A NEW, SIMPLE PROCEDURE FOR THE ISOLATION OF SIALOGLYCOPROTEINS FROM HUMAN ERYTHROCYTE MEMBRANES OF ABO BLOOD GROUP ACTIVITIES

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1. Introduction

Sialoglycoproteins play a very important role in the functional and structural organization of the human erythrocyte membranes. Whereas the externally located part of glycoproteins functions as a surface carrier for a variety of receptors and blood group antigens [1–7], the internally located part imparts structural stability to the molecule through its interaction with membrane lipids [8]. Most of the available isolation procedures [4,9–11] are either quite laborious and/or result in low yields of purified glycoproteins. Because of increasing interest in function and structure of these integral membrane components, we wish to describe a new simple procedure for the isolation of sialoglycoproteins from A, B or O type human red blood cells and to compare some of their chemical and immunologic properties.

2. Materials and methods

2.1. *Isolation of sialoglycoproteins*

The erythrocyte ghosts were prepared from freshly drawn blood of healthy human donors by the method of Dodge et al. [12]. From each unit of blood (450 ml), 120–150 ml of packed ghosts were obtained with a protein concentration of 3–4 mg/ml. The erythrocyte ghosts were solubilized with Triton X-100 at a final concentration of 5% (v/v) in 10 mM phosphate buffer, pH 4.0, and the mixture was centrifuged in a Beckman model L5-50 ultracentrifuge at 105 000 *g* for 2 h. The supernatant fraction was removed and applied onto a DEAE-cellulose (Bio-Rad Laboratories. Richmond, Calif.; stock #13331) column (2.5 X 20 cm). The column was eluted with 5 mM citrate buffer, pH 4.0, in 0.2% Triton X-100. The retained fraction was eluted with a linear salt gradient formed by mixing 250 ml of 5 mM citrate buffer, pH 4.0, in 0.2% Triton X-100 and 250 ml of 2 M NaCl dissolved in the same buffer. Fractions of 4 ml were collected and monitored for both protein and carbohydrate. The retained fraction was concentrated by ultrafiltration (PM-10 filter, Amicon Corp., Lexington, Mass.) to a final volume of approx. 7.0 ml. In order to remove Triton X-100 from the sialoglycoprotein, the concentrated retained fraction was mixed with an equal volume of 95% ethanol and applied to a Sephadex LH-20 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J., lot #0382) column (2.5 X 100 cm). The column was eluted with 50% aqueous ethanol. The fraction eluted at void volume was dialyzed overnight to remove the ethanol and lyophilized. The yield of sialoglycoproteins from one unit of blood was 15–25 mg.

2.2. *Polyacrylamide gel electrophoresis*

The polyacrylamide gel electrophoresis in the presence of 1% sodium dodecyl sulfate and 8 M urea was performed as previously described [13]. The gels were stained for protein with Coomassie brilliant blue as previously described [13] and for carbohydrate with periodic acid–Schiff reagent according to the procedure of Fairbanks et al. [14].

2.3. *Analytical methods*

Protein was determined by a modification of the Lowry procedure [15] for protein samples containing

Triton X-100. Total neutral carbohydrate was estimated by using phenol–sulfuric acid method of Dubois et al. [16]. Sialic acid was determined by the method of Warren [17]. Individual neutral and amino sugars were determined by gas–liquid chromatography after their conversion to alditol acetates as reported previously [18]. The hydrolysis for neutral sugars was performed with 1.0 N HCl at 100°C for 3 h, and that for the amino sugars with 4 N HCl at 100°C for 6 h. Amino acid analyses were carried out on a Beckman Model 120C amino acid analyzer. Samples were hydrolyzed in evacuated tubes with 5.7 N HCl at 105°C for 24, 48 and 72 h. Because of the overlapping peaks of tryptophan and hexosamines on the amino acid analyzer, tryptophan was determined spectrofluorometrically by the method of Sasaki [19].

2.4. *Immunological methods*

The A, B, M and N blood group activities were determined by the hemagglutination inhibition test with a modification of the micro diluter system of Takatsy [20]. Commercial antibodies used in this study were obtained from Ortho Diagnostics, Inc., Raritan, N.J.

The antiserum to the sialoglycoproteins was prepared by immunizing rabbits with the antigen at weekly intervals for 4 weeks. 5 mg of the sialoglycoproteins were mixed with 1 ml of normal saline and an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.), and injected intraperitoneally into white New Zealand rabbits. One week after the last injection, the rabbits were bled by cardiac puncture. The antiserum gave a positive precipitin line with isolated sialoglycoproteins.

Immunodiffusion and immunoelectrophoresis were performed as previously described [21].

3. Results and discussion

After solubilization of erythrocyte ghosts in 5% Triton X-100 at pH 4.0 and subsequent ultracentrifugation, the soluble supernatant fraction contained 25% of the total membrane protein. Polyacrylamide gel electrophoresis indicated that the major part of sialoglycoproteins was present in the soluble fraction with only a small amount occurring in the insoluble fraction. The selection of DEAE-cellulose column chromatography as a procedure for the separation of sialoglycoproteins was based on our observation of a difference between the isoelectric points of sialoglycoproteins and other membrane proteins.The sialoglycoproteins with a p*I* value less than 3, as determined by isoelectric focusing, were retained by the DEAE-cellu-

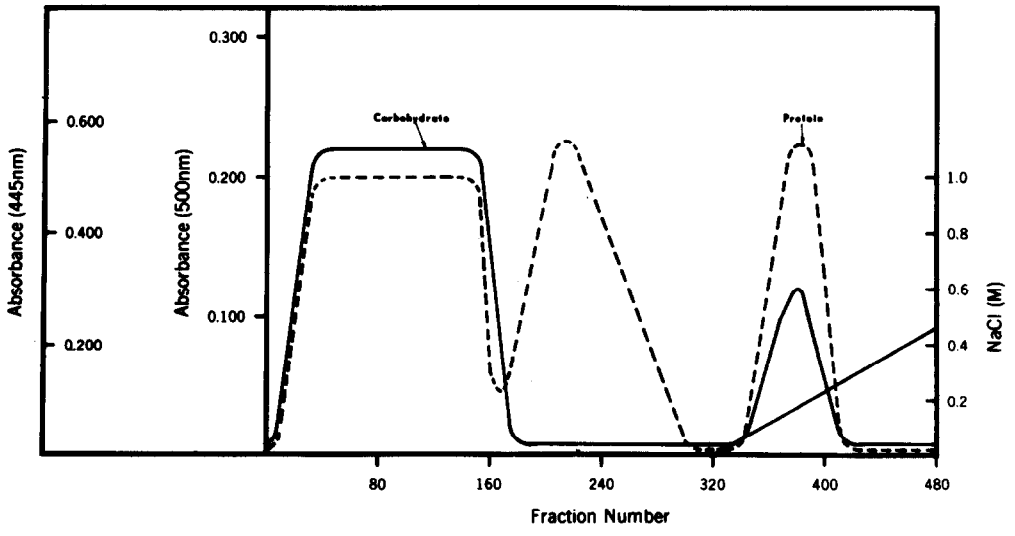

Fig.1. Elution pattern of sialoglycoproteins on DEAE-cellulose. The unretained fraction was eluted with 5 mM citrate buffer, pH 4.0, in 0.2% Triton X-100. The retained fraction was eluted with a linear salt gradient from 0 to 2 M NaCl in the same buffer.

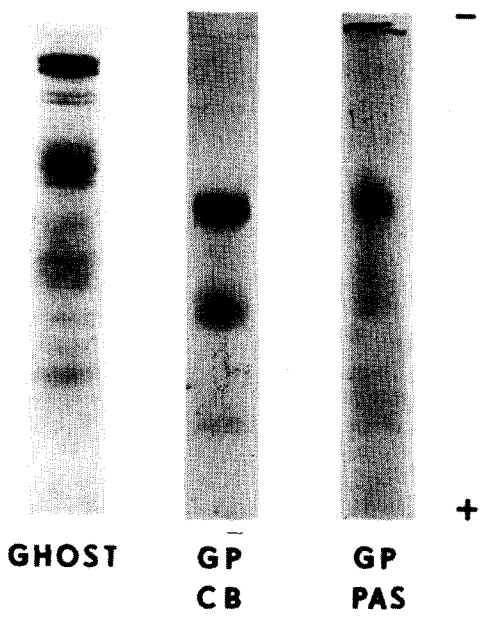

Fig.2. Polyacrylamide-gel electrophoresis of ghost proteins and isolated sialoglycoproteins. Ghost, whole ghosts stained for protein with Coomassie brilliant blue; GP-CB, isolated sialoglycoproteins stained for protein with Coomassie Blue; GP-PAS, isolated sialoglycoproteins stained for carbohydrate with Schiff stain.

lose. All remaining membrane proteins which had p*I* values greater than 3 were unretained. Both the unretained and retained fractions contained protein and carbohydrate. However, the carbohydrate-containing compounds in the unretained fraction were

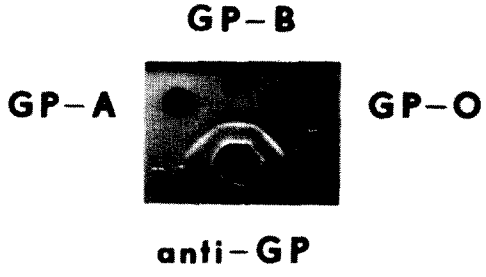

Fig.3. Immunodiffusion pattern of isolated sialoglycoproteins. Antigens are placed in the outer wells and antiserum to sialoglycoproteins in the center well. GP-A, sialoglycoproteins isolated from A type red blood cells; GP-B, sialoglycoproteins isolated from B type red blood cells; GP-O, sialoglycoproteins isolated from O type red blood cells; anti-GP, antiserum to sialoglycoproteins.

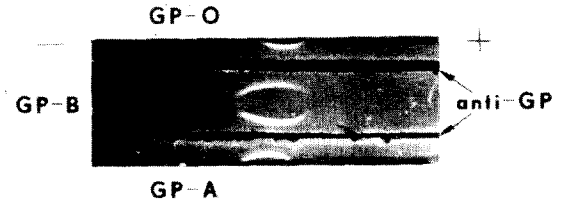

Fig.4. Immunoelectrophoretic pattern of sialoglycoproteins. Antigens are placed in the wells and antiserum is placed in the troughs. The abbreviations are the same as in fig.3.

identified by lipid analysis as glycolipids. The retained fraction was eluted at 0.2 M NaCl concentration estimated by electroconductivity measurement. It only contained sialoglycoproteins. Triton X-100 was separated from sialoglycoproteins by column chromatography on Sephadex LH-20 as described in the section on Materials and methods. The dry weight (15–25 mg) of the sialoglycoproteins accounted for 3–5% of the total ghost protein from one unit of blood.

Gas–liquid chromatography of sialoglycoproteins hydrolysate failed to show the presence of any fatty acids. On polyacrylamide gel electrophoresis, the sialoglycoprotein fraction was characterized by three bands which stained for both protein and carbohydrate

Table 1
Amino acid composition of human erythrocyte membrane sialoglycoproteins

Amino acid	GP-A (Mole %)	GP-B	GP-O
Aspartic acid	4.6	4.6	4.3
Threonine	10.1	11.5	10.6
Serine	9.1	10.0	10.1
Glutamic acid	9.3	9.2	8.8
Proline	5.7	6.3	5.6
Glycine	6.7	6.0	6.3
Alanine	6.9	6.4	6.9
Half-cysteine	0.5	0.2	0.4
Valine	8.5	8.7	8.3
Methionine	1.0	1.2	1.5
Isoleucine	9.2	10.0	9.7
Leucine	8.1	7.6	7.6
Tyrosine	2.7	2.9	2.9
Phenylalanine	2.4	2.0	2.1
Histidine	3.4	3.9	3.8
Lysine	7.2	4.9	6.4
Tryptophan	0.7	0.5	0.5
Arginine	4.6	4.7	4.6

Table 2
Carbohydrate composition of human erythrocyte membrane sialoglycoproteins

Monosaccharide	GP-A (Mole %)	GP-B	GP-O
Fucose	2.0	2.0	1.9
Mannose	2.5	2.8	2.5
Galactose	18.1	23.6	20.0
Glucose	2.5	1.8	2.2
Galactosamine	33.1	26.0	29.3
Glucosamine	10.5	10.1	12.3
Sialic acid	31.3	34.0	32.0

(fig.2). They contained either A or B and M and/or N blood group activities as assayed by hemagglutination inhibition tests. Double diffusion analysis (fig.3) showed that sialoglycoproteins isolated from either A, B or O type red blood cells gave single precipitin lines with an antiserum to sialoglycoprotein with the A activity. On immunoelectrophoresis, the same sialoglycoprotein preparations displayed single precipitin arcs (fig.4).

A comparative study of sialoglycoproteins isolated from blood samples that were either A, B or O type showed that each preparation contained 40% protein and 60% carbohydrate. The amino acid compositions (table 1) of all three sialoglycoproteins were very similar, if not identical. The identity reaction between these sialoglycoproteins in double diffusion analysis (fig.3) suggested either a common protein and/or carbohydrate component. Recent studies (to be published in a separate communication) on the removal of carbohydrate constituents by the Smith degradation of sialoglycoproteins resulted in the isolation of protein moieties which were shown to be chemically and immunologically identical. On the other hand, slight differences in the carbohydrate composition (table 2) of sialoglycoproteins may have reflected the presence of distinct blood group activities.

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