Erythrocyte Invasion and Merozoite Ligand Gene Expression in Severe and Mild *Plasmodium falciparum* Malaria

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Erythrocyte invasion is central to malaria parasite replication and virulence. *Plasmodium falciparum* parasites use different alternative erythrocyte receptors and vary in expression of erythrocyte-binding antigenic (EBA) proteins and reticulocyte-binding protein homologues (Rh). Parasite invasion phenotypes and schizont-stage transcript expression profiles of the 8 eba and Rh protein-coding genes without internal stop codons were determined for 163 clinical isolates cultured ex vivo in The Gambia. There was extensive diversity in ability to invade erythrocytes treated with neuraminidase, trypsin, or chymotrypsin, and severe malaria isolates were less restricted by trypsin treatment than were mild malaria isolates (\(P = .015\)). Expression profiles of the eba and Rh genes showed distinct clusters indicating coordinated alternative transcription. The most divergent of 5 major clusters was dominated by Rh2b, with virtually no expression of eba175 or eba140 genes (which were dominant in the other 4 clusters). Particular transcripts were significantly correlated with parasitemia (Rh5 was positively correlated and eba140 negatively correlated; \(P < .001\) for both) and age of patients (eba181 was positively correlated and eba175 negatively correlated; \(P < .01\) for both) but not with invasion phenotypes or severity of malaria. Severe and mild malaria isolates were also evenly represented across the different expression clusters.

Invasion of erythrocytes by malaria parasites is a complex process [1, 2], and the existence of alternative erythrocyte invasion pathways among cultured lines of the most virulent parasite species *Plasmodium falciparum* has long been recognized [3–5]. Two families of parasite molecules that determine the use of alternative receptors are the erythrocyte-binding antigenic (EBA) proteins and reticulocyte-binding homologue proteins (Rh) [6–11]. The EBA175 and EBA140 ligands bind to erythrocyte receptors glycophorin A and C (GPA and GPC), respectively [12–14]; whereas for some parasites, erythrocyte-binding ligand 1 (EBL-1) can bind to glycophorin B (GPB), for others it is lacking because of an internal stop codon or absence of the gene [15].

The identities of other receptors bound by EBA181 and the Rh proteins are partially characterized by sensitivity to treatment with particular enzymes.

Neuraminidase treatment of erythrocytes removes sialic acids from GPA-C and other receptors, whereas trypsin treatment cleaves several receptors, including GPA and GPC, and chymotrypsin cleaves other proteins, including GPB and band 3 [1]. Parasites that express relatively high levels of Rh1 tend to have sialic acid–dependent invasion [6], but the identity of the neuraminidase-sensitive receptor on the erythrocyte is unknown. In contrast, high levels of expression of Rh2b or Rh4 tend to be associated with sialic acid–independent invasion involving unidentified neuraminidase-resistant receptors [6, 7, 9, 16]. EBA and Rh proteins are targets of invasion-inhibitory antibodies, and parasites may escape some of these invasion-inhibitory effects by switching invasion pathways [17, 18].

Erythrocyte invasion by *P. falciparum* has been in-
vestigated mostly in culture-adapted parasites, but several small studies on clinical isolates have illustrated a wide diversity of invasion phenotypes in India, Brazil, The Gambia, Kenya, Tanzania, and Senegal [19–24]. Differential expression of some of the Rh [19, 20, 25] and EBA ligands [25] has been noted by studying transcripts of ex vivo schizont-stage cultures or examining proteins shed into culture supernatants. The Kenyan study included patients with severe malaria, as well as those with mild malaria, and its findings indicated that the invasion profiles and expression levels of Rh and EBA ligands are not associated with disease severity, although the small number of isolates limited the power to detect differences [24, 25]. The present study on parasite invasion and gene expression in clinical isolates is, to our knowledge, larger than all previous studies combined. It incorporates an analysis of erythrocyte invasion phenotypes in patients with severe or mild clinical malaria recruited during 3 annual malaria transmission seasons in The Gambia and schizont-stage expression profiles of the 3 protein-coding *eba* genes without internal stop codons and all 5 protein-coding Rh genes.

**METHODS**

**Patients with malaria and *P. falciparum* isolates.** The study was approved by the Scientific Coordinating Committee of the Medical Research Council (MRC) in The Gambia, and the Gambian government and MRC Joint Ethics Committee. Written informed consent was obtained from each subject’s parent or guardian before enrollment. Patients with severe malaria presented at the Royal Victoria Teaching Hospital in Banjul, the Jammeh Foundation for Peace Hospital in Serekunda, and the Brikama Health Centre; patients with uncomplicated malaria presented at the Jammeh Foundation for Peace Hospital and the outpatient clinic at MRC Fajara. All are located within 40 km south of Banjul and serve a large urban/perurban conurbation in the coastal area of The Gambia. Subjects aged 1–17 years were recruited during 3 annual malaria seasons (September 2005 to January 2006, September 2006 to January 2007, and September–December 2007). All patients with malaria had axillary temperatures of >37.5°C or a history of fever in the previous 48 h and *P. falciparum* parasite counts >5000 per microliter. Patients with severe malaria also had ≥1 of the following: hemoglobin level ≤6 g/dL, blood glucose level <2.2 mmol/L, lactate level >7 mmol/L, repeated convulsions or a Blantyre coma score of ≤2, or severe prostration. A heparinized venous blood sample (3–5 mL) was obtained from each patient, and plasma was removed. Erythrocytes were then separated from lymphocytes and buffy coat by NycoPrep density gradient centrifugation, washed, and resuspended at 50% hematocrit in Roswell Park Memorial Institute 1640 complete medium (RPMI 1640) (incorporating 25 mmol/L HEPES, 2 mmol/L L-glutamine, 25 mmol/L glucose, 25 mg of gentamicin per liter, 10 mg of hypoxanthine per liter, and 10% human AB serum). DNA was extracted from 100 μL of packed erythrocytes with the QIAamp DNA Blood Mini Kit (Qiagen) and assayed for the presence of single or multiple clones of *P. falciparum* by genotyping the highly polymorphic repeats within *msp1* and *msp2* [26].

**Erythrocyte invasion assays.** Target erythrocytes for invasion were from uninfected laboratory volunteers (blood group O) who had not taken antimalarial drugs in the previous 2 months. Each week, neuraminidase, trypsin, and chymotrypsin treatment of fresh target erythrocytes was performed, and the efficiency of sialic acid and GPA removal was confirmed by specific agglutination tests. Enzyme-treated and control erythrocytes were labeled by incubation, with shaking for 10 min at room temperature with 15 μg/mL sterile fluorescein isothiocyanate (Sigma) solution in RPMI 1640, washed 3 times, and resuspended in complete RPMI 1640 at 2% hematocrit.

In triplicate wells of flat-bottomed 96-well culture plates, 50 μL of target erythrocyte suspension was added to 50 μL of parasitized donor erythrocyte suspension at 2% hematocrit, and the culture was performed in a candle jar at 37°C (with replacement of media after 24 h). After the first complete invasion cycle (48–64 h total in culture), 20 μL from each well was added to 100 μL of phosphate-buffered saline (PBS) containing 10 μg/mL ethidium bromide and incubated, with shaking for 1 h to stain the parasite DNA. Cells were washed once with PBS and resuspended in 200 μL of PBS. In each well, the parasitized cells in 50,000 fluorescein isothiocyanate–labeled erythrocytes were counted by flow cytometry (FACSCalibur; BD), and the mean was then calculated for each treatment. In each assay, the number of nonparasitized cells staining positive for ethidium bromide (mostly reticulocytes) was counted separately and deducted as background. The percentage of inhibition by enzyme treatment was determined as [1 – (proportion of enzyme-treated cells invaded/proportion of untreated cells invaded)] × 100.

**RNA extraction and quantitative transcript analysis.** For parasite RNA preparation, 500 μL of packed erythrocytes from each isolate were cultured in complete RPMI 1640 at 2% hematocrit and in a candle jar at 37°C for up to 48 h until the schizont stage of parasite development; erythrocytes were then resuspended at 50% hematocrit and immediately mixed with 4 volumes of TRIzol reagent (Ambion). Aliquots were stored at −80°C for subsequent RNA extraction using an RNEasy Micro kit (Qiagen). The messenger RNA was reverse transcribed with oligo(dT) using TaqMan reagents (Applied Biosystems), and complementary DNA was quantified in a fluorogenic 5′-nuclease assay on a Rotor-Gene 3000 system (Corbett Life Sciences). Gene-specific TaqMan primers and probe sets for the *Rh1*, *Rh2a*, *Rh2b*, *Rh4* and *ana1* genes were analyzed as described elsewhere [25], as were those for *eba140*, *eba175*, *eba145*, *eba187*, *eba182*, *eba183*, *eba184*, *eba185*, and *eba186*. **Er**
Figure 1. A, Percentage inhibition of invasion due to neuraminidase, trypsin, or chymotrypsin treatment of target erythrocytes for 163 Gambian Plasmodium falciparum isolates, comparing 92 isolates from children with mild malaria and 71 from children with severe malaria. Horizontal bars show mean values within each group. B, Proportions of severe and mild malaria isolates in each of 8 categorical invasion phenotypes defined by the profile of invasion inhibition (>50% or <50%) for all 3 enzymes (eg, for combination A, invasion was inhibited >50% by each of the 3 enzymes).

and eba181 [27]. Rh5 was assayed with forward primer 5'-ACGAAGAATCAAGAAAATAATCTGACGTACT-3', reverse primer 5'-TGTGAATGATCTTTAGCATTATTTGTTTTATATTCTCTTT-3', and probe 5'-CTTCTTCAGTGCTCTTTATGT-3'. Probes were labeled with 6-FAM on the 5' end and a nonfluorescent quencher (MGBNFQ; Applied Biosystems) on the 3' end for single reporter assays. Real-time polymerase chain reactions were performed in 25 μL by using 900 nmol/L concentrations of each primer and 250 nmol/L probe, with 1 cycle at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each run included controls and 3D7 genomic DNA standards, with each sample assayed in duplicate and standard curves generated during each run. The threshold fluorescence value of 0.1 was chosen to determine the cycle threshold, after ensuring that this corresponded to the exponential part of the polymerase chain reaction amplification.

Statistical analyses. For primary analyses, the transcript level of each of the Rh and eba genes was normalized as a proportion of the sum of the transcript levels for the 8 genes in each isolate. For secondary analyses to check the results for individual genes, the transcript level for each gene was examined as a ratio of the transcript level of ama1. Expression profiles were generated by using a heat map, and Ward's clustering was applied to derive a hierarchical cluster analysis of isolate expression profiles, using the BioConductor suite in the R language. Spearman’s ρ was used to assess the nonparametric rank correlation between relative levels of expression, invasion phenotype, patient age, and parasitemia. Mann-Whitney U tests were used to assess whether there were significant differences between groups in the distributions of continuous variables. Statistical analysis was performed using Stata (version 9.0; StataCorp) and SPSS (version 16.0; SPSS) software.

RESULTS

Diversity of erythrocyte invasion phenotypes in clinical malaria isolates. Of the 263 clinical P. falciparum isolates put into in vitro culture, 163 (62%) developed to the schizont stage and successfully reinvaded donor erythrocytes. The proportions were not significantly different between patients with severe malaria (71 [56%] of 126 isolates) and control patients with mild malaria (92 [67%] of 137 isolates). Parasite isolates showed an extremely wide range of dependence on different enzyme-sensitive erythrocyte receptors (Figure 1). Pretreatment of erythrocytes with neuraminidase reduced the relative proportions of cells invaded by a mean of 57.1%, compared with 72.7% for trypsin and 70.1% for chymotrypsin. Isolates from patients with mild malaria were significantly more dependent on trypsin-sensitive receptors than those from patients with severe malaria (P = .015) (Figure 1A). There was no difference between clinical groups in the parasite sensitivity to neuraminidase or chymotrypsin treatment of erythrocytes (P = .26 and .51, respectively).

The mean number of distinct parasite clones detected per isolate (determined by genotyping msp1 and msp2 polymorphic repeat loci) was 1.9 in both severe and mild malaria groups, with 32% of isolates containing a single detectable clone (34% of severe malaria and 30% of mild malaria isolates; P = .6). There was no significant difference between single and multiple genotype isolates in the mean percentage of inhibition by any of the enzyme treatments (P = .7, .8, and .6 for trypsin, neur-
aminidase, and chymotrypsin treatment phenotypes, respectively). Across all isolates, the sensitivity of invasion to trypsin did not correlate with that for either of the other enzyme treatments, whereas neuraminidase sensitivity had a weak positive correlation with chymotrypsin sensitivity (Spearman’s $\rho = 0.23$; $P = .003$).

The combination of sensitivity to the 3 enzymes was then examined by arbitrarily dichotomizing the individual enzyme
profiles with an isolate considered to be “sensitive” if inhibition was >50% than the control and “resistant” if inhibition was <50%. All 8 possible invasion profiles were seen (Figure 1B). The most common profile was characterized by sensitivity to all 3 enzyme treatments (n = 86; profile A in Figure 1B), and the second most common profile was trypsin and chymotrypsin sensitivity with resistance to neuraminidase (n = 32; profile B). Only 3 of the isolates were resistant to all 3 enzymes (profile H).

Expression of Rh and eba genes. Analysis of schizont-stage expression profiles revealed that relative proportions of the 8 eba and Rh gene transcripts varied considerably among the 163 isolates (Figures 2 and 3). The most abundant transcripts were eba140 and eba175, with mean relative proportions of 35% and 31.5%, respectively. The eba181, Rh2a, and Rh2b transcripts showed very similar mean proportions of 8.1%, 7.5%, and 7.1%, respectively. The Rh1 and Rh5 genes showed mean transcript proportions of 4.4% and 4.5%, respectively, and the least abundant transcript was Rh4 (mean proportion, 1.9%).

Cladistic analysis of the expression profiles clustered the 163 isolates into 5 major groups (Figure 2). Group I consists of 13% of the isolates, generally with moderate levels of eba140 and Rh2a and low levels of other transcripts. Group II includes 21% of the isolates and is characterized by high levels of eba140 and moderate levels of eba175. Group III includes 22% of the isolates and has the opposite pattern compared with group II, with high levels of eba140 and moderate levels of eba140. Group IV accounts for 40% of the isolates, and it is characterized by high levels of both eba140 and eba175. The final group, group V, includes only 5% of the isolates but is strikingly different from all the others, because eba140 and eba175 are hardly expressed and the predominant transcript is Rh2b, with varying levels of the other Rh genes (including Rh4, which is rarely expressed outside this cluster). Both severe and mild clinical groups were represented in all the major expression profile clusters, with no significant difference in their distribution; there was also a proportional distribution of single and multiple clone isolates.

Consistent with the major expression clusters revealed by the cladistic analysis, there were strong positive and negative pairwise correlations in the expression of different transcripts (Figure 4). The Rh1, Rh2a, and Rh2b transcript levels are positively correlated (P < .001 for each pairwise comparison), whereas expression of Rh2b was negatively correlated with that of eba175 and eba140.
Figure 5. Correlations between patient age and the relative expression levels of particular schizont-stage transcripts (as a proportion of all 8 eba and Rh gene transcripts measured). A, eba175 is expressed less by parasites from older individuals. B, eba181 is expressed more by parasites from older individuals. These correlations remained but were weaker and not significant when considered as individual transcript ratios to ama1 ($\rho = -0.13$ for eba175 and 0.05 for eba181).

(P<.001). Interestingly, expression of Rh4 was negatively correlated with that of eba175 ($P = .001$) but positively correlated with that of eba181 (Figure 4).

**Associations of eba and Rh gene expression profiles with epidemiological and parasitological variables.** There were no significant differences between severe and mild malaria isolates in the transcript levels of any of the 8 genes considered individually (Figure 3A). However, the proportion of Rh5 was positively correlated with the parasitemia in the patients ($\rho = 0.37; P<.001$), and the proportion of eba140 was negatively correlated with parasitemia ($\rho = -0.30; P<.001$), suggesting that these ligands influence parasite growth rate in vivo (Figure 3B).

The relative levels of expression of particular parasite genes correlated with the ages of the children (Figure 5). The eba175 transcript proportions were significantly lower among older children than among younger children ($\rho = -0.19; P = .01$) (Figure 5A). In contrast, the levels of expression of eba181 were positively associated with increasing age ($\rho = 0.19; P = .01$) (Figure 5B). The expression profiles did not show strong correlations with the parasite invasion phenotypes as assayed by

Figure 6. A, Percentage of inhibition due to neuraminidase (Nm), trypsin (Tr), or chymotrypsin (Ct) treatment of target erythrocytes for 4 samples adapted to culture for ~3 months. Assays were set up at different time points during the period of culture adaptation (days in culture, shown on the X-axes). B, Relative transcript levels of each of the Rh and eba genes in schizont-stage cultures measured at the same time points labeled in A.
the overall neuraminidase, trypsin, or chymotrypsin sensitivity of erythrocyte receptors. Only 2 of 24 tests between 8 gene transcript levels and 3 enzyme-determined invasion phenotype variables showed significant associations at a low level (Rh2a and Rh5 levels correlated negatively with chymotrypsin inhibition of invasion, $\rho = -0.18$ and $-0.19$, respectively; $P = .02$ for both).

Erythrocyte invasion phenotypes and expression of Rh and eba genes during culture adaptation of field isolates. Four single-clone isolates were successfully adapted to culture for $\sim 3$ months, and each remained genotypically distinct and unmixed. Over time in culture, these showed slight variation in dependence on different enzyme-sensitive erythrocyte receptors from that shown after the first round of invasion (Figure 6A). The variation was seen mostly within the first few weeks of growth, and parasites had a stable phenotype before the end of the 3 months of culturing (Figure 6A). Analysis of the schizont-stage expression profiles showed that the relative proportions of the eba and Rh genes remained quite stable throughout the 3-month culture period, with eba175 and eba140 dominating the profile in each of these 4 isolates (Figure 6B).

DISCUSSION

In this large study, although there was a wide diversity of parasite phenotypes in both severe and mild malaria groups, parasites isolated from patients with severe malaria were significantly more able to use trypsin-resistant receptors for erythrocyte invasion. There was also diversity in the sensitivity of invasion to neuraminidase and chymotrypsin but no difference between severe and mild malaria. A Kenyan study showed no significant association between enzyme-sensitive receptor usage and disease severity but included only 12 severe malaria isolates [24]. The diversity of alternative invasion pathways in the mild malaria isolates in our study is consistent with the range shown in an earlier study in The Gambia [21] (Figure 7).

Smaller studies in other populations have shown largely similar phenotypic distributions overall, with a few exceptions (neuraminidase sensitivity was very low in Kenya, as were trypsin and chymotrypsin sensitivity in Brazil) [22, 24] (Figure 7). The weak but significant correlation in invasion inhibition by neuraminidase and chymotrypsin treatments in our study is consistent with results reported in nearby Senegal [20], but we did not replicate the correlation between inhibition by trypsin and inhibition by chymotrypsin reported in Tanzania [19]. Only 3 (2%) of the Gambian isolates showed relatively high ability to invade cells treated by all 3 enzymes (<50% inhibition by each enzyme), a phenotype that is also rare in culture-adapted parasite lines [28].

Previous studies with culture-adapted parasites [9, 29] and field isolates from Kenya and Tanzania [19, 25] have shown that, of the Rh genes, most express predominantly either Rh1 or Rh2a and Rh2b. This pattern was not seen in the present study, in which there were highly significant positive correlations in transcript levels among Rh genes, contrasting with the negative correlations seen with particular eba genes. This finding is more consistent with results from Senegal, where it was
observed that isolates generally expressed Rh1, Rh2a and Rh2b together, rather than as alternatives [20]. The very strong negative correlation between eba175 and Rh4 transcript levels here is notable, consistent with results in Kenya [25] and of experiments in which targeted deletion of eba175 or selection of neuraminidase-treated erythrocytes causes Rh4 transcription to be up-regulated in particular laboratory-adapted lines [7, 30].

Analysis of the 8 eba and Rh genes together showed 5 major expression profile clusters. The 4 largest clusters are generally dominated by eba175 and/or eba140, and the fifth cluster is defined by high levels of Rh2b in the virtual absence of eba140 and eba175 expression. Our study and the Senegalese study [20] showed no strong correlations between the relative expression of these ligands and the enzyme sensitivity of the invasion pathways, and the Kenyan study showed only 1 association of borderline significance (eba175 and neuraminidase sensitivity) [24, 25]. This lack of association may be explained by the crude nature of phenotypes defined by enzyme treatment of erythrocytes, because each enzyme affects not 1 but several receptors. For example, neuraminidase treatment removes sialic acids on GPA, GPB, GPC, and ≥2 other unidentified receptors, so it is unlikely that expression of a single ligand—such as EBA140, which binds to GPC—would correlate strongly with the overall inhibition by neuraminidase treatment.

Some epigenetic changes in expression of eba genes (particularly eba140) in cultured parasite clones have not been associated with a detectable switch in invasion phenotype [31], and a hierarchy of molecular interactions may cause an expressed ligand to be functionally masked by a more dominant ligand [32]. Although most members of the Rh and eba gene families can be experimentally disrupted [1], suggesting some redundancy among them, all attempts to disrupt the Rh5 gene have failed [33]. Interestingly, we found a strong positive correlation between the proportion of Rh5 transcript and the level of parasitemia in the patients, consistent with a hypothesis that Rh5 plays a central role in parasite invasion and replication [11, 33].

The significance of alternative ligand expression should also be considered in the context of acquired immunity. Genetic knockout of particular eba and Rh genes in cultured parasite lines reduced their susceptibility to in vitro inhibition by semimmune Kenyan serum samples [17]. Antibodies to EBA175 were common in those serum samples, as also reported in independent populations studied in the same area [34], elsewhere in Kenya [35], and in The Gambia [36]. EBA175 is a dominantly expressed ligand, important for binding to the major invasion receptor GPA in most laboratory parasite lines, indicating that it defines a favored invasion pathway. In contrast to the other eba genes, sequence polymorphisms in the eba175 gene appear to be maintained by balancing selection [37, 38], so the protein has been inferred to be under immune selection; however, no strong associations have emerged between antibodies and reduced risk of malaria [34–36]. The effects on erythrocyte binding and antibody recognition of structural polymorphism in this and other EBA and Rh proteins need to be more fully understood [11, 14, 15, 39, 40], but it is plausible that immune responses to EBA175 are acquired early in life and select for parasites that express less EBA175 compared with alternative ligands. In support of this hypothesis, we found a negative correlation between the relative levels of expression of eba175 and patient age, whereas none of the other genes showed such a correlation. A vaccine targeting EBA175 in combination with other alternative ligands could be efficient, with Rh5 supported here as a candidate on the basis of a strong positive correlation between relative transcript expression and parasitemia, and Rh2b supported on the basis of its dominant expression in a proportion of isolates.

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