...However, very little is known about the transmission. Reports on the transmission are moderate and strictly seasonal. Study conducted in several endemic areas have demonstrated that asymptomatic infections are frequent. Prolonged carriage of asymptomatic individuals during the dry season is critical for parasite survival. Yet, very little is known about the host/parasite interactions contributing to parasite persistence. In order to study the characteristics of persisting infections during the dry season in regions of malaria, we have genotyped parasites collected from untreated, asymptomatic individuals during the dry season in Ndiop, a Senegalese village with seasonal, mesoendemic malaria. Monthly entomological surveillance did not detect any transmission during that period. Parasite prevalence decreased markedly in the children aged 3–7 years after 7 months of undetected transmission, but was stable in older children and adults throughout the dry season. In all chronically infected individuals, infection complexity remained stable, but there were substantial fluctuations in the allelic distribution within the cohort after 7 months of undetected transmission, contrasting with the stability observed during the preceding rainy season in that village. During chronic asymptomatic parasite carriage, this fluctuation resulted in changes in the allelic distribution within the cohort after 7 months of undetected transmission, contrasting with the stability observed during the preceding rainy season in that village.

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Materials and Methods

Study site and blood collection

N'Diop is a village of about 350 inhabitants situated in Saloum, in the Sahelo-Soudanian region of Senegal. Since 1993, a longitudinal epidemiological and entomological follow-up has been carried out (Röger & Trappe, 1995). The patterns of transmission over 4 consecutive years have been recorded, showing an annual entomological inoculation rate (EIR) ranging from 7 to 63, depending on the year (17 for 1994), and a strictly seasonal transmission where the main vectors are Anopheles gambiae and An. gambiensis (Fontenelle et al., 1997). In the period of interest for this work, transmission was monitored through monthly entomological surveys consisting of 12 man-night captures per month. Malaria transmission occurred from August to October 1994, with an average of 7-5, 8-5 and 1 infected bites/person/month, respectively. The 1994 dry season started early November 1994 and lasted until mid-June 1995. No transmission was detected from November 1994 to August 1995. No single mosquito was captured from 6 December 1994 to 15 June 1995.

Finger-prick blood samples were collected from permanent residents of the village. The samples studied here were part of a longitudinal follow-up of 146 individuals (42-5% of the villagers), who donated blood on successive occasions during a 1-year follow-up. There were 52 children aged <7 years, 41 children aged 7-14 years and 53 aged >15 years. Blood was collected on 12-18 September 1994 (143 individuals), on 10-15 October 1994 (125 individuals), on 13-17 December 1994 (131 individuals), on 9-29 January 1995 (66 individuals), and on 15-20 May 1995 (85 individuals). The age-group distribution was similar for all surveys. Informed consent was obtained from the donors or from the parents. Daily detailed clinical data and treatment courses are available for every subject all through this period. All blood samples were collected and stored as described previously (Trape et al., 1994).

DNA extraction and PCR genotyping

The DNA was extracted from saponin-lysed, thawed red blood cell pellets with proteinase K, followed by phenol-chloroform extractions, as previously described (Contamin et al., 1995). Then, 2 µL of DNA (corresponding to 1 µL of blood) were amplified in a Hybaid thermal reactor (Cera-Labo, Ecquevilly, France) in a final volume of 50 µL containing 200 µM of each deoxyribonucleotide (Pharmacia, Soissons, France), 1 µM of each primer and 2-5 units of Taq polymerase (Pharmacia, Saint-Quentin en Yvelines, France) in 75 mM KCl, 2-25 mM MgCl2 and 15 mM Tris-HCl (pH 9 at room temperature). The primary PCRs were done as previously described (Robert et al., 1996). The product(s) of the primary PCR were examined by agarose gel electrophoresis and ethidium bromide staining. The nested family-specific PCRs were carried out with 1 µL of the primary PCR reaction sample or with a diluted aliquot in order to adjust all nested reactions to a similar template amount. Amplification was done for 17 cycles (5 min at 94°C, 1 min at the appropriate annealing temperature, and 2 min at 72°C) as previously described (Zweryenga et al., 1998). The products of the genotyping PCR were analysed for size polymorphism on a 2% equivalent low-melting agarose gel, containing 0-5% multipurpose agarose and 0-75% infinity agarose enhancer (Appligene-oncogene, Illkirch, France). The DNA was visualized under ultraviolet light after being stained with ethidium bromide.

Prevalence, complexity of infection and statistical analysis

The prevalence was estimated as the percentage of nested PCR-positive isolates within each group considered. A msp1 block 2 and/or msp2-positive reaction was interpreted as an indication for the presence of P. falciparum parasites in that sample, without discriminating gametocytes from trophozoites. Assignment of a PCR fragment to a specific allelic family was based on the result of the secondary PCR using family-specific primers. The distribution of the various msp1 block 2 and msp2 allelic families was estimated by dividing the total number of fragments assigned to one family detected in the typing reactions by the total number of fragments for the locus considered. Within each allelic family, alleles were differentiated by size polymorphism. Comparison of the distributions of prevalences by age by age was made using χ² tests. Yates' corrections were applied when needed.

The complexity of infection (number of bands per infected person), a quantitative variable, was calculated as the average number of distinct fragments per PCR-positive sample. We have considered here the overall 2 loci complexity of the isolates, using the highest number of bands detected in each carrier (whatever the locus). Complexity of infections was analysed using a generalized estimating equation approach that allows the analysis of repeated measures and can be implemented for Poisson responses, using the SPIDA statistical package (SPIDA Version 6, Statistical Computing Laboratory, Eastwood, NSW, Australia, 1992) (Zeger et al., 1986). We used an exchangeable correlation structure in which the correlation between observations made in the same person at different times is assumed to be the same. With this model, the estimated odds ratio can be considered as an estimation of individual relative risk (to have another band) (Zeger et al., 1988). We tried to apply an m-dependence correlation structure to model the correlation of one individual observation with the previous one and the next previous one and so on. This did not reveal any deviation from the exchangeable correlation hypothesis.

Results

P. falciparum prevalence and infection complexity

The parasite prevalence, as evaluated using the msp1 block 2 and msp2 nested PCR was 75%, 68% and 59% in December 1994, January 1995 and May 1995, respectively (131, 66 and 85 individuals recruited, respectively). This is to be compared to a prevalence of 78% and 82% during the preceding transmission season, in September and October 1994, respectively (143, and 125 individuals recruited, respectively). The decrease in prevalence observed during the dry season was statistically significant (p < 0.01). In order to follow the course of natural infections during the dry season, we excluded from the analysis reported here 31 individuals recruited in this cohort who had received an anti-malarial treatment after the October 1994 survey. Twenty villagers were treated at the end of the rainy season (before the December survey) and 11 persons received antimalarials between January and May 1995. They were all febrile and carried low parasite densities. There were 17 children aged <7 years and 14 aged ≥7 years. All individuals were clinically cured. However, subsequent PCR analysis conducted for this work showed that post-treatment cross-sectional blood samples from 19 of these individuals (11 children aged <7 years and 8 aged ≥7 years) were PCR-positive (finding similar to the observations reported by Babiker et al., 1998 and Roper et al., 1998). As treatment had altered the 'natural' course of infection, we excluded the treated individuals for the analysis of chronic carriage reported here. The decreased parasite prevalence noted above at the end of the rainy season for the total population was no longer observed for the untreated group. Parasite prevalence was 76%, 61% and 69% in December 1994, January 1995 and May 1995, respectively (p > 0.3).

Parasite prevalence by age (<7 years, ≥7-14 years and ≥15 years) is shown in Figure 1A. Prevalence was similar in the 3 age-groups in December 1994. There was no
A significant difference in parasite prevalence in the 2 older age-groups for the 3 dry season cross-sectional surveys. However, there was a markedly decreased prevalence in May 1995 in the group aged <7 years. Prevalence was 23% in May as compared to 76% and 50% in this age-group for the December and January surveys, respectively, and as opposed to 89% and 78% for the groups of villagers aged ≥7–14 years and ≥15 years, respectively, during the May 1995 survey \( (P<0.001) \). Thus, there was a specific, marked decrease in prevalence within the untreated, asymptomatic children aged <7 years after 7 months of undetectable transmission.

Figure 1B illustrates the complexity by age in these untreated villagers throughout the dry season. There was no statistically significant difference in isolate complexity in each age-group for the 3 sampling times and between age-groups. A complexity of 1–3–1.6 mspl block 2 and/or msp2 alleles/isolate was observed in the youngest age-group; this figure was 1–4–2.0, and 1–3–1.7 in the \( P. falciparum \) carriers aged ≥7–14 years and ≥15 years, respectively. These data indicate that there was no decrease in complexity with time of interrupted transmission.

**Individual follow-up during the dry season**

Among the untreated individuals sampled during the 3 dry season cross-sectional surveys, blood samples were available for 45 individuals on all 3 occasions, allowing a longitudinal analysis of parasite carriage. As observed by others (BABIKER, 1998), alternating patent and sub-patent infections were detected in many individuals. This is due to the fact that asymptomatic Ndiop villagers usually carry a very low parasite density, which fluctuates above and beneath the detection level (ROGIER & TRAPE, 1995). Table 1 illustrates this phenomenon, showing the fluctuations of PCR patency as estimated using individual reactions or using the combined mspl and/or msp2 reactions. Among the 34 villagers with an mspl PCR-positive blood sample in December 1994, 27 had a PCR-positive blood sample in one or more subsequent survey. Seventeen of these were mspl-positive in May 1995, as well as 5 villagers with a sub-patent PCR in December 1994. The same fluctuating patency was observed for the mspl-based PCR detection. Seven out of the 16 msp2-positive subjects in December 1994 still had an msp2-positive blood sample in May 1995, whereas 12 additional villagers who had an msp2-negative sample earlier had an msp2-positive blood sample in May 1995. As several samples had discordant mspl vs. msp2 positivity, combining both reactions provides a more accurate picture of the infection dynamics. Table 1 shows that among the 37 villagers with an mspl and/or msp2 PCR-positive blood sample in December 1994, 24 still had a PCR-positive blood sample in May 1995. Six
individuals with a PCR-sub-patent infection in December were positive later on. Interestingly, 7/14 of the previously positive individuals who had a PCR-negative blood sample in May 1995 were children aged <7 years. In the <7-years group, 7/9 children were PCR negative in May. In contrast, most of the villagers aged >7 years, namely 26/36 (77%), remained infected in May 1995.

The individual longitudinal follow-up of the msp1 and msp2 genotypes showed substantial fluctuations of the parasite type hosted by the infected villagers over time. Table 2 shows that fluctuating genotypes were observed for 25 and 19 individuals with longitudinal blood samples successfully typed for msp1 and msp2, respectively. The majority of individuals who hosted the same allele throughout the dry season.

Table 2. Fluctuation of PCR-patent infections during the dry season in the cohort of 45 villagers in Ndiop (Senegal) who did not receive any antimalarial treatment and completed the longitudinal follow-up

<table>
<thead>
<tr>
<th>PCR pattern</th>
<th>msp1</th>
<th>msp2</th>
<th>Combined msp1/msp2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dec&lt;-&gt;Jan&lt;-&gt;May&lt;-&gt;</td>
<td>7</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Dec&lt;-&gt;Jan+ May+</td>
<td>10</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>Dec+ Jan+ May+</td>
<td>6</td>
<td>5</td>
<td>19</td>
</tr>
<tr>
<td>Dec+ Jan+ May+</td>
<td>11</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Dec&lt;-&gt;Jan+ May+</td>
<td>2</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Dec&lt;-&gt;Jan+ May+</td>
<td>2</td>
<td>10</td>
<td>5</td>
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<tr>
<td>Dec&lt;-&gt;Jan+ May+</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Dec&lt;-&gt;Jan+ May+</td>
<td>4</td>
<td>8</td>
<td>1</td>
</tr>
</tbody>
</table>

These allelic fluctuations resulted in a significant modification of the parasite population as a whole during the dry season. This is illustrated in Figure 2, which shows the distribution of the 3 msp1 block 2 allelic families (K1, Mad20 and RO33), of the 2 major msp2 allelic families (3D7 and FC27) and of the 2 msp2 hybrid allelic families during the period under study. The prevalence of msp1 and msp2 allelic families in the May 1995 populations differed significantly from the September and October 1994 populations (P < 0.01).

Discussion

The work described here investigated the natural course of parasite carriage during the dry season at the village level in a mesoendemic region, where individuals progressively acquire a premunition. As drug treatment obviously interferes with the 'armed peace' described by Sargent & Parrot (1935), we restricted our analysis to the villagers who had not taken any antimalarial. The design of the longitudinal epidemiological survey is such that drug intake is strictly controlled and regularly monitored by in-vivo drug level measures (Trape et al., 1994; Roger & Trape, 1995). We therefore were in a position to analyse villagers of all age-groups who did not receive or take any antimalarial medication from October 1994 to June 1995 in order to study the infection dynamics and the potential influence of age on parasite persistence.

Interestingly, the infection prevalence, as estimated by nested-PCR, is lower in young children than in adults. Since PCR obviously has a detection limit and investiagates a small volume of blood, we cannot ascertain that the children who became PCR-negative in May 1995 had totally cleared their infection, but rather that, after 7 months of undetected transmission, the individuals who persisted had a fluctuating profile. We also did not receive any antimalarial medication from October 1994 to June 1995 in order to study the infection dynamics and the potential influence of age on parasite persistence.

The longitudinal follow-up of the individuals who hosted the same type of a persisting allele throughout the dry season.
during persistent infections in a proportion of villagers. Therefore, fluctuations in Ndiop seem more pronounced and more generalized than in the Sudanese situation. This difference probably reflects more complex infections in Ndiop due to absence of drug treatment of the villagers studied here and to a higher inoculation rate in Ndiop, where an average of 20 infective bites/year are received by each inhabitant (FONTENELE et al., 1997), as compared to less than 1 infective bite/person/year in Sudan (BABIKER, 1998).

We think that the fluctuations of parasite genotypes observed during the dry season in Ndiop are unlikely to be due to the inoculation of novel parasite types. No single mosquito was captured from early December 1994 to mid-June 1995. While this obviously does not rule out the possibility of an ongoing low-grade transmission during that period, it excludes the possibility of inoculation of novel parasites to such a large proportion of villagers. This interpretation is substantiated by the observation of a decreased prevalence in younger children, the most susceptible group to novel inoculations. Likewise, the observed fluctuations cannot be attributed to import of novel types due to inoculation outside the village. Therefore, fluctuations in Ndiop seem more pronounced and more generalized than in the Sudanese situation.

P. FALCIPARUM DIVERSITY IN SENEGAL

Fig. 2. Comparison of the frequency of the msp1 block 2 and msp2 allelic families in the isolates collected in Ndiop (Senegal) from September 1994 to May 1995.

The fluctuating patent and sub-patent parasitaemia, and the fluctuating genotypes observed during chronic infections here and in Sudan (BABIKER et al., 1998; ROPER et al., 1998), indicate that most infected individuals host more parasite types/clones than detected by the nested PCRs at any given time point and hence that both parasite prevalence and complexity are underestimation at the time of blood collection, was reported in subjects exposed to a large number of infective bites in holoendemic areas such as Dielmo (Senegal) (DAUBERSIES et al., 1996) or Tanzania (FARNERT et al., 1997). Under these conditions, the frequent inoculation of novel parasites results in a high turn-over rate, the average period during which a specific genotype was detected being 2 weeks (DAUBERSIES et al., 1996). The situation investigated here is quite different, as novel inoculations are unlikely or at minimum infrequent. We do not know whether the fluctuations observed here reflect stochastic events due to sequential sampling of an infection with fluctuating genotype ratios or whether they indicate a substantial shift in the dominant types during prolonged chronic carriage. This second scenario predicts that persistence of parasites in a semi-immune host would result in a substantial modification of the local parasite population surviving the dry season in humans. If so, the parasites inoculated during the next transmission season should differ from those circulating the year before. Preliminary analysis collected in September 1995, when transmission had resumed, indicated that indeed msp1 and msp2 allelic frequencies differ from
mated by the technical approach used (ARNOT, 1998). More frequent blood sampling could help solve in part this issue, but there are obvious limitations to the sampling regimen, in particular if close clinical and entomological monitoring is carried out for long periods in a community.

The results reported here show a decreased parasite prevalence in younger children after several months of interrupted transmission and predict a reduced anti-disease immunity at the onset of transmission in the younger age-group. This unexpected finding is interesting, as a decreased concomitant immunity might contribute to the higher risk for younger children of developing clinical malaria once transmission and circulation of many strains including novel ones have resumed.


