PREPARATION AND FURTHER CHARACTERIZATION OF THE MN GLYCOPROTEIN OF HUMAN ERYTHROCYTE MEMBRANES*‡

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The human erythrocyte membrane contains three sialoglycoproteins which can be distinguished by electrophoresis on acrylamide gels in the presence of sodium dodecyl sulfate (SDS)[1] (1, 2)[2]. They appear to extend from the outer surface through the membrane barrier to the interior surface (2, 3). Recently, it was shown that these sialoglycoproteins can be solubilized and recovered almost quantitatively in the aqueous phase after extraction of red cell ghosts with a mixture of chloroform and methanol.[2] In the present report, the preparation of the major glycoprotein, the so-called MN glycoprotein (4–10), from the aqueous phase of chloroform-methanol extracts is described. The partial characterization of this membrane glycoprotein includes the analysis by SDS-acrylamide gel electrophoresis, determination of carbohydrate and amino acid compositions, assays of inhibitory activities for hemagglutination by rabbit M- and N-antisera, influenza virus and *Phaseolus vulgaris* phytohemagglutinin, and assays of its inhibitory activities in various lymphocyte stimulation systems.

Materials and Methods

All chemicals employed in these studies were reagent grade. Protein concentrations were determined by the method of Lowry et al. (11) using bovine pancreatic ribonuclease (Worthington Biochemical Corp., Freehold, N. J.) as standard. The microbiuret method was carried out according to Goa (12). Cholesterol was measured by the method of Zlatkis et al. (13), and phosphorus was assayed by the technique of Bartlett (14). Sialic acid was determined by the method described by Warren (15) after hydrolysis in 0.1 N H_2SO_4 at 80°C for 1 hr. Hexoses were determined with the orcinol method as described by Vasseur (16). Galactose-mannose ratios were obtained after chromatographic separation according to Spiro (17). Hexosamines were measured with Svennerholm's modification (18) of the Elson-Morgan reaction; galactosa-

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[1] *Abbreviations used in this paper:* CM, chloroform-methanol; GP, glycoprotein; PAS, periodic acid-Schiff; PHA, phytohemagglutinin; SDS, sodium dodecyl sulfate.

[2] Hamaguchi, H., and H. Cleve. 1972. Solubilization of human erythrocyte membrane glycoproteins and separation of the MN glycoprotein from a glycoprotein with I, S and A activity. *Biochim. Biophys. Acta.* **278**:271.

mine was assayed by the method of Ludowieg and Benmaman (19). Fucose was measured with a semimicro modification of the method of Dische and Shettles (20). For the chemical analyses the freeze-dried, purified MN glycoprotein was weighed in with a Cahn M 10 electrobalance (Cahn Instruments, Paramount, Calif.). Amino acid analysis was performed on a Beckman Model 120-B automatic amino acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.) using the system of Moore and Stein (21). The protein was hydrolyzed in evacuated sealed tubes in 6 M HCl at 110°C for 22 hr. Methionine was determined after performic acid oxidation according to Moore (22). The least-square numerical method devised by Katz (23) was used for calculating the minimal molecular weight from the amino acid analysis data.

SDS-acrylamide gel electrophoresis was carried out in 1% SDS-7.5% acrylamide gels as described previously (24).[2] The gels were stained with Coomassie brilliant blue (24) or with the periodic acid-Schiff (PAS) reagent according to Zacharius et al. (25).

Blood group activities and activities for myxovirus and phytohemagglutinin receptor sites were measured by hemagglutination inhibition tests. Twofold serial dilutions of antigen-containing materials were tested; the specific inhibitory activities were calculated as the smallest amount of material necessary to inhibit completely hemagglutination at four hemagglutinating units of reagent. The quantities were expressed in micrograms of protein per milliliter according to the values found with the Folin method of Lowry et al. (11). Rabbit antisera for M and N, and human antisera for A and B, were purchased from Behring Diagnostics Inc. (Woodbury, N. Y.). For assay of phytohemagglutinin inhibitory activity, PHA-P (Difco Laboratories, Detroit, Mich.) from *Phaseolus vulgaris* was used. The spot test described by Kornfeld and Kornfeld (26) was employed. Inhibition of myxovirus hemagglutination was determined according to Kathan et al. (4). RI/5+ strain of influenza virus A2 was kindly provided by Dr. Purnell W. Choppin, The Rockefeller University, New York.

Inhibition of the mitogenic response of lymphocytes to various phytohemagglutinins was determined by measuring the incorporation of thymidine-^{3}H into DNA by lymphocytes in short-term cultures to which different amounts of purified MN glycoproteins had been added. 1 ml cultures containing 0.2×10^6 mononuclear cells placed in 13 × 100 mm tissue culture tubes (Falcon Plastics, Oxnard, Calif.) were used. After 72 hr, thymidine-^{3}H incorporation was measured according to Hughes and Caspary (27). *Phaseolus vulgaris* phytohemagglutinins PHA-P (Difco Laboratories, lot No. 550120) and purified phytohemagglutinin (Wellcome Research Laboratories, Beckenham, England, lot No. K. 1360) were used in concentrations of 50 μg/ml and 0.5 μg/ml, respectively. Concanavalin A (K and K Laboratories, Inc., Plainview, N. Y., lot No. 27646) was added at 10 μg/ml.

RESULTS

Preparation of MN Glycoprotein.—150–250 ml of human blood from individual donors, collected in acid-citrate-dextrose and no more than 2 wk old, was used for the preparation of erythrocyte membranes.

Plasma and buffy coat were removed and cells were washed three times with 0.3 M dextrose by centrifugation at +4°C for 15 min at 2000 *g*. Cells were lysed with 9 vol of distilled water and ghosts were sedimented at 12,000 *g* for 20 min. The ghosts were washed four times with 10 mM tris(hydroxymethyl)aminomethane(Tris)-0.1 mM ethylenediaminetetraacetate (EDTA), pH 7.4, by centrifugation at 25,000 *g* for 30 min. Glycoproteins were extracted according to Kornfeld and Kornfeld (26) as described in detail elsewhere.[2] To 1 vol of packed ghosts suspended in Tris-EDTA buffer, 9 vol of a mixture of chloroform-methanol (2:1, v/v) were added with vigorous shaking of the flask. The mixture was stirred for 30 min at room temperature and centrifuged afterwards for 10 min at 1000 *g*. The aqueous phase was carefully aspirated, centrifuged again in order to remove contaminating interphase components, and concentrated

to approximately one-fifth of the volume of the original ghost suspension. The aqueous phase of the chloroform-methanol (CM) extracts was, after reduction and alkylation, fractionated further by gel filtration on Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) columns in the presence of 6 M guanidine hydrochloride (28). To the aqueous phase of CM extracts an equal weight of guanidine hydrochloride was added. Disulfide bonds were reduced by β-mercaptoethanol for 4 hr and alkylation was carried out with 0.1 M iodoacetic acid in the presence of 0.3 M Tris for 1 hr. The solution remained clear throughout this procedure. The reduced and alkylated material was applied with 10% (w/v) sucrose to Sepharose 4B columns prepared with 6 M guanidine hydrochloride; two different sizes were used: 5 cm in diameter and 80 cm in length, or 2.5 cm in diameter and 80 cm in length, respectively. The columns were calibrated with purified polypeptide chains of known molecular weights.

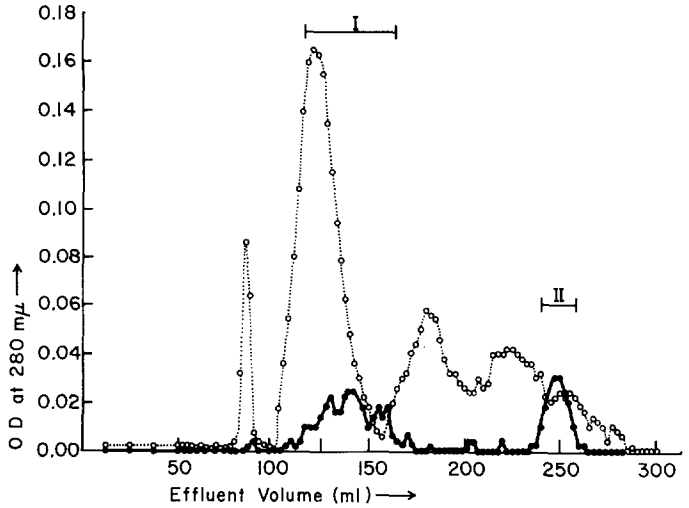

FIG. 1. Gel filtration on Sepharose 4B in 6 M guanidine hydrochloride. O-----O, elution of reduced and alkylated human erythrocyte ghost proteins. ●——●, elution of human erythrocyte membrane glycoproteins from aqueous phase after chloroform-methanol extraction.

Fig. 1 demonstrates a representative elution diagram. The proteins of the aqueous phase of CM extracts are separated into two fractions. The elution pattern of total human erythrocyte ghost proteins on this column is given for comparison (Fig. 1, dotted line). The ghosts were solubilized with 6 M guanidine hydrochloride according to Gwynne and Tanford (28). The red cell ghost proteins were separated into five major fractions. The fractions of the two peaks from the aqueous phase of CM extracts were pooled as indicated, dialyzed for 4 days at +4°C against distilled water, and subsequently concentrated by vacuum ultrafiltration. Analysis of these two fractions were carried out by SDS-polyacrylamide gel electrophoresis (Fig. 2). The aqueous phase of CM extracts contains the glycoproteins GP I, GP II, and GP III and the glycoprotein component B as demonstrated previously.[2] The first peak from the Sepharose 4B-guanidine hydrochloride column is composed of several proteins: some high

molecular weight components penetrate the gel only a few millimeters; these components may represent aggregated glycoproteins. One component migrates slightly slower than GP I; there is also a limited amount of GP I. In addition, substantial amounts of GP II and a fair amount of GP III are found. The second peak contains a single component. Although this material is eluted from the column late, that is at an effluent volume of approximately 250 ml, this glycoprotein GP I migrates relatively slowly on SDS-polyacrylamide gel electrophoresis. In the majority of preparations, the second peak contained only this single component. In the remainder of preparations, this peak contained very

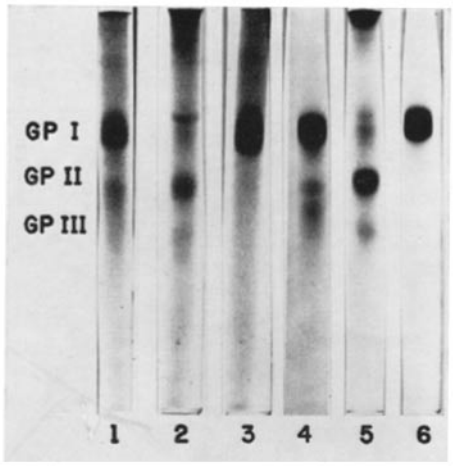

FIG. 2. Electrophoresis on 1% SDS-7.5% acrylamide gels. *1–3* stained by Coomassie brilliant blue: (*1*) aqueous phase of chloroform-methanol extracts, (*2*) fraction I from gel filtration on Sepharose 4B in 6 M guanidine hydrochloride, (*3*) fraction II from gel filtration on Sepharose 4B in 6 M guanidine hydrochloride. *4–6* stained by periodic acid-Schiff (PAS): (*4*) aqueous phase, (*5*) fraction I, (*6*) fraction II. GP, glycoprotein; GP I, II, and III refer to the three glycoproteins present in the aqueous phase of chloroform-methanol extracts.

small amounts of contaminating GP II and GP III which could be disclosed when large amounts of protein (80 μg) were applied to the gels. Purity of these preparations was tested also by polyacrylamide gel electrophoresis without SDS in the presence of 8 M urea, and by electrophoresis on 5% acrylamide gels, without SDS and without urea, using a 5 mM Tris-38 mM glycine buffer, pH 8.3, both as gel buffer and as electrode buffer (24). With both systems single components were observed which, however, gave slightly blurred patterns at the cathodal end of the protein band while the anodal front of the protein bands was sharply defined.

The recovery of purified GP I, the so-called MN glycoprotein, was on the average approximately 5 mg of purified and freeze-dried protein/150 ml blood. It was noticed that the procedure could not be scaled up easily since the capacity

of the Sepharose 4B-6 M guanidine hydrochloride column appeared to be rather limited. Application of larger amounts of proteins from the aqueous phase of of CM extracts did not lead to higher yields of purified MN glycoprotein but to an increase of the first peak which contained relatively large amounts of GP I which were disporprotionately increased, than when smaller quantities of protein were applied to the column.

Analysis of MN Glycoprotein.—The chemical composition of three MN glycoprotein preparations is shown in Table I. Results are given in grams per 100 g dry weight of purified freeze-dried MN glycoprotein. The results of one preparation from each genotype, MM, MN, and NN, are presented. The material contains approximately 40% protein. It is noteworthy that the determination with the phenol reagent of Folin and Ciocalteau gave consistently lower protein estimates than the determination with the biuret reaction. Approximately 40–50% of the material is carbohydrate; approximately 20% of the

TABLE I

Chemical Composition of Human Erythrocyte Membrane Glycoproteins MM, MN, and NN

Constituent	MM	MN	NN
	g/100 g	*g/100 g*	*g/100 g*
Protein (biuret)	38.5	43.0	38.5
Protein (folin)	25.0	26.5	25.5
Sialic acid	17.2	23.4	20.5
Hexosamine	7.9	10.3	7.4
Hexose	10.8	13.7	12.3
Fucose	0.74	0.74	0.60
Phosphorus	0.621	0.120	0.258

total is sialic acid. Traces of phosphorus were detected indicating that these preparations contain phospholipids despite preceding extraction by chloroform-methanol, subsequent treatment with 6 M guanidine hydrochloride, and separation by gel filtration on Sepharose 4B-guanidine hydrochloride columns. The phospholipid content may vary from approximately 20% in the MM preparation to less than 10% in the MN preparation (Table I). Further carbohydrate analysis showed galactosamine concentrations in the MM, MN, and NN preparations of 3.2, 6.4, and 3.4 g/100 g, respectively. The galactose/mannose ratios in the MM, MN, and NN preparations were found to be 3.0, 7.5, and 6.9, respectively.

In Fig. 3 the patterns on 1% SDS-7.5% polyacrylamide gel electrophoresis are shown. Differences in electrophoretic migration rates of the purified glycoproteins of types MM, MN, and NN are not revealed. Fig. 3 also illustrates the varying degrees of purity of different preparations. Whereas the MN glycoprotein shown is free of detectable contaminants, the MM preparation contains one and the NN preparation two minor faster migrating contaminants. Appar-

ent molecular weights were determined from the migration rates according to Weber and Osborn (29). The apparent molecular weight of the MN glycoprotein was with this method found to be 58,000 daltons.

Apparent molecular weights were also estimated from the elution volumes on Sepharose 4B-guanidine hydrochloride columns, as described by Fish, Mann, and Tanford (30). The column was calibrated with purified polypeptide chains; human immunoglobulin G light- and heavy-chains and human haptoglobin 1-1 α- and β-chains were used (Fig. 4). The molecular weight of the MN glycoprotein estimated from the elution volume was found to be 24,000 daltons.

Amino acid composition was determined by duplicate analyses of prepara-

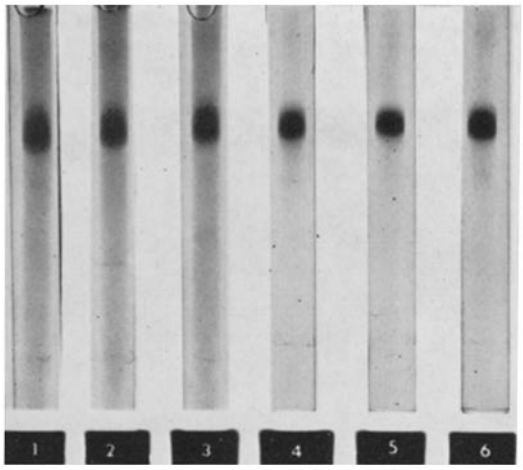

FIG. 3. Electrophoresis on 1% SDS-7.5% acrylamide gels. Purified MN glycoproteins: *1–3* stained by Coomassie brilliant blue; *4–6* stained by PAS reagent. (*1* and *4*) MM glycoprotein, (*2* and *5*) MN glycoprotein, and (*3* and *6*) NN glycoprotein.

tions from three different individuals of each of the three genotypes. The results from these nine preparations are given in Table II. Significant differences among the genotypes MM, MN, and NN were not observed. The content of threonine serine, and glutamine is relatively high; the amount of methionine and phenylalanine is relatively low; half-cysteine residues were not discovered. The amino acid data were used to calculate the minimal molecular weight of the MN glycoprotein (23). The results are given in Fig. 5. The amino acid data are compatible with three different molecular weights for the peptide part of the MN glycoprotein. The molecular weights are 7000, 14,500, and 21,000, respectively. Assuming a peptide content of 40% these values correspond to molecular weights of 17,500, 36,250, and 52,000, respectively for the total glycoprotein. In Table III the number of amino acid residues per peptide has been calculated for the three most compatible molecular weights. The total number of residues was

estimated to be 54, 114, or 164, respectively. The statistical analysis is given by the values of $\Sigma(D_i/R_i)^2 \times 10^4$, where the subscript i represents the ith kind of amino acid residue. D_i is the difference between the analytical composition of an amino acid and its nearest integer for a trial molecular weight, and R_i is moles of i in 10^5 g protein.

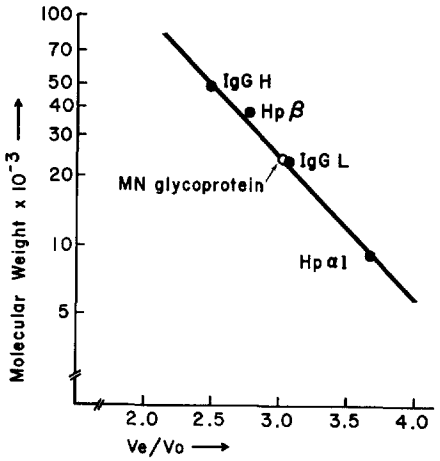

FIG. 4. Determination of apparent molecular weight by gel filtration on Sepharose 4B in 6 M guanidine hydrochloride. For the calibration of the column, reduced and alkylated purified polypeptide chains were used: IgG heavy-chain, haptoglobin β-chain, IgG light-chain, and haptoglobin α1 chain.

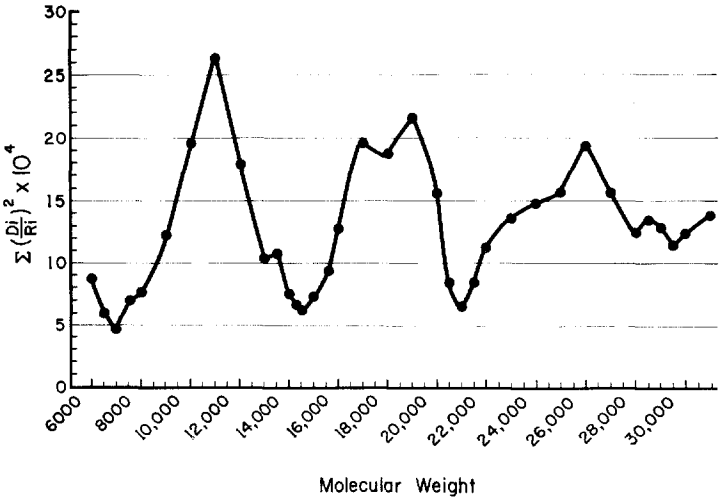

FIG. 5. Calculation of minimal molecular weight by a least-square numerical method using amino acid analysis data. Plot of $\Sigma(D_i/R_i)^2 \times 10^4$ (see text) vs. trial molecular weight for MN glycoprotein.

Immunologic Activities of MN Glycoprotein.—The serologic activities of a total of 14 preparations are summarized in Table IV. Purified MM glycoprotein inhibits rabbit-anti-M serum at a concentration of approximately 5 μg/ml; if very large quantities of MM preparations are employed, 3000 μg/ml, a rabbit-anti-N serum can also be inhibited. Purified NN glycoprotein inhibits a rabbit-anti-N serum at a concentration of approximately 20 μg/ml. The rabbit-anti-M serum cannot be inhibited by this material. Purified preparations from individ-

TABLE II

*Amino Acid Composition of MN Glycoproteins**

	Moles % MM		MN		NN	
	Range	Mean	Range	Mean	Range	Mean
Lys	4.03–4.72	4.51	3.72–5.07	4.21	3.87–4.54	4.23
His	3.60–3.79	3.72	3.36–3.97	3.76	3.30–3.89	3.68
Arg	3.90–4.64	4.29	3.71–4.60	4.21	4.01–4.34	4.20
Asp	6.12–6.62	6.31	5.85–6.33	6.16	6.11–6.53	6.29
Thr	9.84–10.48	10.12	10.20–11.04	10.66	10.0–10.79	10.38
Ser	11.78–13.86	12.68	11.85–12.61	12.23	11.30–12.62	11.96
Glu	10.76–10.97	10.86	9.49–11.01	10.13	11.19–12.06	11.46
Pro	6.26–7.71	6.89	6.79–8.05	7.40	6.86–7.30	7.08
Gly	5.49–6.62	6.05	4.58–5.12	4.89	4.65–5.67	4.95
Ala	4.98–5.31	5.15	4.76–5.27	4.89	4.94–5.45	5.16
Cys/2	0	0	0	0	0	0
Val	7.13–8.00	7.58	7.67–8.52	8.07	6.99–8.23	7.92
Met‡	1.64–2.16	1.81	1.61–2.15	1.97	1.40–1.63	1.51
Ile	6.12–7.51	6.66	7.22–7.57	7.47	6.45–7.67	7.29
Leu	5.13–7.00	5.98	6.32–7.41	6.74	6.64–7.23	6.85
Tyr	3.68–6.70	5.71	4.24–6.47	5.48	3.59–6.48	5.39
Phe	1.46–1.88	1.74	1.55–1.88	1.68	1.59.1.94	1.74

* For each genotype three preparations from three different individuals were analyzed in duplicates. Hydrolysis in 6 N HCl for 22 hr at 110°C.

‡ Met after performic acid oxidation: 2.10 moles per cent (average of two preparations analyzed in duplicates).

uals heterozygous for MN require more than twice the quantity of either homozygous preparation to inhibit M- or N-rabbit antisera. Table IV shows that from the MN preparation approximately 15 μg/ml are required to inhibit the M-, and that approximately 65 μg/ml are necessary to inhibit the N-antiserum. From 5.2 to 42.4 μg/ml of the purified glycoprotein are necessary to inhibit myxovirus hemagglutination. Significant differences between the MN genotypes were not observed, although the quantities required from MM glycoprotein tended to be lower. The purified MN glycoprotein preparations also act as potent inhibitors for *Phaseolus vulgaris* phytohemagglutination. The specific inhibitory activity was found to be approximately 13 μg/ml with a relatively

narrow range from 8.1 to 19.1 μg/ml. Differences in inhibitory activities for PHA were not observed for the three genotypes, MM, MN, and NN. The preparations were also tested for other serologic activities; a total of 15 preparations obtained from donors with blood group A were tested for the presence of the A antigen; 10 preparations were negative for A antigenic activity. In five preparations traces of A activity could be found. The specific inhibitory activities were calculated to be 45, 50, 380, 400, and 1850 μg/ml, respectively. A total

TABLE III

Calculated Number of AA Residues

Assumed molecular weight	7000		14,500		21,000	
Lys	2.32	2	4.81	5	6.97	7
His	2.06	2	4.27	4	6.19	6
Arg	2.31	2	4.78	5	6.92	7
Asp	3.38	3	6.99	7	10.13	10
Thr	5.84	6	12.10	12	17.52	18
Ser	6.71	7	13.89	14	20.12	20
Glu	5.30	5	10.99	11	15.91	16
Pro	4.06	4	8.40	8	12.17	12
Gly	2.84	3	5.89	6	8.53	9
Ala	2.69	3	5.57	6	8.06	8
Cys/2	0	0	0	0	0	0
Val	4.42	4	9.16	9	13.27	13
Met	1.03	1	2.13	2	3.08	3
Ile	4.11	4	8.52	9	12.34	12
Leu	3.70	4	7.66	8	11.09	11
Tyr	2.98	3	6.18	6	8.95	9
Phe	0.92	1	1.90	2	2.76	3
$\Sigma\left(\frac{D_i}{R_i}\right)^2 \times 10^4$	4.80		6.13		6.56	
Total number of AA residues		54		114		164

Data from duplicate analyses of three MN preparations from three different individuals were used.

of 11 preparations were tested for the ability to inhibit hemagglutination by concanavalin A. All 11 preparations were found to be negative. The purified MN glycoprotein does not appear to possess an active receptor for concanavalin A.

Purified MN glycoproteins were tested for inhibition of plant mitogen-induced lymphocyte stimulation in vitro. The results of 12 experiments with the *Phaseolus vulgaris* mitogen PHA-P (Difco Laboratories) are given in Fig. 6. The lymphocyte stimulation induced by a standard dose of PHA-P showed great individual variations. Stimulation as measured by thymidine-^{3}H in-

corporation ranged from 13,500 to 250,000 cpm. Addition of MN glycoprotein also had very variable effects. In some experiments, no significant change was observed. In a few experiments stimulation appeared to be enhanced. In the majority of experiments, however, partial inhibition of lymphocyte stimulation was found. This inhibitory effect appeared to be dose dependent. The variable results could not be related to incompatibilities in the MN system; the MN-type of lymphocyte donor and the MN-type of the erythrocyte glycoprotein preparation were not responsible for the observed variation. When the in-

TABLE IV

Specific Inhibitory Activities of MN Glycoproteins from Human Erythrocytes

	PHA-P (Difco)	Anti-M (rabbit, 7016)	Anti-N (rabbit, 7148)	Influenza virus RI/5+
MM				
A-66839	10.4	5.2	No inhibition	5.2
A-7551-1	15.8	5.2	No inhibition*	10.6
-2	19.1	n.t.	n.t.	n.t.
-3	10.8	4.0	n.t.	10.8
R-70671	10.6	7.1	No inhibition*	14.2
MN				
A-77334-1	10.6	7.2	42.4	42.4
-2	14.7	19.6	78.4	14.8
A-80922	13.6	13.6	36.2	13.6
A-82884-1	11.2	21.6	89.2	29.8
-2	14.2	10.8	85.2	21.2
NN				
A-75056	14.8	No inhibition	29.6	11.0
A-79395-1	16.5	No inhibition	24.4	12.4
-2	8.1	No inhibition	16.2	16.2
C-62971	11.9	No inhibition	15.8	23.8

μg/ml completely inhibiting the agglutination of erythrocytes at four hemagglutinating doses.

n.t., not tested.

* Weak inhibition at 3.0 mg/ml.

dividual variation is disregarded and all results are pooled, average effects can be calculated (Fig. 7). It can be seen that the addition of purified MN glycoprotein results in a partial inhibition of PHA-P-induced lymphocyte stimulation. At a MN glycoprotein concentration of 40 μg/ml stimulation is reduced on the average by approximately 45%. The inhibitory capacity of the erythrocyte MN glycoprotein was also tested for the purified *Phaseolus vulgaris* mitogen (Burroughs Wellcome & Co., Inc., Tuckahoe, N. Y.) (Fig. 8). In these experiments a standard dose of 0.5 μg/ml PHA-BW induced stimulation of 140,000 to 250,000 cpm. Addition of MN glycoprotein to a concentration of

40 μg/ml did not significantly inhibit PHA-BW-induced stimulation of lymphocytes (Fig. 9). Concanavalin A-induced lymphocyte stimulation was studied in four experiments. Addition of MN glycoprotein up to a concentration of 40 μg/ml did not in three out of four experiments inhibit stimulation (Fig. 8). In one experiment partial inhibition was observed. The average results did not indicate a significant influence of the red cell MN glycoprotein on the lymphocyte stimulation induced by concanavalin A (Fig. 9).

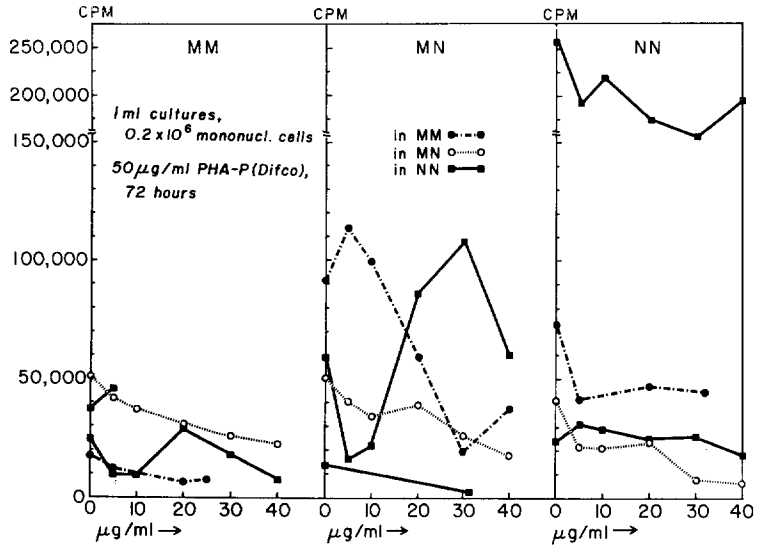

FIG. 6. Inhibition of lymphocyte stimulation induced by *Phaseolus vulgaris* phytohemagglutinin, PHA-P (Difco Laboratories). 50 μg/ml PHA-P were added to 1 ml cultures with 0.2×10^6 mononuclear cells isolated from fresh human peripheral blood. Cultures were kept for 72 hr. Lymphocyte stimulation was measured by thymidine-^{3}H incorporation into DNA; counts per minute are given on the ordinate. Results of 12 experiments are given in three groups according to the MN blood group type of the donor of lymphocytes. On the abscissa the concentrations of MN glycoproteins added to the cultures are plotted. The MN type of the added glycoproteins are given by ●-.-.-.-● for MM, ○-----○ for MN, and ■——■ for NN.

DISCUSSION

By extraction of red cell ghosts with a mixture of chloroform and methanol, erythrocyte membrane glycoproteins are solubilized and recovered in the aqueous phase in high yields and uncontaminated with proteins free of carbohydrate.[2] In the present study it has been shown that the MN glycoprotein can be prepared from this phase by gel filtration on Sepharose 4B-6 M guanidine hydrochloride columns. The purified MN glycoprotein is relatively free from the minor glycoprotein components and, in most preparations, AB blood group activities cannot be demonstrated. The procedure may, thus, offer an advantage

over the widely employed phenol extraction (see ref. 10) and the recently developed solubilization with lithium diiodosalicylate (31). It must be pointed out, however, that our material contained appreciable quantities of lipid.

Analysis of carbohydrate and amino acid compositions of the glycoproteins

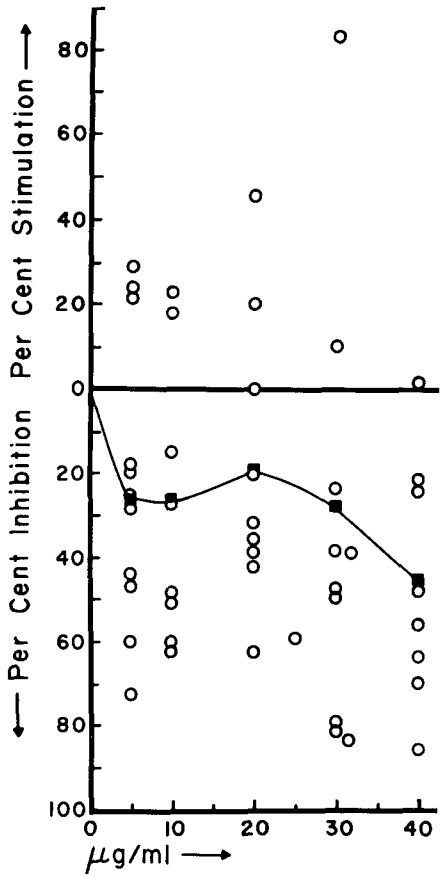

FIG. 7. Influence of MN glycoprotein on the lymphocyte stimulation induced by PHA-P (Difco Laboratories). Results from 12 experiments shown in Fig. 6 are expressed in per cent inhibition and per cent stimulation, respectively. ■—■ is the plot of mean values obtained vs. the concentration of MN glycoprotein added to the cultures.

from the three main MN genotypes did not reveal significant differences confirming previous reports (8, 9). The apparent discrepancies concerning the molecular weight of the MN glycoprotein remain unresolved. Previous ultracentrifugal studies have led to a molecular weight of approximately 31,000 daltons for the momomeric unit (4, 5). More recently, the apparent molecular weight was determined by SDS-polyacrylamide gel electrophoresis and found

to be 55,000 (32). The number of amino acid residues was determined to be 203 (33). In close agreement with these results is the value of 58,000 daltons obtained by us using the same method. Statistical analysis of the amino acid composition, however, suggests a lower molecular weight and a shorter polypeptide chain (Fig. 5 and Table III). If the value of 203 residues per chain proved to be correct, the existence of internal homologies has to be considered. It is interesting to note that the determination of the molecular weight from the elution volume on a Sepharose 4B column in the presence of 6 M guanidine hydrochloride leads to the low value of 24,000. Presumably, the elution of this

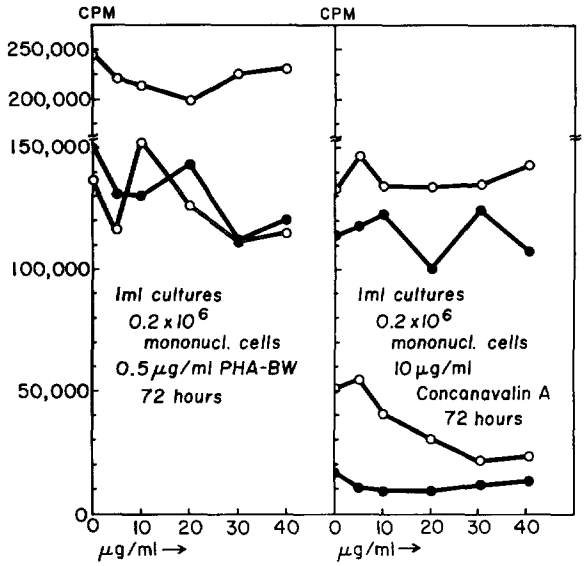

FIG. 8. Influence of MN glycoprotein on the lymphocyte stimulation induced by purified *Phaseolus vulgaris* mitogen (PHA-BW), left side of figure, three experiments, and by concanavalin A, right side of figure, four experiments. Plot as in Fig. 6.

protein very rich in carbohydrate is slightly retarded, but the possibility remains that the values obtained by SDS-acrylamide gel electrophoresis are misleading and higher than the true molecular weight of this protein.

Immunologic analysis demonstrated that in addition to the MN antigens and the receptors for *Vicia graminea* and for myxovirus agglutination the purified MN glycoprotein also contained the receptor for *Phaseolus vulgaris* hemagglutination. The PHA receptor presumably resides in an oligosaccharide side-chain which is structurally different from the oligosaccharide side-chain carrying the MN antigens (10, 26). Highly purified preparations from blood group A donors were free of A activity; the MN glycoprotein also did not contain an active concanavalin A receptor.

The results on the influence of the MN glycoprotein on the PHA-induced

lymphocyte stimulation confirm and extend the initial observations of Kornfeld and Kornfeld (26, 34). The MN glycoprotein inhibits partially the mitogenic activity of unfractionated *Phaseolus vulgaris* phytohemagglutinin. The purified mitogen of *Phaseolus vulgaris* is, however, not inhibited by addition of MN glycoprotein. These results are in agreement with a recent report by Tärnvik and Carlsson (35). The most likely interpretation of our results is the assumption of two different receptor sites on the lymphocyte membrane. One of these

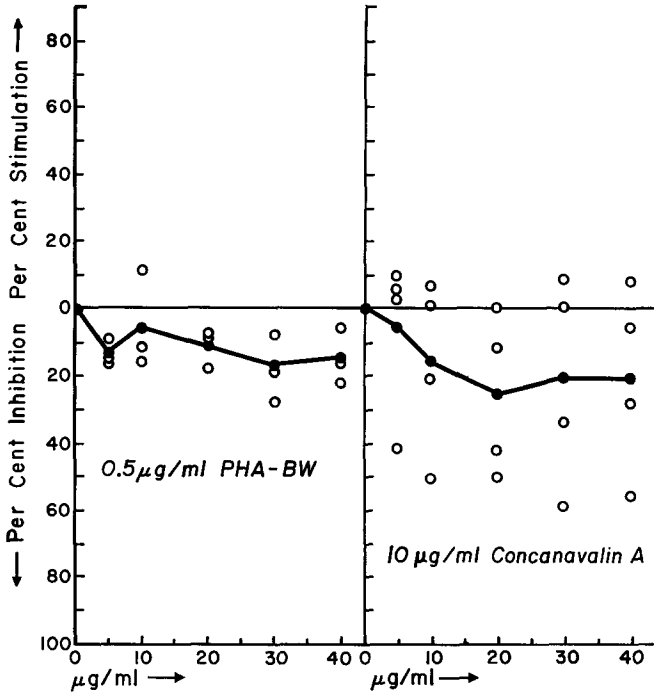

FIG. 9. Influence of MN glycoprotein on the lymphocyte stimulation induced by purified *Phaseolus vulgaris* mitogen (BHA-BW), and by concanavalin A. Plot as in Fig. 7.

receptors reacts with the purified mitogen and is confined to lymphocytes. The other receptor responds to another component in unpurified *Phaseolus vulgaris* preparations. With this reaction the purified MN glycoprotein can interfere, either because similar receptors are present on the red cell MN glycoprotein and on the lymphocyte membrane, or because of binding and inactivation of the mitogen by MN glycoprotein at a site different from that reacting with the lymphocyte receptor.

SUMMARY

Human erythrocyte membrane glycoproteins were solubilized and recovered in the aqueous phase after extraction of red cell ghosts with a mixture of chloro-

form and methanol. The major glycoprotein, the so-called MN glycoprotein, was prepared from this phase by gel filtration on Sepharose 4B columns in 6 M guanidine hydrochloride. By SDS-acrylamide gel electrophoresis the MN glycoproteins from the three major genetic types, MM, MN, and NN, were found to be relatively free from minor glycoprotein contaminants. The carbohydrate and amino acid composition did not reveal significant differences between the three MN genotypes. The specific activities of the purified glycoproteins were determined for inhibition of agglutinating anti-M and anti-N rabbit antisera and for inhibition of myxovirus hemagglutination. It was, furthermore, established that the purified MN glycoproteins contain a receptor for *Phaseolus vulgaris* phytohemagglutination. The red cell MN glycoprotein inhibits partially lymphocyte stimulation induced by unfractionated *Phaseolus vulgaris* phytohemagglutinin; the red cell MN glycoprotein does not influence the lymphocyte stimulation induced by purified *Phaseolus vulgaris* mitogen.

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