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. © 1994 by The American Society of Hematology.

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.Ag.1.2.3. myeloma cells, and hybrid cells were selected by culture in Dulbecco's modified Eagle's medium (DMEM; containing hypoxanthine, thymidine, aminopterin [HAT]) supplemented with 20% fetal bovine serum (FBS) and 10% Y3 culture supernatant in the presence of irradiated 3T3 fibroblasts. BRICs 4 and 10, murine monoclonal antibodies to GPC, were as previously described.1,2 Prozone, trypsin, and chymotrypsin treatment of RBCs were as described elsewhere.3 Immuno blotting was performed as described elsewhere,4 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.5 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 Sephady S300 (Pharmacia) as recommended by the manufacturer. Methods for the purification of Fab fragments, radiolabeling of whole immunoglobulin and Fab fragments, and their use in quantitative binding, functional affinity, and competitive inhibition assays were as previously described.6,7

RESULTS

Characterization of rat monoclonal antibodies BRAC 1 and BRAC 11. BRAC 1 and BRAC 11 agglutinated nor-
WANTITATION OF GLYCOPHORINS C AND D ON RBCs

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. 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.

Fig 1

BRAC 1
BRAC 11

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 $K (\times 10^4 \text{mcg/L})^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRIC 4 IgG</td>
<td>Normal: 62,525 ± 3,961 (16)</td>
<td>1.4 ± 0.7 (4)</td>
</tr>
<tr>
<td>BRIC 10 IgG</td>
<td>Normal: 47,683 ± 4,496 (16)</td>
<td>4.9 ± 1.8 (4)</td>
</tr>
<tr>
<td>BRIC 10 Fab</td>
<td>Normal: 142,463 ± 13,660 (8)</td>
<td>0.67 ± 0.02 (7)</td>
</tr>
<tr>
<td>BRAC 11 IgG</td>
<td>Normal: 29,456 ± 1,065 (5)</td>
<td>2.3 (2)</td>
</tr>
<tr>
<td>BRAC 11 Fab</td>
<td>Normal: 143,410 ± 10,448 (4)</td>
<td>0.12 (2)</td>
</tr>
<tr>
<td>BRAC 1 IgM</td>
<td>Normal: 7,374 ± 1,225 (5)</td>
<td>ND</td>
</tr>
<tr>
<td>BRAC 1 Fab</td>
<td>Normal: 238,721 ± 22,380 (3)</td>
<td>13,380 ± 2,464 (4)</td>
</tr>
</tbody>
</table>

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.
mal 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.\cite{10,11} In contrast, Gerbich-negative cells of the Yus type contain an abnormal GPC, which results from the deletion of exon 2 of the normal GPC gene, which codes for amino acid residues 17 to 35 of normal GPC.\cite{12} 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\cite{13} 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.\cite{21} GPD is a shortened form of GPC (corresponding to residues 22 to 128 of GPC), probably resulting from leaky initiation.\cite{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.\cite{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.\cite{24} These results suggest the location of epitopes for BRAC 1, BRAC 11, BRIC 4, and BRIC 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 BRAC 11 agrees well with that obtained previously.\cite{7} When similar quantitative assays were performed using radioiodinated Fab fragments of BRAC 1, BRAC 11, and BRIC 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 performed competitive inhibition experiments using BRIC 4, BRIC 10, and BRAC 11. The ability of an approximate 10-fold molar excess of unlabeled IgG from BRIC 4, BRIC 10, and BRAC 11 to inhibit the binding of radioiodinated BRIC 4, BRIC 10, and BRAC 11 to normal RBCs was determined. The results demonstrated that while BRIC 4 and BRAC 11 strongly inhibit the binding of each other, BRIC 10 defines a separate epitope (Table 2).

**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 abnor-

---

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

<table>
<thead>
<tr>
<th>125I-Labeled Antibody</th>
<th>BRIC 4</th>
<th>BRIC 10</th>
<th>BRAC 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRIC 4</td>
<td>78 ± 5 (9)</td>
<td>31 ± 11 (9)</td>
<td>71 ± 13 (8)</td>
</tr>
<tr>
<td>BRIC 10</td>
<td>7 ± 6 (6)</td>
<td>82 ± 4 (6)</td>
<td>6 ± 0.6 (4)</td>
</tr>
<tr>
<td>BRAC 11</td>
<td>78 ± 14 (4)</td>
<td>24 ± 4 (4)</td>
<td>90 ± 4 (4)</td>
</tr>
</tbody>
</table>

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.
QUANTITATION OF GLYCOPHORINS C AND D ON RBCs

![Diagram of RBC membrane with labels for BRIC 10, BRAC 11, BRIC 4, BRAC 4, and BRIC 10 with annotations for GPC and GP D.]

**Fig 3.** Diagrammatic representation of GPC and GP D shows the location of epitopes recognized by BRAC 1, BRAC 11, BRIC 4, and BRIC 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 glycophorin 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. 18 Curiously, there is some rather tentative evidence that GPC and GP D 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 quantitate GP 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 GP D 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, 7 and phospholipids 4. The arguments in favor of these other binding sites have been supported by evidence that insufficient GPC and GP D 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, GP D, 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 GP D in a normal RBC. These results do not allow us to distinguish the relative roles of GPC, GP D, band 3, glycophorin A, and phospholipid in binding 4.1 in vivo. Nevertheless, when taken together with other evidence, 6, 9, 10 it seems likely that GPC, GP D, and protein 4.1 interactions play a major role in maintaining the functional integrity of the RBC membrane.

REFERENCES

17. Gardner B, Parsons SF, Merry AH, Anstee DJ: Epitopes on
sialoglycoprotein α: Evidence for heterogeneity in the molecule. Immunology 68:283, 1989


27. Spring FA, Gardner B, Anstee DJ: Evidence that the antigens of the Yt blood group system are located on human erythrocyte acetylcholinesterase. Blood 80:2136, 1992


Quantitation of the number of molecules of glycophorins C and D on normal red blood cells using radioiodinated Fab fragments of monoclonal antibodies

J Smythe, B Gardner and DJ Anstee