MOLECULAR EPIDEMIOLOGY OF MALARIA IN CAMEROON. XII. IN VITRO DRUG ASSAYS AND MOLECULAR SURVEILLANCE OF CHLOROQUINE AND PROGUANIL RESISTANCE

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Abstract. Chloroquine-proguanil combination is one of the options for chemoprophylaxis. The rapid evolution of drug resistance status requires a constant upgrade of epidemiologic data. Due to various difficulties in conducting prospective clinical studies on the prophylactic efficacy of the drug combination, especially in highly chloroquine-resistant zones, in vitro drug sensitivity assays and specific molecular markers for chloroquine (Plasmodium falciparum chloroquine-resistance transporter, pfcr) and cycloguanil (a biologically active metabolite of proguanil; dihydrofolate reductase, dhfr) resistance were evaluated as an alternative approach in this study. Of 116 isolates, 62 (53.4%) were doubly resistant in vitro to chloroquine (IC50 ≥ 100 nM) and cycloguanil (IC50 ≥ 15 nM). Likewise, 62 of 118 isolates (52.5%) carried both the mutant Thr-76 pfcr allele and at least one dhfr mutant allele (1 with a single Asn-108 allele, 8 with double Arg-59 and Asn-108 mutations, and 53 with triple Ile-51, Arg-59, and Asn-108 mutations). The in vitro drug response corresponded with the presence or absence of key mutation(s) in the pfcr and dhfr genes. These results suggest the high proportion of P. falciparum isolates in southern Cameroon that may not respond to chloroquine-proguanil combination.

INTRODUCTION

One of the strategies for malaria control involves the use of chemoprophylaxis for individuals exposed to high risk, including nonimmune travelers from nonendemic regions and pregnant women residing in endemic regions. Drugs recommended for antimalarial prophylaxis depend on several factors, such as the degree of risk, level of chloroquine resistance, tolerance, physiologic conditions, and age. In sub-Saharan Africa, the World Health Organization currently recommends mefloquine as the first drug of choice, followed by doxycycline (second choice), and chloroquine-proguanil combination (third choice) for nonpregnant, nonimmune travelers from nonendemic regions and chemoprophylaxis for individuals exposed to high risk, in-10cluding nonimmune travelers from nonendemic regions and pregnant women residing in endemic regions. Drugs recommended for antimalarial prophylaxis depend on several factors, such as the degree of risk, level of chloroquine resistance, tolerance, physiologic conditions, and age. In sub-Saharan Africa, the World Health Organization currently recommends mefloquine as the first drug of choice, followed by doxycycline (second choice), and chloroquine-proguanil combination (third choice) for nonpregnant, nonimmune temporary visitors.1

The use of chloroquine alone for chemoprophylaxis of nonimmune subjects traveling to sub-Saharan Africa has been seriously compromised in the late 1980s and in the 1990s due to the rapid spread of chloroquine-resistant Plasmodium falciparum. At about the same period, the use of other alternative drugs for prophylaxis, such as sulfadoxine-pyrimethamine and amodiaquine, had been discontinued because of rare but potentially fatal adverse effects.2 In this context, chloroquine-proguanil combination appeared to be a safe, well tolerated, and effective option and was officially recommended for prophylaxis of temporary visitors, as well as for pregnant women residing in endemic areas, in the 1990s. Most studies on the efficacy of the chloroquine-proguanil combination have been based on retrospective surveys or case reports of malaria infections in nonimmune subjects under prophylaxis.3–5 Few prospective studies have been undertaken, which have generally shown a relatively good protection.6,7

Because prospective clinical studies on the current prophylactic efficacy of chloroquine-proguanil are time consuming, costly, and possibly unjustified in some highly chloroquine-resistant areas, alternative methods may be of value to monitor and assess the utility of this drug combination in sub-Saharan Africa. These methods include in vitro drug sensitivity assays and molecular analysis of drug-resistance genes. Previous studies have established the high correlation between the in vitro response to chloroquine and cycloguanil (biologically active metabolite of proguanil) and P. falciparum chloroquine-resistance transporter (pfcr) and dihydrofolate reductase (dhfr) gene mutations, respectively, both in reference clones and field isolates.8–13 Likewise, the key pfcr mutation at codon 76 has been associated with clinical resistance to chloroquine.14 The correlation between dhfr mutations and clinical resistance to proguanil has not been established. In the present study, the efficacy of chloroquine-proguanil combination was indirectly evaluated by an alternative approach based on in vitro drug sensitivity assays and molecular markers with the aim to describe the current trend of drug efficacy in southern Cameroon.

MATERIALS AND METHODS

Patients. After informed consent, venous blood samples were obtained in 2000–2001 from symptomatic children aged >12 years old and adults consulting at the Nlongkak Catholic missionary dispensary if the following criteria were met: the presence of P. falciparum, without other Plasmodium species, at a parasitemia ≥ 0.1%, signs and symptoms of acute uncomplicated malaria, and denial of recent self-medication with an antimalarial drug, confirmed by negative Saker Solomon’s urine test.15 Pregnant women and patients with signs and symptoms of severe and complicated malaria were excluded from the study. All enrolled patients were residents of Yaoundé. The patients were treated with oral amodiaquine and followed by the dispensary staff. This study was approved by the Cameroonian National Ethics Committee and Cameroonian Ministry of Public Health.

In vitro drug sensitivity assay. Chloroquine sulfate and cycloguanil base were provided by Aventis (Antony, France) and AstraZeneca (Rueil-Malmaison, France), respectively. Stock solutions and working solutions were prepared in sterile distilled water. The final concentrations ranged from 25–
1,600 nM for chloroquine (two-fold dilutions in triplicate) and 0.0488–51,200 nM (four-fold dilutions in duplicate) for cycloguanil.

Blood samples were washed with para-aminobenzoic acid (PABA) and folic acid-free RPMI 1640 culture medium (designated NPNF RPMI medium hereafter) three times by centrifugation within 2 to 3 hr after blood extraction. Infected erythrocytes were resuspended in NPNF RPMI medium and standard RPMI medium containing 1 mg/L PABA and 1 mg/L folic acid (both media contained 25 mM HEPES and 25 mM NaHCO₃ buffers) to determine the in vitro activity of cycloguanil and chloroquine, respectively. Culture media were supplemented with 10% human serum. Technical procedures of the isotopic microtest and determination of 50% inhibitory concentrations (IC₅₀), defined as the concentration at which 50% of the parasite growth is inhibited as compared with drug-free control wells, were described in detail previously.¹¹

**Sequence analysis.** Genomic DNA was extracted from 2 mL of red cell pellet, as described previously.¹¹ The region spanning the key pfcrt codon was amplified by polymerase chain reaction, followed by restriction fragment length polymorphism using Apo I endonuclease.¹³,¹⁴ The entire DHFR domain was amplified by polymerase chain reaction, followed by purification of the amplified product, fluorescent labeling, and automatic sequencing (ABI Systems, Perkin Elmer, Les Ulis, France). These procedures were detailed in our previous study.¹¹,¹⁶

**Data interpretation.** The threshold IC₅₀ value for chloroquine resistance was fixed at ≥ 100 nM.¹⁷,¹⁸ This cut-off value is based on the determination of in vitro chloroquine response of African field isolates obtained from malaria-infected, non-immune individuals under chloroquine prophylaxis and semi-immune patients failing to respond to chloroquine treatment. The threshold IC₅₀ for cycloguanil resistance is still undetermined. Although previous studies have arbitrarily fixed the threshold at ≥ 50 nM, more recent studies using the isotopic microtest have suggested that it may lie between 10 and 50 nM.¹¹ Since a reliable molecular marker for cycloguanil resistance exists, an inverse approach was applied to determine the approximate threshold value based on the distribution of cycloguanil IC₅₀ values in respect to the presence of dhfr mutations. Thus, an IC₅₀ value that distinguishes between parasites with wild-type and mutant dhfr was used as the threshold value for in vitro cycloguanil resistance. The in vitro response to chloroquine and cycloguanil was expressed as the geometric mean IC₅₀ and 95% confidence interval.

Wild-type pfcrt and dhfr were defined as DNA sequences that characterize reference clones of *P. falciparum*.⁸,⁹,¹² Mutant pfcrt denotes the Lys-to-Thr amino acid substitution at position 76. Mutant dhfr denotes the presence of at least one amino acid substitution at position 108 (Ser-to-Asn), with possibly two additional substitutions, Asn51Ile and Cys59Arg. Other dhfr mutations at positions 16, 50, 108 (Ser-to-Thr), and 164 have not been observed in Cameroonian isolates.

**Figure 1.** Distribution of chloroquine IC₅₀ values in relation to the key pfcrt codon (K, Lys; T, Thr) at position 76. K76 and T76 are wild-type and mutant alleles, respectively.¹² The hypothetical threshold value (100 nM) for in vitro chloroquine resistance is indicated by the dotted line.
RESULTS

A total of 118 samples were analyzed for \textit{in vitro} response to chloroquine and cycloguanil, and the corresponding DNA sequences of \textit{pfcrt} and \textit{dhfr} were determined. Two drug assays for chloroquine were uninterpretable due to bacterial contamination of the RPMI 1640 medium. The \textit{in vitro} response of these 2 isolates to cycloguanil was available because a different culture medium is used to determine the IC$_{50}$ for cycloguanil. Based on the threshold IC$_{50}$ of $\geq 100$ nM, 49 isolates (42\%) were chloroquine-sensitive (geometric mean, 34.5 nM; 95\% confidence interval, 30.5–38.9 nM; range, 18.6–90.0 nM), and 67 (58\%) were chloroquine-resistant (geometric mean, 209 nM; 95\% confidence interval, 189–231 nM; range, 102–714 nM). Of 118 isolates, 36 (31\%) carried the wild-type Lys-76 \textit{pfcrt} allele, and 68 (58\%) carried the mutant Thr-76 \textit{pfcrt} allele. Fourteen isolates (12\%) had mixed Lys + Thr-76 alleles. The threshold value of 100 nM distinguished between isolates with wild-type and those with mutant \textit{pfcrt} in a large majority of isolates, as illustrated in Figure 1.

The distribution of cycloguanil IC$_{50}$ values in relation to the \textit{dhfr} profiles is presented in Figure 2. Ten isolates had wild-type \textit{dhfr} with no mutation. Of the remaining isolates, 94 were \textit{dhfr} mutants with at least Ser108Asn substitution (two possible additional amino acid substitutions at positions 51 and/ or 59), and 14 had mixed \textit{dhfr} alleles. No new \textit{dhfr} mutation was identified among the isolates. The highest cycloguanil IC$_{50}$ among wild-type isolates and the lowest IC$_{50}$ value among mutants, excluding isolates with mixed \textit{dhfr} alleles, were 9.14 nM and 15.3 nM, respectively. This observation led us to suppose that 15 nM may be the threshold value for \textit{in vitro} cycloguanil resistance in our laboratory. Using this criterion, 18 isolates (10 wild-type and 8 with mixed \textit{dhfr} alleles) were cycloguanil-sensitive (geometric mean, 1.30 nM; 95\% CI, 0.752–2.26 nM; range, 0.249–9.14 nM), and 100 (94 with at least one mutant \textit{dhfr} allele + 6 with mixed \textit{dhfr} alleles) were cycloguanil resistant (geometric mean, 77.1 nM; 95\% CI, 65.0–91.6 nM; range, 15.3–901 nM). Overall, 54 isolates (47\%) were sensitive \textit{in vitro} to at least one of the compounds (Table 1). The analysis of isolates with different combinations of \textit{pfcrt} and \textit{dhfr} alleles showed that 32 isolates (27\%) were of wild-type in either, or both, of the two resistance genes and 62 isolates (53\%) were double mutants (Table 2).

DISCUSSION

The present study indicates that various technical and operational difficulties involved in conducting prospective clinical studies on the prophylactic efficacy of chloroquine-proguanil combination may be partially overcome by resorting to an alternative method to assess the current trend of chloroquine and cycloguanil resistance in an endemic region. Although we must bear in mind that no \textit{in vitro} or molecular methods can entirely substitute for \textit{in vivo} studies at present, and that these results cannot be directly extrapolated to pre-
dict in vivo response, especially on an individual basis, pragmatic considerations lead us to explore indirect methods that may yield relevant information on the epidemiology of drug-resistant malaria. Some of the host factors that are not taken into account by in vitro methods and therefore preclude an accurate prediction of in vivo protective efficacy of the drug combination include wide interindividual variation in pharmacokinetics, pharmacogenetic regulation of hepatic biotransformation of proguanil into its biologically active metabolite, cycloguanil, resulting in a significantly low plasma cycloguanil level in poor metabolizers (proguanil itself has a low blood schizontocidal activity), and tissue schizontocidal action of cycloguanil.19–21

In spite of these inherent limitations of in vitro approaches, the data presented in this study provide useful information on the drug-resistant status of malaria parasites. The isotopic microtest assay can accurately distinguish between sensitive and resistant isolates. The in vitro response to chloroquine can predict the presence of mutant Thr-76 pfcr allele on the basis of the threshold value of 100 nM, and vice versa, in a large majority of isolates. A major confounding factor in testing field isolates by in vitro drug assay seemed to be the presence of multiple parasite populations within an isolate. The in vitro chloroquine response of mixed populations with both wild-type and mutant pfcr cannot be predicted accurately, possibly due to the varying extent of predominance of each population in a given isolate and biologic fitness of each population to multiply under in vitro conditions. In the present study, 12% of the samples had detectable mixed populations with Lys- and Thr-76 pfcr alleles. Using a more polymorphic genetic marker that allows a detailed analysis of the parasite population structure, we have observed a much higher level of mixed P. falciparum infections (57%) among symptomatic patients in Yaoundé.22 Although the presence of mixed parasite populations may lower the correlation between in vitro drug response and pfcr, the latter may serve as a useful molecular marker within the context of an epidemiologic study, in which minor deviations are corrected due to a large sample size.

Comparative studies between clinical response to sulfadoxine-pyrimethamine and dhfr mutations have suggested that a single Ser108Asn mutation does not lead to therapeutic failure and that three mutations, Ile-51, Arg-59, and Asn-108, may be required for clinical resistance to sulfadoxine-pyrimethamine.23–25 Whether this observation is due to the synergistic action of the drug combination, the secondary role played by mutations in the molecular target of sulfadoxine, dihydropteroate synthase, or modification in the transport process of sulfadoxine into the parasites is still being debated. It is not known at present how many dhfr mutations are required to lead to proguanil prophylactic failure in nonimmune subjects. However, if we assume that a single Lys76Thr pfcr mutation and at least one dhfr mutation (Ser108Asn) and/or chloroquine IC50 ≥ 100 nM and cycloguanil IC50 ≥ 15 nM are associated with chloroquine-proguanil prophylactic failure, our results suggest an insufficient protection (about 53%) against the erythrocytic stage of P. falciparum isolates in Yaoundé. Even if we adopt a more stringent theoretical requirement for prophylactic failure by assuming a single Lys76Thr pfcr mutation and triple dhfr mutations, 53 of 118 isolates (45%) would still be expected to display double resistance to the drug combination. These considerations have been partially confirmed by our clinical observation of several cases of prophylactic failure in expatriates residing in Yaoundé, despite the allegation of strict compliance with the daily dose of chloroquine (100 mg) and proguanil (200 mg).

Our results suggest that a high proportion (> 50%) of P. falciparum isolates in Yaoundé is resistant to both chloroquine and cycloguanil, as indicated by in vitro drug sensitivity assays and specific molecular markers. The high level of chloroquine resistance is supported by clinical studies in Yaoundé that have shown a parasitologic failure rate of 49% on day 14 and concluded that chloroquine can no longer be recommended for local populations with acute uncomplicated falciparum malaria.26 This conclusion may be extended to the entire southern and central Cameroon where our recent clinical and molecular data have shown a high prevalence of in vivo chloroquine resistance and triple dhfr mutations.27 Taken together, these data suggest the probable inadequate efficacy of chloroquine-proguanil combination in approximately one half of individuals exposed to infective mosquito bites in southern Cameroon.

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REFERENCES

2. World Health Organization, 1990. Practical chemotherapy of ma-

TABLE 1
Response to chloroquine and cycloguanil determined by pfcr and dhfr sequences

<table>
<thead>
<tr>
<th>Chloroquine response</th>
<th>Cytoxan response*</th>
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<tbody>
<tr>
<td></td>
<td>Sensitive (IC50 &lt; 15 nM)</td>
</tr>
<tr>
<td>Sensitive (IC50 &lt; 100 nM)</td>
<td>13 (11.2%)</td>
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<tr>
<td>Resistant (IC50 ≥ 100 nM)</td>
<td>5 (4.3%)</td>
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</table>

*Results are expressed as the number of Plasmodium falciparum isolates (%). Of 22 Thr-76 pfcr and mutant dhfr, there were 1 single mutant (Asn-108), 2 double mutants (Arg-59 + Asn-108), and 19 triple mutants (Ile-51 + Arg-59 + Asn-108). Of 62 Thr-76, there were 1 single mutant, 8 double mutants, and 53 triple mutants.

TABLE 2

<table>
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<tr>
<th>dhfr allele*</th>
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<tbody>
<tr>
<td>Pfcrt allele</td>
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<td>-------------</td>
</tr>
<tr>
<td>Wild-type Lys-76</td>
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<tr>
<td>Mutant allele Thr-76</td>
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<tr>
<td>Mixed Lys/Thr-76</td>
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*Results are expressed as the number of Plasmodium falciparum isolates (%). Of 22 Thr-76 pfcr and mutant dhfr, there were 1 single mutant (Asn-108), 2 double mutants (Arg-59 + Asn-108), and 19 triple mutants (Ile-51 + Arg-59 + Asn-108). Of 62 Thr-76, there were 1 single mutant, 8 double mutants, and 53 triple mutants.
CHLOROQUINE-PROGUANIL RESISTANCE

387


