IN VITRO EVALUATION OF THE ROLE OF THE DUFFY BLOOD GROUP IN ERYTHROCYTE INVASION BY *PLASMODIUM VIVAX*

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All the clinical and pathologic features of malaria are solely attributable to the parasitic stages of the asexual erythrocytic cycle. The propagation of this cycle in a host is dependent upon extracellular merozoites attaching to and invading susceptible erythrocytes. This attachment and initiation of invasion by malaria merozoites is mediated through specific interactions between parasite receptors and ligand molecules on the erythrocyte plasma membrane (1, 2). The identification of erythrocyte ligands used by invading merozoites is important to understanding this biologic event at the molecular level. Furthermore, this knowledge can aid in the isolation of malaria parasite receptor antigens; potential targets of vaccine induced immunity.

Studies on *Plasmodium knowlesi*, a simian malaria that can invade human erythrocytes, have indicated that an erythrocyte membrane component associated with Duffy blood group determinants is used by this species as a ligand for merozoite invasion (3–5). These studies on a simian malaria became more relevant to human malaria when indirect and retrospective studies in vivo also correlated susceptibility to infection by *Plasmodium vivax*, a human malaria parasite of major importance, with the presence of the Duffy blood group antigens (6, 7).

Direct ligand studies with *P. vivax* could not be done, however, because these parasites were difficult to obtain and could not be cultured in vitro, as has been possible with *Plasmodium falciparum* and *P. knowlesi*. Here we describe a short term invasion assay in vitro for *P. vivax* parasites obtained from squirrel (*Saimiri sciureus*) monkeys. We have made use of this invasion assay to investigate the role of the Duffy blood group as an erythrocyte ligand for *P. vivax* merozoites using human and simian erythrocytes of varying Duffy phenotypes and a mAb against a new Duffy blood group determinant, Fy6 (8).

Materials and Methods

Parasites and Cultures. *P. knowlesi* (H strain) parasites were cryopreserved in liquid nitrogen as ring stage infected erythrocytes from rhesus monkeys (9). Cryopreserved infected erythro-

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cytes for use in invasion assays in vitro were thawed and cultured to the schizont stage in tissue culture medium RPMI 1640 supplemented with 30 mM Hepes, 2 g/liter D-glucose, 50 mg/liter hypoxanthine, and 15% horse serum (HyClone Laboratories, Losan, UT), or 15% human AB serum (complete medium) in an atmosphere of 5% CO_2/5% O_2/90% N_2 at 37°C for 18–20 h.

P. falciparum (FCR-3) parasites were cultured in vitro in human O^+ erythrocytes and RPMI 1640 complete medium with 10% human serum (10). *P. vivax* (Belem) parasites were obtained from squirrel monkeys with synchronous infections when the majority of parasites were at the trophozoite stage. The heparinized blood with added ADP (1 mg/ml) was passed sequentially over acid-washed glass bead (0.11 mM diameter) and Whatman CF11 cellulose columns to remove platelets and leukocytes and then centrifuged on 54% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) to concentrate parasitized erythrocytes to >90% purity (9). Maturation of the parasites to mature schizonts was accomplished in RPMI 1640 supplemented with hypoxanthine (50 mg/liter), D-glucose (2 g/liter) (HyClone Laboratories), Hepes or TES (35 mM), 10% human AB serum, and 10% fetal calf or horse serum (HyClone Laboratories) as above for *P. knowlesi*.

Invasion Assays. *P. falciparum* late trophozoite and early schizont infected erythrocytes were concentrated to 80% parasitemia on plasmagel and adjusted to 10^8/ml in medium. Target cells at 10^8/ml were mixed with parasitized erythrocytes at a ratio of 10:1 and cultured in 0.5 ml volumes in 24-well tissue culture plates. *P. knowlesi* schizont–infected erythrocytes were concentrated to >95% parasitemia on a 54% Percoll cushion (9), adjusted to 5×10^7/ml, mixed with target cells (5×10^7/ml) at a 1:10 ratio, and cultured in 0.5 ml medium volumes in 24-well tissue culture plates. *P. vivax* schizont-infected erythrocytes were mixed with target cells at 1:5 to 1:10 ratios at 5×10^7/ml and cultured as above. Uninfected erythrocytes for use in *P. vivax* invasion assays were first processed on 62% Percoll cushions to increase the percentage of reticulocytes. The less dense erythrocytes at the Percoll interface were used as target cells in assays of invasion. *P. falciparum* cultures were harvested after 12 h for blood film preparation. *P. knowlesi* and *P. vivax* cultures were harvested after 8–10 h. After Giemsa staining of thin film smears, 1,000–2,000 erythrocytes were examined by light microscopy and the number of ring stage parasites was determined. Smears of the infected erythrocytes/target cell mixtures were also made at the start of the invasion assays and the number of ring stage parasites were determined. Any background invasion rates found in these wells were subtracted from the invasion rates determined in the test wells after 8–12 h of incubation.

mAbs and Target Erythrocytes. mAb K6H9 (BG6) recognizes a new determinant (Fy6) on a 43–46-kD erythrocyte membrane component of Duffy-positive erythrocytes that is unrelated to the other recognized Duffy blood group antigen determinants Fy^a, Fy^b, Fy3, and Fy5 (8). mAbs G10 and F7 recognize Kell blood group antigens K-14 and K-2 (k) that are on a 93-kD erythrocyte membrane protein (10). mAb 10-22 recognizes a determinant on erythrocyte glycophorin that is absent from En(a−) and Wr(b−) erythrocytes and mAb 4-21 recognizes a determinant that is absent from Wr(b−) but not En(a−) erythrocytes (Nichols, M., unpublished data). All mAbs were nonagglutinating. Human antisera specific for Fy^a (titer 1:256) was obtained from Ortho Pharmaceuticals (Raritan, NJ) and absorbed with human Duffy-negative erythrocytes before use. Fab fragments were obtained by treating affinity-purified mAb K6H9 with cysteine-activated papain immobilized on agarose beads (Sigma Chemical Co., St. Louis, MO). After digestion, the papain-treated supernatant was processed over a MAPS protein A–affi gel column (Bio-Rad Laboratories, Richmond, CA) to remove the Fc fragments. The Fab preparation of mAb BG6 was judged to be pure by SDS-PAGE and silver strain analysis. Erythrocytes (2×10^9) were sensitized with 2.0 ml of mAbs (25 μg antibody/ml) or 0.5 ml of human anti-Fy^a serum at 25°C for 1–2 h with mixing, washed twice in tissue culture medium, and then used as target cells in invasion assays. Human erythrocytes and simian erythrocytes had been serotyped by M. E. Nichols at the New York Blood Center.

Enzymatic Treatment of Erythrocytes. Erythrocytes (2×10^9) were pretreated with either trypsin (0.5 mg), chymotrypsin (0.5 mg), or neuraminidase (100 mU) for 1 h at 37°C. After enzyme treatment erythrocytes were incubated for 10 min, in 1 ml PBS containing either 1 mg/ml soybean trypsin inhibitor or 5 mM chymostatin for the trypsin- or chymotrypsintreated erythrocytes, respectively and then washed three times in RPMI 1640 tissue culture medium. Control erythrocytes were also incubated for 10 min in either soybean trypsin inhibitor or chymostatin. Neuraminidase (*V. cholerae*), trypsin, and chymotrypsin were purchased from Calbiochem-Behring Corp., San Diego, CA and inhibitors from Sigma Chemical Co., St. Louis, MO.

Results

P. vivax trophozoite–infected erythrocytes were collected from squirrel monkeys with synchronized infections and grown in vitro to fully developed mature schizonts (Fig. 1). Table I shows the results of four successful invasion trials where the in vitro matured parasites were mixed with fresh human erythrocytes, recultured, and examined after an additional 8 to 10 hours. The percentage of schizont-infected erythrocytes at this time has decreased with a corresponding increase in the percentage of erythrocytes showing typical *P. vivax* young ring stage parasites (Fig. 1). Generally, the overall percent parasitemia has actually decreased at this time, but this is incidental to the assessment of invasion that is made by enumeration of new ring stage parasites. These results made it possible to study directly in vitro the role of Duffy blood group antigens as a ligand for invasion by *P. vivax* merozoites.

Human Duffy-positive [Fy(a^+b^+)] and human Duffy-negative [Fy(a^-b^-)] erythrocytes along with various simian erythrocytes were used as target cells for invasion by *P. vivax* and *P. knowlesi* merozoites in vitro. As shown in Table II, *P. vivax* invaded the Fy6 positive erythrocytes of humans and both *Saimiri* and *Aotus* monkeys, but not $Fy6^-$ erythrocytes of humans and rhesus. As has been shown previously (12, 13), *P. knowlesi* invaded the Fy:b^+,3^+,6^- erythrocytes of rhesus, as well as the Fy:6^+ cells of *Saimiri* and *Aotus*, but not human Duffy-negative erythrocytes. A preference for rhesus erythrocytes over human, *Saimiri*, and *Aotus* erythrocytes is shown for *P. knowlesi*.

Treatment of Fy(a^+b^+) human erythrocytes with chymotrypsin abolished susceptibility to invasion by *P. vivax* merozoites with a 91% decrease in invasion in comparison to untreated control erythrocytes. Enzymatic treatment of the human Fy(a^+b^+) erythrocytes with trypsin or neuraminidase had no effect on invasion. Similar results were obtained for *P. knowlesi* as has been reported previously (3, 4).

To further assess the possible role of the Duffy erythrocyte membrane component

TABLE I

In Vitro Invasion of Human Erythrocytes by P. vivax

	Developmental stage* and total parasitemia					
	Immediately after addition of fresh RBCs			8–10 h after addition of fresh RBCs		
Exp.	Sz	R	P	Sz	R	P
		%			%	
1	88	1	9.2	10	86	3.6
2	96	1	11.2	7	93	9.6
3	82	1	14.5	16	82	8.7
4	95	2	12.1	4	96	15.0

* Sz, schizonts; R, ring stages; P, total parasitemia.

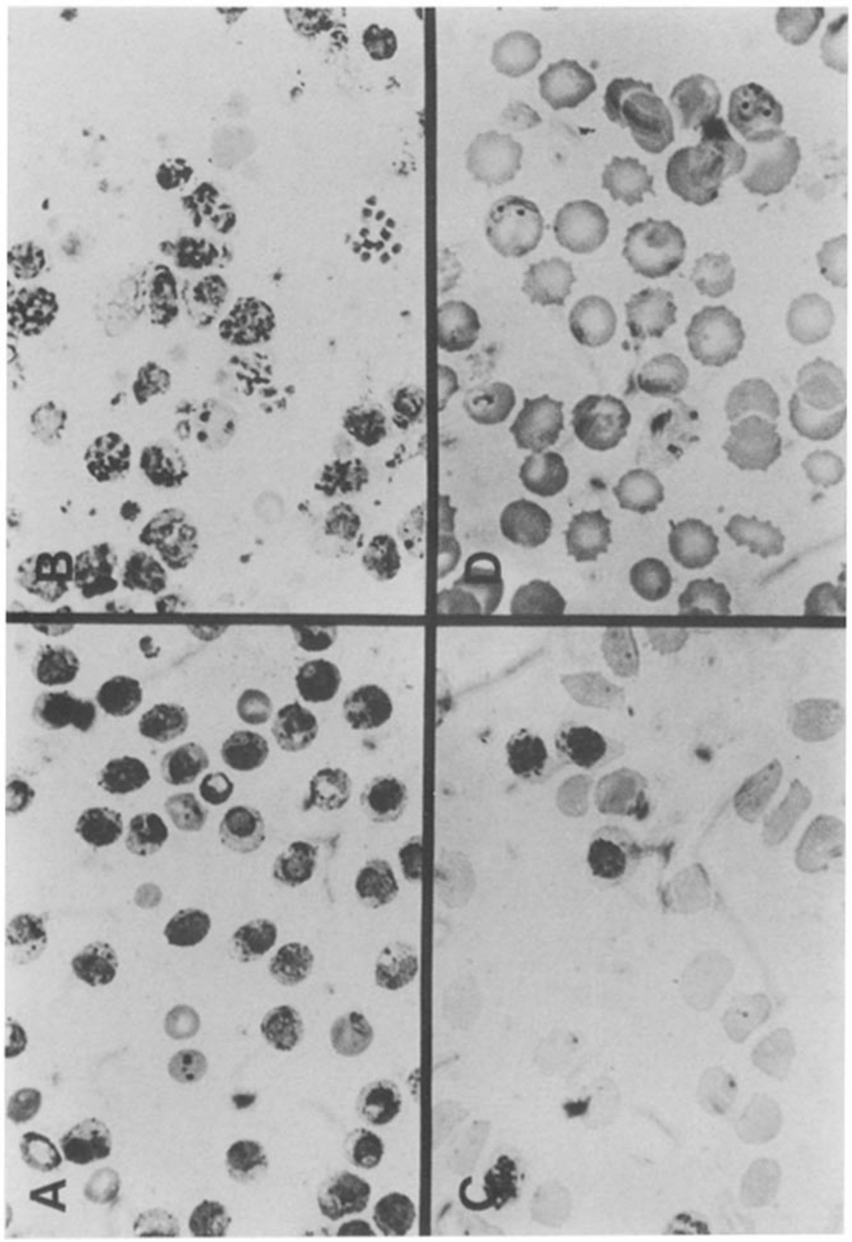

FIGURE 1. (*A*) *P. vivax* late trophozoite stage infected erythrocytes purified on 54% Percoll. (*B*) In vitro maturation of *P. vivax* to mature schizont stage. (*C*) Mature schizont-infected erythrocytes diluted with target cells. (*D*) *P. vivax* cultures 8 h after addition of target erythrocytes.

TABLE II

Invasion of Human and Simian Erythrocytes of Defined Duffy Phenotype by P. vivax and P. knowlesi Merozoites

Erythrocytes species	Duffy phenotype				Invasion rate	
	Fy^a	Fy^b	Fy3	Fy6	*P. vivax*	*P. knowlesi*
					%	
Human	+	+	+	+	8.7	12.8
Human	–	–	–	–	0.1	0.2
Rhesus	–	+	+	–	<0.1	27.4
Saimiri	–	–	+	+	7.8	8.3
Aotus	–	+	+	+	5.1	6.9

TABLE III

Effect of mAbs to Duffy and Other Erythrocyte Antigens on Invasion of Human Erythrocytes by P. vivax, P. knowlesi, and P. falciparum Merozoites

Species of plasmodium	Percent inhibition of invasion* by mAbs against:				
	Duffy (Fy6) determinant (mAb BG6)	Kell (K2) determinant (mAb F7)	Kell (K14) determinant (mAb G10)	En^a determinant (mAb 10-22)	Wr^b determinant (mAb 4-21)
P. vivax	84 ± 9 (4)‡	−5 ± 6 (3)	5 ± 4 (3)	−6 ± 8 (2)	0 ± 6 (2)
P. knowlesi	90 ± 5 (4)	6 ± 5 (3)	−2 ± 6 (3)	7 ± 4 (2)	10 ± 8 (2)
P. falciparum	−7 ± 3 (2)	7 ± 3 (2)	18 ± 13 (2)	88 ± 4 (2)	−5 ± 5 (2)

* Calculated on the basis of ring stage parasitemias in control cultures with unsensitized target erythrocytes.
‡ Number of individual experiments.

as a ligand for *P. vivax* merozoites we sensitized human Duffy-positive erythrocytes [$Fy(a^+b^+)$] with mAb K6H9 (anti-Fy6) or, as controls, mAbs reactive with Kell (K2, K14) or glycophorin [En(a), Wr(b)] determinants for use in invasion assays (Table III). mAb K6H9 inhibited invasion of both *P. vivax* and *P. knowlesi*, 84 and 90% respectively. The control mAbs showed no significant inhibition of invasion of *P. vivax* and *P. knowlesi* merozoites. Conversely, the anti-Fy6 mAb did not inhibit the invasion of *P. falciparum* merozoites, whereas mAb 10-22 [anti-En(a)] did inhibit the invasion of this *Plasmodium* species by 90%. Human $Fy(a^+b^+)$ erythrocytes sensitized with F(ab) fragments of mAb K6H9 also showed a decrease in invasion with both *P. vivax* (88%) and *P. knowlesi* (84.4%).

mAb K6H9 also blocked invasion of *P. vivax* into *Aotus* erythrocytes and *Saimiri* erythrocytes by 41% and 37.8%, respectively, whereas *P. knowlesi* invasion of *Aotus* and *Saimiri* erythrocytes was inhibited 34.2 and 84.7%, respectively. Anti-Fy^a human serum partially inhibited *P. vivax* invasion of $Fy(a^+b^+)$ and $Fy(a^+b^-)$ human erythrocytes 39 and 52%, respectively. Invasion of $Fy(a^-b^+)$ cells incubated with anti-Fy^a serum was not significantly different from nonsensitized control cells.

Discussion

It has been hypothesized that *P. vivax* merozoites use a membrane component associated with the Duffy blood group as a ligand to gain entry into erythrocytes. Studies on Duffy-negative (Fya^-b^-) individuals voluntarily inoculated with *P. vivax* sporozoites or living in areas of high endemnicity found that these subjects never become infected with this human malaria species (6, 7). However, direct *in vitro* experiments with *P. vivax* merozoites were not done as this human malaria parasite could not be easily obtained nor cultured in vitro. We have recently developed a short term in vitro invasion assay for *P. vivax* and produced a mAb antibody, designated K6H9 (BG6), which recognizes an epitope, Fy6, that is unrelated to previously known epitopes on the Duffy blood group antigen (8). Together, these developments provide the first direct evidence that the Duffy antigen does play a role in the invasion of erythrocytes by *P. vivax* merozoites.

Three lines of evidence obtained in vitro indicate that the Duffy blood group antigen serves as a host ligand. First, *P. vivax* did not invade human erythrocytes of the Duffy-negative phenotype, while Duffy-positive erythrocytes were readily infected. *P. vivax* also invaded the erythrocytes of *Aotus* and *Saimiri* (squirrel) monkeys in vitro that lack Fy^a and/or Fy^b Duffy determinants but carry the Fy6 and Fy3 determinants on their cells (8, 12; Table II). In contrast, rhesus erythrocytes which are Fy^b and $Fy3^+$, but lack the Fy6 epitope, are not invaded by *P. vivax*. Second, treatment of Duffy-positive erythrocytes with chymotrypsin, which removes the Duffy determinants Fy^a, Fy^b, Fy6, but not Fy3, inhibits invasion by *P. vivax*. Trypsinization of human Duffy-positive erythrocytes does not remove any Duffy determinants and does not inhibit *P. vivax* invasion.

Third, antibody-mediated inhibition of invasion by mAb K6H9 provided direct evidence that the Duffy antigen serves as a ligand for *P. vivax* merozoites. mAb K6H9 inhibited invasion by both *P. vivax* and *P. knowlesi*, while mAbs against Kell or glycophorin blood group determinants did not block invasion. In addition, the anti-Fy6 mAb did not inhibit *P. falciparum* merozoite invasion. This malaria parasite uses glycophorin as a ligand and invasion was inhibited by the antiglycophorin mAb 10-22. These results indicated the blockage of invasion by mAb K6H9 was specific and did not result from a nonspecific event such as increased erythrocyte membrane rigidity produced by molecular crosslinking (3). Also, since the quantity of Duffy antigen exposed on the erythrocyte surface (12,000 specific antigen sites) is low (8) it is not likely that antibody blockade resulted by preventing the merozoite from interacting with another ligand in the membrane. The specificity of the antibody blockade was further confirmed by an equal inhibition of *P. vivax* and *P. knowlesi* invasion when either intact or F(ab) fragments of anti-Fy6 mAb are used.

The data presented here show that *P. vivax* merozoites use the Duffy glycoprotein (14) as a ligand during the process of invasion. Additionally, previous correlative data in vivo (8) and the in vitro results indicate that the Fy6 epitope or a nearby domain may be the functional site of interaction on the Duffy glycoprotein and a merozoite receptor protein. However, the Duffy ligand probably is not the only erythrocyte component required for *P. vivax* merozoite invasion of erythrocytes. *P. vivax* shows a very strong predilection for invading reticulocytes even though the Duffy glycoprotein is also present on mature erythrocytes. Thus, *P. vivax* may require at least two ligands, the Duffy antigen, and a separate component or characteristic of reticulocytes for the efficient invasion of human erythrocytes. The latter component of reticulocytes could function in initial merozoite attachment whereas the Duffy glycoprotein may be involved in junction formation (15, 16).

Furthermore, the partial inhibition of invasion (40%) of *P. vivax* into *Aotus* and

Saimiri erythrocytes by the anti-Fy6 mAb may even indicate an alternate mechanism of invasion by *P. vivax* in the case of simian erythrocytes. Evidence for the existence of alternate ligands and multiple receptors are known for *P. knowlesi* (5) and has been recently documented for *P. falciparum* (17, 18).

The Duffy Fy^a determinant, and now a new determinant, Fy6, have been localized to an erythrocyte membrane component, a glycoprotein, of ~43 kD (8, 14). Further studies on both the Duffy glycoprotein and the merozoite are needed to more accurately define the molecular interactions that occur during the invasion of erythrocytes by *P. vivax* and *P. knowlesi*. Identification of the corresponding merozoite Duffy receptor(s), potential immunogens in the development of a malaria vaccine, will be essential in defining the mechanism(s) of merozoite erythrocyte invasion at the molecular level.

Summary

A short-term in vitro culture system that allows for significant re-invasion of target erythrocytes by *Plasmodium vivax* was used to study the role of the Duffy blood group antigen as a ligand for merozoite invasion by this human malaria species. Using human Duffy-positive and -negative erythrocytes, various primate erythrocytes, enzymatic modification of erythrocytes, and mAb that defines a new Duffy determinant (Fy6) we conclude that the erythrocyte glycoprotein carrying Duffy determinants is required as a ligand for the invasion of human erythrocytes by *P. vivax* merozoites. Blockade of invasion by Fab fragments of the anti-Fy6 mAb equal to that of the intact molecule and the correlation of *P. vivax* susceptibility with the presence of the Fy6 determinant suggests this epitope or a nearby domain may be an active site on the Duffy glycoprotein. However, as for *P. knowlesi*, there is evidence that an alternate pathway for *P. vivax* invasion of simian erythrocytes may exist.

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