MOLECULAR EPIDEMIOLOGY OF MALARIA IN CAMEROON. XIV. PLASMODIUM FALCIPARUM CHLOROQUINE RESISTANCE TRANSPORTER (PFCRT) GENE SEQUENCES OF ISOLATES BEFORE AND AFTER CHLOROQUINE TREATMENT

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Abstract. Laboratory studies have strongly suggested that the gene coding for Plasmodium falciparum chloroquine resistance transporter (PFCRT) may play a determinant role in chloroquine resistance. A clinical study in Mali also found evidence for selection of the key PFCRT amino acid substitution, Lys76Thr, in patients who fail to respond to chloroquine treatment. To test the hypothesis that in vivo selection of mutant PFCRT alleles occurs after chloroquine treatment, PFCRT and merozoite surface antigen 2 (msa-2) polymorphisms were compared between 61 pretreatment and posttreatment paired samples from children with either clinical or parasitologic failure. There were six wild-type PFCRT alleles, 44 mutant alleles, and 11 mixed alleles among pretreatment isolates. All posttreatment parasites had mutant PFCRT alleles. Recrudescence accounted for 42 of 61 posttreatment infections, while 19 posttreatment infections were due to new infection (including all isolates with Lys-76 before treatment and Thr-76 after treatment). Seven pretreatment isolates with mixed PFCRT alleles had only Thr-76 on recrudescence, providing direct evidence for in vivo selection for mutant PFCRT. Although the presence of mutant PFCRT alleles in pretreatment isolates is not predictive of chloroquine treatment failure, our data support the hypothesis that in vivo selection for recrudescence parasites carrying mutant PFCRT alleles occurs. These results may have important implications for the future surveillance of chloroquine resistance by the use of molecular markers.

INTRODUCTION

Chloroquine is still the first drug of choice for the treatment of acute uncomplicated malaria in many countries in West and Central Africa. In these endemic regions, chloroquine is also prescribed for the chemoprophylaxis of pregnant women. The drug remains highly effective to treat Plasmodium ovale and Plasmodium malariae infections, but has lost its high efficacy against P. falciparum infections. The prevalence rate of chloroquine-resistant P. falciparum is currently being assessed in various parts of the African continent. In Cameroon, recent cumulative data from various sentinel sites throughout the country have shown the rate of chloroquine therapeutic failure > 38% (up to 67% in the littoral region) in southern, central, western, and north-western Cameroon, 27% in the eastern region, and 13–25% in the North.1 Within this context, chloroquine can no longer be recommended for the first-line treatment of uncomplicated falciparum malaria in some regions of the country, and the Cameroonian Ministry of Public Health is advocating the use of amodiaquine as an alternative first-line drug (with sulfadoxine-pyrimethamine as the second-line drug).

The current gold standard for the evaluation of drug-resistant malaria in sub-Saharan Africa is the in vivo test for therapeutic efficacy standardized by the World Health Organization (WHO).2 Clinical assessment of the efficacy of chloroquine and other commonly used antimalarial drugs provides useful data to guide the national drug policy and malaria control programs. However, in vivo response to drug treatment is partly dependent on host factors, including the level of acquired immunity and pharmacokinetic variations. The clinical judgment of therapeutic failure may not necessarily mean the presence of drug-resistant parasites since the persistence or recrudescence of parasitemia may be attributable to causes that are independent of the parasites, such as poor compliance, unreported vomiting, inadequate absorption or biotransformation into biologically active metabolites, and re-infection.

A promising alternative test to assess drug resistance is based on the molecular analysis of resistance genes. Molecular techniques are not only more convenient and rapid to perform on a large number of blood samples in a moderately equipped laboratory, but also yield objective results that are unaffected by various host factors. Point mutations in a newly discovered P. falciparum chloroquine-resistance transporter (PFCRT) gene have been directly associated with in vitro chloroquine-resistant phenotype in laboratory-adapted reference clones.3 More importantly for public health, the key Lys76Thr amino acid substitution was suggested to be a genetic marker associated with in vivo resistance to chloroquine in a clinical study conducted in Mali, where malaria transmission is seasonal.4 In that study, the presence of the key PFCRT mutation before treatment was not predictive of therapeutic failure. However, persistent or recrudescent isolates after treatment were all characterized as PFCRT mutants, providing an indirect evidence for selection of mutant parasites after chloroquine treatment. The aim of the present study was to further evaluate whether selection of mutant PFCRT occurs after chloroquine treatment by comparing paired samples of pre- and posttreatment isolates from symptomatic children treated with chloroquine and assess the importance of the key PFCRT mutation for therapeutic failure within a different epidemiologic context from that of Mali.

PATIENTS AND METHODS

Patients. Symptomatic children aged < 5 years old (< 9 years old in Maroua, a low transmission area) were recruited with informed consent of the accompanying parent or guardian if the following clinical and parasitologic criteria set by the WHO were met: monoinfection with P. falciparum, with parasitemia between 2,000 and 100,000 asexual parasites/μL of
blood, fever, and hematocrit > 15%. Patients with severe malnutrition, clinically evident signs of concomitant febrile diseases, and signs and symptoms of severe and complicated malaria were excluded. The history of recent self-medication with an antimalarial drug was not an exclusion criteria. This study was approved by the Cameroonian Ministry of Public Health and Cameroonian National Ethics Committee.

**Study sites.** Clinical evaluation of chloroquine efficacy was performed during 2000–2001 in study sites where no recent *in vivo* data were available. The detailed outcome of these clinical studies will be published elsewhere. Dispensaries and/or district hospitals were selected from the following localities in Cameroon: Maroua, Ndop, Bafoussam, and Hévécam. Maroua is an urban site in the savannah region in northern Cameroon where malaria transmission is seasonal (approximately September–November). Ndop and Bafoussam are rural and urban sites in northwestern Cameroon, respectively. Hévécam is a rural area in the littoral region. Malaria transmission is intense and occurs throughout the year in Ndop, Bafoussam, and Hévécam, which lie within the southern belt of tropical rain forest.

**Test for therapeutic efficacy.** The WHO protocol for clinical evaluation of antimalarial drug efficacy was described in detail in our previous study. Briefly, each dose of chloroquine (25 mg/kg body weight in three [10 mg/kg, 10 mg/kg, and 5 mg/kg] divided daily doses) was administered under supervision, and patients were observed for at least 30 min for eventual vomiting. All patients were followed by home visits. Clinical and parasitologic responses were evaluated on days 3, 7, and 14 (and any other day in case of clinical aggravation).

In the present study, blood samples were analyzed only if the patient failed to clear parasitemia during the follow-up period beyond day 3. Late treatment failure is defined as either a positive smear and signs and symptoms of severe malaria between day 4 and 14 or positive smear and fever (> 37.5°C axillary temperature) between day 4 and 14. Adequate clinical response with parasitologic failure is defined as positive smear and afebrile on day 14, without previously meeting the criteria of treatment failure. Children responding to treatment failure or adequate clinical response and parasitologic failure on day 14 were treated with oral quinine (25 mg base/kg body weight/day in three divided doses for 5 days).

**Polymerase chain reaction and sequencing.** The nested polymerase chain reaction (PCR) protocols for amplifying a fragment of *PFCRT* spanning the key codon were described in earlier studies. The secondary amplified product was purified by using the High-Pure PCR purification kit (Roche Diagnostics; Meylan, France) and marked with fluorescent nucleotides. The extension product was sequenced by ABI Prism automated DNA sequencer (Perkin Elmer; Les Ulis, France). To determine whether the patient had recrudescent parasites or new infections, merozoite surface antigen 2 (*msa-2*) size polymorphisms were compared between the pre- and posttreatment samples by agarose gel electrophoresis.

**Data interpretation.** The wild-type *PFCRT* was defined as the presence of the following sequences: Cys-72, Met-74, Asn-75, and Lys-76. Mutant *PFCRT* was defined as the presence of Cys-72, Ile-74, Glu-75, and Thr-76. The presence of both alleles in at least one codon denoted mixed infection. Based on the comparison of *msa-2* polymorphisms, persistent or recrudescent parasites after chloroquine treatment were classified as follows: type A recrudescent consisting of identical band size on agarose gel (either one band suggesting infections due to a single parasite population or two or more bands suggesting multiple infections); type B recrudescent, defined as a multiple infection before treatment, indicated by two or more bands and the persistence of a subset of alleles (multiple or single) after treatment; type C recrudescent, defined as the presence of identical bands (single or multiple) in the pre- and posttreatment samples, with an additional band appearing after treatment; and reinfection, defined as the presence of different allele(s).

The comparison of pre- and posttreatment *P. falciparum* isolates may present several possibilities. Infection with isolates carrying wild-type *PFCRT* both before and after chloroquine treatment administered under supervision possibly implies that (i) reinfection occurred, (ii) drug absorption was inadequate, or (iii) *PFCRT* is not involved or is not the only gene involved in chloroquine resistance and may thus be an evidence against the role of *PFCRT* in chloroquine resistance. Wild-type *PFCRT* before treatment and mutant *PFCRT* in recrudescent sample may mean (i) reinfection or (ii) selection (assuming that mixed parasite population was undetected in pre-treatment sample). Mutant *PFCRT* before and after chloroquine treatment may or may not explain *in vivo* therapeutic failure, but is in favor of the possible involvement of *PFCRT* in drug resistance. The presence of either wild-type or mutant *PFCRT* before treatment and mixed alleles at recrudescence possibly implies reinfection or appearance of a minor parasite population undetected in day 0 sample and is also an evidence against the role of *PFCRT* in chloroquine resistance. Mixed infection before treatment and selection of mutant parasites at recrudescence seem to be the best available evidence for the possible role of *PFCRT* in *in vivo* chloroquine resistance. These interpretations may be subject to error in individual patients because of various intervening host factors but may be useful to assess the possible role of *PFCRT* in determining *in vivo* chloroquine resistance if there is a sufficient number of observations.

**RESULTS**

Sixty-one paired samples from patients with either late treatment failure (between day 4 and 14) or adequate clinical response with parasitologic failure (on day 14) were available for molecular analysis: 3 from Maroua, 38 from Ndop, 5 from Bafoussam, and 15 from Hévécam. The limited number of samples from Maroua was due to the relatively high efficacy of chloroquine in the sahelian North. In Bafoussam and Hévécam, the clinical study with chloroquine was terminated prematurely according to the double lot quality assurance sampling method, because of the high proportion of early or late treatment failure. Nineteen posttreatment samples were obtained between day 7 and day 12, and 42 samples were obtained on day 14.

Of 61 pretreatment samples, 6 had wild-type *PFCRT* alleles, 44 carried mutant alleles, and 11 were mixed (Table 1). All 61 posttreatment isolates were characterized to be mutant, with no mixed alleles. No other combination of *PFCRT* alleles at codons 72, 74, 75, and 76 was observed among 122 pre- and posttreatment samples. There was no nucleotide variation in 40 codons flanking codons 72–76. The analysis of *msa-2* polymorphism showed that 35 paired samples were
identical before and after treatment (type A recrudescence), four recrudescent samples had at least one missing band (type B recrudescence), three recrudescent samples had one additional band (type C recrudescence), and 19 paired samples had different patterns (reinfection). Among 44 samples with mutant Thr-76 before and after treatment, 35 were recrudescence (5 type A and 2 type B), and four were due to reinfection. Six isolates with the wild-type Lys-76 on day 0 and mutant Thr-76 in recrudescent parasites were all associated with reinfection.

**DISCUSSION**

The initial clinical study in Mali has shown that the presence of isolates with mutant *PFCRT* allele does not predict chloroquine treatment failure, but the absence of wild-type isolates in any of the recrudescent samples suggested a possible role of *PFCRT* in clinical resistance. The observation that infection with isolates carrying mutant *PFCRT* before treatment does not predict therapeutic failure was confirmed in other studies. To test the hypothesis that *PFCRT* mutants may be selected during chloroquine therapy, we have analyzed the genetic characteristics of pre- and posttreatment paired isolates. Our results suggest that *PFCRT* may be involved in in vivo resistance to chloroquine. As in the Malian study, all recrudescent isolates in our study carried the mutant Thr-76 allele. Among isolates with mixed *PFCRT* before treatment and Thr-76 after treatment, some provided evidence that the underlying mechanism that explains this phenomenon may be selection, i.e., diminution in the number of parasite populations, as determined by msa-2 polymorphisms, after treatment. Furthermore, mixed *PFCRT* before treatment was always replaced by pure Thr-76 allele in case of reinfection. All new infections occurring on day 14 in patients infected with isolates carrying Thr-76 on day 0 were due to mutant isolates. Likewise, pretreatment Lys-76 to posttreatment Thr-76 switch in day 14 isolates was explained by reinfection, and not selection of parasite populations. Taken together, these observations support the hypothesis that wild-type isolates may be selected against and/or may be unable to infect patients with therapeutic or subtherapeutic plasma level of chloroquine during 14 days after treatment.

The data in our study were obtained by direct sequencing of PCR products. Although DNA sequencing may not always detect mixed alleles, especially when one of the alleles is represented by a minor parasite population, our results are similar to those of the Malian study, in which either nested PCR or restriction fragment length polymorphism was performed to analyze the key *PFCRT* codon. The proportion of Malian isolates attributable to true recrudescence and reinfection was not studied. Furthermore, the obvious difference between the Sahelian climate in Mali (Maroua, Cameroon) and tropical rain forest in the southern half of Cameroon does not allow a direct comparison of the epidemiology of drug-resistant malaria and malaria transmission in these two countries. Nonetheless, our data on 61 Cameroonian isolates are in agreement with those of Djimde et al. (*N* = 60 Malian isolates) in that recrudescent isolates obtained after chloroquine treatment are characterized to have mutant *PFCRT* alleles, without exception. In Yaoundé, Cameroon, 42% (*N* = 111) of randomly selected pretreatment isolates had wild-type *PFCRT* allele; in Mali, 59% (*N* = 116) of samples before treatment were of wild-type. If *PFCRT* were not involved at all in clinical chloroquine resistance, wild-type *PFCRT* allele should have been randomly distributed to about one-half of the recrudescent parasites in Malian and Cameroonian studies, which was not the case.

Further clinical studies will be necessary to define the usefulness of *PFCRT* as a molecular marker for chloroquine resistance and identify host factors that may influence the clinical outcome to drug treatment and explain why certain pa-
tients infected with parasites carrying PFCRT mutations are able to clear parasitemia. Although acquired immunity may intervene, Cameroonian and Malian studies were conducted in young children in whom protective immunity is not expected to be totally acquired, in particular in Mali and northern Cameroon where malaria transmission is seasonal and in young children aged < 5 years old. Despite the inability of PFCRT mutations to predict clinical response to chloroquine, there is some evidence for the selection of recrudescent parasites carrying mutant PFCRT alleles. Furthermore, experimental and field data have provided a strong evidence that PFCRT is the best available candidate gene, or at least one of the candidate genes, associated with chloroquine-resistant phenotype.

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