Evidence That the Antigens of the Yt Blood Group System Are Located on Human Erythrocyte Acetylcholinesterase

By Frances A. Spring, Brigitte Gardner, and David J. Anstee

The Yt blood group system comprises two antigens, Yta and Ytb. Human anti-Yta and human anti-Ytb immune precipitate a component of the same apparent molecular weight as acetylcholinesterase from radioiodinated erythrocytes of appropriate Yt phenotype. Immune precipitates obtained with anti-Yta and anti-Ytb contained acetylcholinesterase activity. In contrast, immune precipitates obtained with human anti-Gy⁶ and murine monoclonal anti-CD55, which identify other glycosylphosphatidylinositol-linked erythrocyte surface proteins, did not have acetylcholinesterase activity. Quantitative binding assays using murine monoclonal antiacetylcholinesterase antibodies (AE-1 and AE-2) gave 3,000 to 5,000 binding sites/cell for IgG and 7,000 to 10,000 sites/cell for Fab fragments. Endo F digestion of immune precipitates obtained with AE-1 and anti-Yta indicated that approximately 10% of the enzyme comprises N-glycans. These results indicate that the Yt antigens define an inherited polymorphism on erythrocyte acetylcholinesterase and that the recent assignment of the Yt blood group locus to the long arm of chromosome 7 (Zelinski et al, Genomics 11:165, 1991) provisionally identifies the position of the acetylcholinesterase gene.

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ACETYLCOLINESTERASE (AChE; EC 3.1.1.7) is a well-characterized enzyme² found at neuromuscular junctions, on erythrocytes, and on some mature peripheral blood leukocytes. It plays a key role in cholinergic neurotransmission, but its function on erythrocytes and leukocytes is unknown. In human erythrocytes, the enzyme is predominantly expressed as a disulphide-bonded dimer, although low levels of monomer are also present. The enzyme, a glycoprotein, is attached to the plasma membrane through a glycosylphosphatidylinositol (GPI) tail. Erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNH) have a gross deficiency of GPI-linked proteins, including AChE.

The Yt blood group system comprises two antigens, Yta³ and Ytb⁴. Most Europeans are Yta(a+) and approximately 8% are Ytb(b−); there are thus three phenotypes, Yta(b+), Yta(b−), and Ytb(b−), of which Yta(b+) is the most common. Inheritance of the phenotype Yta(b−) has not been described, but patients with the acquired disorder, paroxysmal nocturnal hemoglobinuria (PNH), are reported to have a proportion of Yta(b−) erythrocytes in their peripheral blood.⁵

We report evidence suggesting that the Yta and Ytb antigens are located on AChE and use monoclonal antibodies (MoAbs) to AChE to quantitate the number of AChE molecules per erythrocyte.

MATERIALS AND METHODS

Erythrocytes were obtained from the South Western Regional Transfusion Centre, Bristol, UK. Erythrocytes were treated with trypsin as described.⁶ Treatment of erythrocytes with 6% (wt/vol) 2-aminoethylisothiouronium bromide (AET) was as described.⁷ Human sera containing anti-Yta were obtained from J. Judd (University of Michigan, Ann Arbor, MI) (235) and J. Poole (International Blood Group Reference Laboratory, Bristol, UK) (DW). Human anti-Ytb (1144) was from J. Judd. Anti-Gy⁶ (GL) was from Dr R. Herron (Wessex Regional Transfusion Centre, Southampton, UK). Hybridomas AE-1 and AE-2 were obtained from the American Type Culture Collection (Rockville, MD). NBTS/BRIC 230 (BRIC 230) was as described.⁸ IgG and Fab fragments of MoAbs were prepared as described.⁹ The number of AChE molecules per erythrocyte and the functional affinity constants were determined as described elsewhere. Human anti-Yta, anti-Ytb, and anti-Gy⁶ were purified by absorption onto antigen-positive cells followed by acid elution from digitonin-lysed red blood cell (RBC) stroma.¹⁰ The specificity of the eluted antibody was confirmed by hemagglutination with erythrocytes of known Yt phenotype. Immune precipitation using human or murine antibodies was performed on unlabeled or radiiodinated erythrocytes of known Yt phenotype following the method of Moore et al.¹¹ Immune complexes used for AChE assay were prepared from packed erythrocytes (500 µL) incubated with antibody (5 mL) at 4°C overnight. Membranes were solubilized in phosphate-buffered saline (PBS), pH 7.2, containing either 1% or 2% (wt/vol) Triton X-100, 2 mmol/L phenylmethylsulphonyl fluoride, and 5 mmol/L K₂ EDTA (TX-buffer). Immunoblotting was performed as described using 13% (wt/vol) acrylamide with a 3% (wt/vol) overlay under reducing or nonreducing conditions. Autoradiography of dried gels was performed using Hyperfilm-MP (Amersham International Ltd, Aylesbury, Bucks, UK). Immunoblotting was performed as described using 5% (wt/vol) bovine milk powder as the blocking agent, Immobilon P membranes (Millipore Ltd, Harrow, Middlesex, UK), and antihuman Ig or antimouse Ig (DAKO Ltd, Buckinghamshire, UK) as appropriate.

Acetylcholinesterase was assayed as described.¹² Test sample (erythrocyte membranes, Triton solubilized membrane fraction, or immune complex) was mixed with 10 mmol/L dithiobisnitrobenzoic acid 100 µL (BDH, Poole, UK) in 0.1 mol/L phosphate buffer, pH 8.0 (2 mL), and the change in optical density at 412 nm recorded at 30-second intervals over a 5-minute period after the addition of acetylthiocholine (50 µL, 10 mmol/L, in 0.1 mol/L phosphate buffer, pH 8.0).

From the South Western Regional Blood Transfusion Centre, Bristol, UK; the Department of Haematology, St Mary's Hospital, London, UK; and the International Blood Group Reference Laboratory, Bristol, UK.

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Address reprint requests to Frances A. Spring, PhD, Department of Immunohematology, South Western Regional Blood Transfusion Centre, Southmead Road, Bristol, BS10 5ND UK.

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addition of 75 mmol/L thioccholine iodide (20 μL; Sigma Ltd, Poole, UK).

RESULTS

Immune precipitation with human anti-Yta, human anti-Ytb, and murine monoclonal anti-AChE (AE-1, AE-2). Immune precipitation experiments from radioiodinated erythrocytes of appropriate Yt phenotype were performed with human anti-Yta and anti-Ytb. The components of these immune precipitates were electrophoretically separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under either reducing or nonreducing conditions. The resultant autoradiographs are illustrated in Figs 1 and 2. Both anti-Yta and anti-Ytb specifically precipitated a component of molecular weight (Mr) 160,000 under nonreducing conditions (Fig 1A, lane c, and Fig 2, lane c, respectively), and of Mr 72,000 under reducing conditions (Fig 1B, lane e, and Fig 2, lane g, respectively) from the appropriate cells.

AE-1 and AE-2 were used in immune precipitation experiments performed in parallel with those using anti-Yta and anti-Ytb. The results show a major component of Mr
Fig 2. Autoradiographs of immune precipitates prepared with AE-1 and human anti-Yf. Precipitates prepared with AE-1 from Yt(a+b−) RBCs (a and e) and Yt(a−b+) RBCs (b and f). Precipitates prepared with anti-Yf(1144) from Yt(a−b+) RBCs (c and g) and Yt(a+b−) RBCs (d and h). Lanes a through h were from the same experiment separated in gels of 10% acrylamide with a 3% overlay run under either nonreducing (a through d) or reducing (e through h) conditions. Autoradiography was for 3 weeks (a through d) or 4 weeks (e through h).

160,000 under nonreducing conditions (Fig 1A, lanes a and b, and Fig 2, lanes a and b) and Mr 72,000 under reducing conditions (Fig 1B, lanes a and c, and Fig 2, lanes e and f). The figures illustrate data for AE-1 for Yt(a+b−) and Yt(a−b+) RBCs; AE-2 gave identical results (F.A.S., data not shown). When immune precipitates obtained using anti-Yt\textsuperscript{a} and anti-Yt\textsuperscript{b} were directly compared on the same gel with those obtained using AE-1, the results clearly showed that components specifically precipitated by anti-Yt\textsuperscript{a} and anti-Yt\textsuperscript{b} were of the same electrophoretic mobility as those precipitated by AE-1 under both reducing and nonreducing conditions (Figs 1 and 2). Immune precipitates prepared from trypsin-treated erythrocytes using both AE-1 and anti-Yt\textsuperscript{a} showed a major band of Mr 64,000, with some undigested material remaining at Mr 72,000 (under reducing conditions, Fig 1A, lanes e through h). When immune precipitates prepared with AE-1 and anti-Yt\textsuperscript{a} were digested overnight with an Endo F preparation, subsequent autoradiography of the electrophoretically separated material showed identical results with the two antibodies. In both cases a band of Mr 64,000 was observed (Fig 1B, lanes b, d, and f, reducing conditions), in comparison with a band of Mr 72,000 in the untreated material (Fig 1B, lanes a, c, and e).

Assay of AChE activity in immune precipitates obtained with murine MoAbs to AChE and human anti-Yt\textsuperscript{a} and anti-Yt\textsuperscript{b}. To determine whether the Yt blood group antigens were carried on AChE or on another protein that exhibited the same electrophoretic mobility under nonreducing and reducing conditions, the immune precipitates obtained with anti-Yt\textsuperscript{a} and anti-Yt\textsuperscript{b} were assayed for AChE activity (see Materials and Methods). The ability of anti-Yt\textsuperscript{a} and anti-Yt\textsuperscript{b} to precipitate AChE activity was compared with that of the MoAbs AE-1 and AE-2 and antibodies to two other GPI-linked erythrocyte membrane proteins, the Gy\textsuperscript{a}/Hy glycoprotein\textsuperscript{16} and CD55.\textsuperscript{17} The Gy\textsuperscript{a}/Hy glycoprotein was precipitated with human anti-Gy\textsuperscript{a} and CD55 with murine MoAb BRIC 230. A representative experiment is shown in Fig 3. In this experiment, antibody AE-2 precipitated only 8% of the AChE activity obtained with AE-1. This is likely to reflect the known inhibitory action of AE-2 on AChE.\textsuperscript{18} Human anti-Yt\textsuperscript{a} (235) and anti-Yt\textsuperscript{b} precipitated 22% and 48%, respectively, of the activity brought down by AE-1, whereas anti-Gy\textsuperscript{a} and BRIC 230 precipitates contained no detectable AChE activity. Treatment of erythrocytes with AET dramatically reduced (by 88%) the AChE activity of AE-1 precipitates. Confirmation that the Gy\textsuperscript{a}/Hy glycoprotein and CD55 were precipitated under the conditions of this experiment was obtained by immunoblotting the electrophoretically separated components of the immune precipitates with human anti-Gy\textsuperscript{a} and BRIC 230, respectively (data not shown). Additional experiments were also performed with radioiodinated erythrocytes and the resulting immune complexes analyzed by autoradiography after SDS-PAGE. The results clearly showed that those immune precipitates that contained the Mr 160,000 component (nonreducing conditions) and the Mr 72,000 component (reducing conditions) also have AChE activity (F.A.S., data not shown). Two additional experiments were performed with anti-Yt\textsuperscript{a} (DW) and these yielded 40% and 54%
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Functional affinity constants were also determined. The results are presented in Table 1. Site numbers in the range of 2,964 to 4,833 were obtained with IgG, whereas Fab fragments gave values approximately twofold higher. The Yt phenotype of the target erythrocytes did not seem to significantly influence the number of binding sites for IgG or Fab fragments.

### DISCUSSION

The results show that human anti-Yt\(^a\) and anti-Yt\(^b\) precipitate a component of Mr 160,000 (nonreducing conditions) or Mr 72,000 (reducing conditions) from radioiodinated erythrocytes of appropriate Yt phenotype. Murine MoAbs to human erythrocyte AChE precipitated components of the same Mr as anti-Yt\(^a\) and anti-Yt\(^b\) under both reducing and nonreducing conditions. These results, which strongly suggest that the Yt antigens are expressed on AChE, were further supported by experiments involving trypsin-treated RBCs and Endo F-treated immune precipitates in which AE-1 and anti-Yt\(^a\) also gave identical results. Endo F digestion of immune precipitates obtained with AE-1 and anti-Yt\(^a\) indicated that approximately 10% of the protein is N-glycan. This value is consistent with previous estimates based on composition/analysis. Such a hypothesis is consistent with other observations that indicate that both AChE and Yt antigens are sensitive to reduction by sulphydryl reagents, that both AChE and Yt antigens are carried on GPI-linked molecules, and that both the Yt\(^a\) antigen and AChE are sensitive to chymotrypsin treatment of intact erythrocytes but resistant to trypsin treatment.

Quantitative binding experiments using radioiodinated IgG and Fab fragments of AE-1 and AE-2 showed that Fab fragments gave values approximately twofold higher than those obtained for IgG. It is possible that this difference in binding of IgG and Fab fragments is related to the...
occurrence of AChE as a disulphide-bonded homodimer. Hence, only one IgG molecule per homodimer is bound because of steric hindrance. In this case, the values obtained with Fab fragments are a measure of the total number of AChE monomers in the RBC membrane. We have observed similar twofold increases in Fab binding compared with IgG on several occasions using antibodies to other erythrocyte membrane proteins (glycophorin A, CD44, CD58, and CD59). Whereas there is some evidence that glycophorin A occurs as a dimer, information regarding CD44, CD58, and CD59 is lacking. These results lend support to the hypothesis that these proteins also occur as homodimers. Because the antigens Yt\(^a\) and Yt\(^b\) are likely to be located on AChE (discussed previously), the measurements of the abundance of AChE on erythrocytes are also a measure of the abundance of Yt\(^a\) and Yt\(^b\) antigens.

The Yt blood group locus has recently been assigned to the long arm (q22.1-q22.3) of chromosome 7. The association of the Yt blood group antigens with acetylcholinesterase allows the provisional assignment of the erythrocyte acetylcholinesterase gene to the same locus. Deletions involving the 7q22 region have frequently been noted in the hematopoietic cells of patients with myelodysplastic syndromes and acute nonlymphocytic leukemia. These results suggest that DNA probes for the AChE gene may be useful in the analysis of these disorders.

Inheritance of the “null” phenotype, Yt(a-b-), has not been reported, although the phenotype is expressed on the defective RBC population of patients with PNH and inherited partial AChE deficiency has been described. Comparable “null” phenotypes are known in several other blood group systems and in these cases the affected erythrocytes have relatively mild functional abnormalities. The apparent absence of the Yt(a-b-) phenotype suggests the possibility that inherited AChE deficiency might have severe consequences for the growth and differentiation of hematopoietic cells. Evidence that cholinergic agents are potent initiators of DNA synthesis in murine hematopoietic stem cells is consistent with such an important functional role.

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NOTE ADDED IN PROOF

Since this report was accepted for publication, Getman et al have reported the localization of the acetylcholinesterase gene to 7q22.

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