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Note

Reactivity of glycosidase-treated, blood-group M and N glycopeptides with lectins*,†

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Blood-group M and N antigens from human erythrocyte membranes (glycophorin A) are complex glycoproteins containing about 15 *O*-glycosyl oligosaccharide chains and one *N*-glycosyl chain per mol. wt.[1] 30 000. Albeit the antigenic differences between blood-group M and N glycoproteins depend on the amino acid sequence of their polypeptide chains[2–4], the carbohydrate portion of these glycoproteins is involved in their antigenic properties[5], and also in interactions with lectins[6,7]. *Diplococcus pneumoniae* secrete several exo- and endo-glycosidases[8–13] including α-neuraminidase (EC 3.2.1.18), β-D-galactosidase (EC 3.2.1.23), *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30), endo-*N*-acetyl-β-D-glucosaminidase (EC 3.2.1.96), and endo-*N*-acetyl-α-D-galactosaminidase (EC 3.2.1.97), but neither α-L-fucosidase nor α- or β-D-mannosidase. Lack of these two enzymes in the mixture of enzymes should result in the formation of some fragments of the *N*-glycosyl chain in the form of oligosaccharide(s), including the L-fucose-containing receptor for *Evonymus europaeus* lectin[7], by treatment of blood-group M and N glycoproteins or glycopeptides. Moreover, such treatment may give extensively deglycosylated polypeptide chains for comparative immunological studies. The effect of the mixture of *D. pneumoniae* glycosidases on tryptic M and N glycopeptides is described herein.

EXPERIMENTAL

Glycoproteins and glycopeptides. — Blood-group M and N glycoproteins were isolated from the membranes of outdated human OM and ON erythrocytes,

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respectively, by the phenol-extraction procedure[14], and purified by Bio-Gel P-300 gel filtration[15], monitored by $NaDodSO_4$–polyacrylamide gel electrophoresis[16]. Fractions containing pure M or N glycoproteins were pooled, dialyzed against 50% ethanol, then against distilled water, and lyophilized. The NH_2-terminal tryptic M and N glycopeptides were obtained as described previously[2,17].

Diplococcus pneumoniae *glycosidases.* — *D. pneumoniae* type 1 were grown in 3% Tood Hewith Broth (5 L) (Difco Labs., U.S.A.) for 3 days at 37°. All further operations were performed at 4°. The culture supernatant (1 L) was treated with solid ammonium sulfate to 80% saturation and kept overnight. The protein sediment was dissolved in 0.1M Tris · HCl buffer pH 7.5, containing 0.1M sodium chloride and 0.03% sodium azide, and was dialyzed exhaustively against this buffer. The retentate was fractionated on a Sephadex G-200 column in the same buffer. The column eluates were monitored for absorbance at 280 nm and for neuraminidase, β-D-galactosidase, and *N*-acetyl-β-D-glucosaminidase activities. All enzymes were found in the first protein peak eluted at 40–50% column volume. The activity of endoglycosidases was not tested, but it is known that they are eluted from Sephadex G-200 together with exoglycosidases[10,11]. The active fractions were pooled and concentrated by ultrafiltration. The solution obtained (28 mL) had a protein concentration of 4.8 mg/mL and showed the following exoglycosidase activities: β-D-galactosidase, 0.13 unit/mL; *N*-acetyl-β-D-glucosaminidase, 1.1 unit/mL, and α-neuraminidase, 0.22 unit/mL. No proteolytic activity and no *N*-acetyl-α-D-galactosaminidase activity were detected in this preparation. This crude enzyme fraction was stored in small portions at −20°.

Enzyme assays. — Activities of β-D-galactosidase, *N*-acetyl-β-D-glucosaminidase, and *N*-acetyl-α-D-galactosaminidase were determined by the method described by Hughes and Jeanloz[8,9] using as substrates *o*-nitrophenyl β-D-galactopyranoside, *p*-nitrophenyl 2-acetamido-2-deoxy-D-glucopyranoside, and *p*-nitrophenyl 2-acetamido-2-deoxy-α-D-galactopyranoside, respectively. The neuraminidase activity was estimated for a solution in 0.1M acetate buffer, pH 6.0, with glycophorin A and α_1-acid glycoprotein as substrates; the released sialic acid was determined by the thiobarbituric acid method of Warren[18]. The unit of activity was the amount of enzyme which released 1 μmol of the respective sugar within 1 min. The proteolytic activity was determined by the method of Kunitz[19], after 3-h incubation of the enzyme sample with casein and hemoglobin.

Analytical methods. — Protein was determined by the method of Lowry *et al.*[20] with bovine serum albumin as a reference protein, free sialic acid by the thiobarbituric acid method of Warren[18], total sialic acid by the periodate–resorcinol method of Jourdian *et al.*[21], and free *N*-acetylhexosamine by the method of Reissig *et al.*[22], with 2-acetamido-2-deoxy-D-galactose and -D-glucose as standards. Neutral sugars were determined by the phenol–sulfuric acid method[23], and total hexosamines by the method of Ludowieg and Benmaman[24] after hydrolysis with 4M hydrochloric acid for 4 h at 100°.

For g.l.c. determination, samples were hydrolyzed with M sulfuric acid for 8

h at 100°, D-xylose being added as an internal standard, and the hydrolyzates were made neutral with AG 3-X4A(OH^-) resin. The sugars were transformed into alditol acetates[25] that were quantitatively determined with a Varian 2100 gas chromatograph in a metal column (0.3 × 300 cm) filled with 3% OV-225 coated on GasChrom Q (100–200 mesh) at a constant temperature of 180° for neutral sugars, and 220° for hexosamines.

Cleavage of α-sialyl residues from glycopeptides. — α-Sialyl residues were removed by hydrolysis[26] of glycopeptides in 25mM sulfuric acid for 4 h at 60°, followed by gel filtration on Bio-Gel P-2.

Reactions with lectins. — The interactions of glycopeptides with lectins was tested by the hemagglutination inhibition assay described previously[27]. Human blood-group O erythrocytes and the following lectins were used: wheat-germ agglutinin (WGA, purchased from Pharmacia, Sweden), *Phaseolus vulgaris* lectin (Bacto-Phytohemagglutinin P from Difco Labs., U.S.A.), and *Evonymus europaeus* lectin obtained from Dr. J. Petryniak[7] from our department. The inhibitory activity was expressed as a minimal concentration of glycopeptide at which a complete inhibition of 4 agglutinating units was achieved.

RESULTS AND DISCUSSION

In the degradation of M and N tryptic glycopeptides with *D. pneumoniae* glycosidases, the crude enzyme fraction (0.2 mL) was added to the 1% glycopeptide solution (5 mL) in 0.1M citrate–phosphate buffer (pH 5.5), and the samples were incubated at 37° under toluene. After time intervals, as indicated in Fig. 1, aliquots (50 μL) were withdrawn for determination of free sialic acid and *N*-acetylhexosamines, and further portions of the enzyme solution (0.2 mL) were added. The release of sialic acid seemed to be complete within 24 h (see Fig. 1). The release of *N*-acetylhexosamines proceeded more slowly and was completed after 6–7 days of incubation. When M and N glycoproteins were treated with *D. pneumoniae* glycosidases under the same conditions, removal of sialic acid was complete within 48 h, but the maximal release of other carbohydrates approached only ~one-third of the amount released from the equivalent portion of glycopeptides (results not shown).

The products of treatment of M and N glycopeptides with *D. pneumoniae* glycosidases were fractionated by gel filtration on a Sephadex G-200 column (see Fig. 2). In a control experiment under the same conditions, the enzyme solution showed no carbohydrate at the position corresponding to the position of elution of glycopeptide-degradation products (measured by the phenol–sulfuric acid method) and negligible u.v. absorbance. Fractions A and B from the Sephadex G-200 column containing aglyco(deglycosylated)glycopeptide and released carbohydrates, respectively, were pooled. Analysis (see Table I) showed that all sialic acid and >80% of neutral sugars and hexosamines had been removed.

The fractions A containing aglyco M or N glycopeptide (Fig. 2) were desalted

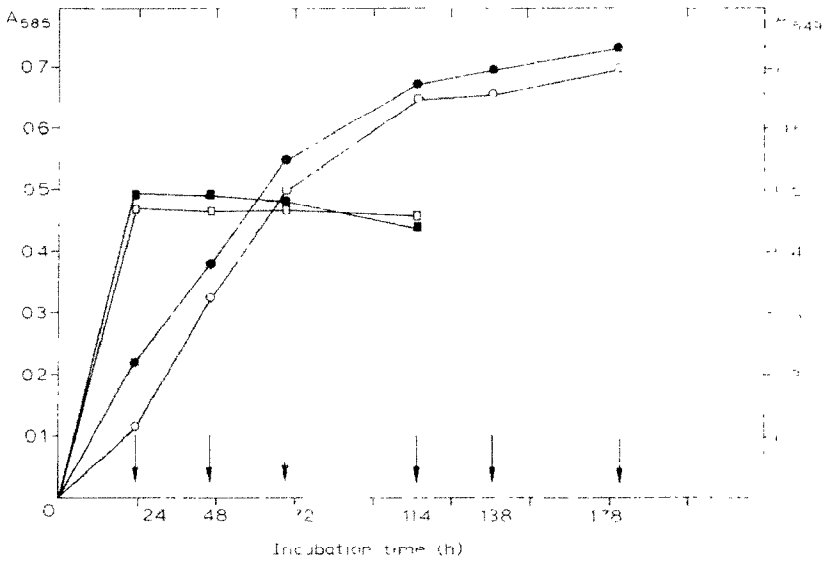

Fig. 1. Release of sialic acid (■, □) and *N*-acetylhexosamines (●, ○) during incubation of M (■, ●) and N (□, ○) glycopeptides with *D. pneumoniae* glycosidases. At indicated time intervals, 5- and 20-μL samples were withdrawn for determination of free sialic acid[18] and *N*-acetylhexosamines[22], respectively. Arrows indicate addition of 0.2 mL of enzyme solution; values are not corrected for dilution of samples

by gel filtration on Bio-Gel P-2 and lyophilized. From 50-mg portions of starting M and N glycopeptides containing 20% protein (on the weight basis of lyophilized samples), 8.2 mg and 8.6 mg of respective aglycoglycopeptides that contained 80% of protein were obtained. Comparison of the carbohydrate composition of un-

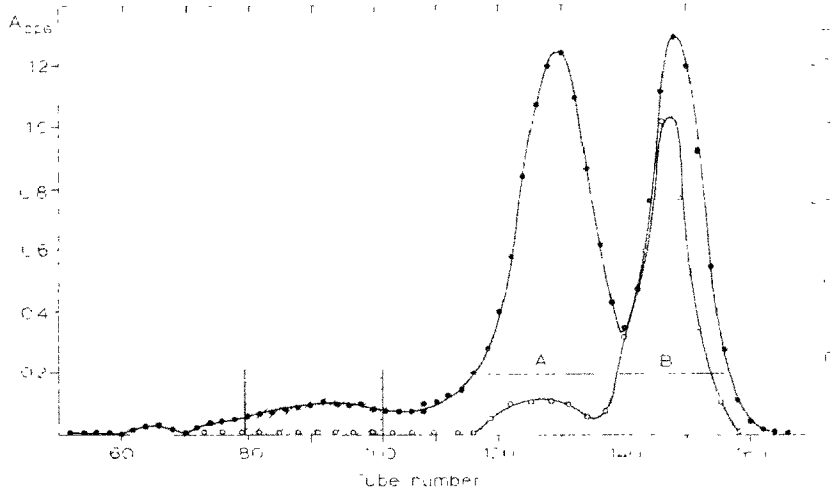

Fig. 2. Fractionation of products of enzymic degradation of M glycopeptide on a column (2.6 × 100 cm) of Sephadex G-200 in 0.1M acetate buffer, pH 5.8; fractions (3.2 mL) were monitored by absorbance at 226 nm (●), and colorimetric[19] determination of neutral sugar (○) (100-μL samples). Hatched area indicates fractions showing neuraminidase and β-galactosidase activity. Fractions A and B were pooled. Fractionation of the enzyme-treated N glycopeptide gave an identical pattern

TABLE I

DISTRIBUTION OF CARBOHYDRATE COMPONENTS IN THE POOLED FRACTIONS A AND B[a] OBTAINED FROM TRYPTIC BLOOD-GROUP M AND N GLYCOPEPTIDES TREATED WITH *D pneumoniae* GLYCOSIDASES[b]

Carbohydrate components	*Glycopeptide M (mg)*		*Glycopeptide N (mg)*	
	A	*B*	*A*	*B*
Sialic acid	0	7.9	0	10.0
Neutral sugars	1.7	7.0	1.9	6.8
Hexosamine total	1.3	6.2	1.8	7.0
N-Acetylhexosamines without hydrolysis	0	14.1[c]	0	14.5[c]

[a]See Fig. 2. [b]Colorimetric methods described in Experimental section were used. [c]*N*-Acetylhexosamines were determined by the method of Reissig *et al.*[22] with 2-acetamido-2-deoxy-D-galactose as standard; 2-acetamido-2-deoxy-D-glucose gave an absorbance higher by ~three-fold than that of 2-acetamido-2-deoxy-D-galactose, and 2-acetamido-2-deoxy-D-galactose substituted at O-3 gave an absorption two-fold higher than that of the free unsubstituted compound[28]

treated and aglycoglycopeptides (Table II) ascertained that all sialic acid and most of other carbohydrates had been removed by the action of the glycosidases.

The effect of deglycosylation on the interaction of M and N glycopeptides with some lectins is shown in Table III. The asialoglycopeptides inhibited weakly *P. vulgaris* and *E. europaeus* lectins. The enzymic deglycosylation of glycopeptides destroyed their activity toward *P. vulgaris* lectin, whereas the ability to inhibit *E. europaeus* lectin was increased. None of the glycopeptides inhibited wheat-germ agglutinin.

TABLE II

CARBOHYDRATE COMPOSITION OF UNTREATED AND DEGLYCOSYLATED M AND N GLYCOPEPTIDES[a]

Component	*Glycopeptide M*				*Glycopeptide N*			
	Untreated		*Deglycosylated*		*Untreated*		*Deglycosylated*	
	%	*μmol*	*%*	*μmol*	*%*	*μmol*	*%*	*μmol*
Sialic acid[b]	35.6	47.0	0	0	36.8	49.3	0	0
Fucose[c,d]	3.5	8.9	3.5	2.6	3.2	8.0	2.8	2.1
Mannose[c]	2.5	5.6	2.1	1.4	2.6	6.1	1.4	0.9
Galactose[c]	16.2	36.7	5.6	3.8	15.3	35.2	6.5	4.5
2-Acetamido-2-deoxyglucose[c]	5.5	10.1	3.5	1.9	5.8	10.8	4.2	2.3
2-Acetamido-2-deoxygalactose[c]	11.2	22.1	2.1	1.2	12.2	22.8	4.5	2.5
Protein[e]	24.5		83.1		24.1		80.6	

[a]Values are given as percent, taking a sum of carbohydrates and protein as 100%, and as μmol/10 mg of protein. [b]Determined colorimetrically by the periodate–resorcinol method[21]. [c]Determined by g.l.c. [d]Values for fucose are probably too high due to an artifact having the same retention time. [e]Determined by the Lowry[20] method

TABLE III

INHIBITORY ACTIVITY OF UNTREATED ASIALO AND AGLYCO M AND N GLYCOPEPTIDES TOWARD LECTINS[a]

Glycopeptide	*Lectins*		
	E. europaeus	P. vulgaris	*Wheat germ*
M	>2[b]	1	1
Asialo M	2	1	>2
Aglyco M	1	>4	>4
N	>2	2	1
Asialo N	2	2	>2
Aglyco N	0.5	>4	>4

[a]Expressed as a minimal concentration of glycopeptide protein (mg/mL) giving a complete hemagglutination inhibition [b]No inhibition at the concentration given.

The mixture of *D. pneumoniae* glycosidases deglycosylated extensively M and N tryptic glycopeptides, but gave only a partial deglycosylation of M and N glycoproteins. The *O*-glycosyl chains of M and N glycopeptides were completely desialylated and their disaccharide unit was released in >90% by endo-*N*-acetyl-α-D-galactosaminidase. The *N*-glycosyl chain of M and N glycopeptides also was degraded to a significant extent, but no fucose and free mannose were detected among the products released by the enzymic reaction. The deglycosylated glycopeptides obtained contained both L-fucose and *N*-acetylglucosamine, but also contained small proportions of mannose and galactose, indicating that not all *N*-glycosyl chains were exhaustively degraded. The receptors for *E. europaeus* lectin, located on the *N*-glycosyl chain[7], were not removed by the action of *D. pneumoniae* glycosidases and were, even, exposed in the deglycosylated glycopeptides, in contrast to the receptors for *P. vulgaris* lectin. The *E. europaeus* lectin reacts with L-fucose-containing receptors and Petryniak *et al.* reported that the sequence α-L-Fuc→D-Gal is necessary for this interaction[7,29]. The present results suggest that (a) either *E. europaeus* lectin reacts with α-L-Fuc→D-GlcNAc groups, or (b) the *N*-glycosyl chain on the minor portion of glycopeptide molecules has a structure different from that assumed[5,6] (*e.g.*, an α-L-Fuc→D-Gal group at the nonreducing end), which is resistant to *D. pneumoniae* glycosidases. Identification of the *E. europaeus* lectin receptor of glycophorin A will be facilitated by use the enzymically degraded glycopeptides.

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