THE AMINO ACIDS OF M AND N BLOOD GROUP GLYCOPEPTIDES ARE DIFFERENT

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Received April 13,1977

SUMMARY: The major glycopeptides isolated from tryptic digests of M and N blood group glycoproteins of erythrocytes have different N-terminal amino acids, serine and leucine, respectively. They also differ elsewhere in that the blood group N glycopeptide has a glutamic acid residue in place of a glycine residue of the M glycopeptide, presumably at position 5 of both peptides.

The major glycoprotein of human erythrocyte membranes (glycophorin) carries M and N blood group determinants (1, 2). In spite of the fact that knowledge of the structure of this glycoprotein is fairly advanced, no chemical difference has been found between glycoproteins derived from M and N red blood cells. The effect of modifications of amino groups of M and N glycoproteins on their M, N, and N_{Vg} activities (3-6) strongly suggests that the structural difference between the antigens is confined to their polypeptide chains, and not to their carbohydrate portion. However, this proposal needs direct chemical evidence.

The major glycopeptide obtained by tryptic digestion of M or N glycoprotein contains most of the glycoprotein oligosaccharide chains (1, 2, 7-10) and retains its specific M or N serologic activity (7, 9). The glycopeptide is the N-terminal fragment of the glycoprotein, extending out from the erythrocyte membrane (1, 2, 8, 11). The glycopeptide does not aggregate in water solution and therefore is more easily purified than the highly aggregated glycoprotein. In the present communication we report the results of amino acid analysis and determination of N-terminal amino acids in purified N-terminal glycopeptides derived from M and N glycoproteins.

MATERIALS AND METHODS

Preparation of glycoproteins — Outdated blood group O erythrocytes were obtained from the Blood Transfusion Center in Wroclaw, and their MN phenotype was determined. Glycoprotein was isolated from a pool of 10-15 blood units of M or N erythrocytes by the phenol-water extraction of membranes (12).

ISSN 0006-291X

Isolation of glycopeptides — The glycopeptides were obtained by the procedure described previously (9), with some modifications. A 2% solution of M or N glycoprotein, containing 11.5 mM $CaCl_2$ and 0.02% trypsin (twice crystallized, salt-free product of Sigma Chemical Co., USA) was incubated under toluene for 2 hours at 37°, and then centrifuged at 50,000 x g for 30 minutes. Trichloroacetic acid (TCA) was added to the supernatant fluid to a final concentration of 5%, and the precipitate was removed by centrifugation. The supernatant fluid was extracted several times with ether to remove TCA, concentrated *in vacuo* in a rotatory evaporator, and lyophilized. The glycopeptides were fractionated by gel filtration on Sephadex G-200 in 0.05 M pyridine-acetate buffer, pH 5.3. The main carbohydrate-containing peak, retarded on Sephadex G-200, was rechromatographed on a column of Sephadex G-50 in the same buffer. The final purification of the major glycopeptide was performed by ion-exchange chromatography on DEAE-Sephadex A-50 equilibrated and eluted with 0.02 M phosphate buffer, pH 7.0. The glycopeptide was retained on the column, and its elution was completed using a linear gradient formed by equal volumes of 0.02 M phosphate buffer and of the same buffer containing 0.5 M NaCl. The major glycopeptide was eluted at 0.1-0.2 M concentration of NaCl. The glycopeptide was desalted by gel filtration on Bio-Gel P-6 and lyophilized.

Determination of N-terminal amino acids — The glycopeptide was treated with dansylchloride (DNS-Cl, BDH Chemicals, Ltd., U.K.) by the procedure described by Gray (13). The DNS-glycopeptide was hydrolyzed in 6 N HCl for 6 and 16 hours at 105°. The DNS-amino acids were identified according to the method of Gros and Labouesse (14), by bidimensional thin-layer chromatography (TLC), using TLC plates precoated with silica gel 60 (Merck, W. Germany). The following solvent systems were used: (i) benzene : pyridine : acetic acid (80 : 20 : 5, v/v/v) and (ii) toluene : 2-chloroethanol : 25% ammonia (30 : 50 : 20, v/v/v). The amount of sample applied to any one TLC plate corresponded to 1-2 nanomoles of the glycopeptide, assuming a molecular weight of 10,000-15,000 (2, 10). Thin-layer chromatography and a mixture of standard DNS-amino acids (Serva, W. Germany) and the cochromatography of the samples examined with some standard DNS-amino acids were performed under the same conditions.

Determination of amino acid composition — The glycopeptides were hydrolyzed with 6 N HCl for 24, 48, and 72 hours at 110° in ampoules sealed under vacuum. The hydrolysates were analyzed in an automatic amino acid analyzer (Locarte, England). The amount of each amino acid was corrected for destruction during hydrolysis by extrapolation to 0 time.

Determination of the blood group activity — The M and N blood group activities were measured by inhibition of hemagglutination, as described elsewhere (6). Rabbit anti-M and anti-N immune sera produced by Behringwerke AG (W. Germany) were used.

RESULTS AND DISCUSSION

The purified M and N blood group glycopeptides have carbohydrate compositions and other properties corresponding to those described in other papers (1, 2, 9, 10). They show specific M or N blood group activity (Table I), which is lower than the activity of untreated glycoproteins (7, 9), most probably due to the fact that the glycopeptides are not aggregated.

TABLE I

MN blood group activity and N-terminal amino acids of the M and N blood group glycopeptides

Sample	Glycopeptide MN phenotype	Inhibitory activity [a] M	Inhibitory activity [a] N	N-terminal amino acids
1	M	1.2	⩾ 10	serine (leucine)
2	M	1.2	> 10	serine (leucine)
3	N	> 10	0.6	leucine
4	N	> 10	2.5	leucine

[a] Activity is expressed as the lowest concentration of glycopeptide (mg/ml) giving complete inhibition of an equal volume of antiserum (titer 1 : 8); > 10 and ⩾ 10 means no inhibition or partial inhibition, respectively, at 10 mg/ml.

The M and N glycopeptides were dansylated, and the results showed their purity. Only ε-DNS-lysine and DNS-leucine were found in the hydrolyzate of dansylated N glycopeptide, whereas dansylation of M glycopeptide produced ε-DNS-lysine and DNS-serine, accompanied by a trace of DNS-leucine. Therefore, serine and leucine are N-terminal amino acids of M and N glycopeptides, respectively. Identical results were obtained with another pair of glycopeptides, obtained from other preparations of M and N glycoproteins (Table I). The difference in N-terminal amino acids between M and N glycopeptides is obviously connected with their blood group specificity, as each glycopeptide preparation is obtained from pooled erythrocytes of 10-15 persons, excluding the possibility of individual differences. As the glycopeptides investigated are N-terminal fragments of the glycoproteins (2, 8, 11), serine and leucine are the N-terminal amino acids of M and N glycoproteins, respectively.

The small amount of N-terminal leucine in M glycopeptide may be ascribed either to an impurity present in both M and N glycopeptides and unrelated to

TABLE II

Amino acid composition of M and N glycopeptides

	M glycopeptide		N glycopeptide	
Amino acid	% by weight	moles/mole Ile	% by weight	moles/mole Ile
Lys	1.7	1.9	1.5	1.7
His	1.7	1.8	1.7	1.9
Arg	1.7	1.6	1.6	1.5
Asx	2.0	2.6	2.0	2.6
Thr	6.0	8.4	5.4	7.5
Ser	5.0	7.9	4.5	7.1
Glx	0.9	1.0	1.7	1.8
Pro	0.4	0.5	nd	nd
Gly	0.5	1.0	0.0	0.0
Ala	1.0	1.8	1.0	1.8
Val	1.6	2.2	1.3	1.9
Met	0.7	0.7	0.3	0.4
Ile	0.8	1.0	0.8	1.0
Leu	0.1	0.1	0.9	1.1
Tyr	nd	nd	nd	nd
Phe	0.0	0.0	0.0	0.0
Total	24.1		22.7	

MN specificity, or to small amounts of N-specific determinants in the preparation of M glycopeptide. The existence of "buried" N determinants in M glycoprotein is known, but after tryptic digestion of M glycoprotein, the N activity is recovered in the aggregated fraction, separate from the major M glycopeptide (7, 9). However, the available method of determination of MN activity by inhibition of hemagglutination is not sensitive enough to determine whether or not the separation of both kinds of determinants is complete.

The amino acid composition of the two glycopeptides (Table II) agrees with the N-terminal amino acid analysis in that the M glycopeptide has more serine

than the N glycopeptide, while the N glycopeptide has more leucine than the M glycopeptide. In addition, the two glycopeptides differ in their content of glycine and glutamic acid: the M glycopeptide has one glycine residue and one glutamic acid residue per residue of isoleucine, while the corresponding numbers in the N glycopeptide are 0 and 2. From the published amino acid sequence of pooled M and N glycopeptides (2), it is likely the M glycopeptide has a glycine residue at position 5, while the N glycopeptide has a glutamic acid residue at position 5.

The results of the present investigation are consistent with our earlier finding of N-terminal leucine and serine in MN glycoproteins by dinitrophenylation procedure (3). Segrest *et al.* (15) determined the N-terminal amino acids in the three preparations of glycoprotein obtained from erythrocytes of single individuals. In two they found serine and leucine, and in one leucine only, but the MN phenotype of these individuals was not determined.

The results reported here demonstrate that the presence of serine or leucine at the amino terminal end of the major glycoprotein of human erythrocyte membranes and its content of glycine and glutamic acid is related to its M or N blood group activity. This is the first distinct chemical difference found between M and N blood group antigens and provides direct evidence for the proposal that these antigens have different polypeptide chains (6).

ACKNOWLEDGMENTS

This work was supported by grant No. 10.5. of the Polish Academy of Sciences, and by NIH PL-480 Research Agreement No. 05-001-0.

REFERENCES

1. Winzler, R. J. (1970) in *Blood and Tissue Antigens* (Aminoff, D., ed.), pp. 117-126, Academic Press, New York and London.
2. Tomita, M., and Marchesi, V. T. (1975) *Proc. Natl. Acad. Sci. U.S.A.* 72, 2964-2968.
3. Lisowska, E., and Morawiecki, A. (1967) *Eur. J. Biochem.* 3, 237-241.
4. Dahr, W., Uhlenbruck, G., and Bird, G. W. G. (1975) *Vox Sang.* 28, 389-391.
5. Lisowska, E., and Duk, M. (1975) *Vox Sang.* 28, 392-397.
6. Lisowska, E., and Duk, M. (1975) *Eur. J. Biochem.* 54, 469-474.
7. Baranowski, T., and Lisowska, E. (1963) *Arch. Immunol. Ther. Exp.* 11, 631-640.

8. Jackson, R. L., Segrest, J. P., Kahane, I., and Marchesi, V. T. (1973) *Biochemistry* 12, 3131-3138.
9. Lisowska, E., and Jeanloz, R. W. (1973) *Carbohydr. Res.* 29, 181-191.
10. Javaid, J. J., and Winzler, R. J. (1974) *Biochemistry* 13, 3639-3642.
11. Marchesi, V. T., Tillack, T. W., Jackson, R. L., Segrest, J. P., and Scott, R. E. (1972) *Proc. Natl. Acad. Sci. U.S.A.* 69, 1445-1449.
12. Baranowski, T., Lisowska, E., Morawiecki, A., Romanowska, E., and Strozecka, K. (1959) *Arch. Immunol. Ther. Exp.* 7, 15-27.
13. Gray, R. W. (1972) *Methods Enzymol.* 25, 121-138.
14. Gros, C., and Labouesse, B. (1969) *Eur. J. Biochem.* 7, 463-470.
15. Segrest, J. P., Kahane, I., Jackson, R. L., and Marchesi, V. T. (1973) *Arch. Biochem. Biophys.* 155, 167-183.