NEW EMERGING \textit{PLASMODIUM FALCIPARUM} GENOTYPES IN CHILDREN DURING THE TRANSITION PHASE FROM ASYMPTOMATIC PARASITEMIA TO MALARIA

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Abstract. Semi-immunity against \textit{Plasmodium falciparum} occurs after many infections. In areas of high malaria transmission, the prevalence of asymptomatic parasite carriers increases with age. We investigated \textit{P. falciparum} genotypes in a cohort of asymptomatic carriers who were followed until they became symptomatic. Blood spots on filter paper and blood smears were collected daily from 10 children in Lambaréné, Gabon. The parasite genotypes present on successive days were determined by a polymerase chain reaction using the polymorphic region of the merozoite surface antigen-2 for typing. The same parasite genotypes persisted in eight out of ten children and parasite densities were low throughout the asymptomatic phase indicating inhibition of parasite growth. Appearance of symptoms was associated with an increase in parasitemia and appearance of novel parasite genotypes. The results suggest that the parasites causing a clinical episode are those against which a child has not yet mounted an efficient protective immune response.

INTRODUCTION

Humans develop a partially protective immunity against \textit{Plasmodium falciparum} malaria after repeated infections. In Africa, adolescents 12–15 years of age and older usually acquire the semi-immune status that protects against disease, although young children may acquire resistance against severe disease after very few exposures to \textit{P. falciparum}.\textsuperscript{1}

In high transmission areas, asymptomatic carriers, i.e., individuals harboring parasites without symptoms, are numerous, even among older children. The parasite burden can consist of numerous subpopulations that are stable in complexity,\textsuperscript{2} but can show some degree of diversity from day to day.\textsuperscript{3} Studies in Senegal demonstrated a high complexity of infection based on the genotypic pattern of the parasites in asymptomatic carriers.\textsuperscript{4} Parasite multiplication is thus controlled even if the parasites represent different strains. Factors in both the host and the parasite might be involved in this process. Human genetic polymorphisms that influence the course of an infection include the sickle cell trait\textsuperscript{5} and NOS2 Lambaréné.\textsuperscript{6} Conversely, certain parasite types may cause more severe symptoms than others. This finding, however, has a strong geographic component since different parasite genotypes were associated with disease severity in different regions.\textsuperscript{7,8}

We were interested to know whether a parasite population changes within the host when an asymptomatic individual becomes symptomatic. Therefore, we followed the profile of parasite genotypes in asymptomatic parasiticemic children until they became ill.

PATIENTS AND METHODS

The study took place in Lambaréné, Gabon, a typical Central African rain forest area where \textit{P. falciparum} infection is hyperendemic.\textsuperscript{9} Ten children 5–11 years old were enrolled. They were part of a study cohort of 200 children followed by our center in Lambaréné since 1995.\textsuperscript{10} This study was approved by the Ethics Committee of the International Foundation for the Albert Schweitzer Hospital in Lambaréné. The design, objectives, and initial results of this study have been previously reported.\textsuperscript{10,11}

The inclusion criterion for the present study was a thick blood smear positive for \textit{P. falciparum}, with no symptoms, for at least five days. Asymptomatic parasiticemic children were detected during the active follow-up visits carried out every second week. The asymptomatic children were always visited daily at 6:00 PM until symptoms appeared because of the known circadian rhythm of malarial fever.\textsuperscript{12} After seven days of asymptomatic parasitemia, the children were visited every second or third day. Symptoms were defined as fever (a rectal temperature $\geq 38.3^\circ C$) and facultatively other symptoms that could be related to malaria. At each visit the children were questioned about symptoms, a clinical examination was performed, a thick blood smear for microscopic analysis was taken, and 50 $\mu l$ of blood from the same fingerprick was spotted onto filter paper. The blood on the filter paper was dried and stored in individual plastic bags at 4°C for subsequent analysis by a polymerase chain reaction (PCR). The rectal temperature was measured using a digital thermometer. When the child became symptomatic blood was taken for laboratory analysis and the patient was given standard therapy with sulfadoxine/pyrimethamine.\textsuperscript{13}

For preparation of \textit{P. falciparum} DNA, a small piece of filter paper containing dried blood was cut out and soaked in 1 ml of phosphate-buffered saline (PBS) and saponin (0.05%) at room temperature overnight until the blood was dissolved. The brown solution was removed, and the sample was washed again in PBS and then resuspended in 200 $\mu l$ of lysis buffer (40 mM Tris-HCl, pH 8.0, 80 mM EDTA, 2% sodium dodecyl sulfate). The DNA was purified using a DNA purification kit (Qiagen, Hilden, Germany).

The PCR was performed on a Rapid Cycler (Idaho Technologies, Idaho, MI) using standard procedures.\textsuperscript{14} The nested PCR was necessary to increase sensitivity of \textit{P. falciparum} detection. Genotyping of merozoite antigen-2 (MSA-2) was performed and DNA fragments were visualized after electrophoresis on a 1.5% agarose gel using standard procedures.\textsuperscript{15}

The PCR conditions are given as $[\text{denaturing temperature (denaturing time)}] - [\text{annealing temperature (annealing time)}] - [\text{extension temperature (extension time)}].$
extension temperature (extension time) in °C (seconds). The first round PCR was done with T4: 5'-GAA GGT AAT TAA AAC ATT GTG TGC-3' (94°C for 5 sec, 50°C for 5 sec, and 72°C for 15 sec) and T8: 5'-CTA GAA CCA TGC ATA TGT CC-3' (94°C for 5 sec, 50°C for 5 sec, and 72°C for 15 sec). The differentiation between the MSA-2 families was done with T4 and 3d7rev: 5'-GCT TGT TCA GGT TGT GCA GT-3' (94°C for 5 sec, 52°C for 5 sec, and 72°C for 15 sec) and Fe27rev: 5'-CTC TTC TCC TTT ACC GTC TG-3' (94°C for 5 sec, 52°C for 5 sec, and 72°C for 15 sec). Thirty cycles were carried out in first amplification and 20 cycles were carried out in the second amplification. All PCRs were carried out in a total volume of 30 μl. Positive and negative controls were run in parallel with each test sample.

RESULTS

Ten children met our inclusion criteria and were enrolled into the study. Seven of them were girls. Their mean ± SD age was 7.1 ± 0.6 years. The duration of the asymptomatic course varied between 7 and 38 days.

The daily parasitemia varied from child to child. It remained low and in eight children the parasite densities peaked with the occurrence of symptoms. There was a dominant parasite genotype in eight cases. An increase in parasitemia was coupled with the occurrence of new variants and appearance of symptoms. Nevertheless, in two children (52S and 65M), the appearance of new variants causing symptoms was not associated with an increase in parasitemia. The delay between the last documented malarial attack and the reinfection leading to asymptomatic parasitemia was eight months for one child (subject 80M) and one to four months for the others. Hemoglobin levels ranged between 9.2 and 12.3 g/dl when the children became symptomatic. Two children were carriers of the sickle cell trait.

The characterization of the parasite strains with a single genetic marker was sufficient to show the course of the infection. Both allelic families of MSA-2 were found in all children, and extensive size polymorphism was observed.

Child 80S (Figure 1) experienced eight days of asymptomatic infection with only one parasite of the Fe27 family. On day 9 the child became symptomatic and a new Fe27 variant and an additional 3D7 strain appeared. A second child (8M, Figure 2) followed the same course: after 32 days of harboring a particular Fe27 strain, the child became symptomatic with two new Fe27 variants and one new 3D7 strain. In five children (36M, 80M, 52S, 58M, and 48M; Figures 3–7), the parasites during the asymptomatic phase were of the 3D7 variant.

![Figure 1. Parasite densities and genotyping analyses of finger-prick blood samples from a child (80S) with an asymptomatic parasitemia. The upper panel shows the course of the parasitemia (parasites/μl) over the time period given in days on the x-axis; parasitemias estimated on successive days are connected by a line. The middle and lower panels show the polymerase chain reaction products specific for the two different merozoite surface antigen-2 families: the 3D7 family in the middle panel and the Fe27 in the lower panel. Lane 9 represents the symptomatic phase. A kilobase (kb) marker was also used. + indicates the positive control for the respective strain; - indicates the negative control. The arrow points to a band representing a minor fraction of 3D7 parasites.](image1)

![Figure 2. Parasite densities and genotyping analyses of finger-prick blood samples from a child (8M) with an asymptomatic parasitemia. For more information, see Figure 1.](image2)
The children became symptomatic with emerging Fc27 parasites.

In three cases (65M, 54S, and 17S; Figures 8–10), a less clear pattern was observed, although at the time children became symptomatic new parasite genotypes also appeared in those cases.

**DISCUSSION**

A high complexity, with a high degree of fluctuation, of parasite strains of *P. falciparum* has been shown to be present in asymptomatic infections in earlier studies. 3, 4 Eight of our children presented, in contrast, a rather constant parasite sub-population profile. However, when children became symptomatic, a new emerging parasite genotype appeared in the blood of these patients.

Asymptomatic *P. falciparum* infection is quite common in adolescents and adults, since protective immune responses against *P. falciparum* malaria seem to be acquired only slowly after many infections. However, asymptomatic episodes with *P. falciparum* parasitemia start to occur from approximately five years of age onwards in our study area. This is exactly the age range (5–11 years) when protective immune responses become effective enough to control parasite multiplication. Thus, children have episodes of variable periods during which they are parasitemic but asymptomatic. However, based on our own data, 10 we know that many of those children become
FIGURE 6. Parasite densities and genotyping analyses of finger-prick blood samples from a child (58M) with an asymptomatic parasitemia. For more information, see Figure 1.

FIGURE 7. Parasite densities and genotyping analyses of finger-prick blood samples from a child (48M) with an asymptomatic parasitemia. The arrow indicates a weak band with a new Fc27 genotype. For more information, see Figure 1.

FIGURE 8. Parasite densities and genotyping analyses of finger-prick blood samples from a child (65M) with an asymptomatic parasitemia. For more information, see Figure 1.

FIGURE 9. Parasite densities and genotyping analyses of finger-prick blood samples from a child (54S) with an asymptomatic parasitemia. For more information, see Figure 1.
symptomatic. In the present study, the parasite densities were relatively low throughout the course of the asymptomatic phase, reflecting a certain degree of inhibition of parasite growth. Thus, the immune response already developed by the children maintained specific parasite genotypes under the threshold at which they would induce disease. In addition, in some cases new types that occurred during the asymptomatic phase also could be controlled.

Lambaréné, Gabon, the site of our study, is an area that is hyperendemic for *P. falciparum,* with an entomologic inoculation rate of approximately 50. However, it is unclear to what extent our observations can be extrapolated to other malaria-endemic areas. We do not know how parasite strains in areas of lower malaria endemicity behave in asymptomatic subjects.

Increasing parasitemia was associated in eight cases with rapid multiplication of a particular parasite type probably results from a lack of an immune response with the appropriate specificity. This is reflected in the children in whom new variants arise with the onset of symptoms or shortly before them. It is likely that these strains represent new infections since malaria transmission in the Lambaréné area is approximately 50 infected bites per year. The cases studied here are part of a study cohort of 200 children followed since 1995. Other individuals who experienced successive symptomatic infections were always infected by a different *P. falciparum* genotype.

We conclude that the parasites causing clinical episodes obviously are those against which a child had not yet mounted an efficient immune response. We have not determined in this study the relative role of antibodies with specificity for variant antigens of *P. falciparum.* Such antibodies are known to influence the outcome of infection.

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