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Glycophorin B as an EBA-175 independent *Plasmodium falciparum* receptor of human erythrocytes

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Abstract

Invasion of erythrocytes by malaria parasites involves multiple receptor-ligand interactions. To elucidate these pathways, we made use of four parasite clones with differing specificities for invasion, erythrocytes that are mutant for either glycophorin A or B, and enzyme modification of the erythrocyte surface with neuraminidase and trypsin. Neuraminidase alone abolishes invasion of two parasite clones (Dd2, FCR3/A2); these invade after trypsin treatment alone. A third clone (7G8) is unable to invade trypsin-treated erythrocytes. The fourth clone (HB3) can invade after either neuraminidase or trypsin treatment. The receptor for invasion of trypsin-treated erythrocytes was explored in two ways: treatment of trypsin-treated normal cells with neuraminidase, and trypsin treatment of glycophorin B-deficient cells. Both treatments eliminated invasion by all clones, indicating that the trypsin-independent pathway uses sialic acid and glycophorin B. To identify parasite proteins involved in the different pathways, erythrocyte binding assays were performed with soluble parasite proteins from each clone. Based on binding assays using erythrocytes that lack glycophorin A, the parasite protein known as EBA-175 appears to bind predominantly to glycophorin A. In contrast, the glycophorin B pathway does not appear to involve EBA-175, as binding of EBA-175 was similarly reduced to trypsin-treated normal and trypsin-treated glycophorin B-deficient erythrocytes. Thus, the glycophorin B-dependent, sialic acid-dependent invasion of trypsin-treated normal erythrocytes uses a different parasite ligand, indicating two or more sialic-dependent pathways for invasion. Clone 7G8, which cannot invade trypsin-treated erythrocytes, may be missing the ligand for invasion via glycophorin B. Redundancy in the invasion process may give a selective advantage to parasites that must survive in polymorphic human populations.

Key words: Erythrocyte invasion; Ligand; *Plasmodium falciparum*; Receptor

1. Introduction

Plasmodium falciparum invades and multiplies in erythrocytes. Parasite ligands of the invasive-stage merozoite interact with erythrocyte recep-

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tors to mediate invasion. Sialylated molecules on the erythrocyte surface are erythrocyte receptors, as neuraminidase treatment of erythrocytes reduces or eliminates invasion, depending on the parasite clone studied [1–6]. Parasite ligands able to bind erythrocyte sialylated molecules have been identified using soluble proteins present in malaria culture supernatants for the binding assay [7]. Indeed, those parasite proteins that bind normal erythrocytes but fail to bind neuraminidase-treated erythrocytes are candidates for these ligands. To date, two proteins have been identified that fit this pattern: the erythrocyte binding protein of 175 kDa (EBA-175) [7] and the major surface protein on the merozoite (MSP-1) [8].

Previous work also demonstrated that a trypsin-sensitive pathway that was sialic acid-independent is used by the parasite to invade erythrocytes [5,6]. This conclusion derived from the fact that invasion by one parasite clone, 7G8, into neuraminidase-treated erythrocytes was minimally affected, whereas there was no invasion into trypsin-treated erythrocytes. Most parasite clones, however, invade trypsin-treated erythrocytes, although efficiency of invasion is much reduced [6,9,10]. The receptor molecules involved in residual invasion of trypsin-treated erythrocytes were explored by the study of invasion of trypsin-treated erythrocytes deficient in glycophorin B, an erythrocyte glycoprotein that is not cleaved by trypsin [11]. *P. falciparum* was unable to invade trypsin-treated glycophorin B-deficient erythrocytes [9,10]. Here, we report the further analysis of the erythrocyte receptors and parasite ligands involved in the residual invasion of trypsin-treated erythrocytes. We show the following: (1) the pathway for invasion of trypsin-treated erythrocytes is sialic acid-dependent; (2) this pathway involves glycophorin B; and (3) the parasite ligand for this pathway does not involve EBA-175.

2. Materials and methods

Plasmodium falciparum parasites. *P. falciparum* clones Dd2 [12], HB3 [13], 7G8 [14], and FCR3/A2 [15] have been described and fingerprinted [16]. In vitro cultivation of the parasites was performed using standard methods [17].

Erythrocytes. Normal erythrocytes were obtained from one donor (SAD) who was blood group MN. The erythrocytes were drawn into citrate-phosphate-dextrose (CPD, Baxter-Fenwall, Deerfield, IL) solution, the serum was removed, and the erythrocytes were washed three times in RPMI 1640 (Gibco, Grand Island, NY). The cells were stored at 40–50% hematocrit in RPMI 1640 at 4°C until used.

En(a−) erythrocytes, which lack glycophorin A were obtained from Osaka, Japan Red Cross from donor. Control erythrocytes were obtained simultaneously and handled similarly to control for transport and storage variables.

S−s−U− erythrocytes that lack glycophorin B were obtained from the Department of Transfusion Medicine, National Institutes of Health (Bethesda, MD). The erythrocytes were washed in RPMI 1640 and stored at 40–50% hematocrit in RPMI 1640 at 4°C until used.

Enzymatic treatment of erythrocytes. 10^8 erythrocytes ml^{-1} RPMI 1640 with 1 mg ml^{-1} TPCK-treated trypsin (Sigma, St. Louis, MO) were incubated with rocking at 37°C for 60 min and then washed once in 10 packed-cell volumes of RPMI 1640. 10^8 erythrocytes ml^{-1} were suspended in RPMI 1640 and 0.5 mg ml^{-1} soybean trypsin inhibitor (STI) (Sigma, St. Louis, MO) and incubated at room temperature for 10 min. The cells were washed twice in 10 packed-cell volumes of RPMI 1640. The cells were stored in RPMI 1640 at 4°C until used.

10^8 erythrocytes ml^{-1} RPMI 1640 with 20 units *Vibrio cholerae* neuraminidase (Calbiochem, San Diego, CA) were incubated rocking at 37°C for 60 min, then washed 3 times in 10 packed-cell volumes of RPMI 1640. The cells were stored in RPMI 1640 at 4°C until used.

The efficacy of trypsin and neuraminidase treatment was assessed by the loss of agglutinability of erythrocytes by monoclonal antibodies to blood groups M and N on glycophorin A (M2A1 and 12E.A1, respectively; Gamma Biologics, Houston, TX).

Invasion assays. Cultures with mature asexual stage parasites were enriched either with gelatin flotation [18] or differential centrifugation [19]. The parasites, enriched to greater than 50% parasitemia, were washed with 10 cell volumes of RPMI 1640, counted in a hemocytometer, and mixed with target erythrocytes. 1–2 × 10^6 parasites were mixed with 1–5 × 10^7 target erythrocytes in 1 ml of complete medium (RPMI 1640, 24 mM HEPES, 360 μM hypoxanthine, 24 mM $NaHCO_3$, 10% heat-inactivated human A^+ serum); gassed with 5% CO_2/5% O_2/90% N_2; and incubated for 16–20 h at 37°C in a modular incubator chamber (Billups-Rothenberg, Delmar, CA). Rhesus erythrocytes, refractory to invasion by *P. falciparum*, were included in a separate assay as a control to estimate the invasion into uninfected erythrocytes which had been carried over with the enriched parasites. At the end of the incubation period, the erythrocytes were washed once with 0.5 ml 50% fetal bovine serum in RPMI 1640; a thin smear was made and stained with Giemsa. 1000 erythrocytes were counted to determine the percentage of ring-infected erythrocytes. The percentage of invasion of the test erythrocytes was determined by subtracting the percentage of invasion of rhesus erythrocytes from the percentage of invasion of test erythrocytes.

Erythrocyte binding assays. Metabolic labeling of parasites and erythrocyte binding assays were done similarly to those described [16]. Cultures of mature asexual stage parasites were enriched either with gelatin flotation or differential centrifugation. The parasites, enriched to greater than 50% parasitemia, were washed once in RPMI 1640 and resuspended at 10^7 parasites ml^{-1} in methionine/cysteine-deficient complete media with 1 mCi of ^{35}S-methionine/cysteine mixture (Trans ^{35}S, ICN Radiochemicals, Irvine, CA). The parasites were incubated for 12–16 h at 37°C and then centrifuged at 12 000 × *g* for 20 min at 4°C. The supernatant was recovered and stored in aliquots at −70°C until used.

100 μl of packed erythrocytes was mixed with 400 μl of radiolabeled culture supernatant in a microfuge tube and rocked for 20 min at 37°C. The mix was centrifuged at 14 000 × *g* for 30 s through 500 μl of silicone oil (GE Versilube, Schenectady, NY). The erythrocyte pellet was retrieved from the bottom of the microfuge tube by puncturing the tube with a 19 gauge needle. 18 μl of 1.5 M NaCl was mixed with the erythrocyte pellet, incubated at room temperature for 15 min, and centrifuged at room temperature for 30 s at 14 000 × *g*. The supernatant was retrieved and the volume estimated. 1.25 vols. of 2× sample buffer and 0.25 vols. of 1 M DTT were added, and the sample was heated for 3 min at 95°C. SDS-PAGE and autoradiography were performed. Densitometry data were obtained from the autoradiographs using a LKB ultrascan (LKB Technologies, Sweden).

Statistical methods. Logarithms of the percentage of invasion were subjected to a three-way analysis of variance (enzyme treatment, parasite clone, experiments within clones) [20], and a highly significant enzyme-clone interaction was found ($P<0.0001$). A more detailed analysis of the departures from enzyme-clone additivity was made by applying a Tukey multiple comparison test to the respective clone-control differences within enzyme treatment. The mean log HB3 percentage of invasion in trypsin-treated cells was compared to that in neuraminidase-treated cells with the use of a *t*-test.

3. Results

Invasion of normal human or glycophorin B deficient erythrocytes by various clones: the effect of neuraminidase and/or trypsin treatment. Invasion of neuraminidase-treated normal erythrocytes fell into two categories dependent on the parasite clones studied. Clones HB3 and 7G8 had about a 38% reduction in invasion; clones Dd2 and FCR3/A2 had >90% reduction in invasion (Table 1). The two categories were significantly different ($P<0.01$). The invasion of neuraminidase-treated erythrocytes by HB3 and 7G8 was completely eliminated by trypsin treatment of these neuraminidase-treated erythrocytes, indicating that there was a trypsin-sensitive, sialic acid-

Table 1
The effect of enzyme treatment of normal and glycophorin B-deficient human erythrocytes (RBCs) on invasion by various *P. falciparum* clones

Enzyme treatment:	Neuraminidase		Neuraminidase + trypsin[a]		Trypsin		Trypsin + neuraminidase[a]		Trypsin	
Red cells:	Normal RBCs								Glycophorin B-deficient RBCs[b]	
Receptor status:	X(A,B)[c]		(A,B,X)		B(A,X)		(A,B,X)		(A,B°,X)	
Invasion[d] (% of control)	%	n	%	n	%	n	%	n	%	n
HB3[e]	63	13	2	4	34	13	0	3	0	4
Dd2	0	8	−9	2	42	8	−1	2	0	2
FCR3/A2	6	5	−1	2	36	5	0	2	0	2
7G8	62	8	−3	5	4	8	−1	7	−2	3

[a]The enzymes are listed in the order that they were applied to the erythrocytes. For example, neuraminidase + trypsin indicates that the erythrocytes were treated first with neuraminidase and then with trypsin.

[b]With the exception of two aberrant experiments, the invasion into glycophorin B-deficient erythrocytes was similar to normal erythrocytes (invasion expressed as percentage of invasion into normal erythrocytes: 84% (3 experiments), 106% (3 experiments), 87% (1 experiment), and 104% (2 experiments) for parasite clones HB3, 7G8, FCR3/A2, and Dd2, respectively). The invasion for neuraminidase-treated, glycophorin B-deficient erythrocytes expressed as percentage of invasion into untreated glycophorin B-deficient erythrocytes was 75% (3 experiments), 66% (3 experiments), 1% (2 experiments), and −5% (2 experiments) for parasite clones HB3, 7G8, FCR3/A2, and Dd2, respectively. Thus, the effect of neuraminidase on invasion of glycophorin B-deficient erythrocytes was similar for each parasite clone to the effect of neuraminidase on invasion of normal erythrocytes.

[c]The letters refer to erythrocyte glycophorin A (A), glycophorin B (B), and an unknown sialic acid-independent receptor (X). The letters in parentheses indicate that they are modified or missing (e.g., B° means that the erythrocytes are genetically deficient, S–s–U–).

[d]The invasion data are presented as % of control invasion into untreated, normal erythrocytes and the number (n) of independent experiments. The control invasion rates for HB3, Dd2, FCR3/A2, and 7G8 were 4–33%, 5–15%, 6–37%, and 4–29%, respectively. The statistical tests of invasion of normal enzyme-treated erythrocytes were done by the Tukey multiple comparison tests as follows. Normal erythrocytes: Neuraminidase, HB3 7G8 FCR3/A2 Dd2 $P<0.01$; Trypsin Dd2 FCR3/A2 HB3 7G8 $P<0.01$.

[e]HB3, Dd2, FCR3/A2, and 7G8 are parasite clones.

insensitive receptor on erythrocytes. The biochemical nature of this erythrocyte receptor is unknown. For discussion purposes only, it is referred to as X in Table 1. Possibly, Dd2 and FCR3/A2 are not expressing the parasite ligand for binding to X.

We further studied the invasion of trypsin-treated normal erythrocytes by the four parasite clones. Trypsin treatment of normal erythrocytes cleaves, among other erythrocyte molecules, this unknown receptor X and glycophorin A, which is a receptor for EBA-175 [21] (Fig. 1). The parasite clones in the categories defined by invasion of trypsin-treated normal erythrocytes separated differently than the categories defined by invasion of neuraminidase-treated erythrocytes. Invasion by parasite clones HB3, Dd2, and FCR3/A2 averaged between 34 and 42%; invasion by clone 7G8 averaged 4% (Table 1). The two categories were significantly different ($P<0.01$). The difference between 7G8 and the other clones may reflect an absence of a parasite ligand on 7G8 for binding to trypsin-treated erythrocytes.

The invasion of clones HB3, Dd2, and FCR3/A2 into trypsin-treated normal erythrocytes is eliminated by neuraminidase treatment (Table 1). As glycophorin B is one of the major sialoglycoproteins remaining on trypsin-treated erythrocytes, we examined invasion of trypsin-treated glycophorin B-deficient erythrocytes (blood group S−s−U−). The three parasite clones that were able to invade trypsin-treated normal erythrocytes were unable to invade trypsin-treated glycophorin B-deficient erythrocytes (Table 1). This indicates that glycophorin B has an important role in invasion, at least on trypsin-treated erythro-

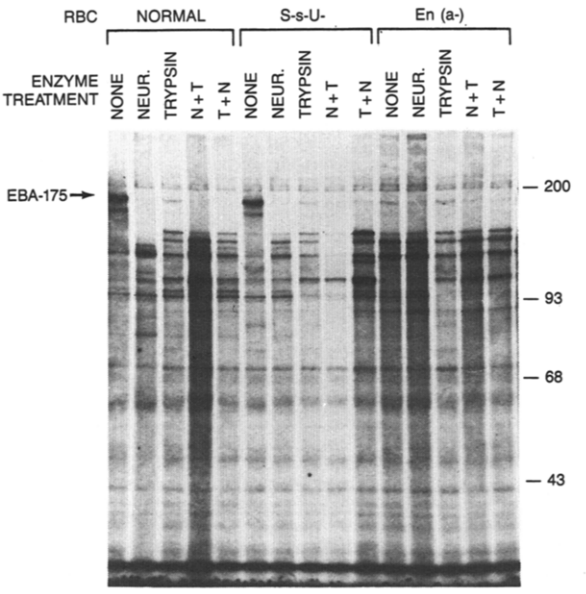

Fig. 1. The effect of neuraminidase (neur.), trypsin, trypsin followed by neuraminidase (T+N), and neuraminidase followed by trypsin (N+T) on binding of soluble parasite proteins (parasite clone HB3) to normal, glycophorin A-deficient (En(a−)), and glycophorin B-deficient (S−s−U−) erythrocytes. The erythrocyte binding protein (EBA-175) is marked by an arrow on the left, and the molecular weight markers in kDa are on the right.

cytes, and probably functions as a receptor. This confirms earlier studies with trypsin-treated erythrocytes [9,10].

What parasite ligand binds glycophorin B? We first monitored whether EBA-175, a sialic acid-binding parasite protein recovered from the different clones, expressed differential binding properties to untreated or enzymatically treated human erythrocytes. Using a binding assay similar to the one previously described [21–23], we found no binding of EBA-175 from parasite clones Dd2 and HB3 to trypsin-treated erythrocytes (Table 2, marked with superscript ^{W}). This method included two steps in separating the erythrocytes from the supernatant containing soluble malarial proteins: (1) centrifugation of the erythrocytes through oil and (2) washing the pellet with medium and centrifuging a second time through oil. The wash was included to remove protein nonspecifically carried through the oil with the pellet during the first centrifugation. Because of the concern that low avidity ligands may be lost during the wash step, the wash step was included only in the two experiments with clone Dd2 and in one experiment with clone HB3 (Table 2, numbers marked with superscript ^{W}).

There was binding of EBA-175 to trypsin-treated normal erythrocytes after only one pass through oil, indicating that there were other sialic acid-dependent binding sites on the trypsin-treated erythrocytes. The signal from EBA-175 was not caused by bulk carryover of culture supernatant, as there was no binding to neuraminidase-treated

Table 2
Low avidity binding of EBA-175 to trypsin-treated normal and glycophorin B-deficient erythrocytes (RBCs)

Clones	Enzyme treatment of normal and glycophorin B-deficient RBCs		
	Neuraminidase	Trypsin	
	Normal RBCs (% of control)	Normal RBCs (% of control)	Glycophorin B-deficient (% of control)
HB3[a]	0^{W}, 0, 0[b]	0^{W}, 7, 0	4
Dd2	0^{W}, 0^{W}	0^{W}, 0^{W}	N.D.[c]
FCR3/A2	7^{H}, 0, 0	27^{H}, 6, 4	2, 2, 4
7G8	4^{H}, 0, 0	10^{H}, 5, 1	9

[a]HB3, Dd2, FCR3/A2, and 7G8 are parasite clones.

[b]The numbers are the percentage binding relative to binding to normal, untreated erythrocytes. The binding of ^{35}S-labeled EBA-175 is determined by scanning autoradiographs of SDS-PAGE gels of radioactive proteins eluted from erythrocytes (see Methods and Fig. 1). The numbers with superscript ^{W} are samples that have been centrifuged through oil twice with a wash in medium after the first centrifugation. The superscript ^{H} refers to 4 erythrocyte binding assays, run on the same day, that were higher than all other data; these are the only samples that showed binding to neuraminidase-treated erythrocytes.

[c]N.D., not done.

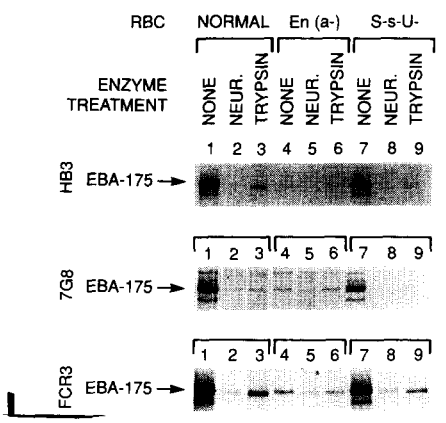

Fig. 2. The effect of neuraminidase (neur.) and trypsin treatment of erythrocytes (RBC) on binding of EBA-175 to normal, glycophorin A-deficient (En(a−)) and glycophorin B-deficient (S−s−U−) erythrocytes. The three parasite clones studied were HB3, 7G8, and FCR3/A2. The data in Table 2 are derived from scans of autoradiographs to determine the percentage binding to the enzyme-treated cells compared to untreated cells. The erythrocyte binding assay was performed as described in Methods, with only one centrifugation through the oil.

erythrocytes except in 2 experiments where the binding to trypsin-treated cells was unusually high (Table 2, superscript ^{H}).

The question as to whether this binding of EBA-175 could account for invasion of trypsin-treated normal erythrocytes by parasite clones HB3, Dd2, and FCR3/A2 was evaluated (Table 2 and Figs. 1 and 2). For each parasite clone, the binding to trypsin-treated normal cells was similar to the binding to trypsin-treated glycophorin B-deficient cells that were completely refractory to invasion. Both showed marked reduction in binding. These results show no correlation between binding of EBA-175 and failure of invasion of trypsin-treated, glycophorin B-deficient erythrocytes, indicating that EBA-175 is not the ligand that mediates invasion *via* glycophorin B. This suggests that a parasite ligand other than the soluble EBA-175 is involved in glycophorin B-dependent invasion.

The erythrocyte protein to which EBA-175 bound was studied by determining the binding to En(a−) erythrocytes that lack glycophorin A. The binding to En(a−) erythrocytes was greatly reduced and was comparable to the binding to trypsin-treated normal erythrocytes (Figs. 1 and 2). Trypsin treatment of En(a−) erythrocytes did not further reduce the binding. These results indicate that EBA-175 binds primarily to glycophorin A.

We searched on the autoradiographs for a protein that bound to trypsin-treated normal erythrocytes but failed to bind trypsin-treated glycophorin B-deficient erythrocytes. No protein was consistently found that bound to trypsin-treated normal erythrocytes and did not bind to trypsin-treated glycophorin B-deficient erythrocytes (data not shown). For example, the protein just below the EBA-175 on trypsin-treated HB3 and FCR3/A2 (Fig. 1) was absent from other experiments with these parasites. In addition, it was not accentuated on En(a−) erythrocytes that have normal glycophorin B. Therefore, using the binding of parasite proteins from the culture supernatants, we were unable to define a protein that bound selectively to glycophorin B.

4. Discussion

Using combinations of parasite clones and enzymatically treated normal or glycophorin B-deficient erythrocytes, we identified multiple ligand-receptor interactions involved in erythrocyte invasion by *P. falciparum*. In addition to erythrocyte receptors that are neuraminidase-sensitive, a trypsin-sensitive receptor has been detected. The present study extends the previous finding that some *P. falciparum* clones invade neuraminidase-treated erythrocytes, while others cannot.

The present data show that invasion of neuraminidase-treated erythrocytes is dependent on trypsin-sensitive receptors on the erythrocyte surface, as trypsin treatment of neuraminidase-treated erythrocytes makes them refractory to invasion by parasite clones HB3 and 7G8. The trypsin-sensitive interaction involves an erythrocyte receptor referred to in this paper as X. It is possible that the clones that are unable to grow in neuraminidase treated erythrocytes (e.g., FCR3/A2 and Dd2) may be missing ligands for the trypsin-sensitive molecules on the erythrocyte. Thus, it was interesting to use the erythrocyte binding assay of

Camus and Hadley [7] to identify the parasite molecules used by different clones of *P. falciparum* for invading human erythrocytes. We were aware that this binding assay does not identify all parasite ligands. It requires release of soluble, binding-competent forms into the culture supernatant. It was successful in identifying the following: the *Plasmodium knowlesi* ligands for the Duffy blood group antigen [22], the *Plasmodium vivax* ligands for the Duffy blood group antigen [24] and for the reticulocyte receptor [25], and the *P. falciparum* ligands for sialic acid [7,8]. It was not possible, with this assay, to identify the *P. knowlesi* ligands involved in invasion of chymotrypsin-treated rhesus erythrocytes [22] and the *P. falciparum* ligands involved in invasion of neuraminidase-treated erythrocytes by the Dd2/NM subclone of Dd2 [16].

Glycophorin B and X: two erythrocyte receptors for binding P. falciparum through unidentified parasite ligands. The erythrocyte-binding antigen of 175 kDa (EBA-175) has been previously identified in parasite culture supernatant through its capacity to bind normal but not to neuraminidase- or trypsin-treated erythroctyes [7]. In the present study, we show that En(a−) erythrocytes that lack glycophorin A have a greatly reduced level of binding roughly equivalent to the binding to trypsin-treated normal erythrocytes, indicating that the binding of EBA-175 is predominantly to glycophorin A.

As trypsin removes both the unknown receptor (X) and glycophorin A, the invasion of trypsin-treated normal erythrocytes suggests that there is a third receptor on the erythrocyte. Trypsin-treated erythrocytes genetically deficient in glycophorin B (blood group S−s−U−) were refractory to invasion [9, 10, and present study], indicating that glycophorin B may act as this third receptor. We have shown that the marked reduction in binding of EBA-175 was similar for trypsin-treated normal and glycophorin B-deficient erythrocytes. This indicates that a parasite ligand other than EBA-175 mediates invasion of trypsin-treated normal erythrocytes. This third ligand appears to require sialic acid, as erythrocytes treated with both trypsin and neuraminidase are completely refractory to invasion. As a region of glycophorin B that has oligosaccharide side chains is identical to glycophorin A, the parasite ligand may bind to both glycophorin B and glycophorin A unless amino acid sequence is also required for specificity. Using the erythrocyte binding assay, we were unable to identify the parasite ligand that bound to trypsin-treated normal erythrocytes but not to trypsin-treated glycophorin B-deficient erythrocytes.

From the results obtained using the erythrocyte binding assay, sialic acid binding proteins of molecular weight lower than the 175 kDa described in the original paper of Camus and Hadley [7] can also be excluded as possible ligands able to bind to glycophorin B. In addition, the major surface protein on merozoites (MSP-1) was found to bind to sialylated molecules on erythrocytes, but trypsin treatment of normal erythrocytes also eliminated binding [8], excluding MSP-1 as the ligand for invasion of trypsin-treated erythrocytes. Thus, the ligands for binding to 2 erythrocyte receptors, glycophorin B and X, remain yet to be identified. A new approach to ligand identification has been the expression of parasite proteins on the surface of COS cells and may be a complementary strategy for identifying the ligands able to bind to glycophorin B and X. As an example, 2 members of the Duffy family of *P. knowlesi* expressed on the surface of COS cells have been found to bind chymotrypsin-treated rhesus erythrocytes (C. Chitnis and L.H. Miller, unpublished data), indicating that these may mediate invasion by an alternative pathway.

How many alternative pathways are used by P. falciparum to invade the broadest range of human erythrocytes? A striking feature of the four parasite clones used in this study is that they are able to utilize different erythrocyte receptors. HB3, capable of invading both neuraminidase-treated erythrocytes and trypsin-treated erythrocytes, can invade through interaction with glycophorin A, glycophorin B, and X. Dd2 and FCR3/A2 invade through interaction with glycophorin A and B, but make little or no use of X. 7G8 is the converse, invading through interaction with glycophorin A and X, but not glycophorin B. These manifold ways by which parasites recognize and invade ery-

throcytes provide redundancy and adaptability to the parasite for this critical step in its erythrocyte stage survival; thus the parasite can adapt to mutations in the host cell receptors. For example, pygmies in Central Africa have a high frequency of blood group S−s−U−, cells deficient in glycophorin B [26]. *P. falciparum* can invade these cells by using glycophorin A. Dantu, another African phenotype that is created by recombination between glycophorin A and B [27], may also have selected parasites that have alternative pathways. This is unlike *P. vivax*, that is unable to infect individuals who are genetically missing the Duffy blood group antigen [28]. In this sense, *P. vivax* is less adaptive than *P. falciparum*.

The three distinct receptor-ligand interactions described in this paper may function in parallel or in series. A parallel interaction may have reduced efficiency as the individual components are removed. If the interactions are in series, then removing any one would completely block invasion unless the step can be bypassed or has an alternative. Different combinations of parallel and series interactions are also possible. The place of each of these and other ligand-receptor interactions in the invasion sequence will be clarified when the molecules involved have been identified.

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References

[1] Miller, L.H., Haynes, J.D., McAuliffe, F.M., Tsugiye, S., Durocher, J.R. and McGinniss, M.H. (1977) Evidence for differences in erythrocyte surface receptors for the malarial parasites, *Plasmodium falciparum* and *Plasmodium knowlesi*. J. Exp. Med. 146, 277–281.

[2] Perkins, M.E. (1981) Inhibitory effects of erythrocyte membrane proteins on the in vitro invasion of the human malarial parasite (*Plasmodium falciparum*) into its host cell. J. Cell Biol. 90, 563–567.

[3] Breur, W.V., Ginsgurg, H. and Cabantchik, Z.I. (1983) An assay for malaria parasite invasion into human erythrocytes. The effects of chemical and enzymatic modification of erythrocyte membrane components. Biochim. Biophys. Acta 755, 262–271.

[4] Mitchell, G.H., Hadley, T.J., McGinniss, M.H., Klotz, F.W. and Miller, L.H. (1986) Invasion of erythrocytes by *Plasmodium falciparum* malaria parasites: evidence for a receptor heterogeneity and two receptors. Blood 67, 1519–1521.

[5] Hadley, T.J., Klotz, F.W., Pasvol, G., Haynes, J.D., McGinniss, M.H., Okubo, Y. and Miller, L H. (1987) *Falciparum* malaria parasites invade erythrocytes that lack glycophorin A nd B (M^kM^k). J. Clin. Invest. 80, 1190–1193.

[6] Perkins, M.E. and Holt, E.H. (1988) Erythrocyte receptor recognition in *Plasmodium falciparum*. Mol. Biochem. Parasitol. 27, 23–34.

[7] Camus, D. and Hadley, T.J. (1985) A *Plasmodium falciparum* antigen that binds to host erythrocytes and merozoites. Science 230, 553–556.

[8] Perkins, M. and Rocco, L.J. (1988) Sialic acid-dependent binding of *Plasmodium falciparum* merozoite surface antigen, Pf200, to human erythrocytes. J. Immunol. 141, 3199–3196.

[9] Howard, R.J., Haynes, J.D., McGinniss, M.H. and Miller, L.H. (1982) Studies on the role of red blood cell glycoproteins as receptors for invasion by *Plasmodium falciparum* merozoites. Mol. Biochem. Parasitol. 6, 303–315.

[10] Pasvol, G., Jungery, M. and Weatherall, D.J. (1982) Glycophorin A as a possible receptor for *Plasmodium falciparum*. Lancet ii, 947–950.

[11] Anstee, D.J. (1982) The blood group MNSs-active sialoglycoproteins. Sem. Hematol. 18, 13–31.

[12] Wellems, T.E., Panton, L.J., Gluzman, I.Y., do Rosario, V.E., Gwadz, R.W., Walker-Jonah, A. and Krogstad, D.J. (1990) Chloroquine resistance not linked to mdr-like genes in *Plasmodium falciparum* clones. Nature 345, 253–255.

[13] Bhasin, V.K. and Trager, W. (1984) Gametocyte-forming and non-gametocyte-forming clones of *Plasmodium falciparum*. Am. J. Trop. Med. Hyg. 33, 534–537.

[14] Burkot, T.R., Williams, J.L. and Schneider, I. (1984) Infectivity to mosquitoes of *Plasmodium falciparum* clones grown in vitro from the same isolate. Trans. R. Soc. Trop. Med. Hyg. 78, 339–341.

[15] Trager, W., Tershakovec, M., Lyandvert, L., Stanley, H. Lanners, N. and Gubert, E. (1981) Clones of the malaria parasite *Plasmodium falciparum* obtained by microscopic selection: their characterization with regard to knobs, chloroquine sensitivity, and formation of gametocytes. Proc. Natl. Acad. Sci. U.S.A. 78, 6527–6530.

[16] Dolan, S.A., Miller, L.H. and Wellems, T.E. (1990) Evidence for a switching mechanism in the invasion of erythrocytes by *Plasmodium falciparum*. J. Clin. Invest. 86, 618–624.

[17] Trager, W. and Jensen, J.B. (1976) Human malaria parasites in continuous culture. Science 193, 673–675.

[18] Jensen, J.B. (1978) Concentration from continuous culture of erythrocytes infected with trophozoites and schizonts of

Plasmodium falciparum. Am. J. Trop. Med. Hyg. 27, 1274–1276.

[19] Aley, S.B., Sherwood, J.A. and Howard, R.J. (1984) Knob-positive and knob-negative *Plasmodium falciparum* differ in antigen expression of strain-specific malarial antigen on the surface of infected erythrocytes. J. Exp. Med. 160, 1585–1590.

[20] Scheffé, H. (1959) The Analysis of Variance, pp. 73–75, 119–124. Wiley, New York.

[21] Orlandi, P., Klotz, F.W. and Haynes, J.D. (1992) A malaria invasion receptor, the 175-kilodalton erythrocyte antigen of *Plasmodium falciparum*, recognizes the terminal Neu5Ac(α2–3)Gal sequence of glycophorin A.J. Cell Biol. 116, 901–909.

[22] Haynes, J.D., Dalton, J.P., Klotz, F.W., McGinniss, M.H., Hadley, T.J., Hudson, D.F. and Miller, L.H. (1988) Receptor-like specificity of a *Plasmodium knowlesi* protein that binds to Duffy antigen ligands on erythrocytes. J. Exp. Med. 167, 1873–1881.

[23] Orlandi, P.A., Sim, B.K.L., Chulay, J.D. and Haynes, J.D. (1990) Characterization of the 175-kilodalton erythrocyte binding antigen of *Plasmodium falciparum*. Mol. Biochem. Parasitol. 40, 285–294.

[24] Werthheimer, S.P. and Barnwell, J.W. (1989) *Plasmodium vivax* interaction with the human Duffy blood group glycoprotein: identification of a parasite receptor-like protein. Exp. Parasitol. 69, 340–350.

[25] Galinski, M.R., Medina, C.C., Ingravallo, P. and Barnwell, J. (1992) A reticulocyte-binding protein complex of *Plasmodium vivax* merozoites. Cell 69, 1213–1226.

[26] Fraser, G.R., Giblett, E.R. and Motulsky, A.G. (1966) Population genetic studies in the Congo. III. Blood groups (ABO, MNSs, Rh, Js^a). Am. J. Human Genet. 18, 546–552.

[27] Unger, P., Procter, J.L., Moulds, J.J., Moulds, M., Blanchard, D., Guizzo, M.C., McCall, L.A., Cartron, J.P. and Dahr, W. (1987) The Dantu erythrocyte phenotype of the NE variety. II. Serology, immunochemistry, genetics, and frequency. Blut 55, 33–43.

[28] Miller, L.H., Mason, S.J., Clyde, D.G. and McGinniss, M.H. (1976) The resistant factor to *Plasmodium vivax* in blacks: the Duffy-blood-group genotype, *FyFy* N. Engl. J. Med. 295, 302–304.