Monoclonal Antibodies Recognizing Epitopes on the Extracellular Face and Intracellular N-Terminus of the Human Erythrocyte Anion Transporter (Band 3) and Their Application to the Analysis of South East Asian Ovalocytes

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This report describes the production and characterization of 13 rodent monoclonal antibodies to the human erythrocyte anion transport protein AE1 (syn. band 3). Eleven antibodies (4 murine and 7 rat) recognize epitopes dependent on the integrity of the third extracellular loop of the protein. Two antibodies (1 murine and 1 rat) recognize epitopes on the N-terminal cytoplasmic domain. Quantitative binding studies using radiiodinated IgG and Fab fragments of antibodies to extracellular epitopes on AE1 ranged from 77,000 to 313,000 molecules bound at saturation. The results indicate that the epitopes recognized by different antibodies vary in their accessibility and suggest that there is heterogeneity in the organization of individual AE1 molecules in the red blood cell membrane. Quantitative binding studies on South East Asian ovalocytes using several antibodies to AE1 and an anti-Wr(+) show a marked reduction in the number of antibody molecules bound at saturation. These results are consistent with the existence of highly cooperative interactions between transmembrane domains of AE1 in normal erythrocytes and the disruption of these interactions in the variant AE1 found in South East Asian ovalocytes. © 1995 by The American Society of Hematology.

THE STRUCTURE AND function of the erythrocyte anion transporter (syn. band 3, AE1) has been reviewed recently. The protein consists of two domains. The 40-kD N-terminal domain is located entirely within the cytoplasm and forms binding sites for intracellular components including hemoglobin and the red blood cell (RBC) skeleton. The 55-kD C-terminal domain is membrane associated, terminates within the cell, and is involved in anion exchange. The complete amino acid sequence of human AE1 has been deduced from cDNA sequence and hydrophathy analysis suggests there may be as many as 14 membrane-spanning regions. There are seven hydrophilic regions that are either located in the outer lipid layer or protrude from the cell as extracellular loops. Various methods of topographical analysis have been used in the search for a reliable structural model. Protein labeling and protease digestion of intact RBCs and "leaky" RBC membranes have been used to determine the membrane orientation and localization of individual amino acid residues and surrounding domains. Such studies have been aided by the use of monoclonal antibodies (MoAbs) that bind to intracellular epitopes on AE1 and MoAbs that bind to exofacial polypeptide epitopes on AE1 would also be useful for this purpose. In addition, antibodies to the extracellular regions of AE1 would be useful tools with which to study the mechanism of anion transport, the topology of AE1 variants, and the implied roles of AE1 in the invasion of RBCs by malarial parasites and in the removal of senescent RBCs via osmoponization with autologous antibodies.

We describe here the production and characterization of 4 mouse and 7 rat MoAbs that react with exofacial epitopes on AE1. The binding of these antibodies is shown to be dependent on the extracellular loop between the 5th and 6th membrane-spanning regions of AE1. We also describe two MoAbs (1 mouse and 1 rat) that bind to intracellular epitopes on the 40-kD N-terminal domain of AE1. Quantitative binding studies using purified radiiodinated IgG and Fab preparations of the antibodies against extracellular epitopes provide evidence that structural heterogeneity exists between AE1 molecules on RBCs and show that some epitopes are altered on the mutant AE1 found in RBCs from individuals with South East Asian ovalocytosis (SAO).

MATERIALS AND METHODS

Normal RBCs, RBCs with rare phenotypes, and primate RBCs were available in-house. RBCs from individuals with SAO were obtained from Prof P. Low (Department of Chemistry, Purdue University, West Lafayette, IN) and from Dr G. Jones (Department of Science, Technology, Mathematics and Education, University of New England, Armadale, Australia). The samples from Dr Jones were from 9 members of the same family (5 with ovalocytosis and 4 with normal RBCs). RBCs from an individual with congenital dyserythropoietic anemia (CDA) type II (HEMPAS) were provided by Prof Alice Maniatis (University of Patras, Patras, Greece).

Hybridoma cell lines secreting murine MoAbs were produced in Balb/c mice by immunizing twice (BRICs 6 and 90), four times (BRIC 71), or five times (BRIC 200) with intact human RBCs (100 µL of 50% RBCs in phosphate-buffered saline [PBS] i.p. in mice immunized with a 1% w/v Triton X-100 soluble fraction prepared from human RBC membranes. The freeze-dried fraction was initially resuspended in PBS and then two 100-µL injections containing 50 µg of material prepared in Freund's complete adjuvant were administered subcutaneously, 2 weeks apart. Two weeks later, 50 µg of material in 100 µL of PBS was injected on 3 consecutive days (IP) and the fusion was performed 1 day later. Hybridoma cell lines secreting rat MoAbs BRACs 14, 15, 17, 18, 21, 23, 25, and 66 were produced by immunizing LOU rats twice with intact human RBCs (200 µL of 50% RBCs in PBS, pH 7.4) by IP injections 2 weeks apart, as previously described. Mouse MoAbs to Wr(+) (BRICs 14 and 9311) and to glycoporphin A (GPA; R18') were as previously described. Dot-blot screening of culture supernatants and antibody

isotyping were performed essentially as described. BRIC 6 was mouse IgG3 and BRICs 71, 90, 169, and 200 were mouse IgG1. BRAC 14 was rat IgG1, BRACs 15 and 25 rat IgG2a, and BRACs 17, 18, 21, 23, and 66 were rat IgG2b.

The treatments of RBCs with pronase, trypsin, chymotrypsin, sialidase, and aminoethylisothiouronium bromide (AET) were as described. The treatment of RBCs with N-tosyl-L-phenyl alanine chloromethylketone (TPCK)-treated trypsin under low ionic strength (LISS) conditions was as described. RBCs were also treated sequentially with chymotrypsin and then LISS trypsin. Standard serologic techniques were used and polyspecific rabbit antirat IgG3 and BRACs 71, 90, 169, and 200 were mouse IgG1 antirat IgG3 and BRACs 71, 90, and 200 and BRACs 15, 18, 21, 23, and 25; and rabbit antirat Ig (DAKO Ltd) was used with BRACs 14 and 17. Immune complexes were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography or by immunoblotting with murine antibodies reactive with the intracellular N- and C-termini of AE1, BRIC 169 (described here), or BRIC 130. SDS-PAGE and immunoblotting were as described, except that 5% wt/vol bovine milk powder was used as the blocking agent, Immobilon-P membranes were used, and rabbit antirat peroxidase conjugate (DAKO Ltd) was used with the rat antibodies.

The purification of BRICs 6, 71, 90, and 200 and BRACs 14 and 17 was performed on protein A Sepharose and BRACs 15, 18, 21, 23, and 25 on protein G Sepharose, following the manufacturers’ methods. Size exclusion chromatography was performed using a G3000SW, TSK column (Anachem Ltd, Luton, UK). Purification of Fab fragments, radioiodination of whole IgG and Fab fragments and their use in quantitative binding, and functional affinity and competitive inhibition assays were as previously described.

Indirect immunofluorescence using myeloid (HL60 and U937), lymphoid (RAJI, DAUDI, and MOLT4), and erythroid (HEL and KS62) cells lines and normal peripheral blood cells (lymphocytes, granulocytes, and platelets) was performed using a fluorescence-activated cell sorter (FACStar; Becton Dickinson, Oxford, UK).

**RESULTS**

*Initial characterization of MoAbs to intracellular epitopes on AE1.* BRIC 169 and BRAC 66 did not agglutinate human RBCs but reacted by immunoblotting with a broad band migrating with leading edge of 95 kD (Fig 1, lanes a and d). This band was identical to that obtained with BRIC 130 (Fig 1, lane g), a previously described murine MoAb that binds to the intracellular C terminus of AE1. AE1 on intact cells is cleaved by chymotrypsin into 60-kD (N-terminal) and 35-kD (C-terminal) fragments. BRIC 169 and BRAC 66 bound to the 60-kD chymotryptic fragment (Fig 1, lanes b and e), whereas BRIC 130 bound to the 35-kD fragment (Fig 1, lane h). Trypsin treatment of whole RBCs under normal ionic strength conditions does not noticeably affect AE1, but the gentle trypsin treatment of unsealed membranes, as described in the Materials and Methods, cleaves AE1 at Lys360, giving rise to a 55-kD membrane-bound fragment and releasing a 40-kD N-terminal fragment. BRIC 130 detected the 55-kD trypptic fragment (Fig 1, lane i), but BRIC 169 and BRAC 66 did not (Fig 1, lanes c and f). These results suggest that BRIC 169 and BRAC 66 bind to epitopes located on the cytoplasmic 40-kD N-terminal trypptic fragment of AE1.

*Initial characterization of MoAbs to extracellular epitopes on AE1.* Serologic properties of the four murine and seven rat MoAbs to exofacial epitopes on AE1 are given in Table 1. BRIC 6 and BRAC 14 agglutinated normal RBCs directly. BRICs 71, 90, and 200 and BRACs 15, 17, 18, 21, 23, and 25 agglutinated normal RBCs indirectly. All the antibodies failed to agglutinate pronase-treated normal erythrocytes, but agglutinated cells that had been treated with trypsin (normal ionic strength conditions), sialidase, or AET. Hemagglutination titration studies with chymotrypsin-treated cells divided the antibodies into two groups. BRICs 6, 90, and 200 and BRACs 14, 18, 21, and 25 gave markedly reduced titers, whereas BRIC 71 and BRACs 15, 17, and 23 did not (Table 1). Antibodies in the latter group failed to react with RBCs treated sequentially with chymotrypsin and LISS trypsin (Table 1). RBCs of the rare phenotypes Rhnull, Knull, Mnull, En(a−), Lu(a−b−), Fy(a−b−), Jk(a−b−), p, Leach, S-s-U−, and Di(a+b−) were all agglutinated. All the antibodies gave titration scores, with HEMPAS, En(a−), and Mnull cells comparable to those obtained with normal RBCs (data not shown). The antibodies were also tested against a panel of
primate RBCs. All the antibodies agglutinated chimpanzee cells. Seven antibodies agglutinated orantatan RBCs (BRICs 6, 71, and BRACs 15, 17, 18, 21, and 23), 4 antibodies reacted with macaque cells (BRIC 6 and BRACs 17, 23, and 25), and 2 antibodies reacted with patas monkey and baboon cells (BRIC 6 and BRAC 17).

The antibodies did not react with the electrophoretically separated components of human erythrocyte membranes by immunoblotting. Immunoprecipitation from radioiodinated untreated and trypsin-treated RBCs showed a diffuse labeled band of molecular weight (Mr) 95,000 (BRIC 6 in Fig 2, lane a; BRAC 14 in Fig 2, lanes e and f). Confirmation that the band was identical to AE1 was obtained by immunoblotting the electrophoretically separated components of immune precipitates with AE1-specific murine MoAbs BRIC 130 (Fig 2, lanes k through p) and BRIC 169 (data not shown).

AE1 is a member of a family of proteins that have highly homologous membrane domains. AE1 is restricted to RBCs and certain cells in the kidney, whereas nonerythroid anion exchange proteins (AE2 and AE3) are present in a wide variety of cells and tissues.4,5 Four of the rat antibodies (BRACs 17, 18, 21, and 23) reacted by immune fluorescence with normal peripheral blood granulocytes and the myeloid cell lines, HL60 and U937 (data not shown). Murine BRIC 6 reacted weakly with U937 cells. The reactive antibodies no longer bound to U937 cells when Fc receptors were blocked by preincubation with human serum before reactivity with the MoAbs. In studies using radioiodinated U937 cells, BRACs 18, 21, and 23 immunoprecipitated a broad radioiodinated band with a leading edge of 70 kD after SDS-PAGE under reducing conditions (data not shown).

Quantitation of the number of molecules of monoclonal anti-AE1 bound to normal RBCs at saturation and determination of affinity constants. Radioiodinated IgG and Fab fragments, prepared from the 11 antibodies reactive with extracellular AE1 epitopes, were used in quantitative binding assays. The number of epitopes recognized by each antibody was calculated from the maximum number of radioiodinated IgG molecules or Fab fragments bound per RBC at saturation (Scatchard analysis; Fig 3 and Table 2). The number of IgG molecules bound ranged from 77,000 to 313,000 and for Fab fragments from 241,000 to 772,000 (Table 2). Functional affinity constants were determined from Karush plots, as previously described (Fig 3 and Table 2). The affinity constants for IgG were between 4 and 35 times greater than those of Fab fragments.

Competitive binding studies with MoAbs to extracellular epitopes on AE1. Each of the anti-AE1s recognizing extracellular epitopes and two anti-WrP's (BRICs 14 and 93) were examined in competitive inhibition assays (Table 3). The results suggest that the antibodies bind to epitopes in close proximity. Evidence for inhibition of the binding of anti-WrP antibodies was obtained with BRIC 6 and, to a lesser extent, with some of the other AE1 antibodies. Similar results were obtained when Fab fragments were used (data not shown).

Binding of monoclonal anti-AE1, anti-WrP, and anti-GPA to SAO. Recent studies have shown that BRIC 6 reacts with normal AE1 but fails to react with SAO AE1 when it is expressed in Xenopus oocytes. These results prompted us to examine the binding of the AE1 antibodies to SAO erythrocytes. BRIC 6, BRAC 17, and BRAC 25 were chosen as representative of the two antibody groups determined by the agglutination of enzyme treated RBC (Table 1) and because extensive quantitative binding data on normal RBCs was available for them (Table 2 and vide infra). Quantitative binding experiments clearly showed a reduction of between 34% to 54% in the number of molecules of radioiodinated BRIC 6 (IgG or Fab) bound to SAO cells at saturation (Table 4). A similar reduction was observed with radioiodinated BRAC 25 Fab fragments but not BRAC 17 Fab fragments (Table 4). Binding of anti-AE1s was also compared with anti-WrP (BRIC 14) and anti-GPA (R18). Reduced binding of anti-WrP was observed (79% of normal), whereas the results obtained for R18 were not significantly different between normal and SAO cells (Table 4).
Fig 2. Immunoprecipitation by MoAbs reactive with extracellular epitopes of AE1. Lanes a through j are from autoradiographs of radioiodinated material. Lanes a through d are from the same experiment in which lanes a and b contain immunoprecipitates prepared with BRIC 6 from trypsin-treated (lane a) and pronase-treated (lane b) RBCs and lanes c and d are the membranes from trypsin-treated (lane c) and pronase-treated (lane d) RBCs; autoradiography was performed for 2 days. Lanes e through j are from a second experiment with immunoprecipitates prepared with BRAC 14 (lanes e through g) from untreated RBCs (lane e), trypsin-treated RBCs (lane f), and pronase-treated RBCs (lane g) and lanes h through j are the membranes from untreated (lane h), trypsin-treated (lane i), and pronase-treated (lane j) RBCs; autoradiography was performed for 7 days. Lanes k through p are immunoblots with BRIC 130 to immunoprecipitates prepared with BRIC 6 (lanes k through m) and with BRAC 21 (lanes n through p) from untreated RBCs (lanes k and n), trypsin-treated RBCs (lanes l and o), and pronase-treated RBCs (lanes m and p). Lanes a through p were separated under nonreducing conditions. Values for M, are ×105.

DISCUSSION

The initial characterization of antibodies. Hemagglutination, immune precipitation, and immunoblotting studies showed that the 13 antibodies reacted with AE1. BRIC 169 and BRAC 66 were specific for the 40-kD N-terminal cytoplasmic domain, whereas the remainder reacted with extracellular epitopes. Chymotrypsin treatment of intact RBCs cleaves the membrane domain of AE1 after Tyr-553 and Tyr-558. Pronase also cleaves in this region, whereas trypsin under LISS conditions cleaves after Lys-562. The failure of agglutinating antibodies to react with chymotrypsin-treated or chymotrypsin- and LISS trypsin-treated RBCs sug-

A. Scatchard plot

B. Saturation curve

C. Karush plot

Fig 3. Representative Scatchard plot, saturation curve, and Karush plot. The representative Scatchard plot (A), saturation curve (B), and Karush plot (C) were obtained for 125I-labeled BRIC 200 (IgG). The maximum amount of BRIC 200 (0.15 μg) that bound to 6.54 × 108 RBCs was determined from the Scatchard curve x-axis intercept, converted into molecules of antibody, and expressed as antibody binding sites per RBC, assuming a 1:1 binding ratio. Functional affinity constants were determined according to K = ([AbAg]/[Ag] × [Ab]) mol/L. Bound antibody concentrations ([AbAg]) were determined from the bound 125I counts and free antibody ([Ab]) and free antigen ([Ag]) were calculated from known initial concentrations.
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Antibody W Fab W Fab

ERIC

ERIC

ERIC

ERIC

untreated RBCs directly. These isotypes do not have unusual properties from a series of experiments or derived from a single experiment. The results are compiled from a number of experiments on different blood samples taken over a period of approximately a year. In most cases the blood samples were from the same donor.

Other values are either the mean from two experiments or derived from a single experiment. The results are compiled from a number of experiments on different blood samples taken over a period of approximately a year. In most cases the blood samples were from the same donor.

Abbreviation: ND, not determined.

Table 2. Number of Available Binding Sites on RBCs for Anti-AE1 Antibodies and Values of Affinity Constants

<table>
<thead>
<tr>
<th>Antibody</th>
<th>IgG</th>
<th>Fab</th>
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<tbody>
<tr>
<td>BRIC 6</td>
<td>313 ± 28 (13)</td>
<td>614 ± 69 (7)</td>
</tr>
<tr>
<td>BRIC 71</td>
<td>213 ± 12 (6)</td>
<td>746 ± 77 (3)</td>
</tr>
<tr>
<td>BRAC 90</td>
<td>236 (2)</td>
<td>437 (1)</td>
</tr>
<tr>
<td>BRAC 200</td>
<td>90 ± 7 (4)</td>
<td>ND</td>
</tr>
<tr>
<td>BRAC 14</td>
<td>77 ± 12 (3)</td>
<td>ND</td>
</tr>
<tr>
<td>BRAC 15</td>
<td>326 ± 32 (5)</td>
<td>241 (2)</td>
</tr>
<tr>
<td>BRAC 17</td>
<td>176 ± 12 (3)</td>
<td>575 ± 12 (3)</td>
</tr>
<tr>
<td>BRAC 18</td>
<td>100 ± 7 (6)</td>
<td>772 ± 160 (3)</td>
</tr>
<tr>
<td>BRAC 21</td>
<td>108 ± 6 (3)</td>
<td>300 (2)</td>
</tr>
<tr>
<td>BRAC 23</td>
<td>235 ± 28 (4)</td>
<td>ND</td>
</tr>
<tr>
<td>BRAC 25</td>
<td>141 ± 22 (5)</td>
<td>351 ± 36 (9)</td>
</tr>
</tbody>
</table>

Mean ± SD values are given for three or more measurements (the number of determinations is given in parentheses). Other values are either the mean from two experiments or derived from a single experiment. The results are compiled from a number of experiments on different blood samples taken over a period of approximately a year. In most cases the blood samples were from the same donor.

Abbreviation: ND, not determined.

Table 3. Inhibition of 125I-Labeled Purified Igs With Unlabeled Antibodies

<table>
<thead>
<tr>
<th>Unlabeled Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bric 6</td>
</tr>
<tr>
<td>Anti-AE1</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>-</td>
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<tr>
<td>+</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>Anti-Wp</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>+</td>
</tr>
</tbody>
</table>

Inhibition of binding of 125I-labeled IgG in the presence of a 10-fold molar excess of unlabeled IgG.

Abbreviations: +, greater than 50% inhibition; (+), 10% to 50% inhibition; -, less than 10% inhibition; ND, not determined.

Table 4. Maximum Uptake of 125I-labeled IgG and Fab Fragments by Normal and Ovalocytic RBCs

<table>
<thead>
<tr>
<th>Labeled Antibodies</th>
<th>Site Numbers × 10²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Ovalocytes</td>
</tr>
<tr>
<td>AE1</td>
<td></td>
</tr>
<tr>
<td>BRIC 6</td>
<td>286 ± 30 (4)</td>
</tr>
<tr>
<td>Fab</td>
<td>568 (2)</td>
</tr>
<tr>
<td>BRAC 17</td>
<td>578 (2)</td>
</tr>
<tr>
<td>BRAC 25</td>
<td>337 ± 28 (6)</td>
</tr>
<tr>
<td>Wr⁺</td>
<td>890 ± 70 (6)</td>
</tr>
<tr>
<td>GPA</td>
<td>1,010 ± 67 (5)</td>
</tr>
</tbody>
</table>

Mean ± SD values are given for three or more measurements. (The number of determinations is given in parentheses.) The results for each normal and SAO sample with a given antibody were obtained at the same time under identical conditions and are thus directly comparable. The binding affinities calculated for each antibody with normal and SAO cells did not differ significantly.

(data not shown). There is some evidence that mouse IgG3 forms noncovalent dimers via Fc-Fc interactions when antibody binding occurs.²⁸ It is possible that BRIC 6 agglutinates RBCs through this mechanism and that rat IgG1 (BRAC 14) may interact in a similar manner.

Immunofluorescence experiments showed that BRACs 17, 18, 21, and 23 and BRIC 6 bind to myeloid cells and BRACs 18, 21, and 23 were shown to immune precipitate a component of 70 kD from U937 cells. It seems likely that these antibodies were binding via their Fc domains and precipitating Fc receptor I (FcRI) known to be present on U937 cells.²⁹ This conclusion is supported by the observation that human
serum inhibited the binding of MoAbs to U937 cells. BRACs 17, 18, 21, and 23 are IgG2b, the only rat isotype that contains the Leu-Leu-Gly-Gly-Pro motif (aa 234 to 238) believed to be essential for human FcRI interaction. Mouse IgG3 (BRIC 6) also binds weakly to human FcR1. Granulocytes do not express FcRI, but they do express FcRII, a medium-affinity receptor that binds human IgG1 and IgG3, the isotypes most similar to rat IgG2b; this may explain why the rat antibodies bound to granulocytes.

Quantitation of the number of anti-AE1 molecules bound to RBCs at saturation. The number of molecules of AE1 on normal RBCs is estimated to be of the order of 10⁶ / cell. The results obtained with anti-AE1 showed that the number of binding sites/RBC were generally lower for IgG than for Fab fragments of the same antibody (Table 2). These differences are probably attributable to steric hindrance, which prevents access of the larger IgG molecules to all available epitopes. We have noticed that site numbers determined with some MoAbs may vary when different preparations of the antibodies are labeled with different ¹²⁵I preparations; therefore, results obtained with a single labeled antibody preparation should be treated with caution. The data for BRIC 6, BRAC 17, and BRAC 25 presented in Table 2 were obtained from several different antibody preparations (4, 3, and 2, respectively) and several different radioiodinations (6, 3, and 4, respectively) and the results were reproducible for each antibody. The data show considerable variation in the number of epitopes accessible to IgG or Fab fragments prepared from different anti-AE1s. We have previously observed a similar wide range of values with preparations from different monoclonal anti-GPAs, but in this case the variation could be explained, at least in part, by heterogeneity of O-glycosylation on this glycoprotein. AE1 does not possess O-glycans and the single N-glycan does not appear to be a prerequisite for binding of antibodies described here because HEMPAS cells, which have a truncated N-glycan on AE1, and En(a−) or M⁰⁰⁰⁰ M⁰⁰⁰⁰ cells, which have an abnormally large N-glycan on AE1, both give normal reactions with anti-AE1s. Our results suggest that there is some heterogeneity in the organization of individual AE1 molecules in the RBC membrane such that certain epitopes are not accessible to all molecules. It is possible that fatty acylation could influence the organization of different AE1 molecules in normal cells. Alternatively, and perhaps more likely, the accessibility of epitopes on AE1 may be influenced by the oligomeric state of the protein. Approximately 70% of the AE1 in normal RBCs is in the form of a dimer, with the remainder consisting of tetramer and higher oligomers. The proportion of tetramer and higher oligomers increases with RBC age (22% in young cells and 56% in old cells). It is possible that those antibodies giving unexpectedly low numbers of AE1 binding sites (Fab fragments of BRAC 15, 21, and 25; Table 2) preferentially recognize tetrameric AE1. If this is so, then it might be anticipated that old RBCs would have a higher number of binding sites for these antibodies than young RBCs. Experiments designed to address this possibility are in progress.

Epitope mapping of monoclonal anti-AE1s. The results of competitive inhibition experiments suggest that the anti-AE1s recognize a heterogeneous group of related epitopes (Table 3). All of these epitopes depend on the integrity of the third extracellular loop of AE1 because all are lost after chymotrypsin treatment or a combination of chymotrypsin and trypsin treatment under low ionic strength conditions (vide supra).

Interpretation of these data is complicated by the possibility discussed above that antibody binding is influenced by the oligomeric state of AE1 in the RBC membrane. Nevertheless, it is interesting to note that some anti-AE1s, notably BRIC 6, can impede the binding of anti-Wr² (Table 3). The Wr² epitope is defined by interaction between AE1 and GPA and is dependent on GLU 658 in the fourth extracellular loop of AE1.

Altered expression of AE1 epitopes on SAO. Several recent studies of SAO have shown that the phenotype results from inheritance of an abnormal form of AE1 that lacks 9 amino acids (400-408) at the junction of the N-terminal cytoplasmic domain and the first transmembrane domain. This abnormal AE1 (AE1 SAO), which has only been described in heterozygotes, is thought to confer some protection against malarial parasitemia. It is presumed that homozygosity for AE1 SAO would be a lethal condition because AE1 SAO does not function as an anion transporter²⁴,²⁵ SAO RBCs are more rigid than normal RBCs and this has been related to the observation that AE1 SAO has a higher tetramer:dimer ratio than normal AE1 (50% compared with 30%). The reduction in binding of BRIC 6 and BRAC 25 to SAO cells (Table 4) appears too large to be accounted for by alterations in the dimer:tetramer ratio of AE1 on these cells. Because the antibodies to extracellular AE1 each recognize epitopes that depend on the integrity of the third extracellular loop of normal AE1 (vide supra), the reduced number of BRIC 6 and BRAC 25 epitopes found in the binding studies with SAO RBCs suggest that subtle changes in the folding of this loop occur in AE1 SAO.

The anion transport inhibitor di-isothiocyanato-dihydrostilbene disulphonate (H₂DIDS) fails to bind AE1 SAO. The H₂DIDS binding site is located in the fifth transmembrane domain at Lys-539 or Lys-542. Therefore, it is possible that the changes in the folding of AE1 SAO that give rise to the loss of the H₂DIDS binding site also alter the accessibility of the BRIC 6 and BRAC 25 epitopes. At alkaline pH, H₂DIDS cross-links Lys539 with Lys851, which is predicted to be between transmembrane domains 13 and 14 on extracellular loop 7. The Di² blood group antigen is associated with increased binding of H₂DIDS and has recently been shown to correlate with a Pro854-Leu change on the 7th extracellular loop of AE1. In this context it is interesting to note that the Di² antigen is reported to be severely depressed in SAO cells. These and other results suggest that changes in the extracellular region between transmembrane domains 13 and 14 and in transmembrane domain 14 itself can modify the environment of Lys-539.

There are other abnormalities associated with SAO cells. Weakened reactions of the polyclonal antibodies made by En(a−) individuals (anti-GPA and anti-Wr³) have been reported. Our results (Table 4) confirm previous observa-
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...that the number of Wr3 antigen sites is significantly reduced on SAO cells, although the results for GPA are less conclusive (GPA site numbers were only marginally reduced on some SAO cells).

Taken together, these results suggest that the mutation that gives rise to the deletion of residues 400-408 at the junction of the N-terminal cytoplasmic domain with the first transmembrane domain of AE1 affects the accessibility of antigens associated with extracellular loops formed by the 5th/6th (BRIC 6), 7th/8th (WF7), and 13th/14th (Di4) transmembrane regions. Such a conclusion is consistent with other reports45,49 that conclude that a highly cooperative interaction exists between transmembrane domains in AE1 such that a change in one domain can disrupt its interaction with others. These interactions between apparently remote regions of AE1 may occur if AE1 adopts a circular or helical conformation in addition to forming oligomers in the RBC membrane.

The evidence presented by Booth et al47 suggests that the abnormalities found in SAO cells are not confined to AE1. In particular, they note weakened expression of Rh antigens and other antigens located on proteins now known to be associated with Rh (LW, S, s, and U). Preliminary studies on SAO cells using MoAb BRIC 69, which binds to the Rh 30-kD polypeptides,50 show a 33% reduction in the site numbers found on SAO cells in comparison with normal cells, suggesting that the Rh polypeptides may be intimately associated with AE1 in these cells. These results raise the interesting possibility that AE1 is closely associated with other integral membrane proteins (in addition to GPA) in the normal RBC and that the mutation in AE1 SAO also effects changes in the organization of these proteins on SAO cells. The S and s antigens are on glycophorin B (GPB) and, because GPB forms heterodimers with GPA that are stable under the conditions of SDS-PAGE,51 a large membrane complex comprising AE1, GPA, GPB, and Rh proteins seems possible.

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