

Duffy-null promoter heterozygosity reduces DARC expression and abrogates adhesion of the *P. vivax* ligand required for blood-stage infection

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Abstract The Duffy blood group antigen is an essential receptor for *Plasmodium vivax* entry into erythrocytes in a process mediated by the parasite ligand, the Duffy binding protein (DBP). Recently, individuals living in a malaria endemic region of Papua New Guinea were identified as heterozygous for a new allele conferring Duffy negativity, which results in 50% less Duffy antigen on their erythrocytes. We demonstrate that DBP adherence to erythrocytes is significantly reduced for erythrocytes from heterozygous individuals who carry one Duffy antigen negativity allele. These data provide evidence that emergence of this new allelic form of Duffy negativity is correlated with resistance against vivax malaria. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Malaria; Duffy binding protein; Duffy antigen receptor for chemokines; Genetic selection; *Plasmodium vivax*

1. Introduction

Malaria is a virulent disease caused by the *Plasmodium* parasite, efficiently propagating itself in the blood of its vertebrate host. Innate resistance to malaria infections in humans is conferred by various blood group polymorphisms. A well-documented association between a benign human genetic trait and reduced malaria prevalence involves the Duffy blood group antigen and *Plasmodium vivax* [1,2]. Since individuals who are Duffy-negative are refractory to invasion by *P. vivax* merozoites, it has been presumed that Duffy negativity in humans arose because this imparted a natural resistance to vivax malaria. Field observations from West Africa and Ethiopia have indeed established a strong correlation between absence or low endemicity of vivax malaria and the high prevalence of the Duffy negativity allele [3,4]. In vitro erythrocyte invasion assays showed that Duffy-negative but not

Duffy-positive human erythrocytes were resistant to invasion by *P. knowlesi*, a simian malaria parasite related to *P. vivax* [5]. In addition, experimental infections of human volunteers with *P. vivax* showed that Duffy-negative individuals were resistant to blood-stage infection [6]. These experiments demonstrated that *P. vivax* requires the Duffy blood group antigen for merozoite invasion. In *P. vivax*, the merozoite ligand that interacts with the Duffy blood group antigen was identified as the Duffy binding protein (DBP) [7] and is encoded by a single-copy gene [8]. Experimental analysis of the DBP showed that a cysteine-rich extracellular domain of this protein (so-called region II [9]) was the parasite ligand domain interacting with the Duffy blood group antigen on the host erythrocyte [10]. Additionally, there was a correlation between the ability of the DBP to bind erythrocytes and their susceptibility to invasion by *P. knowlesi* and *P. vivax* merozoites [7,10].

The human Duffy blood group antigen is a transmembrane glycoprotein of 35–43 kDa [11]. The Duffy protein has been identified as a receptor for chemokines and therefore was renamed the Duffy antigen receptor for chemokines (DARC) [12]. Analysis of the encoding gene [13] led to the discovery that the difference between *FY*A* and *FY*B* alleles (*Fy^a* and *Fy^b* antigens, respectively) is due to a single nucleotide polymorphism (G131A), changing a Gly to Asp at residue 44 (*FY*A* and *FY*B*, respectively) [14,15]. Furthermore, the Duffy-negative phenotype observed in black African populations is caused by a single nucleotide polymorphism in the GATA box of the *DARC* promoter (T46C), disrupting a binding site for an erythroid transcription factor that results in a silent *FY*B* allele in erythroid cell lineage [16]. This Duffy-negative phenotype was originally described and only found associated with the *FY*B* allele. The same transition (T46C) in the GATA box was recently identified in a population from Papua New Guinea linked to the *FY*A* allele, and this *FY*A^{null}* mutation appears to have a more recent origin than that of *FY*B^{null}* [17]. All 23 individuals found with this new allele were heterozygous (*FY*A/FY*A^{null}*). Flow cytometric analysis revealed that these individuals expressed half the amount of Duffy antigen on their erythrocytes indicating a gene dosage effect. A definitive conclusion could not be made on whether these heterozygous individuals were less susceptible to infection with *P. vivax*.

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Abbreviations: DARC, Duffy antigen receptor for chemokines; DBP, Duffy binding protein; EBA-175, erythrocyte binding protein-175; HSVgD1, herpes simplex virus glycoprotein D1

An *in vitro* binding assay has proved successful in studying the interactions between the Duffy antigen present on the erythrocyte surface and its parasite ligand [10,18]. In this assay, the *P. vivax* DBP ligand domain expressed on the surface of COS cells mediates cytoadherence to Duffy-positive erythrocytes. Using this assay, we found a significant reduction of binding of heterozygous Duffy-negative vs. homozygous Duffy-positive erythrocytes.

2. Materials and methods

2.1. Blood samples

Blood samples were collected in Papua New Guinea into sodium EDTA and shipped on wet ice to Cleveland. An aliquot of each sample was then shipped overnight on wet ice to Notre Dame and used in the binding assays approximately 2 weeks after collection. Additional samples were collected at the American Red Cross Blood Service as tube segments from blood collection bags, using citrate phosphate dextrose as an anticoagulant. These samples were stored under optimal conditions and used within 5 days of collection. All blood samples were collected after obtaining informed consent following protocols approved by the institutional review boards of Case Western Reserve University and University Hospitals of Cleveland, the American Red Cross of Cleveland and the University of Notre Dame.

2.2. Duffy antigen characterization

Surface expression of the Duffy antigen was quantitated on a subset of the blood samples by flow cytometry using a Fy⁶ monoclonal antibody as reported previously. Expression levels were consistent with those reported previously for the homozygous and heterozygous genotypes [17].

2.3. Binding constructs

2.3.1. pRE4-DBP_{II}. The pRE4 construct containing *P. vivax* dbp region II was described earlier [10]. The vector contains the open reading frame (ORF) of the herpes simplex virus glycoprotein D1 (HSVgD1), a type I integral membrane protein and expression is driven by the Rous sarcoma virus long terminal repeat. The HSVgD1 ORF contains unique *Apa*I and *Pvu*II restriction sites where PvDBP_{II} was cloned as a HSVgD1 chimera.

2.3.2. pEGFP-DBP_{II}. A second HSVgD1/DBP_{II} plasmid expressed this chimeric protein as a recombinant fusion at the N-terminus of green fluorescent protein (GFP) as described previously [18].

2.3.3. pRE4-EBA-175. The pRE4 construct, encoding the region II ligand domain of the *P. falciparum* EBA-175 as a HSVgD1 chimera [19], was used as a control for binding specificity (Fig. 2A,B). All recombinant plasmids were purified using an endotoxin-free plasmid purification kit (Qiagen).

2.4. Fluorescence microscopy

Surface expression of COS-7 cells transfected with pRE4-DBP_{II} was

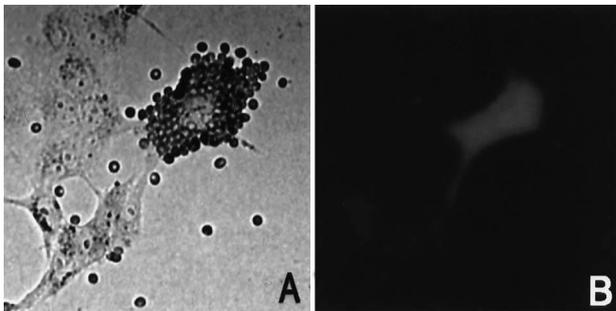


Fig. 1. Erythrocyte binding to *P. vivax* DBP_{II} expressed on COS-7 cells as an N-terminal GFP fusion protein. A: Rosette of human erythrocytes adherent to only the COS-7 cell expressing DBP_{II}. B: Cell-associated fluorescence due to the expression of the GFP part of the fusion protein confirmed erythrocyte cytoadherence was associated with DBP_{II} expression.

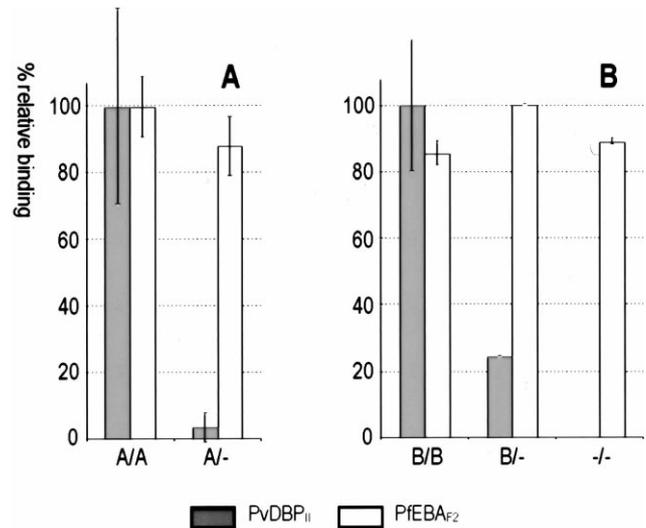


Fig. 2. Binding to *P. vivax* DBP and *P. falciparum* EBA175 ligand domains expressed on the surface of COS-7 cells (pRE4 plasmid) by human erythrocytes with different Duffy antigen genotypes. Percentage binding was expressed relative to the erythrocyte genotype showing the highest number of rosettes for each binding construct. A: Erythrocytes from Papua New Guinea. B: Erythrocytes from North Americans. A/A: *FY**A/*FY**A; A/-: *FY**A/*FY**A^{null}; B/B: *FY**B/*FY**B; B/-: *FY**B/*FY**B^{null}; -/-: *FY**B^{null}/*FY**B^{null}.

detected by using monoclonal antibodies 1D3 and DL6 against HSVgD1 epitopes remaining in the expression construct [20]. COS-7 cells transfected with pEGFP-DBP_{II} were observed with an epifluorescence Nikon microscope (Fig. 1B).

2.5. Transfection of COS-7 cells and binding assays

COS-7 cells were transferred into 6-well culture plates and then transfected with plasmid DNA (1 µg per well) using Lipofectamine (Gibco BRL) in Dulbecco's modified Eagle's medium (DMEM) (Sigma). At 24 h after transfection, medium was replaced by DMEM with 10% fetal bovine serum and cells were incubated for a further 24 h. After this treatment the transfected cells were incubated for 2 h at room temperature with human erythrocytes of different Duffy phenotypes (1% final suspension, previously washed in DMEM without serum). Cells were washed three times with phosphate-buffered saline to remove non-adherent erythrocytes. In 30 fields of 200× magnification, rosettes of adherent erythrocytes were scored as positive when erythrocytes bound to more than 50% of the cell surface.

2.6. Statistical analysis

Statistical analyses were performed using StatView, version 4.51 (Abacus Concepts, Inc., Berkeley, CA, USA).

3. Results

P. vivax DBP region II (PvDBP_{II}) mediates adhesion to human erythrocytes that have DARC on their surface. Erythrocyte adhesion by region II of the *P. falciparum* EBA-175 (PfEBA_{II}) depends on a sialic acid-dependent receptor on glycoprotein A [19]. Erythrocyte cytoadherence activity of both parasite ligand domains can be assayed when expressed on the surface of COS-7 cells as chimeric molecules with the HSVgD1. Human erythrocytes representing different Duffy blood group alleles were overlaid on the transfected COS-7 cells and cytoadherence activity was compared (Fig. 1A). The PvDBP_{II} expressed on the surface of COS-7 cells did not bind homozygous Duffy-negative erythrocytes (Fig. 2B). A significant decline in binding activity was observed with the heterozygous Duffy-positive/negative genotypes, both *FY**A/*FY**A^{null} and *FY**B/*FY**B^{null}, when compared to the homozy-

gous Duffy-positive erythrocytes (FY^*A/FY^*A) and (FY^*B/FY^*B) (Fig. 2A,B). In contrast, erythrocyte cytoadherence to PfEBA_{II} was not affected by any Duffy phenotype, (FY^*A/FY^*A^{null}), (FY^*B/FY^*B^{null}), (FY^*A/FY^*A), (FY^*B/FY^*B), and $FY^*B^{null}/FY^*B^{null}$ (Fig. 2A,B and data not shown).

A more sensitive assay was then used to confirm the significant difference of DBP erythrocyte cytoadherence for Duffy-positive homozygotes (FY^*B or FY^*A) versus heterozygous (FY^*A or FY^*B and FY^*A^{null} or FY^*B^{null}) donor erythrocytes. The PvDBP_{II} expressed as a recombinant fusion protein to the N-terminus of the GFP allowed better assessment of DBP cytoadherence activity [18]. Erythrocytes originating from North American volunteers and processed under optimal conditions were used in the cytoadherence assay as an added assurance of accurately measuring differences in DBP cytoadherence properties for the different Duffy phenotypes irrespective of their FY^*A or FY^*B alleles. In these assays, cytoadherence between the DBP ligand domain and erythrocytes from Duffy promoter heterozygous donors was significantly reduced (Fig. 3). As expected, erythrocytes from the homozygous Duffy-negative individuals did not bind DBP (Fig. 3).

4. Discussion

Malaria is an important selective force for human genetic adaptations due to the sustained, lethal impact that it has had on human populations around the world. Homozygosity for the Duffy-negative blood group antigen confers complete resistance to vivax malaria. Nevertheless, it is unclear whether selective pressure of vivax malaria alone was the main cause for the emergence and fixation of the $FY^*B^{null}/FY^*B^{null}$ genotype in much of West Africa where vivax malaria is absent. Indeed there is little doubt that malaria caused by *P. vivax* is not as directly lethal as *P. falciparum*, but a fulminant *P. vivax* infection still causes serious morbidity especially in those

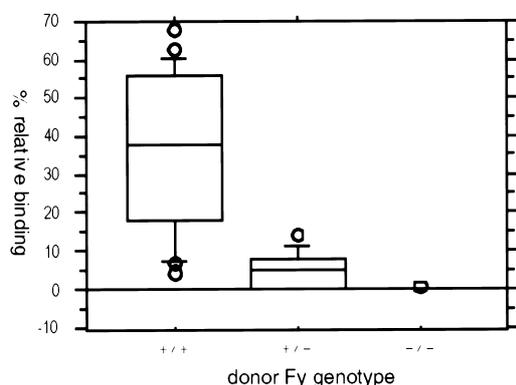


Fig. 3. Binding to *P. vivax* DBP_{II} expressed on the surface of COS-7 cells (pEGFP plasmid) by human erythrocytes from North American volunteers of different Duffy antigen negativity genotypes (regardless of FY^*A and FY^*B alleles). Binding is expressed as the relative number (%) of erythrocyte rosettes per individual sample compared to a separate positive control. The box plots depict percentiles for percent binding of erythrocytes for each FY promoter genotype, 90th (upper whisker), 75th (upper boundary of box), 50th (median, central line in box), 35th (lower boundary of box), and 10th (lower whisker). A Kruskal–Wallis test, evaluating the difference among all three genotypes, gave $P > 0.0001$. A Mann–Whitney test, evaluating the two genotypes of +/+ to +/-, also gave $P > 0.0001$.

living at the minimum subsistence level. The emergence of an independent Duffy-negative blood group allele in a region of Papua New Guinea highly endemic for vivax malaria lends credence to the theory that vivax malaria can act as a selective agent [17].

Genetic epidemiological surveys conducted in Papua New Guinea have so far only found individuals in whom the FY^*A^{null} allele has been heterozygous with FY^*A . This new FY^*A/FY^*A^{null} genotype conferred a 50% decrease for Duffy antigen expression on their erythrocytes when compared to individuals who are homozygous Duffy-positive. When previous studies compared erythrocytes with different Duffy phenotypes from various hosts, a correlation was shown between erythrocyte invasion of a particular type of erythrocyte and its potential to bind the *P. vivax* DBP [7]. A similar phenomenon was observed with the *P. knowlesi* DBP cytoadherence activity and merozoite invasion efficiency.

Previous studies have confirmed that heterozygous Duffy-negative individuals remain susceptible to infections by *P. vivax* [21]. Using a DBP-specific in vitro cytoadherence assay we demonstrated that erythrocytes from FY^*A/FY^*A^{null} Papua New Guineans show a significant reduction in binding to the DBP ligand when compared to erythrocytes from FY^*A/FY^*A Papua New Guinea donors (Fig. 2A). The same was observed when using erythrocytes from heterozygous (FY^*B^{null}/FY^*B , FY^*B^{null}/FY^*A) North American donors (Figs. 2B and 3). These results support the previous observation [17] of an association between the heterozygous FY^*A/FY^*A^{null} genotype and a reduced prevalence of *P. vivax* infection. The prevalence of infection in the small study population was not statistically significant and probably will require a larger study population and inclusion of additional clinical parameters.

It is too early to affirm that the Duffy-negative allele was selected by the vivax malaria endemicity in the Papua New Guinea study population. Nevertheless, our results suggest that even at the heterozygous state the Duffy-negative allele can confer a quantifiable resistance advantage against *P. vivax*. Our previous study demonstrated that naturally acquired and vaccine-elicited antibodies blocked erythrocyte cytoadherence of the *P. vivax* DBP [18]. These data showed that a naturally acquired immunological response against the *P. vivax* DBP can effectively block the vital receptor binding function of this merozoite protein. Unfortunately, such an effective high titer anti-DBP inhibitory response was found to be relatively rare (~10%) even among residents living in highly endemic regions of Papua New Guinea. Instead, individuals from this region were characterized by a poorly inhibitory, low titer antibody response [22]. Overall, our studies suggest that the heterozygous FY^*A/FY^*A^{null} genotype may enhance efficacy of a poorly inhibitory anti-DBP antibody response. Potentially, FY^*A/FY^*A^{null} individuals are then better able to control the *P. vivax* growth rate and suffer less morbidity from infection.

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