Erythrocyte Invasion Phenotypes of Plasmodium falciparum in The Gambia

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In vitro experimentation with Plasmodium falciparum has determined that a number of different receptor-ligand interactions are involved in the invasion of erythrocytes. Most culture-adapted parasite isolates use a mechanism of invasion that depends primarily on the erythrocyte sialoglycoprotein glycophorin A (GYPA) and erythrocyte-binding antigen 175 (EBA-175) of the parasite blood-stage merozoite. However, a minority of culture-adapted parasites and a majority of Indian field isolates can apparently invade by other means. Here, erythrocyte invasion phenotypes of P. falciparum field isolates in Africa were studied. For 38 Gambian isolates, invasion of neuraminidase-treated and trypsin-treated erythrocytes was inhibited, on average, by more than 60 and 85%, respectively, indicating a high level of dependence on sialic acid and trypsin-sensitive proteins on the erythrocyte surface. These results support the hypothesis that African P. falciparum parasites use GYP A as a primary receptor for invasion. However, the considerable variation among isolates confirms the idea that alternative receptors are also used by many parasites. Three amino acid polymorphisms in the GYP A-binding region of EBA-175 (region II) were not significantly associated with invasion phenotype. There was variation among isolates in the selectivity index (i.e., a statistical tendency toward aggregation or multiple invasions of host erythrocytes), but this variation did not correlate with enzyme-determined invasion phenotype or with eba-175 alleles. Overall, these invasion phenotypes in Africa support a vaccine strategy of inhibiting EBA-175 binding to GYP A but suggest that parasites with alternative phenotypes would be selected for if this strategy were used alone.

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that apparently correlates with invasion phenotype, albeit in a small number of isolates.

There are major differences in the invasion phenotypes of culture-adapted isolates of *P. falciparum*. Previous work (4, 7, 19, 26) characterized the sensitivity of diverse cultured isolates to neuraminidase- and trypsin-sensitive receptors on the erythrocyte surface. Invasion by most of these parasites was trypsin sensitive (reducing invasion by >50%), but sensitivity to neuraminidase varied more widely (reducing invasion by 20 to 100%) (4). Although these isolates represented a broad collection of culture-adapted isolates, it is not clear how representative their invasion phenotypes are of *P. falciparum* in human infections. Recent work on a small number of field isolates from India (23) showed that a majority of the isolates (12 out of 15) were able to invade and grow quite effectively in both neuraminidase- and trypsin-treated erythrocytes, calling into question the primacy of GYPA as a receptor in natural populations.

*P. falciparum* merozoites are able to invade a large proportion of the erythrocyte population in a human host, enabling infections to reach very high levels of parasitemia; in contrast, *P. vivax* preferentially invades reticulocytes, the younger erythrocytes (20). However, there is evidence from studies in Thailand that the invasion process may not be random in many *P. falciparum* infections (6, 31). The degree of aggregation of parasites in erythrocytes with multiple parasite invasions, the selectivity index (SI), has been shown to correlate inversely with the severity of malaria disease (6, 31). The molecular interactions that underlie this SI are unknown, but culture assays suggest that they are to some extent parasite determined (6) and thus are potential virulence determinants. It is likely that they relate to receptor-ligand interactions involved in invasion and therefore may be related to parasite invasion phenotypes assayed by enzyme treatment of erythrocytes.

The parasite ligands necessary for erythrocyte invasion are potential candidates for malaria vaccines (16, 29). As the majority of global *P. falciparum* malaria cases are in sub-Saharan Africa (11), it is most important to study erythrocyte invasion by African parasites. Here, invasion phenotypes of field isolates from patients in The Gambia were characterized. In vitro assays of the first cycle of invasion of normal and neuraminidase- and trypsin-treated erythrocytes were performed with fresh parasite isolates. The results indicated that the majority of parasites in The Gambia use a neuraminidase- and trypsin-sensitive invasion pathway, although there is also considerable use of alternative invasion pathways. Investigation of the parasite *eba*-175 alleles and SI in vivo showed no correlation with in vitro invasion phenotypes.

**MATERIALS AND METHODS**

**P. falciparum field isolates.** A 2-ml heparinized venous blood sample was obtained from each of 64 malaria patients presenting at the outpatient clinic of the MRC Hospital in Fajara, The Gambia. All patients or their guardians gave informed consent, and the study was approved by the Joint Gambian Government/MRC Ethics Committee. Samples were collected over a 5-week period from 22 October to 23 November 2001, during the end of the peak of the annual malaria transmission season. Peripheral blood parasitemia was calculated for each patient as an inclusion criterion for allowing informative culture assays to be performed. Samples were obtained from patients only if their parasitemia levels were greater than 100 parasites per high-power (>×1,000) microscopic field in a thick smear (10), which corresponded to a parasitemia level of about 3% infected erythrocytes in a thin smear. Patients were excluded if they reported having taken an antimalaria treatment during the preceding 2 weeks in an interview. A thin blood smear was prepared to determine the percentage of parasitized erythrocytes and the SI, based on the number of multiple parasite invasions per erythrocyte (see below). Twenty microliters of blood was spotted onto glass fiber filter paper and kept dry at +4°C to be used later for parasite genotyping. The remaining blood sample was washed three times at 500 × g for 5 min in prewarmed RPMI 1640 Invitrogen, Life Technologies, Paisley, United Kingdom) to remove the leukocytes and plasma. The washed, packed, parasitized erythrocytes were resuspended in RPMI 1640 complete medium (containing 25 mM HEPES, 4 mM sodium bicarbonate, penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% AB-positive serum) at a 10% hematocrit and triturated. The remaining blood sample was washed three times in RPMI 1640. Soybean trypsin inhibitor (0.5 mg/ml) (Sigma) was added to the trypsin-treated cells, and the mixture was shaken for 10 min at room temperature to inactivate the enzyme. The cells were washed three times in RPMI 1640. Control (non-enzyme-treated) cells were washed in the same manner as enzyme-treated cells. The efficiency of the enzyme treatments in removing GYPA from the erythrocyte surface was assayed by agglutination tests with monoclonal antibodies directed against the M and N epitopes of GYPA (Biotest, Solihull, United Kingdom).

**Enzymatic treatment of erythrocytes.** Blood (O positive, Rhesus factor positive, MN [as determined by agglutination with monoclonal antibodies; see below]) from a single malaria-negative donor who had not taken antimalaria drugs during the preceding 2 months was washed as described above. A 0.1-ml portion of the resulting packed erythrocytes (approximately 10^9 cells) was treated with 20 μl of neuraminidase (Sigma, Grillingham, United Kingdom) or 1 mg of tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma) ml^-1 in a 1-ml final volume of RPMI 1640 as previously described (4, 23). These mixtures were incubated for 1 h at 37°C with periodic shaking and then were washed three times in RPMI 1640. Soybean trypsin inhibitor (0.5 mg ml^-1) (Sigma) was added to the trypsin-treated cells, and the mixture was shaken for 10 min at room temperature to inactivate the enzyme. The cells were washed three times in RPMI 1640. Control (non-enzyme-treated) cells were washed in the same manner as enzyme-treated cells. The efficiency of the enzyme treatments in removing GYPA from the erythrocyte surface was assayed by agglutination tests with monoclonal antibodies directed against the M and N epitopes of GYPA (Biotest, Solihull, United Kingdom).

**Fluorescence labeling of target erythrocytes.** In accordance with the method of Lamont et al. (17), 4 μg of filtered FITC (Sigma/ml) in phosphate-buffered saline solution at pH 7.4 (Sigma) was added to washed enzyme-treated and untreated erythrocytes. These mixtures were incubated with shaking for 10 min at room temperature to label target cells, which were then washed three times in RPMI 1640 to remove all unincorporated FITC. In practice, it was not possible to completely dissolve all of the FITC in phosphate-buffered saline; thus, the final concentration in the filtered solution was less than 4 μg/ml. This detail did not affect the ability to obtain fluorescent cells. Labeled erythrocytes were resuspended in RPMI 1640 complete medium at a 10% hematocrit.

**In vitro invasion assays.** For invasion assays, 10 μl of target cell suspension (approximately 10^9 enzyme-treated or untreated control erythrocytes) was added to 10 μl of parasitized donor cell suspension in each of triplicate wells in flat-bottom 96-well microtiter plates (BD Falcon). Prewarmed RPMI 1640 complete medium was added to 200 μl, and the plates were cultured at 37°C in a candle jar with replacement of fresh medium after 24 h. After one invasion cycle, 48 to 54 h in total, a thin smear of a sample from each of the triplicate wells was fixed in methanol and incubated in 4 μg of ethidium bromide (Sigma) ml^-1 for 15 min to stain parasite DNA. Slides were washed, air dried, and examined with a vertical fluorescence microscope (Leica Microsystems) at a magnification of ×1,000 under oil immersion. The number of parasites in at least 1,000 FITC-labeled erythrocytes in each of triplicate wells was scored (nonlabeled cells were not counted), and the mean was calculated for each treatment. Percent inhibition by enzyme treatment was determined as [1 − ([proportion of enzyme-treated cells invaded)/(proportion of untreated cells invaded)]) × 100 (4).

**In vivo parasitemia and SI.** Thin smears of samples from each patient were stained with Giemsa stain and examined at a magnification of ×1,000 to determine parasitemia (the number of parasitized erythrocytes counted among at least 1,000 erythrocytes) and the number of single and multiple infections of erythrocytes (counted among at least 300 infected erythrocytes). To avoid potential biases, all counts were determined for all cells in full microscope fields (thus, the precise numbers of cells counted were greater than or equal to the minimum numbers of 1,000 and 300, respectively). Erythrocytes with multiple infections were defined as those containing two or more individual ring- or trophozoite-stage parasites. The observed number of erythrocytes with multiple infections was compared to the number expected if parasites were randomly distributed. The random probabilities of erythrocytes being infected with single and multiple parasites were calculated according to a Poisson distribution, (e^{-μ}μ^x)/x!, where μ is the mean number of parasites per erythrocyte [−ln(1 − parasitemia)] and x is the number of parasites per erythrocyte; the values obtained were then used to calculate the expected class of parasite counts (i.e., with 0, 1, 2, or ≥3 parasites per cell). The difference between the observed and the expected numbers of erythrocytes with single and multiple infections (the pooled
number of pooled erythrocytes with two or more parasites) was tested by using the $\chi^2$ statistic with the Yates correction calculated for each individual. The degree of deviation of the observed distribution from that randomly expected was calculated as the SI (observed number of cells with multiple infections/parasitemia in enzyme-treated cells/parasitemia in control cells) $\times 100$.

Invasion phenotypes of Gambian field isolates. A total of 64 $P. falciparum$ isolates were cultured, and of these, 38 (59%) were tested for association with the enzyme-determined invasion phenotype by using Mann-Whitney U tests (these treat the values of percent inhibition of invasion in a nonparametric manner). Tests of associations between the multiplicity of parasite genotypes within an isolate (1 or more) and the degree of deviation of the observed distribution from that randomly expected were also performed by using Mann-Whitney U tests.

The geometric mean SI for each parasite isolate in vivo was calculated and compared to values reported previously in Thailand (6, 31). Differences in the mean and variance of parasite SI values between isolates with parasitemias of $<5\%$, $5$ to $10\%$, and $>10\%$ were tested by using Mann-Whitney U tests and SPSS version 10.0.

RESULTS

Invasion phenotypes of Gambian field isolates. A total of 64 $P. falciparum$ isolates were cultured, and of these, 38 (59%)
successfully grew and invaded target erythrocytes in vitro (above 2% parasitemia postinvasion). That a large number of the isolates failed to grow was not unexpected and was probably due to many patients not reporting prior antimalarial treatment. The 64 isolates were from 31 male and 33 female patients (1 to 36 years old; average, 5 years). Inhibition of invasion by treatment of erythrocytes with neuraminidase varied between 21 and 87% for different parasite isolates (Table 1 and Fig. 1a), with a mean percent inhibition of 60%. There was also variation in inhibition by trypsin treatment of erythrocytes, with a range of 32 to 99% (Fig. 1b), but the mean percent inhibition was much higher, at 85%. The percent inhibition of each isolate caused by each enzyme treatment was plotted on two axes (Fig. 2) and compared with data for laboratory-maintained isolates from previous studies (4). This analysis showed that the distribution of sensitivity to both enzymes was similar to the distribution characterized for laboratory-maintained isolates from diverse geographical origins. The majority of parasite isolates were sensitive to both enzyme treatments (>50% inhibition), indicating that they depend largely on the presence of sialic acid groups and trypsin-sensitive proteins on the erythrocyte surface for successful invasion.

**Single- and multiple-clone infections.** *P. falciparum* isolates frequently contain multiple distinct genotypes, and so it is possible that invasion phenotypes measured in vitro are often a composite of different parasite types. The presence of single- and multiple-clone infections in each blood sample was determined by PCR amplification of two polymorphic loci, *msp-1* block 2 and *msp-2* block 3, markers that vary considerably among parasite isolates and that provide a relatively stringent way of detecting multiple genotypes within an infected blood sample (9). Of the 38 blood samples assayed for invasion phenotypes, 17 (45%) were determined to have a single detectable parasite genotype, with the rest having two or more genotypes (some having evidence of at least four genotypes). Figure 3a shows the distribution of invasion phenotypes for multiple-clone (two genotypes or more) and single-clone infections. There was no difference in the mean percent inhibition by neuraminidase treatment in single-clone (mean inhibition, 62%) and multiple-clone (mean inhibition, 58%) infections (*P* value, 0.56, as determined by a Mann-Whitney U test). Similarly, there was no difference in the mean percent inhibition by trypsin treatment in single-clone (mean inhibition, 89%) and multiple-clone (mean inhibition, 84%) infections (*P* value, 0.42, as determined by a Mann-Whitney U test).

**Tests of association between invasion phenotypes and sequences of eba-175 and Pfhrp1.** Parasite isolates were typed for three nonsynonymous SNPs at codon positions 577, 584, and
592 of *eba*-175 region II. No association was found between alleles at any of the three SNP loci and invasion phenotypes (Fig. 3b to d) (*P* value for all comparisons, >0.05, as determined by Mann-Whitney U tests). All 38 parasite isolates had an in-frame sequence of *Pfnbp1*, i.e., with a C(A10)T sequence and not a C(A11)T sequence at nucleotide position 8300, which was reported to result in a frameshift stop codon in clone 3D7 (28); these data precluded any test of association between *Pfnbp1* type and invasion phenotype here.

**SI and tests for correlation with invasion phenotype.** Of the thin-film smears of samples from the 64 patients enrolled in the study, 48 (75%) had counts of erythrocytes with single and multiple infections compatible with a random (Poisson) distribution of parasites. In 15 other isolates (23% of the total), significantly more erythrocytes with multiple infections were observed than expected (*P* value for each isolate, >0.05, as determined by chi-square goodness of fit) (Table 2). A single isolate (JB17) had fewer erythrocytes with multiple infections than expected (Table 2). Overall, the geometric mean SI (which determines the magnitude and direction of the tendency toward erythrocytes with multiple infections) was 1.28 (range, 0.39 to 2.74), indicating a fairly moderate tendency in this direction. The average SI for the 38 isolates that successfully grew in vitro was very similar (1.26). Nonrandomness of invasion did not correlate with parasitemia, as no differences were found among the mean SI values for infections with <5%, 5 to 10%, and >10% parasitemia (*P* value for all comparisons, >0.05, as determined by a Mann-Whitney U test) or in their variance (*P* value, 0.790, as determined by a single-factor analysis of variance of the mean SI values for the different levels of parasitemia). The average SI of 1.28 corresponds to the conceptual estimate that about 78% of circulating erythrocytes (the reciprocal of the SI, i.e., 1/1.28) are available to parasites for invasion.

There was no significant association between the SI and percent inhibition of invasion by neuraminidase or trypsin treatment of target erythrocytes for all 38 Gambian isolates that were successfully cultured (*R*² = 0.002, *P* = 0.81, or *R*² = 0.004, *P* = 0.71, respectively) (Fig. 4). Similarly, there was no association between the SI and *eba*-175 region II SNPs at codons 577, 584, and 592 (*P* value for all comparisons, >0.05, as determined by a Mann-Whitney U test). Thus, if the SI is due to parasite invasion phenotypes, then the receptors responsible for selectivity on erythrocytes are probably not sensitive to neuraminidase or trypsin treatment, and the parasite determinants do not appear to involve these polymorphisms in *eba*-175.

**DISCUSSION**

Erythrocyte invasion assays with Gambian *P. falciparum* field isolates demonstrated that there is considerable variability in
the dependence of parasites on the presence of enzyme-sensitive receptors on the erythrocyte surface for invasion. This variability is as extensive as that characterized for laboratory-maintained isolates (4) and for field isolates from India (23).

However, the majority of Gambian parasites do depend on neuraminidase- and trypsin-sensitive receptors, as invasion is inhibited, on average, by 60 and 85% in neuraminidase- and trypsin-treated erythrocytes, respectively. These results indicate two features of wild *P. falciparum* parasites in Africa. First, they are consistent with the hypothesis that the majority of parasites do use the GYPA receptor to invade erythrocytes. These results provide support for the development of a vaccine that elicits antibodies to EBA-175 (the GYPA-binding ligand), which could block erythrocyte invasion via this pathway (16). However, an equally important feature is that none of the parasite isolates are completely dependent on this route of invasion, since none showed 100% inhibition by both enzyme treatments (despite the removal of the GYPA receptor, as determined by antibody agglutination). These results suggest that although a vaccine directed against the GYPA invasion pathway might be partially successful, it could select for parasites using alternative receptor-ligand interactions. These results support the view that an effective vaccine will need to be a multivalent entity to target more than one parasite vulnerability (29).

A previous attempt to correlate parasite genotype with invasion phenotype did not find a correlation between major sequence polymorphisms in *P. falciparum* merozoite antigens and the ability to invade enzyme-treated erythrocytes (4). Typing of the 38 field isolates here for three amino acid polymorphisms in the putative binding region of the F2 subdomain of region II in EBA-175 also showed no correlation with parasite invasion phenotype. This result suggests that amino acid polymorphisms in EBA-175 region II might not be functionally important with respect to binding to GYPA. However, they could be adaptive if they facilitate evasion of host immune responses; this scenario might explain the recent finding that EBA-175 region II shows significant evidence of being under positive diversifying selection within *P. falciparum* (3a, 24).
Typing of the 38 isolates for a region of the Pfmdr1 gene reported to have a stop codon in clone 3D7 (potentially conferring trypsin sensitivity of invasion) (28) showed that Gambian parasites all had a read-through allele. The possibility remains that Pfmdr1 is truncated in some isolates due to an unidentified stop codon (as in another trypsin-sensitive laboratory isolate, 7G8).

The average SI for all 64 Gambian Plasmodium falciparum infections sampled here was 1.28. If we assume that this index reflects the selectivity of erythrocyte invasion in human infections, i.e., that, on average, 78% (~1/1.28) of circulating erythrocytes are invaded, then most Plasmodium falciparum parasites should be able to invade the majority of erythrocytes. This SI is similar to SIs obtained for P. falciparum infections with comparable levels of parasitemia (~2%) in Thailand (6, 31). The Thai studies reported that the SI was inversely associated with the level of parasitemia. However, the majority of this trend was due to lower-parasitemia infections (~2%) that tended to have higher SI values (geometric mean, 2.44) (6, 31). Further studies of lower-density infections are needed to confirm whether this trend is consistent.

The molecular basis of parasite or host determinants of the SI is as yet unknown. The results reported here show an absence of any correlation with the sensitivity of invasion to enzyme treatment of target erythrocytes. These results suggest that the SI is not simply determined by differences in the abilities of parasites to utilize sialic acid or trypsin-sensitive proteins as receptors on the erythrocyte surface. Furthermore, the SI does not appear to be affected by amino acid polymorphisms in the F2 domain of EBA-175 region II. Further investigations of the molecular basis of SI phenotypes could examine variations in other merozoite antigens, in particular, homologues of EBA-175 (1) and PfNBP1 (28).

Gambian field isolates use erythrocyte receptors that are neuraminidase and trypsin sensitive as the major means of erythrocyte invasion. Since GYPA is the dominant sialoglycoprotein on the erythrocyte surface, the data presented here suggest that it is the primary receptor for erythrocyte invasion in West African parasites. Thus, antibodies against the erythrocyte-binding ligand EBA-175 may be effective against these parasites. Antibodies to region II of EBA-175 are common in children in The Gambia, and high levels of these antibodies show a weak association with a lower prospective risk of malaria (22). The role of a homologous antigen, EBA-140, in invasion by wild isolates is also allowed for by these results, since its putative receptor, GYPB (18), is also sensitive to the effects of neuraminidase and trypsin. Despite the variability in the dependence of invasion on neuraminidase- and trypsin-sensitive receptors in Gambian isolates, no parasite isolate is completely dependent on their presence. This finding argues that a vaccine that elicits antibodies against EBA-175 (thereby blocking invasion via GYPA) or EBA-140 (thereby blocking invasion via GYPB) might be initially successful, but protective immune responses to other antigens would also be essential for long-term success.

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