Genetic Analysis of Circulating and Sequestered Populations of *Plasmodium falciparum* in Fatal Pediatric Malaria

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Falciparum malaria is characterized by cytoadherence of host erythrocytes containing mature asexual-stage parasites and the consequent sequestration of these forms in tissue microvasculature. A postmortem study of pediatric malaria provided us with the opportunity to compare the genetic complexity of circulating and sequestered *Plasmodium falciparum* populations, in patients with fatal cerebral malaria (CM) versus control subjects with incidental *P. falciparum* parasitemia who died of causes other than malaria. Parasite genotypes identified in peripheral blood collected at the time of admission to the hospital constituted a subset of those detected in the tissues at death. Despite a higher tissue burden of parasitized erythrocytes in patients with CM than in parasitemic control subjects, parasite populations in tissues from patients with CM were less genetically complex, and the genotypes were more homogeneously distributed throughout the body, than in patients with incidental infection. Our findings support the notion that CM is associated with the emergence of a small number of dominant genotypes in an infected individual.

Cytoadherence is a specific interaction between endothelial receptors lining microvessel walls and *Plasmodium falciparum* ligands expressed on the surface of infected erythrocytes. Sequestration in the microvasculature via cytoadherence is a characteristic histopathological feature of fatal malaria, and it is thought to play a crucial role in the pathogenesis of that disease. As *P. falciparum* parasites circulate throughout the body and mature to cytoadherent stages, sequestration occurs in the brain, gut, lung, and other tissues in all infected individuals, whether they have symptomatic disease or not [1, 2]. Although all *P. falciparum*-infected erythrocytes (pRBCs) appear to be capable of cytoadherence, parasites that differ phenotypically or genetically can promote adherence to different receptors or combinations of receptors [3, 4]. This has been shown in vitro to result in differential adherence to endothelia derived from different human tissues [5, 6]. Although the density of sequestered pRBCs in a particular tissue is influenced by vascularity, tissue-specific expression of distinct combinations of endothelial receptors may select for parasites that express particular surface ligands and, thereby, may influence the density of sequestration at the vessel level. In theory, erythrocytes infected with genetically variant parasites could differentially sequester in the body. An example of tissue-specific sequestration is seen in malaria during pregnancy, when specific *P. falciparum* ligands exclusively mediate adherence to the placental receptors chondroitin sulfate A and hyaluronic acid [7–10]. Whether this phenomenon oc-
Table 1. Clinical details and final diagnoses.

<table>
<thead>
<tr>
<th>Study group, diagnosis, case no.</th>
<th>Age, months</th>
<th>Type of CM</th>
<th>Time to death, h:min</th>
<th>Level of parasitemia, parasites/μL peripheral blood</th>
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<tr>
<td></td>
<td></td>
<td>CM</td>
<td>At admission</td>
<td>Final</td>
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<tr>
<td>CM</td>
<td></td>
<td>CM</td>
<td></td>
<td></td>
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<tr>
<td>34</td>
<td>70</td>
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<td>74,319</td>
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<td>114</td>
<td>2</td>
<td>14:45</td>
<td>6712</td>
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<td>38</td>
<td>84</td>
<td>1</td>
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<td>39</td>
<td>18</td>
<td>2</td>
<td>17:40</td>
<td>6030</td>
</tr>
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<td>55</td>
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<td>33</td>
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<td>4716</td>
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<tr>
<td>Viral pneumonia</td>
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<td>17</td>
<td>0:45</td>
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</tr>
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</table>

* Types of cerebral malaria (CM): 1, only parasitized erythrocytes in brain microvasculature; 2, additional hemorrhage, accumulation of white cells, and thrombi.

b Patient had Salmonella meningitis.

curs in other tissues has not yet been investigated in vivo, owing to the inaccessibility of sequestered pRBCs during life. Skin [11] and rectal mucosa are potentially accessible, but these may not allow a comprehensive sampling of the parasite strains present in the body.

In regions where the rate of malaria transmission is high, it is common for children to host asymptomatic *P. falciparum* infections that consist of complex mixtures of genotypes that appear periodically in the circulation and in a pattern consistent with the 48-h *P. falciparum* life cycle [12]. In the absence of treatment, the appearance and disappearance of genotypes is generally believed to be a consequence of individual strains sequestering during mature stages and recirculating with each new asexual cycle. If these observations are relevant to symptomatic disease, a single sample from peripheral blood may reveal only a subset of infecting genotypes.

The appearance of clinical symptoms generally coincides with the appearance of new genetic types [13]. In symptomatic and severe disease, genotypes are mainly retained or lost over time, and the appearance of novel genotypes occurs in a minority of cases [14–17]. This rarity of emerging genotypes is influenced in part by treatment, although quinine, which is used in many studies, has a slow onset of action [18].

Malaria causes >40% of deaths in Malawian children <2 years old, and cerebral malaria (CM) is a common manifestation [19]. Children in Blantyre have an average of 2.8 episodes of symptomatic malaria per year, and malaria transmission is stable and intense, which results in genetically heterogeneous infections [9, 16, 20]. Owing to a high prevalence of incidental parasitemia, many children who die of other diseases have *P. falciparum* parasitemia at the time of death. An ongoing post-mortem study in Malawi provides the opportunity to examine sequestered pRBCs. In contrast to longitudinal studies, post-mortem analysis provides a snapshot of malarial infection, at a single point in time, throughout the body. Parasites in every part of the body can be sampled simultaneously, which allows the true genetic complexity of an infection to be ascertained.

We examined circulating and sequestered parasites in chil-
dren who died while infected with *P. falciparum*; for some of these children, malaria was the cause of death; for others, parasitemia was incidental to or not the main cause of death. We analyzed blood and tissues for *P. falciparum* types at the merozoite surface protein (*msp*) 1 and 2 loci and examined the complexity of infection and the distribution of genetically variant parasites.

**MATERIALS AND METHODS**

**Clinical samples.** The present study is part of a larger, ongoing postmortem study that was initiated in 1996. Archived samples from 5 February 1999–15 March 2001 were used for the work described in the present article. Patients were admitted to the pediatric research ward and were classified during life as having CM, severe malarial anemia (SMA), or cerebral malaria and anemia (CM + SMA). The clinical case definition for CM is a Blantyre coma score of ≤2, peripheral *P. falciparum* parasitemia, and no other identifiable cause of coma. SMA was defined as peripheral *P. falciparum* parasitemia, a hematocrit level of <15% at any time during hospitalization, and consciousness until ≤2 h before death. Children with CM + SMA met the case definition for CM and had a hematocrit level of ≤15%.

During the period of the study, 512 patients were admitted to the ward, and 91 of them died. Postmortem examination was requested for 63 patients, and informed consent was obtained from parents or guardians in 24 cases. The 19 children who were 6 months–9 years old were selected for the study (table 1); inclusion criteria were peripheral parasitemia of >50 parasites/μL at the time of admission and availability of a complete array of properly preserved tissue samples. One apasitemic patient with meningitis was also sampled as a negative control. All patients with malaria were treated with quinine; details of diagnosis and treatment have been described elsewhere [21–23].

In 8 of the 19 patients autopsied, either the autopsy itself or the subsequent histological studies revealed a nonmalarial cause of death (table 1); the parasitemia in these patients was assumed to be incidental to the cause of death. In the other 11 patients, no anatomic cause of death was identified; 10 of these patients were assumed to have died of autopsy-confirmed CM, and 1 of them was assumed to have died of autopsy-confirmed SMA. These designations were made before the analyses described in the present article were undertaken. Hereafter, CM, CM + SMA, and SMA will refer to autopsy-confirmed diagnoses.

Peripheral venous blood was sampled at the time of admission and was stored in 8 mol/L guanidine hydrochloride at 4°C. The time between admission and death was 45 min–2.5 days, with a median of 18.5 h, and autopsies were performed at a median of 6 ± 5 h after death. Tissue samples were snap frozen in liquid nitrogen in frozen tissue matrix (OCT compound; Tissue-Tek) at the time of autopsy and were either stored at −80°C or fixed in 10% buffered formalin and processed using routine paraffin embedding for histological examination. The present study was reviewed and approved by the ethics committees at the College of Medicine, University of Malawi; Michigan State University; and the University of Liverpool.

**Histopathological analysis and parasite quantitation.** Tissue sections were cut and stained with hematoxylin-eosin. In sections of brain (parietal lobe, pons, and cerebellum), parasites were quantified as described elsewhere [23]. In other tissues, the number of PRBCs (early-trophozoite to late-schizont stages) was quantified in a total of 10 high-power fields (HPFs). To facilitate comparisons between the counts from the method used for brain samples (based on counting at least 100 cross-sectioned capillaries) and those for other tissues (based on counted capillary contents in 10 HPF), the normalized brain counts (total parasites/100 vessels) were divided by 4 (using an average of 2.5 capillaries/HPFs).

**DNA extraction and genotyping.** Genomic DNA was purified from 200 μL of peripheral blood by use of a standard phenol:chloroform extraction protocol. Approximately 0.5 g of frozen tissue was ground in liquid nitrogen, and DNA was isolated by phenol:chloroform:isoamyl alcohol (25:24:1) extraction and ethanol precipitation. Genotyping for *msp1* and *msp2* was performed with 1 μL of DNA, by the method of Snounou et al. [24]. Briefly, a primary polymerase chain reaction (PCR) amplified the variable block 2 of *msp1* and block 3 of *msp2*. Nested PCRs amplified 3 *msp1* alleles (MAD20, K1, and R033) and 2 *msp2* alleles (FC27 and IC).

Products were electrophoresed in 2% agarose and were scored on a UV transilluminator as described elsewhere [25]. Because the genotyping method for *msp1* and *msp2* can be highly subjective [26], a conservative approach was adopted. Bands within 10 bp in size were considered to be the same, and any band that was <10% the intensity of any other band in the same lane was excluded. All 13 samples from each patient were analyzed simultaneously, and the analyses were repeated for confirmation. The number of detected genotypes was defined as the highest number of products amplified at either locus.

**Statistical analysis.** For statistical analysis, cases were grouped as either CM with or without anemia or as parasitemic control subjects (PCs) who had diagnoses of pneumonia or other causes of death. Data from the patient with only SMA and the apasitemic control subject were not included in the calculations. Statistical differences in *P. falciparum* genetic diversity, both between diagnosis groups and between tissue types, were investigated by repeated-measures analysis of variance, with diagnosis as the between-subject variable and tissue type as the within-subject variable. All other calculations used 2-
RESULTS

Clinical findings. Of the 20 patients studied, 19 had peripheral parasitemia at admission, and 1 was an aparasitemic patient included as a negative control. The pathological findings for the 10 patients with CM were determined as described elsewhere [23]; in 2 of these 10 patients, the brain microvasculature contained only pRBCs, whereas the other 8 patients also exhibited associated hemorrhages, accumulation of white cells, and thrombi. Anemia was a contributing factor in 5 deaths (all patients with CM + SMA), and SMA was the main cause of death in 1 patient. The most common diagnosis in PCs was pneumonia.

Parasite burden. All patients with CM and all PCs were infected with *P. falciparum*. Peripheral blood smears were assayed every 6 h during treatment, to quantify the number of parasites remaining in circulation. The parasitemia level decreased in 11 patients and increased slightly in 2 (table 1). The level of *P. falciparum* parasitemia at admission was 597–782,320 parasites/μL.

After autopsy, the number of pRBCs in the tissues was assessed by light microscopy. The brain microvasculature of patients with CM was highly parasitized (figure 1). Despite high levels of circulating parasitemia in some PCs, few mature pRBCs were observed in the tissues of 2 of them, and none were observed in 6 of them. On average, in all tissues, the mean parasite count in children with CM was higher than that in PCs, significantly so (*P* <.05) in all 3 brain sites and the gut (figure 2A). There were dense accumulations of pRBCs in the spleens of patients with CM, particularly in patient 34 (figures 1 and 2A). There was no correlation between peripheral parasitemia and the number of mature pRBCs in the tissues.

The 1 patient with SMA had a very high level of peripheral parasitemia, at both admission and death, and had dense accumulations of pRBCs in the gut and spleen (figure 1). This patient could not be classified as either having CM or being a PC—and so was not included in statistical analysis—but was included as a comparative case of SMA in the absence of CM.

Genetic analysis. DNA samples obtained from peripheral blood, from 5 sites in the brain, and from 7 other tissues were analyzed by genotyping the single-copy *msp1* and *msp2* genes. The range of genotypes observed in a single patient was 2–9, although that within individual tissues was 0–7. As has been seen in other studies, the level of genetic diversity was very high, which precluded any analysis of genotype clustering between groups.

The average number of circulating genotypes was 2.4 ± 1.4 (range, 1–6). Among patients, mixed infections in the brain extended from a mean of 2.2 ± 0.9 genotypes in the caudate to 2.8 ± 1.3 genotypes in the frontal lobe (range among sites, 0–6). In patients with CM, there was little variation in the average number of genotypes amplified from tissues outside...
patients with CM without anemia and PCs or between patients with CM with anemia and those without anemia. These disparities in genetic diversity do not reflect differences in the age of patients, because this was only a factor within the CM group (67 ± 36 months in patients with CM + SMA vs. 21 ± 11 months in patients with CM without anemia; P = .042).

Figure 3 depicts the distribution of each genotype within each of the 19 patients. Genotypes amplified from peripheral blood were generally detected in most tissues. Only patient 52 had peripheral blood genotypes not amplified from any tissue, and 3 patients (38, 49, and 54) had peripheral blood genotypes amplified in only 1 tissue sample. For patients 52, 38, and 49, this observation of single (or no) amplification could be explained by the extended interval between the collection of peripheral blood during hospital admission and the time of death (table 1). Alternatively, these genotypes may be in low abundance and, therefore, inconsistently amplified. We did not find that particular msp1 or msp2 genotypes were amplified only in brain sites, nor were any other patterns in distribution of individual genotypes identified.

**Negative control.** An asexual patient with meningitis was examined as a negative control for the PCR. No amplification was observed in any tissue, apart from the liver, in which a single msp2 FC27 allele was detected. Histological examination revealed considerable numbers of pigment-laden monocytes in the tissues (data not shown). Because pRBCs were not detected in any histological section (figure 1), we concluded that the child had recently had a malaria infection and that a remnant of this had been amplified from the liver.

**DISCUSSION**

The main question that we investigated in the present study was whether there is evidence of restricted distribution of genetically variant *P. falciparum* in the tissues of the human host. To answer this, we compared, in children with fatal malarial disease and in *P. falciparum*-infected hosts who died of causes other than CM, parasite populations from peripheral blood, multiple sites in the brain, and 7 other tissues.

With one exception, *msp* genotypes amplified from peripheral blood were also detected in ≥1 tissue, usually at a majority of sites. Additional genotypes were amplified from the tissues in both disease groups; in patients with CM, the few extra genotypes were distributed relatively homogeneously throughout the tissues, whereas PCs had more-complex infections, with most genotypes dispersed heterogeneously through a subset of tissues.

We analyzed 5 sites in the brain to determine whether particular types of *P. falciparum* were detected there but were absent from other tissues, which would provide evidence of brain-specific sequestration. We found no compelling evidence of this, nor were there any obvious patterns in the distribution of individual *msp* genotypes in other tissues examined. We conclude
Figure 3. Distribution of *Plasmodium falciparum* merozoite surface protein (*msp*) 1 and 2 genotypes in the tissues of 19 parasitemic children. Each chart represents data on a single patient; the charts are grouped on the basis of autopsy-confirmed diagnosis. The patient no. is above each chart and tissue site is on left. Each shaded square represents a genotype detected by polymerase chain reaction. Vertical alignment represents genotypes of the same electrophoretic mobility within a chart, but not between charts. Light gray shading denotes *msp1* alleles, and dark gray shading denotes *msp2* alleles. Final diagnoses for deaths from other causes are provided in table 1. B-bg, brain basal ganglia (caudate); B-c, cerebellum (tonsils); B-fl, brain frontal lobe; B-p, pons (brain stem); B-pl, brain parietal lobe; Gut, jejunum and right colon of gut; Hrt, left ventricle of heart; Kdy, right kidney; Lng, right upper lung; Lvr, liver; PB, peripheral blood; PM, pectoralis muscle; Spl, spleen.
that specific genetic populations of pRBCs, as defined in terms of *P. falciparum msp1* and *msp2* genotypes, are not associated with preferential sequestration in the brain in children with malaria.

In comparison with the high levels of sequestered pRBCs in patients with CM, most PCs had few or no sequestered pRBCs in any of the sampled tissue sites. Despite this, genetically mixed *P. falciparum* populations were commonly observed in PCs and in some tissues from patients with CM in whom no intact pRBCs were observed. The low level of sequestered or circulating pRBCs in PCs could explain why the distribution of individual types is diffuse and random—specific genotypes at low levels might be detected inconsistently. This could be seen particularly in patients 45 and 47, who were admitted with high levels of peripheral parasitemia that decreased by >99.8% before death. Alternative explanations for this pattern include the possibility of amplification of DNA from killed parasites or of detection of gametocyte populations. We did not observe *P. falciparum* sexual stages in any of the sequential peripheral blood samples or any tissue samples. However, pigment-laden monocytes were observed in all patients in whom genetically mixed infections were detected (data not shown), including in the liver of our “aparasitemic” control subject.

It is possible that CM infections are not actually less complex than asymptomatic parasitemia but that, instead, 1 or 2 genotypes are present at such high numbers that they completely dominate the infection and prevent the detection of other strains by PCR. The PCR method used in the present study does not provide any relative quantification of the different strains, nor does it always allow the detection of minority populations. It also does not distinguish between circulating and sequestered parasites. However, the level of peripheral parasitemia in most patients with CM decreased substantially during treatment, whereas microscopy results demonstrated high levels of mature pRBCs remaining in the tissues at death. In terms of pathogenesis, dominant types detected in the present study may be more adept at specific types of sequestration, such as those seen in pregnancy-associated malaria. An alternative explanation is provided by recent reports, which have demonstrated that specific genetic populations of pRBCs, as defined in terms of *P. falciparum msp1* and *msp2* genotypes, are not associated with preferential sequestration in the brain in children with malaria.

The high diversity of genetic types, up to 9 in a single child, and the common presence of malarial pigment in the tissues are suggestive of chronic carriage of *P. falciparum* by these children. Longitudinal studies have noted recurrences of *P. falciparum* genetic types in the peripheral blood of asymptomatic children over time [12, 28, 29]. Although, owing to their low density, chronic carriage of genetic types may not be consistently detected in the circulation, they are more likely to be detected in the tissues, where they are concentrated via cytoadherence to vascular beds.

The lack of a differential *msp1/msp2* genotype distribution in the tissues can be explained by evidence that merozoite surface protein functions in the invasion of host erythrocytes (reviewed in [30]); therefore, tissue tropism of parasites expressing variants of these genes would not be expected. Because of the unequal expression of endothelial receptors in the tissues [1], cytoadherence ligands may provide a more relevant target for restricted distribution of *P. falciparum* types. Differential sequestration is more likely to result from the expression of different forms of *P. falciparum* erythrocyte membrane protein 1, an antigenically variant protein family that has been implicated in cytoadherence. Owing to the extreme heterogeneity and selective transcription of this protein family, expression studies would be required for this investigation.

Pediatric postmortem studies are, by their nature, difficult to perform; thus, the number of samples available to the present study was limited. For this reason, data from only 18 patients were included in statistical analyses, and, therefore, the present study’s observations do not have the power of those from a larger study and are mainly of a descriptive nature.

With the exception of patient 54, who died shortly after admission, all parasitemic patients were treated with quinine, and many had received antimalarial treatment before admission. Quinine arrests the development of parasites at schizogony; circulating parasites are unaffected until maturation and sequestration, at which point they are arrested in the tissues [31]. We observed that, in the patients with CM in whom pRBCs were detected in most tissues—that is, patients 32, 34, and 42—death occurred rapidly (i.e., within only 4 h after admission). This interval was longer—between 11 and 38 h—for the remaining patients with CM, all of whom had few pRBCs in their tissues (apart from the liver and spleen). These findings suggest that the removal of pRBCs from tissues does occur during treatment with quinine. Previous studies that attempted to quantify the sequestered parasite load after antimalarial treatment found no suitable markers for sequestered parasites [31, 32]; an expansion of our study, to investigate this question in vivo, may provide insights into host response to *P. falciparum* infection during treatment with quinine.

In conclusion, we have found that children who die of CM have dense *P. falciparum* sequestration in brain microvasculature and, less consistently, in other tissues. The genetic complexity of *P. falciparum* is comparable throughout the body, and circulating genotypes form a subset of those present in the tissues. Patients who die with asymptomatic parasitemia carry little or no sequestered parasite load, although the complexity of infection is equal to or greater than that seen in tissues from patients with CM. The genotypes may be circulating or remnant forms, as is demonstrated by their heterogeneous distribution...
throughout the body. These results imply that there is extensive chronic carriage of *P. falciparum*, as indicated by the relatively high numbers of genotypes detected in tissues. Our findings support the hypothesis that there is restricted distribution of specific parasite populations in tissues, but a definitive demonstration will require further studies that are able to define the pRBC phenotypes more closely linked to the parasite’s adhesive behavior.

Acknowledgments

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References


