MOLECULAR EPIDEMIOLOGY OF MALARIA IN CAMEROON.
IX. CHARACTERISTICS OF RECRUDESCENT AND PERSISTENT
PLASMODIUM FALCIPARUM INFECTIONS AFTER CHLOROQUINE OR
AMODIAQUINE TREATMENT IN CHILDREN

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Abstract. In the absence of a firmly established gene responsible for chloroquine and amodiaquine resistance in Plasmodium falciparum, surveillance of resistance to these first-line drugs in Cameroon needs to be performed by in vivo or in vitro tests for drug resistance. These 2 methodological approaches to define drug resistance were shown to be complementary and concordant in a majority of cases at our study sites, but discordant results may be observed in a few cases, probably as a result of acquired immunity and low plasma drug levels. To further examine the nature of recrudescent and persistent parasitemia after treatment with chloroquine or amodiaquine, the clinical response of children aged < 5 years, presumably with insufficient immune response, was assessed, and the in vitro response of the corresponding isolates was determined if treatment or parasitological failure occurred. Genotyping of pretreatment and posttreatment isolates was performed by polymerase chain reaction to distinguish between recrudescence and reinfection. Plasma drug levels were measured at the time of therapeutic failure by high-performance liquid chromatography. All cases of therapeutic or parasitological failure observed on or before Day 14 were due to the persistence or recrudescence of the original parasite populations present before treatment, with or without selection and appearance of new populations. Most parasites were characterized by elevated 50% inhibitory concentrations for chloroquine and amodiaquine at the time of clinical or parasitological failure. In some children, recrudescence was explained by the absence of drug in the plasma. The simultaneous analysis of clinical and in vitro responses, plasma drug level measurement, and genotyping may yield results that may explain the reasons for therapeutic failure, help establish the threshold level for in vitro resistance, and provide a set of more accurate tools to describe the epidemiology of drug-resistant P. falciparum while awaiting for the identification of the chloroquine and amodiaquine resistance gene or genes.

INTRODUCTION

Despite the increasing prevalence of drug resistance, chloroquine is still recommended for the first-line treatment of acute uncomplicated Plasmodium falciparum malarial infections in many Central and West African countries.1 In Cameroon, both chloroquine and amodiaquine are first-line drugs of choice for malaria treatment (Ministry of Public Health, Yaoundé, Cameroon, unpublished data). However, in Yaoundé, the capital city of Cameroon, where long-term in vitro and clinical studies have been conducted, chloroquine can no longer be recommended for P. falciparum infections due to a high rate of therapeutic failure, which is supported by a similarly high proportion of chloroquine-resistant isolates in vitro.2-4 Amodiaquine has thus replaced chloroquine for the first-line treatment of uncomplicated malarial infections in Yaoundé.5,6 In other areas of Cameroon, clinical studies are currently being undertaken to evaluate the therapeutic efficacy of chloroquine.

Two methods have been used to define drug resistance in the past: in vivo test and in vitro drug sensitivity assay. The in vivo test of drug resistance evaluates the therapeutic efficacy of an antimalarial drug by administering the standard dose and after the parasitological or clinical response in either asymptomatic or symptomatic patients.5,8 The in vitro drug assay determines the ability of the parasites to grow in different concentrations of the test compound. Our previous studies have shown that although there is a moderate to good agreement between the in vitro response of P. falciparum isolates and clinical response of the corresponding patients treated with chloroquine, discordance between in vitro and in vivo responses may be observed in up to 20% of cases.4,9 Some of these discordant cases were due to either reinfection or relatively lower plasma drug levels,4,10 but the role of acquired immunity was not excluded because these previous studies were based on symptomatic malaria-infected patients aged ≥ 5 years.

In the present study, we have further examined the clinical response and characteristics of recrudescent parasites after chloroquine or amodiaquine treatment in children < 5 years old. Because of the absence of a standardized biological test to quantitate the level of acquired immune response to malaria parasites, malaria-infected, symptomatic children aged < 5 years were assumed to be nonimmune to malarial infections.11 We have also analyzed the threshold level of in vitro drug resistance in relation to the in vivo response and the presence or absence of drugs in the plasma.

MATERIALS AND METHODS

Study sites. Yaoundé is the capital city of Cameroon and is situated in the tropical rain forest region. Eseka and Bertoua are urban sites located within the southern belt of tropical rain forest area in Cameroon, ~ 120 km to the west and 350 km to the east of Yaoundé, respectively. Malaria transmission is intense and continuous throughout the year in rural areas but is much less intense in urban sites in southern Cameroon, with peak seasons corresponding to the rainy sea-
tions (March–May and September–November). There are no previous epidemiological data on the drug resistance status in Eseka and Bertoua.

**Patients.** As specified in the World Health Organization (WHO) protocol for the evaluation of therapeutic efficacy in areas of high transmission, patients consulting spontaneously at health centers were enrolled if they satisfied the following criteria: age ≤ 5 years, positive blood smear with parasitemia between 2,000 and 100,000 asexual parasites per microliter of blood, *P. falciparum* infection (without other *Plasmodium* species) with no other associated bacterial or viral infections, and fever at the time of consultation. In addition, informed consent was obtained. Patients with signs and symptoms of severe and complicated malaria were excluded.

The history of a recent intake of antimalarial drugs was not an exclusion criterion. This study was approved by the Cameroonian Ministry of Public Health.

**In vivo test.** The WHO standardized 14-day test of therapeutic efficacy was used to assess the therapeutic efficacy of chloroquine. The patients were treated with the standard dose of chloroquine (25 mg base/kg body weight in 3 divided daily doses) under supervision by the observation of drug intake, inspection of oral cavity, and exclusion of eventual vomiting for at least 30 min after drug administration. Patients were monitored on Days 0, 1, 2, 3, 4, 7, and 14. The patients’ guardians were strongly encouraged to return to the health center at any time during the 14-day period for aggravation or persistence of signs and symptoms. In vivo responses were classified into 3 groups according to the WHO scheme.

Early treatment failure (ETF) was defined as positive blood smear and signs 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; or positive smear on Day 3 (parasite density ≥ 25% of pretreatment density). Late treatment failure (LTF) was defined as positive smear and either signs of severe malaria or fever between Days 4 and 14. Adequate clinical response (ACR) was defined as negative smear on Day 14 (with or without fever) or apyrexia (with positive or negative smear) during the follow-up. Patients with an ACR with a positive smear on Day 3 (parasite density > 25% of pretreatment density) and fever on Day 2; positive smear and fever on Day 3; or positive smear on Day 3 (parasite density ≥ 25% of pretreatment density) were categorized as clinical success but parasitological failure.

**In vitro assay.** The isotopic microtest developed by Desjardins and others was performed on posttreatment isolates without previous adaptation to *in vitro* culture conditions. Pretreatment venous blood samples were not available for drug sensitivity assay. Infected erythrocytes were washed 3 times in RPMI 1640 medium by centrifugation (2,000 × g for 10 min). An aliquot of 1 mL of red blood cell pellet was used to extract parasite DNA. Infected erythrocytes were suspended in 15 mL of ice-cold NET buffer (150 mM NaCl, 10 mM EDTA, and 50 mM Tris, pH 7.5) and lysed with 0.015% saponin. The lysate was centrifuged at 2,000 × g for 10 min, and the pellet was transferred to a 1.5-mL microfuge tube and suspended in 500 μL of NET buffer. The mixture was treated with 1% N-lauroylsarcosine (Sigma) and RNase A (100 μg/mL) at 37°C for 1 hr and proteinase K (200 μg/mL) at 50°C for 1 hr. Parasite DNA was extracted 3 times in equilibrated phenol (pH 8), phenol-chloroform-isoamyl alcohol (v/v/v 25:24:1), and chloroform-isoamyl alcohol (v/v 24:1), and was precipitated by the addition of 0.3 M sodium acetate and cold absolute ethanol. The extracted DNA was air-dried and resuspended in TE buffer (10 mM Tris, 1 mM EDTA). Parasite DNA was stored at −20°C until use.

**Genotyping of *P. falciparum* isolates.** DNA fragments of 3 polymorphic markers, circumsporozoite protein (*csp*), merozoite surface antigen-1 (*msa-1*), and merozoite surface antigen-2 (*msa-2*) genes, were amplified by nested polymerase chain reaction (PCR). Each of the 3 loci was amplified separately. The following species-specific oligonucleotide primers were designed from published sequences of the complete gene sequences: CSP-1, 5'-AAATTACAAATCCATGATGA-GAAATTAGC-3' (forward primer), CSP-2, 5'-GATGTTGTTTCTTTAATTTAAGGACAAG-3' (reverse primer), MSA1-1, 5'-ATGAAGGTAATTAAAACATTGTCTATTA-3' (forward primer), MSA1-2, 5'-TTATATGAATATG-GCAAAGATAAAAACAG-3' (reverse primer), MSA1-3, 5'-ATGAAGGATCATATTCTTTATTTATTGCTATTTA-3' (forward primer), and MSA2-1, 5'-TTATATGAATATG-GCAAAGATAAAAACAG-3' (reverse primer). These primers were designed to amplify the entire *csp* and *msa-2* genes and block 2 of the *msa-1* gene.

The reaction mixture for the primary PCR consisted of DNA templates (~200 ng of genomic DNA for DNA extracted from a 1-mL aliquot of red cell pellet or 10 μL of DNA extracted from 3 MM Whatmann filter papers), 15 μL of 10× PCR buffer (10 mM Tris, 1 mM EDTA, and 50 mM Tris, pH 7.5), 3 μL of 25 mM MgCl2, 2 μL of 10 mM dNTPs, 1 μL of 50 μM primers, 0.5 μL of 5 U/μL Taq polymerase, and sterile distilled water. The PCR program consisted of an initial denaturation at 94°C for 3 min, followed by 30 cycles of 30 sec each at 94°C, 45 sec at 45°C, and 1 min at 72°C, and a final extension at 72°C for 10 min.
pmol of forward and reverse primers, buffer (50 mM KCl, 10 mM Tris, pH 8.3), 1.5 mM MgCl₂, 200 μM deoxyribonucleoside triphosphates (dNTP), and 1 unit of Taq 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 amplification product (1–4 μL) from the primary PCR was used to perform the secondary, nested PCR with the following internal primers: 5′- ATATAGTAGATCAGTTG-GAGA-3′ (forward primer) and 5′- GCATATTGAGCTTG-TGCCA-3′ (reverse primer) for csp, 5′- TAAATATAGCAACACATTCTAAACAATGC-3′ (forward primer) and 5′- CCACAAAACTCTGTAGTTACAAAAGAA-3′ (reverse primer) for msa-2, and 5′- GAGATGCAATGTTGACAAGG-3′ (forward primer) and 5′- GTTCTTATTAGTGAAACAAG-3′ (reverse primer) for msa-1. The same amplification program as that for the primary amplification was used. The amplified DNA fragments were resolved by electrophoresis in a 1.8% agarose gel, stained with ethidium bromide, and visualized under ultraviolet transillumination.

**Plasma drug concentrations.** Venous blood samples were collected and centrifuged at 2,000 × g for 10 min. Plasma samples were stored at −80°C until assay. Chloroquine, monodesethylchloroquine (major human metabolite of chloroquine), amodiaquine, and monodesethylamodiaquine (major human metabolite of amodiaquine) concentrations were measured simultaneously in posttreatment samples by a solid-phase extraction procedure and a high-performance liquid chromatographic method adapted from the technique described by Puissant and others. Plasma (1 mL) was transferred onto Bond Elut C18, 500 mg, 2.8 cm³ extraction column (Merck Eurolab, Fontenay-sous-Bois, France), eluted, and evaporated to dryness. The residue was reconstituted with 100 μL of 0.1 N HCl, and a reverse-phase chromatography was performed on a C-18 analytical column (Symmetry C-18 column, 250 by 4.6 mm, 5-μm particle size; Waters, Millipore, Milford, MA). The mobile phase consisted of acetonitrile/methanol/0.005 M heptanesulfonic acid mixture (v/v/v 20:13:67). The flow rate was adjusted to 1.2 mL/min. The ultraviolet detector was set to 242 nm. The limit of detection for all 4 compounds was 2.5 ng/mL.

**Data analysis.** Paired samples of *P. falciparum*-infected erythrocytes were obtained from patients with either an ACR with positive smear on Day 14 (parasitological failure) or therapeutic failure (early or late) to either chloroquine or amodiaquine within 14 days after the start of antimalarial treatment. Persistent parasitemia was observed in patients requiring an alternative treatment on or before Day 3 as a result of an ETF. Recrudescence was defined as the initial presence of fever and positive blood smear on Day 0, fever and parasite clearance on or before Day 4, then reappearance of parasitemia, with or without fever, on or before Day 14. This definition of recrudescence roughly corresponds to the LTF of the new WHO classification of antimalarial drug response. However, in some patients responding with LTF, a considerable diminution of the parasite density (< 25% of the pretreatment parasite density) was observed on Day 2, Day 3, or both, but a negative smear was not obtained, primarily because daily blood examinations are not performed until Day 7 in the new WHO protocol. In these patients, it was assumed that parasite clearance occurred before they returned for consultation with signs and symptoms of malaria.

As in our previous study, 3 types of recrudescent parasitemia were distinguished. Type A recrudescent was defined as the presence of identical patterns with either single alleles at all 3 loci, suggesting monoclonal infections, or multiple alleles at 1, 2, or 3 loci, suggesting multiclonal infections. Type B recrudescent was defined as a multiclonal infection before treatment, indicated by ≥ 2 bands in at least 1 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 pretreatment and posttreatment samples, with an additional band appearing after treatment. The biological possibilities that give rise to these different types of recrudescence have been described by Snounou and Beck. Reinfection was defined as the presence of different alleles at all 3 loci.

The sample size for the clinical studies was determined by the double lot quality assurance method. This sampling method allowed the termination of the study on the basis of a small sample size if the initial results indicated a very high level of treatment failure.

**RESULTS**

A total of 160 children with acute uncomplicated *falciparum* malaria were treated with chloroquine (*n* = 70; 16 in Esake and 54 in Bertoua) or amodiaquine (*n* = 90; 43 in Yaoundé and 47 in Esake). Because of the high rate of treatment failure with chloroquine, the clinical study was terminated prematurely in Esake in accordance with the double-lot quality assurance method. Thirty-nine children (14 in Esake and 25 in Bertoua) either failed to respond to chloroquine therapy or had an ACR with a positive smear on Day 14 (parasitological failure). The other patients had an ACR with negative smears on Day 14. Venous blood samples were obtained with the guardians’ consent from 21 children at the time of clinical or parasitological failure. Venipuncture was not performed in some patients because of difficulties in transporting blood samples to Yaoundé from Bertoua.

Of 21 children responding with clinical or parasitological failure, 11 experienced persistent parasitemia (ETF) and 10 experienced recrudescence (LTF or ACR with positive smear) (Table 1). The recrudescent or persistent parasitemia ranged 0.1–6.5%. Persistent parasitemia until Day 4 was associated with the presence of pretreatment and posttreatment isolates with identical polymorphic patterns defined by csp, msa-1, and msa-2 genes. In 2 persistent isolates on Day 3, an additional band was observed, suggesting the presence of a new population of isolates that was undetected in the pretreatment sample. In one paired sample, there were multiple
bands before treatment, suggesting the presence of multiclonal populations, and an identical size pattern, less one band, was seen in the posttherapeutic sample on Day 4, probably due to the selection of parasite populations.

Similarly, recrudescent parasites obtained on Day 7 or Day 14 from patients responding with LTF or ACR with positive smear had identical polymorphic patterns as the pretreatment isolates, with either an appearance of an additional band or disappearance of a band. Of 21 posttreatment samples from the chloroquine-treated group, 19 yielded interpretable in vitro results (Figure 1). An additional sample from one patient (Patient EPC2) who had positive smears on Days 7 and 14 yielded an interpretable result (a total of 20 data points in Figure 1). All recrudescent isolates from chloroquine-treated patients had elevated chloroquine IC₅₀ values (range, 25.6–115 nM) in posttherapeutic isolates. In 2 patients (1 in Eseka, whose isolates were identical (Type A recrudescence), posttherapeutic isolates were identical to that of pretreatment isolates, with an evidence for selection of parasite populations after amodiaquine therapy (Type B recrudescence). The Day 14 recrudescent isolate from Patient 13 was not characterized because of technical errors, but the Day 34 isolate from the same patient who returned for consultation because of recurrent fever was shown to be different from that of Day 0 isolate. The relationship between the plasma concentrations of monodesethylamodiaquine and amodiaquine and chloroquine IC₅₀ values for recrudescent isolates is illustrated in Figure 2.

In vitro drug sensitivity assay was performed to determine both chloroquine and monodesethylamodiaquine IC₅₀ in 8 of 11 available posttreatment blood samples (a total of 16 data points in Figure 2). In vitro assays were not performed for 3 isolates (isolates 13, 25, and 74) because of insufficient parasitemia (< 0.1%). The IC₅₀ values for amodiaquine ranged 25.6–115 nM in posttherapeutic isolates. In 2 patients with no detectable drug in their plasma, the IC₅₀ values were 59.3 nM and 112 nM. The parent compound, amodiaquine, was not detected in any of the posttreatment plasma samples.

### DISCUSSION

The in vivo and in vitro tests of drug resistance measure different parameters and do not always yield concordant results. One of the reasons for in vivo–in vitro discordance results from the interference of the acquired immune system in patients residing in hyperendemic zones. Because these patients may be able to eliminate residual, drug-resistant parasites after a therapeutic or subtherapeutic dose of an antimalarial drug, treatment is more effective in semi-immune
than in nonimmune patients.\textsuperscript{20,21} Thus, the results of the \textit{in vivo} test conducted in a hyperendemic area may underestimate the degree of “real” drug resistance.

In the present study, this confounding factor was minimized by restricting our study population to children aged < 5 years, in whom a protective antimalarial immunity has not yet been fully developed.\textsuperscript{11} The validity of our assumption that young Cameroonian children aged < 5 years have not acquired immunity is supported by the observation that in older children aged > 5 years and adult residents of Yaoundé, there was no clinical or parasitological failure after

\begin{table}[h]
\centering
\caption{Characteristics of recrudescent malaria after amodiaquine treatment}
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Isolate no. & \textit{In vivo} response\textsuperscript{†} & Day & Parasitemia at time of failure (%) & Genotyping\textsuperscript{‡} \\
\hline

Yaoundé & & & & \\
10 & ACR + PF & 14 & 0.2 & Type B recrudescence \\
13 & ACR + PF & 14 & 0.02 & ND \\
 & & 34 & & Reinfection \\
22 & ACR + PF & 10 & 1.0 & Type B recrudescence \\
25 & ACR + PF & 7 & 0.005 & Type A recrudescence \\
46 & ACR + PF & 14 & 0.1 & ND \\
59 & ACR + PF & 14 & 0.4 & Type B recrudescence \\
61 & ACR + PF & 14 & 0.1 & Type B recrudescence \\
74 & ACR + PF & 14 & 0.2 & Type B recrudescence \\
 & & 14 & 0.04 & Type B recrudescence \\

Eséka & & & & \\
EPC11 & ACR + PF & 19 & 0.3 & Type A recrudescence \\
B02/10 & ACR + PF & 14 & 0.6 & Type A recrudescence \\
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\end{tabular}
\end{table}
amodiaquine therapy, whereas in the present study, 21% of young children residing in Yaoundé had an ACR with positive smear on Day 14 (parasitological failure). Furthermore, we have shown that in the patient population comprising older children aged > 15 years and adults in Yaoundé, the absence of therapeutic failure to amodiaquine therapy was not in agreement with the in vitro drug assay results, which indicated the presence of amodiaquine-resistant parasites (defined as monodesethylamodiaquine IC_{50} > 60 nM) in 18% of the isolates tested between 1994 and 1997.5

Another factor that may lead to discordant results is wide interindividual variations of pharmacokinetics, in particular plasma drug levels.22 In our previous studies, we have not observed any significant difference in the plasma levels of amodiaquine, sulfadoxine, and pyrimethamine between adults and children aged 5–15 years responding with ACR and those with therapeutic failure.4,6,23 Although our data in the present study do not allow comparison between young, nonimmune children with ACR and those with therapeutic failure, it was evident that none of the patients with recrudescence malaria had detectable chloroquine in their plasma on Day 14. In one patient, chloroquine was undetectable on Day 7. In another patient with persistent parasitemia, chloroquine was not detected on Day 3. The elimination half-life of chloroquine has been estimated to be ~ 6–10 days.24 The patients with no detectable plasma chloroquine on Days 3 or 7 may have experienced vomiting that went unreported. The nondetection of chloroquine on Days 7 or 14 may also suggest that these patients had metabolized and eliminated the drug more rapidly than the average patient population. Thus, an insufficient maintenance of therapeutic plasma drug level for at least 3 parasite cycles (> 6 days) may explain recrudescence in these patients.22 In contrast, persistent parasitemia until Days 3 or 4 and the presence of chloroquine in the plasma may be ascribed to drug resistance, even though a minimal plasmatic therapeutic level has not been well defined for chloroquine.

Our experimental work has shown that a brief exposure (up to 30 min) of fresh isolates obtained from Saker-Solomons urine test negative patients to chloroquine or amodiaquine, followed by several thorough washings of the infected erythrocytes just before performing the in vitro drug assay, diminishes the IC_{50} values, as compared with the IC_{50} values obtained with unexposed isolates (Ringwald P, unpublished data). This observation is probably due to a partial inhibition of parasite growth before the performance of drug assays, requiring less amount of drug to kill the parasites in vitro during the 48-hr incubation. By analogy, we may question whether the IC_{50} values of isolates obtained from patients with residual amounts of chloroquine or amodiaquine, from either self-medication or treatment failure, reflect the “real” values. Likewise, the threshold values for drug resistance estimated from the IC_{50} values determined for isolates obtained from tourists with prophylactic failure are not based on solid evidence for resistance.55 In this latter study, the threshold value was estimated by calculating the mean IC_{50} values of various isolates and adding 2 standard deviations. The IC_{50} values determined for monoclonal isolates obtained from patients who have been treated and failed to respond on Days 7 or 14 and in whom drugs are no longer detectable by high-performance liquid chromatography may be an alternative source of data that may provide a basis to establish the threshold resistance value for in vitro resistance. Although the number of isolates that satisfied this criterion is very limited, it may be suggested that on the basis of the present findings, the threshold IC_{50} values for chloroquine and amodiaquine are at least 148 and 59 nM, respectively. More extensive field studies involving a simultaneous analysis of the in vitro resistance and therapeutic failure, as well as plasma drug level measurement and genotyping (the quadruple approach), are required to be able to ascribe cases of treatment failure to drug resistance, and not to confounding factors, such as interindividual pharmacokinetic variations and reinfections, and are required to estimate the threshold level for in vitro resistance.

Other confounding factors, such as intake of other antimalarial drugs during the follow-up period, may also need to be excluded by specific high-performance liquid chromatographic assays designed to detect sulfadoxine-pyrimethamine and quinine in Africa. As an increasing number of advanced African research centers are joining efforts to equip their laboratories and train young scientists, the technical procedures used in this study should become available in the near future to provide more analytical tools to understand the dynamics driving the spread of drug-resistant Plasmodium falciparum in Africa. In the absence of a firmly established resistance gene for chloroquine and amodiaquine, the “quadruple approach” may be one of the best available means to define the epidemiology of chloroquine- and amodiaquine-resistant P. falciparum.

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