Quantitation of the Number of Molecules of Glycophorins C and D on Normal Red Blood Cells Using Radioiodinated Fab Fragments of Monoclonal Antibodies

By Jon Smythe, Brigitte Gardner, and David J. Anstee

Two rat monoclonal antibodies (BRAC 1 and BRAC 11) have been produced. BRAC 1 recognizes an epitope common to the human erythrocyte membrane glycoproteins glycophorin C (GPC) and glycophorin D (GPD). BRAC 11 is specific for GPC. Fab fragments of these antibodies and BRIC 10, a murine monoclonal anti-GPC, were radioiodinated and used in quantitative binding assays to measure the number of GPC and GPD molecules on normal erythrocytes. Fab fragments of BRAC 11 and BRIC 10 gave values of 143,000 molecules GPC per red blood cell (RBC). Fab fragments of BRAC 1 gave 225,000 molecules of GPC and GPD per RBC. These results indicate that GPC and GPD together are sufficiently abundant to provide membrane attachment sites for all of the protein 4.1 in normal RBCs.

The shape and deformability of the mature human erythrocyte is controlled by a flexible two-dimensional lattice of proteins, which together comprise the membrane skeleton. The major components of the skeleton are spectrin, actin, ankyrin, and protein 4.1. The skeleton is attached to the cytoplasmic face of the lipid bilayer through interactions involving ankyrin and protein 4.1. It is known that ankyrin interacts with the N-terminal cytoplasmic domain of the integral membrane protein, band 3. The way in which protein 4.1 binds to the plasma membrane is less clearly understood. Evidence has been presented to support interaction of protein 4.1 with band 3, glycophorin A, glycophorin C (GPC) and glycophorin D (GPD), and phospholipid. The relative importance of these interactions remains unclear. However, there is strong evidence in support of a role for GPC and GPD in vivo. GPC and GPD are present in skeletons prepared from normal erythrocytes, but are absent from skeletons prepared from erythrocytes of individuals with hereditary elliptocytosis resulting from homozygous protein 4.1 deficiency. Erythrocyte membranes of the Leach phenotype, which have a total deficiency of GPC and GPD, lack a membrane binding site for protein 4.1. Since in contrast to normal membranes, protein 4.1 is readily extracted from Leach phenotype membranes under the low ionic strength conditions used to isolate spectrin.

One problem concerning interpretation of the in vivo significance of GPC and GPD as membrane binding sites for protein 4.1 has been the apparent difference between the number of protein 4.1 molecules per red blood cell (RBC) (200,000) and those reported for GPC (50,000). This numerical difference has led to the suggestion that a significant proportion of protein 4.1 in normal erythrocyte membranes must be bound to sites other than GPC and GPD. The value of 50,000 molecules of GPC per RBC was obtained using radioiodinated murine monoclonal IgG anti-GPC. This value is a minimum estimate, since accessibility to the relevant epitope on all GPC molecules may be impeded by steric hindrance resulting from the size of whole IgG molecules. In this report, we have measured the abundance of GPC using Fab fragments of rat and murine monoclonal anti-GPC antibodies. We also describe the use of Fab fragments from a rat monoclonal antibody that recognizes an epitope common to GPC and GPD.

MATERIALS AND METHODS

Human RBCs were obtained from Blood Services South West, Bristol, UK. Rat monoclonal antibodies (BRACs 1 and 11) were produced in LOU rats in response to two intraperitoneal injections of human RBCs (0.2 ml of 50% suspension in phosphate-buffered saline [PBS], pH 7) 24 days apart. Three days after the second immunization, splenocytes were fused with Y3.Ag1.2.3. myeloma cells, as described elsewhere, as was the treatment of RBCs with sialidase and with 2 aminoethylisothiouronium bromide (AET).

Immunoblotting was performed as described elsewhere, except that 5% w/v bovine milk powder was used as the blocking agent. Immobilon-P membranes (Millipore Ltd, Watford, UK) were used, and rabbit anti-rat peroxidase conjugate (DAKO, High Wycombe, UK) was used with the BRAC antibodies. The rat monoclonal antibody isotypes were determined using a dot-blot method essentially as described by McDougal et al. IgG was purified from cell-culture supernatant using Protein A Sepharose (Pharmacia, Milton Keynes, UK) and IgM was purified using ion exchange on Q Sepharose fast-flow followed by gel filtration through Sephacryl S300 (Pharmacia) as recommended by the manufacturer. Methods for the purification of Fab fragments, radiolabeling of whole immunglobulin and Fab fragments, and the use in quantitative binding, functional affinity, and competitive inhibition assays were as previously described.

RESULTS

Characterization of rat monoclonal antibodies BRAC 1 and BRAC 11. BRAC 1 and BRAC 11 agglutinated nor-
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Origin - MW 40K - MW 32K - MW 30K - Fig 1.

Immunoblotting with BRAC 1 and BRAC 11. Erythrocyte membrane components were separated under nonreducing conditions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% (wt/vol) acrylamide gels with a 3% (wt/vol) overlay.31 Lanes a and f, normal erythrocyte membranes. Lanes b and g, membranes from Leach phenotype erythrocytes. Lanes c and h, membranes from trypsin-treated normal erythrocytes. Lane d, membranes from Gerbich-negative erythrocytes of the Yus type. Lane e, membranes from Gerbich-negative erythrocytes of the Ge phenotype.

Analysis of BRAC 1 and BRAC 11 by immunoblotting showed that BRAC 1 bound to both GPC and GPD in normal RBC membranes, but failed to react with membranes of the Leach phenotype and gave very weak reactivity with trypsin-treated normal membranes (Fig 1a through c). BRAC 1 also reacted with the abnormal GPC found in Gerbich-negative RBCs of the Yus type, but not with the abnormal GPC found in Gerbich-negative RBCs of the Ge type (Fig 1d and e). BRAC 11 bound only to GPC in normal RBCs and failed to react with membranes of the Leach phenotype or trypsin-treated normal membranes (Fig 1f through h).

Quantitative binding experiments using rat and murine monoclonal antibodies to GPC and GPD. Rat monoclonal antibodies BRAC 1 and BRAC 11 and murine monoclonal antibody BRIC 10 were purified and Fab fragments prepared from each antibody. The purified antibodies and Fab fragments were radiiodinated and used in quantitative binding assays. The number of molecules of GPC and GPD per cell were calculated from the maximum number of antibody molecules or Fab fragments bound per RBC at saturation (Scatchard analysis; Fig 2 and Table 1). Values obtained with BRAC 1 gave site numbers for GPC plus GPD averaging 7,000 per RBC using IgM and 238,000 (corrected to 225,000 assuming binding to Leach phenotype RBCs [Table 1] represents nonspecific binding) using Fab frag-

Table 1. Number of Available Binding Sites on RBCs for Anti-GPC (BRAC 11, BRIC 4, BRIC 10) and Anti-GPC/D (BRAC 1) and Values of Affinity Constants

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Sites per RBC</th>
<th>Affinity K (× 10^4 mol/L)</th>
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<tbody>
<tr>
<td>BRIC 4 IgG</td>
<td>Normal 62,525 ± 3,961</td>
<td>Leach 3,000 (1)</td>
</tr>
<tr>
<td>BRIC 10 IgG</td>
<td>47,683 ± 4,496 (16)</td>
<td>4,000 (2)</td>
</tr>
<tr>
<td>BRIC 10 Fab</td>
<td>142,463 ± 13,660 (8)</td>
<td>ND</td>
</tr>
<tr>
<td>BRAC 11 IgG</td>
<td>29,456 ± 1,065 (5)</td>
<td>ND</td>
</tr>
<tr>
<td>BRAC 11 Fab</td>
<td>143,410 ± 10,448 (4)</td>
<td>ND</td>
</tr>
<tr>
<td>BRAC 1 IgM</td>
<td>7,374 ± 1,225 (5)</td>
<td>ND</td>
</tr>
<tr>
<td>BRAC 1 Fab</td>
<td>238,721 ± 22,380 (3)</td>
<td>13,380 ± 2,464 (4)</td>
</tr>
</tbody>
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Mean ± SD values are given for three or more measurements (the number of determinations is given in parentheses). All other values are either the mean from two experiments or derived from a single experiment. Abbreviation: ND, not determined.
DISCUSSION

The results of agglutination tests and immunoblotting clearly show that BRAC 1 recognizes an epitope common to GPC and GPD. The position of this epitope was further defined by immunoblotting of membranes from Gerbich-negative RBCs of the Ge type and the Yus type (Fig 1d and e). Gerbich-negative cells of the Ge type contain an abnormal GPC resulting from the deletion of exon 3 of the normal GPC gene, which codes for amino acid residues 36 to 63 in normal GPC. BRAC 1 bound to the abnormal GPC from Yus type cells, but not to that from Ge type cells, indicating that the epitope recognized by BRAC 1 on normal GPC is located in the region of residues 36 to 63. Trypsin treatment of normal RBCs cleaves GPC at Arg-48 and since the reactivity of BRAC 1 is markedly reduced after trypsin treatment of normal RBCs, it seems likely that the epitope recognized resides in the region of residues 36 to 48 of normal GPC. GPC and GPD are products of the same gene."21 GPD is a shortened form of GPC (corresponding to residues 22 to 128 of GPC), probably resulting from leaky initiation."22 Amino acid residues 15 to 27 of GPD correspond to residues 36 to 48 of GPC, and this region would be expected to contain the epitope recognized by BRAC 1 on GPD. A murine monoclonal antibody (NaM 19-3C4) of the same specificity as BRAC 1 has recently been described by Loirat et al."23

BRAC 11 is specific for GPC (Fig 1f through h) and so must recognize an epitope in the region of residues 1 to 21 of GPC. Competitive inhibition experiments demonstrated that BRAC 11 sees a related epitope to that recognized by BRIC 4. BRIC 10, unlike BRIC 4, requires the free amino group at the N-terminus of GPC for reactivity."24 These results suggest the location of epitopes for BRAC 1, BRAC 11, BRIC 10, and BRAC 10 as depicted in Fig 3.

Quantitative binding experiments gave results of 7,000, 29,500, and 48,000 GPC molecules per RBC for BRAC 1 (IgM), BRAC 11 (IgG), and BRIC 10 (IgG), respectively. The value obtained for BRIC 10 agrees well with that obtained previously."7 When similar quantitative assays were performed using radioiodinated Fab fragments of BRAC 1, BRIC 11, and BRAC 10, the results (Fig 2 and Table 1) demonstrated a dramatic increase in the number of epitopes and hence GPC and GPD molecules bound. Fab fragments of the two anti-GPC antibodies (BRAC 11 and BRIC 10) gave values in the region of 143,000 molecules bound per cell, while the anti-GPC plus GPD antibody BRAC 1 gave values of approximately 225,000 molecules per cell. These results suggest that there is considerable steric constraint on the binding of whole antibody molecules. We have previously

Table 2. Inhibition of Binding of 125I-Labeled Anti-GPC to RBCs by Unlabeled Antibodies

<table>
<thead>
<tr>
<th>125I-Labeled Antibody</th>
<th>Inhibition by Unlabeled Antibody (%)</th>
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<tr>
<td>BRIC 4</td>
<td>78 ± 15 (9)</td>
</tr>
<tr>
<td>BRIC 10</td>
<td>72 ± 8 (6)</td>
</tr>
<tr>
<td>BRAC 11</td>
<td>78 ± 14 (4)</td>
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125I-labeled IgG (0.1 μg) was incubated with RBCs (2 to 5 μL) and unlabeled IgG at 10-fold molar excess. Results are mean values ± SD for the percentage inhibition. The number of determinations is given in parentheses.
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![Diagram of Red Cell Lipid Bilayer]

Fig 3. Diagrammatic representation of GPC and GPD shows the location of epitopes recognized by BRAC 1, BRAC 11, BRAC 4, and BRAC 10.

observed a twofold increase in the binding of Fab fragments over IgG with antibodies to CD44, CD58,25,26 acetylcholinesterase,27 and some antibodies against glycoporin A,17 but only in the case of some monoclonal antibodies to the Kell glycoprotein have we seen Fab to IgG ratios of the magnitude observed in the present study.28 Curiously, there is some rather tentative evidence that GPC and GPD may be associated with the Kell glycoprotein, since RBCs of the Leach phenotype and Gerbich-negative cells of the Ge type show weakened expression of Kell blood group antigens.29 The marked degree of steric hindrance observed when IgG is used to quantify GPC suggests that GPC molecules may be clustered in the RBC membrane. Such a concept would be consistent with a model of the RBC skeleton in which several (two to 12) spectrin dimers, and therefore band 4.1 molecules (one 4.1 molecule per spectrin dimer), bind to a single actin core.30

Although there is considerable indirect evidence in support of the hypothesis that GPC and GPD provide membrane attachment sites for protein 4.1 in normal RBCs,6,7,9,10 others have suggested a major role for band 3,24 GPA,2 and phospholipids.4 The arguments in favor of these other binding sites have been supported by evidence that insufficient GPC and GPD are present in normal RBCs to accommodate all of the protein 4.1 molecules.7 In this report, we have sought to address directly this particular question. Our results show that GPC, GPD, and protein 4.1 are present in similar amounts, and that it is therefore theoretically possible for all protein 4.1 molecules to interact with GPC and GPD in a normal RBC. These results do not allow us to distinguish the relative roles of GPC, GPD, band 3, glycoporin A, and phospholipid in binding 4.1 in vivo. Nevertheless, when taken together with other evidence,6,9,10 it seems likely that GPC, GPD, and protein 4.1 interactions play a major role in maintaining the functional integrity of the RBC membrane.

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