Human Erythrocyte Acetylcholinesterase Bears the Yt⁺ Blood Group Antigen and Is Reduced or Absent in the Yt(a⁻b⁻) Phenotype

By Neeraja Rao, Carolyn F. Whitsett, Sarah M. Oxendine, and Marilyn J. Telen

The Cartwright (Yt) blood group antigens have previously been shown likely to reside on a phosphatidylinositol-linked erythrocyte membrane protein. In this study, an unusual individual whose red blood cells (RBCs) were of the previously unreported Yt(a⁻b⁻) phenotype were used, along with normal Yt(a⁺) cells, to investigate serologically and biochemically the relationship of the Yt⁺ antigen to known phosphatidylinositol-linked erythrocyte proteins. Yt(a⁻b⁻) RBCs expressed normal amounts of various phosphatidylinositol-linked proteins except acetylcholinesterase. Further, human anti-Yt⁺ reacted with acetylcholinesterase in immunoprecipitation and immunoblotting studies. Thus, acetylcholinesterase is now identified as the protein bearing the Yt blood group antigens.

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The Cartwright (Yt) blood group system comprises two antigens: Yt⁺ and Yt⁻.¹ The Yt⁺ antigen occurs in 99.7% of blood donors, while the antithetical antigen Yt⁻ occurs in only 0.3% of donors. Nevertheless, anti-Yt⁺, made by the 0.3% of the population that is Yt(a⁻b+), is not rare and may have significant ability to destroy transfused homologous Yt⁺ red blood cells (RBCs). No persons with Yt(a⁻b⁻) RBCs have been previously described.

Until recently, little was known about the biochemistry of the Yt antigens, except that they could be denatured by combinations of sulfhydryl reagents and proteases,¹ implying a protein backbone. However, study of RBCs from patients with paroxysmal nocturnal hemoglobinuria (PNH) has shown that the Yt antigens were likely to reside on a phosphatidylinositol (PI)-anchored protein, as abnormal complement-sensitive cells of PNH patients failed to express these antigens.² It is well recognized that complement-sensitive PNH erythrocytes fail to express proteins anchored to the membrane in this way, although the defect responsible for this deficiency has not yet been defined.³

Human blood cells express a wide variety of PI-anchored proteins subserving diverse functions.⁴ PI-anchored proteins expressed by erythrocytes include decay accelerating factor (DAF; CD55),⁵ membrane inhibitor of reactive lysis (MIRL; CD55),⁵ lymphocyte function associated antigen-3 (LFA-3; CD58),⁶,⁷ acetylcholinesterase (AChE),⁸-¹¹ NAD⁺ glycohydrolase,¹² JMH protein (p76),¹³ and the protein that bears NAD⁺ glycohydrolase have not been previously shown to bear blood group antigen activity.

The purpose of this study was to identify the PI-anchored protein carrying Cartwright (Yt) blood group antigens and to characterize a newly described Yt(a⁻b⁻) phenotype. Using biochemical and serologic techniques to study both normal Yt(a⁺b⁻) and rare Yt(a⁻b⁻) erythrocytes, we have now demonstrated that the common Yt⁺ antigen resides on erythrocyte AChE.

MATERIALS AND METHODS

Blood cells and sera. For most studies, whole blood from healthy normal donors was collected using tripotassium EDTA as anticoagulant and was stored in acid citrate dextrose at 4°C for up to 1 week. Erythrocytes (E) were then washed in phosphate-buffered saline (PBS, pH 7.4) before use. Immunoprecipitation studies were performed on freshly drawn, unstored cell samples.

The Yt(a⁻b⁻) RBCs (donor WH) used in this study were identified as having that phenotype by a number of different reference laboratories, and detailed serologic studies will be described elsewhere. A number of different Yt⁺ antisera were used in various parts of this study. Two different antisera for Yt⁺ were used for immunohistochemical analyses: These were kind gifts from Rock Oyen (New York Blood Center, New York) and from Kathy Skradski (Minneapolis Memorial Blood Center, Minneapolis, MN). For some radioimmunoprecipitations, human antisera were partially purified by adsorption onto random donor erythrocytes and eluted by using Elu-KitIII (gift of Gamma Biologicals, Houston, TX), according to manufacturer's instructions. For negative controls, normal sera not containing detectable alloantibodies were similarly processed. A large excess of albumin was then added to each eluted antibody to equalize protein content and to prevent degradation of small quantities of purified antibody.

All the monoclonal antibodies (MoAbs) used in this study have been previously described. The anti-AChE antibodies AE1 and AE2²¹ were obtained from hybridoma cells supplied by the American Type Culture Collection (Rockville, MD). AE3 and AE4 antibodies were the kind gift of Dr Douglas Fambrough (Johns Hopkins University, Baltimore, MD). Anti-LFA-3²⁵ was a gift of Dr Timothy Springer (Dana Farber Cancer Institute, Boston, MA), and anti-JMH antibody bi²⁴ and anti-DAF antibody 3.3 were gifts of Dr Robert Knowles (Ortho Pharmaceuticals, Raritan, NJ). Anti-CD55 antibody 2/24D⁴ was provided by Dr Anne Fletcher (Red Cross Blood Transfusion Service, Sydney, Australia).

Radioimmunoassays. Antibody reactivity with random donor as well as the variant Yt(a⁻b⁻) E was measured by radioimmunoassay (RIA) as previously described.¹² All erythrocytes were assayed in triplicate for antigen expression at comparable cell counts (determined with an ELT8; Ortho Diagnostics, Raritan, NJ). Yt(a⁻b⁻) E, along with cells from normal donors, were tested for their ability to bind antibodies to various PI-linked proteins such as DAF, LFA-3, MIRL, and JMH as well as four MoAbs AE1, AE2, AE3, and AE4 to different...
AChE epitopes. P3x63Ag8 ascitic fluid, as well as buffer alone, was used as negative control.

**Enzyme assays.** AChE activity was measured at room temperature using the assay of Ellman et al.\(^2\) with 1 mmol/L acetylthiocholine iodide and 250 μmol/L 5,5′-dithiobis(2-nitrobenzoic acid) in 100 mmol/L sodium phosphate buffer, pH 7.4. After the reaction was stopped with absolute alcohol, optical density (OD) was measured spectrophotometrically at 412 nm (Spectronic 1001; Milton Roy Co, Rochester, NY).

Radioimmunoprecipitation. Erythrocytes were obtained from EDTA-anticoagulated whole blood. They were washed three times in PBS and were labeled with \(^{125}\)I by using Iodo-Gen (Pierce, Rockford, IL), as described previously.\(^{26}\) Immunoprecipitation with anti-Yt\(^a\) and AE4 antibodies was then performed according to previously described procedures.\(^{28,29}\) For immunoprecipitation with human antiserum, 5 × 10\(^9\) washed, radiolabeled cells were incubated with 400 μL of adsorbed and eluted Yt\(^a\) antibody or a similar preparation made from nonreactive serum, for 1 hour at 22°C, washed, and then lysed with hypotonic buffer.\(^{30}\) Membranes from 5 × 10\(^6\) cells were then solubilized in 400 μL of 0.015 mol/L NaCl, 0.05 mol/L Tris, 0.005 mol/L EDTA, pH 7.4 with 1% (vol/vol) Nonidet P-40 (Sigma, St Louis, MO), 0.003 mol/L phenylmethylsulfonyl fluoride (PMSF), and 0.1% gelatin (Rip buffer) for 30 minutes at 4°C. After centrifugation to remove unsolubilized material, supernates were then used for immunoprecipitation. "Preclearing" was accomplished by two incubations of this membrane protein solution with 200 μL of gelatin-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) suspended in Rip buffer. Immune complexes were then precipitated by using Sepharose beads conjugated to anti-human antibody (Cappel, West Chester, PA). Elution of immunoprecipitated protein and analysis by polyacrylamide gel electrophoresis (PAGE) and autoradiography were then accomplished as described previously.\(^{28,29}\) Radioimmunoprecipitation and analysis with the use of MoAb AE4 and P3x63/Ag8 ascitic fluid (negative control) were performed simultaneously to control for AChE level of expression.\(^{15}\)

**Dot-blot analysis.** Serial dilutions of purified AChE were applied to nitrocellulose and allowed to bind for 2 hours at 22°C. The membrane was then washed with Tris-buffered saline containing 0.05% Tween 20 and incubated with either human anti-Yt\(^a\) or normal human serum overnight at 4°C. Washing and detection of antibody binding was then performed using an alkaline phosphatase-anti-IgG conjugate and the 5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium (BCIP/NBT) chromogenic substrate (Promega, Madison, WI).\(^{16}\)

**RESULTS**

**Identification of Yt(a−b−) propositus.** A 60-year-old white man, with cardiomyopathy and previous aortic valve replacement, was undergoing evaluation for heart transplant. He had no history of neurologic dysfunction. Routine pretransfusion antibody screening for RBC alloantibodies showed presence of an antibody reactive with all cells but the patient’s. Family members RBCs were incompatible. The patient’s cells had a weakly positive Yt(a−b−) reaction with all cells tested except complement-sensitive PNH RBCs, suggesting that it recognized a PI-anchored structure. Chromium survival studies confirmed apparent presence of anti-Yt\(^a\) in that both Yt(a+b−) and Yt(a−b+) cells had reduced survival: 74% and 12% of Yt(a+b−) cells survived 1 and 24 hours, respectively, whereas 75% and 46% of Yt(a+b+) cells survived 1 and 24 hours; 96% of autologous cells survived at 24 hours.

**Expression of PI-linked proteins by Yt(a−b−) erythrocytes.** Expression of various PI-linked proteins was examined by RIA, using a variety of monoclonal antisera. In initial studies, Yt(a−b−) RBCs bound antibodies to LFA-3, CD59, JMH protein, and DAF in amounts similar to those bound by normal controls (Table 1). However, in the same study, 4% as much AE1 antibody to AChE was bound by Yt(a−b−) cells as by random donor cells (Table 1). When binding of other anti-AChE MoAbs was measured and compared with control cells, the amount of binding seen was 17%, 8%, and 17% of normal for antibodies AE2, AE3, and AE4. AE2 antibody recognizes an epitope that overlaps but is not identical to that which binds AE1, while the AE3 and AE4 antibodies recognize two independent epitopes of AChE.\(^{21}\) Antibody AE4 was then used to compare expression of AChE by Yt(a−b−) cells with that of a larger number of random donors. When compared with 13 additional normal cell samples, the Yt(a−b−) cells bound only 10% as much AE4 antibody as did the normal cells (64 cpm v 643 cpm ± 35 SEM). Thus, Yt(a−b−) cells expressed a level of AChE antigenic activity more than 4 SDs below the mean for normal individuals.

**AChE enzyme activity.** To confirm that lack of AChE antigenic activity correlated with lack of AChE enzyme activity, a colorimetric assay of AChE was performed.\(^{15,27}\) Yt(a−b−) cells expressed 15% as much AChE enzyme activity as did normal, sample age-matched controls (OD\(_{412}\) of 0.058 ± 0.035 for three normal controls). Thus, the level of AChE enzyme activity correlated with AChE antigenic activity.

**Immunochemochemical studies of Yt(a−b−) RBCs.** To confirm further that AChE was absent on Yt(a−b−) RBCs, immunoprecipitation studies were performed using variant and normal cells in parallel. As shown in Fig 1A, AChE could be immunoprecipitated by antibody AE4 from the lysate of normal radiolabeled RBC membranes but not from Yt(a−b−) cell membrane lysate.

**Immunoprecipitation and dot-blot studies using human anti-Yt\(^a\).** Confirmation of the relationship between AChE and the high-frequency antigen Yt\(^a\) was obtained using human anti-Yt\(^a\) in immunoprecipitation studies. In experiments performed using human anti-Yt\(^a\) and murine monoclonal anti-AChE in parallel (Fig 1B), all three antisera immunoprecipitated a single protein band of approximately 160 Kd

| Table 1. Expression of PI-Linked Proteins by Yt(a−b−) Erythrocytes |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | LFA-3 | MIRL | JMH | DAF | AChE |
| Yt(a−b−)         | 1,820 | 2,284 | 872 | 1,759 | 19   |
| Normal no. 1     | 1,427 | 2,396 | 802 | 1,720 | 543  |
| Normal no. 2     | 1,743 | 2,674 | 630 | 1,731 | 278  |
ACETYLCHOLINESTERASE BEARS Yt* ANTIGEN

FIG 1. Immunoprecipitation studies using murine anti-AChE antibody AE4 and human anti-Yt*. (A) Antibody AE4 (lanes 1 and 3) and control murine ascites fluid P3x63/Ag8 (P3) (lanes 2 and 4) were used to immunoprecipitate proteins from lysed radiolabeled Yt(a-b-) erythrocytes (lanes 1 and 2) and normal Yt(a+) erythrocytes (lanes 3 and 4), and the results were analyzed under nonreducing conditions. AE4 precipitated AChE (Mr approximately 160,000) only from the Yt(a+) cells. (B) Two different examples of human anti-Yt* (lanes 1 and 2), as well as nonreactive human serum (lane 3) and murine antibody AE4 (lane 4), were used in immunoprecipitation experiments with normal Yt(a+) erythrocytes. Both examples of anti-Yt* precipitated a 160,000-Da band (lanes 1 and 2) of similar molecular weight to the protein immunoprecipitated by antibody AE4 (lane 4). Nonreactive human serum (lane 3) did not precipitate a similar band, nor did murine myeloma protein P3 (data not shown). The protein of approximately 92,500 Da in lanes 1 through 3 is band 3, nonspecifically immunoprecipitated by all human sera.

(ANALYZED UNDER NONREDUCING CONDITIONS), WHEREAS CONTROL SERA (NORMAL HUMAN SERUM AND MURINE P3 MYELOMA PROTEIN) FAILED TO PRECIPITATE SUCH PROTEIN BANDS.

BECAUSE RBC MEMBRANES CONTAIN ONLY VERY SMALL NUMBERS OF AChE MOLECULES, A FACT THAT MAKES WESTERN BLOTTING OF MEMBRANE PROTEINS FOR AChE DIFFICULT, AND, BECAUSE HUMAN ANTI-Yt* IMMUNOPRECIPITATES PROTEIN ONLY WHEN ANTIBODY IS APPLIED TO INTACT CELLS, A THIRD TECHNIQUE WAS USED TO PROVE THE IDENTITY OF THE PROTEIN RECOGNIZED BY ANTI-Yt*.

PHYSICOCHEMICALLY PURIFIED AChE was applied to nitrocellulose at varying concentrations, and the bound protein was reacted with both human anti-Yt* as well as normal human serum. The anti-Yt* reacted with AChE in this system, whereas the normal serum did not (Fig 2).

DISCUSSION

This study shows that deficient expression of human RBC AChE and normal expression of other PI-anchored proteins are associated with the first reported example of the Yt(a-b-) blood group phenotype. Furthermore, human anti-Yt*, the antibody directed against the high-frequency Yt antigen, recognizes AChE in immunoprecipitation and immunoblotting experiments. Thus, this study confirms previous work demonstrating that both the Yt* and Yt* antigens were absent from the affected cells of patients with paroxysmal nocturnal hemoglobinuria and thus were probably resident on a PI-anchored membrane protein. While this report was being prepared, confirmation of this work was presented by Spring and Anstee.
In addition, recent mapping of the Yt blood group system to chromosome 7q now provides a locus for the human AChE gene. Extensive study and follow-up of the Yt(a−b−) propositus (to be reported elsewhere) has shown that this patient developed the Yt(a−b−) phenotype in conjunction with an antibody probably directed to the AChE molecule. Over many months, expression of Yt⁰, as well as of AChE, increased, so that Yt⁰ became detectable, although weakly so, by routine blood bank methods 4 months after initial study. Quantitation of AChE at the end of the follow-up period showed increased expression of AChE to 57% of normal levels concomitantly with appearance of the Yt⁰ antigen. Loss of expression of blood group antigens while an autoantibody to the antigen or molecule is present has been described previously and is perhaps most common in the acquired JMH-negative phenotype. However, it is important to note that the function of AChE on RBCs is unknown and that isolated hereditary erythrocyte AChE deficiency is at least theoretically possible. Recent work has shown that RBC AChE uses a small exon not expressed in other tissues; it is this exon that provides the sequence necessary for posttranslational cleavage and attachment of a PI anchor. Thus, a mutation in that exon might be predicted to cause isolated AChE deficiency. However, genetic studies have not been performed in the current study because of the transient nature of the Yt(a−b−) phenotype.

In summary, PI-anchored proteins have now been confirmed to bear antigens of at least four different blood groups: Cromer, JMH, Holley/Gregory (Hy/Gp⁰), and Cartwright (Yt). In addition, Dombrock antigens appear to reside on a PI-linked protein. Utilization of human antisera to these proteins of the erythrocyte membrane. Baillieres Clinical Haematol 4: 849, 1991

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N Rao, CF Whitsett, SM Oxendine and MJ Telen

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