CONCISE COMMUNICATION

Dynamics of Gametocytes among Plasmodium falciparum Clones in Natural Infections in an Area of Highly Seasonal Transmission

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The dynamics of gametocyte production in Plasmodium falciparum clones were studied in inhabitants of an area of highly seasonal malaria transmission in eastern Sudan. Reverse-transcriptase polymerase chain reaction was used to detect expression of 2 genes that encode gametocyte-specific proteins, pfs25 and pfg377, in parasites sampled from individuals throughout one year. Some patients who acquired infections during the wet season were found to harbor sub-patent gametocytemia through the following dry season in the apparent absence of mosquito transmission. Genotyping of parasites in multiclonal infections showed considerable fluctuation of gametocyte production by individual clones. The gametocytes present at the end of the dry season provide the most probable source of the genetically complex cyclical malaria outbreaks following the rainy season in this region.

The gametocyte-producing capacity of individual Plasmodium falciparum clones in natural infections in humans is central to their evolutionary success, especially in areas of low and seasonal transmission. Cross-mating among genetically different gametocyte-producing clones allows the parasite to continually produce new genotypes that can potentially circumvent control measures against the parasites. Such a process can evidently occur only if multiple clones within an infection can produce infectious gametocytes at the same time. P. falciparum isolates have been found to vary significantly in their capacity to produce gametocytes in vitro [1] and in vivo [2]. However, there is no information currently available on gametocyte production by individual clones in natural infections of P. falciparum, whether multiple or clonal.

We investigated gametocyte production among clones in P. falciparum–infected inhabitants of a village in eastern Sudan over a single year. Our studies there over the past 10 years demonstrated that infections that occur immediately after mosquito transmission in the rainy season are genetically diverse and frequently multiclonal. No 2 inhabitants have been found to harbor infections with identical multilocus genotypes [3, 4]. This high level of diversity suggests that the parasites derive from a large parasite reservoir existing prior to the beginning of malaria transmission. Recent work demonstrated that some patients do not clear parasites after treatment but maintain chronic subpatent asymptomatic infections throughout the dry season [4, 5]. For the present study, we monitored the dynamics of gametocytes among P. falciparum–infected patients in eastern Sudan over a 12-month period, using reverse-transcriptase (RT) polymerase chain reaction (PCR) of 2 genes, pfs25 and pfg377, expressed only in gametocytes.

Methods

Study area. This study was carried out in Asar village in eastern Sudan. In this area, malaria endemicity is low, and transmission is markedly seasonal (September through December), following brief annual rains. The rest of the year remains hot and dry and is considered to be free of malaria transmission. P. falciparum is the predominant parasite species, and Anopheles arabiensis is the main mosquito vector. The mean annual entomological inoculation rate is 1 infective bite per person every 2 years [6].

Patients and surveys. Cross-sectional surveys of inhabitants of Asar for P. falciparum infections were carried out during the transmission season (October 1998), the beginning of the dry season (January 1999), the mid–dry season (June 1999), the pretransmission period (August 1999), and the next transmission season (October 1999), at which times 680, 390, 273, 260, and 736 patients were ex-
amined, respectively. Thick and thin blood smears were stained with Giemsa’s stain and examined microscopically. Parasites were counted per 300 leukocytes, assuming a mean of 6000 leukocytes/μL of blood for inhabitants of this area [7].

**Collection of blood samples and preparation of DNA and RNA.** In October 1998, all patients who were microscopically found to have malaria were treated with the standard curative dose of chloroquine (25 mg/kg). Immediately before treatment, ~2 mL of venous blood was collected from these patients, as well as from people with no visible parasites but who had a recent history (previous 2–3 weeks) of infection. The blood samples were left at room temperature for 2 h, to allow exflagellation and microgamete formation to occur, after which they were processed and stored for RNA isolation [8]. Total RNA was isolated using the High Pure RNA Isolation kit (Roche). At the same time, 20 μL of blood was spotted onto filter paper and stored for genomic DNA isolation. The DNA was prepared using InstaGene Matrix (Bio-Rad) [4]. In subsequent surveys in 1999, blood samples were collected in the same way from the same inhabitants, whether or not parasites were detectable by microscopy.

**Detection and characterization of pfs25 and pfg377 genes.** The presence of pfs25 and pfg377 in the blood samples was determined by PCR of genomic DNA and by RT-PCR of RNA. Since these genes are expressed only in *P. falciparum* gametocytes [8, 9], the presence of mRNA specific for them indicates the presence of gametocytes among the parasites being examined. The absence of such mRNA indicates that the infection consists only of asexual forms.

Reverse transcription and subsequent amplification of cDNA of the *pfs25* gene (RT-PCR) was carried out in a single tube. The conditions and primers used have been described elsewhere [8]. RT-PCR of *pfg377* was performed in a 2-step reaction using the GenAmp RNA PCR core kit (Roche). *pfg377* contains 4 regions with repetitive sequences, the most polymorphic being region 3, which encodes 7 degenerate amino acid repeats; alleles of this gene thus vary by multiples of 21 bp [9]. For precise sizing of *pfg377* alleles in the present study, nested PCR was done, using a fluorescent-labeled primer (377R3D2). The PCR products were run on an ABI automated sequencer, and the polyacrylamide gel images then were analyzed with Genescan and Genotyper software (all from Applied Biosystems).

*pfs25* is less polymorphic than *pfg377* but is more sensitive for the detection of gametocyte RNA by RT-PCR (authors’ unpublished data). *pfs25* was therefore used to determine initially whether subpatent gametocytes were present in a blood sample, and, if they were, *pfg377* then was used to examine the diversity of alleles among them.

### Results

**Seasonal prevalence of asexual forms and gametocytes by microscopy.** Table 1 shows the number of patients exhibiting patent *P. falciparum* parasitemia and gametocytemia (i.e., that visible by microscopy). Among the 680 patients examined during the transmission season of 1998, 42 (6.2%) harbored patent gametocytemia. The number of infections fell dramatically during the course of the dry season, and none of the patients exhibited patent gametocytemia at the end of the dry season in August 1999 (table 1).

**Prevalence and persistence of subpatent gametocytes.** Blood samples taken in October–November 1998 from 44 patients who were gametocyte negative by microscopy showed that 23 (52.3%) actually had subpatent gametocytes detectable by RT-PCR of *pfs25* (table 1). These patients had a history of malaria and had received chloroquine treatment within the previous 2 weeks.

Similar findings were made at the remaining sampling times (table 1). The prevalence of gametocyte carriers revealed by RT-PCR declined as the dry season progressed. At the last sampling point (August 1999) before the start of the new transmission season, none of the 260 patients examined by microscopy had visible gametocytes, but 6 (12.2%) of 49 patients examined by RT-PCR of *pfs25* carried subpatent gametocytes.

Among patients with persisting subpatent gametocytemia, only the first blood samples taken in October 1998 (and, in one case, the second sample taken in January 1999) showed gametocytes visible by microscopy. All subsequent gametocytemia in these patients during the subsequent dry season was detectable by RT-PCR only.

**Gametocytes of individual *P. falciparum* clones.** Some infections containing gametocytes at each sampling point were typed for *pfg377* alleles by conventional PCR and by RT-PCR. In most infections, the *pfg377* alleles detected in mRNA (gametocytes) were identical to those in genomic DNA. However, in longitudinal samples from the same individuals, different gametocyte genotypes often were detected at different sampling points during the dry season. Table 2 shows data obtained from 5 patients, examined in all surveys, demonstrating the multiple carriage and fluctuation of different gametocyte genotypes over time. On 7 (41%) of 17 occasions, these patients were found to harbor >1 gametocyte genotype.

### Table 1. Asexual forms and gametocytes of *Plasmodium falciparum* parasites detected by microscopy and subpatent gametocytemia detected by reverse-transcriptase polymerase chain reaction (RT-PCR) of *pfs25* among inhabitants of Asar village, Sudan, between October 1998 and October 1999.

<table>
<thead>
<tr>
<th>Date of survey</th>
<th>No. of patients examined by microscopy</th>
<th>No. (%) with visible parasites</th>
<th>No. (%) with visible gametocytes</th>
<th>No. of patients examined by RT-PCR</th>
<th>No. (%) with gametocytes detected by RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct/Nov 1998</td>
<td>680</td>
<td>362 (53.2)</td>
<td>42 (6.2)</td>
<td>44</td>
<td>23 (52.3)</td>
</tr>
<tr>
<td>Jan/Feb 1999</td>
<td>390</td>
<td>52 (13.3)</td>
<td>11 (2.8)</td>
<td>24</td>
<td>8 (33.3)</td>
</tr>
<tr>
<td>Jun 1999</td>
<td>273</td>
<td>5 (1.8)</td>
<td>3 (1.1)</td>
<td>71</td>
<td>19 (26.8)</td>
</tr>
<tr>
<td>Aug 1999</td>
<td>260</td>
<td>5 (1.9)</td>
<td>0 (0.0)</td>
<td>49</td>
<td>6 (12.2)</td>
</tr>
<tr>
<td>Oct/Nov 1999</td>
<td>736</td>
<td>360 (48.9)</td>
<td>33 (4.5)</td>
<td>58</td>
<td>14 (25.5)</td>
</tr>
</tbody>
</table>


alleles in gametocyte RNA and total genomic DNA, detected at different sampling points in one patient (AS180), are illustrated in figure 1 and detailed in table 2. The first sample, taken in October 1998, showed a single allele of \textit{pfg377} (denoted D) that was identical in both genomic DNA (PCR) and gametocyte RNA (RT-PCR), showing that the clone containing this allele was producing gametocytes. In the following sample of January 1999, 2 alleles (D and E) were found, but only 1 of these (D) was detectable in gametocyte RNA by RT-PCR. We interpret this as indicating the presence of 2 clones, 1 of which (D) was producing gametocytes, whereas the second (E) existed only as asexual forms. In June 1999, clones with new alleles (B and C) appeared, in addition to allele D, which was still present. The major gametocyte genotype was B; a weak D allele was also present, detectable only by fluorescence in polyacrylamide sequencing gels (data not shown). Genotype D, however, was the predominant PCR product detectable in the genomic DNA, showing that this clone was present mostly as asexual forms at this time. Unexpectedly, genotype B was undetectable in the genomic DNA sample. The most likely explanation is that this clone was present only as very low numbers of gametocytes, below the sensitivity of PCR of genomic DNA; RT-PCR amplification of RNA is considerably more sensitive than PCR amplification of a single copy gene in genomic DNA. In the final sample of August 1999, only genotype B was detected in both gametocyte and genomic DNA.

\textbf{Discussion}

The present study has revealed the most probable sources of the annual epidemics of \textit{P. falciparum} infection that occur in areas of highly seasonal malaria transmission. This parasite, retained asymptomatically in subpatent infections among some inhabitants of Asar village during the lengthy dry season, consistently produces gametocytes in the absence of fresh infection. Although we have not formally proved that these gametocytes are infectious to mosquitoes, there are several previous studies demonstrating that mosquitoes can be infected from people not exhibiting patent parasitemia [10]. Our work has also shown that natural \textit{P. falciparum} clones have a high capacity to produce gametocytes, a process that will influence the frequency of crossing between clones in mosquitoes and, hence, the generation of novel parasite strains by recombination.

There are natural variations in the capacity of different \textit{P. falciparum} clones for gametocytogenesis, but the biological basis of this process is not clear. In vitro culture, variations in levels of gametocytes occur that differ from one clone to another and that may remain stable over many asexual multiplications [1, 11].

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
\hline
\textbf{AS/133} & & & & & \\
\hline
Parasites/\textmu L & 0 & 0 & 0 & 0 & 0 \\
Gametocytes/\textmu L & 180 & 0 & 0 & 0 & 0 \\
\textit{pfg377} alleles in genomic DNA & D & C & C & — & — \\
\textit{pfg377} alleles in gametocyte RNA & C, D & C, D & C & — & — \\
\hline
\textbf{AS/151} & & & & & \\
\hline
Parasites/\textmu L & 300 & 120 & 0 & 0 & 0 \\
Gametocytes/\textmu L & 60 & 0 & 0 & 0 & 0 \\
\textit{pfg377} alleles in genomic DNA & C & D & D & — & — \\
\textit{pfg377} alleles in gametocyte RNA & C & D & B & — & — \\
\hline
\textbf{AS/180} & & & & & \\
\hline
Parasites/\textmu L & 3300 & 240 & 0 & 0 & 0 \\
Gametocytes/\textmu L & 30 & 360 & 0 & 0 & 0 \\
\textit{pfg377} alleles in genomic DNA & D & D, E & C, D & B & — \\
\textit{pfg377} alleles in gametocyte RNA & D & D & B, D & B & — \\
\hline
\textbf{AS/192} & & & & & \\
\hline
Parasites/\textmu L & 0 & 90 & 0 & 0 & 0 \\
Gametocytes/\textmu L & 120 & 0 & 0 & 0 & 0 \\
\textit{pfg377} alleles in genomic DNA & D & D & D, B & — & — \\
\textit{pfg377} alleles in gametocyte RNA & D & D & D & — & — \\
\hline
\textbf{AS/204} & & & & & \\
\hline
Parasites/\textmu L & 0 & 0 & 0 & 0 & 0 \\
Gametocytes/\textmu L & 0 & 0 & 0 & 0 & 0 \\
\textit{pfg377} alleles in genomic DNA & C & A, C & C & — & B \\
\textit{pfg377} alleles in gametocyte RNA & C & C, D & C & — & B \\
\hline
\end{tabular}
\caption{Alleles of \textit{Plasmodium falciparum} pfg377 gene in genomic DNA and gametocyte RNA of 5 patients with persistent subpatent parasitemia between October 1998 and October 1999, by date.}
\end{table}

\textbf{NOTE.} Alleles of 270, 310, 330, 351, and 371 bp are denoted as A, B, C, D, and E, respectively. Dashes (—) indicate that the patient was negative by polymerase chain reaction (PCR) or reverse-transcriptase PCR.
Thus variations may be due to changes in gene expression or gene deletions during the artificial conditions of prolonged culture. In natural infections, similar variation has been observed among *P. falciparum* infections. It has been suggested that, once a clone begins gametocyte production, it continues this process with every schizogony [12]; older *P. falciparum* infections have been found to have a high level of gametocyte production, from low-level parasitemia, compared with younger infections [13].

Gametocyte production by individual *P. falciparum* clones in nature has not been studied previously. It has been suggested that gametocytogenesis could be favored in conditions where asexual growth is limited (e.g., by drug pressure) [14]. Similarly, it is possible that immune responses that regulate chronic asymptomatic parasitemia may favor gametocyte production to maximize the parasites' chances of transmission. It has been demonstrated that, once *P. falciparum* switches to gametocyte production, it may continue this process for a long period of time [12]. However, the length of such a process in natural infections is not known. In the present study, consistent gametocytemia was retained as a chronic infection for >9 months. However, a steady decline in prevalence of subpatent gametocytemia among the inhabitants of Asar during the dry season was observed.

Gametocytes found late in the dry season could have a long life span, persisting as mature forms for many weeks, or they could have only a short life span but could be produced regularly during an infection from an underlying population of asexually dividing parasites. Smallley and Sinden [15] produced evidence that gametocytes have a half-life of only 2.4 days but that they may persist for up to 20 days. The present study does not provide direct information on this subject, because of the difficulties of distinguishing subpatent asexual stages from gametocytes. However, during the course of our work, we found some chronic subpatent infections with no evidence of gametocyte production. Presumably, these are long-lasting, asexually reproducing forms that could subsequently become the sources of subpatent gametocytes. Sampling of such clones at more frequent intervals than was done here would answer this question. It was of interest that some of the clones we examined contained gametocytes at the start of the study, following the acute phase and drug treatment, and continued gametocyte production during the dry season. This conforms with the observation of Smalley et al. [13] that older *P. falciparum* infections have a high gametocyte production capacity, compared with younger infections.

An important novel finding is that multiple gametocyte genotypes coexist together in the same infection and fluctuate during the dry season. The majority of infections showed gametocyte genotypes identical to those of all parasite genotypes, including asexual forms, in the same infection. However, not all multiple clones produced gametocytes simultaneously. Currently, we are conducting a closer longitudinal monitoring of individual patients in Asar over the dry season, which will give better information on the dynamics of such multiple gametocyte genotypes in the same infection. Such data will allow a precise estimate of the extent of gametocyte genotype multiplicity, especially at the end of the dry season and the beginning of the next transmission season, when the emerging mosquitoes can feed on such gametocyte carriers. The low frequency of simultaneous carriage of multiple gametocyte genotypes, as seen in Asar, may reflect the limited number of clones present per infection and the high level of inbreeding among the parasites, compared with that in an area with high multiclonal infection, such as Tanzania [6].

It seems to be clear that the pretransmission gametocytes among the inhabitants of Asar detected in this study are the source of mosquito infection at the start of the transmission season. Gametocyte-targeted control strategy during the dry season could thus have a strong impact on malaria morbidity and mortality in this area.
Acknowledgments

The present study would not have been possible without the cooperation of the villagers of Asar, the Malaria research group of the Biochemistry Department (Faculty of Medicine, Khartoum University, Khartoum, Sudan), the Malaria Administration of the Sudanese Ministry of Health, and the staff of Gedaref Hospital (Gedaref City, Sudan). We thank Richard Carter, Margaret Mackinnon, and Paul Hunt for help and comments on the manuscript.

References