Expression of the Major Sialoglycoprotein (Glycophorin) on Erythroid Cells in Human Bone Marrow

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The major sialoglycoprotein of human erythrocyte membranes (glycophorin) is one of the best-characterized integral membrane glycoproteins. It contains about 60% carbohydrate and penetrates the membrane. The amino acid sequence has been established and shows some interesting features: the NH₂ terminal is hydrophilic and is located on the outside, the middle portion of the polypeptide is hydrophobic, and the COOH terminal is again enriched in hydrophilic amino acids. Glycophorin possibly interacts with peripheral proteins on the cytoplasmic surface of the membrane.

In contrast to the large body of information on the structure of glycophorin, almost nothing is known about its synthesis and expression in bone marrow cells. It is not known which cells in the erythrocyte lineage actively synthesize the protein.

Recently we and others have found that erythrocytes of the rare human blood group En(a−) lack glycophorin. We took advantage of this fact to produce a specific antiglycophorin antiserum. Rabbits were immunized with a crude preparation of glycophorin and the resulting antiserum absorbed with erythrocyte membranes of the En(a−) blood group. This antiserum was em...
employed to study (by a staphylococcal protein A technique) at which stage of erythrocyte differentiation glycophorin appears at the cell plasma membrane.

MATERIALS AND METHODS

Chemicals and enzymes. Acrylamide and \( N,N'\)-methylenebisacrylamide were obtained from Eastman Kodak, Rochester, N.Y. Na\(^{3}H\)\(_{4}\) (8.6 Ci/m mole) was purchased from the Radiochemical Centre, Amersham, England. Neuraminidase (\(Vibrio cholerae\), 500 U/ml) was obtained from Behringwerke, Marburg-Lahn, Germany. Galactose oxidase (200 U/ml) was obtained from Kabi, Stockholm, Sweden. The neuraminidase and galactose oxidase preparations did not contain measurable proteolytic activities when assayed as described previously.12

Cells. Normal human erythrocytes (AB Rh\(^{+}\)) and En(a\(^{-}\)) erythrocytes, from patient G.W. (AB Rh\(^{+}\)), were obtained through the Finnish Red Cross Blood Transfusion Service, Helsinki. Bone marrow aspirates from patients with nonmalignant diseases with no obvious disturbances in erythropoiesis were obtained from Helsinki University Hospital through Dr. P. Vuopio.

The suspension of bone marrow cells was depleted from most of the erythrocytes and mature granulocytes by centrifugation on a one-step Ficoll-Isopaque density gradient.19 The cells used for surface radiolabeling were further incubated with a 0.84\(^{\circ}\), aqueous solution of NH\(_{4}\)Cl 0.017 M Tris pH 7.45 for 10 min at 37\(^{\circ}\)C to remove contaminating erythrocytes. After this treatment no mature erythrocytes were left.

Protein A-containing Staphylococcus aureus strain Cowan I was obtained from Dr. P. Landwall (Statens Bakteriologiska Laboratorium, Stockholm, Sweden) and cultivated as previously described.20 The bacteria were collected immediately after the logarithmic growth phase, heat killed at 80\(^{\circ}\)C for 10 min, and washed three times in 0.15 M NaCl 0.01 M sodium phosphate pH 7.4 (PBS). They were then fixed by heating in 5\(^{\circ}\), trichloroacetic acid at 70\(^{\circ}\)C for 10 min and washed three times in PBS. The bacteria were stored in PBS 0.02\(^{\circ}\), sodium azide at 4\(^{\circ}\)C. Before use they were washed in 0.05\(^{\circ}\), Triton X-100 0.15 M NaCl 5 mM EDTA 0.02\(^{\circ}\), sodium azide pH 7.4 (buffer A) and suspended as a 10\(^{\circ}\), suspension in this buffer. One hundred microliters of the preparation bound IgG from 6 \(\mu\)l of rabbit serum.

Cell surface labeling and solubilization of membranes. Erythrocytes (0.5 ml packed cells) or \(100 \times 10^{6}\) bone marrow cells were washed three times in PBS and suspended in 1 ml of Dulbecco’s PBS containing Ca\(^{2+}\). Then 25 \(\mu\)l of the neuraminidase and 25 \(\mu\)l of the galactose oxidase preparations were added to the tubes; these were incubated at 37\(^{\circ}\)C with continuous gentle shaking. Control tubes received no enzymes. The cells were then washed twice with PBS and suspended in 1 ml PBS. To each tube 0.5 mCi Na\(^{3}H\)\(_{4}\) was added, and the samples were incubated at room temperature for 30 min. The cells were subsequently washed three times in PBS. Membranes were isolated from the erythrocytes as previously described.18 Samples for immune precipitations were obtained by dissolving the labeled erythrocyte membranes or bone marrow cells in PBS containing 1\(^{\circ}\), Triton X-100 1\(^{\circ}\), ethanol and 2 mM phenylmethylsulfonylfluoride (as a protease inhibitor) at 0\(^{\circ}\)C followed by centrifugation at 2000 \(g\) for 10 min. Aliquots were counted for radioactivity in a Wallac-LKB Liquid Scintillation Counter model 81000.

Production of antiglycophorin antiserum. A glycophorin preparation was isolated from normal erythrocytes by chloroform-methanol extraction.21 One major band was present on gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) with an apparent molecular weight of 85,000 daltons. Rabbits were immunized subcutaneously with 0.5 mg of this preparation in 0.5 ml PBS emulsified with 2 ml Freund adjuvant (Difco) with 2-wk intervals. Ten days after the last injection the rabbits were bled and the serum collected; 1 ml of this serum was absorbed three times with 1 ml of packed En(a\(^{-}\)) erythrocyte membranes at 4\(^{\circ}\)C for 24 hr. After the last absorption the serum was centrifuged at 100,000 \(g\) for 1 hr and stored at 4\(^{\circ}\)C with 0.02\(^{\circ}\), sodium azide.

Immunoprecipitation. To aliquots of Triton X-100 solubilized \(^{3}H\)-labeled normal or En(a\(^{-}\)) erythrocyte membranes and bone marrow cells was added 5 \(\mu\)g mouse IgG and 5 \(\mu\)l rabbit anti-mouse IgG prepared by standard techniques. All subsequent incubations were done at 0\(^{\circ}\)C. After 1 hr, 100 \(\mu\)l of the staphylococcal suspension was added and the tubes incubated for 30 min. These were then centrifuged and the supernatant solutions recovered. To identical amounts of supernatant solutions were added either 5 \(\mu\)l antiglycophorin antiserum or preimmunization...
Fig. 1. PAGE patterns of ³H-labeled erythrocyte membranes and immunoprecipitations with antiglycophorin antiserum. (A) Pattern of normal erythrocytes labeled after treatment with neuraminidase and galactose oxidase (25 µg protein). (B) Pattern of the same sample as in A but containing 5 µg protein. (C) Pattern of membranes from En(a⁻) erythrocytes, labeled after treatment with neuraminidase and galactose oxidase. (D) Pattern obtained from normal erythrocyte membranes after treatment with antiglycophorin antiserum and protein A-containing staphylococci. (E) Pattern obtained from En(a⁻) membranes after treatment with antiglycophorin antiserum and protein A-containing staphylococci. (F) Pattern obtained from normal erythrocyte membranes after treatment with preimmunization serum and staphylococci. BPB, position of the marker dye bromphenol blue. B3, position of band 3.
serum, and the tubes were incubated for 2 hr. Then 0.2 ml of the staphylococcal suspension was added, and the incubation was continued for 1 hr. The bacteria were then washed three times in buffer A and the proteins eluted from the staphylococci by boiling in 1% SDS.

Polycrylamide gel electrophoresis (PAGE). Samples for electrophoresis were prepared by boiling in the sample buffer of Laemmli. Cylindrical gels were run in the presence of SDS with 8% acrylamide in the separating gel. The gels were then sliced into 2-mm slices and treated with NCS (Amersham/Searle) solubilizer and the slices counted in a toluene-based scintillation fluid.

Incubation of bone marrow cells and erythrocytes with antiglycophorin antiserum and protein A-containing staphylococci. Bone marrow cells or erythrocytes (20–50 x 10^6) were suspended in 0.1 ml cold HEPES buffered Hanks' basic salt solution (HBSS) containing 0.02% sodium azide and 1%, bovine serum albumin. Then 10 μl antiglycophorin antiserum or 10 μl preimmunization serum from the same rabbit was added. After 30 min on ice, the cells were washed twice with 10 ml HBSS and suspended in 0.1 ml of the same medium; then 5 μl packed staphylococci were added. After incubation for 30 min at room temperature with intermittent shaking, the suspension was washed three times at 600 g for 5 min to remove loose bacteria, and cell smears were prepared by use of a Shandon cytocentrifuge. Cells binding five or more staphylococci at their cell surfaces were considered positive. The smears were stained with the Lephene modification of the peroxidase reaction to detect cells containing hemoglobin and counterstained with May-Grünwald-Giemsa stain.

RESULTS

Reaction of antiglycophorin antiserum with surface-labeled erythrocytes. When normal erythrocytes were labeled with 3H after treatment with neuraminidase and galactose oxidase, the membranes isolated, solubilized in SDS, and run on PAGE, three major protein peaks corresponding to the major carbohydrate-containing sialoglycoproteins were obtained (Fig. 1A). Other glycoproteins contain much less galactose/N-acetylgalactosamine and were therefore relatively weakly labeled. The dimer of glycophorin migrates as peak PAS 1 and the monomer in peak PAS 2. When a smaller amount of membrane is electrophoresed, PAS 2 becomes the dominating peak (Fig. 1B). In contrast to normal erythrocytes, En(a–) erythrocytes do not contain glycophorin (Fig. 1C). When normal labeled erythrocyte membranes were solubilized with Triton X-100 and precipitated with antiglycophorin antiserum and staphylococci and run on PAGE, the pattern of Fig. 1D was obtained; only the PAS 2 peak was seen. No peak was obtained from En(a–) membranes with antiglycophorin antiserum (Fig. 1E) or from normal membranes with preimmunization serum (Fig. 1F).

Visualization of bone marrow cells expressing surface glycophorin with S. aureus Cowan I rosettes. The erythrocytes and normoblasts in bone marrow pretreated with the antiglycophorin antiserum were heavily coated with staphylococci (Fig. 3). When the bone marrow smears were stained for hemo-
Fig. 2. PAGE patterns of 3H-labeled bone marrow cell proteins obtained after treatment with antiglycophorin antiserum and protein A-containing staphylococci. (A) Pattern obtained from bone marrow cells with antiglycophorin antiserum. (B) Pattern obtained from bone marrow cells with preimmunization serum.

Fig. 3. Cytocentrifuged smear of bone marrow cells pretreated with antiglycophorin antiserum and S. aureus Cowan I. Smear was stained for hemoglobin with LePhene's peroxidase reaction and counterstained with May-Grünwald-Giemsa stain. It was photomicrographed using a red interference filter to accentuate the green benzidine reaction. A, pronormoblast; B, basophilic normoblast; C, erythrophilic normoblasts; D, mature erythrocytes; E, myelocyte; F, metamyelocytes. Strong binding of staphylococci to B, C, and D is clearly seen. B, C, and D were stained green. The few staphylococci bound to the cell F represents nonspecific binding, which was occasionally seen with both antiserum and preimmunization serum.
Fig. 4. Specificity of staphylococcal rosette technique. Cytocentrifuged smears stained and photomicrographed as described for Fig. 3. A, normal erythrocytes plus antiglycophorin antiserum; B, normal erythrocytes plus rabbit preimmunization serum; C, En(a−) erythrocytes plus antiglycophorin antiserum.

globin with the peroxidase-benzidine reaction and counterstained with May-Grünwald-Giemsa stain and analyzed, the surface expression of glycophorin apparently coincided with the presence of hemoglobin in the erythroid cell lineage. The pronormoblasts were negative for hemoglobin staining and did not form rosettes. The basophilic normoblasts showed weak hemoglobin reaction and binding of staphylococci. Strong binding could be seen on cells representing the subsequent stages of erythrocyte maturation. No rosettes were found in bone marrows pretreated with normal rabbit serum (Table 1). Significant binding of staphylococci to bone marrow cells outside the erythrocyte lineage was not observed. Cells of the monocyte and granulocyte series, which are rich in IgG Fc receptors, showed only occasional binding of staphylococci (Fig. 3F), presumably because of aggregated IgG present in the sera. These experiments were repeated four times with different bone marrow samples, and each time more than 100 erythroid cells were examined. Pronormoblasts were consistently negative, whereas the subsequent stages of erythroid differentiation were always positive.

Table 1. Surface Binding of Staphylococci Cowan I to Erythroid Cells in Bone Marrow
After Treatment With Antiglycophorin Antiserum or With Preimmunization Serum

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Antiglycophorin Antiserum (Staphylococci/Cell ± SD*)</th>
<th>Preimmunization Serum (Staphylococci/100 Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes (normocytes + reticulocytes)</td>
<td>19.3 ± 5.8</td>
<td>4</td>
</tr>
<tr>
<td>Oxophilic normoblasts</td>
<td>14.1 ± 3.2</td>
<td>8</td>
</tr>
<tr>
<td>Polychromatic normoblasts</td>
<td>12.3 ± 3.0</td>
<td>5</td>
</tr>
<tr>
<td>Basophilic normoblasts</td>
<td>10.0 ± 5.3</td>
<td>7</td>
</tr>
<tr>
<td>Pronormoblasts</td>
<td>0.3 ± 0.7</td>
<td>5</td>
</tr>
</tbody>
</table>

* More than 20 cells of each type were scored.
We labeled surface glycoproteins of normal and En(a-) erythrocytes by the neuraminidase-galactose oxidase/Na\(_{2}\)H\(_4\) technique. The major protein peaks obtained from normal erythrocytes corresponded to the sialoglycoproteins PAS 1, PAS 2 and PAS 3. By use of labeled cells we could establish the specificity of the antiglycophorin antiserum. Interestingly, only the monomer form of glycophorin (PAS 2) is seen on PAGE in the presence of 5DS owing to efficient disaggregation of the glycophorin dimer (PAS 1) at low concentrations of protein (see Figs. 1 A and 1 B).

It has been established that the NH\(_2\)-terminal portion of glycophorin is located outside the lipid bilayer. This part of the polypeptide contains the MN antigens and receptors for influenza virus and various lectins. The specific antiglycophorin antiserum obtained after absorption with En(a-) membranes clearly reacts with the external part of the glycophorin polypeptide and is different from the antiserum of Cotmore et al., which was made against the COOH-terminal portion of glycophorin and in fact reacted only with the cytoplasmic surface of the membrane.

To the best of our knowledge no studies have been made on the synthesis of glycophorin. As a first step in this direction we used antiglycophorin antiserum to establish which cells in the human bone marrow express glycophorin on their surfaces. For this purpose we adapted a rosette technique using S. aureus Cowan I strain, which contains surface-bound protein A. Protein A has a high affinity for the Fc portion of IgG. The great advantage of this technique as compared to, e.g., immunofluorescence staining is that this can be combined with conventional staining and histochemistry, which allows the identification of the particular surface antigen-carrying cell from permanent preparations. The presence of almost any surface antigen could probably be analyzed in this way. One additional advantage with the rosette technique is that the large size of the cell-staphylococci complexes should allow the selective recovery of the antigen-containing cell by size and/or density fractionation methods. The sensitivity of this technique is similar to that of direct immunofluorescence.

From this study it is clear that the basophilic normoblasts contain glycophorin at their surface. These cells also contain hemoglobin. Glycophorin is abundantly present in the later stages of erythrocyte differentiation. The pro normoblasts, however, do not form rosettes, nor do they contain hemoglobin as detected by the peroxidase reaction. Thus the onset of hemoglobin synthesis and the expression of glycophorin at the cell surface seem to occur at the same stage of erythroid differentiation. Bone marrow cells of other cell lineages never contain glycophorin at their surface, as shown by the absence of specific staphylococcal binding. This protein is evidently specific for the erythrocyte lineage.

The molecular mechanisms involved in the biosynthesis of glycophorin are unknown. The possibility exists that it is synthesized as a precursor before it is expressed on the cell surface. By use of monospecific antisera such aspects are now amenable to analysis.
ACKNOWLEDGMENT

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REFERENCES

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