Arg89Cys Substitution Results in Very Low Membrane Expression of the Duffy Antigen/Receptor for Chemokines in Fy<sup>x</sup> Individuals

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The Duffy (FY) blood group antigens are carried by the DARC glycoprotein, a widely expressed chemokine receptor. The molecular basis of the Fy<sup>A</sup>/Fy<sup>B</sup> and Fy(a-b-) polymorphisms has been clarified, but little is known about the Fy<sup>x</sup> antigen and the FY<sup>x</sup>* allele associated with weak expression of Fy<sup>A</sup>, Fy<sup>3</sup>, Fy<sup>5</sup>, and Fy<sup>6</sup> antigens. We analyzed here the structure and expression of the FY gene in 4 Fy(a-b<sup>weak</sup>) individuals. As compared with Fy(a+b<sup>+</sup>) controls, the Fy(a-b<sup>weak</sup>) red blood cell membranes contained residual amount of DARC polypeptide and these cells were poorly bound by anti-Fy antibodies and chemokines. The Fy gene from Fy(a-b<sup>+</sup>) and Fy(a-b<sup>weak</sup>) individuals differed by one substitution, C286T. The resulting Arg89Cys amino acid change reduced the binding of anti-Fy antibodies and chemokines to DARC transfectants. We concluded that the Fy<sup>b</sup> donors carried the FY<sup>x</sup>* allele at the FY locus and that the Fy<sup>x</sup> antigen corresponds to highly reduced expression of a grossly normal Fy<sup>A</sup> polypeptide caused by the Arg89Cys substitution. Because FY is a single copy gene, this defect should also affect DARC expression in nonerythroid cells. Because the Fy<sup>x</sup> phenotype is not associated with apparent clinical consequences, we discussed these findings in the light of the putative roles of DARC in various tissues. Finally, we developed a Fy<sup>x</sup> DNA typing assay that should be useful for genetic studies and clinical transfusion medicine.

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sian families. These investigators postulated that the Fy\textsuperscript{weak} phenotype is conveyed by a fourth allele at the FY locus with a frequency of 2%, which they named FY\textsuperscript{X} because the total gene product was not certain. Further serological studies indicated that expression of FY\textsubscript{a}, FY\textsubscript{b}, and FY\textsubscript{6} antigens is also depressed in FY\textsuperscript{X} RBCs from donors with the putative genotype FY\textsuperscript{X}/FY\textsuperscript{X}.\textsuperscript{24, 25} Although it has been proposed that FY\textsubscript{4} should correspond to a quantitative rather than a qualitative variant of FY\textsubscript{a}, the molecular nature, the biological properties, and the genetic background of the FY\textsubscript{a} antigen and of the FY\textsuperscript{X} allele, constituted one of the dark holes in the study of the Duffy blood group system. To clarify these issues, we analyzed the structure and expression of the FY locus from 4 FY\textsuperscript{a-b\textsuperscript{weak}} donors. We identified a mutation that accounts for very low DAR expression and chemokine binding to RBCs and transfected eukaryotic cells. Our results, together with the previous characterization of FY\textsubscript{A}, FY\textsubscript{B}, and FY\textsubscript{A-Fy}, provide the definitive proof that the Duffy blood group system is controlled by four alleles.

**MATERIALS AND METHODS**

**Blood samples.** Blood samples from healthy donors were collected on EDTA and the Fy phenotypes were determined by agglutination studies using the antiglobulin gel test (Diamed SA, Morat, Switzerland) and by subsequent adsorption/elution studies, when necessary. The FY\textsuperscript{a-b\textsuperscript{weak}} donors BE.T (and family members) and donors SEV and BAR were obtained from the Etablissement de Transfusion Sanguine of the Centre National de Référence pour les Groupes Sanguins (CNRGS; Paris, France), respectively. The FY\textsuperscript{a-b\textsuperscript{weak}} blood sample TAR was collected at the Centre Regional de Transfusion Sanguine of Toulouse (Toulouse, France).

**Materials.** \textsuperscript{125}I-labeled IL-8, MGSA, and RANTES (specific activity, 2,200 Ci/mmol) were obtained from DuPont NEN (Boston, MA). Unlabeled and fluorescent fluorescein isothiocyanate (FITC)-conjugated recombinant IL-8\textsuperscript{\beta} were donated by Dr A. Proudfoot (Glaxo Wellcome Research and Development, Geneva, Switzerland). The anti-Fy\textsubscript{b} (i3A), anti-Fy\textsubscript{3} (CRC-512), and anti-Fya MoAbs were kindly provided by Dr D. Blanchard (CRTS, Nantes, France), Dr Makato Uchikawa (Japanese Red Cross, Tokyo, Japan), and Dr F. Buffiere (ETS, Bordeaux, France), respectively. Anti-Fy\textsuperscript{a} and anti-Fy\textsuperscript{b} human polyclonal antibodies were from Ortho Diagnostic Systems (Raritan, NJ). The anti-p55 rabbit polyclonal antibody, prepared by immunizing rabbit with synthetic peptide residues 28 to 47 of the human p55 protein,\textsuperscript{30} was donated by Dr P. Bailly (INTS, Paris, France).

**Polymerase chain reaction (PCR) amplification of FY gene and transcripts.** Total RNAs were extracted from 400 µL of whole peripheral blood by the miniscule acid-phenol-guainumidum method.\textsuperscript{31} The pellet was resuspended in 50 µL H\textsubscript{2}O, and 10 µL was used to produce first cDNA strands using the first-strand cDNA synthesis kit (Pharmacia, Uppsala, Sweden). One sixth of the cDNA products were enzymatically amplified between primers FY\textsubscript{100} (5'-GAACACAAACGTGCCATGGGGAATCTGGT\textsuperscript{G}-3') (sense, positions -15 to +15), and FY\textsubscript{99} (5'-GGGAGAGAGACCGGTGTTCTCAAGCT\textsuperscript{G3'} (antisense, positions +102 to +992). Genomic DNA (200 ng) isolated from whole blood was amplified between primers FY\textsubscript{94} (5'-AACAGC- GTCCCTAACCAG-3') (sense, positions -1253 to -126), and FY\textsubscript{99}. Thirty cycles of PCR were performed as follows: 1 minute at 94°C, 1 minute at 58°C, and 2 minutes at 72°C. PCR products were subcloned in plasmid vector and sequenced by the dye-sequencing chain termination method using an ALFExpress automatic DNA sequencing instrument (Pharmacia).

**Restriction analysis of PCR-amplified genomic sequences.** For the FY\textsuperscript{X} restriction fragment length polymorphism (RFLP) assay, the PCR reactions were performed with 200 ng of leukocyte DNA between primers FY\textsubscript{7}, 5'-ACTCTGGACTGCCCTTCCTTC-3' (sense, positions +176 to +195), and FY\textsubscript{57}, 5'-GGGCGAAGGCTGAGCCA-3' (antisense, positions +428 to +411), under the following conditions: 30 cycles of 40 seconds at 94°C, 1 minute at 58°C, and 45 seconds at 72°C. PCR products were purified on Ultrafree-MC (30,000) filter units (Millipore, Bedford, MA) and one third was digested for 2 hours with 20 U of the Acc I restriction enzyme. Restriction fragments were directly analyzed in 12% acrylamide minigels. FY\textsuperscript{A-FY\textsuperscript{B}} genotyping was performed as described,\textsuperscript{9} FY\textsuperscript{A-Fy\textsuperscript{b}} typing was performed essentially as described,\textsuperscript{26} except that the reverse primer P\textsubscript{39} was changed to FY\textsubscript{97}, 5'-TTTGCACAGATGTCCCATG-3' (position +40 to +27), to better separate the FY\textsuperscript{A-Fy\textsuperscript{b}} specific Sty I restriction fragment (64 bp) generated by the T-46C mutation from other fragments.

**Construction of FY expression plasmid and mutagenesis.** pcDNA-3 expression vector (Invitrogen BV, Leek, The Netherlands) carrying the coding sequence of the major isoform (spliced) of FY\textsubscript{a} was described elsewhere.\textsuperscript{32} Mutagenesis was subsequently performed on the recombinant plasmids by the use of PCR primers carrying appropriate nucleotide substitutions and the Quick Change Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Inserts of the mutated plasmids were sequenced as described above.

**Cell culture and transfection.** COS-7 cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in Iscove medium supplemented with 10% fetal calf serum and 50 µg/mL penicillin and streptomycin. Cells (3 × 10\textsuperscript{5}/assay) were transfected with 10 µg of recombinant plasmid using Lipofectin reagent (Life Technologies, SARNL, Cergy-Pontoise, France). Transiently transfected COS-7 cells were analyzed after 36 hours of culture.

**Flow cytometry analysis.** Expression of Duffy antigens on RBCs or transfected cell lines was measured on a FACSscan flow cytometer (Becton Dickinson, San Jose, CA) using anti-Fy\textsubscript{b}, anti-Fy\textsubscript{a}, and anti-Fy\textsubscript{3} MoAbs. Cells (3 × 10\textsuperscript{5}) were incubated for 60 minutes at 22°C with appropriate dilution of antibody in 0.15 mol/L phosphate-buffered saline (PBS). After washing with PBS supplemented with 0.5% bovine serum albumin (BSA), the cell suspension was incubated with fluorescein-conjugated antimiouse or antihuman IgG (H+L) (Immunotech, Marseille, France). After another washing step, 0.1 ng of propidium iodide (PI) was finally added to 1 mL of cell suspension. PI-positive cells (dead cells) were excluded from analysis. Fy\textsubscript{a-b} RBCs or mock-transfected cells and irrelevant mouse and human MoAbs were used as negative controls.

**Receptor binding assay.** RBCs (10\textsuperscript{7}) and COS-7 cell transfecants (10\textsuperscript{5}) were analyzed for their ability to bind \textsuperscript{125}I-chemokines as previously described.\textsuperscript{32} Cells transfected with the pcDNA3 vector alone were used as negative controls.

The binding of FITC-conjugated IL-8 to RBCs was directly measured by flow cytometry analysis.

**Protein chemistry.** RBCs membranes from FY\textsuperscript{b}-typed donors were prepared by hypotonic lysis.\textsuperscript{33} For Western blot analysis, RBC membrane proteins (50 µg) were separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)\textsuperscript{34} using a Novex apparatus (San Francisco, CA) and transferred to nitrocellulose sheets. The blot was incubated with an appropriate dilution of the i3A anti-Fy 6 MoAb and then with antimouse IgG peroxidase-tagged antibody (Biosis, Coupvray, France). Finally, the immunoblot was stained with the ECL chemiluminescent system from Amersham (Bucks, UK) and exposed to x-ray film.

**RESULTS**

**Duffy antigen expression on FY\textsuperscript{a-b\textsuperscript{weak}} RBCs.** In agglutination studies with polyclonal anti-Fy\textsubscript{a} reagents, RBCs from donors BAR and SEV were unreactive with the anti-Fy\textsubscript{a} antibodies and gave positive reactions with some anti-Fy\textsubscript{b} antisera, but with an agglutination titer fivefold lower than those
given by Fy(a−b+) and Fy(a+b+) controls. RBCs from donors TAR and BE.T were unreactive with all anti-Fya and anti-Fyb reagents and thus were initially considered as Fy(a−b−) (data not shown). However, in subsequent adsorption-elution experiments, these two RBC samples yielded an antibody with Fyb specificity. From these analysis, donors BAR, SEV, TAR, and BE.T were phenotyped Fy(a−b+). The altered expression of Fyb on these RBC samples was better shown by flow cytometry analysis (Table 1). When compared with the Fy(a−b+), Fy(a+b+), Fy(a+b−), and Fy(a−b−) controls, a faint fluorescence signal was detected on the Fybweak RBCs, ranging from 3% to 7.7% of that obtained with Fy(a−b+) (FY*B/*B) control RBCs. Similarly, the use of anti-Fyb and anti-Fy6 monoclonal antibodies (MoAbs) also showed a drastic alteration of Fy3 and Fy6 antigen expression on the Fybweak RBCs. The decreased anti-Fy6 binding capacity were strictly correlated between the FY*X genotype and the antibody binding capacity of Fyb antigens. From these analysis, donors BAR, SEV, TAR, and BE.T samples being 50% of that in BAR membranes (Fig 2B). A signal was detected in membranes from Fyb− controls. DARC was not detected in these membranes had a barely detectable signal, equivalent to less than 5% of the normal intensity, with the signal in SEV and BE.T samples being 50% of that in BAR membranes (Fig 2B).

## Table 1. Serological Characteristics of the Duffy Samples as Determined by Flow Cytometry Analysis

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Fy+a</th>
<th>Fy+b</th>
<th>Fy3</th>
<th>Fy6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Samples</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAR</td>
<td>0</td>
<td>140</td>
<td>470</td>
<td>250</td>
</tr>
<tr>
<td>SEV</td>
<td>0</td>
<td>33</td>
<td>220</td>
<td>150</td>
</tr>
<tr>
<td>TAR</td>
<td>0</td>
<td>80</td>
<td>230</td>
<td>150</td>
</tr>
<tr>
<td>BE.T</td>
<td>0</td>
<td>50</td>
<td>275</td>
<td>190</td>
</tr>
<tr>
<td>BE.L</td>
<td>1600</td>
<td>0</td>
<td>2300</td>
<td>1560</td>
</tr>
<tr>
<td>BE.C</td>
<td>1800</td>
<td>110</td>
<td>2950</td>
<td>1990</td>
</tr>
</tbody>
</table>

*FITC-equivalent/cell.
†Site number/cell.
As a control, all samples exhibited normal amounts of the p55 peripheral protein (Fig 2C).

FY gene and transcript analysis. RNAs extracted from whole blood of the 4 Fy(a−bweak) donors and from a Fy(a−b+) control were reverse transcribed to cDNA and used as template to amplify the entire coding region for the major (spliced) isoform of DARC between primers FY100 and FY99. A PCR product of the expected size (1,036 bp) was obtained in all samples (data not shown) and subcloned in plasmid vector. Sequence analysis of several clones from each Fy(a−bweak) sample showed one kind of cDNA that differed from the common FY*B allele by only one substitution, C286T (taken as the erythroid cap site 21; Fig 3). A survey of the literature indicated that T286 has never been found in the FY*A, FY*B, or FY*Fy alleles of donors with common Duffy phenotypes. The C286T nucleotide change resulted in the amino-acid substitution Arg to Cys at position 89 of the DARC polypeptide. It is noteworthy that all Fybweak cDNA clones carried a G at nucleotide 319, whereas G or A, resulting in Ala100Thr substitution, was identified at this position by sequencing the FY*B or FY*A alleles from Fy(a−b+) or Fy(a+b+) donors7,20 (and our unpublished data).

The C286T nucleotide substitution was confirmed at the genomic level after amplification of the FY gene, including intron 1 and 1,253 nucleotides in 5’ of the erythroid cap site. However, in contrast to the cDNA analysis, sequence analysis of several genomic clones suggested homozygosity for donor BAR and showed the heterozygosity of donors SEV, BE.T, and TAR. One species of clones from SEV, BE.T, and TAR samples carried a normal Fyb coding sequence and exhibited the T-46C mutation in the promoter region typical of FY*Fy and was previously shown to abolish the erythroid expression of this bone marrow silent FY*B allele.21 Accordingly, this allele could not be shown by cDNA analysis of erythroid cells. Conversely, the second species of clones exhibited the C286T substitution but no additional polymorphism compared with the noncoding

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Table 2. Chemokine Binding to RBCs

<table>
<thead>
<tr>
<th>Samples</th>
<th>IL-8</th>
<th>MGSA</th>
<th>RANTES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>BAR</td>
<td>23.5</td>
<td>5.7</td>
<td>176</td>
</tr>
<tr>
<td>SEV</td>
<td>16</td>
<td>5</td>
<td>NT</td>
</tr>
<tr>
<td>Controls (n = 3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fy a−b+</td>
<td>84-86</td>
<td>11-12</td>
<td>380-390</td>
</tr>
<tr>
<td>Fy a−b−</td>
<td>4-4.5</td>
<td>3.6-3.7</td>
<td>3.5-3.8</td>
</tr>
</tbody>
</table>

(A) 10^8 RBCs were incubated with 0.5 nmol/L 125I-labeled chemokine. Results are expressed as bound cpm (×10^3). (B) Same as (A), but 200 nmol/L of cold ligand was added.

Abbreviation: NT, not tested.

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Fig 2. Immunoblot analysis of RBC membrane proteins. Total membrane proteins (60 µg) separated on SDS-PAGE were blotted on nitrocellulose sheets and incubated with the murine anti-Fy6 MoAb i3A and with a rabbit anti-p55 antibody. The DARC glycopolypeptides and p55 proteins were visualized by chemiluminescence autoradiography using goat antimouse or goat antirabbit IgG conjugated to horseradish peroxidase, as second antibodies, respectively. The 35- to 45-kD DARC glycopolypeptide forms aggregates migrating as bands of 90 and 200 kD.22
**STRUCTURE AND EXPRESSION OF THE FY*X ALLELE**

Fig 3. Schematic comparative structure of the DARC cDNAs isolated from whole blood of Fy(a-b+) and Fy(a-bweak) donors. The full-length major isoform (spliced) of the FY transcripts was isolated by RT-PCR. The nucleotide sequences of Fy(a-b+) and Fy(a-bweak) cDNA clones were identical, except for the C286T substitution (+1 taken as the erythroid cap site) resulting in the Arg89Cys polymorphism on the FY transcript. G was found at nucleotide position 319 in all clones from four unrelated Fy(a-bweak) donors, whereas G or A, resulting in Ala100Thr amino acid change, could be found in the FY*B allele from Fy(a-b+) and Fy(a-b+) donors. The nucleotide residue found at the Fy/Fy-associated polymorphic position (G125A) is indicated.

**DISCUSSION**

The Fy\(^s\) antigen. Whereas the previous analyses of the Fy\(^s\) phenotype were based on barely quantitative agglutination studies, we have performed flow cytometry and chemokine binding analysis to accurately estimate the variation of Fy antigen expression on RBCs from different phenotypes. Using HD50 agglutination assay and human polyclonal antisera, Habibi et al\(^{27}\) previously found a full correlation between Fy\(^b\) and Fy3 depression on Fy\(^b\) RBCs. We confirmed these results with the four Fy(a-bweak) samples, which indicated that these cells exhibited a typical Fy\(^b\) phenotype, as previously defined.\(^{23,27}\) Fy5 expression could not be analyzed because anti-Fy5 was not available. However, we demonstrated that there is also a full correlation between Fy3 and Fy6 expression on RBCs of different phenotypes, including Fy\(^c\) cells (\(R = .98\), data not shown). Furthermore, by the use of murine MoAbs that allow antigen site density to be precisely estimated, we calculated that, as compared with Fy\(^*\)B/B control cells, Fy\(^*\)X/X RBCs expressed only 12% of Fy3 and Fy6 antigens. The decrease in Fy\(^b\) expression, as measured with human anti-Fy\(^b\) antisera, was slightly higher, but the polyclonal nature of the reagent did not allow an accurate calculation of Fy\(^b\) site numbers.

Apart from its biological relevance (see below), chemokine binding to RBCs also represents a powerful approach in the comparison of DARC expression on RBCs with different phenotypes. It has been previously shown that chemokines from both C-C and C-X-C classes bind to RBCs from the three common Fy-positive phenotypes, but not to Fy-negative RBCs.\(^{7}\) We show here that these chemokines bind also to Fy\(^s\) RBCs, but that the Fy\(^*\)X/X RBCs could only bind 20% to 30% of the amount of chemokines that bind to Fy\(^*\)B/B RBCs. It is noteworthy that both flow cytometry and chemokine binding analysis allowed us to discriminate between Fy\(^*\)X/X and Fy\(^*\)Fy/x RBCs and that cells mistyped as Fy\(^s\) could be clearly reclassified as Fy-positive.

Recent structure-function analysis of DARC showed that the binding of the various ligands used in this study involved different domains of DARC. The Fy6 epitope has been precisely mapped by synthetic peptide/pins technology and mutagenesis analysis to the heptapeptide comprising residues Q19-25 of the...
The epitopes recognized by anti-Fy* and anti-Fy3 have not been fully characterized. However, the Fy* epitope is thought to encompass the polymorphic G42D position in the NH-2 terminal region associated with the Fy a/Fy b antigenic polymorphism and analysis of chimeric receptors indicated that the third extracellular loop of DARC is necessary for the binding of anti-Fy3. We have recently demonstrated that the Fy6, Fy*a, and Fy3 epitopes are all involved in the binding of chemokines and that the close association of the first and fourth extracellular domains of DARC by a disulfide bond is required for ligand binding, because it may create a chemokine binding pocket. Thus, the ability of Fy* RBCs to bind all the anti-Fy antibodies and chemokines tested indicated that the overall structure of the DARC polypeptide expressed at the membrane of these cells is most likely not altered. Conversely, the parallel decrease of the binding capacity for all ligands together with the highly reduced amount of DARC polypeptide detected in Fy* RBC membrane
by Western blot analysis strongly suggested that the Fya antigen represents a poorly expressed but grossly normal Fyb antigen.

The FY*X allele. Not only has the structure of the FY*X allele not been previously characterized, but whether “Fy” weak reactions lies with FyA being the tail-end expression of Fyb or with it being the expression of a fourth allele, “FyX” also remained an unresolved matter of controversy. Sequence analysis of the FY transcripts showed that the 4 Fy(a−b<sup>weak</sup>) donors investigated in this study carry the same allele, the coding sequence of which differs from that of a normal FY*B allele by a single substitution, C286T. Further sequence analysis of the FY gene did not show mutation in the intronic sequence or in the promoter region that could account for transcriptional or posttranscriptional alteration of FY expression. Conversely, the C286T mutation is most likely causative of low expression of DARC in Fy(a−b<sup>weak</sup>) erythroid cells, because expression of the FY gene linked to an heterologous promoter demonstrated that the construct with the Fyb<sup>weak</sup> sequence was expressed about twofold to threefold less in COS-7 cells compared with the Fyb<sup>+</sup> construct.

These results provide the definitive proof that the Fyb<sup>weak</sup> phenotype is conveyed by a fourth allele at the FY locus, FY*X (GenBank accession no. AF055992), which is as distinct from the FY*B allele as is the FY*A allele in Africans and Afro-Americans. However, the present characterization of FY*X, together with that of FY*A, FY*B<sup>9,18−20</sup> and FY*B<sup>21,22</sup> and Caucasians<sup>20</sup> does not complete the elucidation of the molecular genetic basis of the Duffy blood group system. Indeed, the lack of mutation in the sequence encoding for the minor, unspliced, isoformal DARC in 2 unrelated Fyb<sup>weak</sup> donors investigated by Mallinson et al<sup>20</sup> suggested that the Fyb<sup>weak</sup> phenotype might arise from at least two different genetic mechanisms. However, the sequences of the promoter, exon 1, and intron 1 of FY was not elucidated when these investigators performed their study. Hence, it remains to be determined whether mutation in the 5′ part of FY accounts for quantitative or qualitative alteration of Fyb expression in the 2 Fyb<sup>weak</sup> donors studied. A further complexity of the Duffy blood group system was recently suggested by Shimizu et al<sup>18</sup> in the course of a serotyping study of 434 individuals from several Thai ethnic groups. The investigators emphasized the presence in 8 individuals of a weak-Fya antigen that has never been described before. It is anticipated that these studies could lead to the characterization of a fifth allele at the FY locus, FY*A<sup>weak</sup>, which should be to FY*A what FY*X is to FY*B.

FY*X DNA typing in clinical and transfusion medicine. A FY*X DNA typing based on the C286T substitution has been developed that, together with the previously published FY*A, FY*B, and FY*Fy genotyping, can discriminate between alleles associated with normal (FY*B), bone marrow silent (FY*Fy), and weak (FY*X) expression of the Fyb antigen and made a full FY genotyping of a large series of donors easy to perform. Because FY*B/FyB and FY*B/Fy*X RBCs are generally indistinguishable and because Fya is often undetected in FY*A/FyX and FY*B/Fy*X RBCs by standard serological methods, the frequency of FY*X will certainly appear to be much higher than previously reported (0.015)<sup>39</sup> when population studies are performed using DNA typing. Interestingly, by using the FY*B genotyping test, Murphy et al<sup>40</sup> have already reported that weakened expression of the Fyb antigen is responsible for 12% of discrepancies between the genotypically (FY*A/B) and serologically [Fya(a−b−)] determined Fyb status of 109 Caucasian donors. It is assumed that complete FY genotyping could show the presence of FY*X in most of these discrepancies.

In the present study, FY genotyping was used to demonstrate that the inheritance of the silent FY*X allele accounts for apparent paternity exclusion in family BE. Similarly, FY*X DNA typing will be useful to investigate apparent anomalous inheritance within the Duffy system in Caucasian families, which in most cases is due to weakened expression of Fyb.<sup>23</sup>

Anti-Fya and, to a lesser extent, anti-Fyb antibodies can cause HDN, with some of them being fatal,<sup>1</sup> and a recent study has pointed out the clinical value of antenatal Fya/Fyb genotyping in pregnancies at risk of HDN due to anti-Fya.<sup>41</sup> It is expected that the Fya genotyping test will also prove to be useful in the management of pregnancies at risk of Fyb hemolytic disease by discriminating between fetuses with normal or weakened expression of the Fyb antigen when the mother exhibits high titer of anti-Fyb<sup>b</sup>.

Even though no transfusion reaction related symptoms have yet been observed when blood units with weakened expression of Fyb were transfused to Fyb<sup>-</sup>-negative patients, FY DNA typing would have important implications with respect to the good practice of blood transfusion.<sup>40</sup> In that respect, the newly
described Fy\(^{a}\) DNA test should be performed to ascertain that Fy\(^{b}\)-negative patients, with a high titer of anti-Fy\(^{b}\), will be transfused by true Fy\(^{b}\)-negative blood units and not by Fy\(^{b}\)weak samples serologically mistyped as Fy\(^{b}\)-negative. Secondly, it is important that RBCs serving as reference for blood bankers are better characterized, both at the phenotypic and genetic levels.

**Genotype-phenotype relationship.** The C286T substitution results in an Arg89Cys amino acid change that is predicted to occur in the first cytoplasmic loop of the DARC polypeptide, according to the current seven transmembrane domain topological model.\(^7\) Thus, the elucidation of the Fy\(^{a}\)-associated substitution represents the first study that highlights the critical role of intracellularly exposed residues in the expression of DARC. The cytoplasmic localization of the Fy\(^{a}\)-specific amino acid (Cys89) fits well with the fact that antibodies specific to Fy\(^{a}\) RBCs have never been characterized.

Because our data suggest that the overall structure of the membrane-expressed Fy\(^{a}\) polypeptide is similar to that of the other allelic forms of DARC (see above), we assume that the presence of a Cysteine residue at position 89 of the Fy\(^{a}\) polypeptide is not associated with an additional disulfide bond that would significantly modify the tertiary structure of DARC.

In agreement with the positive inside rule,\(^42\) the three short intracellular loops connecting the seven hydrophobic transmembrane helix of DARC contain several positively charged residues (Arg, Lys) that might be in the vicinity of the polar groups of the phospholipid molecules along the cytoplasmic side of the lipid bilayer. It has been experimentally demonstrated that basic amino acids, through their interaction with negatively charged phospholipids, are critical in the blocking of the translocation of loops across the membrane and thereby should contribute to the control of membrane topology (van Klompenburg et al\(^43\) and references herein). Accordingly, we suggest that, by modifying the positive charge of the first intracellular loop, the lack of Arg89, a residue conserved (or replaced by the homologous charged residue Histidine) in the DARC related polypeptides from 11 nonhuman primate species, mouse, and cow\(^{18,44}\) (and our unpublished results), might result in an insufficient insertion of the Fy\(^{a}\) polypeptide in the cell membrane and thereby account for the very low cell surface expression of DARC in Fy\(^{a}\) cells.

**The Fy\(^{a}\) phenotype and the elusive DARC function.** The precise function of DARC in immunobiology and neurobiology remains uncertain. Because a murine DARC-like protein with conserved chemokine binding properties has been recently characterized,\(^44\) it is expected that DARC-deficient mice will be obtained and will help in the understanding of the biological role of DARC. Another approach to determine how much DARC is important in normal and pathological human physiology is to look for a potential correlation between the clinical status and the Duffy phenotypes of a large serie of donors. Hence, the fact that a large majority of blacks do not express DARC on their RBCs without apparent clinical consequence strongly suggested that DARC on RBCs is dispensable.\(^5,45\) On the other hand, the demonstration that, in these Fy\(^{a}(a-b-\)) individuals, the downregulation of DARC was restricted to erythroid cells\(^{6,11,21}\) and thus should represent an adaptative response to resist malaria, reinforced the hypothesis of an important role of DARC in nonerythroid tissues. Conversely, this suggestion became more difficult to support when it was demonstrated that at least 3 apparently healthy Fy\(^{a}(a-b-\)) Caucasians carry genomic mutations that should results in the lack of DARC in all cells and tissues.\(^46\) However, significant, albeit reduced expression of Fy\(^{a}\) and Fy6 antigens (~40% as compared with control) were achieved when one of these defective genes that carry a 14-bp deletion resulting in a premature stop signal at codon 118 was transfected in COS-7 cells, but, as expected from structure function/analysis (see above), Fy3 was not expressed and chemokine did not bind to this protein lacking extracellular loops 2 and 3 (our unpublished results). Taken together, these results indicated that, even though a truncated DARC-related protein might be expressed in nonerythroid cells of these rare Caucasian Fy\(^{a}(a-b-\)) donors, these donors failed to express a functional chemokine receptor in all their tissues without detectable adverse consequences.

Importantly, one finding from the present study, as substantiated by transfectant analysis, is that the substitution identified in the Fy\(^{a}\)X allele is also predicted to alter DARC expression in all tissues without deleterious consequences for normal physiology. If DARC is involved in transduction of a signal across the membrane upon chemokine binding, by a still undetermined pathway, it is not unlikely that its residual level in homozygous Fy\(^{a}\) cells could be sufficient to support normal function. However, it has thus been demonstrated that mutants of the IL-8 RA receptor that poorly bind IL-8 could mediate signal transduction in a way similar to how wild-type receptor does.\(^46\)

In conclusion, the elucidation of the molecular basis of the Fy\(^{a}\) phenotype adds one more degree of complexity to the genetics of the Duffy blood group system, but again raises questions about the importance of DARC in erythroid and nonerythroid tissues. Indeed, if one tries to link up the genetics with biology, it must be postulated that, whatever the precise function of DARC, other structures might operate when it is poorly expressed or absent. Similar compensatory mechanisms have been postulated to account for the observation that inactivation of the water channel AQP-1 gene in individuals with the Colton nul [Co\(^{a}(a-b-\)] blood group phenotype is not associated with any apparent clinical consequence.\(^47\) Obviously, the challenge now is to develop direct experimental approaches to elucidate the biological role of DARC.

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