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PRESENCE OF INFLUENZA VIRUS-REACTIVE GLYCOPHORINS OTHER THAN GLYCOPHORIN A IN HUMAN ERYTHROCYTE MEMBRANES

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The reactivities against seven hemagglutinins including influenza A and B viruses of six sialoglycoprotein (glycophorin) fractions, M-Frs.1~3 and N-Frs.1~3, separated from human OMs and ONs erythrocyte membranes by a combination of the LIS-phenol method and gel filtration were studied. The serological results show that M-Fr.1 and N-Fr.1 were glycophorins A^M and A^N, respectively, the reactivities against influenza A and B viruses of both M-Fr.2 and N-Fr.2 which contained glycophorins B and C were remarkably higher than that of glycophorin A and the reactivities against influenza viruses of both M-Fr.3 and N-Fr.3 which contained glycophorins B and D were considerably lower than that of glycophorin A.

Influenza A and B viruses agglutinate human erythrocytes regardless of their blood groups (1). Influenza virus receptor on the surface of human erythrocyte is known to be glycophorin A which is the main sialoglycoprotein of human erythrocyte membrane and exhibits blood group MN activity (2,3,4). The reactive site for influenza viruses of glycophorin A is situated on the MN-active sialoglycopeptide moiety which is liberated from glycophorin A by treatment with trypsin or chymotrypsin (5,6). On the other hand, in 1961, Uhlenbruck noted that some proteases such as trypsin, ficin, bromelin and papain, did not decrease the influenza virus receptor activity of human erythrocytes (7). Twenty years after, Onuma et al.(8) reported that treatment of human erythrocytes with pronase resulted in a reduction of the influenza virus receptor activity of their erythrocytes and simultaneous release of sialoglycopeptides with a weak influenza virus receptor activity. Mishima et al. (9) then separated a sialoglycopeptide with a relatively high influenza virus receptor activity from the membranes of pronase-treated human erythrocytes. These findings seem to suggest that there are some sialoglycoproteins (glycophorins) different from glycophorin A in human erythrocyte membranes as influenza virus receptors, and these glycophorins are hidden within glycophorin A on human erythrocyte surface and exposed by the protease treatments.

In this work, we describe some of chemical properties and reactivities against several hemagglutinins including influenza A and B viruses of glycophorins separated from human OMs and ONs erythrocyte membranes by the combined use of the lithium diiodosalicylate (LIS)-phenol method and gel filtration in the presence of non-ionic detergent.

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MATERIALS AND METHODS

Materials.- Amphitol N20 (lauryl dimethylamine N-oxide, non-ionic detergent) was obtained from KAO Co. Ltd. (Tokyo, Japan). Bio-Gel A1.5m was purchased from Bio-Rad laboratories (Richmond, CA, USA). Blood group anti-M, -N -S and -s sera and mouse anti-human IgG serum were obtained from Ortho Diagnostic System Inc. (Norcross, GA, USA). *Vicia unijuga* anti-N lectin was prepared from their leaves by the method of Yanagi et al.(10). Influenza A and B viruses used were PR-8 and Osaka strains, respectively.

Chemical composition analysis.- Sialic acid was determined colorimetrically by the method of Jourdian et al (11). Glucose, mannose, galactose, fucose, N-acetylglucosamine and N-acetylgalactosamine were determined by gas-liquid chromatography using a Shimadzu GC-9AM according to the method of Metz et al. (12). Individual amino acids were measured with a Hitachi L-8500 high speed amino acid autoanalyzer according to the method described by Yanagi et al. (10).

Sodium dodecylsulfate (SDS)-polyacrylamide slab gel electrophoresis (PAGE).-SDS-PAGE was carried out according to the method of Yanagi et al. (10). PAS (periodic acid-Schiff staining)-positive bands in SDS-PAGE of Frs.1~3 of the LIS-phenol extract obtained from human OMs or ONs erythrocyte membranes were identified by reference to the Rf values of glycophorins A, B, C and D found in SDS-PAGE by Anstee and Tanner (13) and Anstee (14) and measured densitometrically with a Shimadzu CS-9000 (dual-weavelength flying-spot scanner).

Hemagglutination inhibition test.- Hemagglutination inhibition tests (10) with anti-M and -N sera, *Vicia unijuga* anti-N lectin and influenza A and B viruses were carried out by the saline test. Hemagglutination inhibition test (15) with anti-S and -s sera was performed by the indirect anti-globulin test.

Preparation of sialoglycoproteins (glycophorins) from human erythrocyte membranes.- Sialoglycoprotein mixture (LIS-phenol extract) was extracted from human OMs or ONs erythrocyte membranes by the LIS-phenol method (16). Twenty mg of the LIS-phenol extract was dissolved in 3ml of buffer A (0.05M phosphate buffer, pH 7.5, containing 0.15M NaCl, 0.05% Amphitol N20 and 0.1% NaN_3) containing 5% mercaptoethanol and allowed to stand for 24hr at 4°C. The solution was applied to a Bio-Gel A1.5m column (2.0 x 90cm) previously equilibrated with buffer A. Chromatography was carried out in a flow rate of 9ml/hr at 4°C and 3 ml fractions were collected. The fractions showing absorbances at 280 nm (for protein), 750 nm (for protein (17)), 490 nm (the phenol-sulfuric acid reaction for neutral sugar (18)) and 670 nm (the periodate-resorcinol reaction for sialic acid (11)) were pooled, dialyzed fully against distilled water and lyophilized. The dry residues were used as samples in the chemical composition analysis, SDS-PAGE and hemagglutination inhibition test.

RESULTS AND DISCUSSION

Two sialoglycoprotein mixtures (the LIS-phenol extracts) which were obtained from human OMs and ONs erythrocyte membranes by the LIS-phenol method (16), were subjected to gel filtration using Bio-Gel A1.5m in the presence of Amphitol N20 according to the method of Furthmayr et al. (19) and separated into three fractions, M-Frs.1~3 and N-Frs.1~3, respectively, as presented in Fig.1. The carbohydrate composition analyses and yields (Table 1) of M-Frs.1~3 and N-Frs.1~3 show that these six fractions were sialoglycoprotein (glycophorin) fractions containing 38.78, 20.07, 18.67, 44.80, 26.68 and 20.20% carbohydrate, respectively and among these fractions, M-Fr.1 and N-Fr.1 were the main sialoglycoprotein of the LIS-phenol extracts from human OMs and ONs erythrocyte membranes, respectively and the others were minor fractions. SDS-PAGE of M-Frs.1~3 and N-Frs.1~3 are, in the lump, shown in Fig.2 The results of SDS-PAGE of M-Frs.1~3 and N-Frs.1~3 show that M-Fr.1 and N-Fr.1 each contained only glycophorin A and M-Fr.2 and N-Fr.2 each contained glycophorins A and B, a complex of their glycophorins in small quantities and glycophorin C and also M-Fr.3 and N-Fr.3 each contained a complex of glycophorins A and B and glycophorins B and D in fairly small quantities.

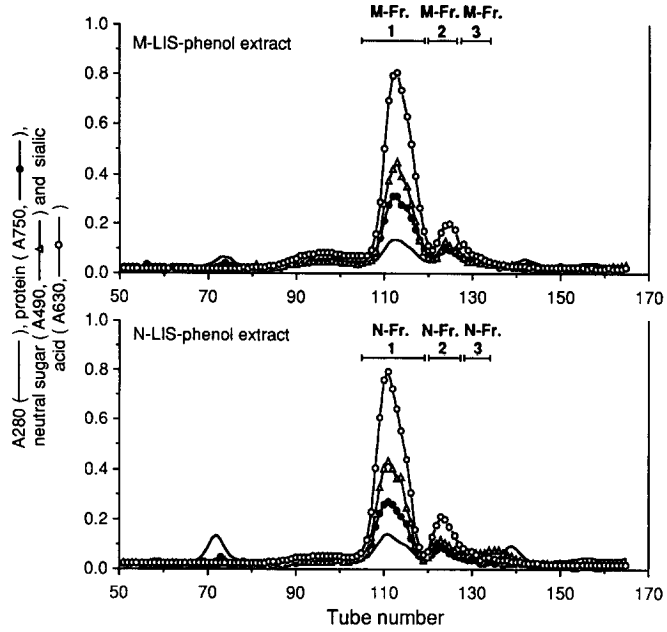

Fig. 1. Bio-Gel A1.5m column chromatography of M- and N-LIS-phenol extracts prepared from human OMs and ONs erythrocyte membranes.

Table 1. Yields and chemical compositions of M-Frs.1~3 and N-Frs.1~3

Fraction	M-Fr.1	M-Fr.2	M-Fr.3	N-Fr.1	N-Fr.2	N-Fr.3
Yield						
mg/100mg of LIS-phenol extract	56.55	11.61	5.96	57.59	12.35	5.11
mg/100mg of erythrocyte membrane	2.81	0.58	0.29	2.48	0.53	0.22
Carbohydrate composition (w/w, %)						
Sialic acid	20.06	16.23	8.02	22.97	15.77	9.72
Glucose	0.15	0.04	0.87	0.79	0.70	0.95
Mannose	1.17	0.42	1.33	2.18	2.23	1.69
Galactose	6.63	3.83	2.75	7.22	4.34	3.13
Fucose	0.64	0.58	0.69	0.97	0.74	0.86
N-acetylglucosamine	2.89	1.51	3.02	3.51	0.76	2.15
N-acetylgalactosamine	7.24	4.46	1.99	7.16	2.14	1.70
Amino acid composition (mole/100mole)						
Aspartic acid	6.45	6.80	7.71	6.42	6.33	7.60
Threonine	11.49	10.77	10.29	11.15	11.46	9.70
Serine	13.88	11.61	11.50	12.63	11.60	12.78
Glutamic acid	11.46	9.36	11.01	12.25	9.52	11.69
Glycine	5.06	8.15	9.75	5.01	7.57	10.86
Alanine	4.96	9.68	10.42	5.09	8.37	10.47
Cysteine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Valine	8.03	5.61	4.70	7.98	6.18	4.61
Methionine	1.06	3.87	3.37	1.09	3.79	2.82
Isoleucine	7.29	6.08	4.88	7.47	6.27	4.62
Leucine	5.43	6.64	6.25	6.31	7.19	5.66
Tyrosine	3.01	3.25	3.22	3.00	3.12	2.98
Phenylalanine	1.59	2.09	2.24	1.63	2.01	2.10
Lysine	4.11	2.91	3.18	3.52	2.73	3.00
Histidine	4.06	2.50	2.57	3.19	2.51	2.37
Arginine	4.14	4.23	3.88	4.44	4.40	3.71
Proline	7.96	6.43	5.02	7.82	6.93	5.00

n.d.: not detected

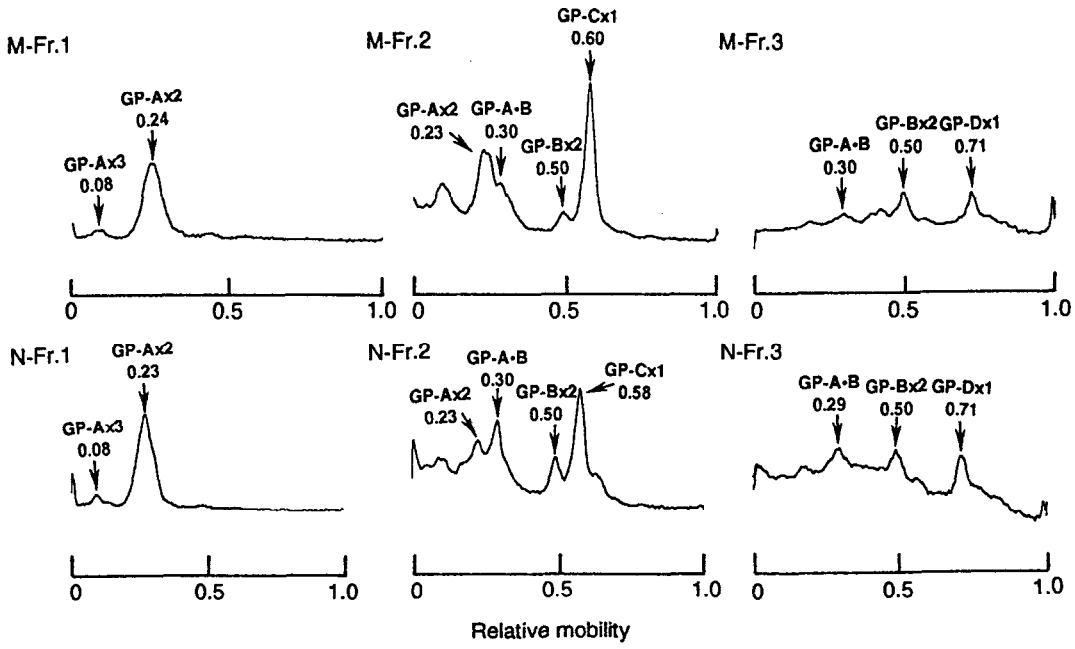

Fig. 2. SDS-PAGE of M-Frs.1~3 and N-Frs.1~3.

Twenty mg of M-Fr.1 and N-Fr.1, 30mg of M-Fr.2 and N-Fr.2 and 50mg of M-Fr.3 and N-Fr.3, were loaded on slab gels, respectively. Abbreviations are as follows: GP-Ax3, trimer of glycophorin (GP) A; GP-Ax2, dimer of GP-A; GP-Bx2, dimer of GP-B; GP-Bx1, monomer of GP-B; GP-Cx1, monomer of GP-C; GP-Dx1, monomer of GP-D; GP-A•B, a complex of glycophorins A and B.

The reactivities of M-Frs.1~3 and N-Frs.1~3 against anti-M, -N, -S and-s sera, *Vicia unijuga* anti-N lectin and influenza A and B viruses are presented in Table 2. M-Fr.1 showed bothM activity and influenza virus receptor activity and N-Fr.1 exhibited both N activity and influenza virus receptor activity. From the results of SDS-PAGE of both M-Fr.1 and N-Fr.1 described above and the serological studies of both the fractions described here, it is certain that M-Fr.1 was glycophorin A^M with influenza virus receptor activity and similarly, N-Fr.1 was glycophorin A^N with influenza virus receptor activity. This is in fair agreement with the findings

Table 2. Hemagglutination inhibition activities[1] of M-Frs.1~3 and N-Frs.1~3

Fraction / Hemagglutinin[2]	M-Fr. 1	M-Fr. 2	M-Fr. 3	N-Fr. 1	N-Fr. 2	N-Fr. 3
Anti-M serum	156	n.i.	n.i.	n.i.	n.i.	n.i.
Anti-N serum	n.i.	313	1,250	313	313	1,250
Anti-S serum	n.i.	n.i.	n.i.	n.i.	n.i.	n.i.
Anti-s serum	n.i.	20	313	n.i.	20	625
Vicia unijuga anti-N lectin	n.i.	313	1,250	313	313	2,500
Influenza A virus	313	156	1,250	313	156	1,250
Influenza B virus	313	39	156	313	39	156

1: hemagglutination inhibition activity was indicated as minimum concentration (μg/ml) of fraction giving complete inhibition
2: titer=8
n.i.: no inhibition of hemagglutination at 10,000 μg/ml

of Furthmayr et al. (19) and the description of Furthmayr (4) that the main sialoglycoprotein separated from the LIS-phenol extract of human erythrocyte membranes by gel filtration in the presence of non-ionic detergent is glycophorin A with MN blood group activity and influenza virus receptor activity. Both M-Fr.2 and N-Fr.2 showed Ns activity and influenza virus receptor activity which was remarkably higher than the influenza virus receptor activity of glycophorin A (Table 2). On the basis of the results of SDS-PAGE of M-Fr.2 and N-Fr.2 described above and the serological studies of both the fractions described here and considering the findings of Anstee (14), Furthmayr (20) and Dahr et al. (21) that glycophorin B expresses NSs antigens, but glycophorin C has not MNSs activity, it is probable that both M-Fr.2 and N-Fr.2 comprised glycophorin A, glycophorin B with Ns activity, their complex in small quantities and glycophorin C without having MNSs activity and that the influenza virus receptor activities of glycophorin B and/or glycophorin C are higher than that of glycophorin A. Both M-Fr.3 and N-Fr.3 exhibited Ns activity and influenza virus receptor activity, but the serological activities of both the fractions were remarkably lower than those of both M-Fr.2 and N-Fr.2 (Table 2). From the results of SDS-PAGE of both M-Fr.3 and N-Fr.3, the serological studies of both the fractions and the findings of Anstee (14), Furthmayr (20) and Dahr et al. (21) that glycophorin B carries NSs antigens and glycophorin D has not MNSs activity, it is reasonable to infer that both the fractions consisted of a complex of glycophorins A and B, glycophorin B with Ns activity and glycophorin D without having MNSs activity and that the influenza virus receptor activities of glycophorins B and/or glycophorin D are considerably lower than that of glycophorin A.

From the above-mentioned results, it is concluded that human erythrocyte membranes comprise glycophorin A and two kinds of glycophorins different from glycophorin A as influenza viruses-reactive components, namely, one of these kinds is glycophorins possessing influenza virus receptor activity which is remarkably higher than the influenza virus receptor activity of glycophorin A and the other is glycophorins exhibiting influenza virus receptor activity which is considerably lower than the influenza virus receptor activity of glycophorin A.

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