

Coordinate Glycosylation and Cell Surface Expression of Glycophorin A During Normal Human Erythropoiesis

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The expression of two epitopes on glycophorin A (GPA) during erythroid development was examined on normal human bone marrow using quantitative flow cytometry. The highly correlated binding of two monoclonal antibodies, one sensitive and the other insensitive to glycosylation, indicated that the two epitopes were coordinately expressed during erythroid development. Both antigens

GLYCOPHORIN A (GPA) is the major sialoglycoprotein of the human erythrocyte membrane.^{1,2} This protein is restricted to the erythroid lineage.³ Cell surface expression of GPA is first detected at the erythroblast stage of development, after the cells can no longer form erythroid colonies.^{3,4} GPA occurs in two allelic forms corresponding to the (M) and (N) antigens of the MN blood group.^{5,6} The (M) and (N) antigens are identical except for two amino acid differences in the amino terminal region, but O-linked tetrasaccharides in this region are required for the binding of many (M) and (N) specific antibodies.⁷⁻⁹

Biochemical analyses of cell lines (K562 and HEL) stimulated to undergo maturation and comparisons of abnormal with normal bone marrow suggested that GPA acquired an increased number of O-linked tetrasaccharides when the erythroid cells matured.¹⁰ These conclusions were supported by experiments using antibodies that detected (M) and (N) blood group antigens.¹¹

In studying the expression of GPA during normal erythropoiesis, we showed that GPA reached maximal expression at the basophilic normoblast stage and remained in a constant amount per cell throughout further maturation to the erythrocyte.⁴ The reported late glycosylation of GPA during erythrocyte development was difficult to explain in light of this invariance of amount of GPA on the maturing erythroid cells. Therefore, we investigated the correlated expression of two epitopes of GPA during erythropoiesis using monoclonal antibodies, one epitope was insensitive to glycosylation while the other required intact tetrasaccharides.

MATERIALS AND METHODS

Monoclonal antibodies. Anti-GPA (M) (9A3) binds only to type (M) glycophorin A while anti-GPA (M/N) (10F7) identifies both types (M) and (N) GPA.¹²⁻¹⁴ Fluorescein isothiocyanate (FITC) was used to prepare directly conjugated 10F7.¹⁵ Rat anti-mouse kappa phycoerythrin (PE) and isotype control IgG₁ FITC was obtained from Becton Dickinson Immunocytometry Systems, Mountain View, CA.¹⁶

Cell preparation and immunofluorescence. Bone marrow aspirates were obtained from three consenting normal adult volunteers. Collection of samples was performed after approval by the Institutional Review Board, as approved by the Department of Health and Human Services. The low-density leukocytes (1.077 g/mL) were stained using indirect immunofluorescent as described.⁴ Briefly, 10⁶ cells were incubated sequentially with anti-GPA(M), rat anti-mouse kappa PE, 10% normal mouse serum, and anti-GPA(M/N) FITC with a wash interspersed between each step. As a negative control an IgG₁ FITC monoclonal antibody with irrelevant specificity was used

reached a maximum expression during the early normoblast stage and were maintained at a constant amount per cell throughout further maturation to erythrocytes. These data suggest that glycosylation of GPA, as detected by antibodies recognizing blood group (M) and (N) antigens, does not increase during erythroid maturation.

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in place of the anti-GPA (M/N). The cells were fixed in 1% paraformaldehyde for later analysis.¹⁷ Some bone marrow cells were incubated with 0.5 IU neuraminidase for one hour at 37°C (Sigma, St Louis) before immunofluorescence staining.¹² The data from three separate experiments gave identical results.

Flow cytometry. Quantitative fluorescence analyses were performed on a FACS 440 (Becton Dickinson) using the filter combinations previously described.¹⁸ Linear amplifiers were used for the light scattering signals, while 4-decade logarithmic amplifiers were used for the fluorescence signals. A minimum of 20,000 events were collected in list mode with contour levels drawn at 2, 4, 8, 16, 32, 64, and 128.

RESULTS

All immunofluorescence analyses were performed by gating on either the "Lymph" or the "Blast" light scattering populations identified for normal bone marrow (Fig 1). No staining above background was found for either the anti-GPA (M) specific or the anti-GPA (M/N) binding antibody on cells which were in the "Gran" light scattering window (data not shown).

The indirect immunofluorescence staining with the anti-GPA (M) specific antibody used rat anti-mouse kappa PE as a second step. A distinct population of labeled cells was observed in both the "Lymph" and "Blast" light scattering windows (Fig 2A). The lack of staining with the negative control, IgG₁ FITC, indicated that there was complete blocking of the free valencies of the rat anti-mouse kappa PE by normal mouse serum used in the staining protocol.

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Submitted April 10, 1987; accepted August 17, 1987.

Supported in part by Grants No. CA 32318 and CA 06973 from the US Public Health Service and R 811819-02 from the US Environmental Protection Agency. Curt I. Civin is a Scholar of the Leukemia Society of America.

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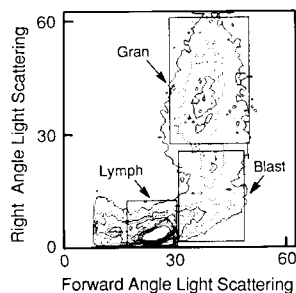


Fig 1. Correlation of forward and right angle light scattering of low density human bone marrow after immunofluorescence staining and fixation. Three populations of cells are identifiable in this plot: "Lymph" including lymphocytes, mature erythrocytes, and late normoblasts; "Blast" including early normoblasts, erythroblasts, monocytes, monoblasts, and myeloblasts; "Gran" exclusively neutrophil lineage from promyelocyte to mature neutrophil.⁴ The fluorescence data in Fig 2 were gated on either the "Lymph" or "Blast" light scattering window. The differences observed between this light scattering pattern and that previously reported are a consequence of the fixation of the cells.⁴

When the anti-GPA (M) and anti-GPA (M/N) antibodies were used together to stain the sample, they bound to the same cells (Fig 2B). There were no cells that stained with the anti-GPA (M) (9A3) antibody that did not also bind the anti-GPA (M/N) (10F7) antibody and vice versa. In addition, there was a tight quantitative correlation of binding of the two antibodies on the cells (Fig 2B, "Blast"). The intensity of fluorescence of the anti-GPA (M) staining was the same whether or not the anti-GPA (M/N) was bound to the cells. These data suggest that the two antibodies identified the same antigen but bound at different epitopes¹⁹ since they did not inhibit the attachment of the other antibody.

An aliquot of the same bone marrow preparation was treated with neuraminidase (which selectively removes sialic acid residues) before immunofluorescence labeling by the

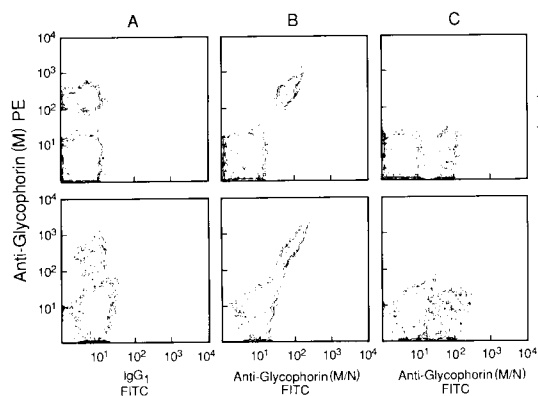


Fig 2. Correlation between anti-GPA (M) specific and anti-GPA (M/N) reactive monoclonal antibodies on normal human bone marrow. (A) Comparison of anti-GPA (M) specific antibody with a negative control of IgG₁ FITC. (B) Fluorescence analysis of marrow cells double labeled with anti-GPA (M) and anti-GPA (M/N) antibodies. (C) Fluorescence analysis of marrow cells treated with neuraminidase before labeling with both anti-GPA (M) and anti-GPA (M/N) antibodies.

two anti-GPA antibodies. Binding of anti-GPA (M) was lost with the removal of the terminal sialic acid residues while no difference in intensity of anti-GPA (M/N) binding (Fig 2C). This illustrated the absolute requirement of the terminal sialic acids for the binding of the (M) specific antibody. This enzymatic treatment had no effect on the binding of the anti-GPA (M/N) antibody.

DISCUSSION

Both of the anti-GPA monoclonal antibodies used in this study recognize the amino terminal peptide of the glycophorin A molecule with essentially no reactivity with glycophorin B.¹² The anti-GPA (M) specific antibody (9A3) requires the terminal sialic acid residues for binding, as shown previously on erythrocytes¹² and, in this study, on less mature erythroid lineage cells (Fig 2C). The binding of the anti-GPA (M/N) antibody (10F7) is unaffected by neuraminidase treatment, both on mature erythrocytes and on immature erythroid cells (Fig 2C).¹² Anti-GPA (M/N) also reacts with cells which lack all but the first carbohydrate residue of the tetrasaccharides on the GPA.¹³ These data suggest that the anti-GPA (M/N) probably binds to a glycosylation insensitive site somewhat removed from the *N*-terminus.¹²

The highly correlated expression of the two epitopes shown in Fig 2B suggests that the two antibodies identify not only the same populations but that the two epitopes were coordinately expressed on those cells. Since all maturational stages of erythroid development are represented in the normal bone marrow, these data indicate that the two epitopes appear at the same time during erythroid maturation. Thus, GPA is sialated at the time that it is first expressed on the cell surface.

The morphological analysis of cells sorted on the basis of GPA intensity indicated that GPA first appears during the erythroblast stage.⁴ Cells with intermediate amounts of GPA were >90% erythroblasts while those expressing maximal amounts of GPA were predominantly normoblasts. The less mature normoblasts were larger than the more mature cells and could be distinguished by light scattering differences. Since both the anti-GPA (M/N) and anti-GPA (M) epitopes follow the same pattern of expression during maturation, it can be concluded that there is no change in the sialic acid residues on these epitopes on GPA after the erythroblast stage. The anti-GPA (M/N) and the anti-GPA (M) epitopes both reach a maximum amount per cell and remain constant during further maturation of the cells to erythrocytes.

If there were glycosylation during the later normoblast stages of maturation, a discordance in immunofluorescence staining would be observed, as has been demonstrated with other erythroid cell surface antigens such as transferrin receptor and CD45 (common leukocyte antigen).⁴

The observations by Ekblom et al may have resulted from less precise quantitation of the binding of their monoclonal antibodies and their rabbit antiserum.^{10,11} In addition, the flow cytometric experiments detected only cell surface antigens while immunofluorescence staining after fixation on a slide identified both cell surface and cytoplasmic antigens.¹¹

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