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Glycosylation Pattern of Kappa Light Chains in Massive Cutaneous Hyalinosis

ANTTI KONTIOLA[1], C P J MAURY[2], ANNA-MAIJA TEPPO[2], JUHA VIITALA[1] and JOHAN JÄRNEFELT[1]

[1]*Department of Medical Chemistry and* [2]*Fourth Department of Medicine, University of Helsinki, SF-00170 Helsinki, Finland*

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In the light of the hypothesis that the kappa light chain accumulation in massive cutaneous hyalinosis (MCH) is related to an abnormal glycosylation pattern, we analyzed the oligosaccharide structures of the cold precipitable kappa light chains excreted in the urine of an MCH patient.

The MCH kappa chains contain about 25% carbohydate. Concanavalin A-Sepharose affinity chromatography of the glycopeptides obtained from pronase digests showed that the bulk of the glycopeptides (82%) did not bind to the column; 16% had a weak affinity, and 2% a strong affinity. Methylation analyses indicated that the unbound fraction consisted mainly of tetra-antennary glycopeptides, but tri-antennary and bisecting structures were also found. Most of the weakly-bound glycopeptides had a bi-antennary carbohydrate structure.

Massive cutaneous hyalinosis (MCH) represents a new type of hyalin disease which differs from colloid milium, cutaneous amyloidosis and lipoid proteinosis [1, 2]. It is characterized by nonfibrillar, Congo-red negative, periodic acid-Schiff positive extracellular deposits in the dermis and subcutaneous tissues and, to a lesser extent, in the intestine. Recent biochemical and immunochemical studies have revealed a realtionship between MCH and the gammopathies [2, 3]. A major expression of the disease is the dermal accumulation of monoclonal kappa light chains, and MCH can be classified as a light chain deposition disease.

In MCH, kappa light chains are also excreted in the urine where the major part of them are cold precipitable and have a high content of carbohydrate. Preud'Homme and associates [4] have demonstrated the production of abnormal kappa light chains that were probably glycosylated in two cases of nonamyloid visceral kappa light chain deposition. In view of these findings, we hypothesized that the mechanism causing the accumulation of kappa light chains in MCH may be related to their abnormal glycosylation. We therefore conducted a closer study of the carbohydrate structures of the kappa

light chains in MCH. Our results demonstrate a unique glycosylation pattern of urinary cold precipitable kappa light chains, a major part of the carbohydrate has a complex, tetra-antennary structure which is in contrast to the bi-antennary structures found in myeloma kappa and lambda light chains [5, 6, 7].

Materials and Methods

Reagents

Concanavalin-A-Sepharose, Sephadex G-25, G-50 Fine gels, Blue-Sepharose CL-6B and Blue Dextran were from Pharmacia (Uppsala, Sweden). Pronase, fetuin, transferrin, bovine serum albumin and Dalton mark VII-L were from Sigma Chemical Co. (St. Louis, MO, USA). α_1-Acid glycoprotein was a generous gift from Dr. G. Myllylä, Finnish Red Cross Blood Transfusion Service (Helsinki, Finland). ^{3}H-Acetic anhydride was from Amersham International (Amersham, UK). *Vibrio cholerae* neuraminidase was from Behringwerke AG (Marburg, W. Germany).

Preparation of Glycopeptides

^{3}H-Labeled tetra-antennary, tri-antennary and bi-antennary glycopeptides were prepared from α_1-acid glycoprotein, fetuin and transferrin, respectively [8, 9]. Asialotransferrin glycopeptides were prepared by incubating transferrin glycopeptides in Tris-HCl buffer (pH 6.8) containing neuraminidase (5U/0.2 ml) at 37°C for 24 h.

Kappa chains were isolated from the urine of one patient with MCH [2] and of two patients with IgG-kappa myeloma. The MCH kappa chains were precipitated from 0.1 M NaOH neutralized (pH 7) urine at 4°C overnight. After centrifugation the precipitate was carefully washed with ice-cold 0.05 M sodium phosphate buffer (pH 7.3) and further purified by agarose gel electrophoresis in 0.075 M barbital-sodium barbital buffer (pH 8.6) [10]. The myeloma kappa chains were precipitated from the urine of two patients with half-saturated ammonium sulphate. Blue-Sepharose chromatography in 0.05 M Tris-HCl (pH 7.0) containing 0.1 M KCl was used to remove albumin [11]. Kappa chains were further purified by preparative agarose gel electrophoresis as above [10].

Whole kappa chains were digested with pronase [9] and glycopeptides were isolated by gel filtration on Sephadex G-25 (20 cm × 1 cm, bed volume 16 ml) in 10 mM pyridine acetic acid buffer (pH 5.0). The glycopeptides were *N*[^{3}H] acetylated in their peptide moiety with [^{3}H]acetic anhydride in 0.1 M $NaHCO_3$ at room temperature for 30 min. The *N*[^{3}H]acetylated glycopeptides were separated from free radioactivity by lyophilizing three times and by gel filtration as above.

The glycopeptides from the MCH kappa chains were fractionated by Concanavalin-A affinity chromatography (bed volume 3 ml) [12]. The unbound fraction obtained from Con-A was subjected to mild alkaline degradation [13] and further purified by Sephadex G-50 gel filtration (1 × 46 cm, bed volume 36 ml) in 0.1 M pyridine acetic acid buffer (pH 5.0). The weakly and strongly bound fractions were also purified by gel filtration on Sephadex G-50.

Glycopeptides from myeloma patients were not fractionated by Con-A.

Table 1. Carbohydrate composition of kappa chains. The values are expressed as nmol/mg protein (M1 and M2 are myeloma kappa chains).

	MCH	M1	M2
Fucose	64	n.d.[a]	n.d.
Mannose	228	3.9	13
Galactose	286	4.8	18
N-Acetylgalactosamine	224	0.6	2.6
N-Acetylglucosamine	260	3.3	13
N-Acetylneuraminic acid	118	2.8	11
% Carbohydrate	25	0.3	1.2

[a] Background too noisy to detect fucose by GC.

Analytical Methods

Protein was determined according to the method of Lowry *et al.* [14] as modified by Hartree [15].

The molecular size of glycopeptides was estimated by Sephadex G-50 gel filtration [16], using *N*-glycosidic glycopeptides of transferrin, asialotransferrin, fetuin and α_1-acid glycoprotein as standards. Radioactivity was monitored by LKB minibeta 1211 and 1212 liquid scintillators.

Monosaccharides were analyzed after methanolysis by gas chromatography [17, 18, 19] using a packed SE-30 column. *N*-Acetylneuraminic acid was analyzed colorimetrically [20, 21].

The substitution patterns of the monosaccharides were determined by mass fragmentography on a Hewlett-Packard 5992A mass-spectrometer. Glycopeptides were permethylated by the modified [22] method of Hakomori [23], and after acetolysis/acid hydrolysis the partially methylated alditol acetate derivatives of hexoses were analyzed in a 3% OV-210 column using a temperature gradient from 184°C to 210°C at a rate of 1°C/min, and detected m/z values of 117, 161, 189 and 233 [24]. Detection of the partially methylated hexosaminitols was carried out at m/z values of 116, 158 and 170 after separation in a 2.2% OV-101 column at 220°C [22, 24].

Polyacrylamide gel electrophoresis [25] was performed using a 5-20% gradient of polyacrylamide and Coomassie Blue Staining. Molecular weight was estimated [26] comparing the mobility of the samples with known standards.

Amino acid composition was determined with a Kontron Liquimat III amino acid analyzer after hydrolysis in 6 M HCl at 120°C in a nitrogen atmosphere for 18 h.

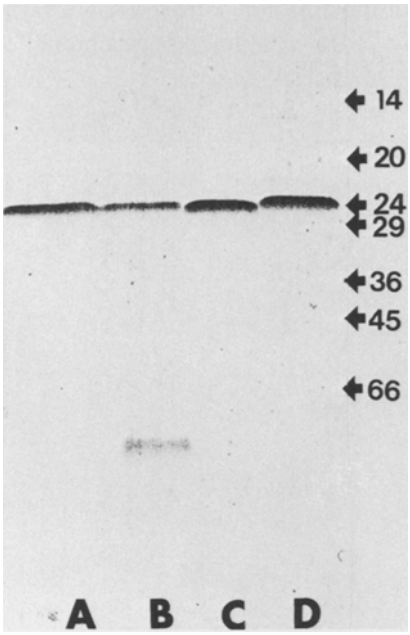

Figure 1. Polyacrylamide gel electrophoresis of MCH and myeloma kappa chains. A: from the urine of the MCH patient. B: from tissue deposits from the MCH patient. C: myeloma 1. D: myeloma 2. Molecular weight standards are shown (kDa).

Results

Carbohydrate Composition and Molecular Weight

The carbohydrate content of the MCH kappa-chains was greater than that of kappa chains from two patients with multiple myeloma (Table 1). The molecular weight of the studied kappa chains was about 24 000 as determined by polyacrylamide gel electrophoresis (Fig. 1).

Concanavalin A Affinity Chromatography and Sephadex G-50 Gel Filtration

The glycopeptides were fractionated on Con-A-Sepharose. Most of the carbohydrate did not bind to Con-A and eluted with buffer in fraction A (82%). A smaller amount bound weakly and eluted with 20 mM α-methylmannoside in fraction B (16%) and some bound strongly in fraction C (2%). Sephadex G-50 gel filtration (Fig. 2) indicates that fraction A had the same hydrodynamic volume as glycopeptides from α_1-acid glycoprotein and fetuin, which contain tetra-antennary and tri-antennary complex type oligosaccharides. Fraction B eluted from G-50 in about the same volume as transferrin glycopeptides, which are bi-antennary. Results of fraction C are not presented.

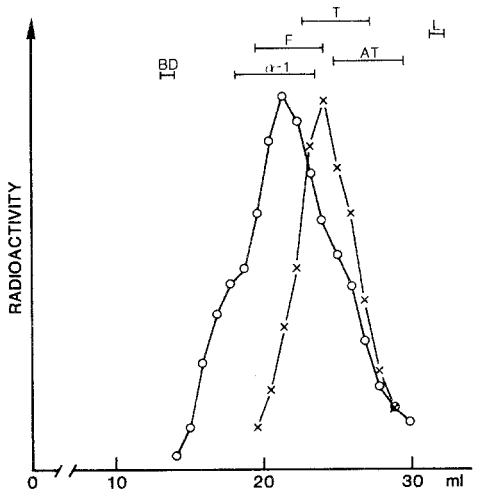

Figure 2. Estimation of molecular weights of the Con-A unbound (○) and Con-A bound (×) glycopeptides of MCH kappa chains. Fractions were analyzed for radioactivity. The glycopeptides were chromatographed on a Sephadex G-50 column (see the Materials and Methods section). For comparison the elution volumes of blue dextran (BD), α_1-acid glycopeptide (α-1, M_r = 4400), fetuin glycopeptide (F, M_r = 3400), transferrin glycopeptide (T, M_r =2400), asialotransferrin glycopeptide (AT, M_r = 1800) and lactose (L, M_r = 360) are shown.

Methylation Analysis

The results of the methylation analysis are summarized in Table 2.

In fraction A, the almost equal number of 2,4-di-*O* and 2,6-di-*O*-substituted mannose residues and only small amounts of 2-mono-*O*-substituted mannose suggest a tetra-antennary structure. The existence of 3,4,6-tri-*O*-substituted mannose and terminal *N*-acetylglucosamine indicates a small proportion of bisecting structures i.e. terminal *N*-acetylglucosamine linked to proximal mannose.

In fraction B there was mainly 2-mono-*O*-substituted mannose apart from the branching 3,6-di-*O*-substituted mannose. Fraction B thus consisted mainly of bi-antennary structures.

Terminal fucose was found in both fractions and was probably 6-*O*-linked to the proximal *N*-acetylglucosamine.

The methylation data on glycopeptides from two myeloma patients are also included in Table 2. As there were less 2,4-di-*O*- and 2,6-di-*O*-subsituted mannose residues than in the glycopeptides of the MCH patient, the glycopeptides contained fewer branched glycans.

The methylation data indicate a great variety of oligosaccharide structures (Fig. 3).

Amino Acid Composition

The amino acid composition of MCH and myeloma kappa chains are presented in Table 3.

Table 2. Relative amounts of substituted sugars in fractions A and B of MCH kappa chain glycopeptides and of total glycopeptides from two myeloma patients. Values are expressed as mol/3 mol mannose. The relatively low amount of *N*-acetylglucosamine is probably due to the resistance of the core *N*, *N'*-diacetylchitobiose unit to hydrolysis (M1=myeloma 1, M2=myeloma 2).

Compound	Glycosidic linkage	A (82%)	B (16%)	M1	M2
Fucose					
2,3,4-Tri-*O*-methyl	terminal	1.0	0.7	0.5	0.7
Galactose					
2,3,4,6-Tetra-*O*-methyl	terminal	0.5	0.4	0.4	0.7
2,4,6-Tri-*O*-methyl	3	2.0	0.9	1.7	1.7
2,3,4-Tri-*O*-methyl	6	1.7	0.7	1.9	1.4
total		4.2	2.0	4.0	3.8
Mannose					
3,4,6-Tri-*O*-methyl	2	0.2	1.4	1.7	1.2
3,6-Di-*O*-methyl	2 and 4	0.9	0.3	0.3	0.5
2,4-Di-*O*-methyl	3 and 6	0.9	1.2	0.8	0.9
3,4-Di-*O*-methyl	2 and 6	0.8	0.1	0.1	0.3
2-Mono-*O*-methyl	3, 4 and 6	0.2	0.0	0.1	0.1
total		3.0	3.0	3.0	3.0
N-Acetylglucosamine					
3,4,6-Tri-*O*-methyl	terminal	0.7	0.1	0.1	0.2
3,6-Di-*O*-methyl	4	3.4	1.5	2.3	2.4
4,6-Di-*O*-methyl	3	0.1	0.0	0.0	0.0
6-Mono-*O*-methyl	3 and 4	0.0	0.0	0.1	0.2
3-Mono-*O*-methyl	4 and 6	0.5	0.3	0.3	0.3
total		4.7	1.9	2.8	3.1
N-Acetylneuraminic acid					
total		2.5	1.3	2.2	2.5

Discussion

Immunoglobulin light chains are normally not glycosylated. However, carbohydrate has been found in several Bence Jones proteins. These myeloma light chains are monoclonal and are often formed by extensive sequence alterations in the variable regions, creating an Asn-X-Thr/Ser sequence for the attachment of carbohydrate [5-7, 27-29]. The carbohydrate structures found have been of the complex bi-antennary type, each light chain contains one oligosaccharide of the bi-antennary type [5-7].

In this study we showed that each cold precipitable urinary kappa chain in the MCH patient contained approximately two tetra-antennary complex type oligosaccharides. Tetra-antennary oligosaccharides have not previously been described in immunoglobulin subunits. In order to attach two oligosaccharides of this type, the sequence must have been altered to provide two sites with the Asn-X-Ser/Thr sequence. In the absence of sequence data, we cannot deduce the type of alterations responsible for the creation of these two sites. The MCH and myeloma kappa chains had identical mobility in

Table 3. Amino acid composition of MCH and myeloma kappa chains (% of total amino acid composition).

	MCH	M1	M2
Glycine	10.0	5.7	6.0
Alanine	8.5	6.4	6.1
Valine	8.2	7.3	5.6
Leucine	9.5	8.7	9.2
Isoleucine	3.2	2.5	2.9
Phenylalanine	5.2	4.0	3.9
Proline	5.2	6.2	6.9
Serine	9.7	14.5	12.4
Threonine	7.7	8.5	10.6
Cysteine	2.2	1.6	1.5
Methionine	0.0	0.2	0.2
Tyrosine	3.9	3.8	5.0
Aspartic/Glutamic acid	1.8	11.0	10.1
Asparagine	10.5	8.5	8.2
Lysine	5.5	6.2	6.3
Arginine	4.9	3.2	2.6
Histidine	2.2	0.9	1.8
Phenylserine	1.6	0.5	0.6

polyacrylamide electrophoresis (Fig. 1) although the MCH chain had 25% carbohydrate and myeloma chains less than 1%. Therefore, it is possible that there is a deletion in the MCH kappa chain. A deletion of 81 amino acids has been reported in a glycosylated light chain [7].

The presence of *N*-acetylgalactosamine (Table 1) suggests the presence of *O*-linked carbohydrate chains, which have been found in light chains [7] but they were not characterized in this study.

The unique feature of the oligosaccharides of the MCH kappa chains is their tetra-antennary structure. The regulation of the biosynthesis of such structures is very complex, and depends on both the presentation of the correct acceptor structures to glycosyltransferases and the presence of these enzymes [30, 31]. Also the rate of passage through the Golgi apparatus may play an important role.

It is interesting that the glycosylated kappa chains of MCH form multiple, extracellular cutaneous deposits. The reason for their deposition extracellularly as well as their cold insolubility is not clear, but it is possible that these properties are related to their carbohydrate moieties.

Our preliminary data (unpublished) show that in MCH the extracellularly deposited kappa chains are also highly glycosylated and have a monosaccharide composition similar to that of urinary kappa chains but they are less sialylated. In general, light chains are cleared from plasma primarily through glomerular filtration, tubular catabolism and urinary excretion [32]. In renal failure, light chains accumulate in plasma and may consist of deposited tissues. The MCH patient, however, had normal renal function, a factor which excludes this possibility. Preud'Homme *et al.* [4] have described

		MCH kappa
S-G-GN-6 S-G-GN-2M-6 (GN)-4 M-GN-GN- S-G-GN-4M-3 S-G-GN-2M	Tetra-antennary	75%
S-G-GN-2M-6 (GN)-4 M-GN-GN- S-G-GN-4M-3 S-G-GN-2M	Tri-antennary	10%
S-G-GN-2M-6 (GN)-4 M-GN-GN- S-G-GN-2M-3	Bi-antennary	15%

Figure 3. Summary of the postulated structures and estimation of their relative amounts according to methylation data. Linkages of mannose residues are shown. Peripheral arms might lack *N*-acetylneuraminic acid or galactose residues which is not shown. (M = mannose, G = galactose, F = fucose, GN = *N*-acetylglucosamine, S = *N*-acetylneuraminic acid).

the production of abnormal, probably glycosylated, kappa light chains that accumulated in the visceral organs in two patient with non-amyloid light chain deposition disease. The kappa chains were secreted as polymers. It is noteworthy that these patients did not excrete any appreciable amounts of kappa chains in the urine.

The site of synthesis of the abnormal kappa chains in MCH is not known, but local production remains a possibility. The hyalin deposits are surrounded by an infiltrate consisting principally of plasma cells, and in electron microscopy most of the cells have a well-developed endoplasmic reticulum and extended Golgi vesicles [1], suggesting active protein synthesis. In addition to glycosylated kappa chains, the hyalin deposits contain a sialylated mannose-rich glycoprotein (M_r 90 000), which has adhesive properties [2]. The possibility should be considered that the kappa chains are produced locally in the subcutaneous tissues and intestines (sites of hyalin deposits) and are to a great extent retained at these sites because of the high affinity to mannosylglycoprotein of the storage material.

In conclusion, our results demonstrate the presence of kappa light chains with approximately two tetra-antennary complex type carbohydrate structures per molecule in the urine of an MCH patient. Such structures have not previously been reported in immunoglobulin light chains. It is noteworthy that our patient's disease does not meet the criteria for myeloma or amyloidosis; for the present it may be classified as a kappa light chain deposition disease [33].

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