Invasion Pathways and Malaria Severity in Kenyan *Plasmodium falciparum* Clinical Isolates

Anne-Marie Deans,1 Susana Nery,2 David J. Conway,2 Oscar Kai,3 Kevin Marsh,3 and J. Alexandra Rowe1,*

1 Institute of Immunology and Infection Research, University of Edinburgh, Edinburgh EH9 3JT, United Kingdom; 2 London School of Hygiene and Tropical Medicine, London, United Kingdom; and 3 KEMRI/Wellcome Laboratories, Kilifi, Kenya

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Erythrocyte invasion by *Plasmodium falciparum* occurs through multiple pathways that can be studied in vitro by examining the invasion of erythrocytes treated with enzymes such as neuraminidase, trypsin, and chymotrypsin. We have studied the invasion pathways used by 31 Kenyan *P. falciparum* isolates from children with uncomplicated or severe malaria. Six distinct invasion profiles were detected, out of eight possible profiles. The majority of isolates (23 of 31) showed neuraminidase-resistant, trypsin-sensitive invasion, characteristic of the pathway mediated by an unknown parasite ligand and erythrocyte receptor “X.” The neuraminidase-sensitive, trypsin-sensitive phenotype consistent with invasion mediated by the binding of parasite ligand erythrocyte binding antigen 175 to glycophorin A, the most common invasion profile in a previous study of Gambian field isolates, was seen in only 3 of 31 Kenyan isolates. No particular invasion profile was associated with severe *P. falciparum* malaria, and there was no significant difference in the levels of inhibition by the various enzyme treatments between isolates from children with severe malaria and those from children with uncomplicated malaria (P, >0.1 for all enzymes; Mann-Whitney U test). These results do not support the hypothesis that differences in invasion phenotypes play an important role in malaria virulence and indicate that considerable gaps remain in our knowledge of the molecular basis of invasion pathways in natural *P. falciparum* infections.
isolates used a neuraminidase- and trypsin-resistant pathway (21), whereas among isolates from The Gambia and Brazil, the neuraminidase- and trypsin-sensitive invasion profile was predominant (30 of 38 isolates in The Gambia and 7 of 14 isolates in Brazil) (1, 15). The study in The Gambia indicated that the majority of isolates probably invaded through the EBA-175/GPA pathway (or the EBA-140/GPC pathway, as the GPC receptor is also neuraminidase and trypsin sensitive) (1), but the Brazilian study showed that 5 of the 7 Brazilian isolates with this enzyme sensitivity receptor phenotype could invade erythrocytes which lacked GPA (15). In addition, approximately a third (5 of 14) of the Brazilian isolates used trypsin-resistant receptors, which is an unusual phenotype elsewhere (15).

All of the above-cited studies were based on field isolates collected from patients with uncomplicated malaria (1, 15, 21). Parasite isolates from patients with severe P. falciparum malaria have not been studied previously, and it is unknown whether isolates from patients with severe malaria may use a specific invasion pathway or possibly a larger range of invasion pathways than isolates from patients with mild malaria. We therefore investigated the invasion profiles of Kenyan field isolates collected from children diagnosed with either uncomplicated or severe P. falciparum malaria and tested for an association between the invasion profile and disease severity.

### MATERIALS AND METHODS

**Parasite isolates.** Parasite isolates were collected from patients with P. falciparum infections attending Kilibi District Hospital, Kilibi, Kenya, from December 2003 to January 2004 and June to August 2004. At this site, malaria transmission is seasonal (June to August and December to February), with the average number of infective bites per individual estimated at approximately 10 to 30 per year (13). Children with cerebral malaria (those that could not be roused from a comatose state with a Blantyre score of 5) were removed, and the erythrocytes were washed three times in incomplete RPMI 1640 medium (incRPMI; RPMI 1640 medium plus 25 mM HEPES, 2 mM glutamine, 25 mM glucose, and 25 μg of gentamicin/ml, pH 7.2 to 7.4). The parasites were cultured as described below.

**Parasite genotyping.** For DNA analysis, approximately 200 μl of each parasite culture (equivalent to 5 μl of packed erythrocytes) was sampled before growth and DNA was extracted by using the QIAamp DNA blood mini kit (QIAGEN, United Kingdom). To test for the presence of multiple clones of P. falciparum among the isolates, the highly polymorphic repeat sequences in two loci (msp1 block 2 and msp2 block 3) were amplified by using a nested PCR method with allele type-specific internal primers (28).

**SI.** The selectivity index (SI) is a measure of whether invasion is a random process or whether more multiple invasions occur than would be expected if invasion proceeded by chance alone. An increase in the number of multiple invasions is thought to occur when only a subset of the total red blood cell (RBC) population is available for invasion (27). An SI of 1 indicates the random invasion of RBCs, whereas an SI of, for example, 2 indicates twofold more multiply infected RBCs than would be expected if invasion occurred by chance alone. To assess the SI, thin smears from the blood sample collected from each patient were prepared and stained with Giemsa. Three hundred ring-infected erythrocytes per slide were counted, and the number of rings in each erythrocyte was recorded. The SI was calculated as described in detail previously (27).

**Parasite cultures.** Field isolates were cultured in complete RPMI 1640 medium (incRPMI) plus 10% pooled human AB serum. Cultures were set up at a hematocrit value of 1 to 2% with blood group O+ erythrocytes and incubated at 37°C with 3% CO2, 1% O2, and 96% N2. Cultures were monitored for 18 to 36 h by using Giemsa smears, and only those isolates with normal morphology that matured to the schizont (segmenter) stage were included in the study. Two laboratory strains, P. falciparum clone 7G8 (11) and strain Dd2 (6), were studied as controls for the invasion inhibition assays. The laboratory strains were cultured in complete RPMI 1640 medium as described above, except that 10% pooled human O serum. Laboratory strains were synchronized by sorbitol lysis (14).

**Donor erythrocytes.** Donor erythrocytes for the field isolate invasion assays were collected from a single Caucasian donor (blood group O+). Ten milliliters of whole blood was collected into 2 ml of acid citrate dextrose, mixed to prevent coagulation, and stored at 4°C for a maximum of 2 weeks. Before use, the white blood cells were removed by layering 6 ml of whole blood over 5 ml of Lymphoprep (Axis Shield) and centrifuging for 20 min at 400 x g. The erythrocyte pellet was washed twice with 10 ml of incRPMI, resuspended at a 50% hematocrit value, and used within 1 week. O+ erythrocytes for experiments with laboratory strains were obtained from the Scottish Blood Transfusion Service. They were treated as described above.

**Treatment of erythrocytes.** Frozen aliquots of trypsin, chymotrypsin, and the soybean trypsin inhibitor were prepared. The enzymes were dissolved in incRPMI to the required concentrations, as follows: trypsin, 1 mg/ml; chymotrypsin, 1 mg/ml; and soybean trypsin inhibitor, 0.5 mg/ml. They were then frozen in 1-ml aliquots at −20°C. The neuraminidase (Vibrio cholerae neuraminidase; Calbiochem) was reconstituted in distilled H2O and stored at 4°C according to the manufacturer's instructions. The enzyme treatment was based on protocols described previously (1). An aliquot of each of the required enzymes was thawed. A tube with 50 μl of neuraminidase in 1 ml of incRPMI was prepared, and one control tube with 1 ml of incRPMI was prepared. To each tube containing 1 ml of an enzyme mixture (neuraminidase, chymotrypsin, or trypsin) or incRPMI alone (control), a volume of 100 μl of packed donor erythrocytes was added. The tubes were incubated on a rotating wheel for 1 h at 37°C. After that time, all samples were washed once with 1 ml of incRPMI and then soybean trypsin inhibitor was added to the trypsin and chymotrypsin samples to inhibit the enzymes. IncRPMI was added to the control and neuraminidase samples. The samples were incubated on a rotating wheel for 10 min at room temperature and then washed three times before the erythrocytes were resuspended at a 50% hematocrit value in incRPMI. The enzyme-treated erythrocytes and controls were stored at 4°C and used for a maximum of 1 week, after which samples were freshly prepared.

### Table 1. Invasion profiles of P. falciparum laboratory strains

<table>
<thead>
<tr>
<th>Invasion profile</th>
<th>Parasite ligand</th>
<th>RBC receptor</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NmS T8 CT</td>
<td>BAEBL/BAE-140</td>
<td>GPA</td>
<td>22, 26</td>
</tr>
<tr>
<td>NmS T8 CT</td>
<td>JESEBL/EA-181</td>
<td>Receptor E</td>
<td>9</td>
</tr>
<tr>
<td>NmS T8 CT</td>
<td>PiRh1</td>
<td>Receptor Y</td>
<td>23, 31</td>
</tr>
<tr>
<td>NmR T8 CT</td>
<td>Receptor X</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>NmR T8 CT</td>
<td>MSP1</td>
<td>Band 3</td>
<td>10</td>
</tr>
<tr>
<td>NmR T7 CT</td>
<td>AMA1</td>
<td>X</td>
<td>12</td>
</tr>
<tr>
<td>NmR T7 CT</td>
<td>PiRh4</td>
<td>?</td>
<td>30</td>
</tr>
</tbody>
</table>

*a* Nm, neuraminidase; *T*, trypsin; *CT*, chymotrypsin; *S*, sensitive; *R*, resistant. 

*b* Pf, P. falciparum.
antibody (Biotest [UK] Ltd.), anti-S antibody (Biotest [UK] Ltd.), or peanut
Arachis hypogaea) lectin (Sigma; reconstituted in phosphate-buffered saline and
stored in aliquots at −20°C) in a Pyrex tube. The samples in the tubes were
mixed, and the tubes were covered with Parafilm. The samples were incubated for
5 min (anti-S antibody) or 30 min (anti-M antibody and peanut lectin) at
room temperature. The anti-S antibody samples were centrifuged for 1 min at
150 × g before the agglutination was assessed. To check for agglutination, the
pellets were gently dislodged and the agglutination was assessed by eye and scored
(one large agglutinate, +++; small agglutinates, +; and no agglutinates, −). Trypsin
activity was shown by the complete loss of anti-M-mediated agglutination, neuramin-
diase activity by the gain of peanut lectin-mediated agglutination, chymotryp-
sin activity by the complete loss of anti-S-mediated agglutination, and neuramin-
diase activity was shown by the gain of peanut lectin-mediated agglutination after enzyme
treatment.

Invasion assays. The assay to determine the parasite multiplication rate
(PMR) in the first cycle of in vitro culture was set up as described previously (3,
5). Invasion inhibition assays were carried out with two laboratory strains (7G8
and Dd2) in three independent experiments and with the Kenyan field isolates in
a single experiment. All parasites were cultured as described above until the
mature schizont stage. The schizont samples were enriched by centrifugation
in three independent experiments and with the Kenyan field isolates in
a single experiment. All parasites were cultured as described above until the
treatment.

Invasion profiles of Kenyan P. falciparum iso-
lates. The invasion profiles of 31 Kenyan field isolates in the
first cycle of in vitro growth were examined. These isolates
were a subset of those studied previously to examine the rela-
tionships among the PMR, the erythrocyte SI, and malaria
severity (5). The inhibition of the invasion of neuraminidase-
treated erythrocytes by Kenyan field isolates varied from
−32.4% (indicating an increase in invasion) to 100% (i.e., no
ring-infected erythrocytes were counted), with a median of
12.8%. The inhibition of the invasion of trypsin-treated eryth-
rocytes varied from 28 to 100%, with a median of 89.1%. The
inhibition of the invasion of chymotrypsin-treated erythrocytes
ranged from −66.7 to 100%, with a median of 62.8%.

The invasion profiles of the field isolates were defined by the
sensitivity of the isolates to invasion inhibition by neuramin-
diase, trypsin, and chymotrypsin. Isolates exhibiting levels of
inhibition above 50% compared to the control (no enzyme
treatment) were defined as sensitive, and those with levels
below 50% were defined as resistant according to the common
definition criteria (1, 15). The Kenyan isolates showed six out
of a possible eight invasion profiles (Table 2). The most com-
mon profile, seen in 18 of 31 isolates, was neuraminidase-
resistant and trypsin- and chymotrypsin-sensitive invasion,
which matches the profile of the laboratory strain 7G8 (Table
2) (6). Five of the Kenyan isolates also showed neuraminidase
resistance and trypsin sensitivity but were chymotrypsin resis-
tant. Three of the Kenyan isolates were sensitive to all three
enzymes, indicating a pathway whose molecular basis is com-
pletely unknown and that has been identified previously in only
one Brazilian field isolate (15). Three of the isolates showed no
sensitivity to chymotrypsin and were neuraminidase and tryp-
sin sensitive, exhibiting the invasion profile predominant
among samples of Brazilian isolates and Gambian isolates
(although the chymotrypsin sensitivity of the Gambian isolates is
unknown) (1, 15). One isolate showed the same profile as the
laboratory strain Dd2 (6) and was neuraminidase sensitive and
chymotrypsin and trypsin resistant. Finally, one isolate was
resistant to both neuraminidase and trypsin treatment but was
sensitive to chymotrypsin treatment. Two more profiles are
theoretically possible but were not seen in this study (resis-
tance to all three enzymes and a neuraminidase-sensitive, tryp-
sin-resistant, and chymotrypsin-sensitive profile).

The minimum number of parasite genotypes per isolate from
each patient was estimated for isolates from 12 patients,
and as expected for an area with moderately high levels of
malaria transmission, multiple infections were common (mean
number of genotypes overall, 2.2; mean number of genotypes
in isolates from patients with severe malaria [n = 6], 1.5; and
mean number of genotypes in isolates from patients with un-
complicated malaria [n = 6], 2.8). Four of six patients with
severe malaria had single infections, whereas none of the pa-
tients with uncomplicated malaria had single infections.
The presence of multiple genotypes within an isolate from a single
patient has the potential to complicate the interpretation of
invasion profile data, because the profile seen may be a com-
posite of different parasite types. In a mixed-clone infection, it
is unclear whether a single genotype tends to dominate the
infection or whether the isolate consists of roughly equal pro-
portions of the different clones. A previous study found no
significant difference in the invasion profiles corresponding to
single-clone infections and multiple-clone infections, suggest-
ing that the presence of mixed-clone infections does not nes-
sessarily confound the data (1). In addition, for practical rea-
sons, the examination of only single-clone infections in areas
with moderate to high levels of transmission would severely
restrict the number of samples available and would exclude
potentially important data.

Invasion profiles of Kenyan isolates and malaria severity.
Previous studies of the invasion pathways used by field isolates
have been carried out with isolates taken from patients with
uncomplicated malaria. It is unknown whether isolates from
patients with severe P. falciparum malaria may invade by a
specific invasion pathway. We therefore compared the levels of
inhibition of the invasion of enzyme-treated erythrocytes by
isolates collected from patients with severe and uncomplicated
malaria. Nineteen of the isolates were from children with un-
complicated malaria (mean age, 46.3 months [standard devia-
Invasion profiles and other parasite variables. We determined the SIs (a measure of whether invasion occurs randomly in any available erythrocyte or is selective for a subset of erythrocytes) (27), PMRs (5), and rosette frequencies (a parasite virulence-associated factor) (24, 25) for the isolates included in this study as part of a larger study of PMRs and clinical malaria isolate phenotypes described elsewhere (5). None of these variables correlated significantly with the level of inhibition by any of the enzyme treatments ($P > 0.1$; Spearman’s rank correlation).

**ABO blood group and invasion phenotype.** We found a significant difference in neuraminidase sensitivity among isolates taken from patients of different ABO blood group types.
ways. For example, a vaccine based on EBA-175 and/or EBA-140 may not be as effective in Kenya as it would be in The Gambia. Furthermore, the redundancy of invasion pathways used by field isolates indicates that a vaccine based on only one invasion ligand may not greatly inhibit *P. falciparum* invasion and growth but may instead select for parasites which invade by different mechanisms.

The invasion patterns of the majority of the Kenyan isolates (23 of 31) could be broadly described as neuraminidase-resistant and trypsin sensitive. This profile was originally described for the laboratory strain 7G8, and it was inferred that this profile indicated the existence of an erythrocyte receptor “X” (6, 11). The finding that this pathway occurs commonly in clinical malaria isolates in Kenya argues that further research to delineate the molecular interactions underlying this pathway is of major importance. Indeed, it is likely that X is not only one receptor, as invasion by this pathway may be either sensitive or resistant to the treatment of erythrocytes with chymotrypsin. The parasite ligand(s) involved in the neuraminidase-resistant, trypsin-sensitive pathway is also unclear, although one possible candidate is the Rh4 protein that mediates neuraminidase-resistant, chymotrypsin-resistant invasion (30) (the trypsin sensitivity of Rh4-mediated invasion has not yet been described).

The work described here constitutes the first study to examine the invasion profiles of field isolates collected from children with severe *P. falciparum* malaria. Although no significant association between the invasion profiles and disease severity was seen in this study, the sample size was small (only 12 isolates were from children with assorted severe malaria syndromes), so we cannot rule out that a difference may be seen in a larger study, and further work is required. However, the lack of an observed association between invasion profiles and disease severity is consistent with the findings of a previous study of a larger group of isolates collected at the same study site. No significant differences in the multiplication rates or the red cell SIs between isolates from children with uncomplicated malaria and those with severe malaria were observed (5), in contrast with previous results from Thailand (3). Due to the

![FIG. 1. Percentages of inhibition of erythrocyte invasion by Kenyan field isolates from children with uncomplicated (black bars) or severe (gray bars) *P. falciparum* malaria.](image1)

![FIG. 2. Percentages of inhibition of the invasion of neuraminidase-treated erythrocytes by Kenyan field isolates from children with the ABO blood group A (n = 5), B (n = 6), or O (n = 17). The median levels of inhibition are shown; error bars represent third quartile values. Isolates from patients with blood group B were more inhibited by the treatment of erythrocytes with neuraminidase than those from patients with blood group A or O (P = 0.02; Kruskal-Wallis test).](image2)
strong association between the PMR and disease severity in Thailand, a study of the invasion profiles of Thai field isolates, examining whether the high multiplication rates displayed by isolates from patients with severe malaria can be associated with overall less sensitivity to the enzyme treatment of erythrocytes or with a specific preferred invasion profile, may provide further insight.

As knowledge of the P. falciparum ligands involved in invasion increases and technological advances allow their study in field samples, it is important to characterize the expression levels of putative ligands and correlate these findings with invasion phenotypes. Thirteen of the samples analyzed in this study were also included in a recent study of the expression profiles of four Rh genes (those encoding Rh1, Rh2a, Rh2b, and Rh4) and the EBA-175 gene (20). Although the numbers are small and, therefore, this analysis should be regarded as preliminary, a positive correlation between the relative levels of EBA-175 and sensitivity to the treatment of erythrocytes with neuromidase was found, consistent with the known neuromidase sensitivity of the EBA-175 receptor GPA (26).

One unexpected finding from this study was a significant difference in the levels of inhibition by neuromidase treatment depending on the ABO blood group of the host from which the parasites were isolated. Isolates from patients with blood group B were the most inhibited by the treatment of erythrocytes with neuromidase compared to isolates from patients with blood group A or O (Fig. 2) \( (P = 0.02; \text{Kruskal-Wallis test}) \). This finding suggests that the host ABO type may influence the invasion phenotype of the parasite. It remains unclear whether these results occurred by chance, as the sample size was small and the study was not designed to look at the invasion inhibition in relation to the host ABO blood group type, but these findings may merit further investigation.

In summary, this work shows that a variety of invasion pathways are used by Kenyan field isolates and that there is no single discernible pathway specifically associated with severe P. falciparum malaria. Further studies in a variety of geographical areas with isolates associated with various disease syndromes, along with parallel studies examining the transcription of parasite invasion ligands (20), will be required to fully characterize the diversity in invasion pathways and indicate if these pathways may be effectively targeted by vaccines based on the relevant ligands.

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