Rapid Turnover of *Plasmodium falciparum* Population in Asymptomatic Individuals Living in a High Transmission Area

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Abstract. A polymerase chain reaction (PCR) typing technique, based on the amplification of polymorphic regions from the merozoite surface protein 1 (MSP-1) and MSP-2 *Plasmodium falciparum* genes, was used to characterize parasites collected in a longitudinal study of asymptomatic carriers of malaria parasites living in two distinct epidemiologic situations. Blood samples were collected from children and adults living in the village of Dielmo, Senegal, when malaria transmission was 3-6 infective bites/week/individual. For each individual, every sample collected at two-week intervals over a period of three months showed a specific PCR pattern. Changes involved both appearance and disappearance of specific alleles. Analysis of blood samples collected at a few-days interval showed that modifications of the PCR patterns occurred rapidly. Most alleles were detected over a period of 2-3 weeks, but some alleles could be detected only for a few days. The frequent modifications of the PCR patterns indicate significant changes in allelic balance over time, and importantly, this was observed both in children and adults. These results strongly contrast with the stability of the parasite types harbored by asymptomatic individuals living in Pikine, Senegal during a period in which malaria transmission was interrupted, and therefore suggest that the rapid turnover observed in Dielmo may reflect the introduction of new parasite populations by mosquitoes.

Protective immunity against *Plasmodium falciparum* malaria is acquired slowly after a large number of infections and its maintenance requires a sustained exposure to infected mosquitoes. Adults living in holoendemic and hyperendemic malaria areas possess a status of premunition, resulting in a high prevalence of individuals carrying low-grade parasitemia without presenting clinical symptoms. In these regions, asymptomatic carriage can be observed in more than 90% of individuals, as demonstrated by highly sensitive detection techniques such as the polymerase chain reaction (PCR). This peculiar immune status is generally described as the successive and/or concomitant involvement of two distinct immune phenomena: 1) a clinical or anti-disease immunity that enables the carriage of important parasite loads in the absence of symptoms and that develops rapidly during childhood, and 2) an anti-parasite immunity, responsible for a marked reduction of the parasite densities after the age of 15-20. It is still unclear how these two mechanisms interact or complement one another and result in asymptomatic carriage of the parasite.

*Plasmodium falciparum* isolates are known to be composed of complex mixtures of parasite populations, as demonstrated by isoenzyme patterns, two-dimensional gels, monoclonal antibody studies, or PCR analysis of polymorphic markers. However, since most samples characterized so far have been collected from symptomatic patients, very little is known about the complexity and behavior of parasite populations harbored by constantly exposed asymptomatic individuals from holoendemic regions. In 1990, an in-depth, follow-up study was initiated on the entire population of the village of Dielmo in Senegal where transmission is high and perennial, thus permitting not only precise information on entomologic parameters in this village, but also a documented survey of the epidemiologic, clinical, parasitologic, and immunologic history of each inhabitant. A cross-sectional survey of asymptomatic residents from this village demonstrated that the complexity of the infections decreases in an age-dependent manner in this village, in parallel with the appearance of an immune protection and a reduction of parasite density. Furthermore, this showed that acquired anti-parasite immunity not only reduces parasite density, but also limits the number of parasite genotypes infecting an individual at a given time. However, little is known about how these infections evolve over a long period of time.

We performed a longitudinal analysis of the parasite populations infecting asymptomatic individuals living in Dielmo. We also studied longitudinal samples from chronic carriers living in a low-endemicity urban area during a period of nontransmission. Parasites were characterized using a PCR technique based on amplification of polymorphic merozoite surface protein 1 (MSP-1) block 2 and MSP-2 central domain. The sensitivity and the potential limitations in the analysis of samples containing several alleles have been detailed in a previous report. The data reported here provide new information on the dynamics of host-parasite relationships, on anti-parasite immunity, and on parasite evasion mechanisms.

MATERIALS AND METHODS

Study site, choice of blood donors, and collection of isolates. All blood samples were collected with the informed consent of the donors or their parents. The highly endemic site chosen was the village of Dielmo, Senegal, 280 km southeast of Dakar, where malaria transmission is perennial due to the presence of a stream, but is maximal during the rainy season that occurs between July and October. The first follow-up study was conducted between June and September 1990 (individuals with identification numbers 07/06, 10/05, 11/13, 13/03, and 29/06) and the second one between August...
and September 1992 (individuals with identification numbers 06/01, 04/01, and 18/11). During both these periods, the entomologic inoculation rate was 3–6 infective bites/individual/week.7 Studied subjects were selected on the criteria of a positive thick blood smear at enrollment and the absence of gametocytes during the follow-up study. None of these individuals presented any clinically patent malaria episode in the course of, and for at least one month prior to, the survey.

The third follow-up study was performed in the district of Pikine, Senegal, a Dakar suburb. Samples were collected between January and February 1993, i.e., during a nontransmission period.8,9 Blood donors (individuals with identification numbers 407, 512, and 385) were randomly chosen among patients with malaria coming in for consultation in December 1992 who were treated with chloroquine and who showed subsequent asymptomatic chronic carriage of parasites despite treatment.

All samples were collected and stored as previously described.10 Parasite density was determined by microscopic examination of Giemsa-stained thick blood smears.11

**Extraction of DNA and PCR conditions.** The DNA from approximately 10⁶ red blood cells was phenol-extracted as previously described and resuspended in 70 µL of water.8 Five microliters were amplified according to the manufacturer’s recommendations (Amersham, Buckinghamshire, United Kingdom) in an Hybrid automated heat block (Cera-Labo, Ecquevilly, France) for 35 cycles (94°C for 2 sec, 55°C for 1 min, 72°C for 2 min). The PCR analysis used here was based on the amplification of highly polymorphic regions from two markers: block 2 of MSP-1 and the repeated region of MSP-2.10,11 The primers used were 1) for MSP-1: MSP-1.A (5'-AAG CTT TAG AAG ATG CAG TAT TGA C-3') and MSP-1.B (5'-ATT CAT TAA TTT CTT CAT ATC CAT C-3'); and 2) for MSP-2: MSP-2.2 (5'-AAC GAA T1C ATA AAC AAT GCT TAT AAT AGT ATG-3') and MSP-2.3 (5'-GAT GAA T1C TAG AAG CAT GCA TAT GTG CAT G-3'). Detailed procedures are described by Commin and others.12

**PCR analysis and hybridization conditions.** Ten microliters of PCR mixture were loaded on agarose gel (FMC Bioproducts, Rockland, ME) containing 55°C agarose gel (FMC Bioproducts, Rockland, ME) containing 55°C.

**RESULTS**

**Longitudinal analysis of asymptomatic Dielmo villagers during a period of intense transmission. Two-week samplings.** We first analyzed the complexity of parasite populations harbored by constantly exposed asymptomatic carriers and the duration of carriage of specific parasite genotypes. Figures 1 and 2 show the MSP-1/MSP-2 PCR products amplified from blood samples collected every 2–4 weeks during the 1990 rainy season from individuals between three and 26 years of age (see figure legends). Both polymorphic markers indicate that these asymptomatic infections were complex, since in some cases up to 5–6 bands could be visualized in one sample with a single pair of primers. In all cases, the PCR patterns obtained with the samples collected from a single individual were all distinct from one another, indicating substantial changes in the composition of the parasite populations in the peripheral circulation. It is important to stress that the modifications of these PCR patterns involved both the appearance and disappearance of certain amplified products, which were not related to modifications in the parasite densities. A typical example is shown in Figure 1, in which samples from a 3.5-year-old child have been analyzed. The 440-basepair (bp) band amplified from the sample collected on day 1 was no longer detected in the next sample collected on day 16. The amplification of two new bands (470 bp and 525 bp) on day 16, while the parasite density on that day was lower than on day 1, indicates that the dominant parasite genotypes on day 16 were different from those prevailing on day 1. The detection of these two new bands reflects either recent inoculation(s) or an increase in the parasites that had previously been carried at a lower density. The two bands amplified from the sample collected on day 16 could no longer be detected in the samples collected on day 29 and day 43, which reflects at least a significant decrease in the density of the corresponding parasites. Likewise, the amplification of two novel alleles (360 bp and 480 bp) from the sample collected on day 29, concomitant with a four-fold increase in parasitemia, can be attributed to either a novel infection or a significant increase in the parasites previously carried at a lower density. The 360-bp band was still detected, with a lesser intensity, on day 43 although it could not be detected on day 57. Additionally, two new genotypes (420 bp and 515 bp) were detected on day 43. Since they were absent from the products generated from the samples collected previously, it is likely that they resulted from a recent inoculation. Similarly, on day 57, three novel parasite populations were visualized, while the three former ones were no longer amplified. The comparison between the profiles obtained on day 43 and day 57 is particularly informative, since the parasite densities were similar and, therefore, the distinct patterns obtained reflect totally different parasite populations. This clearly suggests a rapid turnover of the parasites detectable in the peripheral circulation.

Similarly, rapidly changing patterns were observed in each of the four individuals analyzed in Figure 2. Indeed, both MSP-1 and MSP-2 profiles differed from one sample to the next. Note that parasite densities in the samples from individuals 07/06, 10/05 and 11/13 were in the same range. This further suggests that the modifications observed reflect intrinsic differences in the parasite populations present at the various time points in the same individual.

**Sampling at 2–4-day intervals.** Our results led us to investigate in greater detail the turnover rate of the parasite populations present in asymptomatic individuals. A second longitudinal study with samples taken at shorter time inter-
Indeed, detection of a 640-bp FC27 allele fluctuated from an asymptomatic teenager shown in Figure 4 ranged from day 12 to day 39. Another FC27 allele (730 bp) was also detected with varying intensity from day 12 to day 29 and was no longer detected thereafter, while a new IND/3D7 allele of the same size was amplified. Another IND/3D7 allele (700 bp) was detected on days 12, 13, 16–24, and 50. It is interesting to note that on day 14, the three parasite genotypes detected in this individual decreased under the threshold of sensitivity of both PCR and thick smear examination, although they were apparently still present since they became detectable again two days later. In agreement with the results of the first follow-up (Figure 1 and 2), samples collected at 12-day intervals generated different PCR patterns: none of the genotypes amplified on day 1 were still detectable on day 12; similarly on day 50, four previously undetected genotypes were observed; together with two alleles (a 700-bp, IND/3D7-type and an 590-bp, unassigned allele) that had been previously detected.

The results presented in Figure 5 also show fluctuating MSP-1 and MSP-2 patterns. In contrast with the previous examples, most of the genotypes observed in the samples collected between day 12 and day 15 (comprising a total of five distinct MSP-1 alleles and five distinct MSP-2 alleles) were no longer detected thereafter. Other parasite genotypes were detected from day 22 onward, and in addition, a novel MSP-2 allele was amplified from the samples collected from
days 31–37. Therefore, the patterns observed for this individual reconfirm that in general, there were significant differences between samples collected at 10–15-day intervals. An interesting point was that distinct parasite populations evolve with quite different kinetics. The 580-bp FC27 allele was consistently detected throughout the 50-day survey. On the contrary, other populations apparently decreased rapidly, as indicated by the significantly different patterns observed for both markers in the samples collected two days apart, i.e., on day 13 and day 15.

Analysis of the parasite genotypes in asymptomatic individuals in the absence of transmission: the Pikine study. To evaluate the influence of new parasite inoculations by mosquitoes on the turnover rate of blood parasite populations, a third series of longitudinal studies was carried out in Pikine, a suburb of Dakar, at a time when transmission was interrupted. Samples were collected from three teenagers, identified as chronic parasite carriers and recovering, with no symptoms, from a previous malaria attack.

Figure 6 shows the PCR patterns obtained with five samples from each donor, collected every 1–2 weeks, over a total period of five weeks. In contrast to the Dielmo study, a single band was visualized in each sample with both markers, indicating a relatively homogeneous parasite population. With the MSP-1 marker, hybridization with allele-specific probes revealed two very faint bands with the samples from both donors. The patterns remained perfectly stable, showing the same single bands over the five-week survey, with no appearance of any additional amplified bands. The only modification concerned the intensity of the amplifications, which likely reflects the important fluctuations in parasite densities observed microscopically during the survey (Figure
Figure 4. Polymerase chain reaction (PCR) typing of merozoite surface protein 2 (MSP-2) (repeats) alleles in successive blood samples collected from a 16-year-old asymptomatic Dielmo, Senegal villager during the 1992 rainy season (individual code 18/11). A, analysis of the PCR products by ethidium bromide staining of the agarose gel. B, results of hybridization with family-specific probes (IND/3D7 and FC27) used sequentially on the same filter. C, schematic representation of the alleles detected in the various blood samples; codes for each family are as indicated. D, days of blood sampling and corresponding parasite densities. For definitions of abbreviations, see Figure 3.

Figure 5. Polymerase chain reaction typing of merozoite surface protein 1 (MSP-1) (block 2) and MSP-2 (repeats) alleles in successive blood samples collected from a 49-year-old asymptomatic Dielmo, Senegal villager during the 1992 rainy season (individual code 06/01). A, results of hybridization with family-specific probes (K1, MAD20, or RO33 for MSP-1 and IND/3D7 or FC27 for MSP-2) used sequentially on the same filter. B, schematic representation of the alleles detected in the various blood samples. Codes for each family are as indicated. C, days of blood sampling and corresponding parasite densities. The MSP-1 ethidium bromide--stained bands were of insufficient intensity to be shown here. For definitions of abbreviations, see Figure 3.
MSP-1
A K1
MAD20
RO33
B

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<th>day of sampling</th>
<th>1  2  3  4  5  6  7  8  9  10 11 12 13 14 15 16 17 18 19 20</th>
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<td>parasites/100</td>
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<td>leukocytes</td>
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MSP-2
A IND/3D7
FC27
B

C

TURNOVER OF P. FALCIPARUM POPULATIONS
9 Point mutations or minor changes in sequence could not be investigated by the approach used here because this would require DNA sequencing. Nevertheless, our results show that no major allelic changes were observed with these markers, i.e., that the parasites were propagated for a long time without major modification at these loci. This does not rule out the possibility that modifications occurred at other loci, such as those involved in clonal antigenic variation and recently identified. In very low transmission areas such as Pikine, children and adults do not acquire protection against malaria and are likely to have a clinical episode upon subsequent inoculation. Nevertheless, they are able to support long-term infections by the same parasite. The relationship between strain-specific and strain-independent anti-parasite immunity to clinical immunity in these individuals clearly warrants a detailed investigation.

6B). However, it should be stressed that in contrast to Dielmo, these large variations were not associated with any modification of the PCR patterns, indicating that the same populations were propagated for a long time in the absence of novel inoculations by mosquitoes.

**DISCUSSION**

In our study conducted in Pikine, we have observed that in the absence of transmission by mosquitoes, PCR patterns obtained with both MSP-1 and MSP-2 primers are simple and remain stable over a period of five weeks. The simplicity of these patterns is consistent with the low endemicity in this region; the low inoculation rate implying that a malaria attack would most likely result from a single infective bite. Furthermore, the three subjects studied here had received a chloroquine treatment resulting in asymptomatic Rf-type resistant infections, which can further explain the homogeneity of the parasites harbored. The stability of the PCR patterns was illustrated by the fact that for both genetic markers, no modification of the profiles was observed over a period of five weeks. This was also observed for amplified thrombospondin anonymous protein and circumsporozoite polymorphic regions. Point mutations or minor changes in sequence could not be investigated by the approach used here because this would require DNA sequencing. Nevertheless, our results show that no major allelic changes were observed with these markers, i.e., that the parasites were propagated for a long time without major modification at these loci. This does not rule out the possibility that modifications occurred at other loci, such as those involved in clonal antigenic variation and recently identified. In very low transmission areas such as Pikine, children and adults do not acquire protection against malaria and are likely to have a clinical episode upon subsequent inoculation. Nevertheless, they are able to support long-term infections by the same parasite. The relationship between strain-specific and strain-independent anti-parasite immunity to clinical immunity in these individuals clearly warrants a detailed investigation.

The results of the longitudinal study performed in Dielmo during the 1990 rainy season showed important and consistent modifications of the PCR patterns with both markers and for all individuals in a time as short as 2–3 weeks, a phenomenon involving both appearance and disappearance of amplification products. Kinetics of parasite turnover were further studied using a closer follow-up conducted during the summer of 1992 on one teenager and two adults. In some cases, PCR patterns changed considerably within only a few days, even though several genotypes could be detected for up to 15 days or even 50 days in one case. Two distinct interpretations can be proposed. First, as stable patterns prevail in the absence of transmission while changing patterns are observed in a period of intense transmission, this rapid turnover may reflect frequent renewal of parasite populations resulting from sporozoite inoculations. In that case, the turnover rate of parasite populations detected in the peripheral blood of Dielmo villagers would indicate that asymptomatic carriage consists of sequential waves of inoculation and partial or total parasite clearance. Since the cumulative exposure...
to malaria differs considerably between Dielmo and Pikine inhabitants, the question of whether elimination results from a strain-specific immune mechanism or from competition of distinct strains for the same niche has yet to be resolved. An alternative interpretation would be that the appearance and disappearance of amplification products may essentially reflect fluctuations of the allele ratios,\(^2\) and thus that the observed modifications of PCR patterns result from fluctuations in the relative proportion of distinct parasite populations maintained in long-term infections. Modifications of the density of a specific genotype in the peripheral circulation could be due to strain competition, to strain-specific immune response, and/or to the temporary sequestration in the deep vasculature. These two interpretations of the rapidly changing PCR patterns are not mutually exclusive. Further investigations are needed to determine whether the constant turnover represents successive waves of infection/elimination or whether it corresponds to asynchronous fluctuations of long-term infections in which mosquito inoculations represent a minor contribution.

Our data indicate that there are major quantitative and qualitative variations in the parasite antigen alleles harbored by asymptomatic individuals. In the few cases analyzed, different dynamics were observed and no general picture could be drawn. Indeed, the period over which a specific allele could be detected was highly variable, ranging from a single day to 50 consecutive days. Some alleles were fluctuating concomitantly while others had apparently independent and asynchronous fates. The parasites present in a given asymptomatic individual can therefore be considered as a patchwork of subpopulations that will constantly present different antigens to the immune system.

Another important observation was that the turnover of parasites was as active in adults as in 3-8-year-old children. In Dielmo, as in other holoendemic areas, the mean parasite density is significantly lower in adults than in children less than 15.\(^7\) These children can harbor important parasite loads, while remaining asymptomatic during prolonged periods of time. This is usually interpreted as indicating that the anti-disease immunity develops more rapidly that the anti-parasite immunity.\(^2\) The significant reduction (or even elimination) of many distinct genotypes that we observed here over short periods of time suggests that the children may also possess an efficient anti-parasite immunity. However, children still carry high parasite loads and have a significant probability of experiencing a clinical episode. This suggests that the immune response developed by children provides the possibility to maintain most parasite populations under the threshold of pathogenicity. In the light of our results, it would be important to determine whether this apparently strain-specific anti-parasite response mounted by children is an early manifestation of the phenomenon that will ultimately lead to premunition or whether it constitutes a distinct type of immunity.

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[Image 8x-0 to 591x847]

DAUBERIES AND OTHERS


