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Molecular Basis of In Vivo Resistance to Sulfadoxine-Pyrimethamine in African Adult Patients Infected with *Plasmodium falciparum* Malaria Parasites

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In vitro sulfadoxine and pyrimethamine resistance has been associated with point mutations in the dihydropteroate synthase and dihydrofolate reductase domains, respectively, but the in vivo relevance of these point mutations has not been well established. To analyze the correlation between genotype and phenotype, 10 Cameroonian adult patients were treated with sulfadoxine-pyrimethamine and followed up for 28 days. After losses to follow-up ($n = 1$) or elimination of DNA samples due to mixed parasite populations with pyrimethamine-sensitive and pyrimethamine-resistant profiles ($n = 3$), parasite genomic DNA from day 0 blood samples of six patients were analyzed by DNA sequencing. Three patients who were cured had isolates characterized by a wild-type or mutant dihydrofolate reductase gene (with one or two mutations) and a wild-type dihydropteroate synthase gene. Three other patients who failed to respond to sulfadoxine-pyrimethamine treatment carried isolates with triple dihydrofolate reductase gene mutations and either a wild-type or a mutant dihydropteroate synthase gene. Three dihydrofolate reductase gene codons (51, 59, and 108) may be reliable genetic markers that can accurately predict the clinical outcome of sulfadoxine-pyrimethamine treatment in Africa.

The spread of chloroquine-resistant *Plasmodium falciparum* has led to increasing use of a sulfadoxine-pyrimethamine combination to treat acute, uncomplicated malaria in many African countries (12, 22, 32). Sulfadoxine-pyrimethamine is one of the few effective and cheap alternative drugs that have the advantages of excellent compliance (single oral dose therapy), good tolerance, and relatively few side effects. However, its potential drawback is the rapid emergence of resistant parasites when sulfadoxine-pyrimethamine is massively employed, as demonstrated in Southeast Asia, where this drug combination is no longer effective (30, 31). To prolong the clinical efficacy of the drug as much as possible until other affordable drugs are available, it is of the utmost importance to understand the molecular basis of sulfadoxine-pyrimethamine resistance.

The molecular targets of each of the drug components have been identified: the dihydrofolate reductase (DHFR) domain of the bifunctional DHFR-thymidylate synthase (TS) enzyme for pyrimethamine and the dihydropteroate synthase (DHPS) domain of the bifunctional 7,8-dihydro-6-hydroxymethylpterin pyrophosphokinase (PPPK)-DHPS enzyme for sulfadoxine (6, 10, 23). These two enzymes are essential for the folic acid biosynthetic pathway. Pyrimethamine and sulfadoxine are analogs of dihydrofolate and *p*-aminobenzoic acid, respectively, which inhibit folate synthesis sequentially. Resistance to sulfadoxine-pyrimethamine, assessed individually by in vitro drug sensitivity tests, has been shown to be associated with distinct point mutations in the *P. falciparum* DHFR-TS and PPPK-DHPS genes. In vitro pyrimethamine resistance is almost always associated with the key Ser-to-Asn mutation at residue 108 of the DHFR domain (1, 2, 9–11, 13–15, 35). Higher in vitro levels of resistance result from the presence of ancillary mutations at residues 51 (Asn→Ile), 59 (Cys→Arg), and/or 164 (Ile→Leu). The association between the DHFR genotype and the pyrimethamine resistance phenotype is further supported by site-directed mutagenesis and transfection experiments (18–20, 25, 34). Similarly, in vitro sulfadoxine resistance seems to be associated with an Ala-to-Gly mutation at residue 437 of the DHPS domain. Higher levels of sulfadoxine resistance are associated with additional mutations at residues 581 (Ala→Gly), 436 (Ser→Phe), and 613 (Ala→Ser) (5, 6, 15, 23, 24, 26, 28).

Because previous studies have attempted to correlate the DHPS and DHFR genotypes separately with in vitro resistance to sulfadoxine and pyrimethamine, respectively, it is not clear to what extent point mutations in the DHFR-TS and PPPK-DHPS genes play a role in in vivo sulfadoxine-pyrimethamine resistance (10, 28). To assess the relevance of nucleotide substitutions in the two *P. falciparum* genes to in vivo resistance to sulfadoxine-pyrimethamine, we have determined the DNA sequences of the DHFR-TS and PPPK-DHPS genes and correlated these findings with patients' responses to sulfadoxine-pyrimethamine.

MATERIALS AND METHODS

Patients and treatment. Ten Cameroonian adult patients residing in Yaoundé, Cameroon, were enrolled in the study if the following criteria were met: fever and other symptoms associated with acute, uncomplicated falciparum malaria, a positive blood smear (>5,000 asexual parasites/μl of blood), a negative Saker-Solomons urine test for 4-aminoquinolines, and informed consent (16). Patients presenting signs and symptoms of severe and complicated malaria, as defined by the World Health Organization (WHO) (29), and pregnant women were excluded. Venous blood samples were obtained before treatment. Three tablets of sulfadoxine-pyrimethamine (1,500 mg of sulfadoxine, 75 mg of pyrimethamine) were administered on day 0. Patients were followed up on days 1, 2, 3, 7, 14, 21, and 28 by clinical evaluation and thick blood smears. The study was approved by the Cameroonian National Ethics Committee and the Cameroonian Ministry of Health.

Therapeutic responses. Therapeutic response was graded by using the 1996 revised WHO classification (33). "Early treatment failure" (the rough equivalent

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TABLE 1. Clinical and laboratory data on Cameroonian patients treated with sulfadoxine-pyrimethamine

Patient	Age (yr)	Sex[a]	Wt (kg)	Parasitemia (no. of parasites/μl of blood)								In vivo response[b]	In vitro IC_{50} of pyrimethamine (nM)	Hemoglobin (g/dl)		
				Day 0	Day 1	Day 2	Day 3	Day 7	Day 14	Day 21	Day 28			Day 0	Day 7	Day 14
1030	15	F	61	130,400	7,380	0	0	0	0	0	0	ACR	0.3	9.5	8.0	11.4
1027	42	M	60	9,000	8,650	0	0	0	0	0	0	ACR		15.5	15.0	14.5
1012	24	F	61	71,200	84,750	37	0	0	0	0	0	ACR	368	12.7	10.2	11.4
1014	23	F	56	12,900	15,100	0	0	0	32		12,840	LTF	2,160	6.9	6.9	9.7
1037	37	M	70	129,000	38,800	81	17					ETF	2,480	15.1	11.8	12.2
1015	16	M	55	21,800	28,100	770	50,400					ETF		6.8	6.6	8.5

[a] F, female; M, male.
[b] Classification was done by the in vivo test developed by the WHO in 1996 (33). ACR, adequate clinical response; LTF, late treatment failure; ETF, early treatment failure.

of the former resistance grades RII and RIII) refers to one of the following conditions during the first 3 days of follow-up: (i) a positive smear and signs and symptoms of severe malaria; (ii) fever and an increase in parasitemia, compared with the pretreatment parasitemia, on day 2; (iii) fever and a positive smear on day 3; or (iv) parasitemia on day 3 that is ≥25% of the pretreatment parasitemia. "Late treatment failure" (the rough equivalent of the former RII and early RI resistance grades) is defined by the following conditions between days 4 and 14: (i) a positive smear and signs and symptoms of severe malaria or (ii) fever and a positive smear. "Adequate clinical response" (equivalent to the former sensitive [S] and late RI resistance grades) refers to either (i) a negative smear on day 14, with or without fever, or (ii) apyrexia during the follow-up period, without previously meeting any of the criteria of early or late treatment failure. Patients who failed to respond favorably to sulfadoxine-pyrimethamine were treated with halofantrine.

In vitro drug sensitivity. In vitro assays were performed with day 0 venous blood samples to determine pyrimethamine sensitivity. The technique was based on a modified in vitro drug sensitivity test using an enriched-lipid and albumin mixture (Albumax I; Gibco BRL, Paisley, United Kingdom), instead of human serum, *p*-aminobenzoic acid- and folate-free RPMI 1640 medium, and 96-well tissue culture plates (17). Parasite growth in various drug concentrations was assessed by the incorporation of tritium-labeled hypoxanthine. The results were expressed as the 50% inhibitory concentration (IC_{50}), defined as the concentration corresponding to 50% hypoxanthine incorporation compared with drug-free control wells. The levels of in vitro response to pyrimethamine were defined as sensitive (IC_{50}, <100 nM), moderately resistant (IC_{50}, 100 to 2,000 nM), and highly resistant (IC_{50}, >2,000 nM) (4). An in vitro assay using sulfadoxine did not yield consistent, reproducible results due to its poor solubility in water and alcohol (28), and in vitro data on sulfadoxine were excluded from the analysis.

DNA extraction and sequencing. Parasite DNA was extracted from blood samples obtained on day 0 as described previously (3). PCR-restriction fragment length polymorphism was performed with parasite genomic DNA to exclude mixed DHFR 108 alleles (8). Previous studies have demonstrated that a Ser-to-Asn substitution at position 108 is the first mutation that appears in a pyrimethamine-resistant strain and that other, ancillary mutations, notably, at positions 51, 59, and 164, arise from stepwise selection of the Asn-108 mutants (1, 2, 9–11, 14, 15, 35). It has also been demonstrated by mutagenesis that Asn-108 mutant DHFR displays a high catalytic activity that confers clear biochemical advantages on parasites under pyrimethamine drug pressure (20). Our methodological approach thus excludes parasite subpopulations with mixed phenotypes for pyrimethamine sensitivity. Likewise, mixed subpopulations that are homogeneous at position 108 and are of the wild type (Ser-108) have the same genotype. However, mixed subpopulations that are homogeneous at position 108 and are of the mutant type (Asn-108) may be heterogeneous at ancillary sites.

Genomic DNA samples with pure Ser-108 or Asn-108 were further analyzed. PCR amplification of the entire DHFR domain or part of the DHPS domain was performed with *Pwo* DNA polymerase, which possesses a proofreading activity. The amplification products were phosphorylated by T4 polynucleotide kinase, ligated into blunt-end vector pMOS Blue (Amersham International, Buckinghamshire, United Kingdom), and transformed into XL-1 Blue competent cells. The recombinant plasmids were extracted by the alkaline lysis method, and DNA sequences were determined by the dideoxy-chain termination method.

RESULTS

Of the 10 patients enrolled, 1 was lost to follow-up on day 4. This patient was afebrile and had a negative blood smear on day 3. Three patients were carriers of mixed isolates with both Ser and Asn-108 DHFR alleles. DNA samples from these four patients were not analyzed further. The clinical features, therapeutic responses, and in vitro pyrimethamine sensitivities of the six remaining patients and their corresponding isolates are presented in Table 1.

Three patients had an adequate clinical response to sulfadoxine-pyrimethamine, and the other three failed to respond to treatment. The genotypes of the corresponding *P. falciparum* isolates are summarized in Table 2. Patients who responded favorably to the sulfadoxine-pyrimethamine combination carried either *P. falciparum* isolates with the wild-type genotype in both the DHFR and DHPS domains (patient 1030) or isolates with wild-type DHPS and mutant DHFR (Asn-108 with or without Arg-59; patients 1012 and 1027). The IC_{50} of pyrimethamine for the isolate obtained from patient 1030 was low (0.3 nM), indicating high in vitro sensitivity to pyrimethamine. This isolate had wild-type DHFR. Isolate 1012, which displayed two DHFR mutations (Asn-108 and Arg-59), was moderately resistant to pyrimethamine in vitro (IC_{50}, 368 nM). All three patients (1014, 1015, and 1037) who failed to respond to sulfadoxine-pyrimethamine treatment had isolates with three mutations in the DHFR domain (Ile-51, Arg-59, and Asn-108) and either wild-type or mutant DHPS. The triple DHFR mutations were associated with high-level pyrimethamine resistance in vitro (IC_{50}, >2,000 nM).

DISCUSSION

A single Asn-108 DHFR mutation is the key nucleotide change that confers moderate in vitro resistance to pyrimethamine (2, 10, 19). A second mutation at either residue 51 or 59 confers a higher level of pyrimethamine resistance in vitro (1, 9, 11, 13–15, 35). Our results suggest that since sulfadoxine and pyrimethamine are synergistic (7), pyrimethamine resistance due to the presence of up to two DHFR mutations, including key residue 108, is offset by the synergistic interaction of the

TABLE 2. Relationship between *P. falciparum* DHFR and DHPS gene sequences and clinical responses to sulfadoxine-pyrimethamine treatment in African adult patients

Isolate	Response[a]	DHFR residue:					DHPS residue:				
		16	51	59	108	164	436	437	540	581	613
1030	ACR	Ala	Asn	Cys	Ser	Ile	Ala	Ala	Lys	Ala	Ala
1027	ACR	Ala	Asn	Cys	**Asn**[b]	Ile	Ala	Ala	Lys	Ala	Ala
1012	ACR	Ala	Asn	**Arg**	**Asn**	Ile	Ser	Ala	Lys	Ala	Ala
1014	LTF	Ala	**Ile**	**Arg**	**Asn**	Ile	Ala	Ala	Lys	Ala	Ala
1037	ETF	Ala	**Ile**	**Arg**	**Asn**	Ile	Ala	Ala	Lys	Ala	Ala
1015	ETF	Ala	**Ile**	**Arg**	**Asn**	Ile	Ser	**Gly**	Lys	Ala	Ala

[a] The clinical and parasitological responses, adequate clinical response (ACR), late treatment failure (LTF), and early treatment failure (ETF), were defined by the WHO (33).
[b] Residues in boldface are mutations.

drug combination if the DHPS component is of the wild type and therefore sensitive to sulfadoxine.

In contrast, three sulfadoxine-pyrimethamine-resistant cases (1014, 1015, and 1037) illustrate that triple mutations in DHFR confer a high-level pyrimethamine resistance that may not be overcome by the synergistic effect of sulfadoxine. Patient 1014 responded with late treatment failure (early RI resistance). The isolate displayed triple mutations in DHFR and wild-type DHPS. Parasitemia was cleared on day 2, and thick blood smears were negative on days 2, 3, and 7. The blood smear became positive on day 14 (32 asexual parasites/μl of blood), but since the patient remained asymptomatic, she was not treated until day 28, when parasitemia (12,840 parasites/μl) was accompanied by fever (37.9°C). Like patient 1014, patient 1037 had parasites with triple DHFR mutations and wild-type DHPS. This patient responded with early treatment failure (RII resistance). There is no obvious difference in the in vitro pyrimethamine resistance level (IC_{50}, 2,160 versus 2,480 nM) and the genotype between isolates 1014 and 1037 that may explain the in vivo resistance level difference, with the possible exception of the initial parasitemia (0.5 versus 2.4%) and body weight (56 versus 70 kg). The latter two factors may be responsible for the differential pharmacodynamic features of these patients. The higher body weight and parasitemia in patient 1037 imply a relatively lower drug concentration in relation to the parasite load, assuming that drug absorption was comparable in the two patients. Another possibility that may explain the differential responses of the two patients to sulfadoxine-pyrimethamine treatment is reinfection. In patient 1014, the initial clearance of asexual parasitemia, followed by the reappearance of asexual parasites, may be due to either recrudescence of the original infection or a new infection. Triple mutations in DHFR (residues 51, 59, and 108) plus moderate sulfadoxine resistance (Gly-437) (patient 1015) resulted in early treatment failure. This patient presented fluctuating parasitemia and recurrent fever between days 0 and 3. Among the patients enrolled in this study, this was the only patient with high-level parasitemia on day 3.

Initial studies that attempted to correlate in vitro sulfadoxine resistance and DHPS mutations were not conclusive (5, 6, 23, 26). More convincing data that directly correlate DHPS point mutations and in vitro sulfadoxine resistance were recently obtained by using the progeny of a cross between sulfadoxine-sensitive HB3 and sulfadoxine-resistant Dd2 or site-directed mutagenesis (24, 28). These studies have shown the key role played by amino acid residue 437. In laboratory-adapted *P. falciparum* clones, the wild-type codon at position 436 is Ser, while the mutant codon is Phe (6, 23, 26, 28). Ala-436 has been reported in one sulfadoxine-sensitive Tak 9/96 Thai clone (6). In our previous study on Cameroonian isolates, 21 of 32 parasites had Ala-436, while there were 10 of 32 isolates with Ser-436 and 1 isolate with Phe-436 (5). The role of Ala-436 in determining sulfadoxine sensitivity has not been defined in laboratory clones, and our clinical data support the hypothesis that Ala-436 or Ser-436 does not play a role in determining sulfadoxine sensitivity and that both may be considered alternative wild-type codons. A similar conclusion was reached by Wang et al. (27), who examined the DHPS genotypes of 141 field isolates.

As for pyrimethamine resistance, several studies on field isolates and clones of *P. falciparum*, as well as biochemical analysis of the affinity between pyrimethamine and recombinant DHFR, have provided solid evidence for a direct correlation between DHFR point mutations and in vitro pyrimethamine resistance (1, 2, 9–11, 14, 15, 35). In studies conducted in Kenya and Papua New Guinea, pyrimethamine-resistant, fresh *P. falciparum* isolates were shown to possess the mutant DHFR residue Asn-108 and either mutant Ile-51 or Arg-59 (11, 15). In the Kenyan study, the IC_{50}s for pyrimethamine were 0.3 to 5.1 nM for wild-type isolates and 184 to 785 nM for isolates with double mutations. These in vitro results are in agreement with our in vitro findings. Although these two studies were based on in vitro drug sensitivity, it may be predicted from our data that patients enrolled in these studies should have been cured if they were treated with sulfadoxine-pyrimethamine. In contrast, triple DHFR mutations were common in Vietnamese and Cambodian isolates (1, 27, 35). In agreement with the epidemiology of drug resistance, Southeast Asia is known for a high rate of clinical failure with sulfadoxine-pyrimethamine, as evidenced by a recent clinical trial in Burma showing a 70% failure rate (21). Moreover, a recent study on *P. falciparum* isolates from East Africa has shown that parasites that were cleared after sulfadoxine-pyrimethamine treatment presented wild-type DHPS and DHFR domains or a double DHFR mutation (Asn-108 and Arg-59) and either wild-type or mutant DHPS (Gly-437 and Glu-540) (27). In the same study, most of the patients who failed to clear asexual parasitemia were infected with recrudescent isolates carrying a double or triple DHFR mutation (Ile-51 and/or Arg-59 and Asn-108) with either wild-type or mutant DHPS. Our results are thus in agreement with those of Wang et al. (27).

Genotypic and phenotypic studies on the DHFR-TS and PPPK-DHPS gene sequences and sulfadoxine-pyrimethamine sensitivity have so far been conducted independently of each component by using in vitro models of *P. falciparum*. In vitro proof that the phenotype and genotype are associated has not been obtained, partly because the relevance of in vitro sulfadoxine-pyrimethamine resistance to in vivo resistance has not been established. Our study was thus conducted with the aim to determine to what extent the *P. falciparum* genotype corresponds with the phenotype. To address this problem without the possible interference of mixed alleles, clinical isolates presenting either pure Ser-108 or Asn-108 were analyzed in this study. Three patients had mixed parasite populations. Two of the three patients were cured with sulfadoxine-pyrimethamine. The other patient failed to clear the parasitemia after a 28-day follow-up (late RI resistance). Full understanding of the in vivo sensitivity or resistance of mixed parasite populations requires cloning of initial parasites to separate individual parasite clones for DNA sequencing, as well as quantitative estimation of the proportion of sensitive and resistant parasite populations in a patient.

Our results demonstrate that in vitro pyrimethamine resistance and in vivo sulfadoxine-pyrimethamine resistance are directly associated with the number of point mutations in the DHFR-TS gene. Moderate in vitro pyrimethamine resistance is associated with one or two DHFR point mutations (Asn-108 and Ile-51 or Arg-59), while high in vitro pyrimethamine resistance is associated with triple DHFR mutations (Asn-108, Ile-51, and Arg-59). In vivo, one or two DHFR mutations (moderate pyrimethamine resistance) and wild-type DHPS did not affect clinical curing by sulfadoxine-pyrimethamine treatment in Cameroonian adult patients, most probably due to the synergistic action of the drug combination. In vivo resistance was observed when a patient carried isolates with triple DHFR mutations, with or without mutant DHPS associated with moderate sulfadoxine resistance. It is not known whether, and to what extent, acquired immunity enhances parasite clearance, largely because there is no reliable quantitative biological marker that reflects the degree of immunity. Our results should therefore not be extrapolated to nonimmune individuals from areas where the disease is not endemic or to indigenous chil-

dren. Furthermore, the role of DHPS mutations in conferring in vivo resistance to sulfadoxine was not observed in a small number of patients in our study. The true importance of DHPS mutations can only be determined in a study involving a larger number of patients treated with sulfadoxine-pyrimethamine in parallel with a reliable and reproducible in vitro assay using sulfadoxine.

The resistance genes of other currently used antimalarial drugs (chloroquine, amodiaquine, quinine, mefloquine, halofantrine, artemisinin derivatives, and atovaquone) have not been identified with certitude, requiring classical means (in vivo and in vitro tests) to monitor drug efficacy retrospectively. Molecular analysis of the DHFR-TS and PPPK-DHPS genes is thus the first technical tool that enables clinicians to predict patient response to antimalarial treatment.

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