Am. J. Trop. Med. Hyg., 62(2), 2000, pp. 271–276

MOLECULAR EPIDEMIOLOGY OF MALARIA IN YAOUNDE, CAMEROON. VI. SEQUENCE VARIATIONS IN THE *PLASMODIUM FALCIPARUM* DIHYDROFOLATE REDUCTASE–THYMIDYLATE SYNTHASE GENE AND *IN VITRO* RESISTANCE TO PYRIMETHAMINE AND CYCLOGUANIL

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Abstract. Pyrimethamine and cycloguanil, the major human metabolite of proguanil, are inhibitors of dihydrofolate reductase that play a key role in the treatment and prevention of chloroquine-resistant *Plasmodium falciparum* infections in sub-Saharan Africa. Resistance to these antifolate drugs has emerged in some areas of Africa. Earlier molecular studies have demonstrated that point mutations at key positions of the dihydrofolate reductase–thymidylate synthase gene are strongly associated with antifolate resistance. However, whether the same or distinct mutations are involved in the development of resistance to both pyrimethamine and cycloguanil has not been well established in naturally occurring *P. falciparum* isolates. In this study, the *in vitro* responses to both antifolate drugs were measured in 42 Cameroonian isolates and compared with the complete sequence of the dihydrofolate reductase domain of the gene (from 34 of 42 isolates) to analyze the genotype that may distinguish between pyrimethamine and cycloguanil resistance. The wild-type profile (n = 11 isolates) was associated with low 50% inhibitory concentrations (IC_{50}s) ranging from 0.32 to 21.4 nanamole for pyrimethamine and 0.60–6.40 nM for cycloguanil. Mutant isolates had at least one amino acid substitution, Asn-108. Only three mutant codons were observed among the antifolate-resistant isolates: Ile-51, Arg-59, and Asn-108. The increasing number of point mutations was associated with an increasing level of pyrimethamine IC_{50} and, to a much lesser extent, cycloguanil IC_{50}. These results support a partial cross-resistance between pyrimethamine and cycloguanil that is based on similar amino acid substitutions in dihydrofolate reductase and suggest that two or three mutations, including at least Asn-108, may be necessary for cycloguanil resistance, whereas a single Asn-108 mutation is sufficient for pyrimethamine resistance.

Pyrimethamine and proguanil are dihydrofolate reductase (DHFR) inhibitors that continue to play a major role in the chemotherapy of chloroquine-resistant *Plasmodium falciparum*.[1] Initially used as a monotherapy in the 1950s and 1960s, both of these DHFR inhibitors are currently used in combination with another antimalarial drug to delay the emergence of resistant strains and/or to enhance their specific activity against the malaria parasites.[2–4] Sulfadoxine-pyrimethamine is indicated for the treatment of acute, uncomplicated malarial attacks due to chloroquine-resistant *P. falciparum* infections in areas where this drug combination remains generally effective, such as in most of the African continent. Proguanil, in combination with chloroquine, is recommended for chemoprophylaxis in nonimmune individuals traveling to certain areas where malaria is endemic and in pregnant women residing in malaria-endemic zones.[5] Proguanil is also used for the treatment of multidrug-resistant *P. falciparum* infections in combination with a new antiprotozoan drug, atovaquone.[6,7]

Pyrimethamine and cycloguanil, the biologically active human metabolite of proguanil, share similar chemical structures and inhibit the same molecular target.[8,9] These two features suggest a potential for cross-resistance between the drugs. However, earlier *in vivo* and *in vitro* studies have drawn contradictory conclusions.[2] Another approach based on the analysis of the genetic mechanism of antifolate resistance may help resolve this question. Comparison of the *P. falciparum* dihydrofolate reductase–thymidylate synthase (*dhfr-ts*) gene sequences from several reference clones has suggested genetic profiles that are associated with pyrimethamine resistance and cycloguanil resistance.[10,11] According to these studies, a Ser-to-Asn substitution at position 108 confers resistance to pyrimethamine, while a Ser-to-Thr substitution at position 108, associated with a Ala-to-Val substitution at position 16, confers resistance to cycloguanil. Ancillary mutations at positions 51, 59, and/or 164 are associated with elevated levels of antifolate resistance.

These molecular criteria for antifolate resistance have not been fully confirmed in naturally occurring isolates of *P. falciparum*. In our previous studies on Cameroonian isolates, the key *dhfr* codon was compared with the phenotype defined by the *in vitro* sensitivity or resistance to pyrimethamine.[12] In the present study, the *in vitro* responses to both pyrimethamine and cycloguanil were determined for clinical isolates obtained in Cameroon and the DHFR domain of the *dhfr-ts* gene was fully sequenced, with the aim to establish whether there is a particular genotype that defines pyrimethamine and cycloguanil resistance.

PATIENTS, MATERIALS, AND METHODS

Patients. Most of the patients participated in the clinical trials conducted in Yaounde, Cameroon between 1996 and 1998. Inclusion criteria included an age ≥5 years old, fever at consultation (or history of fever within the past 24 hr), a monoinfection with *P. falciparum* based on the microscopic examination of Giemsa-stained thin and thick blood smears, a parasite density >5,000 asexual parasites/μl of blood, and no recent history of self-medication with antimalarial drugs, as confirmed by a negative Saker-Solomons urine test result.[13] Patients with signs and symptoms of severe and complicated malaria, as defined by the World Health Organization,[14] were excluded. Depending on the clinical conditions, the patients were treated with the first-line (amodiaquine),

second-line (sulfadoxine-pyrimethamine), or third-line drug (quinine) used in Cameroon. Informed consent was obtained from the patient or the patient's guardian in the case of children. The study was approved by the Cameroonian National Ethics Committee and the Cameroonian Ministry of Public Health.

Parasite DNA. Forty-two clinical isolates of *P. falciparum* were obtained by venipuncture before treatment. Venous blood samples (5–10 ml of whole blood) were collected in a tube coated with an anticoagulant (EDTA) (Vacutainer; Terumo Europe NV, Leuven, Belgium) and washed three times in folate- and *p*-aminobenzoic acid-free RPMI 1640 medium by centrifugation (2,000 × *g* for 10 min) within 3 hr after blood collection. An aliquot of 1.5–2 ml of the red blood cell pellet was used to extract parasite DNA (contaminated with human leukocyte DNA).

Infected erythrocytes were suspended in 15 ml of ice-cold NET buffer (150 mM NaCl, 10 mM EDTA, 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 Chemical Co., St. Louis, MO) 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 three 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 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.

Polymerase chain reaction. The entire DHFR domain of the *dhfr-ts* gene of *P. falciparum* was amplified by the polymerase chain reaction using the PTC-100 thermal cycler (MJ Research, Watertown, MA) under the following conditions: approximately 200 ng of genomic DNA, 15 pmol of primers 5′-ATGATGGAACAAGTCTGCGACGTTTTCGAT-3′ (sense) and 5′-TTCATTTAACATTTTATTATTCGTTTTC-TT-3′ (anti-sense), buffer (25 mM KCl, 5 mM $(NH_4)_2SO_4$, 10 mM Tris, pH 8.8), 2 mM $MgSO_4$, 200 μM dNTP, and 1 unit of *Pwo* DNA polymerase (Roche Diagnostics, Meylan, France) in a 50-μl reaction at 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 in all cycles, for a total of 30 cycles. The primers were designed on the basis of the complete *P. falciparum dhfr-ts* sequence.[15,16] Five microliters of the amplification product were loaded on a 1.2% agarose gel, subjected to electrophoresis, stained with ethidium bromide, and visualized under ultraviolet transillumination to confirm the presence of the 708-basepair DNA fragment.

Sequencing of DNA. One of the primers was phosphorylated at its 5′-end by incubating 1 nanomole of primer in a mixture containing 0.5 mM ATP, buffer (10 mM $MgCl_2$, 5 mM dithiothreitol, 70 mM Tris-HCl, pH 7.6), and 20 units of T4 polynucleotide kinase (New England Biolabs, Inc., Beverly, MA), in a volume of 100 μl at 37°C for 15 min. The kinase was heat-inactivated at 65°C for 20 min. The 5′-end-labeled primer was used to perform the polymerase chain reaction described above. The amplified products were purified by glass beads (Jetsorb; Genomed Inc., Research Triangle Park, NC) and treated with lambda exonuclease (Gibco-BRL Life Technologies, Cergy Pontoise, France) to generate single-stranded DNA. The single-stranded template was used to sequence the amplified product by the dideoxy chain termination reaction.[17]

According to the previous studies, 5 amino acids at positions 16, 51, 59, 108, and 164 of the *dhfr-ts* gene undergo mutational changes.[10,11,18] The wild-type genotype, designated the 3D7-type profile, was defined as Ala-16/Asn-51/Cys-59/Ser-108/Ile-164. The mutant genotype with a single Ser-to-Asn-108 mutation was designated the HB3-type profile. The mutant genotypes with a double mutation were designated the K1-type (Asn-108/Arg-59), the 7G8-type (Asn-108/Ile-51), or the FCR3-type (Val-16/Thr-108). The mutant genotype with triple mutations Asn-108/Arg-59/Ile-51 was designated the W2-type. The genotype with quadruple mutations Asn-108/Arg-59/Ile-51/Leu-164 was designated the Cambodian type.[19]

***In vitro* assay.** *In vitro* drug sensitivity assays were performed on the clinical isolates without prior adaptation to the *in vitro* culture conditions. Infected erythrocytes were suspended in the complete folate- and *p*-aminobenzoic acid-free RPMI 1640 medium consisting of 10% non-immune human serum, 25 mM HEPES, 25 mM $NaHCO_3$ at a hematocrit of 1.5% and an initial parasitemia of 0.2–1.0%. If the blood sample had a parasitemia >1.0%, fresh uninfected, type A^+ erythrocytes were added to adjust the parasitemia to 0.6%.

The isotopic microtest developed by Desjardins and others[20] was used in this study.[21] Two hundred microliters of the suspension of infected erythrocytes were distributed in each well of 96-well tissue culture plates. The parasites were incubated at 37°C in 5% CO_2 for 18 hr. ^{3}H-hypoxanthine (1 μCi/well; Amersham International, Plc., Buckinghamshire, United Kingdom) was added to assess parasite growth. After an additional 48 hr of incubation, the plates were frozen to terminate the *in vitro* drug sensitivity assay. The plates were thawed, and the contents of each well were collected on glass-fiber filter papers, washed, and dried using a cell harvester. The filter disks were transferred into scintillation tubes, and 2 ml of scintillation cocktail (Organic Counting Scintillant®; Amersham International, Plc.) were added. The incorporation of ^{3}H-hypoxanthine was quantitated using a liquid scintillation counter (Wallac 1409; Pharmacia, Uppsala, Sweden).

The 50% inhibitory concentration (IC_{50}), defined as the drug concentration corresponding to 50% of the uptake of ^{3}H-hypoxanthine measured in the drug-free control wells, was determined by non-linear regression analysis of logarithm of concentrations plotted against the parasite growth inhibition. The best-fitting sigmoid curve was derived by using the Prism™ software (GraphPad Software, Inc., San Diego, CA). The pyrimethamine IC_{50} values were classified as sensitive (<100 nM), moderately resistant (100–2,000 nM), and highly resistant (>2,000 nM), as in our previous studies.[22,23] Similarly, the IC_{50} values for cycloguanil were classified as sensitive (<50 nM), moderately resistant (50–500 nM), and highly resistant (>500 nM). These threshold values have been defined arbitrarily to separate three distinct *in vitro* responses observed in our earlier studies.[22,23] More recently, Nzila-Mounda and others have also observed three

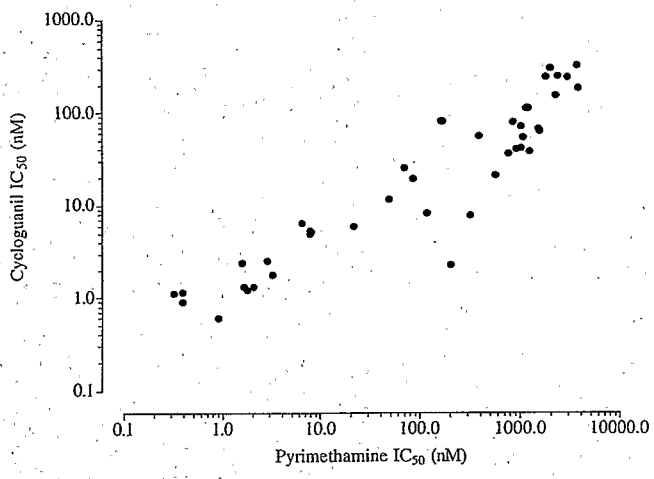

FIGURE 1. *In vitro* responses of Cameroonian isolates of *Plasmodium falciparum* to pyrimethamine and cycloguanil. The *in vitro* response was arbitrarily classified into three groups: sensitive (pyrimethamine 50% inhibitory concentration [IC_{50}] < 100 nM, cycloguanil IC_{50} < 50 nM), moderately resistant (pyrimethamine IC_{50} = 100–2,000 nM, cycloguanil IC_{50} = 50–500 nM), and highly resistant (pyrimethamine IC_{50} > 2,000 nM, cycloguanil IC_{50} > 500 nM). The IC_{50} values are highly correlated (r = 0.935, $P < 0.05$).

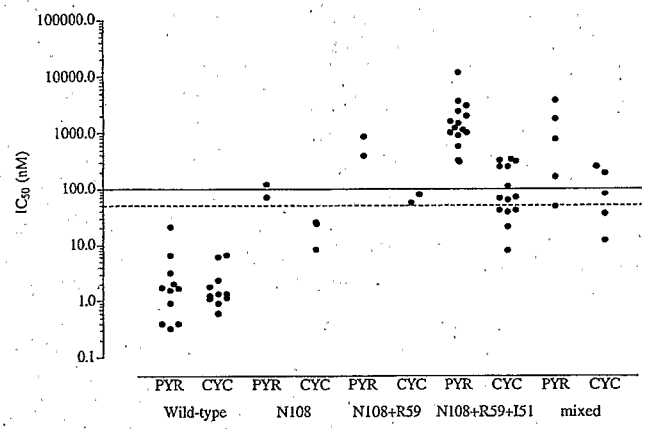

FIGURE 2. Relationship between the phenotype determined by *in vitro* drug sensitivity assays and expressed as the 50% inhibitory concentration (IC_{50}) of pyrimethamine (PYR) and cycloguanil (CYC), and the dihydrofolate reductase genotype, defined by either the absence of mutations (wild-type) or presence of point mutations Asn-108 (N108), Asn-108 and Arg-59 (N108 + R59), and Asn-108, Arg-59, and Ile-51 (N108 + R59 + I51). Mixed refers to the presence of one or more mixed alleles at positions 51, 59, and/or 108. The solid (corresponding to 100 nM) and dotted (corresponding to 50 nM) lines are hypothetical cut-off levels for *in vitro* pyrimethamine and cycloguanil resistance, respectively.[22,23]

distinct groups of Kenyan isolates based on the IC_{50} of antifolate drugs.[24] The validity of these cut-off values, in particular their possible relevance to *in vivo* responses, has not been established. Data were expressed as geometric mean IC_{50} and range. Correlation of the logarithmic values of IC_{50} for pyrimethamine and cycloguanil was calculated by a linear regression analysis. Data were analyzed by using the Statview software (Abacus Concepts, Inc., Calabasas, CA).

RESULTS

The *in vitro* activity of pyrimethamine and cycloguanil was determined for 42 clinical isolates. Using the earlier classification of *in vitro* responses to antifolate drugs, 18 isolates were pyrimethamine-sensitive (geometric mean IC_{50} = 4.01 nM, range = 0.32–84.8 nM) (Figure 1). All of these 18 pyrimethamine-sensitive isolates were also sensitive to cycloguanil (mean IC_{50} = 3.03 nM, range = 0.6–25.8 nM). A total of 26 isolates were cycloguanil-sensitive (mean IC_{50} = 5.17 nM, range = 0.6–42 nM). Of 42 isolates, 18 and 16 were moderately resistant to pyrimethamine (mean IC_{50} = 713 nM, range = 119–1,978 nM) and cycloguanil (mean IC_{50} = 133 nM, range = 54.4–333 nM), respectively. Six isolates were highly resistant to pyrimethamine (mean IC_{50} = 3,730 nM, range = 2,280–11,700 nM). None of the isolates was highly resistant to cycloguanil (IC_{50} > 500 nM). The *in vitro* responses to pyrimethamine and cycloguanil were highly correlated (r = 0.935, $P < 0.05$).

The complete sequence of the DHFR domain of the *dhfr-ts* gene was determined in 34 isolates. Amino acid substitutions occurred at positions 51, 59, and 108. Eleven isolates with the wild-type (3D7-type) profile had geometric mean (range) pyrimethamine IC_{50} and cycloguanil IC_{50} values of 1.60 nM (0.32–21.4 nM) and 1.64 nM (0.60–6.40 nM), respectively (Figure 2). Two isolates had a single mutation (HB3-type profile) at amino acid residue 108. The IC_{50} values of pyrimethamine (70.6 nM and 119 nM) and cycloguanil (8.1 nM and 25.8 nM) for these two HB3-type isolates were higher than the range of values for the wild-type isolates. The Arg-59/Asn-108 double mutation (K1-type profile) was found in two isolates with even higher IC_{50} values for both pyrimethamine (396 nM and 854 nM) and cycloguanil (56.5 nM and 78.7 nM). The triple mutation Ile-51/Arg-59/Asn-108 (W2-type) was detected in 14 isolates presenting elevated IC_{50} values for pyrimethamine and cycloguanil. The IC_{50} values of five isolates with mixed alleles were within the range of values displayed by mutant isolates. None of the isolates had the mutant codons Val-16, Thr-108, and Leu-164.

DISCUSSION

As in the previous studies, the *in vitro* response of pyrimethamine and cycloguanil was highly correlated.[22–25] These results are in agreement with the fact that pyrimethamine and cycloguanil share similar chemical structures and inhibit the same enzyme.[8,9] Eleven isolates were characterized by the wild-type *dhfr* profile and exhibited low IC_{50} values. These data allow us to deduce that the phenotype and wild-type genotype correspond to the antifolate-sensitive pattern. The presence of one (Asn-108) or two (Asn-108 + Arg-59) mutations in the *dhfr-ts* gene was clearly associated with an increasing level of pyrimethamine IC_{50} values. Although the mean pyrimethamine IC_{50} values for isolates with a triple mutation were higher than the IC_{50} values for isolates with a double mutation, the latter values were within the range of values observed in isolates with a triple mutation. Our data thus suggest a stepwise increment in the level of resistance to pyrimethamine that is directly related to the presence and number of point mutations, but there was no clear-cut difference in the level of resistance between the isolates

with double or triple mutations. As for cycloguanil, the relationship between the HB3-type single mutation and cycloguanil resistance was not evident from our data. However, the K1-type double mutations (Asn-108 + Arg-59) and the W2-type triple mutations (Asn-108 + Arg-59 + Ile-51) showed some degree of association with an elevated IC_{50} for cycloguanil.

When the range of IC_{50} values for pyrimethamine is compared between the wild-type isolates (0.32–21.4 nM) and mutant isolates with one (71 and 119 nM) to three point mutations (323–11,700 nM), it can be deduced that the cut-off point for *in vitro* pyrimethamine resistance is between 22 and 70 nM. The cut-off value for *in vitro* cycloguanil resistance cannot be estimated in the present study due to the wide dispersion of the IC_{50} and the existence of some mutant isolates with IC_{50} values between 10 and 50 nM.

The approximate cut-off value for pyrimethamine resistance determined in the present study is lower than the values fixed arbitrarily in our previous studies.[19,22] In fact, the IC_{50} values vary widely from one laboratory to another. The major underlying reason may be due to the difference in the assay methods. Thus, there are several technical problems in the *in vitro* assays that need to be resolved before the cut-off value can be defined. *In vitro* assays for antifolate drugs are performed with a culture medium that is either poor or devoid of folic acid and *p*-aminobenzoic acid, both of which antagonize the schizontocidal action of these drugs.[26–28] The culture medium that is devoid of folic acid and *p*-aminobenzoic acid does not yield an optimal parasite growth in fresh clinical isolates, and serum sources influence the IC_{50} values.[29] Although the folate- and *p*-aminobenzoic acid-free RPMI 1640 culture medium was used in our study, each batch of serum contains variable levels of these two components. These parameters may explain the different levels of IC_{50} values for antifolate drugs determined in the previous studies.[19,22–24,27,30,31] A standardized isotopic *in vitro* assay is required so that the *in vitro* responses obtained in one study can be compared with those of other studies. The standardization of the *in vitro* assay needs to address the problem of serum or serum substitute to be used, the type of culture medium, tissue culture plates (including the volume of erythrocyte suspension per well), incubation period, hematocrit, parasitemia, and oxygen content of the incubator. Because the number of resistant isolates is limited in this study, a greater number of isolates should be studied, using a standardized *in vitro* assay, to define the threshold IC_{50} value with more precision. It may also be necessary to exclude mixed infections by analyzing polymorphic genetic markers, such as merozoite surface antigen genes and circumsporozoite protein gene, for the correlation of genotype and phenotype.

Our complete DNA sequencing failed to detect mutations or insertions other than in the amino acid residues that were previously identified to be variable. In some South American isolates, a 5 amino acid insertion after codon 30, designated the "Bolivia repeat," and Cys-to-Arg substitution in codon 50 are present but do not seem to influence the drug sensitivity phenotype.[8,32,33] In addition, the mutant Leu-164 codon seems to be relatively common in the Amazon basin, and the mutant Thr-108 codon was reported in 11% of the field samples from Colombia.[33,34] However, in the study of Giraldo and others,[34] *in vitro* and/or *in vivo* sensitivity tests were not performed to determine the effect that the Thr-108 codon may have in antifolate resistance.

In the present study, we did not observe any distinct genetic profile that may be associated with cycloguanil resistance, independently of pyrimethamine resistance. In our previous study, we showed that the presence of quadruple mutations (Asn-108, Ile-51, Arg-59, Leu-164) in some Cambodian isolates is associated with a high level of antifolate resistance, in particular with an elevated IC_{50} for cycloguanil.[19] None of the African isolates studied so far displayed the Leu-164 codon.[19,24,30,32,35,36] Thus, contrary to the molecular criteria set by Foote and others[10] and Peterson and others,[11] we failed to identify a differential *in vitro* response associated with a unique genotype for cycloguanil resistance.

Several hypotheses may explain the absence of a distinct genotype associated with cycloguanil resistance in our study. First, cycloguanil resistance is absent in Africa. This hypothesis is unlikely. Several clinical studies conducted in Africa have shown the inevitable decrease of efficacy when proguanil or cycloguanil was used as a monotherapy or monoprophylaxis over several weeks to several months.[2] Moreover, recent studies have reported several cases of prophylactic failure in nonimmune patients under chloroquine-proguanil prophylaxis.[37–39] Second, the methodology for the determination of cycloguanil-sensitive or cycloguanil-resistant phenotype may not be sufficiently precise, as mentioned previously. If the phenotype cannot be determined with precision using a standardized assay, the interpretation of the results of *dhfr-ts* gene analysis may lead to erroneous conclusions. Third, there may be geographic differences in the genotype associated with cycloguanil resistance. For example, in multidrug-resistant Cambodian isolates, quadruple mutations were observed frequently.[19] Fourth, there may be no difference in the genotype associated with pyrimethamine resistance and cycloguanil resistance, at least in African isolates, except for a higher number of mutations required for cycloguanil resistance.

The last hypothesis seems to be the most likely explanation, based on the available phenotype and genotype data on African isolates. The *in vitro* responses to pyrimethamine and cycloguanil are highly correlated, and their levels, especially that of pyrimethamine, are directly associated with the number of mutations in the *dhfr-ts* gene. In the study conducted by Parzy and others,[30] the widely different IC_{50} levels for cycloguanil (>300-fold difference between the most sensitive and the most resistant isolates) were explained solely on the basis of an increasing number of point mutations in codons 51, 59, and 108, as in our study. No additional, new mutation was detected by a full-length sequencing of the gene for 28 isolates in their study. Similarly, sequence analysis of DHFR alleles in other studies has shown that antifolate resistance in African field isolates was attributable to mutational changes in only three codons (51, 59, and 108).[24,32,35]

These considerations seem to suggest that the differential molecular criteria for pyrimethamine resistance (Asn-108) and cycloguanil resistance (Thr-108/Val-16), which were established on the basis of the genotype of several reference clones of *P. falciparum*,[10,11] may not be applicable to the field isolates. The basis of the differential response may be

the number of mutations in the *dhfr-ts* gene, at least for African isolates of *P. falciparum.* This possibility is supported by experimental studies involving the transformation of wild-type *P. falciparum* and *Saccharomyces cerevisiae* strains with recombinant vectors carrying different types and combinations of *dhfr* mutations.[8,40] Thus, a search for a single mutation at codon 108 of the gene seems to be appropriate for detecting pyrimethamine resistance but inadequate to detect cycloguanil resistance, as suggested by relatively low predictive values of the mutation for *in vitro* cycloguanil resistance.[41,42] These findings may have an important implication for the design of epidemiologic surveillance of drug resistance by genotype analysis, especially in areas, such as in east Africa, where antifolate drugs are rapidly losing effectiveness for both chemoprophylaxis and treatment.[24,43]

Acknowledgments: We thank Sisters Solange Menard and Marie-Solange Oko and their nursing and laboratory staff at the Nlongkak Catholic missionary dispensary for precious aid in recruiting patients.

Financial support: The study was supported by Zeneca Pharma (La Defense, France), the Agence Universitaire de la Francophonie, and the French Ministry of Cooperation and Development.

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ISSN=0002-9637

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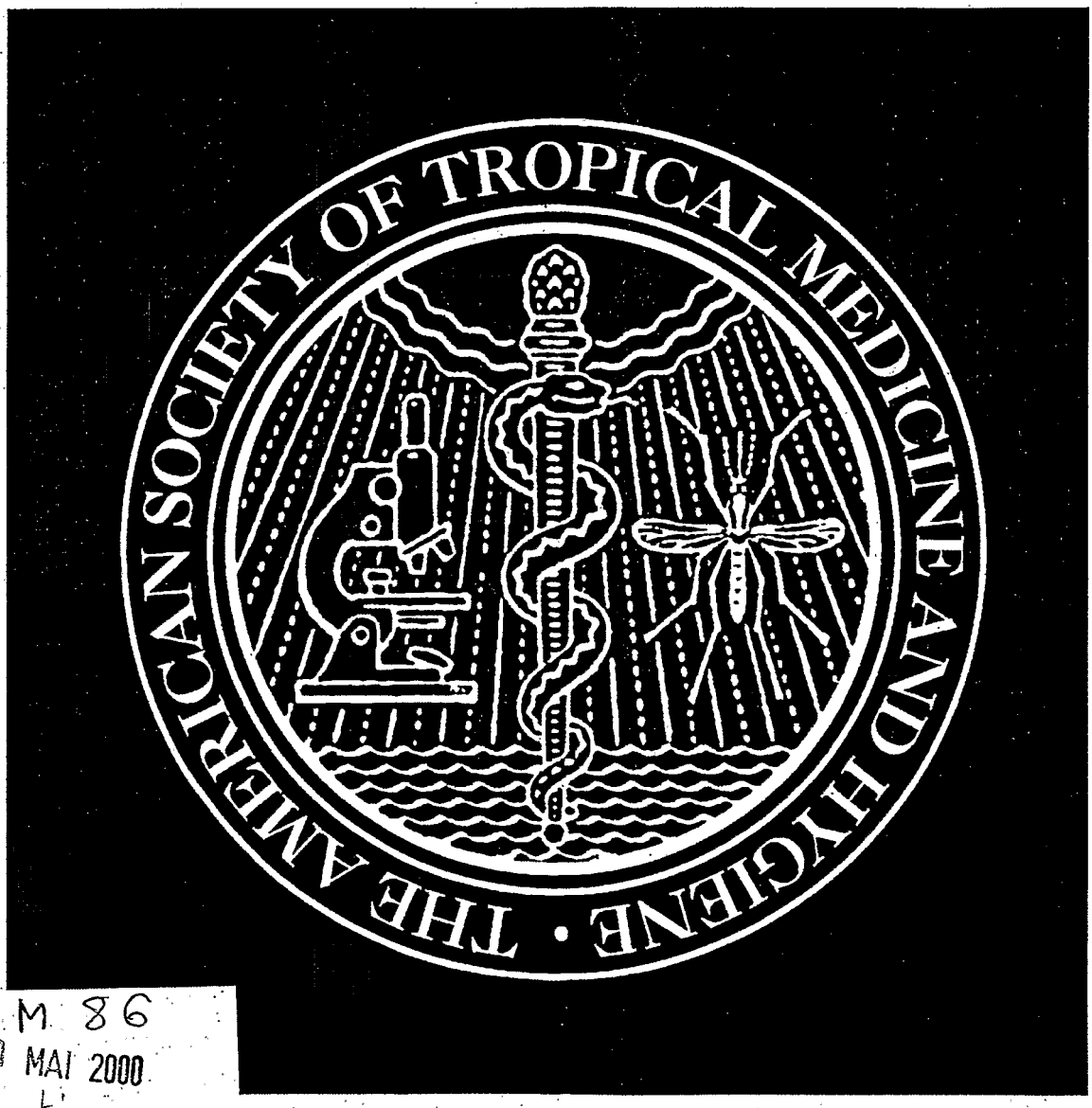
The American Journal of TROPICAL MEDICINE & HYGIENE



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