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Two Monoclonal Antibodies Highly Specific for the Blood Group N Determinant

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Two monoclonal IgM antibodies, 179K and 35/5F, obtained following immunization of mice with A_2,MN or O,MN human erythrocytes, agglutinate NN and MN red cells strongly, and MM erythrocytes weakly. As shown by hemagglutination inhibition and solid phase ELISA, both antibodies are highly specific for the blood group N determinant. They react with N glycoprotein, its amino-terminal glycopeptides, and with Ss glycoprotein (glycophorin B), which carries the blood group N determinant. They fail to react with M glycoprotein, M glycoprotein-derived glycopeptides, or with internal glycopeptides derived from N glycoprotein. Reaction of the antibodies with N glycoprotein is abolished by desialylation, periodate oxidation/borohydride reduction, or *N*-acetylation of the glycoprotein. Thus, the antibodies are specific for an epitope which includes sialylated oligosaccharide chain(s) and is located in the region of the amino-terminal leucine residue of N glycoprotein. MMU^- erythrocytes, lacking both blood group N and Ss glycophorin are non-reactive. Agglutination of MMU^+erythrocytes by the anti-N antibodies occurs *via* interaction with glycophorin B and correlates with the Ss phenotype of red cells: MM,S erythrocytes are usually more strongly agglutinated than MM,ss cells. The agglutination of MM erythrocytes decreases markedly as the pH is increased from 6 to 8, while agglutination of NN red cells is much less affected by shifts in pH over this range. As a result, both monoclonal antibodies are highly anti-N specific typing reagents when the agglutination assay is carried out at pH 8.

The MN blood group system reflects genetically determined variation in amino acid sequence of the polypeptide chain of the major sialoglycoprotein (glycophorin A) of human erythrocyte membranes: glycoprotein M contains serine and glycine, whereas glycoprotein N has leucine and glutamic acid residues at positions 1 and 5, respectively [1-4]. In both glycoproteins, the serine residue at position 2 and the threonine residues at positions 3 and 4 are *O*-glycosylated [5, 6]. Although no blood group-dependent differences in the structure or distribution of oligosaccharide chains have been found between M and N glycoproteins [3, 7-9], the carbohydrate portion contributes to their antigenic properties [10 and refs. therein]. The minor red cell membrane glycoprotein (glycophorin B) carries blood group Ss determinants [6, 11] and is identical with N glycoprotein in the first 25 amino acid residues of its NH_2-terminal portion in individuals of all M and N blood groups [3, 6].

The structural complexity of M and N antigenic determinants gives rise to the formation of a variety of antibodies which may be directed against different epitopes of these determinants. Antibodies contained in polyclonal rabbit sera and rare human anti-M and anti-N sera react differentially with M and N antigens whose sialic acid residues or amino groups have been chemically modified and with erythrocytes carrying variant forms of glycophorin A or B (see discussion). The varying reactivities of these polyclonal antisera suggest that the predominant antibodies they contain are different. The hybridoma technique offers the possibility of obtaining anti-M or anti-N monoclonal antibodies (MoAbs) with different individual "subspecificities" that should allow a more precise characterization of antigenic sites within the genetically differentiated portion of glycophorin A. Several monoclonal antibodies against glycophorin A have already been obtained and some of them showed anti-M or anti-N specificities [12-16]. In this report we describe two new highly specific monoclonal anti-N antibodies which were obtained independently in Bethesda (MoAb 179K) and in Lund (MoAb 35/5F) and have almost identical properties.

Materials and Methods

Immunization and Preparation of Hybridomas

Balb/cj female mice (Jackson Laboratories, Bar Harbor, MA, USA), 12 weeks old, were injected intraperitoneally with 1 ml of a 10% suspension of O,MN human erythrocytes in Dulbecco's phosphate buffered saline pH 7.5 (PBS)(M. A. Bioproducts, Walkersville, MD, USA) and after 2 weeks were boosted with a second injection. Four days later the mice received 0.4 ml of the same erythrocyte suspension by injection into the tail vein. Three days after the final injection, splenocytes from immunized mice were fused at a ratio of 5:1 with SP2/0 plasmacytoma cells according to the method described by Nowinski *et al.* [17], using 50% polyethylene glycol (PEG-1000, Sigma, St. Louis, MO, USA). Subsequent cloning was performed by the limiting dilution technique in 96-well microtiter plates with Balb/c thymocytes as feeder cells, at 10^5/well. Hybridomas were selected by hemagglutination tests described below, using a panel of native and papain-treated blood group O,MM and O,NN red cells. After double cloning a monoclonal hybridoma

cell line producing anti-N agglutinin and designated 179K was established. The cells were grown as ascites tumors in Balb/c mice.

A hybridoma cell line producing another anti-N antibody was obtained in a similar way after immunization of 5-9 week old female Balb/cABom mice (obtained from Gl Bomholtgaard, Ry, Denmark) with A_2,MN erythrocytes. Immunization, fusion, and cloning procedures have been described [18]. The established monoclonal cell line 35/5F was grown as ascites tumors in mice treated with Pristane® (Aldrich, Beerse, Belgium), or in expanded cell culture *in vitro* under standard conditions [18].

Isotype Determination

The antibody isotype was determined by double immunodiffusion in agarose, using rabbit anti-mouse IgGl, IgG2a, IgG2b, IgG3 and IgM sera from Miles (Elkhart, IN, USA).

Blood Group M, N and Ss Glycoproteins

Sialoglycoproteins were prepared from outdated O,MM and O,NN erythrocytes by phenol/saline extraction of erythrocyte membranes [19]. The crude glycoproteins were fractionated on Ultrogel AcA44 (LKB, Uppsala, Sweden) equilibrated with 0.05 M pyridine-acetate buffer pH 5.3 containing 1% sodium dodecylsulfate (SDS) (Sigma). The column fractions were monitored for absorbance at 280 nm, for neutral sugar content by the phenol/sulfuric acid method [20], and by SDS-polyacrylamide gel electrophoresis [21]. Fractions containing PAS-1 and PAS-2 electrophoretic bands, corresponding to dimeric and monomeric glycophorin A, were pooled, dialyzed against 50% ethanol to remove SDS, dialyzed against distilled water, and lyophilized. Those column fractions enriched in Ss glycoprotein (glycophorin B) were also pooled and rechromatographed on Ultrogel AcA54 under the same conditions. Fractions shown by electrophoresis to contain pure glycophorin B (predominantly PAS-3 band) were pooled and treated as described above for M and N glycoproteins.

Tryptic and Chymotryptic Glycopeptides of M and N Glycoproteins

The blood group M and N glycoproteins were digested with trypsin (Worthington, Freehold, NJ USA) for 2 h at 37°C and the digests were fractionated on Sephadex G-200 [1] to give a rough separation of NH_2-terminal glycopeptides designated T1/T2 (a mixture of two glycopeptides with amino acid residues 1-39 and 1-30/31) from an internal glycopeptide T3 (amino acid residues 40-61). The T1/T2 glycopeptides were further purified on DEAE-Sephadex A-50 as described previously [1]. Fractions from the Sephadex G-200 column containing the glycopeptide T3 were pooled and the glycopeptide was further purified by gel filtration on Sephadex G-75, and then by ion-exchange chromatography on DEAE-Sephadex.

Digestion of M and N glycoproteins with chymotrypsin (Worthington) was carried out in 0.1 M sodium phosphate buffer pH 7.0 for 24 h at 37°C. The glycopeptides CH1, CH2 and CH3, containing amino acid residues 1-64, 1-34 and 35-64, respectively, were isolated

by gel filtration on an Ultrogel AcA54 column equilibrated with 0.1 M ammonium acetate pH 6.8 [5].

Identification and assessment of purity of all glycopeptides was based on analysis of their amino acid and carbohydrate compositions and NH_2-terminal amino acids [1] compared with the amino acid sequence, carbohydrate composition and sites of glycosylation, and proteolytic cleavage sites of glycophorin A [5].

Modification of Blood Group N Glycoprotein

Glycoprotein N was desialylated by treatment with 0.5 U neuraminidase from *Clostridium perfringens* (Sigma) in 0.03 M sodium acetate buffer pH 5.5 and 0.01 M $CaCl_2$ for 2 h at 37°C. Desialylation was also performed by hydrolysis of the glycoprotein in 0.025 M sulfuric acid for 5 h at 60°C, followed by neutralization with NaOH, dialysis and lyophilization. *N*-Acetylation was accomplished by treating 1 ml of 0.2% glycoprotein solution with five 10 μl portions of 10% acetic anhydride in ethanol at about 10 min intervals. After each addition of acetic anhydride the pH was adjusted to 7.5-8 with 0.01 N NaOH. The sample was dialyzed and lyophilized. Periodate oxidation was carried out by treating 2 ml of 0.1% glycoprotein solution with 0.05 M sodium periodate at pH 6 for 10 h at 4°C. The sample was then treated with an equal volume of 1% $NaBH_4$ for 1 h at room temperature. The excess $NaBH_4$ was decomposed with 5% acetic acid and the sample was dialyzed and lyophilized.

Erythrocytes and their Enzymic Modifications

A standard panel of red cells of different phenotypes was kindly provided by the NIH Blood Bank and the Blood Bank, University Hospital, Lund. Protease treatment of red cells was carried out by incubating 250 μl of packed cells resuspended in an equal volume of PBS containing 1 mg TPCK-trypsin or chymotrypsin (Worthington) for 2 h at 37°C. Red cells were desialylated by incubating 1 ml of a 20% suspension of cells in 0.15 M NaCl containing 0.04 U neuraminidase (Sigma) for 30 min at 37°C. The effectiveness of desialylation was checked by determination of sialic acid in the supernatant by the method of Jourdian *et al.* [22], and by testing the agglutinability of erythrocytes by peanut lectin (Sigma). The amount of sialic acid released under these conditions was about 100 μg per ml packed red cells. The asialo-erythrocytes were agglutinated by peanut lectin at concentrations of lectin below 1 μg/ml.

Hemagglutination and Hemagglutination Inhibition

Hemagglutination was performed in 96-well V-bottom microtiter plates by incubating 25 μl of a 1% suspension of erythrocytes with serially diluted 50 μl aliquots of hybridoma culture supernatant or ascitic fluid. Plates were read macroscopically after 1 h at room temperature. Hemagglutination inhibition assays were carried out by preincubating 25 μl of a solution containing antibody at two doubling dilutions below the endpoint titer with an equal volume of serially diluted inhibitor. After 1 h at room temperature, 25 μl of 1% red cell suspension was added and agglutination was read after 1 h.

Alternatively, agglutination was tested on glass macroplates, using 40 μl of serially diluted antibody solution and 20 μl 5% red cell suspension. The results were read macroscopically after 10 min at room temperature. For inhibition of agglutination, 20 μl samples of antibody solution diluted as given above were mixed with 20 μl aliquots of serially diluted inhibitor and left for 10 min at room temperature. Then 20 μl of 5% erythrocyte suspension was added and agglutination was read after 10 min. The first and second versions of the assay were used for testing 179K and 35/5F, respectively. Some assays were performed in parallel with both MoAbs using identical procedures. Unless otherwise specified, the assays were carried out in Dulbecco's PBS which was used for dilutions and suspending the erythrocytes.

Enzyme-linked Immunosorbent Assays (ELISA)

ELISA was carried out in microtiter plates (Dynatech, Immunolon, Plochingen, W. Germany) coated with purified M or N glycoprotein by incubating a 2 μg/100 μl solution in 0.05 M sodium carbonate buffer pH 9.6 in each well overnight at 4°C. Wells were washed three times with PBS, pH 7.5 containing 0.05% Tween 20, and antibody (100 μl/well) was serially diluted in triplicate in the same buffer. After incubation for 30 min at room temperature with gentle shaking, polyvalent rabbit anti-mouse immunoglobulins-alkaline phosphatase (Dakopatts, Copenhagen, Denmark) was added at 100 μl/well. The plates were incubated as before and washed 4 times. A solution containing *p*-nitrophenyl phosphate, di-sodium (Sigma 104 Phosphatase Substrate Tablets) dissolved in substrate buffer (0.05 M sodium carbonate buffer plus 1 mM $MgCl_2$, pH 9.6, 1 tablet/5 ml) and 100 μl of this solution was added to each well. Plates were scanned at 405 nm using a microtiter plate scanner (LKB Multiscan) at time 0 and after 30 min intervals until the increase of absorbance with time was linear.

Dot immunobinding assay on nitrocellulose was performed according to the procedure of Beyer [23]. M and N glycoprotein solutions were serially diluted and applied as dots (10-0.3 μg/2 μl dot) on nitro-cellulose filters (BA 85, 0.45μ, Schleicher and Schuell, Keene, NH, USA). The filters were consecutively incubated with 35/5F undiluted culture supernatant fluid, rabbit anti-mouse IgM antibody (Miles) diluted 1:200, Protein A-peroxidase conjugate (Sigma) diluted 1:500, and the substrate prepared by dissolving 20 mg 3-aminoethylcarbazole in 2.5 ml dimethylformamide and mixing with 50 ml 0.15 M sodium acetate buffer pH 5.5 containing 25 μl 30% H_2O_2.

Results

Both antibodies 179K and 35/5F are of the IgM isotype. They strongly agglutinate all blood group N-positive (NN and MN) erythrocytes (Table 1). Agglutination of MM erythrocytes is weaker and correlates with the Ss phenotype: most red cells of SS or Ss phenotype are agglutinated more strongly than erythrocytes of ss phenotype (Table 1). Erythrocytes of the MMS^-s^-U phenotype, which lack glycophorin B completely [11] are not agglutinated at all by the potent 179K ascitic fluid.

Table 1. Agglutinating titers of two monoclonal antibodies with erythrocytes of various MNSs phenotypes.

Erythrocytes	Antibodies	
	179K ascitic fluid	35/5F culture supernatant
NN, all Ss types	1/800 000—1/1 600 000[a]	1/500—1/1000
MN, all Ss types	not tested	1/500 — 1/1000
MM SS or MM Ss	1/12 800	1/4 — 1/64
MM ss	1/1000	neg — 1/8
MM $S^-s^-U^-$	neg[b]	not tested

[a] The range of titers is given when 5—10 samples of erythrocytes with indicated phenotype were tested.
[b] "neg" denotes lack of agglutination.

These results suggest that both MoAbs have anti-N specificity and agglutinate MM erythrocytes due to the interaction with Ss glycoprotein which carries the blood group N determinant [2, 3, 6]. This conclusion is further supported by the findings that treatment of erythrocytes with trypsin and chymotrypsin produces differential effects on their agglutinability (Table 2). Trypsin selectively digests glycophorin A on red cell surfaces and chymotrypsin preferentially digests glycophorin B [24, 25]. Treatment of NN red cells with trypsin or chymotrypsin decreases their agglutinability by both MoAbs in the same manner as treatment of MM erythrocytes with chymotrypsin. However, tryptic digestion of MM erythrocytes causes increased agglutination, due to degradation of M glycoprotein (glycophorin A) and better exposure of N-like Ss glycoprotein (glycophorin B). All erythrocytes tested lose their agglutinability after neuraminidase treatment, indicating that both MoAbs require sialic acid residues on antigenic determinants (Table 2).

The anti-N specificities of the MoAbs were further confirmed by a solid phase ELISA using microtiter plates coated with purified M or N glycoprotein. Both antibodies bound N glycoprotein, but failed to bind M glycoprotein over a wide range of antibody concentrations (Fig. 1). The same result was obtained with antibody 35/5F in "dot" ELISA assays on nitrocellulose. The color spots were distinctly visible with N glycoprotein present in amounts of 10-0.6 μg per dot, while no reaction was detectable with M glycoprotein at 10 μg per dot (results not shown).

To define the specificity of the MoAbs in more detail, we studied inhibition of the hemagglutination reaction by various preparations of modified and unmodified M and N glycoproteins and their glycopeptides. The results of hemagglutination inhibition are essentially the same for the MoAbs 179K and 35/5F (Table 3). Some differences in apparent activities of inhibitors may have resulted from testing them in separate laboratories under slightly different conditions (see Materials and Methods). Both MoAbs are inhibited by N glycoprotein, its NH_2-terminal glycopeptides, and by Ss glycoprotein which shows inhibitory activity equal to that of N glycoprotein. M glycoprotein, all its glyco-

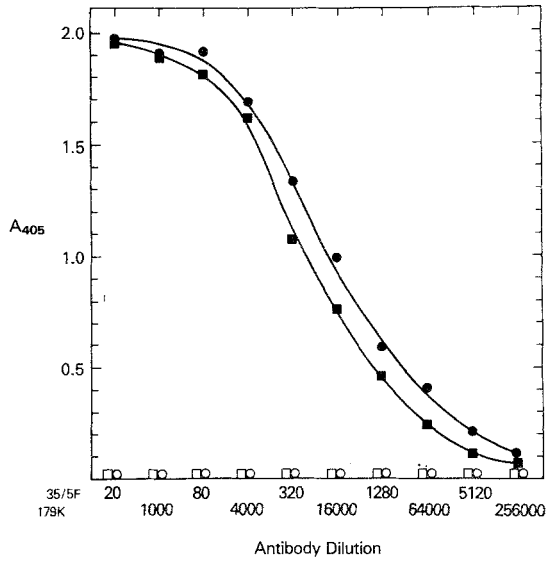

Figure 1. Binding of monoclonal antibodies 179K (□, ■) and 35/5F (○, ●) to blood group M and N glycoproteins, measured by the microtiter plate ELISA described in the Materials and Methods section. Binding to N glycoprotein-coated wells (■, ●) and to M glycoprotein-coated wells (□, ○) was recorded after incubating the enzyme reaction for 1h.

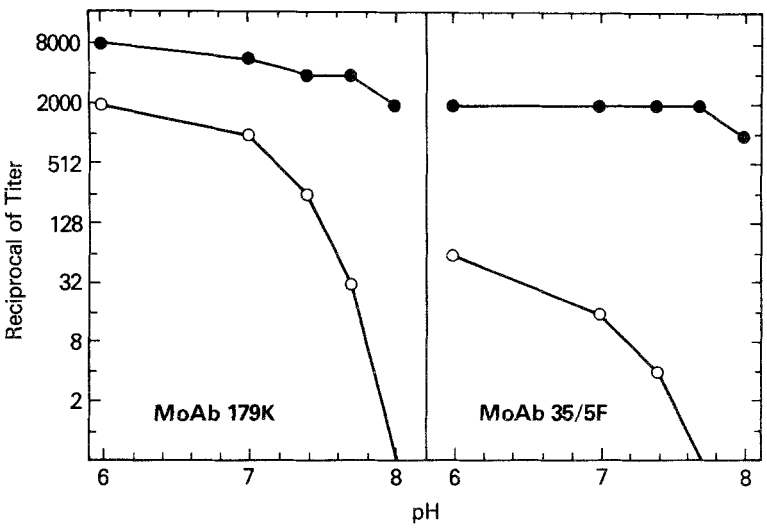

Figure 2. The effect of pH on agglutination of NN, Ss (●) and MM, SS (○) erythrocytes by monoclonal antibodies. Ascitic fluids 179K and 35/5F were serially diluted with 0.1 M sodium phosphate buffer of the indicated pH and tested using a microtiter agglutination assay (see the Materials and Methods section).

Table 2. Effect of enzymatic treatments of MM and NN red cells on their agglutinability by the monoclonal anti-N antibodies 179K and 35/5F.

Antibody	Red cell phenotype	Titer			
		Untreated Red Cells	Red Cells treated with		
			Trypsin	Chymotrypsin	Neuraminidase
179K ascitic fluid	NN S	1/800 000	1/26 000	1/13 000	1/2
	NN ss	1/1 600 000	1/26 000	1/3 200	1/2
	MM S	1/13 000	1/100 000	1/2	1/2
	MM ss	1/1000	1/13 000	1/2	1/2
35/5F culture supernatant	NN ss	1/500	1/8	1/2	neg[a]
	MM S	1/8	1/64	neg	neg
	MM ss	neg	1/4	neg	neg

[a] "neg" denotes lack of agglutination.

peptides, and the internal glycopeptides derived from N glycoprotein are inactive (Table 3).

Desialylation or periodate oxidation of N glycoprotein totally abolishes its interaction with the MoAbs (Table 3). The only components oxidized within the blood group N determinant are sialic acid residues, which are transformed into seven-carbon derivatives [26-28]. Therefore, our results suggest that both MoAbs, like the previously studied polyclonal anti-M and anti-MN sera [28-31], require sialic acid residues with carbons 7-9 intact for full binding activity. Both anti-N MoAbs also fail to react with *N*-acetylated blood group N glycoprotein (Table 3). A similar requirement for unmodified amino groups in these antigens was previously found for most polyclonal anti-M and anti-N sera [9, 30, 32-35].

As intact amino groups appear to be required for antigenic activity of M and N glycoproteins, we studied the pH-dependence of agglutination of MM and NN erythrocytes by the MoAbs. Ascitic fluids of 179K and 35/5F were serially diluted in 0.1 M sodium phosphate buffers in the range of pH 6-8, and agglutinating titers were estimated after addition of erythrocytes suspended in saline (Fig. 2). The strongest agglutination of both types of erythrocytes is observed at pH 6. Agglutination of NN erythrocytes is only slightly affected by pH, the titer at pH 8 being only two- to four-fold lower than at pH 6.0. However, agglutination of MM erythrocytes is distinctly pH-dependent: MoAbs 179K and 35/5F agglutinate MM erythrocytes at pH 6.0 at the titer 1/2000 and 1/64, respectively, but neither antibody agglutinates MM erythrocytes at pH 8.0. Similar results were obtained with several samples of NN and MM red cells. Therefore, these MoAbs are highly specific typing reagents when agglutination is carried out at slightly alkaline pH.

Table 3. Minimum concentration (μg/ml) of untreated or modified red cell sialoglycoproteins or their glycopeptides required to inhibit agglutination of NN red cells by monoclonal antibodies 179K and 35/5F.

Inhibitor		Inhibitor concentration (μg/ml)	
		Antibody 179K	Antibody 35/5F
Glycoprotein M		>5000[a]	>5000
Glycoprotein N:			
untreated		15 (40)[b]	40
desialylated		>5000	>5000
N-acetylated		5000	5000
periodate/borohydride[c]		>5000	>5000
Glycoprotein Ss		(40)	40
Tryptic glycopeptides:			
T1/T2(1-30/31+1-39)[d]	M	>5000	>10000
	N	300	2500
T3 (40-61)	N	n.t.[e]	>10000
Chymotryptic glycopeptides:			
CH1 (1-64)	M	>5000	n.t.
	N	300	n.t.
CH2 (1-34)	M	>5000	n.t.
	N	600	n.t.
CH3 (35-64)	M or N	>5000	n.t.

[a] Denotes that no inhibition was observed at the concentration given.
[b] Values in parentheses indicate the results obtained when MoAbs 179K and 35/5F were tested in parallel (see Materials and Methods).
[c] Indicates that the glycoprotein was oxidized with periodate and reduced with sodium borohydride as described in the text.
[d] Numbers in parentheses indicate amino acid residues of glycopeptides from M or N glycoproteins.
[e] n.t. = not tested.

Discussion

The MoAbs 179K and 35/5F are highly specific for the blood group N determinant. They react strongly with glycoproteins and glycopeptides carrying this determinant but not with purified M glycoprotein and its glycopeptides when assayed by inhibition of hemagglutination, or in two kinds of ELISA. However, our results confirm that even highly specific anti-N reagents cannot be absolutely specific for N-positive erythrocytes because all erythrocytes except those of the very rare $S^-s^-U^-$ phenotype carry the Ss glycoprotein which bears the blood group N determinant [2, 3, 6]. Interestingly, we noticed a correlation between agglutinability of various MM erythrocytes by the anti-N MoAbs and the Ss phenotype of red cells. Dahr *et al.* [36, 37] reported that staining of the electro-

phoretic band PAS-3, representing Ss glycoprotein, is about 1.5 times more intense in red cell glycoprotein preparations from SS individuals than in preparations from ss individuals. Thus, the stronger agglutinability of MMS^+ red cells as compared with MMss cells, probably results from S^+erythrocytes carrying more copies of glycophorin B per cell. Monoclonal anti-N antibodies may be useful to study this problem further.

Both MoAbs 179K and 35/5F require free amino groups in blood group N determinants. The sharp decrease in agglutinability of MM erythrocytes over the pH range 6-8 is probably due to titration of amino groups in the N antigen carried on glycophorin B, rather than to direct effects of pH on the antibody. This conclusion is supported by the lack of any strong effect of pH on agglutination of NN erythrocytes and by results obtained with anti-N *Vicia graminea* lectin [38]. Unlike anti-M and anti-N antibodies, this lectin reacts better with N and M glycoproteins with blocked amino groups and binding of the lectin to NN and MM erythrocytes increases markedly with increasing pH in the range of 6-8. In the agglutination reaction of *V. graminea* lectin with NN erythrocytes, which contain a large number of N antigenic sites carried on both the N glycoprotein (0.5-2 $\times$ 10^6 copies per cell) and Ss glycoprotein, increasing the pH from 6 to 8 produces little effect. However, for MM erythrocytes, whose only N antigen is carried by the Ss glycoprotein (0.7-1 $\times$ 10^5 copies per cell), agglutination increases markedly over the pH range 6-8. These observations imply that a critical number of non-ionized N antigenic sites are necessary for hemagglutination of red cells by *V. graminea* lectin and that the critical number of sites is reached during titration of MMS cells from pH 6 to 8. When the total number of sites per cell is large, as in NN cells, the critical number of sites apparently exists over the entire pH range from 6-8.

Both the anti-N MoAbs we have studied require distinctly higher concentrations of N glycopeptides than of N glycoprotein for inhibition of hemagglutination, a finding that agrees with our previous experience with many polyclonal anti-M and anti-N sera. However, there may be exceptions to this rule since monoclonal anti-M antibodies obtained by Bigbee *et al.* [13] were equally well inhibited by M glycoprotein and tryptic M glycopeptides. The higher activity of M and N glycoproteins compared to their glycopeptides is probably due to their aggregated state in aqueous buffers [39] and to their polyvalency. Recent ^{13}C-NMR studies indicate that proteolytic degradation of glycophorin A does not significantly affect the conformation of its NH_2-terminal portion and that secondary structure is preserved even in small NH_2-terminal glycopeptides [9, 40, 41].

The fact that M and N glycoproteins differ at two positions of the polypeptide chain (Table 4) suggests that each of the glycoproteins contains two blood group-specific epitopes in the region of the first and fifth amino acid residues which are here tentatively designated M_{ser}, M_{gly}, N_{leu}, N_{glu}. This possibility has also been suggested by the results of studies on the interaction of polyclonal anti-M and anti-N sera with some rare erythrocytes carrying variant MN antigens. There are many variant antigens related to the MNSs blood group system [47] and the structures of some of them have been elucidated (Table 4). For example, the NH_2-terminal portion of glycophorin A from M^c erythrocytes has a structure of intermediate type between M and N antigens (Ser...Glu, Table 4). M^c cells are agglutinated by most anti-M and a minority of anti-N sera [48, 49], indicating that most polyclonal anti-M and anti-N sera are of anti-M_{ser} and anti-N_{leu} types, respectively. However, there are also less common antisera containing predominantly antibo-

Table 4. Structures of amino-terminal portions of M,N glycoprotein (Glycophorin A) and Ss glycoprotein (N-like, glycophorin B) containing common and variant forms of the M and N antigens.

Blood group determinant	Glycophorin that carries determinant	Amino-terminal structure	References
M	A	0^a 0 0 | | | Ser-Ser-Thr-Thr-Gly-	1-4,6
N	A and B	0 0 0 | | | Leu-Ser-Thr-Thr-Glu-	1-4,6
M^c	A	0 0 0 | | | Ser-Ser-Thr-Thr-Glu-	42,43
M^g	A	Leu-Ser-Thr-Asn-Glu-	43-45
He (Henshaw)	B	0 0 0 | | | Try-Ser-Thr-Ser-Gly-	46

[a] "0" indicates the presence of an *O*-linked oligosaccharide chain with the structure: NeuAcα2-3Galβ1-3 (NeuAcα2-6)GalNAcβ1-*O*-Ser(Thr).

dies against the internal portion of the M or N determinant, i.e. anti-M_{gly} or anti-N_{glu}. Some anti-M sera also agglutinate NN, Henshaw (He)-positive erythrocytes. Anti-M and anti-He specificities in these sera are inseparable and designated anti-M^e [50-52]. The structure of the Henshaw antigen (Table 4) suggests that anti-M^e is an anti-M of anti-M_{gly} type. It is noteworthy that He and M^g variant antigens with amino acid residues not present in M and N determinants (Table 4) give rise to anti-He and anti-M^g antibodies which do not cross-react with MN antigens, but a specific anti-M^c antibody has not been found so far [47].

Modifications of amino groups abolish the reactions of M and N glycoproteins with the respective antisera [30, 32-34] and the loss of activity is specifically determined by modification of the alpha-amino group of NH_2-terminal amino acid residues [9, 35]. However, a few anti-M sera react equally well with untreated and *N*-acetylated M glycoprotein [33, and E. Lisowska unpublished]. These data suggest that the more common anti-M_{ser} and anti-N_{leu} antibodies require a free amino group at the NH_2-terminal serine or leucine residue, respectively, whereas the less frequent anti-M_{gly} and anti-N_{glu} antibodies bind their antigens whether or not the amino group is substituted.

Other variations in specificity among anti-M and anti-N antibodies may result from recognition of different regions of the carbohydrate portion in the antigenic epitopes. Although it has been firmly established that anti-M and anti-N sera fail to react with desialylated antigens [9, 53-57], Judd *et al.* [58] and Issitt and Wilkinson [59] described a number of human and rabbit anti-M and anti-N sera reacting equally well with native and desialylated erythrocytes.

All these differences between polyclonal anti-M and anti-N sera could be more readily defined by characterizing individual reagents within a large panel of monoclonal antibodies against the M and N antigens. Several anti-M and anti-N monoclonal antibodies described so far [12-16] have been characterized to different degrees. All were NeuAc-dependent and all antibodies classified as anti-N reacted with MM red cells. Their specificities were assessed by the lack of agglutination of $MMS^-s^-U^-$ erythrocytes and by the effects of proteolytic treatments of erythrocytes on their agglutinability. Dependence of the antigen on amino groups was tested only by Bigbee *et al.* [13]. Their two anti-M MoAbs 6A7 and 9A3 were not sensitive to *N*-acetylation of MM erythrocytes. According to the above considerations they should have anti-M_{gly} specificity and should not react with M^c antigen. However, only antibody 6A7 does not react with M^c antigen [14] and therefore appears to bind a complex epitope which is altered when glutamic acid is substituted for glycine at position 5. In contrast, antibody 9A3 reacts equally well with MM and M^c antigens [14] and thus may recognize the M_{ser} determinant, but the contribution of the amino terminal serine residue to binding apparently does not include the alpha-amino group. The MoAb 8A2 failed to react with *N*-acetylated erythrocytes, but the anti-N specificity of this antibody is not certain since it binds to MM red cell-derived glycophorin A electophoretic band PAS-1 [13]. The pH-dependence was checked only for two anti-N MoAbs obtained by Fraser *et al.* [12]. These authors found a rather sharp dependence of agglutination on pH which was similar for MM, MN and NN erythrocytes, with a maximum at pH 8.5. This result was entirely different from those described in this paper and may reflect a strong pH-sensitivity of the antibody molecules studied by Fraser *et al.* [12]. Unfortunately, these antibodies were not tested with *N*-acetylated M and N antigens. Ochiai *et al.* [60], described an anti-glycophorin A MoAb which was not blood group-specific but showed some preference in reaction with M glycoprotein. Their results suggest that some MoAbs may be directed against an epitope comprising one of the blood group-related amino acid residues, but this residue is not immunodominant.

In conclusion, it would be interesting if all MoAbs with established anti-M or anti-N specificity were tested for their dependence on amino groups of the antigen and for the reactivity with variant red cells, e.g. M^c or NN,He. It should be useful to establish whether the amino group-dependence of an anti-N or anti-M MoAb could be a simple indicator of whether or not the MoAb is directed against the epitope in the region of the first (Ser or Leu) or fifth (Gly or Glu) amino acid residue of glycophorin A, especially in view of the properties of MoAb 9A3 [13, 14]. Our anti-N MoAbs 179K and 35/5F have not been tested yet with variant red cells. However, based on the results described in this paper, they seem to fall into the most common category of anti-N_{leu}, NeuAc-dependent antibodies. Characterization of a greater number of monoclonal anti-M and anti-N antibodies should show whether the suggestions discussed here are correct.

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