Relationship of the Human Erythrocyte Wr\textsuperscript{b} Antigen to an Interaction Between Glycophorin A and Band 3

By Marilyn J. Telen and Joel A. Chasis

The Wr\textsuperscript{a} antigen is a high-frequency human erythrocyte antigen invariably absent from En (a\textsuperscript{--}) erythrocytes, which lack glycophorin A. However, glycophorin A from En (a\textsuperscript{+}) Wr (a\textsuperscript{+} b\textsuperscript{--}) red cells has an amino acid sequence identical to that of glycophorin A from Wr (b\textsuperscript{+}) erythrocytes. Evidence has suggested that the Wr\textsuperscript{b} antigen may require the interaction of glycophorin A with either a lipid moiety or with another erythrocyte-integral membrane protein, band 3. We have investigated the role of band 3 in Wr\textsuperscript{b} expression using murine monoclonal antibodies (MoAbs) with Wr\textsuperscript{b} specificity. These antibodies reacted by radioimmunoassay (RIA) only with cells expressing both glycophorin A and band 3. In immunoprecipitation studies, Wr\textsuperscript{b} antibodies immunoprecipitated both band 3 and glycophorin A, while antibodies specific for band 3 or glycophorin A precipitated only the protein with which they were reactive. These data strongly suggest that band 3 is the other membrane component necessary for expression of Wr\textsuperscript{b} and that band 3 and glycophorin A are closely associated in the erythrocyte membrane.

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membrane deformability, antibodies were first purified using affinity chromatography. Affinity columns were prepared by coupling Affi-10 (Bio-Rad Laboratories, Richmond, CA) with purified glycophorin A, kindly provided by Dr Heinz Furthmayr (Stanford University, Stanford, CA). After coupling, the ability of these columns to absorb out antibodies to glycophorin A was confirmed using 10F7, a well-characterized anticglycoprotein antibody. Antibodies were bound to the columns in phosphate-buffered saline (PBS), pH 7.4, and eluted in HCl-glycine, pH 2.2. Column fractions were monitored spectrophotometrically.

Cell lines, media, and reagents. K562 and HEL erythroleukemia cell lines, obtained from Dr Russell Kaufman, Duke University, and from the American Type Culture Collection (Rockville, MD), respectively, were grown in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS, MA Bioproducts, Walkervale, MD).

Cell preparation. Whole-blood samples were drawn from normal individuals after obtaining informed consent. Erythrocytes used for radioimmunoassays (RIAs) and radioimmunoprecipitation studies were collected in acid citrate dextrose and then washed in PBS prior to use; erythrocytes used for deformability studies were washed in 5 mmol/L Tris, 140 mmol/L NaCl, pH 7.4. Cell suspensions were counted using an ELT-8 (Ortho Diagnostic Systems, Inc, Raritan, NJ). When thawed with cells from rare blood group phenotypes (eg, M^0M^0) were used, thawed control cells were tested simultaneously.

Immunoassay and immunoprecipitation. The reactivity of red blood cells (RBCs) with MoAbs was tested quantitatively by RIA under conditions of saturating antibody concentrations, as previously described. All red cells were used in RIAs at similar concentrations (10^5/mL) in each assay. All RIAs were performed in triplicate. Nucleated cells were assayed for reactivity with various antibodies at concentrations of 2 x 10^5/mL. The binding of antibodies to nucleated cells was detected by subsequent binding of fluorescein-labeled goat antimouse IgG (TAGO Inc, Burlingame, CA), as analyzed by a FacScan (Becton-Dickinson, San Jose, CA).

Immunoprecipitation experiments were done as previously described. Briefly, cell surface proteins of intact erythrocytes were radiolabeled using Iodo-Gen (Pierce Biochemical Co, Rockford, IL). Erythrocyte membrane ghosts were then prepared by the method of Dodge et al and solubilized in Tris-buffered saline containing 1 mmol/L ethylene diamine tetra-acetic acid (EDTA). 0.1% gelatin, and either 1% deoxycholate or 1% Nonidet-P40. Generally, 5 μL of ascitic fluid or 50 μL of tissue culture supernate containing MoAbs were added to lysate prepared from 4 x 10^8 erythrocytes. Immune complexes were isolated using formalin-fixed staphylococci-bearing Protein A, in the case of E6, or similar Staphylococci precoated with antimouse Ig antibodies in the cases of 10-22 and 4-21. Results were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography.

Deformability studies. To measure red blood cell deformability, washed red cells were resuspended in dextran (40,000 mol wt, 25 g/100 mL in 10 mmol/L phosphate buffer, pH 7.4; Pharmacia Fine Chemicals, Uppsala, Sweden) and examined by ektacytometry, a laser diffraction method previously described. In brief, suspended cells were exposed in the ektacytometer to an increasing shear stress (0 to 125 dynes/cm²) and the change in their laser diffraction pattern from circle to ellipse measured. This photometric measurement produced a signal-designated deformability index that quantitated cell ellipticity.
Table 2. Reactivity of MoAbs With Normal Wr (b+) and En (a+)

<table>
<thead>
<tr>
<th>Cell Phenotype</th>
<th>Antibody Used</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>E6</td>
</tr>
<tr>
<td>Wr (b+)†</td>
<td>3,694</td>
</tr>
<tr>
<td>Wr (b+)</td>
<td>3,643</td>
</tr>
<tr>
<td>Wr (a+b-) (M. Fr.)</td>
<td>3,276</td>
</tr>
<tr>
<td>Wr (a+b-)</td>
<td>233</td>
</tr>
</tbody>
</table>

Reactivity measured by cpm bound: cpm bound = cpm [125I]-labeled Fab′, sheep antimouse Ig.

*Murine ascites fluid from the myeloma cell line P3 × 63/Ag8, used in preparation of MoAb-producing cell lines. Tissue culture supernate from the same cell line gave comparable results.

† Reactivity with three representative Wr (b+) erythrocyte samples is demonstrated. More than 30 such samples have been tested.

Reactivity of MoAbs with erythroleukemia cell lines.

To examine whether band 3 played a role in Wr expression, the binding of the three Wr antibodies to K562 and HEL cells was quantitated by flow cytometric analysis of immunofluorescence. These two erythroleukemia lines were used because they have been shown to express glycophorin A but not band 3.22 As shown in Table 3, antiglycophorin antibodies E3 and E4 reacted with both K562 and HEL cell lines, confirming that expression of glycophorin A was easily detectable on these cells. In contrast, MoAbs E6, 10-22, and 4-21 showed minimal or no reactivity with either the K562 or HEL cell lines. These data suggest that band 3 may be necessary for the binding of Wr antibodies.

Immunochromatographic characterization of proteins reactive with MoAbs.

Immunoprecipitation assays using antibody E6 and erythrocyte membrane proteins solubilized in 1% deoxycholate, a slightly ionic detergent, resulted in precipitation of band 3 (Fig 1A). However, it has previously been shown, using radiolabeled erythrocyte membrane proteins solubilized in the nonionic detergent NP-40, that addition of E6 resulted in specific immunoprecipitation of large quantities of band 3 as well as somewhat lesser amounts of glycophorin A. 10 Concurrent immunoprecipitation experi-

Table 3. Reactivity of MoAbs With Human Erythroleukemia Cell Lines

<table>
<thead>
<tr>
<th>Antibody</th>
<th>% Cells Reactive by Immunofluorescence</th>
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<tbody>
<tr>
<td></td>
<td>K562</td>
</tr>
<tr>
<td>E3 (antiglycophorin A + B)</td>
<td>69.7</td>
</tr>
<tr>
<td>E4 (antiglycophorin A)</td>
<td>28.1</td>
</tr>
<tr>
<td>E6 (anti-WrB)</td>
<td>12.0*</td>
</tr>
<tr>
<td>10-22 (anti-WrB)</td>
<td>0</td>
</tr>
<tr>
<td>4-21 (anti-WrB)</td>
<td>0</td>
</tr>
</tbody>
</table>

*E6 reactivity with K562 and HEL cells, when graphed by cell number and fluorescence intensity, appeared comparable with simultaneously run negative controls because cells counted as positive were only very weakly so.
ments using antibody TE10, directed against band 3, produced no similar coprecipitation of glycophorin A (Figure 1B). Of note is the difference in width of band 3 immunoprecipitated by E6 and TE-10. The narrowness of the band observed after treatment with E6 suggests that only a subset of band 3 molecules was immunoprecipitated by the anti-Wr\(^{b}\) antibody. E3 antibody, specific for glycophorins A and B, precipitated glycophorins A and B but no band 3 from NP-40 lysates. Numerous MoAbs to glycophorin A used under identical conditions did not precipitate detectable quantities of band 3. Hence neither band 3 nor glycophorin A were nonspecifically precipitated under these experimental conditions. We therefore compared the ability of the three Wr\(^{b}\)-related murine MoAbs to precipitate membrane proteins solubilized in NP-40. As shown in Fig 2, antibodies 10-22 and 4-21 produced the same pattern of immunoprecipitated bands as antibody E6. At this time it is unclear why there is a difference in the relative quantities of band 3 and glycophorin A precipitated by the three antibodies; however, the presence of both polypeptides in the precipitates implies an association between band 3 and glycophorin A.

It is interesting to note that antibody 4-21 produced the same immunoprecipitation pattern as antibodies E6 and 10-22, further suggesting that the antigen recognized by antibody 4-21 is related to Wr\(^{b}\) expression. To examine in more detail how antibody 4-21 binds to erythrocytes that lack glycophorin A, we performed immunoprecipitation experiments using En (a−) Wr (a−b−) erythrocytes. In these studies antibody 4-21 precipitated small amounts of band 3 (data not shown). These results imply that the observed agglutination of En (a−) erythrocytes by antibody 4-21 was produced by antibody binding to the transmembrane protein band 3. Considered together, the results of experiments with antibody 4-21 suggest that band 3 plays a role in Wr\(^{b}\) expression.

Effect of antibody binding on membrane deformability. Previous studies have shown that binding of antibodies with specificity for glycophorin A induced a decrease in erythrocyte membrane deformability, while binding of antibodies to A, B, and Rh blood group antigens, which recognize other cell-surface components, had no effect on deformability.\(^{23,24}\) Therefore, we measured the effect of E6 binding on red blood cell deformability. When the deformability index was plotted as a continuous function of applied shear stress, the curve generated reached a plateau at approximately 0.7 for normal red cells, as seen in Fig 3A. Antibody E6 binding induced a decreased deformability index. To quantitate the extent of decreased deformability induced by E6 binding, the shear stress was plotted on a log scale and the deformability index on a linear scale (Fig 3B). Because the lines are parallel, one can see that red blood cells treated with E6 required 43-fold greater shear stress than nontreated control cells to reach equivalent deformation at all points along the curve. This result indicates that E6-treated red cells had 0.025×normal deformability. Doubling the amount of E6 did not further change the deformability, indicating that we had achieved the maximum effect of E6 binding on deformability (data not shown).

The deformability of E6-treated erythrocytes was then compared to that of cells treated with 10F7, a well-characterized antibody specific for glycophorin A (Fig 4). While untreated control cells had a deformability of 100%, erythrocytes pretreated with E6 had a relative deformability of 2.5%, and those pretreated with antibody 10F7 had a relative deformability of 8.9%. Thus E6 induced a dramatic decrease in deformability similar to that observed with ligands to glycophorin A. When E6 and 10F7 were incubated with En (a−) erythrocytes, which lack glycophorin A, erythrocyte deformability did not decrease significantly (Fig 4). These observations are consistent with the binding and immunoochemical data and imply that E6 requires the presence of glycophorin A for binding to intact cells.

To determine whether the rigidity-inducing effect of E6 could be absorbed out with glycophorin A, we passed the antibody over a glycophorin A affinity column. No antibody bound specifically to the column, as determined by monitoring the acid eluate spectrophotometrically. Furthermore, the ability of the E6 preparation to decrease deformability was maintained after passage over the column. When we tested the effects of antibodies 10-22 and 4-21 on deformability, we found that they, like E6, decreased deformability and that this effect was also not altered by passage over a glycophorin A affinity column (data not shown). We conclude from these data that E6, 10-22, and 4-21 do not bind to glycophorin A in the purified form and may require glycophorin A and band 3 in their native, conformational relationship for binding. Whether the antibody-induced decrease in membrane deformability is mediated through band 3 or through glycophorin A remains unclear, in part because the effect on deformability of band 3-specific antibodies is unknown. This set of experiments has been impossible to perform because, to our knowledge, no band 3 antibody has been produced that binds to intact, nonenzymatically treated erythrocytes.
Fig 3. Deformability of erythrocytes after incubation with E6 antibody. In panel A the deformability index is plotted against the shear stress applied to erythrocyte membranes. Erythrocytes without antibody achieved a maximum deformability index of approximately 0.7, while erythrocytes incubated with antibody E6 achieved maximum deformability index of 0.5. Results did not change when the amount of antibody used was increased (data not shown), indicating that these results represent the maximal antibody effect. In panel B, deformability index is plotted on a linear scale, while shear stress is plotted on a log scale. The data produce two linear curves, indicating that erythrocytes incubated with E6 required 43 times more shear stress to reach a deformability equivalent to that of cells not incubated with antibody. This relationship was constant at all levels of shear stress tested.

DISCUSSION

The Wr<sup>a</sup> and Wr<sup>b</sup> antigens have presented an interesting puzzle to serologists for many years. MoAbs E6 and 10-22 were useful probes for exploring the determinants of Wr<sup>b</sup> antigen expression, since both demonstrated anti-Wr<sup>b</sup> specificity in that they reacted with random donor erythrocytes but failed to react with Wr<sup>(a+b−)</sup> and En<sup>(a−)</sup> erythrocytes. The finding that neither of these antibodies bound to K562 and HEL human erythroleukemia cell lines, which do not express band 3, indicated that the presence of glycophorin A in apparently normal orientation on the cell surface was not sufficient for Wr<sup>b</sup> expression. Although glycophorin A on K562 and HEL cells may be underglycosylated, abnormal glycosylation is an unlikely explanation for the absence of Wr<sup>b</sup>, since the glycosylated domains of glycophorin A are not essential for expression of Wr<sup>b</sup> antigen. The binding data obtained with K562 and HEL cells thus support the hypothesis that a second membrane moiety is required for expression of Wr<sup>b</sup> and that band 3 may be this component. The presence of both band 3 and glycophorin A in the immunoprecipitates further implies a role for both of these polypeptides in Wr<sup>b</sup> expression.

At this time we do not fully understand the specificity of antibody 4-21. On the one hand it is similar to antibodies E6 and 10-22 in that it immunoprecipitates both band 3 and glycophorin A, it decreases membrane deformability, and it does not bind to K562 and HEL cell lines. On the other hand, although antibody 4-21 has been reported by several laboratories to fail to react with Wr<sup>(a+b−)</sup> erythrocytes, this study, as well as others (eg, participants in the First International Workshop on Monoclonal Antibodies Against Red Cells and Related Antigens), found that it reacted weakly when tested with fresh rather than frozen, thawed Wr<sup>(a+b−)</sup> cells. Furthermore, in deformability studies using Wr<sup>(a+b−)</sup> erythrocytes, no change in deformability was seen with antibodies E6 and 10-22, while a slight decrease in deformability was observed with 4-21, confirming weak reactivity of antibody 4-21 with Wr<sup>(a+b−)</sup> cells. Most interesting, however, is the normal reactivity of 4-21 with En<sup>(a−)</sup> erythrocytes, suggesting that if antibody 4-21 does recognize a determinant related to the Wr<sup>b</sup> antigen, it must recognize one independent of glycophorin A expression. Due to the extreme rarity of En<sup>(a−)</sup> red cells and the necessity of fresh cells for such studies, we were able to perform only one immunoprecipitation experiment with En<sup>(a−)</sup> red cells. When antibodies 4-21 and TE-10 were used in parallel,
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Fig 4. Effect of antibody binding on normal and En (a−) erythrocytes. When erythrocytes were incubated in buffer alone or in buffer containing antibody, erythrocytes treated with antibody E6 had a relative deformability of 2.5%, and those treated with antibody 10F7 (a well-characterized antibody to glycophorin A) had a relative deformability of 8.9%. When En (a−) erythrocytes were tested in similar experiments, antibodies E6 and 10F7 caused no significant decrease in deformability.

TE-10 precipitated large amounts of band 3 protein while 4-21 precipitated small amounts of band 3. We conclude from this very preliminary data that the 4-21 epitope may be on band 3 but that in the absence of glycophorin A, antibody 4-21 has low affinity for its epitope under the experimental conditions used.

Taken together the antibody binding, immunoprecipitation, and membrane deformability data support the hypothesis that band 3 and glycophorin A are both involved in Wr<sup>b</sup> antigen expression and that a polymorphism of band 3, which affects band 3-glycophorin A interaction, may constitute the primary polymorphism responsible for expression of the Wr<sup>b</sup> antigen. For this model to be valid, molecules of band 3 and glycophorin A must be in close proximity to one another either at the external or cytoplasmic surface of the lipid bilayer or within the bilayer. An association between the two polypeptides has been suggested by Nigg et al., who reported that antiglycophorin A antibodies markedly reduced the rotational diffusion of band 3 and concluded that band 3 and glycophorin A might form a complex in the erythrocyte membrane. Although there is considerable knowledge about the structural organization of both band 3 and glycophorin A, little is known about the spatial relationships between these two proteins. Band 3 associates with the cytoplasmic skeletal protein network through an interaction between its aminoterminal domain and the skeletal protein, ankyrin. However, not all copies of band 3 can associate with ankyrin, since there are approximately 1 million copies of band 3 per erythrocyte and only 2 × 10<sup>4</sup> copies of ankyrin. Although protein–protein interactions of the remaining copies of band 3 have not been fully characterized, one potential interaction is an association with the skeletal protein 4.1. Interestingly, protein 4.1 has a high-affinity binding site on glycophorin A. Since both band 3 and glycophorin appear capable of interaction with the same skeletal protein component, at least a certain number of copies of each of these two transmembrane proteins may be in close proximity to one another. The possibility that only certain subpopulations of each of these polypeptides is involved in Wr<sup>b</sup> expression is supported by our finding that anti-Wr<sup>b</sup> precipitates contain a narrower band 3 on SDS gels than the immunoprecipitates obtained with the band 3-specific antibody TE-10. If, indeed, molecules of glycophorin A and band 3 are adjacent to one another, an electrostatic interaction might occur between negatively charged sialic acid residues on the extracellular domain of glycophorin A and positively charged extracellular band 3 loops between helices 5-6, 7-8, and 9-10.

Conclusive evidence that a polymorphism of band 3 is responsible for expression of the Wr<sup>b</sup> antigen must await sequencing of band 3 or its gene from Wr<sup>b</sup> erythrocytes or nucleated cells, respectively. However, the data in the present study strongly suggest that band 3 and glycophorin A are both involved in Wr<sup>b</sup> antigen expression and that these two proteins are closely associated in the erythrocyte membrane.

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REFERENCES

1. Wren MR, Issitt PD: Evidence that Wr<sup>a</sup> and Wr<sup>b</sup> are antithetical. Transfusion 28:113, 1988

2. Issitt PD, Pavone BG, Wagstaff W, Goldfinger D: The phenotypes En (a−), Wr<sup>a</sup>(a− b−), and En (a+), Wr<sup>a</sup>(a+ b−), and further studies on the Wr and En blood group systems. Transfusion 16:396, 1976


7. Wren MR, Issitt PD, Dahr W: Additional data on the relationship of Wr<sup>a</sup> to Wr<sup>b</sup>: Antigen measurement using enzyme-linked antiglobulin tests and further data on the structure of Wr<sup>b</sup>. Transfusion 24:425, 1984 (abstr)


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