Expression of Duffy antigen receptor for chemokines during reticulocyte maturation: using a CD71 flow cytometric technique to identify reticulocytes

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Flow cytometric methods commonly used to identify reticulocytes are of limited usefulness in malarious areas, since RNA staining also detects plasmodia. An important antigen expressed on reticulocytes is Duffy antigen receptor for chemokines (DARC, also known as Fy), the receptor for *Plasmodium vivax.* An early marker for reticulocytes is CD71 (transferrin receptor). We have been interested in CD71 as an alternative marker for reticulocytes in the context of Fy expression. Flow cytometry was used to determine the expression of Fy on CD71-positive and -negative reticulocytes and to correlate serology and genotype. A reduction of 13 percent was seen in Fy6 expression between CD71-positive reticulocytes and RNA-positive reticulocytes. CD71 disappears early during reticulocyte maturation, while Fy6 expression is relatively preserved. CD71 is an alternative to staining for RNA for reticulocyte assays relating to Fy6 expression. ***Immunohematology* 2005;21:15–20.**

Key Words: DARC, Fy, reticulocyte, CD71, transferrin receptor, ORF, flow cytometry

With the discovery of the Duffy system, it became apparent there are two codominant alleles, *Fya* and *Fyb,* that correspond to the two codominant antigens, Fy^a and Fy^b, respectively.[1] However, in many of those of African descent, including African Americans, neither antigen is expressed.[2] These individuals have been described as Duffy negative, with the allelic designation *Fy.* Subsequently, it has been shown that this phenotype, which expresses neither Duffy antigen receptor for chemokines (DARC) mRNA nor protein, is caused by a nucleotide polymorphism in the GATA-1 binding site of the promoter regions: a single T to C substitution at nucleotide -46.[3] This change in the promoter region prevents downstream transcription of the open reading frame (ORF). It is noteworthy, though, that DARC is expressed on other tissues, including brain, kidney, and endothelial cells of post-capillary venules, even in those with the Duffy-negative phenotype.[4] That is, in the Duffy-negative phenotype, Fy is silent only in the erythroid lineage.

Other Duffy-related epitopes have been discovered serologically. DARC follows the general structure of chemokine receptors, having seven transmembrane-spanning helices. Anti-Fy3 antibody attaches to the third extracellular loop in a pocket where chemokines themselves attach to their receptor.[5] It was initially described as a human antibody but now a mouse monoclonal is available. Anti-Fy4 and anti-Fy5 are rare and clinically unimportant human antibodies.[1] Anti-Fy6 is a mouse monoclonal antibody that attaches to the first extracellular domain of DARC, in an area thought to correspond to the binding site of *Plasmodium vivax* merozoites.[6,7]

The functional role of Fy on RBCs is not known, but it has been speculated that it acts as a "sink" for chemokines, removing them from the circulation.[8] The absence of Fy antigens or proteins on the RBCs of Africans, resulting in the serologic Fy(a–b–) phenotype, however, has no discernible ill effect. Similarly, no ill effect has been seen in other rare examples, of Caucasians with mutations leading to lack of functional Fy expression on all tissues.[9] Whether consequent elevated chemokine levels in these individuals might have a protective effect against malaria or other infectious diseases is a question for future investigation. There is certainly evidence that chemokine levels may vary according to Fy phenotype, as do outcomes in some disease models.[10,11]

P. vivax is known to preferentially invade reticulocytes. It is proposed that this is due to the presence of a second receptor on the surface of reticulocytes[12] that is perhaps not present on more mature RBCs. However, no second receptor has yet been identified. Recently we developed a flow cytometric assay to measure the relative amount of Fy6 on reticulocytes and demonstrated an increased expression of Fy6 on these cells compared to mature RBCs.[13] Expression of the Fy6 epitope was higher (49 ± 19%) on reticulocytes than on mature RBCs, regardless of donor genotype ($p < 0.0001$).[13] We have speculated that this may be due to relative differences in size, membrane structure, RNA activity, or some combination of these.

The transferrin receptor (CD71) is a transmembrane glycoprotein involved in iron metabolism, specifically the cellular uptake of transferrin, during erythrocyte ontogeny. Its persistent presence, after release from the bone marrow, defines an early population of reticulocytes.[14] Therefore, the relative expression of Fy6 on CD71-positive and -negative reticulocytes may define the extent and timing of loss of Fy6 from maturing reticulocytes, all of which stain with thiazole orange (TO), the stain commonly used to identify reticulocytes in flow cytometric assays. Because TO also stains the RNA of any plasmodia present, we wished to evaluate the use of CD71 as an alternative marker for reticulocytes in this assay. Used in this way, it may be an acceptable alternative labeling method for samples from areas where malaria is endemic. The main objective of this study was to develop such a method and specifically look at the profile of DARC expression on reticulocytes less mature than those that do not express CD71, to see if that pattern was consistent with DARC's role as a "sink" for chemokines in the circulation, as theorized, or with its having an important role in RBC ontogeny in the bone marrow.

Materials and Methods

Samples

Samples were obtained from bag segments prepared from units of whole blood donated at the American Red Cross Blood Services, Northern Ohio Region. Racial designation was by donor self-identification.

Methods

RBC phenotyping for Fy^a and Fy^b was performed using standard blood bank reagents and methods. DNA was isolated from samples using the QIA blood kit (Qiagen, Santa Clarita, CA). Genotyping of the FY promoter and open reading frame polymorphisms was performed by PCR-RFLP strategies.[13]

Flow cytometry studies were done using an anti-Fy6 antibody (NYBC-BG6), kindly provided by John Barnwell, Centers for Disease Control and Prevention, Atlanta, Georgia. This antibody binds to the extracellular portion of the Fy glycoprotein involved in interaction of the reticulocyte surface with the *P. vivax* merozoite ligand.[7] This antibody was conjugated directly to a phycoerythrin (PE) label (ProZyme, San Leandro, CA) according to the manufacturer's instructions. Prior experiments were done to determine the optimum concentration and incubation time for labeling erythrocytes. A 5 µL aliquot of blood was washed three times in 50 µL of 2% BSA/PBS with 0.1% sodium azide. The RBCs were then incubated with 50 µL of a 1:50 dilution of PE-labeled anti-Fy6 for 15 minutes at 37°C. Cells were again washed twice with 2% BSA/PBS with 0.1% sodium azide. A second mouse antibody against human CD71 (Caltag, Burlingame, CA) labeled with TC (Tricolor) was added at a dilution of 1:50 and incubated for 15 minutes. The RBCs were then washed twice in PBS and resuspended in TO solution (Becton Dickinson, San Jose, CA) for 30 minutes to stain reticulocytes, according to the manufacturer's specifications. Flow cytometry readings were performed using a Coulter Elite instrument (Coulter Corp., Miami, FL) equipped with a 488-nm air-cooled argon ion laser at 15 mW. TO, PE, and TC fluorescence was collected with band pass filters at 525 nm, 575 nm, and 675 nm, respectively. Compensation was applied at the level of the hardware after the appropriate initial experiments, i.e., after controls were run, negative fluorescent signals were adjusted until orthogonal. Forward scatter, side scatter, and fluorescence data were analyzed. A count of 5000 TO-positive cells was taken per sample. This was approximately 1 to 2 percent of the total number of cells assayed for each individual. Experiments were standardized using Quantum Simply Cellular Beads (Flow Cytometry Standards Corp., San Juan, PR) and Immunobrite beads (Coulter) according to the manufacturers' instructions. Analysis was undertaken using Win MD 2.8 software (Dr. Joe Trotter, BD Biosciences, accessed at http://facs.scripps.edu/software.html), using the quadrant tool to gate upon the populations of interest. Care was taken in the use of the quadrant tool to reduce background.

Correlation coefficients for mean fluorescence were calculated using Pearson's bivariate analysis, and statistical analysis was undertaken with the SPSS 10 software package (SPSS Inc., Chicago, IL).

Results

Genotyping was performed on samples from 33 donors. Samples were classified according to promoter and ORF polymorphisms. Twenty were homozygous for the wild-type promoter, six were heterozygous, and seven were homozygous for the promoter polymorphism. In the group homozygous for the wild-type promoter, ten were *FY*A/FY*A* for the ORF, one was *FY*A/FY*B,* and nine were *FY*B/FY*B.* All but three of these donors identified themselves as white; the others self-identified as African American. Among the six persons who were heterozygous for the promoter polymorphism, two were *FY*A/FY*B* for the ORF and four were *FY*B/FY*B.* Five of these donors were self-identified as African American and one as white. As expected, all seven who were homozygous for the promoter polymorphism were *FY*B/FY*B.* These individuals were all self-identified as African American. Serologic findings were consistent with genotyping in these donors.

Characteristic flow cytometric dot plots obtained for RBCs from individuals with each of the three different promoter genotypes are shown in Figure 1. The X axis demonstrates the amount of PE fluorescence (Fy6) and the Y axis the reticulocyte markers as measured by TO fluorescence (RNA) or TC fluorescence (CD71). Graphs were prepared with Win 2.8 MD software. Donor A is homozygous for the wild-type promoter, donor B is heterozygous, and donor C is homozygous for the GATA-1 promoter polymorphism (i.e., having no Fy expression on RBCs). In the first column, TO expression is a marker for reticulocytes. In the second column, TC is used as the marker for the presence of CD71. PE fluorescence represents Fy6 expression. A vertical line has been drawn to delineate the main population of cells. Values from the upper left quadrant of each panel therefore represent reticulocytes, and values from the lower left quadrant represent mature RBCs.

The flow cytometry and genotyping results for all 33 subjects are summarized in Table 1 (mature RBCs) and Table 2 (reticulocytes). They show a high degree of correlation between the two methods of measuring Fy expression. Table 3 shows the percentage of each population labeled with TC (CD71-positive) and TO (RNA-positive). The mean reduction in Fy expression between early, CD71-positive reticulocytes and the population of reticulocytes as a whole was only 13 percent. Therefore, there is a strong degree of correlation between the results of the two flow cytometric assays for Fy6 expression on reticulocytes.

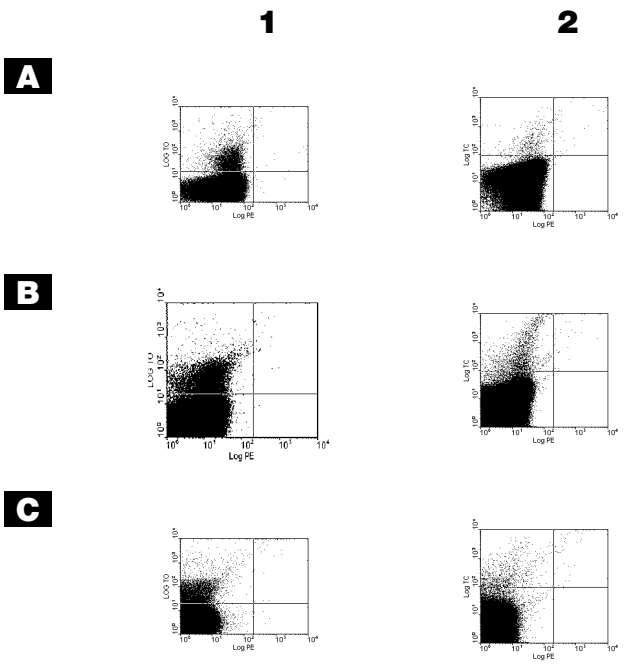

Fig. 1. Representative flow cytometric curves for RBCs from donors with and without Fy expression on RBCs. The panels in row **(A)** represent a serologically Duffy-positive individual who is homozygous for the wild-type promoter. The panels in row **(B)** represent a donor heterozygous for that polymorphism; those in row **(C)** show a serologically negative individual who is homozygous for the promoter polymorphism. Panels in the first column show PE fluorescence, which represents the Fy6 epitope versus TO. Panels in the second column show PE versus TC, which represents CD71, or the transferrin receptor, for the same individuals. The mean fluorescent intensity (MFI) of the left upper quadrant has been used as representative of double-labeled cells to reduce background. These MFI values for the top panel are 32.7 and 30.7 (TO/PE and TC/PE respectively), for the middle 10.0 and 14.0, and for the bottom 1.9 and 3.6, respectively.

Discussion

The key finding from this study was that DARC expression is relatively preserved during reticulocyte maturation. Comparing immature reticulocytes and the total reticulocyte population (at least four-fifths of which are CD71 negative), Fy6 expression is relatively stable in the life of the reticulocyte, falling by a mean of 13 percent as CD71 is lost. This is in keeping with previously published data showing the antigen appears late in the bone marrow during ontogeny of RBCs.[15]

Table 1. Level of Fy6 expression on mature RBCs according to Fy promoter and ORF genotype as measured by MFI

Promoter genotype	ORF genotype	Number	Mature RBCs RNA (TO) negative MFI ± SD	Mature RBCs CD71 (TC) negative MFI ± SD	Correlation coefficient (Pearson's bivariate)
Homozygous wild-type promoter	*FY*A/FY*A*	10	14.55 ± 4.95	14.66 ± 4.99	
	*FY*A/FY*B*	1	6.2	6.2	
	*FY*B/FY*B*	9	10.38 ± 2.57	10.44 ± 2.58	
	All	20	12.26 ± 4.56	12.34 ± 4.60	1.00
Heterozygous wild-type promoter	*FY*A/FY*A*	0	–	–	
	*FY*A/FY*B*	4	5.63 ± 0.98	5.73 ± 0.98	
	*FY*B/FY*B*	2	3.75 ± 0.35	3.75 ± 0.35	
	All	6	5.12 ± 1.20	5.07 ± 1.28	1.00
Total Fy positive		26	10.5 ± 5.08	10.66 ± 5.11	1.00
Homozygous promoter polymorphism (Fy negative)	*FY*B/FY*B*	7	1.57 ± 0.10	1.57 ± 0.10	1.00

Table 2. Level of Fy6 expression on reticulocytes according to Fy promoter and ORF genotype as measured by MFI

Promoter genotype	ORF genotype	Number	Reticulocytes RNA (TO) positive MFI ± SD	Reticulocytes CD71 (TC) positive MFI ± SD	Correlation coefficient TO+ versus TC+ populations
Homozygous wild-type promoter	*FY*A/FY*A*	10	24.25 ± 8.30	24.37 ± 11.82	
	*FY*A/FY*B*	1	9.9	15.2	
	*FY*B/FY*B*	9	19.42 ± 3.33	24.25 ± 8.30	
	All	20	21.36 ± 7.10	23.91 ± 9.13	0.771
Heterozygous wild-type promoter	*FY*A/FY*A*	0	–	–	
	*FY*A/FY*B*	4	10.63 ± 1.04	13.53 ± 2.35	
	*FY*B/FY*B*	2	7.9 ± 0.00	12.35 ± 0.49	
	All	6	9.72 ± 1.62	13.13 ± 1.93	0.469
Total Fy positive		26	18.67 ± 7.99	21.42 ± 9.25	0.831
Homozygous promoter polymorphism (Fy negative)	*FY*B/FY*B*	7	2.04 ± 0.26	4.2 ± 1.2	

Table 3. Percentage of reticulocytes (TO+) staining positive for CD71 (TC+) by genotype

Promoter genotype	ORF genotype	Number	Race		Percentage of reticulocytes TO+ also CD71 (TC) +	Serology
			White	African American		
Homozygous wild-type promoter	*FY*A/FY*A*	10	10	0	16 ± 7	10 Fy(a+b–)
	*FY*A/FY*B*	1	1	0	19	1 Fy(a+b+)
	*FY*B/FY*B*	9	6	3	13 ± 3	9 Fy(a–b+)
	All	20	17	3	15 ± 5	
Heterozygous wild-type promoter	*FY*A/FY*A*	0	0	0	–	
	*FY*A/FY*B*	4	1	3	15 ± 6	4 Fy(a+b–)
	*FY*B/FY*B*	2	0	2	23 ± 10	2 Fy(a–b+)
	All	6	1	5	18 ± 8	
Total Fy positive		26			16 ± 7	
Homozygous promoter polymorphism (Fy negative)	*FY*B/FY*B*	7		7	18 ± 8	7 Fy(a–b–)

Note: No significant differences are noted.

Assuming a constant rate of reticulocyte release into the circulation and subsequent maturation to RBCs, these results also demonstrate the relatively early disappearance of CD71 from reticulocytes. The loss of CD71 is consistent with previous studies. Although the mean percentage of cells expressing CD71 (15% ± 5%) in the present study is lower than has been reported in some previous studies using similar methods on homozygous wild-type promoter populations,[14] it is consistent with the findings of others. For instance, in a flow cytometry–based study that did not examine Fy expression, Dertinger et al.[16] found it was the youngest 10 to 20 percent of reticulocytes that label with anti-CD71. Thus very large numbers of cells must be

analyzed to obtain a sufficiently large sample of CD71-bearing cells for statistical analysis of their antigen expression.

The persistence of relatively constant quantities of Fy on the RBC surface supports the prevailing theory that DARC's major role is as a "sink" for circulating chemokines. On the other hand, a large difference between the two sets of reticulocytes may have implied a role for Fy6 in RBC development.

Our findings that, first, there is a gene dosage effect associated with the promoter polymorphism and, second, the Fy^b polymorphism in the ORF is independently associated with lower Fy expression, confirmed results previously reported.[13] An incidental finding is the apparent higher expression of DARC on the cells of persons with the Fy(a+b–) phenotype as compared to the Fy(a–b+) phenotype, a finding consistent with previous studies using the same antibody.[13] Whether this is due to a true difference in Fy expression or a difference in antibody affinity is unclear.

This study demonstrates the ease of using three-color flow cytometry to examine RBC antigen expression during maturation. Similar techniques may be used to define subpopulations of maturing reticulocytes in individual patients and to distinguish between mixtures of cell populations, for example, identifying donor or patient reticulocytes in the posttransfusion setting.[17] This technique may be useful when the putative reticulocyte-specific receptor for *P. vivax* malaria is identified and to measure the decline of other RBC surface markers in the circulation. Clearly this technique is unlikely to be of use in African populations who have no Fy expression on RBCs. However, the global burden of malaria includes 70 to 80 million cases annually, about 10 to 20 percent of which occur in Africa. The rest occur predominantly in South America, the Southwest Pacific, and Asia,[18] so there are large populations potentially suitable for study of this technique. In addition, it seems unlikely that in vitro, and perhaps in vivo, RBC exposure to malaria alters DARC antigen expression on RBCs.[19] Consequently, we would not likely see artifactual changes postvenipuncture secondary to malaria. However, it cannot be ruled out, since these studies were performed on healthy blood donors, in whom other factors, such as anemia, malaria, or hemoglobinopathies, might not be significantly different. As DARC is only expressed on erythrocytes, there should be no confounding effect due to the relatively small number of WBCs in the circulation that express CD71 and contain RNA. In addition, of course, the absolute number of WBCs is much smaller than the number of RBCs.

Finally, this study evaluated the utility of CD71 as a reticulocyte marker alternative to RNA labeled by TO. Comparable Fy6 expression on reticulocytes is obtained using staining for either CD71 or RNA, which allows comparison of data from different populations, including those exposed to malaria. The role of transferrin receptor in malaria pathogenesis, if any, is unknown. Studies of soluble levels of transferrin receptor in malaria have led to contradictory results, with levels said to be both lower and higher than expected,[20,21] possibly dependent upon disease severity. The techniques reported here may be a useful adjunct to exploring reticulocyte biology, in the future, with special relevance to malaria.

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References

1. Wasniowska K, Hadley TJ. The Duffy blood group antigen: an update. Transfus Med Rev 1994;8: 281-8.
2. Sanger R, Race RR, Jack J. The Duffy blood groups of New York Negroes: the phenotype Fy (a–b–). Br J Haematol 1955;1:370-4.
3. Tournamille C, Colin Y, Cartron JP, Le Van Kim C. Disruption of a GATA motif in the *Duffy* gene promoter abolishes erythroid gene expression in Duffy-negative individuals. Nat Genet 1995;10: 224-8.
4. Hadley TJ, Peiper SC. From malaria to chemokine receptor: the emerging physiologic role of the Duffy blood group antigen. Blood 1997;89: 3077-91.
5. Tournamille C, Le Van Kim C, Gane P, et al., Close association of the first and fourth extracellular domains of the Duffy antigen/receptor for chemokines by a disulphide bond is required for ligand binding. J Biol Chem 1997;272:16274-80.

6. Nichols ME, Rubenstein P, Barnwell J, et al. A new human Duffy blood group specificity defined by a murine monoclonal antibody. Immunogenetics and association with susceptibility to *Plasmodium vivax*. J Exp Med 1987;166:776-85.
7. Wasniowksa K, Blanchard D, Janvier D, et al. Identification of the Fy6 epitope recognized by two monoclonal antibodies in the n-terminal extracellular portion of the Duffy antigen receptor for chemokines. Mol Immunol 1996;33:917-23.
8. Darbonne WC, Rice GC, Mohler MA, et al. Red blood cells are a sink for interleukin 8, a leukocyte chemotaxin. J Clin Invest 1991;88:1362-9.
9. Mallinson G, Soo KS, Scall TJ, Pisaka M, Anstee DJ. Mutations in the erythrocyte chemokine receptor (Duffy) gene: the molecular basis of the Fya/Fyb antigens and identification of a deletion in the Duffy gene of an apparently healthy individual with the Fy(a–b–) phenotype. Br J Haematol 1995;90:823-9.
10. Akalin E, Neylan JF. The influence of Duffy blood group on renal allograft outcome in African Americans. Transplantation 2003;75:1496-500.
11. Velzing-Aarts FV, Muskiet FA, van der Dijs FP, Duits AJ. High serum interleukin-8 levels in afro-caribbean women with pre-eclampsia. Relations with tumor necrosis factor-alpha, Duffy negative phenotype and von Willebrand factor. Am J Reprod Immunol 2002;48:319-22.
12. Galinski MR, Medina CC, Ingrovallo P, Barnwell JW. A reticulocyte-binding protein complex of *Plasmodium vivax* merozoites. Cell 1992;69: 1213-26.
13. Woolley IJ, Hotmire KA, Sramkoski RM, Zimmerman PA, Kazura JW. Differential expression of the Duffy antigen receptor for chemokines according to RBC age and FY genotype. Transfusion 2000;40:949-53.
14. Serke S, Huhn D. Identification of CD71 (transferrin receptor) expressing erythrocytes by multiparameter-flow-cytometry (MP-FCM): correlation to the quantitation of reticulocytes as determined by conventional microscopy and by MP-FCM using a RNA-staining dye. Br J Haematol 1992;81:432-9.
15. Southcott MJ, Tanner MJ, Anstee DJ. The expression of human blood group antigens during erythropoiesis in a cell culture system. Blood 1999;93:4425-35.
16. Dertinger SD, Torous DK, Hall NE, et al. Enumeration of micronucleated CD71-positive reticulocytes with a single laser flow cytometer. Mutat Res 2002;515:3-14.
17. Perry ES, Moore RH, Berger TA, et al. In vitro and in vivo persistence of reticulocytes from donor red cells. Transfusion 1996;36:318-21.
18. Mendis K, Sina BJ, Marchesinin P, Carter R. The neglected burden of *Plasmodium vivax* malaria. Am J Trop Med Hyg 2001;64(1-2 Suppl):97-106.
19. Chambers DR, Procter J, Muratova O, et al. In vitro RBC exposure to *Plasmodium falciparum* has no effect on RBC antigen expression. Transfus Med 2002;12:213-9.
20. Williams TN, Maitland K, Rees DC, et al. Reduced soluble transferrin receptor concentrations in acute malaria in Vanuatu. Am J Trop Med Hyg 1999;60:875-8.
21. Mockenhaupt FP, May J, Stark K, et al. Serum transferrin receptor levels are increased in asymptomatic and mild *Plasmodium falciparum*-infection. Haematologica 1999;84:869-73.

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