Molecular Epidemiology of Malaria in Yaoundé, Cameroon. VII. Analysis of Recrudescence and Reinfecution in Patients with Uncomplicated Falciparum Malaria

Leonardo K. Basco and Pascal Ringwald

Institut de Recherche pour le Développement (IRD) and Laboratoire de Recherche sur le Paludisme, Laboratoire Associé Francophone 302, Organisation de Coordination pour la lutte contre les Édémies en Afrique Centrale (OCEAC), Yaoundé, Cameroon

Abstract. In an endemic area where malaria transmission is intense and continuous, reappearance of asexual parasites may be ascribed to either recrudescence or reinfection. To distinguish between recrudescence and reinfection after oral treatment with chloroquine, amodiaquine, pyronaridine, sulfadoxine-pyrimethamine, halofantrine, or artesunate, three polymorphic markers (circumsporozoite protein, merozoite surface antigens 1 and 2) from pre-treatment and post-treatment samples were amplified by the polymerase chain reaction, and the in vitro response to chloroquine was determined for comparison. Of 52 paired samples, 22 (42%) were reinfections. Recrudescence occurred more frequently on or before Day 14 (22 of 30 cases, 73%). Except for one case, all reinfections were observed beyond Day 14. The phenotype determination was not sufficiently precise to distinguish between recrudescence and reinfection. Our results suggest that beyond Day 14 (and until Day 42), recrudescence and reinfection cannot be distinguished at our study site unless molecular techniques are used and that some results derived from the polymerase chain reaction need to be compared with the microscopic examination of thick blood smear to exclude gametocyte carriers without asexual parasites after treatment.

INTRODUCTION

In antimalarial chemotherapy, drug resistance is defined as the “ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject.”1 Based on this definition and a 28-day follow-up, parasitologic responses are graded as sensitive (S) or resistant (RI, RII, and RIII). The definitions of RI-RII-RIII have been modified and adapted to several variants of The definitions of RI-RII-RIII have been modified and adapted to several variants of in vivo tests that differ in the duration and frequency of follow-up of asymptomatic or symptomatic patients (7 to 14 days).2,3 Several components of the definition need to be demonstrated to establish resistance: positive blood smear during a regular follow-up, administration of the drug under supervision, measurement of parasitic drug levels, and persistence of the same parasite population as before treatment. The first two criteria may be satisfied with ease at any health care center. The demonstration of an adequate blood level of drugs requires the high-performance liquid chromatography. The fourth component is the key element that must be demonstrated before concluding that the persistence or reappearance of parasites after therapy is due to the same strain that survived in the patient despite correct therapy.

In an endemic area where malaria transmission is intense and perennial, the reappearance (or persistence) of asexual erythrocytic stages of Plasmodium falciparum after a correct therapeutic dose may be attributed to either recrudescence of drug-resistant parasites that were already present before treatment or a new infection. The in vivo response is influenced by the parasite’s resistance profile and the host immune system, which is in turn age-dependent. As part of an effort to define the epidemiology of drug-resistant parasites and evaluate the therapeutic efficacy of antimalarial drugs, it is important to distinguish between recrudescence and reinfection. In the present study, the genotypes of clinical isolates obtained before treatment and those of parasites that reappeared or persisted in the peripheral blood at various times following correct and complete treatment were compared with the aim to differentiate between recrudescence and reinfection. In addition, the phenotype of pre-treatment and post-treatment isolates was compared in some paired samples by in vitro drug sensitivity assays.

PATIENTS, MATERIALS, AND METHODS

Patients. The study was part of randomized clinical trials conducted at the Nlongkak Catholic missionary dispensary in Yaoundé, Cameroon between 1994 and 1998.4,5 The following inclusion criteria were used for enrollment: age ≥ 5 years old, fever at consultation (or history of fever within the past 24 hr), monoinfection with P. falciparum, parasite density > 5,000 asexual parasites/μL of blood (to allow the performance of in vitro drug assays), easy access to the dispensary for daily monitoring, and no recent history of self-medication with antimalarial drugs, as confirmed by a negative Saker-Solomons urine test result.6 Patients with signs and symptoms of severe and complicated malaria, as defined by the World Health Organization (WHO),7 severe anemia (hemoglobin < 5.0 g/dL), or moderate and severe malnutrition were excluded. Informed consent was obtained from either the adult patients themselves or a guardian accompanying the sick children. The study was approved by the Cameroonian National Ethics Committee and the Cameroonian Ministry of Public Health.

The patients were treated with standard oral doses of chloroquine (25 mg/kg of body weight in three divided daily doses), amodiaquine (30 mg/kg of body weight in three divided daily doses), pyronaridine (32 mg/kg in four divided doses over a three-day period), sulfadoxine-pyrimethamine (a single dose of 1.500 mg of sulfadoxine and 75 mg of pyrimethamine in adults, 1 tablet/20 kg of body weight in children), or artesunate (a total of 600 mg over a five-day period in adults) under supervision. For those responding with a therapeutic failure, quinine or halofantrine was ad-
ministered. The patients were followed for at least 14 days according to the test of therapeutic efficacy, developed by WHO in 1996.8

Genotyping of *P. falciparum*. Parasite DNA was extracted from venous blood samples as described in our earlier study.9 The DNA fragments of three polymorphic markers, circumsporozoite protein (CSP), merozoite surface antigen-1 (MSA-1), and merozoite surface antigen-2 (MSA-2) genes, were amplified by the polymerase chain reaction using two methods. In the first method, three pairs of synthetic oligonucleotides flanking the polymorphic regions of CSP, MSA-1, and MSA-2 were used simultaneously in an amplification procedure termed ‘multiplex polymerase chain reaction’ to estimate the degree of allelic diversity in consecutive clinical isolates obtained in Yaoundé.10 In the second method, each of the three loci was amplified separately. This approach allowed an unambiguous counting of the number of polymerase chain reaction products yielded for each locus. The following species-specific oligonucleotide primers were designed from published sequences of the complete gene sequences: CSP-1, 5′-AAATTACAATTCATGATGAGAAAA TTAGC-3′ (forward primer), CSP-2, 5′-GATGTGTCCTTTATCTAAATGAAAGC-3′ (reverse primer), MSA2-1, 5′-ATGAAGGTTAATTTAAAATTGCTATTATA-3′ (forward primer), MSA2-2, 5′-TTATATGAATGCGAAAAAGATAAAAACAGG-3′ (reverse primer), MSA1-1, 5′-GATGAAATTTATGATTAAACTTATGATGAG-3′ (forward primer), and MSA1-2, 5′-TACCGTCTAAATTCCTTGTCACACGATTT TAAG-3′ (reverse primer).11–13

The reaction mixture consisted of approximately 200 ng of genomic DNA, 15 picomoles of forward and reverse primers, buffer (50 mM KCl, 10 mM Tris, pH 8.3), 1.5 mM MgCl2, 200 μM dNTP, and 1 unit of Tag DNA polymerase (Roche Diagnostics, Meylan, France) in a final volume of 50 μl. The PTC-100 thermal cycler (MJ Research, Watertown, MA) was programmed as follows: 94°C for 2 min for the first cycle and 30 sec in subsequent cycles, 50°C for 1 min for the first cycle and 30 sec in subsequent cycles, and 72°C for 1 min for all cycles (2 min for amplifying the entire csp gene), for a total of 30 cycles, followed by a 15 min extension step at 72°C. The amplified DNA fragments were resolved by electrophoresis in a 1.8% agarose gel, stained with ethidium bromide, and visualized under ultraviolet transillumination.

**In vitro drug sensitivity assay.** The isotopic semi-microtest and microtest used in this study were described in previous studies.8,13,15 The suspension (200 μl for the microtest and 700 μl for the semi-microtest) was distributed in each well of the tissue culture plates (96-well plate for the microtest and 24-well plate for the semi-microtest). The parasites were incubated at 37°C in 5% CO2 for 42 hr. 3H-hypoxanthine, 1 μCi/well (Amersham International, Plc., Buckinghamshire, United Kingdom) was added to assess parasite growth. The incorporation of 3H-hypoxanthine was quantitated using a liquid scintillation counter (Wallac 1409; Pharmacia, Uppsala, Sweden). The 50% inhibitory concentration (IC50), defined as the drug concentration corresponding to 50% of the uptake of 3H-hypoxanthine measured in the drug-free control wells, was determined by non-linear regression analysis of logarithm of concentrations plotted against growth inhibition. The threshold IC50 value for *in vitro* resistance to chloroquine was estimated to be ≥ 100 nM.13

**Data interpretation.** Fifty-two paired samples of *P. falciparum*-infected erythrocytes were obtained and analyzed in this study. The recrudescence sample was included in this study if the patient had a positive blood smear, without fever, during the follow-up period on Days 7 or 14 or a second episode of acute uncomplicated malarial infection on or beyond Day 7 and within 100 days after the first (Day 0) infection. Blood samples from patients requiring alternative treatment on or before Day 3 were excluded. These excluded patients satisfied one of the four criteria of an early treatment failure: positive smear and signs and symptoms of severe malaria on Days 1, 2, or 3; positive smear (parasite density > Day 0 density) and fever on Day 2; positive smear and fever on Day 3; and positive smear on Day 3 (parasite density ≥ 25 % of pre-treatment density).8 For some paired samples, there were additional samples from patients with multiple recrudescences. Further recrudescence samples of the patients were included for comparison until Day 300 from the initial drug therapy.

Based on the definitions of RI, RII, and RIII, paired samples were classified into three groups depending on the day when recrudescence parasites were obtained: Group 1, between Day 7 and Day 14; Group 2, between Day 15 and Day 28; and Group 3, beyond Day 29. Recrudescence was defined as the presence of identical amplification products in pre-treatment and post-treatment samples. Type A recrudescence consisted of either single alleles at all three loci, suggesting monoclonal infections, or multiple alleles at 1, 2, or 3 loci, suggesting multiclonal infections. Type B recrudescence was defined as a monoclonal infection before treatment, indicated by two or more bands in at least one locus, and the persistence of a subset of the alleles (multiclonal or monoclonal) after treatment. Type B recrudescence is likely to be due to the selection of drug-resistant populations after drug therapy and the missing band after treatment probably corresponds to the sensitive parasites. Type C recrudescence was defined as the presence of identical bands (monoclonal or multiclonal) in the pre-treatment and post-treatment samples, with an additional band appearing after treatment. The additional allele in type C recrudescence may suggest one of the following possibilities: 1) multiplication and/or selection of the minority of parasite populations present before treatment and undetected by the polymerase chain reaction, 2) appearance of secondary or tertiary broods with delayed or asynchronous development, as compared with the primary brood detected on Day 0, 3) additional parasite populations inoculated by the same or another mosquito few days after the initial infective bite, or 4) gametocyte formation, maturation, and release in the peripheral circulation.16 Type C recrudescence may also be due to a mixture of type A recrudescence and reinfection, but the probability of obtaining amplification products of same sizes at three loci is slight in an area of intense transmission. Reinfection was defined as the presence of different alleles at all three loci. When different alleles were observed at two loci, with an allele at one locus (usually CSP) which appeared to have an identical or similar size, the post-treatment sample was classified as reinfection.
In our preliminary study, consecutive pre-treatment samples were analyzed for allelic differences at the three loci by a multiplex polymerase chain reaction. The amplification products yielded different alleles at the three loci, indicating the wide diversity of *P. falciparum* isolates circulating in Yaoundé among symptomatic patients. The overlapping band sizes obtained from multiclonal infections did not allow a precise analysis of the frequency of each allele in this preliminary approach.

Of 52 paired samples obtained during the clinical trials conducted in Yaoundé, 22 (42%) were reinfections. The other samples were recrudescent, with either identical parasite populations (type A) (n = 10; 19%), selection of parasite populations (type B) (n = 17; 33%), or persistence of initial populations and appearance of other populations (type C) (n = 3; 6%) (Table 1). Recrudescence tended to occur more frequently on or before Day 7 (12 of 30 recrudescent cases, 73%), while reinfections were observed almost exclusively beyond Day 14 (21 of 22 reinfections, 95%). The only case (no. 1055) of reinfection on Day 14 occurred after chloroquine treatment. The patient had a negative blood smear on Day 7 but a positive smear on Day 14. The pre-treatment isolate was resistant *in vitro* (IC$_{50}$ = 253 nM), while the post-treatment isolate was at the borderline (IC$_{50}$ = 106 nM).

The characteristics of the patients and the corresponding isolates obtained at three or more time points, including pre-treatment isolate, are summarized in Table 2. The evolution of parasite genotype within a short time interval after chloroquine treatment was observed in five patients with recrudescence before Day 15. These patients were not treated with an alternative drug until Day 14 or 15 because they were asymptomatic between Day 3 and Day 13. Three of these isolates (no. 157, 1032, and 3009) analyzed at an interval of 1–4 days presented an identical recrudescent genotype. One isolate (no. 156) had type B recrudescence and gametocytes on Day 7 but, on Day 15, had an identical type A recrudescence genotype pattern as that of Day 0. Another isolate (no. 182) had different type B recrudescent genotypes on Day 7 and Day 14. Patients who were treated with amodiaquine, pyronaridine, or sulfadoxine-pyrimethamine and who had several malarial episodes at an interval exceeding seven weeks were infected with new parasite populations. In one of these patients (no. 1062), the sample corresponding to an adequate clinical response with no fever and no detectable asexual parasites on Day 258 (after the third malarial attack on Day 244) but with gametocytes yielded a type B recrudescent genotype. A similar phenomenon was also observed in two other patients treated with sulfadoxine-pyrimethamine. None of these three gametocyte carriers required further treatment.

The phenotype of pre-treatment and post-treatment paired samples was compared if the parasitemia of the post-treatment infection was $\pm 0.1\%$. Chloroquine was chosen as a phenotypic marker for this study. The most significant change in the phenotype occurred when the initially chloroquine-sensitive isolate was characterized to be chloroquine-resistant, or vice versa, at the time of recrudescence or reinfection (Table 3). Such a shift in chloroquine sensitivity/resistance occurred in nine (including one borderline, 106 nM) of 12 infecting isolates as well as in three of 11 recrudescent parasites. Eight of 9 recrudescent isolates after chloroquine treatment were resistant *in vitro* to chloroquine.

**RESULTS**

In our preliminary study, consecutive pre-treatment samples were analyzed for allelic differences at the three loci by a multiplex polymerase chain reaction. The amplification products yielded different alleles at the three loci, indicating the wide diversity of *P. falciparum* isolates circulating in Yaoundé among symptomatic patients. The overlapping band sizes obtained from multiclonal infections did not allow a precise analysis of the frequency of each allele in this preliminary approach.

Of 52 paired samples obtained during the clinical trials conducted in Yaoundé, 22 (42%) were reinfections. The other samples were recrudescent, with either identical parasite populations (type A) (n = 10; 19%), selection of parasite populations (type B) (n = 17; 33%), or persistence of initial populations and appearance of other populations (type C) (n = 3; 6%) (Table 1). Recrudescence tended to occur more frequently on or before Day 7 (12 of 30 recrudescent cases, 73%), while reinfections were observed almost exclusively beyond Day 14 (21 of 22 reinfections, 95%). The only case (no. 1055) of reinfection on Day 14 occurred after chloroquine treatment. The patient had a negative blood smear on Day 7 but a positive smear on Day 14. The pre-treatment isolate was resistant *in vitro* (IC$_{50}$ = 253 nM), while the post-treatment isolate was at the borderline (IC$_{50}$ = 106 nM).

The characteristics of the patients and the corresponding isolates obtained at three or more time points, including pre-treatment isolate, are summarized in Table 2. The evolution of parasite genotype within a short time interval after chloroquine treatment was observed in five patients with recrudescence before Day 15. These patients were not treated with an alternative drug until Day 14 or 15 because they were asymptomatic between Day 3 and Day 13. Three of these isolates (no. 157, 1032, and 3009) analyzed at an interval of 1–4 days presented an identical recrudescent genotype. One isolate (no. 156) had type B recrudescence and gametocytes on Day 7 but, on Day 15, had an identical (type A recrudescence) genotype pattern as that of Day 0. Another isolate (no. 182) had different type B recrudescent genotypes on Day 7 and Day 14. Patients who were treated with amodiaquine, pyronaridine, or sulfadoxine-pyrimethamine and who had several malarial episodes at an interval exceeding seven weeks were infected with new parasite populations. In one of these patients (no. 1062), the sample corresponding to an adequate clinical response with no fever and no detectable asexual parasites on Day 258 (after the third malarial attack on Day 244) but with gametocytes yielded a type B recrudescent genotype. A similar phenomenon was also observed in two other patients treated with sulfadoxine-pyrimethamine. None of these three gametocyte carriers required further treatment.

The phenotype of pre-treatment and post-treatment paired samples was compared if the parasitemia of the post-treatment infection was $\pm 0.1\%$. Chloroquine was chosen as a phenotypic marker for this study. The most significant change in the phenotype occurred when the initially chloroquine-sensitive isolate was characterized to be chloroquine-resistant, or vice versa, at the time of recrudescence or reinfection (Table 3). Such a shift in chloroquine sensitivity/resistance occurred in nine (including one borderline, 106 nM) of 12 infecting isolates as well as in three of 11 recrudescent parasites. Eight of 9 recrudescent isolates after chloroquine treatment were resistant *in vitro* to chloroquine.

**DISCUSSION**

For several decades after the official formulation of the definition of antimalarial drug resistance in 1973, there was no adequate laboratory tool to demonstrate whether a given parasite strain was able to survive and multiply despite correct administration of the drug. To resolve this technical limitation, malaria researchers were usually required to conduct *in vivo* tests of resistance for 28 days in an area where there is no malaria transmission. Otherwise, the reappearance of parasites cannot be attributed to recrudescence or reinfection with certitude. This limitation of *in vivo* tests represented a major disadvantage in areas where malaria transmission occurs throughout the year in the entire country, as in most areas in sub-Saharan Africa.

In the late 1970s and during the 1980s, *in vitro* assay techniques were adapted from the *in vitro* culture method developed by Trager and Jensen to measure drug sensitivity/resistance of *P. falciparum* isolates. However, *in vitro* and *in vivo* responses are not always concordant because host immunity may intervene in *in vivo* tests but not in *in vitro* assays and threshold levels of resistance for *in vitro* tests are arbitrary and may not correlate with *in vivo* response. Furthermore, our results on the comparison of the level of *in vitro* chloroquine sensitivity/resistance of pre-treatment and post-treatment isolates were not decisive in distinguishing recrudescence from reinfection. The primary underlying reason for the poor performance of the comparison of phenotype may be related to the presence of multiple *P. falciparum* populations before treatment that may undergo selection or modify the proportion of different parasite populations after treatment. Drug treatment may kill the drug-
sensitive populations but select resistant parasites, which may persist or reappear in the peripheral blood several days or weeks after the treatment has been completed. This in vivo phenomenon has been reproduced experimentally by cloning or maintaining a clinical isolate in continuous in vitro culture. Another reason is the minimal requirement of 0.1% parasitemia to perform the in vitro assay at the time of recrudescence. Even if this requirement is fulfilled, in vitro assays performed with recrudescent isolates obtained several days to few weeks after treatment may not be able to reproduce the results obtained with the initial isolates due to the presence of antimalarial drugs concentrated within infected erythrocytes. The residual drug in the blood sample tends to lower the IC30 value. Moreover, reinfection cannot be deduced on the sole basis of the chloroquine sensitivity/resistance pattern since there is a probability of approximately 50% to be infected with sensitive or resistant parasites, based on the epidemiology of drug-resistant P. falciparum in Yaoundé.

These technical limitations of in vivo and in vitro tests of drug resistance can be overcome by the polymerase chain reaction. Some investigators have applied nested polymerase chain reaction, hybridization of amplification products with labeled probes, single-strand conformational polymorphism, and DNA sequencing to genotype field isolates of P. falciparum. Our study showed that even a single 30-cycle round of polymerase chain reaction to amplify three independent polymorphic markers provides sufficient data to distinguish recrudescence from reinfection. The genotypic markers can also be amplified in a single reaction tube by multiplex polymerase chain reaction.

The randomized clinical study from which paired samples were obtained for this study included 131 patients treated with chloroquine and followed until at least Day 14, 81 with pyronaridine, 59 with amodiaquine, and 66 with sulfadoxine-pyrimethamine. Recrudescence occurred most frequently after treatment with chloroquine, which is still the first-line drug in Cameroon. This result was expected from our regular monitoring of drug resistance which showed that about 50% of the clinical isolates obtained at the same dispensary in Yaoundé are resistant to chloroquine since 1994. Amodiaquine has been known to be a highly effective alternative first-line drug in Cameroon, as confirmed by our in vitro results. Pyronaridine is also highly active in vitro against Cameroonian isolates and effective in clearing parasitemia in patients. Sulfadoxine-pyrimethamine is a second-line drug in Cameroon and has been shown to have a cure rate of 89%. Thus, the pattern of recrudescent profiles observed in our study closely follows the epidemiology of antimalarial drug resistance in Yaoundé.

Halofantrine and artesunate are new drugs that have been introduced in Cameroon several years ago. Halofantrine has been shown to be effective in a clinical trial conducted in Yaoundé, even with a single cure of 25 mg/kg over a 12-hr

---

**TABLE 2**

Multiple episodes of recrudescence and reinfections occurring in the same patients

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (yr)</th>
<th>Day of infection</th>
<th>Treatment</th>
<th>Parasitemia/μl</th>
<th>Genotype*</th>
</tr>
</thead>
<tbody>
<tr>
<td>156</td>
<td>14</td>
<td>0</td>
<td>Chloroquine</td>
<td>394,000</td>
<td>Recrudescence B</td>
</tr>
<tr>
<td>157</td>
<td>11</td>
<td>0</td>
<td>Chloroquine</td>
<td>455,000</td>
<td>Recrudescence B</td>
</tr>
<tr>
<td>182</td>
<td>7</td>
<td>0</td>
<td>Chloroquine</td>
<td>579,000</td>
<td>Recrudescence B</td>
</tr>
<tr>
<td>1032</td>
<td>23</td>
<td>0</td>
<td>Chloroquine</td>
<td>13,600</td>
<td>Recrudescence C</td>
</tr>
<tr>
<td>3009</td>
<td>11</td>
<td>0</td>
<td>Chloroquine</td>
<td>8,180</td>
<td>Recrudescence B</td>
</tr>
<tr>
<td>1001</td>
<td>17</td>
<td>0</td>
<td>Amodiaquine</td>
<td>55,400</td>
<td>Reinfection 1</td>
</tr>
<tr>
<td>3006</td>
<td>10</td>
<td>0</td>
<td>Amodiaquine</td>
<td>103,000</td>
<td>Reinfection 2</td>
</tr>
<tr>
<td>151</td>
<td>45</td>
<td>0</td>
<td>Pyronaridine</td>
<td>98,800</td>
<td>Recrudescence B</td>
</tr>
<tr>
<td>1062</td>
<td>22</td>
<td>0</td>
<td>SP†</td>
<td>102,000</td>
<td>Reinfection 1</td>
</tr>
</tbody>
</table>

* Recrudescence Type A, identical bands; Type B, selection; Type C, appearance of new bands. Reinfection 1 and 2 refer to two different new infections. Patient no. 1062 (Day 258) had no detectable asexual parasites but carried gametocytes. The presence of gametocytes is not an evidence of recrudescence; but unless microscopic examination is conducted, the polymerase chain reaction alone may lead to a wrong conclusion.

† SP = sulfadoxine-pyrimethamine.
period. In the present study, only one of 56 patients requiring halofantrine for treatment failure with other drugs, essentially chloroquine (n = 48), returned 22 days after halofantrine treatment with symptomatic malaria due to reinfection. Oral artesunate was administered for five days to one patient, who had recrudescent malaria on Day 21. The two patients were cured with five days of quinine. Reappearance of parasites after halofantrine or artesunate treatment requires the dosage of plasmodial drug level few hours or days after drug administration because recrudescence is known to occur due to inadequate drug absorption or rapid elimination.

The results of our study do not agree with the results of some of the previous studies. In the study of Babiker and others, six Sudanese isolates obtained from patients responding with early and late RI chloroquine treatment failure (from Day 9 to Day 30) had identical MSA-1 and MSA-2 alleles, leading the investigators to conclude that RI chloroquine resistance is due to recrudescence. Using the same markers, a similar observation was made on 12 recrudescent isolates obtained 14–21 days after chloroquine or amodiaquine therapy in Papua New Guinea. None of the parasites was characterized as reinfection in that study. In Thailand, recrudescence accounted for 38% of post-treatment reappearance of parasitemia up to Day 62 in nonpregnant patients, and new infections were detected as early as 21 days post-treatment. However, most Thai patients were treated with quinine, mefloquine, and/or artemisinin derivatives. The long elimination half-life of mefloquine and the rapid elimination of quinine and artemisinin derivatives are expected to prolong and shorten the recrudescence period, respectively. Moreover, in Thailand and Sudan, malaria transmission is seasonal and/or hypoendemic, suggesting a relatively lower risk of reinfection as compared with the tropical rain forest region in Africa. These differences preclude a direct comparison of results with those of our study. In Cameroonian patients residing in Yaoundé, reinfections were clearly present between Days 15 and 28 after chloroquine, amodiaquine, or sulfadoxine-pyrimethamine treatment. Similar observations were made in other African countries, such as Tanzania and Gabon, described as holoendemic areas.

While the definition of resistance is sufficiently precise from the operational viewpoint, the time-period of the grading system that allows a distinction between late and early RI seems to be arbitrary and possibly variable depending on the pharmacokinetics of antimalarial drugs, risk of exposure to new infections, and degree of acquired immunity. Despite these shortcomings, Day 14 as the arbitrary cut-off point for true recrudescence after chloroquine therapy may be acceptable for practical reasons in Yaoundé at present. However, between Day 14 and Day 28, recrudescence and reinfection cannot be distinguished by clinical and parasitologic response to chloroquine, and we need to resort to molecular techniques to genotype the isolates. Other standard antimalarial drugs and new drugs are still generally effective in Cameroon, which means that isolates from any patient who returns with symptomatic malaria after treatment need to be genotyped and compared with the pre-treatment isolate as part of the surveillance program of antimalarial drug resistance and test of therapeutic efficacy.

Acknowledgments: We thank Sisters Solange Menard and Marie-Solange Oko and their nursing and laboratory staff at the Nlongkak

### Table 3

Comparison of the in vitro chloroquine response between pre-treatment and post-treatment isolates

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Treatment</th>
<th>Recrudescence day</th>
<th>Genotype†</th>
<th>Chloroquine IC₅₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Pre-treatment</td>
</tr>
<tr>
<td>157</td>
<td>Chloroquine</td>
<td>11</td>
<td>Recrudescence B</td>
<td>159</td>
</tr>
<tr>
<td>143</td>
<td>Chloroquine</td>
<td>14</td>
<td>Recrudescence A</td>
<td>151</td>
</tr>
<tr>
<td>CQ9</td>
<td>Chloroquine</td>
<td>14</td>
<td>Recrudescence A</td>
<td>117</td>
</tr>
<tr>
<td>182</td>
<td>Chloroquine</td>
<td>14</td>
<td>Recrudescence B</td>
<td>24.4</td>
</tr>
<tr>
<td>1019</td>
<td>Chloroquine</td>
<td>14</td>
<td>Recrudescence B</td>
<td>287</td>
</tr>
<tr>
<td>175</td>
<td>Chloroquine</td>
<td>14</td>
<td>Recrudescence C</td>
<td>102</td>
</tr>
<tr>
<td>156</td>
<td>Chloroquine</td>
<td>15</td>
<td>Recrudescence A</td>
<td>163</td>
</tr>
<tr>
<td>CQ11</td>
<td>Chloroquine</td>
<td>15</td>
<td>Recrudescence B</td>
<td>37.4</td>
</tr>
<tr>
<td>147</td>
<td>Chloroquine</td>
<td>22</td>
<td>Recrudescence A</td>
<td>278</td>
</tr>
<tr>
<td>180</td>
<td>Chloroquine</td>
<td>23</td>
<td>Reinfecction</td>
<td>45.6</td>
</tr>
<tr>
<td>CQ17</td>
<td>Chloroquine</td>
<td>21</td>
<td>Reinfecction</td>
<td>81.1</td>
</tr>
<tr>
<td>1055</td>
<td>Chloroquine</td>
<td>14</td>
<td>Reinfecction</td>
<td>253</td>
</tr>
<tr>
<td>1060</td>
<td>Amodiaquine</td>
<td>28</td>
<td>Reinfecction</td>
<td>39.4</td>
</tr>
<tr>
<td>1104</td>
<td>Amodiaquine</td>
<td>42</td>
<td>Reinfecction</td>
<td>55.2</td>
</tr>
<tr>
<td>133</td>
<td>Pyronaridine</td>
<td>42</td>
<td>Recrudescence B</td>
<td>58.9</td>
</tr>
<tr>
<td>7</td>
<td>Pyronaridine</td>
<td>63</td>
<td>Reinfecction</td>
<td>94.0</td>
</tr>
<tr>
<td>113</td>
<td>Pyronaridine</td>
<td>50</td>
<td>Reinfecction</td>
<td>310</td>
</tr>
<tr>
<td>161</td>
<td>Pyronaridine</td>
<td>51</td>
<td>Reinfecction</td>
<td>474</td>
</tr>
<tr>
<td>185</td>
<td>Pyronaridine</td>
<td>36</td>
<td>Reinfecction</td>
<td>391</td>
</tr>
<tr>
<td>1014</td>
<td>Sulfadoxine-pyrimethamine</td>
<td>20</td>
<td>Recrudescence A</td>
<td>184</td>
</tr>
<tr>
<td>3063</td>
<td>Sulfadoxine-pyrimethamine</td>
<td>80</td>
<td>Reinfecction</td>
<td>355</td>
</tr>
<tr>
<td>106</td>
<td>Halofantrine</td>
<td>22</td>
<td>Reinfecction</td>
<td>355</td>
</tr>
</tbody>
</table>

| HAR          | Artesunate | 20                | Recrudescence A | 186 | 316 |

* In addition to chloroquine, paired 50% inhibitory concentration (IC₅₀) values of the drugs used for treatment were determined for the following isolates: no. 1060, monodesethylamodiaquine (active metabolite of amodiaquine) IC₅₀ values = 16.4 nM vs 54.4 nM; no. 1104, monodesethylamodiaquine IC₅₀ values = 19.0 nM vs 9.02 nM; no. 133, pyronaridine IC₅₀ values = 3.03 nM vs 8.40 nM; no. 113, pyronaridine IC₅₀ values = 3.50 nM vs 1.33 nM; no. 161, pyronaridine IC₅₀ values = 7.93 nM vs 5.37 nM; no. 1014, pyrimethamine IC₅₀ values = 2.180 nM vs 281 nM; and no. 3063, pyrimethamine IC₅₀ values = 291 nM vs 236 nM. The threshold IC₅₀ values for in vitro resistance to chloroquine, monodesethylamodiaquine, and pyrimethamine were arbitrarily set at ≥ 100 nM, ≥ 60 nM, and ≥ 100 nM, respectively. The threshold value for pyronaridine is undetermined.

† Recrudescence Type: A = identical bands; Type B = selection; Type C = appearance of new bands.
Catholic missionary dispensary for precious aid in recruiting pa-
tients.
Financial support: The clinical studies were supported by Agence
Universitaire de la Francophonie and the French Ministry of Foreign
Affairs (Department of Cooperation).
Authors’ addresses: Leonardo Basco and Pascal Ringwald, OCEAC/IRD, BP 288, Yaoundé, Cameroon. (Present address for Pascal Ring-
wald: Cluster of Communicable Diseases, Surveillance and Re-
sp. Ant-i Infective Drug Resistance Surveillance and Contain-
ment, World Health Organization, CH-1211 Geneva 27, Switzer-
land.)

REFERENCES
1. World Health Organization, 1973. Chemotherapy of malaria and
resistance to antimalarials: report of a WHO Scientific Group.
World Health Organ Tech Rep Ser 529.
2. Bruce-Chwatt LJ, Black RH, Canfield CJ, Clyde DF; Peters W; Wer-
3. Rieckmann KH, 1990. Monitoring the response of malaria in-
ronaridine versus chloroquine for acute uncomplicated falcipar-
5. Mount DL, Nahlen BL, Patchen LC, Churchill FC, 1989. Ad-
aptations of the Sakor-Solomons test: simple, reliable colo-
metric field assays for chloroquine and its metabolites in
6. Warrell DA, Molyneux ME, Beales PF, 1990. Severe and com-
7. Basco LK, Ringwald P, 1996. Assessment of therapeutic ef-
ciency for uncomplicated falciparum malaria in areas with in-
WHO/MAL/96/1077.
laria in Yaoundé, Cameroon. I. Analysis of point mutations in
the dihydrofolate reductase-thymidylate synthase gene of
falciparum: a simple polymerase chain reaction method for
differentiating strains. Exp Parasi tol 75: 207–212.
Hockmeyer WT, Maloy WL, Haynes JD, Schneider I, Roberts D,
Sanders GS, Reddy PE, Diggs CL, Miller LH, 1984. Struc-
ture and hy-
dropathy profile of the major merozoite surface antigen
(gp195) of the Uganda-Palo Alto isolate. Exp Parasi tol 67:
1–11.
11. Smyth JA, Coppel RL, Day KP, Martin RK, Oduolu AMJ,
Kemp DJ, Anders RF, 1991. Structural diversity in the Plas-
modium falciparum merozoite surface antigen 1. Proc Natl
Acad Sci USA 88: 1751–1755.
Quantitative assessment of antimalarial activity in vitro by a
semiautomated microdilution technique. Antimicrob Agents
Chemother 16: 710–718.
vitro tests and an in vivo test for chloroquine resistance in
Plasmodium falciparum clinical isolates. J Clin Microbiol 36:
243–247.
the assessment of recrudescence or reinfection after antimalarial
15. Trager W, Jensen B, 1976. Human malaria parasites in contin-
laria parasites. Wer nsdorfer WH, McGregor IA, eds. Malaria:
Principles and Practice of Malariology. Edinburgh: Churchill
Livingstone, 1765–1800.
tests of resistance in patients treated with chloroquine in Ya-
isolates before and after adaptation to continuous culture. Exp
Parasitol 56: 9–14.
19. Thaihtong S, Beale GH, Fenton B, McBride JS, do Rosario V,
Walker A, Walliker D, 1984. Clonal diversity in a single iso-
late of the malaria parasite Plasmodium falciparum. Trans R
aram: recrudescence of parasites in culture. Exp Parasitol
81: 556–563.
Chongsuphajisiddhi T, Mellouk S, Langsley G, 1998. A pri-
mary malarial infection is composed of a very wide range of
genetically diverse but related parasites. J Clin Invest 101:
22. Mercereau-Puijalon O, Fandeur T, Bonnefoy S, Jacqemot C,
Sarthou JL, 1991. A study of the genomic diversity of Plas-
modium falciparum in Senegal. 2. Typing by the polymerase
D, 1994. Genetic evidence that RI chloroquine resistance of
Plasmodium falciparum is caused by recrudescence of resis-
polymorphism analysis differentiates Plasmodium falciparum
treatment failures from re-infections. Mol Biochem Parasitol
79: 167–175.
Evidence that recurrent Plasmodium falciparum infection is
cured by recrudescence of resistant parasites. Am J Trop Med
26. Belger I, Marshal VM, Reeder JC, Hunt JA, Mgone CS, Beck HP,
1997. Sequence diversity and molecular evolution of the
merozoite surface antigen 2 of Plasmodium falciparum. J Mol
27. Ranford-Cartwright LC, Taylor J, Umashanthar T, Taylor LH,
Babiker HA, Lell B, Schmidt-Ort JR, Lehman LG, Walliker
parasites in a Plasmodium falciparum drug efficacy trial in
line treatment of malaria in Yaoundé, Cameroon: presumptive
evidence from activity in vitro and cross-resistance patterns.
activity of pyronaridine, alone and in combination with other
antimalarial drugs, against Plasmodium falciparum. Antimi-
Chimientoiresistance de P. falciparum en milieu urbain à Yaoun-
dé, Cameroun. Part 2: Evaluation de l’efficacité de
l’amodiaquine et de l’association sulfadoxine-pyriméthamine
pour le traitement de l’accès palustre simple à Plasmodium
falciparum à Yaoundé. Trop Med Int Hlth 8: 620–627.
crironized halofantrine in semi-immune patients with acute un-
complicated falciparum malaria in Cameroon. Antimicrob
malarial activity, pharmacokinetic properties and therapeutic
33. Bouchaud O, Basco LK, Gillotin C, Gimenez F, Ramilariosa
O, Genissel B, Bouvet E, Farinotti R, Le Bras J, Coulaud JP,
1994. Clinical efficacy and pharmacokinetics of micrized


