RECEPTOR-LIKE SPECIFICITY OF A *PLASMODIUM KNOWLESI* MALARIAL PROTEIN THAT BINDS TO DUFFY ANTIGEN LIGANDS ON ERYTHROCYTES

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Human erythrocytes that lack the Duffy blood group antigens are refractory to infection by *Plasmodium knowlesi* malaria parasites in vitro and *Plasmodium vivax* in vivo, suggesting that these antigens are used as ligands for invasion of erythrocytes by merozoites (1–5). This specificity has been the prototype for understanding malaria parasite invasion of erythrocytes. The molecule that carries a Duffy determinant was characterized recently as a 35–45-kD glycoprotein (6), but no parasite molecule with specificity for Duffy has been described. A study with *Plasmodium falciparum* demonstrated that after release of merozoites from cultured schizont-infected erythrocytes, a 175-kD protein accumulates in the culture supernatant. This molecule binds to both Duffy-positive and Duffy-negative human erythrocytes that are invaded, but does not bind to neuraminidase- or trypsin-treated human erythrocytes that are refractory to invasion by *P. falciparum* (7). We used modifications of methods of Camus and Hadley (reference 7, Haynes, J. D., manuscript in preparation) to determine whether *P. knowlesi* uses a receptor analogous to that of *P. falciparum* but with the appropriate specificity for Duffy blood group antigen. Erythrocytes were incubated with the metabolically labeled proteins in culture supernatant, centrifuged through oil, and the bound proteins were eluted with salt. We now describe a 135-kD protein synthesized by *P. knowlesi* that bound to Duffy blood group-positive, but not to Duffy blood group-negative, human erythrocytes. It seems likely that the 135-kD Duffy binding protein is one of the receptors responsible for invasion of *P. knowlesi* merozoites into Duffy-positive erythrocytes.

Materials and Methods

Parasites and Labeled Culture Supernatants. Schizont-infected rhesus erythrocytes (Malaysian H strain of *P. knowlesi*) were enriched and metabolically labeled at 1.5×10^7/ml with 100 μCi/ml [^{35}S]methionine in methionine-deficient RPMI-1640 culture medium (3, 8) with 1% rabbit serum. The parasites were cultured for 6–10 h to allow the maturation of schizonts,

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the release of merozoites, and the release of molecules from merozoites. There were <10% uninfected erythrocytes in the culture. A control culture of uninfected rhesus erythrocytes was similarly prepared. Culture supernatant was centrifuged (1,000 *g* for 15 min, then 10,000 *g* for 20 min) before freezing at −70°C. Culture supernatant of *P. falciparum* schizonts (Camp strain) was obtained after labeling with 50 μCi/ml [^{3}H]isoleucine in medium containing 0.03 mM isoleucine and 10% human serum (7).

Erythrocytes and Pretreatments with Enzymes. Erythrocytes were collected in acid citrate dextrose solution and stored at 4°C for up to 3 wk. Washed erythrocytes were treated with tosyl-phenylalanine-chloromethyl-ketone (TPCK)-trypsin or tosyl-lysine-chloromethyl-ketone (TLCK)-chymotrypsin followed by soybean trypsin inhibitor (STI) or PMSF, (Sigma Chemical Co., St. Louis, MO), or control treatment with inhibitor alone (6, 7). The treated erythrocytes were extensively washed before using in binding and invasion studies.

Duffy Phenotype Determination. The Duffy-positive (Fy^a and Fy^b) and Duffy-negative (FyFy) phenotypes of untreated human and rabbit erythrocytes were determined by standard blood banking methods. Duffy phenotypes of rhesus (*Macaca mulatta*) and of enzyme-treated erythrocytes were determined by the depletion or by the adsorption and elution of Duffy antibodies (1, 3, 9).

Binding to and Elution from Erythrocytes. Washed erythrocytes (100 μl) and culture supernatant (400 μl) were mixed for 30 min at room temperature. The suspension was layered over silicone oil (GE Versilube F50) and centrifuged for 30 s at 13,000 *g*. The erythrocyte pellet was washed once with culture medium, mixed with 18 μl of 1.5 M NaCl, and eluted 10 min at room temperature, then centrifuged at 13,000 *g*. For the *P. falciparum* binding study, 60 μl of erythrocytes were centrifuged through silicone oil but not washed. The supernatant eluate was heated with an equal volume of 2× SDS sample buffer, electrophoresed on a 5–10% linear gradient polyacrylamide gel (Fig. 1), or a 7.5% gel (Figs. 2, 3, and 5) and processed for autoradiography (8). Prestained molecular weight markers (Bethesda Research Laboratories, Rockville, MD) were electrophoresed on the same gel.

Invasion of Erythrocytes. The ability of *P. knowlesi* to invade erythrocytes was examined in 10% FCS containing medium (3, 5) for duplicate cultures with the same erythrocytes used concurrently in the binding and elution studies.

Competitive Binding of Anti-Duffy Antibodies and the 135-kD Protein. The anti-Fy^6 monoclonal IgG1 antibody (NYBC-BG6) was a gift of John Barnwell (New York University, New York, NY) and Pablo Rubenstein, (New York Blood Center, New York, NY) (10). The MOPC21 mouse myeloma IgG1 control was a gift of Richard Asofsky (National Institutes of Health, Bethesda, MD). Human Fy^b erythrocytes or rhesus erythrocytes (75 μl) were suspended in 120 μl RPMI-1640 medium containing 0.25% wt/vol BSA and either 10 μg anti-Fy^6 or 10 μg MOPC21 IgG1. After 2 h of rocking at room temperature, 400 μl of radiolabeled *P. knowlesi* culture supernatant was added and rocking was continued another 30 min. The erythrocytes were washed and eluted, and electrophoresis was carried out as described above. Similar experiments were performed with human Fy^a and Fy^b erythrocytes and a high titer anti-Fy^a serum (6).

Electroblotting and Probing. Procedures were modified from reference 6. Erythrocyte ghosts from Fy^a, Fy^b, and FyFy erythrocytes were extracted in an equal volume of 0.06 M Tris (pH 7.5), 0.5 M NaCl, and 0.2% deoxycholate, and were centrifuged at 10,000 *g* for 20 min. The supernatant was added to an equal volume of 2× SDS sample buffer without heating, electrophoresed on 10% polyacrylamide gels, and electrophoretically transferred to nitrocellulose paper. The blots were blocked for 1 h with 0.3% Tween-20 in PBS and another hour with 1% rabbit serum added. The blots were probed with [^{35}S]methionine-labeled 135-kD protein eluted from 4 ml of Fy^b erythrocytes. The eluate was supplemented with 1% rabbit serum, dialyzed extensively against PBS, and brought to a final volume of 5 ml with PBS containing 1% rabbit serum. The blots were incubated in this solution for 2 h at room temperature with shaking, rinsed five times with cold PBS, dried, and exposed to x-ray film. Duplicate blots were reacted with anti-Fy^a antibody and visualized using alkaline phosphatase-conjugated anti-human IgG (Promega Biotec, Madison, WI).

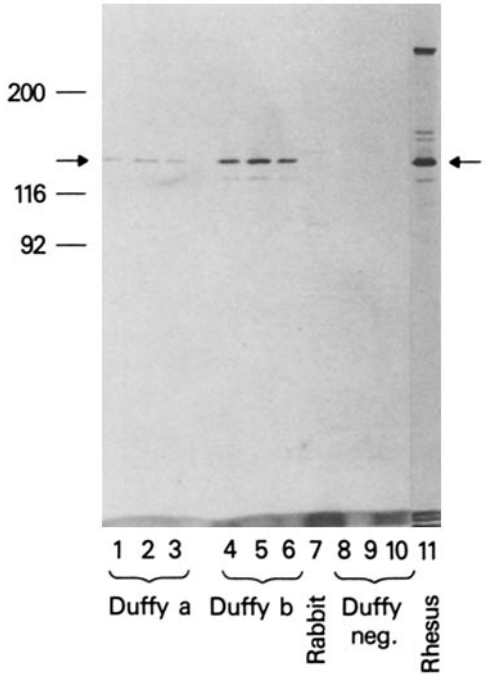

FIGURE 1. Metabolically radiolabeled proteins from *P. knowlesi* culture supernatants that bind to and are eluted from erythrocytes then analyzed by electrophoresis and autoradiography. Binding to erythrocytes from three Duffy a people (lanes 1–3), three Duffy b people (lanes 4–6), one Duffy-negative rabbit (lane *7*), three Duffy-negative people (lanes *8–10*), and one Duffy b rhesus monkey (lane *11*). The 135-kD protein is indicated by the arrow on the right and molecular weight markers are indicated on the left.

Results

A 135-kD protein from cultured *P. knowlesi* bound to and was eluted from Duffy a (Fy^a)- or Duffy b (Fy^b)-positive human erythrocytes but not Duffy-negative (FyFy) human erythrocytes (Fig. 1). The 135-kD protein was a minor fraction of the total [^{35}S]methionine-radiolabeled molecules in culture supernatants (Fig. 2). The 135-kD protein migrated faster than a previously described 140-kD antigen (8) run on the same gel (not shown). A minor band of 120 kD was also present. Control [^{35}S]methionine-labeled rhesus erythrocyte cultures without the parasite did not produce significant amounts of radiolabeled culture supernatant proteins, and no 135-kD protein binding to Duffy-positive erythrocytes was detected from such control cultures (data not shown).

The 135-kD molecule also bound to rhesus erythrocytes (Fig. 1), which are Fy^b (3). It did not bind to rabbit erythrocytes (Fig. 1) or guinea pig erythrocytes (data not shown), which are Duffy-negative and are not invaded by *P. knowlesi* (3). Rhesus erythrocytes bound several additional molecules, including a band of >200 kD that did not bind to rabbit or human erythrocytes (Fig. 1).

The binding of the 135-kD protein to human Fy^b erythrocytes (Fig. 3) was blocked by an mAb against a newly described Fy^6 determinant present on human Duffy-positive erythrocytes (10). Anti-Fy^6 antibody did not block the 135-kD protein binding to rhesus erythrocytes (Fig. 3) that lack the Fy^6 determinant (10). Controls with myeloma antibody of the same IgG subtype and with rhesus erythrocytes indicated that the antibodies did not interfere nonspecifically with binding. Simi-

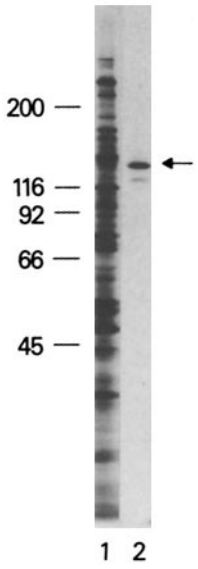

Figure 2. Comparison of the radiolabeled molecules in total culture supernatant (lane *1*) and those binding to human Duffy b erythrocytes (lane *2*).

larly, specific blocking of binding to Fy^a erythrocytes but not to Fy^b erythrocytes was seen in experiments with human Fy^a and Fy^b erythrocytes and anti-Fy^a antisera (data not shown).

The 135-kD protein consistently distinguished between Fy^a and Fy^b human erythrocytes. The quantity of 135-kD protein eluted from Fy^b was always greater than that from Fy^a (Fig. 1 and data not shown). Furthermore, when the culture supernatant was preadsorbed with Fy^a or Fy^b erythrocytes and then assayed for 135-kD protein binding to rhesus erythrocytes, a greater amounts of this 135-kD protein was depleted by the Fy^b erythrocytes (data not shown). This specificity indicates that the molecules carrying the Duffy blood group antigens are themselves the ligands for the binding of the receptor. To show this definitively, the 135-kD protein was eluted from Fy^b erythrocytes and used to probe erythrocyte membrane proteins that had been electrophoretically separated and transferred to nitrocellulose (Fig. 4). The 135-kD P. knowlesi protein bound to the 35–45-kD region in lanes containing Duffy-positive erythrocyte proteins, but not to those containing Duffy-negative erythrocyte proteins. Again, greater binding was observed to the Fy^b antigen than to the Fy^a antigen. As a control, parallel lanes probed with an anti-Fy^a serum demon-

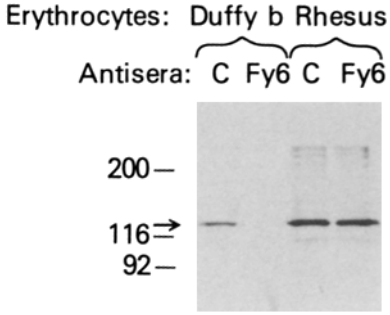

Figure 3. Blocking of binding of the 135-kD protein by competition with anti-Duffy antibody. Erythrocytes were incubated with anti-Fy^6 mAb (Fy 6), or control antibody (*C*), then incubated with radiolabeled *P. knowlesi* culture supernatants, washed, and eluted. Duffy b erythrocytes were human Fy^b erythrocytes that also carried the Fy^6 determinant (10). Rhesus erythrocytes carried the Fy^b but lacked the Fy^6 determinant (10). The position of the 135-kD protein is noted by the arrow.

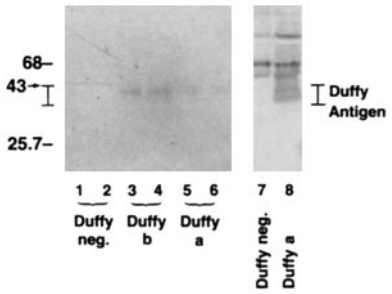

Figure 4. Binding of the 135-kD protein to Duffy blood group antigens on blots of electrophoretically separated erythrocyte membrane proteins. Purified 135-kD protein was incubated with blots from erythrocytes that were Duffy-negative (lanes *1* and *2*), Duffy b (lanes *3* and *4*), or Duffy a (lanes *5* and *6*); and anti-Duffy a antibody was incubated with blots from Duffy-negative (lane *7*) or Duffy a erythrocytes (lane *8*).

strated, as previously shown (6), that Duffy antigen is carried on a 35–45-kD erythrocyte glycoprotein.

Cleavage of the Duffy antigen on human erythrocytes by chymotrypsin treatment eliminated binding of the 135-kD molecule to the treated erythrocytes, and the

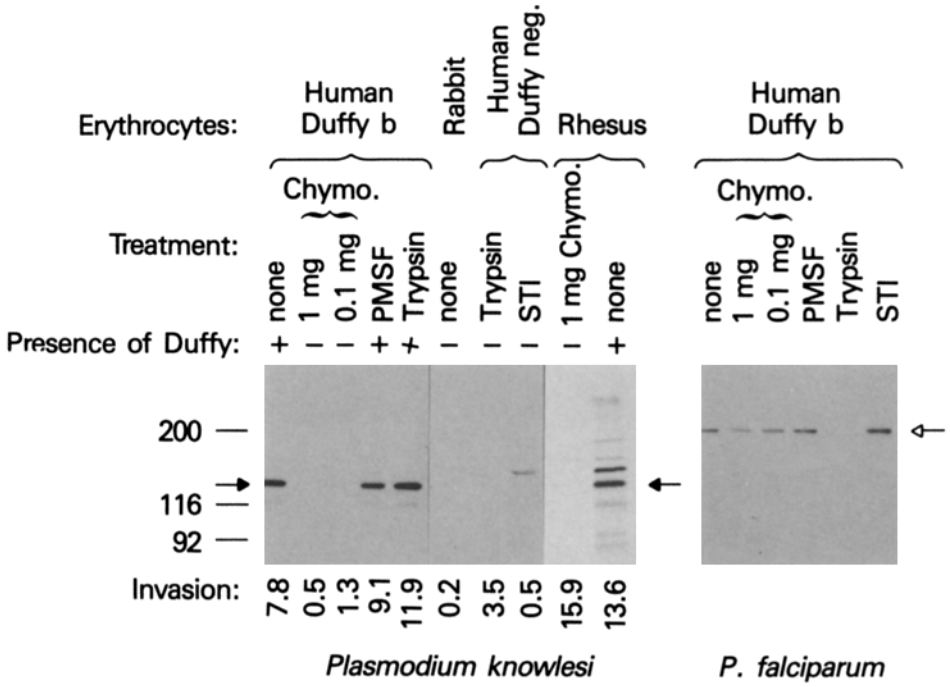

Figure 5. Binding and elution of the *P. knowlesi* 135-kD protein (*solid arrow*) or the *P. falciparum* 175-kD protein (*open arrow*) to enzyme-treated erythrocytes. (*Left*) [^{35}S]methionine-labeled molecules from *P. knowlesi* culture supernatant that bound to and eluted from different erythrocytes: Duffy b human erythrocytes either untreated (*none*), treated with 1 mg/ml chymotrypsin, 0.1 mg/ml chymotrypsin (*0.1 mg chymo.*), 5×10^{-4} M PMSF, or 1 mg/ml trypsin; Duffy-negative rabbit erythrocytes; Duffy-negative human erythrocytes treated with trypsin or with 1 mg/ml STI; rhesus erythrocytes (Duffy b) either treated with 1 mg/ml chymotrypsin (*1 mg chymo.*) or untreated (*none*). The + or – above a lane indicates the presence or absence of Duffy antigen (1, 3, 9). The numbers below the lanes are percentages of erythrocytes invaded by *P. knowlesi*. (*Right*) [^{3}H]isoleucine-labeled molecules from *P. falciparum* culture supernatant that bound to and eluted from Duffy b human erythrocytes either untreated (*none*), treated with 1 mg/ml chymotrypsin (*1 mg chymo.*), 0.1 mg/ml chymotrypsin (*0.1 mg chymo.*), PMSF, trypsin, or STI.

chymotrypsin-treated erythrocytes were refractory to invasion by *P. knowlesi* (Fig. 5 and reference 5). These chymotrypsin-treated erythrocytes were normally invaded by *P. falciparum* and still bound the 175-kD receptor from *P. falciparum* (Fig. 5 and reference 7). Conversely, trypsin-treated Duffy-positive erythrocytes bound the receptor for Duffy from *P. knowlesi* but not the 175-kD receptor from *P. falciparum*. Trypsin does not cleave Duffy blood group antigen; the treated erythrocytes are invaded normally by *P. knowlesi*. The invasion of additional erythrocytes by *P. knowlesi* not shown in Fig.5 are 9.2% for STI-treated Duffy b human, 0.4% for Duffy-negative human, and 12.9% for PMSF-treated rhesus erythrocytes. Invasion of treated erythrocytes by *P. falciparum* parasites is reduced to 10–25% of control by 1 mg/ml trypsin and unaffected by 0.1 mg/ml chymotrypsin (5, 11). For each species of parasite, in each of these instances, invasion paralleled the binding of the respective putative receptor molecule.

Despite the specificity of the Duffy receptor–ligand interaction, invasion occurs under unusual circumstances independently of this interaction. *P. knowlesi* merozoites invade trypsin-treated Duffy-negative human erythrocytes and chymotrypsin-treated rhesus erythrocytes (3, 5), both of which lack the Duffy antigen and fail to bind the 135-kD receptor for Duffy (Fig. 5).

Discussion

The correspondence between the binding of the 135-kD *P. knowlesi* protein and the invasion of Duffy-positive erythrocytes suggests that the 135-kD *P. knowlesi* protein is a receptor for invasion. Whether its binding to a Duffy ligand on a Duffy-positive erythrocyte prepares the erythrocyte for invasion, acts as an adhesion molecule between the erythrocyte and the merozoite, transmits a signal to the merozoite activating it for invasion, or initiates some combination of these remains to be determined. The specificity of the 135-kD putative receptor is emphasized not only by its lack of binding to Duffy-negative (FyFy) erythrocytes (Figs. 1 and 5), but also by the consistent demonstration of greater binding to Fy^b than to Fy^a, both to antigen in situ on erythrocytes and electrophorectically separated on blots (Figs. 1 and 4). The fact that experiments to date do not show any great difference in the rate of invasion of Fy^a and Fy^b erythrocytes may indicate that a threshold binding of the 135 kd protein less than the possible maximum is sufficient for invasion under these experimental conditions. Nevertheless, otherwise normal Duffy-negative human erythrocytes do not bind the 135-kD protein and are not invaded.

Anti-Fy^a serum blocks invasion of Fy^a erythrocytes by *P. knowlesi* (3) and blocks the binding of the 135-kD protein. Available anti-Fy^b antisera were not strong enough to block invasion or binding, but here we show blocking of the 135-kD protein binding to Fy^b erythrocytes by an anti-Fy^6 mAb that reacts with a newly described Duffy Fy^6 determinant that is independent of Fy^a and Fy^b (Fig. 3 and reference 10).

It is unlikely that the binding of the 135-kD protein to a Duffy determinant on the erythrocyte participates in the initial attachment of the *P. knowlesi* merozoite to the erythrocyte. The initial attachment occurs with Duffy-negative (FyFy) human erythrocytes; however, the initial attachment does not progress to the apical junction formation that is seen when the erythrocyte is Duffy-positive (12).

It is not known why the 135-kD protein is found in the culture supernatant after merozoite release from infected erythrocytes. The 135-kD putative receptor may

be a secreted molecule or it may dissociate from the merozoite surface. In either case, the 135-kD protein is only a small fraction of the total labeled parasite proteins released into the culture media, and the selectivity of its binding to erythrocytes is remarkable (Fig. 2).

Duffy-positive erythrocytes that have been altered by pretreatment with chymotrypsin or trypsin are differentially invaded by *P. knowlesi* and *P. falciparum* (11). Here we demonstrated that cleavage of the Duffy antigen by chymotrypsin prevented binding of the 135-kD protein of *P. knowlesi*, as well as prevented invasion by *P. knowlesi* (Fig. 5). On the other hand, cleavage of glycophorins by trypsin did not prevent binding of the 135-kD protein, nor did it prevent invasion by *P. knowlesi*. As additional controls, it was shown that the chymotrypsin treatment did not nonspecifically prevent binding; the 175-kD putative receptor of *P. falciparum* still bound and its merozoites still invaded chymotrypsin-treated erythrocytes. Conversely, trypsin treatment prevented the binding of the 175-kD putative receptor of *P. falciparum*. We conclude that chymotrypsin or trypsin treatment of human erythrocytes differentially alters ligands recognized by the two putative receptors. This is another example of the correspondence of binding of the 135-kD protein and invasion by *P. knowlesi*.

However, there are two examples of altered erythrocytes that are invaded by *P. knowlesi* that do not bind the 135-kD protein. First, human Duffy-negative (FyFy) erythrocytes that have been treated with trypsin still do not bind the 135-kD protein but have gained some susceptibility to invasion (Fig. 5). Second, rhesus erythrocytes that have been treated with chymotrypsin can still be invaded, although they become Duffy-negative and no longer bind the 135-kD protein (Fig. 5). Such enzyme treatments may expose cryptic non-Duffy ligands, allowing invasion to bypass the normal sequence requiring binding of the 135-kD protein, or allowing invasion to proceed by an alternative pathway.

In a somewhat similar observation, cleavage of sialic acid from erythrocytes prevents invasion by many strains of *P. falciparum*, but some strains of *P. falciparum* parasites can invade sialic acid-deficient erythrocytes (5, 13, 14), though they possess a 175-kD receptor with the usual requirement for a sialic acid-dependent ligand on the erythrocytes (Hadley, T. J., et al., unpublished data). Thus, it appears that malaria parasites can bypass certain steps or use alternative pathways for invasion. Analogies to the serum complement cascades may be valid. Alternative pathways may be used by the parasite to invade different host erythrocytes and to be available as a backup system for invasion if the host's antibodies should interfere with one or the other. Other possible receptors for invasion have been reviewed (5) and include a 66-/44-/42-kD *P. knowlesi* merozoite surface protein (15).

The fact that the parasite can invade some altered erythrocytes without the interaction of this putative receptor (the 135-kD protein) and ligand (the Duffy antigen) probably reflects the selective advantage that alternative mechanisms for invasion impart to the parasite. Thus, in designing an antimalarial vaccine directed against receptors used for invasion it would be desirable to include several receptor proteins.

Summary

A 135-kD parasite protein, a minor component of the *Plasmodium knowlesi* malaria radiolabeled proteins released into culture supernatant at the time of merozoite release and reinvasion, specifically bound to human erythrocytes that are invaded and

carry a Duffy blood group determinant (Fy^a or Fy^b), but did not bind to human erythrocytes that are not invaded and do not carry a Duffy determinant (FyFy). Specific anti-Duffy antibodies blocked the binding of the 135-kD protein to erythrocytes carrying that specific Duffy determinant. Purified 135-kD protein bound specifically to the 35–45-kD Duffy glycoprotein on a blot of electrophoretically separated membrane proteins from Fy^a and Fy^b erythrocytes but not from FyFy erythrocytes. Binding of the 135-kD protein was consistently greater to Fy^b than to Fy^a both on the blot and on intact erythrocytes. The 135-kD protein also bound to rhesus erythrocytes that are Fy^b and are invaded, but not to rabbit or guinea pig erythrocytes that are Duffy-negative and are not invaded. Cleavage of the Duffy determinant by pretreating Fy^b human erythrocytes with chymotrypsin greatly reduced both invasion and binding of the 135-kD protein, whereas pretreating Fy^b erythrocytes with trypsin had little effect on the Duffy antigen,the 135-kD protein binding, or on invasion. However, instances of invasion of other enzyme-treated erythrocytes that are Duffy-negative and do not bind the 135-kD protein suggest that alternative pathways for invasion do exist.

We thank J. Chulay, M. Zegans, K. Pinkston, D. Camus, L. Reyes, C. Diggs, M. Mallet, D. Finbloom, G. Schatz, and I. Green for helpful discussions and support. The views of the authors do not purport to reflect the position of the United States Army or the Department of Defense.

Received for publication 29 October 1987 and in revised form 29 February 1988.

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