**CONCISE REPORT**

**Late Expression of M and N Antigens on Glycophorin A During Erythroid Differentiation**

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The M/N blood groups are carried by the major human red cell sialoglycoprotein, glycophorin A. O-glycosidic carbohydrate is needed for the activity, but the M/N specificity is due to amino acid replacements in the NH2-terminal portion of the molecule. We have used monoclonal antibodies specific for M and N blood groups to study their expression during normal erythropoiesis. Here we report that the M/N blood group activities are very weakly or not at all expressed before the polychromatic normoblast stage.

The M and N blood group antigens are primarily carried by glycophorin A, the major sialoglycoprotein of the human erythrocyte membrane. The minor sialoglycoprotein, glycophorin B, is N-active irrespective of the M/N blood group. Glycophorin A is expressed on the surface membrane of the earliest morphologically recognizable erythroid cells, proerythroblasts, and it is a useful marker for both normal and malignant erythroid precursors.

The structural differences between the M and N antigens are based on the amino acid sequence polymorphism within the amino terminal region of the glycophorin A polypeptide. Glycophorin A contains serine and glycine at positions 1 and 2, while glycophorin A contains leucine and glutamic acid at the corresponding positions. Although the compositions of the carbohydrate moieties of the M and N types of glycophorin A appear identical, the oligosaccharides are essential for the M/N antigenicities because these are abolished by removal of sialic acids and can be regained by sialylation.

Recently, monoclonal antibodies which recognize epitopes on the M and N antigens have become available. We have used such antibodies to determine the stages of erythroid maturation at which the M and N antigens are expressed in normal bone marrow.

**MATERIALS AND METHODS**

Cells from normal human bone marrow were obtained during routine examinations of bone marrow donors before transplantation. Alternatively, pieces of ribs which had been resected during open thoracic surgery at Helsinki University Hospital were used as a source of bone marrow cells. The bone marrow cells were suspended in phosphate-buffered saline (PBS), and the mononuclear cells were isolated by Ficoll-Isopaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. In some cases, the erythroid cells were enriched further by the removal of Fe-receptor containing myeloid and monocyctic cells by passage through an Ig-anti-Ig column.

Blood samples were obtained with informed consent from healthy volunteers. The typing of M and N antigens was performed by the Finnish Red Cross Blood Transfusion Service, Helsinki.

Specific rabbit anti-glycophorin A antiserum was prepared as previously described and rendered specific by absorption with En(a−) red cell membranes, which lack glycophorin A. The hybridoma cell clones which produce monoclonal antibodies 8159 (6A7) and 8161 (8A2) against the M and N forms of glycophorin A, respectively, were obtained from the American Type Culture Collection (Rockville, Md.). Ascites fluid produced in Pristane (Sigma, St Louis)-treated BALB/c mice by the 8159 clone-agglutinated homozygous M type erythrocytes at a dilution of 1:5,120 to 1:10,240, and ascites produced by the 8161 clone-agglutinated homozygous N erythrocytes at a dilution of 1:5,120.

Cytocentrifuge smears from the bone marrow cells were prepared using a Shandon (Shandon Southern Products, Cheshire, England) cytocentrifuge. For morphological analysis, the slides were stained with May-Grünwald-Giemsa (MGG). For immunofluorescent staining, the cytocentrifuge smears were fixed in cold methanol (−20 °C) for ten minutes.

Double immunostaining was made by incubating the smears with the monoclonal antibodies 8159 or 8161 followed by Staphylococcus aureus protein A containing sheep anti-mouse IgG and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated sheep anti-rabbit Ig antigens (Cappel Laboratories, Cochranville, Pa) were used as second antibodies. In control stainings, irrelevant monoclonal mouse IgG and normal rabbit Ig were used instead of the first antibodies.

For semiquantitative analysis of antibody binding to different maturational stages of erythroid cells, the bone marrow cells were treated in suspension with rabbit anti-glycophorin A antiserum or the monoclonal antibodies 8159 and 8161. Cells with antibodies bound to their surface antigens were allowed to form rosettes with protein A containing Staphylococcus aureus Cowan I strain as described. The cells were centrifuged onto glass slides and stained with MGG. Irrelevant monoclonal IgG or normal rabbit Ig instead of the antibody or antiserum was used in control rosettes. The number of staphylococci bound to the morphologically recognizable erythroid cells was counted. At least 50 cells of each maturation stage were scored.

For immunoprecipitation, the cell-surface glycoconjugates were radioactively labeled. Sialic acid residues were radiolabeled by oxidation with sodium metaperiodate at 0 °C followed by reduction with NaB3H4. Immuno-precipitations of the surface-labeled membrane lysates were made with monoclonal antibodies 8159 or 8161, followed by rabbit anti-mouse Ig using the protein A containing S'...
RESULTS

Surface-labeled homozygous M and N erythrocytes were immunoprecipitated with the monoclonal antibodies 8159 and 8161 and the molecules recognized by these antibodies were identified by polyacrylamide slab gel electrophoresis. In homozygous N red cells, no bands were precipitated with the anti-M antibody (Fig 1B). Using the anti-N antibody (8161), the glycophorin A monomer and dimer molecules were obtained from homozygous N red cells (Fig 1C), but not from MM red cells (Fig 1E). Similarly, the anti-M antibody reacted with glycophorin A from M cells (Fig 1D).

The binding of the anti-glycophorin A antiserum and the monoclonal anti-M and anti-N antibodies to bone marrow erythroid cells heterozygous for the M and N antigens was analyzed by the *Staphylococcus aureus* Cowan I rosetting technique (Fig 2). Rabbit anti-glycophorin A antiserum reacted strongly with the earliest morphologically recognizable erythroid cells, proerythroblasts, and basophilic normoblasts (Fig 3). In contrast, the monoclonal anti-M and anti-N antibodies showed weak reactivity with the proerythroblasts and basophilic normoblasts. Polychromatic normoblasts and more mature erythroid cells showed strong reactivity with both the rabbit anti-glycophorin A and the monoclonal anti-M and anti-N antibodies. The epitopes recognized by the anti-M and anti-N antibodies appeared at approximately the same stage of the erythroid differentiation.

In indirect immunofluorescence, rabbit anti-glycophorin A antiserum stained large nucleated erythroid precursors (Fig 4A). The monoclonal anti-M and anti-N antibodies reacted with mature erythrocytes and relatively small nucleated red cell precursors (normoblasts), while the largest immature glycophorin A-containing cells did not stain with the monoclonal antibodies (Fig 4B).

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**Fig 1.** Polyacrylamide slab gel to show specificity of monoclonal anti-M and anti-N antibodies. (A) Surface glycoprotein pattern of RBCs labeled by the periodate/NaB₃H₄ technique; (B) pattern of immune precipitate obtained from ³H-labeled NN cells with anti-M antibodies; (C) pattern obtained from ³H-labeled NN cells with anti-N antibodies; (D) pattern obtained from ³H-labeled MM cells with anti-M antibodies; (E) pattern obtained from ³H-labeled MM cells with anti-N antibodies. GPA-D, glycophorin A dimer; GPA-M, glycophorin A monomer; GPB-D, glycophorin B dimer; GPB-M, glycophorin B monomer. Note that GPA-M and GPB-D have the same electrophoretic migration rates and that GPB is N-active irrespective of the MN blood group.

**Fig 2.** The binding of monoclonal anti-N antibody 8161 to bone marrow cells as assessed by the *S aureus* Cowan I rosetting technique. Erythroid cells are marked by letters. A, proerythroblast; C, polychromatophilic normoblast; E, erythrocyte. May-Grünwald-Giemsa stain (original magnification ×100; current magnification ×64).

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*aureus* Cowan I strain. Electrophoresis on 8% polyacrylamide slab gel was performed according to Laemmli. The gels were fixed and treated for fluorography as described.

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**Fig 3.** The binding of rabbit anti-glycophorin A antiserum (R anti-GPA-A) and monoclonal anti-M (8159) and anti-N (8161) antibodies to bone marrow erythroid cells. Vertical bars indicate the number of staphylococci per cell (mean ± SD). Aa, proerythroblast; Bb, basophilic normoblast; Cc, polychromatophilic normoblast; Dd, orthochromatic normoblast; Ee, erythrocyte. Capital letters indicate binding of the relevant antibodies and small letters indicate binding of control immunoglobulins from the same species as the antibodies.
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Fig 4. Double immunofluorescence staining of bone marrow erythroid cells. (A) Cells stained with rabbit anti-glycophorin A antiserum followed by TRITC-conjugated sheep anti-rabbit Ig. (B) The same field stained with monoclonal anti-M antibody 8159 followed by FITC-conjugated sheep anti-mouse IgG.

DISCUSSION

The monoclonal anti-M antibody 8159 has been reported to react specifically with the M type of glycophorin A. Our findings from the immunoprecipitation of homozygous M and N erythrocytes are in agreement with these observations. Bigbee et al used an immunoblotting technique and reported that the anti-N antibody (8161) reacted with both the M and N types of glycophorin. We did not observe any cross-reactivity in immunoprecipitation of this antibody with the glycophorin AM. Instead, the 8161 antibody specifically precipitated glycophorin AN.

In addition, the monoclonal anti-N antibody precipitated glycophorin B from both M and N types of erythrocytes as described earlier from immunoblotting studies. Both M and N erythrocytes contain some N antigen activity in glycophorin B, whose first 23 amino acids are identical with those of glycophorin AN.

Glycophorin A has been shown to be a specific and early erythroid marker present in the membrane of proerythroblasts. The epitopes recognized by the monoclonal anti-M and anti-N antibodies appear on the surface of erythroid cells clearly later, at the polychromatic normoblast stage as demonstrated by the double immunofluorescence and the S. aureus Cowan I rosetting technique.

Carbohydrates are known to contribute to the antigenic determinants recognized by the M/N-specific monoclonal antibodies 8159 and 8161. The external, NH2-terminal portion of the glycophorin A molecule from mature red cells is highly glycosylated, containing one N-glycosidic oligosaccharide at ASN-26 and 15 O-glycosidic oligosaccharides. The relatively late expression of blood group M and N determinants, as compared to the appearance of the glycophorin A molecule identified by the rabbit anti-glycophorin A antiserum, apparently reflects changes in the glycosylation of glycophorin A during erythroid maturation. The present results are consistent with our recent finding that glycophorin A molecules of early erythroid precursors are less O-glycosylated than those from more mature erythrocytes.

Since the present results show that the carbohydrate changes leading to expression of the M and N blood group antigens occur relatively late during erythroid maturation, monoclonal antibodies reacting with epitopes on glycophorin A to which carbohydrates contribute may not be adequate for phenotyping early erythroblastic maturation.

REFERENCES

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