INTRODUCTION

Infections in *Plasmodium* spp. can only be transmitted from the vertebrate host to the mosquito through the male and female gametocytes that developed from committed asexual parasites in the vertebrate host obtained from the blood meal by the mosquito. In the mosquito, the female *Plasmodium falciparum* gametocyte (macrogametocyte) release one gamete, whereas the male gametocyte (microgametocyte) may release up to eight gametes (Sinden *et al.*, 1978). Exflagellation of the microgamete and fertilization of the macrogamete by the microgamete form the zygote that later develops to the sporozoite. The sporozoite is eventually injected through the mosquito bite to the vertebrate host when the female mosquito obtains a blood meal. Immature but not mature *P. falciparum* gametocytes which develop from proliferating asexual parasites in man can be eradicated by a number of antimalarial drugs including chloroquine (CQ) and pyrimethamine-sulphadoxine (PS) (Sinden, 1982, 1983). If CQ promptly and completely clears asexual parasites following treatment of CQ-sensitive (CQ-S) *P. falciparum* infections, then further recruitment of gametocyte is completely inhibited and the changes in the mature gametocytes in circulation can be conveniently followed (Smalley & Sinden, 1977).

In West Africa, children are the most susceptible to *falciparum* malaria. In these children gametocyte rate...
prior to drug treatment may be up to 17% (von Seidelein et al., 2001) or even higher (Robert et al., 2000). In much of West Africa, CQ still remains the drug of first choice but despite the sex ratio being crucial to transmission success by the mosquito (Robert et al., 1996; Paul et al., 2002), there is little or no recent information on gametocyte sex ratios or their changes following CQ treatment in this at risk group.

The aims of the present study were: to evaluate the changes in *P. falciparum* gametocytaemia during treatment of CQ-S infections in children, and to determine if there are differences in the changes involving macro- and micro-gametocytes during treatment with CQ.

**PATIENTS AND METHODS**

**PATIENTS**

The study took place at the University College Hospital in Ibadan, a hyperendemic area for malaria in southwestern Nigeria (Salako et al., 1990), between August 2000 and December 2002 and was approved by the local ethics committee.

The details of the clinical component of the study are as previously described (Sowunmi, 2002, 2003; Sowunmi & Fateye, 2003a). Briefly, children with symptoms compatible with acute falciparum malaria who fulfilled the following criteria were enlisted in the study: age below 120 months, pure *P. falciparum* parasitaemia greater than 2,000 asexual forms/µl blood, gametocytaemia ≥ 12 sexual forms/µl blood negative urine tests for antimalarial drugs (Dill-Glazko and lignin tests), absence of concomitant illness, no evidence of severe malaria and written informed consent given by parents or guardians. An important additional criterion was: following CQ treatment clearance of parasitaemia within 72 h and with no recurrence within 672 h of follow-up.

After detailed clinical and parasitological assessment, CQ was given orally at a dose of 30 mg/kg of the base over 3 d, that is, 10 mg/kg at presentation (day 0, 0 h) and daily for additional two days. The patients were observed for three hours to ensure that the drug was not vomited. Follow-up with clinical and parasitological evaluation was at 24, 48, 72, 96, 120, 144, 168, 336, 504 and 672 h. Clinical evaluation consisted of a general clinical examination including measurement of weight, core temperature and physical examination. Thick and thin blood films prepared from a finger prick were Giemsa-stained and quantification of parasitaemia was done as previously described (Sowunmi, 2002, 2003; Sowunmi & Fateye, 2003a). Giemsa-stained blood films were examined by light microscopy under an oil-immersion objective, at × 1,000 magnification, by two independent assessors. Parasitaemia in thick films was estimated by counting asexual parasites relative to 1,000 leukocytes, or 500 asexual forms, whichever occurred first. From this figure, the parasite density was calculated assuming a leukocyte count of 6,000/µl of blood.

Parasite deoxyribonucleic acid (DNA) was extracted by methanol fixation and heat extraction (Plowe et al., 1995) from blood samples collected on to filter paper at 0, 24, 48, 72, 96, 120, 144, 168, 336, 504 and 672 h and the merozoite-surface-protein (MSP1 and MSP2) the K1, MAD20, and RO33 allelic families of MSP1, the ICI and FC27 allelic families of MSP2, and region II of glutamine-rich-protein (GLURP) were analysed, using the primer sequence and PCR conditions described by Snounou & Beck (1999). The regions of the CQ resistance transporter (*pfCRT*) and the parasite’s multidrug resistant 1 (*pfmdr1*) genes surrounding the polymorphisms of interest in pre- and post-treatment samples were amplified by nested polymerase chain reaction (PCR) (Djimde et al., 2001) and analysed by RFLP, so that the *pfCRT* (GenBank accession number AF030694) K76T and *pfmdr1* (GenBank accession number X56851) N86Y and D1246Y mutations could be detected, if present, as previously described (Happi et al., 2003).

**QUANTIFICATION OF GAMETOCYTAEMIA AND DETERMINATION OF GAMETOCYTE SEX**

Gametocytaemia was quantified as previously described (Sowunmi, 2002, 2003; Sowunmi & Fateye, 2003a) at 0, 24, 48, 72, 96, 120, 144, 168, 336, 504 and 672 h. Gametocytes were counted in thick blood films against 1,000 leukocytes assuming an average leukocyte count of 6,000/µl of blood. Gametocytes were sexed if gametocytaemia was ≥ 12 sexual forms/µl. Gametocyte sex determination was based on following criteria (Carter & Graves, 1988; Robert et al., 1996): males are smaller than females; the nucleus is bigger in males than females; the ends of the cells are round in males and angular in females; the cytoplasm stains pale purple in males and deep blue in females; and the granules of malaria pigment are centrally located in females and more widely scattered in males. Gametocyte sex ratio was defined as the proportion of gametocytes in peripheral blood that were microgametocytes (Pickering et al., 2000).

**KINETICS OF MICRO-AND MACROGAMETOCYTAEMIA**

Gametocyte kinetic parameters were estimated from gametocyte and gametocyte densities (gametocyte and gametocyte sex concentrations or gametocytaemias) by a non-compartmental method using the computer programme Turbo Ken (Clinical Pharmacology Group, University of Southampton, UK, through the courtesy of Professor A.G. Renwick).
gametocyte kinetic parameters were calculated from the curve of gametocytaemia by using the real times of sampling from each patient. Areas under the curves of gametocytaemia versus time until the last detectable gametocyte concentration ($C_tgm$), (AUC$_{gm\text{last}}$), were calculated using the trapezoidal method. Area under the gametocytaemia-time from zero to infinity (AUC$_{gmO-w}$) was calculated by adding to AUC$_{gm\text{last}}$ the extrapolated AUC$_{gm}$ calculated as $C_tgm/k_{el}$, the elimination rate constant derived from the semilogarithmic plot of gametocytaemia versus time (visual inspection of the final part of the gametocytaemia - time curve was used to identify the elimination phase). $C_{maxgm}$ (maximum gametocytaemia) and $t_{maxgm}$ (time to $C_{maxgm}$) were noted directly from the data. Terminal elimination half-life, $t_{1/2b}$, was calculated as $0.693/k_{el}$. The volume of blood completely cleared of gametocytes (CLB$_{gm}$) was calculated as the gametocytaemia at enrolment/AUC$_{gm}$.

**STATISTICAL ANALYSIS**

Data were analysed using version 6 of the Epi-Info software (Anon., 1994). Normally distributed, continuous data were compared by Student's t-tests and analysis of variance (ANOVA). Data not conforming to a normal distribution were compared by the Mann-Whitney U-tests and the Kruskal-Wallis tests (or by Wilcoxon rank sum test). All tests of significance were two-tailed. $P$-values of $< 0.05$ were taken to indicate significant differences. The values presented below are generally means and standard deviations (sd) or standard error (se).

**RESULTS**

A total of 96 children with acute symptomatic uncomplicated falciparum malaria was initially evaluated. Gametocytes were seen in peripheral blood in 15 children at presentation but in only 10 children did parasitaemia clear within 72 h of commencing CQ treatment. One of the 10 children was excluded because of uncontrolled additional antimalarial intake during follow-up. The demographic and other characteristics at enrolment and the responses to CQ treatment of the nine patients who completed the study are shown in Table I. Parasite DNA was demonstrable at 0-48 h only confirming clearance of asexual parasitaemia within 72 h of commencing CQ treatment. The parasites from all nine children did not carry mutant alleles of pfcr or pfmdr1.

A total of 78 gametocytes was counted on day 0 (0 h) and 34, 64, 106, 74, 74, 55, 53, 20, and 10, gametocytes were counted at 24, 48, 48, 96, 120, 144, 168, 336 and 504 h, respectively. Of these, gametocytes could be sexed in 70, 27, 56, 99, 79, 79, 58, 58, 22, 10 and 4 at 0, 24, 48, 72, 96, 120, 144, 168, 336, 504 and 672 h, respectively. Gametocytes were found in one patient at 672 h.

The temporal changes in gametocytaemia are shown in Figure 1. The individual model output data are shown in Table II. Following peak gametocytaemia, gametocytes persisted in blood for 72-504 h with a terminal elimination half-life of 43.2 ± 20.4, range 13.1-206 h. Figure 2 shows the prevalence and the temporal changes in sex ratios during follow-up. Pre-treatment sex ratio was female-biased in three and male-biased in six children. Overall, the pre-treatment mean sex ratio was...
male-biased and it remained so throughout the duration of the study (Fig. 2). In the three children with pre-treatment female-biased sex ratio, there was a change to male-biased sex ratio by day 7 and these remained so till day 14. In these three children, a sex ratio of 0.5 was attained on days 2, 4 and 6. In the remaining six children, the pre-treatment male-biased sex ratio remained unchanged till days 7 and 14. The model output data for macro- and micro-gametocytaemias are shown in Table III. Both peak macro- and micro-gametocytaemia occurred between 0-120 h, but compared with macrogametocytaemia, Cmaxgm, t1/2gm and AUCgm were significantly higher for microgametocytaemia and clearance (CLBgm) was significantly slower.

Table II. - Output data of a non-compartment model of the changes in gametocytaemia in children enrolled in the study.

<table>
<thead>
<tr>
<th>No</th>
<th>Age (y)/Sex</th>
<th>Cmaxap (/pl)</th>
<th>Cmaxgm (/pl)</th>
<th>Tmaxgm (h)</th>
<th>t1/2gm (h)</th>
<th>AUCgm (sf/pl.h)</th>
<th>CLBgm (pl/kg/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>151/00</td>
<td>4.1 M</td>
<td>4,860</td>
<td>72</td>
<td>120</td>
<td>22.2</td>
<td>9,400.0</td>
<td>0.00012</td>
</tr>
<tr>
<td>152/00</td>
<td>9.0 M</td>
<td>10,890</td>
<td>36</td>
<td>0</td>
<td>13.1</td>
<td>4,438.5</td>
<td>0.00048</td>
</tr>
<tr>
<td>175/00</td>
<td>9.5 M</td>
<td>15,630</td>
<td>198</td>
<td>72</td>
<td>26.8</td>
<td>24,931.5</td>
<td>0.00013</td>
</tr>
<tr>
<td>200/00</td>
<td>9.5 M</td>
<td>9,390</td>
<td>48</td>
<td>72</td>
<td>206.0</td>
<td>13,352.4</td>
<td>0.00011</td>
</tr>
<tr>
<td>35/01</td>
<td>2.4 M</td>
<td>16,034</td>
<td>48</td>
<td>72</td>
<td>21.6</td>
<td>8,794.4</td>
<td>0.00019</td>
</tr>
<tr>
<td>130/01</td>
<td>7.0 M</td>
<td>24,857</td>
<td>24</td>
<td>0</td>
<td>23.8</td>
<td>4,450.9</td>
<td>0.00024</td>
</tr>
<tr>
<td>39/02</td>
<td>2.7 F</td>
<td>97,684</td>
<td>30</td>
<td>48</td>
<td>26.8</td>
<td>5,274.7</td>
<td>0.00029</td>
</tr>
<tr>
<td>45/02</td>
<td>4.7 M</td>
<td>1,116</td>
<td>60</td>
<td>0</td>
<td>27.0</td>
<td>12,816.1</td>
<td>0.00026</td>
</tr>
<tr>
<td>76/02</td>
<td>4.2 M</td>
<td>36,267</td>
<td>150</td>
<td>0</td>
<td>21.9</td>
<td>17,608.1</td>
<td>0.00065</td>
</tr>
</tbody>
</table>

Cmaxap: peak asexual parasitaemia (occurred in all patients at enrolment); Cmaxgm: peak gametocyte density; Tmaxgm: time to peak gametocyte density; t1/2gm: apparent half-life of gametocytaemia; AUCgm: area under the curve of gametocytaemia versus time; CLBgm: volume of blood completely cleared of gametocytes per unit time; sf: sexual forms; y: year.

DISCUSSION

Given the results of the molecular analyses and the treatment outcome, it seems certain that the asexual parasites and the gametocytes that arose from them were from CQ-S infections and there was no recurrence of sub-patent parasitaemia during the entire duration of the study. The molecular analyses we did became necessary for two reasons: children with clinical cure of their infections by chloroquine can harbour and transmit gametocytes arising from parasites carrying resistance genes (Sutherland et al., 2002a); sub-patent infections can continue to generate more gametocytes resulting in multiple gametocyte genotypes post treatment (Sutherland et al., 2002b).

Although a cohort of asexual parasites destined to become gametocytes will be initiated synchronously at schizogony, it is unlikely that all cohorts of parasites present in a particular patient will be initiated at the same time. This is more so since different stages of asexual parasite development can be readily encountered and recognized in the blood films obtained from patients in this endemic area (Sowunmi & Oduola, 1998). Thus, recruitment of a particular cohort of gametocytes would be synchronous but in most natural infections, maturation of all cohorts should be continuous and therefore asynchronous. By enrolling patients in whom asexual parasitaemia cleared within 72 h of initiating CQ treatment, we tried to ensure that no further recruitment of gametocytes occurred during follow-up of the patients. Thus, only the maturing and mature gametocytes were estimated, and these would, with time, die without replacement (in the absence of asexual parasitaemia during the 28 d period of follow-up). This method allows estimation of gametocyte half life (Smalley & Sinden, 1977; Reece et al., 2003) since
The mean sex ratio at enrolment was male-biased and per child from *P. falciparum* multiple infections with these observations are in keeping with those of earlier and more recent studies in West African children (Smalley, 2003a) and multiple infections are not uncommon. In addition, malaria anaemia is also common and may further contribute to the maleness.

3 - Although considered relatively non-immune (five of the nine children were aged less than five years), immunity could still have influenced the observed sex ratio.

4 - There could have been an early release and subsequent early clearance of macrogametocytes from the circulation before presentation by the children for treatment. Finally, macrogametocytes may have been selectively sequestered in the internal organs because of their relatively large size, before presentation by the children for treatment. Does CQ significantly alter sex ratio during the course of treatment? Or contribute to the observed sex ratio in the cohort of children evaluated? In contrast to our previous finding in children treated with pyrimethamine-sulphadoxine (PS) (Sowunmi & Fateye, 2003b), the sex ratios in the majority of the children remained relatively unchanged during and after CQ treatment. A similar finding has been documented in a group of Gambian children with female-biased sex ratio treated with CQ (Smalley & Sinden, 1977). However, it is possible that CQ may have influenced the change from female to male-biased sex ratio seen in the minority of the children since CQ stimulates erythropoietin.

| Microgametocytaemia | Macrogametocytaemia | P. value*
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>GMGD (1/ul)</td>
<td>48</td>
<td>22</td>
</tr>
<tr>
<td>range</td>
<td>14-158</td>
<td>6-70</td>
</tr>
<tr>
<td>interquartile range</td>
<td>30-84</td>
<td>12-41</td>
</tr>
<tr>
<td>$T_{maxgm}$ (h)</td>
<td>mean ± sd</td>
<td>2.3 ± 0.7</td>
</tr>
<tr>
<td>range</td>
<td>0-6</td>
<td>0-4</td>
</tr>
<tr>
<td>95 % CI</td>
<td>0.6-4.0</td>
<td>0.6-2.8</td>
</tr>
<tr>
<td>AUC (sf/µl/h)</td>
<td>mean ± se</td>
<td>8,347 ± 1,828</td>
</tr>
<tr>
<td>range</td>
<td>2,435-19,102</td>
<td>448-5,564</td>
</tr>
<tr>
<td>95 % CI</td>
<td>4,132-12,563</td>
<td>1,771-4,202</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>mean ± se</td>
<td>41.8 ± 18.7</td>
</tr>
<tr>
<td>range</td>
<td>13.1-191.2</td>
<td>3.4-65.0</td>
</tr>
<tr>
<td>95 % CI</td>
<td>1.4-84.9</td>
<td>3.4-31.4</td>
</tr>
<tr>
<td>CLB$_{gm}$ (µl/kg/h)</td>
<td>mean ± se</td>
<td>0.00025 ± 0.00006</td>
</tr>
<tr>
<td>range</td>
<td>0.00002-0.00006</td>
<td>0.00002-0.000076</td>
</tr>
<tr>
<td>95 % CI</td>
<td>0.00001-0.00004</td>
<td>0.000025-0.000064</td>
</tr>
</tbody>
</table>

GMGD: geometric mean gamocyte density. *paired t-test, *by paired t-test excluding one pair of outliers, 191.2 and 65.0 h for micro- and macrogametocytaemia, respectively, and by * Wilcoxon ranked sum test excluding outliers (*P = 0.004).

$C_{maxgm}$: peak gamocyte density; $T_{maxgm}$: time to peak gamocyte density; AUC$_{gm}$: area under the curve of gametocytaemia versus time; $T_{1/2}$: apparent half-life of gametocytaemia; CLB$_{gm}$: volume of blood completely cleared of gametocytes per unit time; sf: sexual forms.

Table III. – Comparison of output data of a non-compartment model of the changes in macro- and microgametocytaemia.
secretion (El Hassan et al., 1997); but it is also possible that the change was due to the course of infection (Paul et al., 2000).

Gametocytes sex ratios and their disposition have been evaluated before in a limited number of African children (Smalley & Sinden, 1977). However, details of the kinetics of the disposition of sex gametocytes in children have not been provided before now. The finding that macrogametocytes are lost from the circulation faster than microgametocytes was not unexpected. In synchronized Plasmodium falciparum gametocyte culture in vitro, microgametocytes are longer lived (Ponnudurai et al., 1986). Similarly in P. chabaudi infection in black mice, microgametocytes are also longer-lived (Reece et al., 2003). We have also found (Sowunmi & Fateye, 2003b) that following PS treatment of Plasmodium falciparum infections in children, microgametocytamia was significantly higher than macrogametocytamia on days 7 and 14, and microgametocytes are longer lived.

There are possible reasons for the differential clearance of macrogametocytes in our cohort of children: although most of the children were relatively non-immune, immune killing rate may differ between gametocyte sex, as is clearance by the spleen; sex differences in capillary sequestration may occur; finally, a combination of these or other factors may operate. It is not clear whether in chloroquine-resistant (CQ-R) infections there would be alteration in sex ratio or their disposition following CQ treatment, but studies are under way to address this issue. However, gametocyte half-life appears to be longer in CQ-R than in CQ-S infections in children from this endemic area (Sowunmi & Fateye, 2003a).

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REFERENCES


GAMETOCYTAEMIA AND CHLOROQUINE TREATMENT


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