MONOCLONAL ANTIBODIES AGAINST M AND N ANTIGENS

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INTRODUCTION

The MN antigenic determinants reside in the amino-terminal part of glycophorin A (MN glycoprotein) of the human erythrocyte membrane (1-6). The amino-terminal part of glycophorin B (Ss glycoprotein) is structurally identical with that of glycophorin A of NN individuals (2,3,6) and therefore also carries N determinants. The N activity of MM cells is low but still large enough to cause weak reactions with some polyclonal anti-M sera particularly with red cells that also carry the S antigen since they contain more glycophorin B than ss cells (7,8). The MN blood group antigenic determinants consist of a sequence of 5 amino acids (fig 1), and acids 2-4 are substituted with identical tetrasaccharide structures (5,6). Amino acids 1 and 5 are Ser and Gly in M, and Leu and Glu in N antigen, whereas acids 2-4 are identical in both (fig 1).The sugar moiety consists of an internal Gal-GalNAc sequence with two molecules NeuAc attached (fig 1). Most polyclonal reagents recognize the amino-terminal end of the glycoprotein molecule including NeuAc. For this reason the majority of these sera do not react with cells that have been desialylated. Variant M and N antigens exist and the structures of some of them have been elucidated eg M^c and He (fig 1). The variant structures contain amino acid substitutions at positions 1-5 in the polypeptide chain. M^c for instance contains Ser at position 1 as normal M and Glu at position 5 as normal N. This will give rise to atypical reactions with some anti-M and anti-N reagents. It is of great importance particularly in paternity testing to be able to disclose such anomalies and therefore a precise characterization of the epitope recognized by any particular reagent is of interest. In the present study one monoclonal antibody of each specificity were characterized.

FIG 1

BLOOD GROUP	ON GLYCOPHORIN	AMINO-TERMINAL STRUCTURE	REF
M	A	O* O O | | | Ser-Ser-Thr-Thr-Gly-	1-4, 6
N	A and B	O O O | | | Leu-Ser-Thr-Thr-Glu-	1-4, 6
M^c	A	O O O | | | Ser-Ser-Thr-Thr-Glu-	10, 11
He	A	O O O | | | Try-Ser-Thr-Thr-Gly-	10, 11

*O = NeuAcα2-3Galβ1-3[NeuAcα2-6]GalNAc-

MATERIAL AND METHODS

Balb/cABom female mice were injected either with native human A_2, MNSs red cells or with purified glycoprotein prepared from red cell membranes of MM donors.

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The immunization protocol, procedures for cell fusion, cloning and cultivation of growing hybridomas have been described earlier (13).Tissue culture supernatants were tested for hemagglutinating activity against MM and NN red cells in microtiter plates. Hybridomas giving strong and specific reactions were expanded in tissue culture and the culture supernatants were used in further studies.
M,N and Ss glycoproteins and desialylated or N-acetylated derivatives were prepared as described earlier (14).
For hemagglutination studies a panel of red cells of different phenotypes was used. The red cells were used native or after treatment with TPCK-trypsin (Merck), trypsin (Merck) or neuraminidase (Sigma), and suspended in isotonic saline. Tests were performed in tubes or on tiles and read macro-and microscopically.
ELISA was performed in microtiter plates coated with purified M or N glycoproteins or their derivatives as described earlier (14). The same substances were also used for inhibition of antibody binding in ELISA and/or hemagglutination.

RESULTS
Antibody 35/5 F (anti-N):
Native tissue culture supernatant (pH 7.2) gave an immediate and strong agglutination reaction with all NN and MN red cells, and a weaker or no reaction with MM red cells. Stronger reactions were observed with MM cells that also carried the S antigen. The titer with MMS cells from different individuals after serial dilution of the supernatant with PBS at pH 7.2 varied between 1:8 - 1:64. At the same pH the titer with NN red cells from different individuals varied between 1:500 and 1:1000. When the titration was performed with sodium phosphate buffer at pH 6.0 the reactions with MM red cells increased. At pH 7.8, no agglutination was obtained even with MMSS red cells.
Desialylation with neuraminidase completely abolished the reactions with all cells (fig 2). Weak agglutination was obtained with trypsin or chymotrypsin treated NN cells. Chymotrypsin abolished the agglutination reactions with MMS and MMss cells but after trypsinization stronger reactions than in saline were obtained (fig 2).

FIG 2

RED CELL PHENOTYPE	UNTREATED RED CELLS	TITER WITH RED CELLS TREATED WITH TRYPSIN	CHYMOTRYPSIN	NEURAMINIDASE
NNss	1:500	1:8	1:2	neg
MMS	1:8	1:64	neg	neg
MMss	neg	1:4	neg	neg

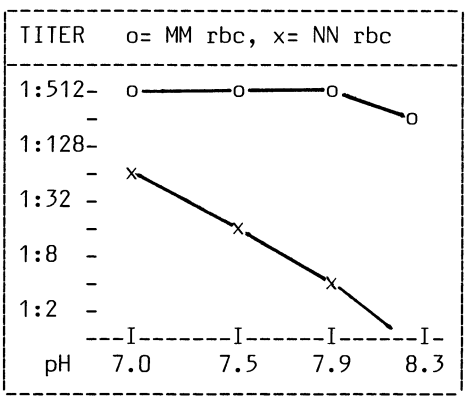
The antibody strongly agglutinated two NN,He+ red cell samples but the reactions were practically abolished after trypsinization of these cells.
The agglutination of N cells could be inhibited (fig 3) by untreated glycoprotein N and Ss but neither by M glycoprotein nor by desialylated, N acetylated or periodate/borohydride treated N glycoprotein.

FIG 3

INHIBITOR	CONC µ G/L
Glycoprotein M	>5 000
Glycoprotein N:	
Untreated	40
Desialylated	>5 000
N-acetylated	5 000
Periodate/borohydride	>5 000
Glycoprotein Ss	40

Antibody 425/2 B (anti-M):
Native tissue culture supernatant (pH 7.2) strongly agglutinated MM and NN red cells. Upon increasing the pH the titer with NN cells decreased and at pH 8.3 no agglutination was observed. With M red cells a slight decrease in titer was observed at pH 8.3 (fig 4). Trypsin treatment almost completely abolished the reactions with MM and NN cells at pH 8.3. Chymotrypsin treatment decreased the titer with MM red cells at pH 8.3, but with NN red cells no reactions were observed at this pH.

FIG 4



After desialylation with neuraminidase, reactions with MM cells at pH 8.3 decreased with increasing neuraminidase concentration. The degree of desialylation was monitored by using anti-T (peanut lectin,Gamma Biologicals). Even at an enzyme concentration of 0.21 mg/ml the antibody gave strong reactions (titer 1:8) with MM red cells.
Two samples of NN,He+ red cells were agglutinated to an intermediate degree (2+) by the antibody. Neither sample was agglutinated by polyclonal anti-M raised in rabbits.
The agglutination of MM cells at pH 8.3 was strongly inhibited by untreated and N-acetylated M glycoprotein but only slightly by the desialylated derivative, and even less with N glycoprotein (fig 5)
In ELISA at pH 8.3 a considerable binding to M glycoprotein was seen whereas only negligible binding to N glycoprotein occured.

FIG 5

pH	RED CELL	INHIBITOR	CONCENTR (µG/ML)
8.3	MM	Glycoprotein M:	
		Untreated	40
		Acetylated	<40
		Desialylated	312
		Glycoprotein N	625

The binding to M glycoprotein could be inhibited by M glycoprotein and its N-acetylated or desialylated derivatives. N glycoprotein or its derivatives did not inhibit the binding to M glycoprotein at pH 8.3.

DISCUSSION
Both antibodies were of the IgM type and gave completely specific reactions in hemagglutination when used at an appropriate pH.
The antibody 35/5 F did not agglutinate desialylated NN red cells and therefore seems to be highly dependent upon the presence of intact sialic acid residues. This was confirmed by the results of the desialylation and periodate/borohydride experiments since such procedures either remove or partially degrade (15-17) the NeuAc residues.
The pH dependency of the antibody indicates that it requires free amino groups for reactivity (20). The amino groups are not ionized (20) at a high pH. Since NN red cells contain a large number of N antigenic sites, carried on both glycoprotein MN and Ss, a slight increase in pH will affect antibody binding only to a small degree. MM red cells, on the other hand only have a small amount of N antigen carried exclusively on Ss glycoprotein. Therefore, an alkaline pH will have a much more

pronounced effect on binding of anti-N to MM than to NN red cells. Furthermore, modification of the amino groups such as acetylation (18-21) abolishes antibody binding.
These findings also indicate that the antibody recognizes an epitope at the amino-terminal end of the polypeptide chain (anti-N_{leu}) since free amino groups do not occur at other sites of the chain.
The anti-N_{leu} type of the antibody is also corroborated by lack of reactivity with trypsinized NN,He+ red cells since trypsin selectively destroys glycophorin A on the red cell surface (22,23).
In contrast to 35/5 F and to most polyclonal anti-M and anti-N reagents of human or animal origin, antibody 425/2 B agglutinates desialylated red cells almost as well as untreated cells.
The epitope recognized by the antibody 425/2 B seems to be located at an interior part of the polypeptide chain, i e the antibody seems to be of the rare anti-M_{gly} type. The agglutination obtained with two NN,He+ samples supports this assumption. Further proof would be non-reactivity with rare red cells of the McMc type since such cells have amino acid Ser at position 1 as normal M but Glu at position 5 as normal N (10,11). However, no such cells were available.
The binding and inhibition experiments also point to an epitope located at an inner portion of the polypeptide chain. The antibody bound strongly to M glycoprotein and its acetylated derivative, and the binding to the untreated glycoprotein was strongly inhibited by the acetylated derivative.
The non-specific binding to N glycoprotein and the ability of this protein and its derivatives to inhibit antibody binding points to an internal epitope which includes amino acid(s) beyond the fifth position in the polypeptide chain since amino acids 6-131 are identical in M and N glycoprotein (6,14). Conformational change supposedly imposed at a higher pH might make the part of the epitope that is common to M and N less available to antibody binding. Such conformational change could also explain the pH dependence of the antibody since blocking of free amino groups by other means such as acetylation does not abolish the antibody activity. Studies are in progress to elucidate these points.

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