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# Detection of a *Plasmodium vivax* Erythrocyte Binding Protein by Flow Cytometry

**Tuan M. Tran,[1] Alberto Moreno,[1] Syed S. Yazdani,[2] Chetan E. Chitnis,[2] John W. Barnwell,[3] and Mary R. Galinski[1,4]***

[1]Emory Vaccine Center, Yerkes National Primate Research Center, Emory University, Atlanta, Georgia
[2]Malaria Research Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India
[3]Malaria Branch, Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia
[4]Department of Medicine, Division of Infectious Diseases, Emory University, Atlanta, Georgia

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**Background:** The malaria parasite *Plasmodium vivax* preferentially invades reticulocytes. It is therefore relevant for vaccine development purposes to identify and characterize *P. vivax* proteins that bind specifically to the surface of reticulocytes. We have developed a two-color flow cytometric erythrocyte binding assay (F-EBA) that has several advantages over traditional erythrocyte binding assays (T-EBAs) used in malaria research. We demonstrate the use of F-EBA using the *P. vivax* Duffy binding protein region II (PvDBP-RII) recombinant protein as a model. This protein binds to all erythrocytes that express the Duffy receptor (Fy) and discriminates binding between normocytes and reticulocytes.
**Methods:** F-EBAs were performed by incubating freshly isolated *Aotus nancymai*, *Macaca mulatta*, *Saimiri boliviensis*, and human erythrocytes with PvDBP-RII, a fluorescent anti-His tag detection antibody, and thiazole orange before flow cytometric analysis. T-EBAs employing immunoblot detection with an anti-His antibody were performed concomitantly.
**Results:** PvDBP-RII bound to *A. nancymai*, *M. mulatta*, and human $Fy^+$ erythrocytes, but not human $Fy^-$ erythrocytes, by F-EBAs and T-EBAs. However, F-EBAs exhibited higher sensitivity and better concordance between experiments compared with T-EBAs.
**Conclusions:** F-EBA is a rapid, simple, and reliable method for quantifying the ability of malaria proteins to bind to the surface of erythrocytes. F-EBA can discriminate binding between erythrocyte subpopulations without enrichment protocols and may be more reliable and sensitive than T-EBAs in identifying novel erythrocyte binding proteins. © 2004 Wiley-Liss, Inc.

**Key terms:** malaria; flow cytometry; *Plasmodium vivax*; reticulocyte; erythrocyte binding assay; Duffy binding protein

Owing to the pressing need for a malaria vaccine and given the current availability of *Plasmodium* genome sequence databases (www.plasmodb.org), there is increased focus on the identification and characterization of parasite proteins that adhere to red blood cells (RBCs), proteins that might be the targets of humoral immunity to prevent invasion of these host cells. Among the *P. vivax* antigens currently being developed as malaria vaccine candidates are several erythrocyte binding proteins that had been identified with traditional erythrocyte binding assays (T-EBAs), namely the *P. vivax* Duffy binding protein (PvDBP), which binds the Duffy glycoprotein Fy (also known as the Duffy chemokine receptor or DARC) at the surface of $Fy^+$ RBCs, and the *P. vivax* reticulocyte binding proteins 1 and 2 (1–6). Counterpart binding proteins exist in *P. falciparum*, which include the erythrocyte binding antigens 175 and 140, which bind to glycophorins A and C, respectively, and the *P. falciparum* normocyte binding proteins (7–13).

PvDBP is currently the most well-characterized *P. vivax* erythrocyte binding protein. Interaction between PvDBP and Duffy glycoprotein is essential for human erythrocyte invasion by *P. vivax* merozoites, making PvDBP a critical target for a *P. vivax* malaria vaccine (1,14). The erythrocyte binding domain of PvDBP has been mapped to a ~350 amino acid conserved, cysteine-rich region, region II (PvDBP-RII), near the aminoterminus (15). This distinct binding region was determined by expressing different

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*Correspondence to: Mary R. Galinski, PhD, Emory Vaccine Center at Yerkes, Emory University, 954 Gatewood Road, Atlanta, GA 30329.
E-mail: galinski@rmy.emory.edu
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domains of the PvDBP on the surface of mammalian cells and testing for their ability to form rosettes by adhering to $Fy^+$ erythrocytes (15). PvDBP-RII is being developed as a vaccine candidate for *P. vivax* malaria. Methods to produce recombinant PvDBP-RII in its correctly folded functional conformation have been developed, and the ability of recombinant PvDBP-RII to bind to human erythrocytes expressing the Duffy antigen has been characterized using T-EBAs (16).

The T-EBA originally described by Camus and Hadley (7) involved incubating erythrocytes with metabolically radiolabeled spent *P. falciparum* culture supernatants, washing away nonadherent supernatant proteins, eluting bound proteins in a high salt solution, and separating the eluted proteins by sodium dodecylsulfate polyacrylamide gel electrophoresis. Detection of putative parasite binding proteins was determined by autoradiography. The original T-EBA was subsequently modified to include a silicone oil step to exclude nonbinding and weakly bound proteins that bind nonspecifically (5,17,18). Bound proteins can also be eluted with NaCl and detected by western immunoblot detection methods using antisera directed against specific proteins (5,16–18). In the study of *P. vivax* proteins, it is especially relevant to investigate reticulocyte-specific binding given the propensity of vivax merozoites to invade immature erythrocytes. In the past, *P. vivax* T-EBA and rosetting assays have been performed with erythrocyte populations enriched for reticulocytes. Such enrichment procedures may involve Percoll gradient density separation or phlebotomy of anemic individuals with elevated reticulocyte counts (3,19). However, these techniques are time and labor intensive. A simpler and more efficient EBA that can be readily adapted for high throughput screening would be advantageous given the availability of malaria genome information and recent advances in malaria proteomics that have, for example, identified more than 250 unannotated merozoite proteins in *P. falciparum* (20–24). Preferably, such a screen would also allow for normalization of binding profiles between different malaria proteins and between different subpopulations of erythrocytes, a feature that is lacking in T-EBAs. The complex process of erythrocyte invasion by *Plasmodium* merozoites makes it reasonable to speculate that additional proteins with adhesive roles remain to be identified.

With the broad goal of improving our understanding of the invasion process and identifying novel adhesive proteins, we have used recombinant PvDBP-RII to develop a novel flow cytometry-based erythrocyte binding assay (F-EBA) that can replace or complement T-EBAs. The known PvDBP characteristics of binding to $Fy^+$, but not to $Fy^-$ RBCs, and to RBCs from different simian species make it a convenient model for testing the specificity of novel erythrocyte binding assays such as the F-EBA. We tested the ability of PvDBP-RII to bind to a panel of human and nonhuman primate erythrocytes by F-EBA and compared those results with results obtained in parallel experiments using T-EBAs. Importantly, F-EBAs allow for comparisons of protein binding to normocytes and reticulocytes without reticulocyte enrichment. Our data support the use of F-EBA as a fast, simple, and reliable method for screening recombinant malaria proteins for their ability to bind erythrocytes.

## MATERIALS AND METHODS

### Preparation of Erythrocytes

Peripheral blood was obtained from volunteers from the Emory Vaccine Center at Emory University (Atlanta, GA, USA) and from *Macaca mulatta*, *Aotus nancymai*, and *Saimiri boliviensis* nonhuman primates by intravenous blood draw into adenosine-citrate-dextrose Vacutainer tubes (ACD Vacutainer; Becton-Dickinson, Franklin Lakes, NJ, USA). Whole blood was stored at 4°C and used within 48 h. Plasma and cells were separated by centrifugation 24 h before the binding assay. The plasma and buffy coat were discarded and the remaining packed cells were washed three times with RPMI 1640 (Cellgro, Herndon, VA, USA). On the day of the assay, RBCs were washed three times in phosphate buffered saline (PBS; Cellgro) containing 1% bovine serum albumin (PBS-BSA). Cells were diluted to a working concentration of $1 \times 10^7$ cells/ml in PBS-BSA.

### Duffy Phenotyping

RBCs from the volunteers were screened for presence of Duffy A ($Fy^{a+}$) or Duffy B ($Fy^{b+}$) antigen by indirect Coombs test using anti-$Fy^a$ and anti-$Fy^b$ globulins (Gamma Biologicals, Houston, TX, USA) according to the manufacturer's protocol. Duffy-positive erythrocytes bearing the $Fy^{a+b-}$, $Fy^{a-b+}$, or $Fy^{a+b+}$ phenotype and the Duffy-negative phenotype $Fy^{a-b-}$ ($Fy^-$) were used in this study.

### Protein Expression

Recombinant PvDBP-RII was expressed with a C-terminal hexa-His tag in *Escherichia coli* BL21 (DE3) strain (Novagen, Madison, WI, USA), purified from inclusion bodies by metal affinity chromatography under denaturing conditions, refolded by rapid dilution, and purified to homogeneity by ion exchange and gel filtration chromatography as described previously (16). For the expression and purification of thioredoxin with a C-terminal hexa-His tag, the pET32b plasmid vector was isolated from *E. coli* DH5α and transformed into *E. coli* BL21 (DE3) (Novagen). Cultures were grown under aerobic conditions at 37°C in Luria-Bertani broth with 50 μg/ml ampicillin until the $OD_{600}$ reached 0.6 to 0.8. The expression was then induced with 1 mM isopropyl-β-D-thiogalactopyranoside. Cells were collected by centrifugation 3 h after induction and resuspended in an ice-cold lysis buffer (10 mM imidazole, 0.3 M NaCl, 50 mM $NaH_2PO_4$, pH 8.0). The resuspended cells were lysed by sonication on ice, and cell debris was removed by centrifugation at 15,000 rpm for 20 min. The supernatant was incubated with Ni-NTA agarose resin (Qiagen, Valencia, CA) for 1 h at 4°C. The resin was collected by centrifugation and washed with a wash buffer (20 mM imidazole, 0.3 M NaCl, 50 mM $NaH_2PO_4$, pH 8.0). The bound protein was eluted from the resin

with an elution buffer (250 mM imidazole, 0.3 M NaCl, 50 mM $NaH_2PO_4$, pH 8.0). The final purification step included gel filtration chromatography using a Sephacryl S-300 column (Amersham Pharmacia Biotech, Piscataway, NJ, USA) equilibrated with 20 mM Tris, pH 8.0 (buffer A). The thioredoxin was loaded on the column, washed with buffer A, and eluted with a step gradient of buffer B (500 mM NaCl in A). Fractions positive for thioredoxin were pooled, concentrated, and dialyzed against PBS.

### Traditional Erythrocyte Binding Assays

The T-EBA used in this study was adapted from the classic binding assays previously described (3,5,7,16–18). Briefly, blood collected in ACD Vacutainer tubes was washed three times in RPMI 1640 before use. In addition, $5 \times 10^8$ RBCs were incubated with 25 μg of protein at room temperature for 4 h. The reaction mixture was layered over 500 μl of 550 silicone oil (Dow Corning, Midland, MI, USA) and centrifuged. The pellet was resuspended in 1× RPMI 1640 and again layered over fresh 550 oil and centrifuged. This step was repeated. Bound proteins were eluted by resuspending cells in 5× RPMI 1640 (Gibco BRL, Grand Island, NY, USA). The suspension was centrifuged to pellet the cells. The supernatant was collected and diluted 1:1 with Laemmli reducing sample buffer (Biorad, Hercules, CA, USA).

Protein samples were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). The membranes were blocked at 25°C for 1 h with blocking buffer (Tris buffered saline [TBS], 3% w/v BSA), washed twice with wash buffer (TBS, 0.2% Triton X-100, 0.05% Tween 20), and incubated with mouse anti-penta-His monoclonal antibody (mAb; Qiagen) diluted 1:1000 in blocking buffer for 1 h at 25°C. The membranes were then washed twice with wash buffer and once with TBS and incubated for 45 min at 25°C with goat anti-mouse immunoglobulin G alkaline phosphate conjugate (Promega, Madison, WI, USA) diluted $1:1 \times 10^5$ in blocking buffer. Membranes were washed three times with wash buffer, and enzymatic detection of the bound protein was carried out with 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium color development substrate kit (Promega). Scanned images of developed membranes were analyzed by densitometry with Image J 1.31l (http://rsb.info.nih.gov/ij/, National Institutes of Health, Bethesda, MD, USA).

### Flow Cytometry–Based Erythrocyte Binding Assays

Nonhuman primate and human erythrocytes of all four Duffy phenotypes were incubated for 4 h at 25°C with 0 to 10 μg of recombinant PvDBP-RII or thioredoxin per $1 \times 10^6$ erythrocytes. Thioredoxin was used as a hexa-His–tagged negative control. Samples were washed twice with 200 μl PBS-BSA and incubated for 1 h at 4°C in the dark with mouse anti-penta-His Alexa Fluor 647 conjugated mAb (Qiagen) diluted 1:25 in PBS-BSA. Samples were washed three times with 200 μl PBS-BSA and incubated with 200 μl thiazole orange (TO) RETIcount reagent (Becton-Dickinson) for 30 min at 25°C. Two hundred thousand total events were acquired per sample using CellQuest software (Becton-Dickinson) on a FACScalibur flow cytometer (Becton-Dickinson). The instrument was calibrated routinely using CALIBRITE beads (Becton-Dickinson) and FACScomp software (Becton-Dickinson), allowing the same voltage settings to be used for experiments performed on different days with minimal variation. TO and Alexa 647 emissions were read in the FL1 and FL4 channels, respectively. Flow cytometric data were analyzed by FlowJo 4.3 (Treestar, Ashland, OR, USA). Forward scatter and side scatter dot plots of total events were used to select a homogeneous erythrocyte population for further analysis. Thioredoxin samples were used to determine background levels of protein binding. Unstained cells and cells singly stained with TO were used to define the normocyte and reticulocyte populations, respectively.

### Inhibition of Binding Experiments

The anti-Fy6 monoclonal antibody K6H9 binds to all $Fy^+$ human erythrocytes ($Fy^{a+b-}$, $Fy^{a-b+}$, $Fy^{a+b+}$) and can inhibit binding of native PvDBP (1,25). $Fy^+$ erythrocytes were preincubated with Fy6 mAb or an immunoglobulin G1 isotype control mAb at concentrations of 0 to 50 μg/ml for 30 min at 37°C before the addition of 2 μg of PvDBP-RII. The rest of the procedure was performed as described above.

### Statistics

Geometric mean fluorescence intensities (MFIs) were used in all statistical analyses. Unpaired and paired Student's *t* test and one-way analysis of variance were used to evaluate the statistical significance of these experiments as appropriate. $P < 0.05$ was considered statistically significant. To compare geometric MFIs between experiments done on different days, the formula ([MFI/baseline MFI] × 100) was used to normalize absolute MFIs, where baseline MFI equals the MFI obtained at a protein concentration of 0. For inhibition of binding studies, the specific binding inhibition percentage was determined with the formula:

$$100 - (100 \times [\text{MFI antibody/MFI no antibody}])$$

## RESULTS

### Flow Cytometric Erythrocyte Binding Assay

**PvDBP-RII binds to $Fy^+$ specifically and with high sensitivity.** The PvDBP-RII His-tagged recombinant protein was used to establish and standardize the F-EBA. A dose response of PvDBP-RII binding was performed to assess the sensitivity of the assay and determine the saturating protein concentration. PvDBP-RII bound to $Fy^+$ RBCs in a dose-responsive manner, saturating at approximately 25 μg/ml (Fig. 1a). A statistical difference in binding between PvDBP-RII and thioredoxin was detected with a concentration as low as 0.1 μg/ml (normalized MFIs of 221 ± 20 and 109 ± 10, respectively; $P < 0.0001$). At a protein concentration of 50 μg/ml, the normalized

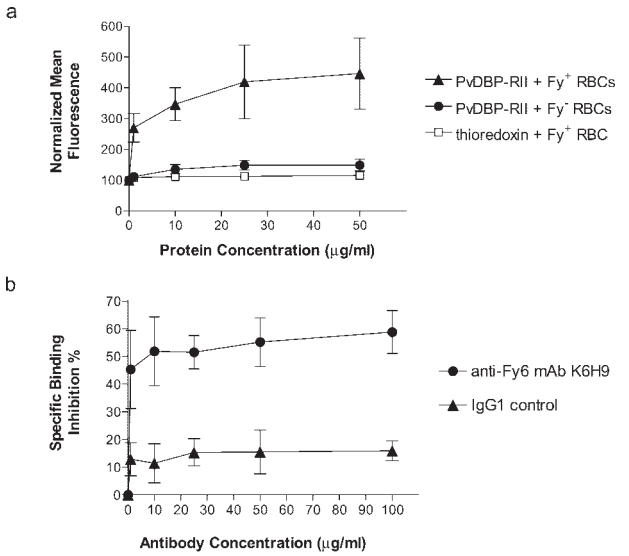


FIG. 1. **a:** Dose-response curve shows PvDBP-RII binding to $Fy^+$ erythrocytes (triangles) or $Fy^-$ erythrocytes (circles). Thioredoxin (squares) was used as a control for nonspecific binding. Erythrocytes were incubated with 0 to 50 μg/ml of protein before flow cytometric detection with an Alex Fluor 647 conjugated anti-penta-His mAb. Results are arithmetic means (±1 standard deviation) of five separate experiments. **b:** Specific binding inhibition curve shows blocking of PvDBP-RII binding with the anti-Fy6 mAb K6H9. Erythrocytes were preincubated with the anti-Fy6 mAb before incubation with 2 μg of PvDBP-RII and subsequent labeling with Alexa Fluor 647 conjugated anti-penta-His mAb (circles). Immunoglobulin G1 (IgG1) isotype mAb was used as a control (triangles). Results are arithmetic mean (±1 standard deviation) of three experiments using $Fy^+$ erythrocytes from the same donor.

MFI for PvDBP-RII binding to $Fy^+$ cells was 447 ± 110, a 3.9-fold increase in binding over thioredoxin (115 ± 6, $P = 0.0008$; Fig. 1a). PvDBP-RII did not bind $Fy^-$ erythrocytes, and thioredoxin did not bind to $Fy^+$ RBCs, and neither differed significantly from baseline MFIs (Fig. 1a).

To confirm specificity of PvDBP-RII binding by F-EBA, we performed inhibition of binding experiments with the anti-Fy6 mAb K6H9. Preincubation of erythrocytes with the anti-Fy6 mAb blocked up to 59% of PvDBP-RII binding to $Fy^+$ erythrocytes in a dose-dependent manner, an effect not observed for the isotype control mAb (Fig. 1b).

To characterize the affinity of PvDBP-RII to the different Fy phenotypes presented by humans, we repeated the assay using $Fy^{a+b-}$, $Fy^{a-b+}$, $Fy^{a+b+}$, and $Fy^{a-b-}$ erythrocytes from different donors. The MFIs of PvDBP-RII binding and dot plot profiles were similar across the $Fy^+$ phenotypes (Table 1 and Fig. 2). One-way analysis of variance was performed on the normalized MFIs across all four groups. $Fy^+$ phenotypes showed increased binding over the $Fy^-$ phenotype ($P < 0.01$ for $Fy^{a+b-}$, $Fy^{a-b+}$, $Fy^{a+b+}$) but did not differ significantly from each other (Table 1).

Figure 3a shows representative gates used in our analysis. Reticulocytes were defined as $TO^+$ erythrocytes, which accounted for 0.7% to 2.6% of all erythrocytes in the samples tested (data not shown). Normocytes were defined as $TO^-$ erythrocytes. PvDBP-RII bound to reticulocytes at 3.3-fold greater levels than normocytes (56.7 ± 8.9 vs. 17.3 ± 3.3, $P < 0.0001$) based on MFI (Fig. 3b). Background mean fluorescence with the negative control thioredoxin was 1.3-fold greater in reticulocytes than in normocytes (4.9 ± 0.8 vs. 3.7 ± 0.6, $P = 0.01$).

**PvDBP-RII binds to erythrocytes from nonhuman primates by F-EBA.** To further assess the specificity of PvDBP-RII, we performed the binding assay with *M. mulatta*, *A. nancymai*, and *S. boliviensis* erythrocytes. *Aotus* and *Saimiri* monkeys, but not *Macaca* monkeys, are susceptible to *P. vivax* infection. Table 1 shows that *Macaca* and *Aotus* erythrocytes bound PvDBP-RII, respectively, at 30.1-fold ($P < 0.001$) and 3.0-fold ($P < 0.01$) greater levels than did $Fy^-$ human erythrocytes (11.7-fold and 1.2-fold greater levels, respectively, than $Fy^{a+b+}$ human erythrocytes). *Saimiri* erythrocytes demonstrated a 1.2-fold increase in normalized mean fluorescence over $Fy^-$ erythrocytes; however, this increase was not statistically significant. Representative shifts in FL4 fluorescence for PvDBP-RII binding are shown in histograms (Fig. 4b).

## T-EBA Binding Profile of PvDBP-RII

To demonstrate that the F-EBA reliably reproduces the results of an established method, we performed T-EBAs concurrently. Five T-EBAs were performed with $Fy^+$, $Fy^-$, *Aotus*, *Saimiri*, and *Macaca* erythrocytes (Fig. 4a shows a representative immunoblot). Different sets of monkeys were used as donors for each experiment. As expected, PvDBP-RII consistently bound to human $Fy^+$ erythrocytes by T-EBA but not to $Fy^-$ erythrocytes (5). *Aotus*, *Macaca*, and *Saimiri* erythrocytes consistently showed positive binding of PvDBP-RII by T-EBA, with *Aotus* erythrocytes showing the most intense binding in four of the five experiments. In these four experiments, the densitometric intensities of the PvDBP-RII bands bound to *Aotus*,

Table 1
*PvDBP-RII Binding to Human and Nonhuman Primate Erythrocytes by Flow Cytometry*

| | n | PvDBP-RII binding, normalized MFI[a] | *P* |
|---|---|---|---|
| Human | | | |
| $Fy^-$ | 5 | 170 ± 24 | n/a |
| $Fy^{a+b-}$ | 4 | 444 ± 61 | <0.001* |
| $Fy^{a-b+}$ | 5 | 387 ± 116 | <0.01* |
| $Fy^{a+b+}$ | 8 | 438 ± 89 | <0.001* |
| Nonhuman primate | | | |
| *Aotus* | 3 | 504 ± 187 | <0.01† |
| *Saimiri* | 3 | 196 ± 30 | 0.29† |
| *Macaca* | 5 | 5110 ± 1950 | <0.0001† |

*One-way analysis of variance was used to compare differences between all human Fy phenotypes ($P = 0.0001$). Subsequently, Tukey's multiple comparison was used for pairwise comparisons. *P* values shown refer to comparisons to $Fy^-$ phenotype. No statistical difference was observed when $Fy^+$ phenotypes were compared with each other.

†Student's *t* test was used to compare differences between means of nonhuman primate erythrocytes and the human $Fy^-$ phenotype.

[a]Values are mean ± standard deviation.

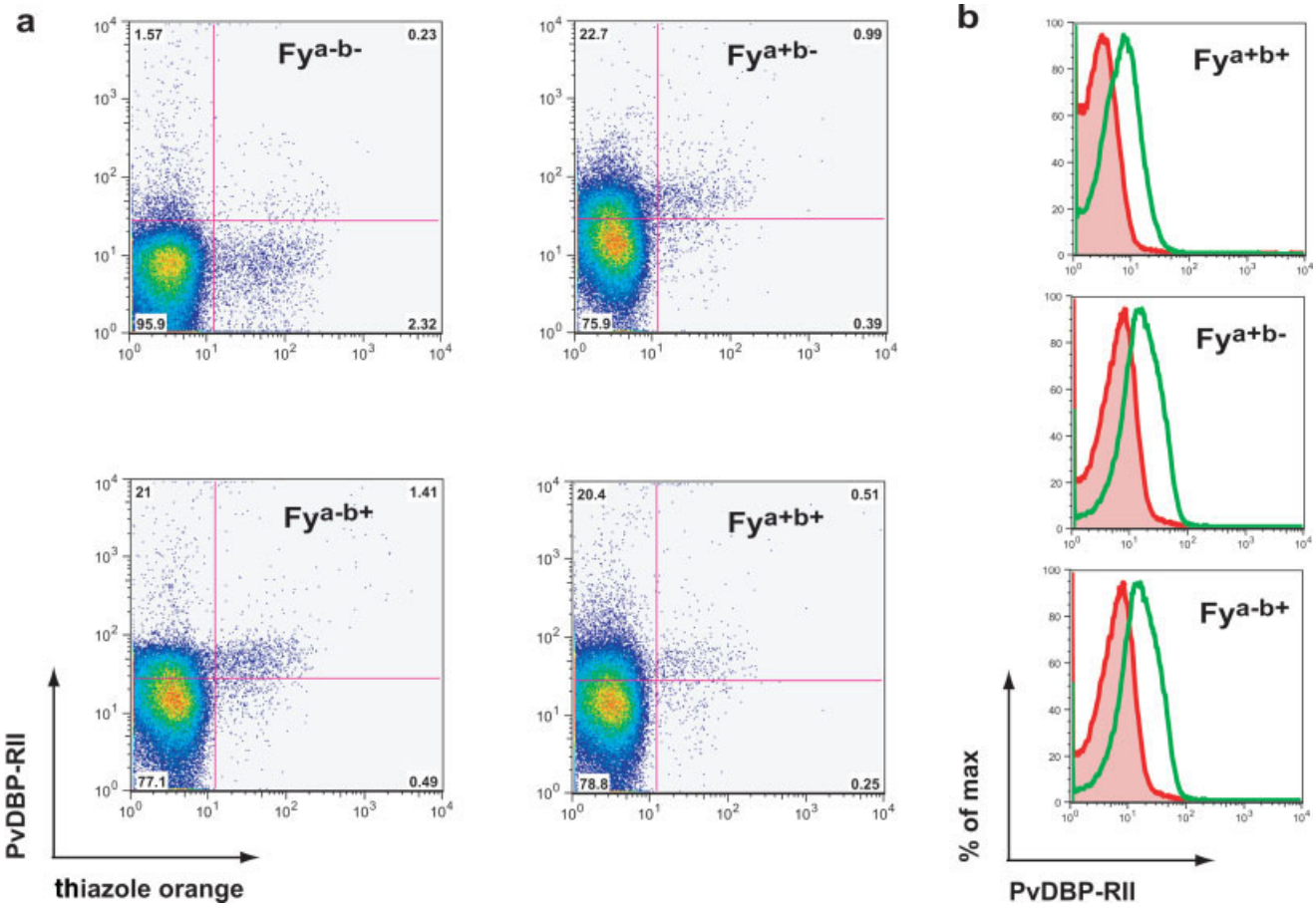


FIG. 2. **a:** Dot plots show binding of PvDBP-RII to erythrocytes from $Fy^{a-b-}$, $Fy^{a-b+}$, $FY^{a+b-}$, and $Fy^{a+b+}$ individuals. Erythrocytes were incubated with 50 μg/ml PvDBP-RII and then labeled with an anti-penta-His mAb Alexa Fluor 647 conjugate and TO. Numbers in quadrants indicate gate frequencies. **b:** Histograms show binding among the three $Fy^+$ phenotypes. Shaded histograms indicate binding of PvDBP-RII to $Fy^-$ erythrocytes. All panels show representative results of at least five samples for each phenotype. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

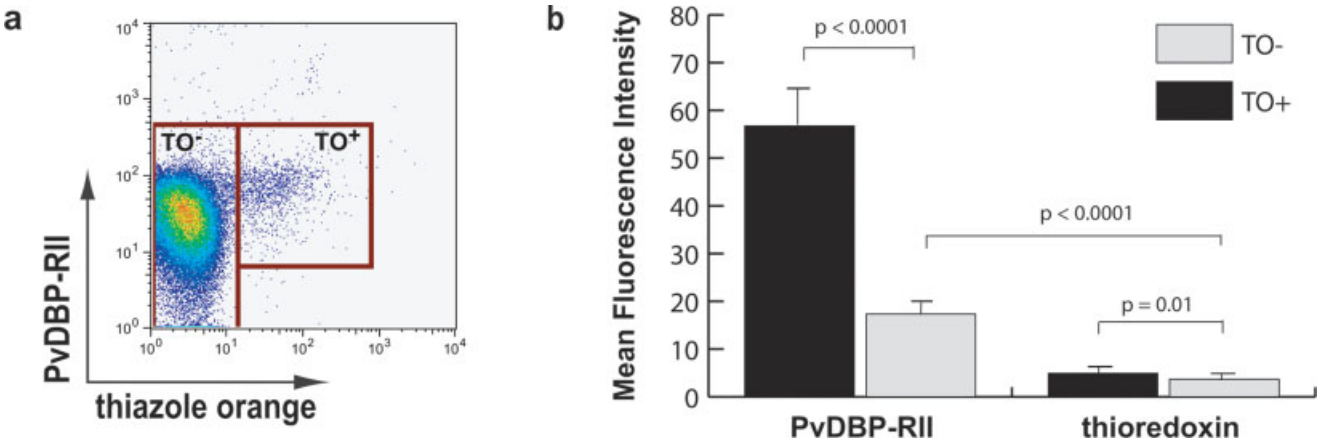


FIG. 3. **a:** Dot plot of normocyte ($TO^-$) and reticulocyte ($TO^+$) gates used in analysis. **b:** Bar chart shows binding of PvDBP-RII and thioredoxin to $TO^-$ normocytes and $TO^+$ reticulocytes. Student's *t* test was used to determine statistical significance. Results are arithmetic means of five separate experiments using erythrocytes form the same $Fy^+$ individual. Error bars indicate one standard deviation from the mean. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

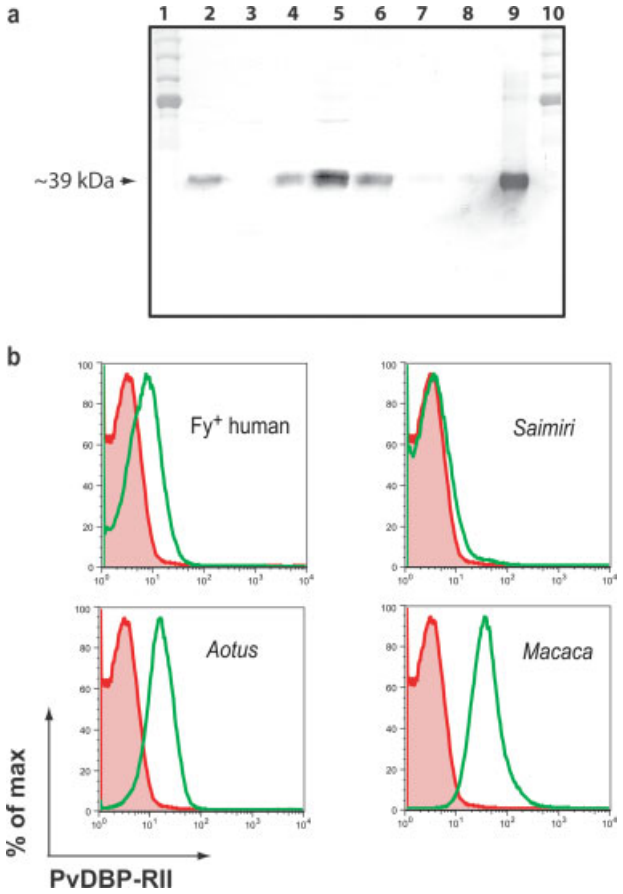


FIG. 4. **a:** Immunoblot of proteins eluted from the surface of erythrocytes. PvDBP-RII was incubated with human and nonhuman primate erythrocytes. Samples used were $Fy^+$ human (lane 2), $Fy^-$ human (lane 3), *Saimiri* monkey (lane 4), *Aorus* monkey (lane 5), and *Macaca* monkey (lane 6). PvDBP-RII was loaded in lane 9 as a positive control, and *Aotus* and *Macaca* erythrocytes were incubated with thioredoxin for lanes 7 and 8, respectively. Lanes 1 and 10 are molecular weight markers. **b:** Histograms of PvDBP-RII binding to human and monkey erythrocytes. All shaded histograms refer to PvDBP-RII binding to $Fy^-$ human erythrocytes. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

*Saimiri*, and *Macaca* erythrocytes were 160 ± 77%, 58 ± 40%, and 117 ± 83%, respectively, of the PvDBP-RII bands bound to human $Fy^+$ erythrocytes. Thus, the order of PvDBP-RII binding from most intense to least intense was *Aotus*, *Macaca*, and human $Fy^+$ erythrocytes and *Saimiri* erythrocytes as determined by densitometry. Statistical analyses were not performed due to the high variance between experiments.

## DISCUSSION

Malaria proteins that bind to erythrocytes have attracted considerable attention because they are major targets for the development of blood-stage vaccines for *P. falciparum* and *P. vivax*. Recent falciparum proteomic studies have identified more than 250 expressed merozoite proteins that are not annotated (22,24). Similar data are forthcoming consequent to the *P. vivax* genome project (21). The functional characterization of such a large number of novel proteins requires a simple, objective, and reliable method to determine erythrocyte binding. This report introduces a flow cytometric method for detecting recombinant malaria proteins specifically bound to the surface of erythrocytes. Our method allows for quantitative comparisons of binding between different recombinant proteins and between phenotypically distinct erythrocyte populations.

In this report, we have shown that PvDBP-RII binds to human erythrocytes of all three $Fy^+$ phenotypes with similar intensity by flow cytometry. These data are consistent with the binding profile for native PvDBP, which binds strongly to erythrocytes of all three $Fy^+$ phenotypes (5). Our results indicate that the binding of PvDBP-RII to $Fy^+$ erythrocytes by F-EBA is specific. PvDBP-RII does not bind to $Fy^-$ erythrocytes by F-EBA (Fig. 1a), an expected finding consistent with previous studies on native and recombinant PvDBP binding using traditional methods (5,16). Similarly, preincubating $Fy^+$ erythrocytes with the Fy6 mAb prevented PvDBP-RII binding (Fig. 1b), as described previously with native PvDBP (1,5).

Although our F-EBA studies on PvDBP-RII and human erythrocytes are consistent with those in the published literature, our data indicate that the recombinant PvDBP-RII exhibits a dissimilar binding tropism from that shown to date for native PvDBP with regard to erythrocytes from nonhuman primates. Native PvDBP has been shown to bind to *Aotus* monkey erythrocytes but not to *Saimiri* monkey erythrocytes, both of which are susceptible to *P. vivax* infection (5). In addition, native PvDBP does not bind to erythrocytes from *Macaca* monkeys, a primate species that is not susceptible to *P. vivax* infection (1,5). Unexpectedly, our results using F-EBAs show that PvDBP-RII binds not only *Aotus* erythrocytes, as expected, but also *Macaca* erythrocytes. PvDBP-RII binding to *Saimiri* erythrocytes is negligible. These data suggest that recombinant, refolded PvDBP-RII, although functionally active, may differ somewhat in conformational structure from the native binding domain in PvDBP and thus could exhibit altered binding specificities. Alternatively, regions outside PvDBP-RII, the binding domain of PvDBP, may influence the fine specificity of binding of PvDBP. Results obtained using F-EBAs correlated with those obtained by T-EBAs performed concomitantly with the same erythrocyte and protein samples. With the exception of *Saimiri* erythrocytes, which did not achieve statistically significant binding of PvDBP-RII by F-EBA but demonstrated weak binding by T-EBA, erythrocytes that demonstrated positive binding by T-EBAs were also positive by F-EBAs. However, both assays showed incongruous results when monkey erythrocytes were ranked according to their relative ability to bind PvDBP-RII. Densitometric analysis showed that *Aotus* erythrocytes consistently bound PvDBP-RII with greater affinity than *Macaca* erythrocytes by T-EBA, the reverse of what was shown by F-EBA. This discrepancy could be due to differences in sample preparation between the two assays, in particular, the use of PBS washes versus a column of oil to remove nonspecific adherents and the de-

tection of adherent proteins bound in situ on the erythrocyte surface versus after elution from the erythrocyte surface. Although results of replicate T-EBAs were qualitatively consistent, comparative quantification of the immunoblots by densitometry yielded high variance between replicates, preventing any meaningful statistical analysis on relative levels of binding between the different monkey erythrocytes.

Because *P. vivax* preferentially invades the reticulocyte subpopulation, it is particularly relevant to compare the binding of malaria proteins between normocytes and reticulocytes. Methods for reticulocyte enrichment have employed Percoll gradient separation techniques or phlebotomy of anemic individuals with high reticulocyte counts (3,19). TO is a commercially available nucleic acid staining reagent that is used routinely in clinical laboratories to enumerate reticulocytes by flow cytometry (26,27). By costaining with TO and an Alexa-conjugated anti-His antibody that specifically detects His-tagged protein bound to the surface of erythrocytes, we were able to compare binding of normocytes and reticulocyte in a single sample taken ex vivo from individuals with normal reticulocyte levels, thus eliminating the need for reticulocyte enrichment. Using TO-stained erythrocytes and flow cytometry, Woolley et al. (28) demonstrated significantly greater Fy expression on the surface of reticulocytes than on more mature erythrocytes. In agreement with this observation, we found that PvDBP-RII binds to reticulocytes at significantly greater levels than normocytes. It has been suggested that the increased surface area of reticulocytes compared with normocytes may account for the increased binding. A recent study used osmotic fragility measurements and flow cytometry to show that the average surface area of normal reticulocytes is $142.4 \pm 2.0\ \mu m^2$, whereas that of normal normocytes is $133.6 \pm 3.0\ \mu m^2$ (29). This change of $9\ \mu m^2$ (6.7%) in cell surface membrane may explain the 1.3-fold increase in reticulocyte binding for thioredoxin but cannot fully explain the 3.3-fold increase in reticulocyte binding for PvDBP-RII. The decrease in Duffy antigen receptor density as the erythrocyte matures may result from a combination of degradation and lack of de novo protein synthesis (28).

One potential application of the F-EBA is the characterization of binding domains of known malaria erythrocyte binding proteins by expression of the protein as recombinant fragments. However, screening of recombinant malaria proteins for erythrocyte binding poses the technical challenge of producing a properly folded protein that mimics the function of the native erythrocyte binding protein, especially when proteins are produced as inclusion bodies in *E. coli*. Refolding procedures to produce a properly folded protein are empirical and require functional validation using the native protein as the standard for comparison. The F-EBA could be used as an efficient tool to determine which products of a matrix-based refolding scheme exhibit an erythrocyte binding pattern most similar to the native protein. Conversely, the flow cytometric erythrocyte binding assay can be adapted to identify novel erythrocyte binding proteins in their native state provided that specific fluorochrome-conjugated monoclonal antibodies are available. The challenge would be increasing the sensitivity of the assay from 0.1 μg/ml achieved in our study to concentrations more representative of native proteins in parasite extracts. With the use of multiwell flow cytometers, the F-EBA can be developed into a high-throughput method for screening malaria proteins and polypeptides for their ability to bind erythrocytes at various stages of maturation and different phenotypic classes of erythrocytes. Our method will facilitate efforts to further characterize the binding domains of known malaria erythrocyte binding proteins and the identification of novel erythrocyte binding proteins.

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