharides conjugated to the heavy chains. In [1] an illustration of such a heterogeneity has been given: oligosaccharides derived from normal as well as from pathological human IgMs could be fractionated by affinity-chromatography into *N*-acetyllactosamine-type and oligomannoside-type carbohydrate chains.

Abbreviations: Fuc, L-fucose; Gal, D-galactose; Man, D-mannose; GlcNAc, *N*-acetyl-D-glucosamine; NeuAc, *N*-acetylneuraminic acid; IgM, immunoglobulin M; Con A, concanavalin A

The structure of the oligosaccharides of immunoglobulins M has been investigated for more than a decade. Meanwhile, their oligomannoside-type structures are rather well established [2–5]; however, as to the *N*-acetyllactosamine type, so far only a structure of the biantennary type was found, either fucosylated [6] or non-fucosylated; the latter forms part of, for example, the J-chain of a pathological human IgM [7]. In addition, for mouse plasmocytoma IgM a glycan of the triantennary type was described [8].

Here we report the primary structure of each of the major glycans of the *N*-acetyllactosamine type, derived from two pathological human IgMs. The structural studies involved hydrazinolysis, followed by determination of carbohydrate composition, methylation analysis including mass-spectrometry, and 500-MHz ^{1}H-NMR spectroscopy.

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2. MATERIALS AND METHODS

Pathological human IgMs were prepared from plasmaphaereses of patients (ZAJ) and (GRA) with Waldenström's macroglobulinemia as in [9]; i.e., IgM(ZAJ) by cryoprecipitation and IgM(GRA) by euglobulin precipitation. NaB^3H_4-reduced oligosaccharides obtained from the native molecules by hydrazinolysis and subsequent reduction were separated by affinity chromatography on a Con A column. The glycans were characterized previously [1], with respect to their binding capacity with Con A [10], as oligomannoside-type and *N*-acetyllactosamine-type chains. The major fractions of the latter type of IgM(ZAJ) (41%) and of IgM(GRA) (58%) were further subfractionated by high-voltage electrophoresis on Whatman 3MM paper in [buffer] pyrimidine–acetic acid–water (18:6:2320, by vol., pH 5.4) at 75 V/cm for 3 h at 4°C. Radioactive strips were cut and eluted with water. Standards used were asialyl (N), monosialyl (MS) and disialyl (DS) biantennary structures [11,12] obtained from human serum transferrin by hydrazinolysis.

Molar ratios of monosaccharides were determined after methanolysis and trifluoroacetylation by gas-liquid chromatography [13]. Exhaustive methylation of reduced oligosaccharides was carried out as in [14]. The methylglycosides obtained by methanolysis of permethylated oligosaccharides were peracetylated and identified by gas-liquid chromatography coupled to mass-spectrometry, as in [15]

For NMR analysis, the reduced oligosaccharide fractions were repeatedly treated with D_2O at pD ~ 7 and room temperature. After each exchange treatment, the materials were lyophilized. Finally, each sample was dissolved in 400 μl D_2O (99.96 atom% D, Aldrich). 500-MHz ^{1}H-NMR spectroscopy was performed on a Bruker WM-500 spectrometer (SON hf-MNR facility, Department of Biophysical Chemistry, Nijmegen University, The Netherlands), operating under control of an Aspect-2000 computer. Experimental details have been described [16]. The probe temperature was kept at 27 (±0.1)°C. Chemical shifts are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), but were actually measured by reference to internal acetone (δ 2.225 in D_2O at 27°C), with an accuracy of 0.002 ppm.

3. RESULTS AND DISCUSSION

3.1. *Separation of* N-*acetyllactosamine-type glycans by high-voltage electrophoresis*

Reduced oligosaccharides obtained from the two pathological IgMs (ZAJ and GRA) were fractionated by affinity chromatography on a Con A column [10] into biantennary *N*-acetyllactosamine-type structures exhibiting a weak affinity for Con A and more complex structures having no affinity for Con A. Each of these fractions was submitted to high-voltage paper electrophoresis, affording 3 subfractions (fig.1). The latter comigrated with the reference compounds, namely,

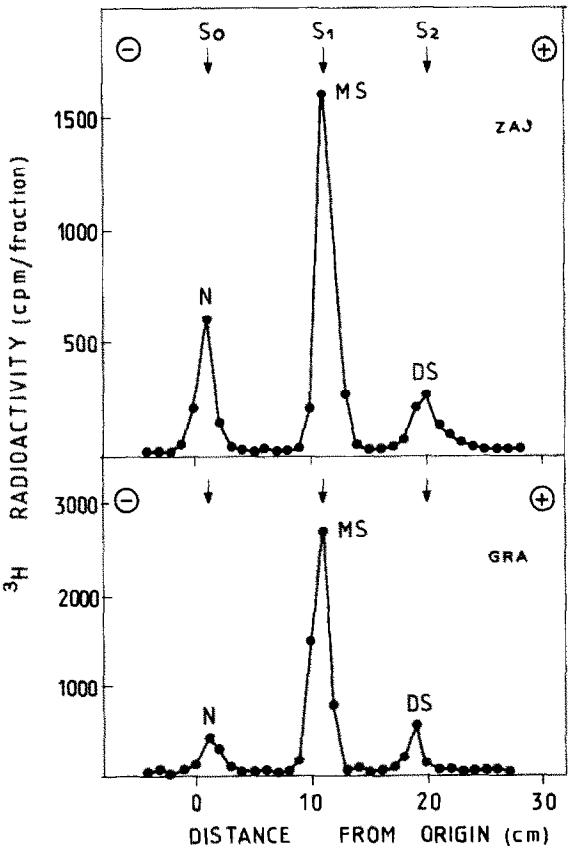


Fig.1. Radioelectropherogram of the NaB^3H_4-reduced oligosaccharides derived from IgM(ZAJ) and from IgM(GRA) by hydrazinolysis (for details, see section 3.1). N, neutral; MS, monosialyl; DS, disialyl oligosaccharide fractions. The arrows indicate the position of the following standards: asialylated (S_0), monosialylated (S_1) and disialylated (S_2) biantennary glycans from human serum transferrin.

Table 1

Molar carbohydrate composition of fractions (MS) obtained by high-voltage electrophoresis from IgM(ZAJ) and IgM(GRA)

Monosaccharide	IgM(ZAJ)	IgM(GRA)
Fuc	1.20	0.74
Gal	1.90	2.00
Man	3.00	3.00
GlcNAc	2.80	4.00
GlcNAcitol	0.73	0.53
Neu Ac	0.79	0.64

The molar ratios were calculated on the basis of 3 mannose residues

the neutral (N), monosialyl (MS) and disialyl (DS) oligosaccharides. For IgM(ZAJ), proportions of these sub-fractions were found to be: N, 23%; MS, 61%; DS, 16%, and for IgM(GRA) they were: N, 15%; MS, 73% DS, 12%.

These fractions appeared homogeneous by thin-layer chromatography (TLC) on silica gel for IgM(ZAJ), but heterogeneous for IgM(GRA).

Table 2

Molar ratios of monosaccharide methyl ethers present in the methanolysates of the permethylated major fractions MS isolated from IgM(ZAJ) and IgM(GRA)

Monosacharide methyl ethers	Fraction MS from IgM(ZAJ)	Fraction MS from IgM(GRA)
(2,3,4)-Me_3-Fuc	0.5	0.4
(2,3,4,6)-Me_4-Gal	0.7	0.7
(2,3,4)-Me_3-Gal	1.0	1.0
(3,4,6)-Me_3-Man	1.5	2.2
(3,6)-Me_2-Man	–	0.0[a]
(3,4)-Me_2-Man	–	0.1
(2,4)-Me_2-Man	0.8	0.1
(2)-Me_1-Man	–	0.9
(1,3,5)-(1,3,5)-Me_3-GlcN(Me)Acitol	0.5	0.4
(3,4,6)-Me_3-GlcN(Me)Ac	–	0.9
(3,6)-Me_2-Glc(Me)Ac	2.2	2.6
(4,7,8,9)-Me_4-Neu(Me)Ac	0.7	0.5

[a] Traces, not determined

The molar ratios were calculated on the basis of 1 residue of (2,3,4)-Me_3-Gal (=2,3,4-tri-*O*-methyl galactose)

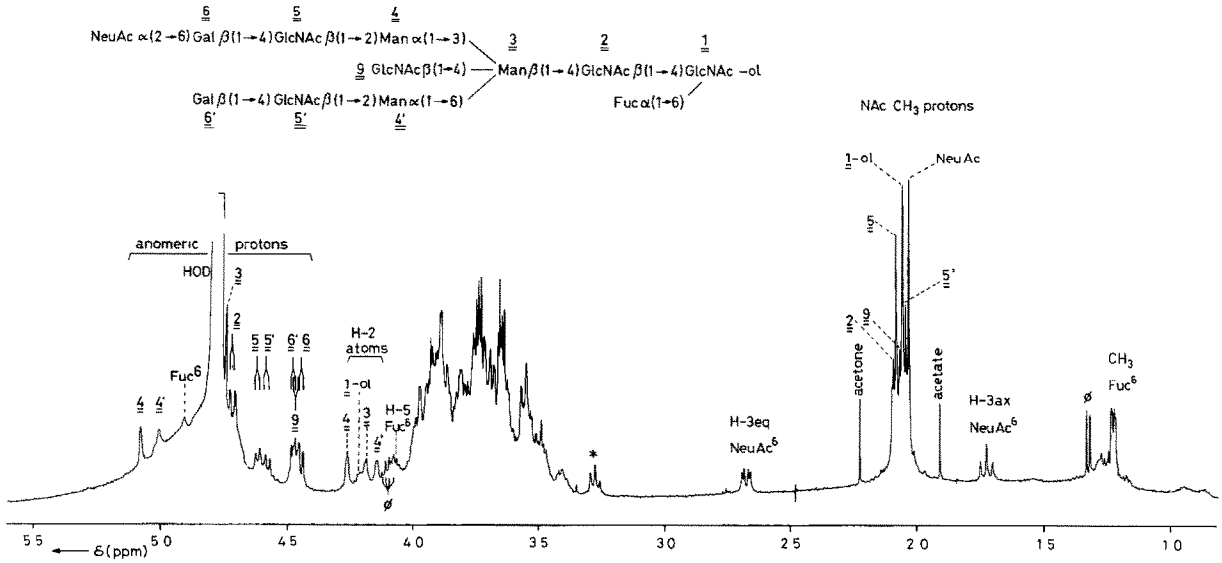


Fig.2. 500-MHz ^{1}H-NMR spectrum (D_2O; pD 7; 27°C) of the monosialyl reduced-oligosaccharide fraction obtained from IgM(GRA). The numbers in the spectrum refer to the corresponding residues in the structure. The appearance of the asterisk-marked doublet-of-doublets at δ 3.27, clearly apart from the bulk of skeleton-proton signals, has been proposed [17] to be correlated with the presence of GlcNAc-9 in the structure. So far, this signal could not be assigned unambiguously.

3.2. *Primary structure determination*

The primary structure of the major fractions MS obtained from IgMs(ZAJ) and (GRA) was established on the basis of sugar composition (table 1), the results of methylation analysis (table 2) and 500-MHz ^{1}H-NMR spectroscopy.

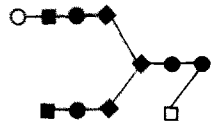
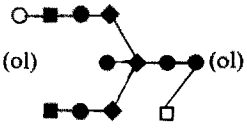
500-MHz ^{1}H-NMR spectra were recorded of neutral D_2O-solutions of the fractions MS obtained from IgM(ZAJ) and IgM(GRA). The spectrum of the latter is depicted in fig.2. The chemical shifts of the structural-reporter groups for both fractions have been compiled in table 3.

The ^{1}H-NMR data for the IgM(ZAJ) preparation point to the presence of a monosialyl biantennary oligosaccharide-alditol of the *N*-acetyllactosamine type. The reasoning is as follows. The reduced GlcNAc-1 lacks an anomeric proton signal, but instead it has its H-2 signal as structural-reporter group at δ 4.220, among the Man H-2 signals. The *N*-acetyl singlet at δ 2.057 is ascribed to this GlcNAc-ol residue (cf. [18–20]). H-1 and the NAc methyl protons of GlcNAc-2, $\beta(1\rightarrow4)$ linked to GlcNAc-1-ol, were found to resonate at δ 4.714 and δ 2.088, respectively (see table 3). The deviations of these values from those reported previously, namely, δH-1 4.637 and δNAc 2.076 [18,19], are attributed to the presence of Fuc in $\alpha(1\rightarrow6)$-linkage to GlcNAc-1-ol. The 4,6-disubstitution of GlcNAc-ol is evident from the results of methylation analysis (table 2). Fuc being linked to C-6 is further substantiated by the magnitude of the downfield shifts of H-1 and NAc of GlcNAc-2 as compared to afucosyl structures (for H-1, $\Delta\delta \sim 0.08$ ppm; for NAc, $\Delta\delta = 0.012$ ppm, cf. [16]). The coupling constant $J_{1,2}$ for Fuc, being 3.8 Hz, indicates the α-anomeric configuration of this linkage. The set of chemical shift values for the Fuc reporter groups, i.e., H-1, H-5 and CH_3, are considerably altered due to the presence of GlcNAc-ol instead of GlcNAc $\beta(1\rightarrow N)$-Asn (cf. table 3 with [16]). The remaining set of chemical shift values listed for IgM(ZAJ) in table 3 shows a very close resemblance with those reported for a corresponding glycopeptide and reducing oligosaccharide (compounds 31 and 27 [16]). This defines the type of branching to the biantennary, and the NeuAc residue to be $\alpha(2\rightarrow6)$-linked to Gal-6 rather than to Gal-6′ [16].

On the basis of similar features in the spectra ob-

Table 3

^{1}H chemical shifts of structural reporter groups of constituent monosaccharides for the monosialylated oligosaccharide-alditols obtained from IgM of patients ZAJ and GRA

Reporter group	Residue[a]	Chemical shift[b,c] in	
		IgM(ZAJ)	IgM(GRA)
			
H-1	GlcNAc-2	4.714	4.716
	Man-3	4.760	4.705
	Man-4	5.136	5.077
	Man-4′	4.924	5.005
	GlcNAc-5	4.605	4.617
	GlcNAc-5′	4.581	4.576
	Gal-6	4.445	4.444
	Gal-6′	4.470	4.476
	GlcNAc-9	–	4.467
	Fuc	4.896	4.904
H-2	GlcNAc-1-ol	4.220	4.214
	Man-3	4.258	4.187
	Man-4	4.197	4.260
	Man-4′	4.110	4.142
H-3ax	NeuAc	1.720	1.722
H-3eq	NeuAc	2.668	2.673
H-5	Fuc	4.071	4.068
CH_3	Fuc	1.224	1.224[d]
NAc	GlcNAc-1-ol	2.057	2.055
	GlcNAc-2	2.088	2.092
	GlcNAc-5	2.071	2.081
	GlcNAc-5′	2.048	2.042
	GlcNAc-9	–	2.066
	NeuAc	2.031	2.031

[a] For numbering of monosaccharide residues and complete structures, see fig.2,3

[b] Chemical shifts are given for neutral solutions at 27°C, in ppm downfield from internal DSS in D_2O

[c] Compounds are represented by schematic structures (cf. [16]); (●—) GlcNAc, (◆—) Man, (■—) Gal, (□—) Fuc, (○—) NeuAc

[d] A doubling of this doublet (δ 1.224 and 1.220) was observed upon resolution-enhancement of the spectrum. So far this feature could not be explained

Fig.3. Primary structures of the major *N*-acetyllactosamine-type glycans isolated from IgM(ZAJ) (A) and IgM(GRA) (B).

tained from IgM(GRA) (see fig.2) and IgM(ZAJ), the presence of the (→4)GlcNAc $\beta(1\rightarrow4)$ [Fuc $\alpha(1\rightarrow6)$]-GlcNAc-ol moiety is concluded. The typical set of Man H-1 and H-2 chemical shifts for IgM(GRA) (see table 3) point to the intersected biantennary type of branching, as was observed in [16,21–23]. The typical parameters of the intersecting GlcNAc-9 residue, namely, its H-1 signal at δ 4.467 ($J_{1,2}$ 8.0 Hz), and its NAc singlet at δ 2.066, are not influenced by the reduced nature of GlcNAc-1. As with IgM(ZAJ), the sialic acid residue could readily be located by NMR in $\alpha(2\rightarrow6)$-linkage to Gal-6 in the $(1\rightarrow3)$-linked branch, because of the specific influences on the chemical shifts of H-1 of Man-4 and Gal-6, and of NAc of GlcNAc-5, as compared to the corresponding asialylated structure [16,21].

The structures of the glycans present in fractions MS from IgM(ZAJ) and IgM(GRA), as deduced by ^{1}H-NMR spectroscopy in combination with sugar and methylation analyses, are presented in fig.3. In addition, it should be noted that for IgM(GRA), methylation results, in particular the finding of (3,6)-Me_2-Man and (3,4)-Me_2-Man (see table 2), suggested tri- and/or tetraantennary oligosaccharides to occur also in fraction MS, besides the major, intersected biantennary glycan. This is consistent with the fact that this fraction does not react with Con A, and may account for the heterogeneity observed by TLC. Such tri- and/or tetraantennary structures were not detected by NMR spectroscopy, probably due to the low content (10%) of that type of glycan. The percentage can be derived from the ratio of (3,4)-Me_2-Man to (2)-Me_1-Man, being 0.1:0.9.

4. CONCLUSIONS

The primary structures proposed for the major *N*-acetyllactosamine-type glycans from pathological IgM(ZAJ) and IgM(GRA) are given in fig.3. They differ with respect to the intersecting GlcNAc residue. The biantennary type of structure has already been described for human IgM [6,7], and for numerous other glycoproteins [24]. The intersected structure has so far been found only in a limited number of glycoproteins e.g., in glycophorin A [25], but mainly in immunoglobulins like human serum IgG [26,27], IgA [28], IgD [29,30] and sIgA [23], and not in human IgM or in mouse plasmocytoma IgM.

With respect to the ^{1}H-NMR features of the compounds, it can be concluded that modification of GlcNAc-1 does not affect the chemical shifts of reporter group signals of residues which are more remote from GlcNAc-1 than the adjacent residues GlcNAc-2 and Fuc. This is in accordance with earlier observations [18,19]. The library of reference data compiled for glycopeptides and reducing oligosaccharides ending in GlcNAc-2 [16] retains its validity, also for reduced oligosaccharides ending in GlcNAc-1-ol. It should be emphasized that well-defined oligosaccharide-alditols prepared by reduction of GlcNAc-1 after having accomplished the release of the oligosaccharide by hydrazinolysis, have to be preferred for structural analysis far above the weakly defined products upon omitting the reduction step [22,31].

The intersecting GlcNAc residue is known to exert a pronounced influence on the conformation and, by consequence, on the biosynthesis of a biantennary oligosaccharide chain [16,32]. It remains a challenge to find out whether the presence of this intersecting GlcNAc in the carbohydrate chains of pathological IgM is somehow correlated with the clinical features of the patient.

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