Published in final edited form as:

J Parasitol. 2010 February ; 96(1): 225–227. doi:10.1645/GE-2281.1.

***Plasmodium vivax* DBP binding to *Aotus nancymae* erythrocytes is Duffy antigen dependent**

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Abstract

Plasmodium vivax is the second leading cause of malaria worldwide. Invasion of human erythrocytes by *P. vivax* merozoites is dependent upon the interaction between the parasite Duffy binding protein (PvDBP) and the erythrocyte Duffy antigen receptor. Therefore, disruption of this vital interaction is an attractive target for therapeutic intervention. Although *Aotus nancymae* is a commonly used primate model for human *P. vivax* infections, it has not been confirmed that the interaction between *Ao. nancymae* erythrocytes and *P. vivax* is Duffy antigen dependent. Our results indicate that normal *Ao. nancymae* erythrocytes readily bind to PvDBP_{II} and that this binding is completely abolished with chymotrypsin treatment of the erythrocytes. Furthermore, the results of our inhibition assays show a dose-dependent decrease in binding with increasing amounts of anti-PvDBP_{II} polyclonal rabbit sera or anti-Fy6 monoclonal antibody. These data indicate that the interaction between *Ao. nancymae* erythrocytes and *P. vivax* DBP_{II} is Duffy antigen dependent, validating this model system for *in vivo* studies of anti-PvDBP inhibition.

Plasmodium vivax results in an estimated 70–80 million cases of malaria annually, with an estimated 2.6 billion people at risk for infection (Mendis et al., 2001; Guerra et al., 2006). *Plasmodium vivax* is seasonally transmitted and produces little sustained immunity, resulting in repeated infections throughout childhood and adulthood. These recurrent infections are rarely lethal, but produce a significant physical, economic and educational burden for affected individuals (Mendis et al., 2001; Fernando et al., 2003). Reports of emerging drug resistance along with reported cases of severe clinical complications due to *P. vivax* infection also point to *P. vivax* as an important target of vaccine development (Baird, 2007; Barcus et al., 2007; Kochar et al., 2009).

Invasion of host erythrocytes by *Plasmodium* merozoites is a complex, multi-step process involving specific interactions between parasite ligands and host cell receptors (Dvorak et al., 1975; Bannister and Dluzewski, 1990; Barnwell and Galinski, 1995; Chitnis and Blackman, 2000; Galinski, 2005). *Plasmodium vivax* invasion of host erythrocytes is dependent on the interaction between the microneme Duffy binding protein (specifically region II, PvDBP_{II}) and the host Duffy antigen receptor (Miller et al., 1976; Haynes et al., 1988; Barnwell et al., 1989; Wertheimer and Barnwell, 1989; Adams et al., 1990; Adams et al., 1992; Chitnis and Miller, 1994). Individuals who do not express the Duffy antigen on their erythrocytes appear completely protected from blood-stage infection by *P. vivax* (Miller et al., 1976). The parasites' reliance on this interaction makes it an attractive target for therapeutic intervention.

Vaccine testing necessitates the use of appropriate animal models. *Plasmodium* parasites infect a wide variety of hosts in a species-specific manner, including rodents, chickens and primates, among others. *Plasmodium vivax* is known to infect a number of primates, including *Aotus nancymae*, a commonly used model for *Plasmodium* vaccine testing (Collins et al., 2005; Darko et al., 2005; Williams et al., 2005; Collins et al., 2006; Makobongo et al., 2006; Singh et al., 2006; Rojas Caraballo et al., 2007). However, invasion of *Ao. nancymae* erythrocytes has not been shown to be Duffy antigen dependent. Confirmation of this interaction is pertinent because *Saimiri boliviensis* monkeys, which are also used as a model of human *Plasmodium* infection, particularly for *P. vivax*, are infected by *P. vivax* in a non-Duffy antigen dependent fashion (Barnwell et al., 1989; Wertheimer and Barnwell, 1989). The DBP ligand does not bind to *Saimiri sp.* erythrocytes. In order to validate the *Aotus* system for *P. vivax* vaccine testing, we characterized the binding interaction between intact *Ao. nancymae* erythrocytes and recombinant PvDBP β II protein expressed on the surface of COS7 cells. Our results indicate that the interaction between *Ao. nancymae* erythrocytes and PvDBP β II is Duffy antigen dependent, validating this model for *in vivo* studies of anti-PvDBP inhibition.

Salvador I strain PvDBP β II DNA was cloned into the pEGFP-N1 plasmid with flanking signal sequences from the herpes simplex virus glycoprotein D1 allowing expression of a green fluorescent protein (GFP) fusion protein on the surface of transiently transfected COS7 cells (Michon et al., 2000). A negative control plasmid, pEGFP-PkDBP β II, was cloned in a similar fashion. Recombinant plasmid DNA was purified using an endotoxin-free plasmid DNA purification system (Qiagen, Valencia, California).

COS7 cells were plated in 24-well plates at a density of 40,000 cells per well and grown for 3 hrs at 37°C, 5% CO₂. The COS7 cells were then transiently transfected with endotoxin-free pEGFP-PvDBP β II DNA (37.5ng/well) using Lipofectamine (Invitrogen, Carlsbad, California) in incomplete Dulbecco's modified Eagle medium (DMEM; Sigma, St. Louis, Missouri). Sixteen hours post-transfection, the medium was replaced with complete DMEM (10% fetal bovine serum (FBS)). Forty-two hours post-transfection, the transfected COS7 cells were incubated with erythrocytes for 2 hrs at room temperature (1% final suspension, previously washed 3 times with incomplete DMEM). Wells were washed 3 times with PBS to remove nonadherent erythrocytes and binding was scored by counting the number of rosettes per 30 fields of view at 200x magnification. Inhibition assays were carried out in the same manner except that transfected COS7 cells were preincubated with antisera for 1 hr at 37°C, 5% CO₂ prior to addition of the erythrocyte suspension.

Aotus nancymae erythrocytes were collected into ACD Solution A. Immediately prior to incubation with transfected COS7 cells the erythrocytes were washed 3 times with incomplete DMEM and added to a final suspension of 1%. Chymotrypsin treatment of erythrocytes was carried out by first washing 3 times with RPMI-1640 and then adding chymotrypsin to a final concentration of 1mg/mL, 50% hematocrit. This suspension was incubated at 37°C for 1 hr with rocking and then rinsed with RPMI-1640. Soybean trypsin inhibitor was added to a final concentration of 0.5mg/mL, 50% hematocrit and incubated at room temperature for 10 min with rocking. Erythrocytes were then washed 2 times with RPMI-1640 before addition to transfected COS7 cells.

Antiserum against recombinant PvDBP β II was produced as previously described (Grimberg et al., 2007) and kindly provided by Dr. Christopher King of Case Western Reserve University. Anti-Fy6 monoclonal antibody (mAb) was produced as previously described (Nichols et al., 1987). Anti-Fy6 mAb was precipitated from ascites with addition of saturated ammonium sulfate to 45% (v/v) and then dissolved in and dialyzed against PBS. This was performed 2 times resulting in a final concentration of 0.45mg/mL. Statistical analyses were performed using the Prism 4 program (GraphPad Software, La Jolla, California). Results were analyzed

using 1-way analysis of variance (ANOVA) and Tukey's post-test. Results from inhibition assays were first normalized to the control as percentages.

In order to determine if *Ao. nancymaae* erythrocytes interact with the parasite produced PvDBP, we initially examined the binding of *Ao. nancymaae* erythrocytes to recombinant PvDBP_{II} as compared to recombinant PkDBP_{II}. PkDBP_{II} is a PvDBP paralogue found in *P. knowlesi*, having more than 70% sequence identity (Adams et al., 1992). PkDBP_{II} does not bind to human erythrocytes, although it readily binds *Rhesus* erythrocytes (Barnwell et al., 1989). Binding assays were carried out by transiently transfecting adherent COS7 cells with either pEGFP-PvDBP_{II} or pEGFP-PkDBP_{II}, incubating with *Ao. nancymaae* erythrocytes and then determining the binding phenotype by counting the number of erythrocyte rosettes. *Aotus nancymaae* erythrocytes showed the same specificity of binding as human erythrocytes, readily binding to PvDBP_{II}, but not at all to PkDBP_{II} (Fig. 1). Chymotrypsin treatment removes the Duffy antigen receptor from erythrocytes (Barnwell et al., 1989). In order to determine if *Ao. nancymaae* erythrocytes interact with PvDBP via the Duffy antigen receptor, we performed binding assays in which we compared untreated erythrocytes with chymotrypsin-treated erythrocytes. Chymotrypsin treatment of *Ao. nancymaae* erythrocytes completely abolished binding to PvDBP_{II} (Fig. 2).

To further show that *Ao. nancymaae* erythrocytes interact specifically with PvDBP, we performed inhibition assays using polyclonal rabbit sera raised against recombinant PvDBP_{II} protein (Grimberg et al., 2007). *Aotus nancymaae* binding to PvDBP_{II} is inhibited by anti-PvDBP_{II} sera in a dose-dependent fashion (Fig. 3). We also performed inhibition assays using anti-Fy6 mAb, which specifically binds to the Duffy antigen receptor. Blocking of the Duffy antigen receptor using anti-Fy6 mAb disrupts binding of *Ao. nancymaae* erythrocytes in a dose-dependent manner (Fig. 4).

Reports of drug resistance in *P. vivax* strains along with reports of severe clinical malaria cases due to *P. vivax*, call for an increased focus on *P. vivax* vaccine development (Baird, 2007; Barcus et al., 2007; Kochar et al., 2009). One of the main targets of *P. vivax* vaccine research is the Duffy binding protein, a parasite ligand required for erythrocyte invasion (Miller et al., 1976; Haynes et al., 1988; Wertheimer and Barnwell, 1989; Adams et al., 1990; Adams et al., 1992; Chitnis and Miller, 1994). Testing of an anti-PvDBP vaccine will require an appropriate animal model. This model will need to have a similar course of disease as human *P. vivax* infections as well as interact with *P. vivax* parasites in a Duffy dependent fashion. *Plasmodium vivax* is capable of infecting a variety of primate erythrocytes, however not all of these infections are dependent on the interaction between PvDBP and the Duffy antigen (i.e. *P. vivax* infection of *Saimiri* monkeys (Barnwell et al., 1989)). Because of this possibility, it is important to confirm that the primate model used for testing anti-PvDBP vaccines is dependent on the PvDBP-Duffy antigen interaction.

We performed a variety of experiments to confirm that *Ao. nancymaae* erythrocytes interact with PvDBP in a Duffy-dependent manner. We found that *Ao. nancymaae* erythrocytes readily bind to PvDBP_{II} expressed on the surface of COS7 cells and chymotrypsin treatment, which removes the Duffy antigen, completely abolishes binding (Figs. 1 & 2). We also determined that specifically blocking either the PvDBP_{II} (with anti-PvDBP_{II} sera) or the Duffy antigen (with anti-Fy6 mAb) inhibits binding of *Ao. nancymaae* erythrocytes in a dose-dependent fashion (Figs. 3 & 4). These results indicate that the interaction of *Ao. nancymaae* erythrocytes with PvDBP is via the Duffy antigen receptor, validating this model for use in testing anti-PvDBP vaccines.

Acknowledgments

We would like to thank Christopher L. King of Case Western Reserve University for providing the anti-PvDBP-II rabbit sera. This work was funded, in part, by a National Institutes of Health grant (R01 AI33656) to J.H.A. and an Arthur J. Schmitt Presidential Fellowship to A.M.M.

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FIGURE 1.

Aotus nancymae erythrocytes bind specifically to PvDBP II. Results are shown for four experiments performed in triplicate.

FIGURE 2.

Chymotrypsin removal of the Duffy antigen receptor abolishes binding of *Aotus nancymaae* erythrocytes to PvDBPII. Results are shown for two experiments performed in triplicate.

FIGURE 3.

Blocking of PvDBPII with polyclonal anti-PvDBPII rabbit sera inhibits binding of *Aotus nancymae* erythrocytes to PvDBPII in a dose-dependent manner. Inhibition is shown as a percentage of the control (sera from a naïve rabbit). Results are shown for four experiments performed in triplicate.

FIGURE 4.

Blocking of the Duffy antigen receptor with anti-Fy6 mAb inhibits binding of *Aotus nancymae* erythrocytes to PvDBPII in a dose-dependent manner. Inhibition of binding is shown as a percentage of the control (no sera). Results are shown for four experiments performed in triplicate.