Short Communication

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Subtle differences in glycosylation of blood group M and N type glycophorin A detected with anti-Tn lectins and confirmed by chemical analysis*

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A higher content of Tn and sialyl-Tn receptors in glycophorin A of blood group N than in that of blood group M was suggested by reactions with anti-Tn lectins. Analysis of β-elimination products of two blood group M and two blood group N preparations by gas liquid chromatography-mass spectrometry showed that GalNAc--ol was detectable in minor amounts in all analyzed samples and its content was higher in the products obtained from desialylated antigens. Moreover, the content of GalNAc--ol detected in blood group N samples was almost twice as high as in respective blood group M samples. Since blood group M and N antigens differ in two amino-acid residues, our results support the existence of sequence-dependent differences in efficiency of substitution of glycophorin GalNAc-Ser/Thr residues with galactose.

Glycophorin A (GPA)[1] is the major sialoglycoprotein of human erythrocyte membranes. It exists in two forms differing in amino-acid residues at positions 1 and 5 of the polypeptide chain that gives rise to M and N blood groups [1]. GPA carries one N-linked and about 15 O-linked oligosaccharide chains. The major O-linked oligosaccharide structure is the tetrasaccharide NeuAcα2-3Galβ1-3[NeuAcα2-6]Gal-NAc-. The tetrasaccharide is accompanied by small amounts of mono-sialylated (trisaccharide) and tri-sialylated (pentasaccharide) chains [2–5]. The Tn antigen was found in persons with a rare syndrome related to deficiency in 3-β-D-galactosyltransferase activity. Erythro-cytes of such persons contain glycophorins with truncated oligosaccharide chains, GalAc-α1-Ser/Thr (Tn receptors) and NeuAcα2-6Gal-NAcα1-Ser/Thr (sialyl-Tn receptors) [6–8]. Anti-Tn antibodies are found to be present at a low level in most human and animal sera. A small amount of Tn receptors in normal asia-loglycophorins was detected by means of natural human antibodies [9], that possibly reflects the microheterogeneity of glycophorin glycosylation. However, the structural studies on the oligosaccharide chains of human glycophorins performed so far did not give unequivocal evidence for the presence of non-galactosylated GalNAc-Ser/Thr residues. The GalNAc-ol and

NeuAcα2-6GalNAc-ol either were not detected in β-elimination products of glycophorins [3, 5] or, if detected as minor components, they were considered to be degradation products of larger chains [2, 4].

Recently, the presence of a minor number of Tn and sialyl-Tn receptors in normal human glycophorins was suggested by studies with lectins and, interestingly, anti-Tn lectins showed stronger reactivity with various blood group N antigen-derived preparations than with their blood group M counterparts. The problem of higher content of Tn receptors in blood group N than M antigen was raised by the studies on *Moluccella laevis* lectin which agglutinates the blood group A or N erythrocytes [10]. The *M. laevis* lectin has been shown to have anti-Tn specificity, since it reacts strongly with asialo-agalactoGPAs and other Tn antigens [11]. Moreover, the *M. laevis* lectin shows much weaker reactivity with asialoGPAs, and the weakest one with untreated GPAs, and in these cases the lectin reacts 4–15 times more strongly with GPA-N than with respective GPA-M preparations [11]. The latter result was confirmed with other anti-Tn lectins [12]. The lectins from *Vicia villosa* and *Salvia sclarea* do not show detectable reactions with untreated erythrocytes or GPA, but they agglutinate asialoerythrocytes (*V. villosa* relatively strongly, *S. sclarea* weakly) and agglutination of ON asialoerythrocytes is distinctly stronger than that of their OM counterparts. The 10–15 times stronger reactivity of *V. villosa* lectin with the blood group N than M antigen was confirmed in experiments with purified asialoGPA-N and -M preparations [12].

These results strongly suggest that GPA-N contains more Tn and sialyl-Tn receptors than does GPA-M. Therefore, we decided to re-investigate the content of unsubstituted GalNAc-residues in GPA-M and GPA-N preparations by gas liquid chromatography/mass spectrometry analysis of carbohydrate components released from these glycoproteins by β-elimination.

MATERIALS AND METHODS

Glycophorins and their fragments. The glycophorins were isolated from the membranes of outdated human OM and ON erythrocytes by phenol-water extraction, and GPA was purified by gel filtration in the presence of sodium dodecyl sulfate [13, 14]. The N-terminal tryptic glycopeptides of GPA-M and GPA-N, containing most of GPA oligosaccharide chains, were isolated by the described procedure [14]. Desialylation of the glycoproteins and glycopeptides was performed by mild acid hydrolysis, in 0.025 M sulfuric acid for 1 h at 80°C.

Isolation of reduced oligosaccharide chains by β-elimination. The GPA and glycopeptide samples (10–20 mg) were degraded in 0.05 M NaOH/1 M NaBH$_4$ in the presence of Cd^{2+} ions at 50°C for 18 h [15]. The excess borohydride was decomposed with 50% acetic acid and the products were fractionated on the Sephadex G-15 column (2 cm × 65 cm) eluted with water.

Gas liquid chromatography-mass spectrometry (g.l.c.-m.s.) analysis. The analyzed samples were supplemented with xylitol as an internal standard, dried by rotatory evaporation and peracetylated in 0.1 ml pyridine/acetic anhydride (1:1, v/v) at 100°C for 40 min. Gas liquid chromatography and identification of the derivatives by m.s. were performed using a Hewlett-Packard 5890 instrument equipped with a mass selective detector. For fractionation a HP-1 column (0.2 mm × 12.5 m) and temperature program 150–230°C (8°C/min) were used. The content of detected components was calculated by comparison of their g.l.c. peaks with the peak of xylitol.

RESULTS AND DISCUSSION

The β-elimination products of GPA were fractionated and desalted on the Sephadex G-15 column and a typical elution profile is shown in Fig. 1. The column eluate was pooled into 3 fractions, containing N-linked glycopeptides, oligosaccharide alditols derived from O-linked chains, and salts, respectively. Analysis of these fractions showed that monosugar alditols were eluted together with salts. Since the salt fraction usually is not analyzed, this may explain why the monosugar alditols escaped detection in our previous experiments [3, 5].

Surprisingly, four monosaccharide alditols were identified by their retention times in g.l.c. (Fig. 2) and mass spectra (not shown): Gal-ol, GalNAc-ol, GlcNAc-ol and ManNAc-ol. The g.l.c.-m.s. analysis was applied to GPA-N and
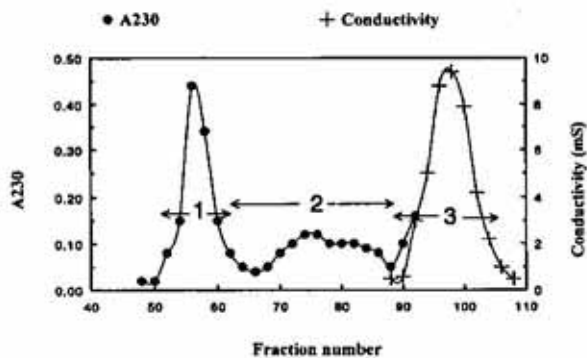
Fig. 1. A typical Sephadex G-15 gel filtration profile of the β-elimination products of GPA.
The column fractions were monitored by determination of absorbance at 230 nm (A_{230}), and of electric conductivity indicating the presence of inorganic salts. 1, 2, and 3 represent the pooled fractions containing as major components glycopeptides with N-glycans, oligosaccharide alditols, and salts, respectively.

GPA-M in untreated and desialylated form and to N-terminal tryptic glycopeptides, untreated and desialylated, derived from other GPA-M and GPA-N preparations (Table 1). GalNAc-ol and Gal-ol were found in all analyzed samples.
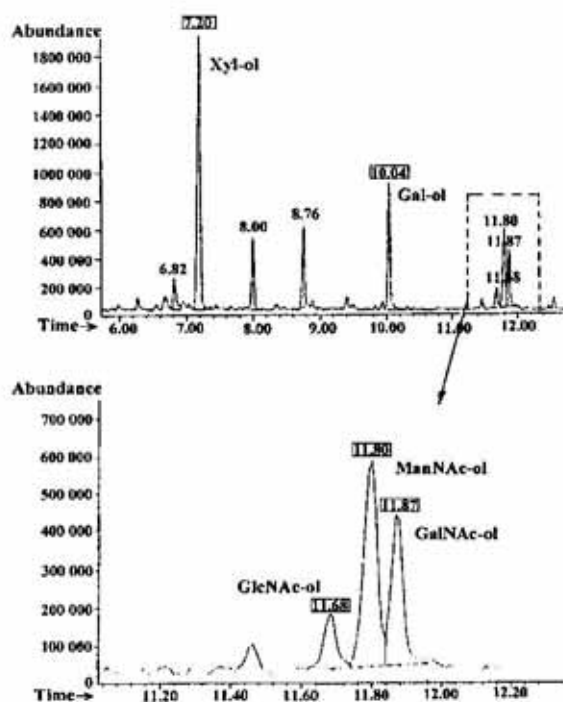

Fig. 2. Gas liquid chromatography of the monosaccharide alditols of GPA-N, present in the salt fraction 3 shown in Fig. 1.
Retention time is given in minutes.

The origin of Gal-ol is unknown; its content was distinctly higher in desialylated samples (Table 1), in agreement with former identification of NeuAc-Gal-ol in β-elimination products of GPA [5]. Small amounts of GlcNAc-ol could derive from other red cell membrane glycoproteins contaminating GPA preparations and known to contain the O-linked GlcNAc residues [16]. This was suggested by the decreased or undetectable contents of GlcNAc-ol in the degradation products of tryptic fragments of GPA which passed additional purification steps. The most surprising component was ManNAc-ol which could be the product of epimerization of GlcNAc-ol. This possibility was suggested by the finding that the content of ManNAc-ol was roughly proportional to the content of GlcNAc-ol, and the presence of ManNAc-ol in desialylated samples indicated that it was not the product of sialic acid degradation.

Our results showed that the content of GalNAc-ol increased after desialylation of the samples and that GalNAc-ol was the only component which was consistently present in blood group N preparations in higher amounts than in their blood group M counterparts (Table 1). Due to the low content of GalNAc-ol (one residue *per* several GPA molecules) and other monosaccharide alditols, the results of their quantitative determination should be treated with caution. However, our present results support the conclusion that a higher reactivity of blood group N than M preparations with anti-Tn lectins is caused by the higher number of Tn receptors in GPA-N. It is possible that a minor number of GalNAc residues, partly substituted with NeuAc, is present in GPA-M and GPA-N due to microheterogeneity of glycosylation. Moreover, there is probably an additional site(s) in the N-terminal fragment of GPA-N, where the enzymatic transfer of Gal to GalNAc is less efficient than in GPA-M due to the blood group N-specific amino-acid sequence. This suggestion is supported by the recent report of Granovsky *et al.* [17] who have found that 3-β-D-galactosyltransferase activity, tested with synthetic peptides containing GalNAc attached to Ser or Thr residues, is dependent on the amino-acid sequence of the substrates.

Our present results showed that the difference between GPA-M and GPA-N in GalNAc- Ser//Thr content is lower than the difference in

Table 1

Monosaccharide alditols found in β-elimination products of GPA-M and GPA-N and blood group M and N tryptic glycopeptides (M-Tr and N-Tr), untreated or desialylated

Sample	GalNAc-ol	Gal-ol	GlcNAc-ol	ManNAc-ol
	(µg/10 mg glycoprotein or glycopeptide)			
GPA-M	6.0	20.0	4.2	15.0
GPA-N	11.3	18.5	4.5	17.5
AsialoGPA-M	16.0	179.0	12.4	48.8
AsialoGPA-N	25.3	133.0	2.9	21.5
M-Tr	13.5	37.0	1.7	10.0
N-Tr	21.6	22.8	0.0	0.0
AsialoM-Tr	21.8	158.0	3.0	18.5
AsialoN-Tr	38.6	74.3	0.0	0.0

their reactivity with anti-Tn lectins [11, 12]. However, the putative blood group N-related Tn receptor, located in the most exposed N-terminal GPA portion, may contribute more efficiently to the reaction with the lectins than other GalNAc-Ser/Thr residues located presumably in the internal portion of GPA.

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