Low-Dose Treatment with Sulfadoxine-Pyrimethamine Combinations Selects for Drug-Resistant *Plasmodium falciparum* Strains

JÜRGEN F. J. KUN,1* LEOPOLD G. LEHMAN,1,2 BERTRAND LEULL,1,2,3 RUPRECHT SCHMIDT-OTT,1,2 AND PETER G. KREMSNER1,2

Department of Parasitology, Institute for Tropical Medicine, University of Tübingen, D-72074 Tübingen, Germany;1 Research Unit, Albert Schweitzer Hospital, Lambaréné, Gabon;2 and Department of Infectious Diseases, Internal Medicine I, University of Vienna, Vienna, Austria3

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A total of 252 children were enrolled in a drug trial to assess the effect of minimal doses of sulfadoxine (Sdx) and pyrimethamine (Pyr). Parasite samples isolated from these patients were analyzed before and after treatment to investigate the level of drug-resistant strains. The parasite genes encoding dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) were assayed for point mutations that are associated with resistance against drugs. Before treatment, Pyr resistance genotypes of the DHFR gene were found in 42% of all samples, 8% of the patients harbored a mixed parasite population and 50% had a sensitive DHFR genotype. In terms of the DHPS gene, we found mutations in 45% of the parasites. Twenty-four percent had a Ser436 mutation, and 26% had a Gly437 mutation. Recrudescent parasites were highly enriched for both Pyr and Sdx strains after treatment (*P* < 0.001 and *P* = 0.029, respectively).

Malaria is endemic in tropical countries around the world. Chemotherapy is the method of choice to combat the malaria parasite *Plasmodium falciparum* during the infection. Over the last few years, however, the parasite has developed resistance against chemotherapy, which is becoming an increasing problem in these areas. In large parts of Africa, formerly highly effective drugs such as chloroquine have become useless (7–9, 21, 22). This might be partly due to the fact that therapeutic dosages were decreased to lower costs or drug therapies were not carried out long enough for the same reason. Both actions result in a suppression of parasites rather than killing, and resistance emerges as a consequence.

The molecular basis for the resistance against antifolate drugs has become clearer within the past few years. Pyrimethamine (Pyr) resistance (PyrR) is linked to mutations in the dihydrofolate reductase (DHFR) gene: the most important is a point mutation affecting amino acid 108 causing a change from Ser to Asn (3, 12, 13, 16). Additional mutations at positions 51 (Asn→Ile), 59 (Cys→Arg), and 164 (Ile→Leu) mediate resistance to higher levels of the drug (5, 6, 13, 17). Mutation experiments with yeast show that DHFR genes from *P. falciparum* with these mutations can induce PyrR to sensitive yeast (2, 23). Mutations in the enzyme dihydropteroate synthase (DHPS) mediate resistance to sulfonamide drugs (e.g., sulfadoxine [Sdx]). Mutations at various sites cause resistance to increasing concentrations of sulfonamides in vitro (20). Position 437 of this enzyme seems to be especially important for the resistance to Sdx (4, 10). This mutation—an exchange of an Ala to a Gly—alters the affinity of the drug to its potential target (17). When Sdx-resistant *P. falciparum* strains are transfected with mutated DHPS genes, the resulting transfectants show resistance against Sdx (18). Additional mutations at positions 436 (Ala→Ser), 450 (Lys→Glu), 581 (Ala→Glu), and 613 (Ala→Ser) mediate resistance to higher dosages of the drug. Drug-resistant strains can be found in areas in which malaria is endemic—sometimes in very high frequency. There are reports of Asian and African parasite populations in which almost every strain investigated carries at least one drug-resistant genotype (19). PyrR or SdxR strains also occur in patients after treatment with the relevant drug (4, 19). Recently, a paper was presented that described the investigation of a population of schoolchildren treated with low doses of either Pyr or Sdx with or without mefloquine (Mef) or with Mef alone to assess a possible synergistic effect of the single components (11). Blood samples taken from all children were used to assess the frequency of resistant genotypes in the study population and the change in the parasite population due to the low-dose treatment. From children who did not respond to the treatment or who became parasitemic again within 28 days, a second sample was taken. Both samples were genotyped for three different polymorphic genes to investigate whether recurring parasites are recrudescences or reinfections in the different samples (14). For the paper presented here, we analyzed the genes coding for DHFR and DHPS to investigate whether these parasites have accumulated mutations that mediate resistance to the drugs used.

MATERIALS AND METHODS

**Patients.** The recruitment of the patients is described in detail in reference 11. Briefly, 252 patients received a single dose of either Mef-Pyr-Sdx, Pyr-Sdx, or Mef in a randomized, double-blind fashion. After exclusion of patients that vomited after intake of the drug or did not fulfill the exact follow-up protocol, 231 patients remained in the study. The patients were monitored every 24 h until they were free of fever, parasites, or any other signs of a malaria infection. Successive examinations were performed 7, 14, 21, and 28 days after treatment. According to the responsiveness of the patients to the therapy, they were classified as follows: nonresponders (NR), i.e., they failed to clear the parasites within 7 days; low-grade resistant (R), i.e., patients cleared the parasites within the first week, but had parasites during the follow-up; or sensitive (S), i.e., they showed clearance of parasites after treatment and no parasites during the follow-up. Blood was taken from all patients before treatment and as soon as parasites recurred. Patients that received only Mef were excluded from the analysis described in this paper. Only samples from patients who received the combination...
Pyr-Sdx (71 persons) or Pyr-Sdx-Mef (74 persons) and who were monitored for a 28-day period or became parasitemic during this time were analyzed. Ethical clearance was obtained from the ethics committee of the International Foundation of the Albert Schweitzer Hospital in Lambaréne, Gabon.

DNA purification was done from the patients’ samples with the Blood Kit from Qiagen (Hilden, Germany). PCR was performed with genomic DNA with Taq polymerase (15) (Perkin-Elmer, Applied Biosystems, Foster City, Calif.) with oligonucleotide primers. The reaction was done under buffer conditions according to the manufacturer’s instructions.

For amplification of the DHFR domain carrying the mutation at codon 108, we used primers Amp1 (5'-TCA TTT TGG TCT CCT TTG TAT 3') and Amp2 (5'-TTA CTA GTA TAT TTG TAC AAG CAC 3'). In some cases, a nested PCR was necessary to overcome low parasitemia; we used the primer SP1 (5'-ATG ATT GAA CAA GTC TGC CAC 3') together with AMP2. The amplification of the DHPS domain that includes mutated sites was done with sulf3 (5'-TCC ATT TGT TGT ATT TGT GAA CCT AAA CGC 3'). If required, a nested PCR was performed with sulf5 paired with Lo2 (5'-CTG GAT TAT TAT TAC AAG CAC 3'). The reaction conditions for the different PCRs were identical. To ensure there was sufficient denaturation of the template, an initial step of 3 min at 94°C was carried out. Then 40 cycles were done, with denaturing for 30 s at 94°C, annealing for 30 s at 55°C, and elongation for 1 min at 72°C. If nested PCR was done, the first round was 30 cycles, followed by a second round of 20 cycles. DNA sequencing was done on an ABI sequencer 373A (Perkin-Elmer, Applied Biosystems).

Statistics. Calculations were done with the StatView program on an IBM-compatible machine. Group comparisons were done by $t$ test; the McNemar test was applied for the analysis of samples before and after treatment.

Below we use the term “resistant” when at least amino acid 108 of DHFR is Asn or amino acid 437 of DHPS is Gly.

## RESULTS

Parasitological findings. The patients could be classified into three different groups. One group consisted of 96 individuals that were completely cured with no subsequent parasitemia during 4 weeks of follow-up. We will refer to this group as the S group; it included 69% of the patients. A second group of 33 children developed recrudescent parasitemia during the follow-up, which we refer to as the R group (24% of the patients). The last group, the NR group, was made up of 10 patients (7%) who did not clear parasites from the blood. The type of treatment had no effect on the occurrence of reinfections. There was no statistical significance in the distribution between the S, R, or NR individuals regarding the combination therapy used. In the S group, we had 48 patients with Sdx-Pyr and 48 patients treated with Sdx-Pyr-Mef. In the R group, 13 patients were treated with Sdx-Pyr versus 20 treated with Sdx-Pyr-Mef. In the NR group, we observed a 4 versus 6 Mef-treated—non-Mef-treated distribution. Thus, the addition of Mef into the formula had no effect in this trial.

DHFR genotyping. We obtained PCR products for the DHFR gene fragment from 179 samples. These include 139 samples collected before treatment and 40 from reappearing or persisting parasites. From 139 samples collected on day 0, we found 69 (50%) sensitive and 59 (42%) resistant DHFR genotypes, as judged by the presence of an Asn codon at position 108. Another 11 (8%) samples contained mixed populations of sequences. When we compared the distributions of genotypes between the groups, we found that 85% had Ser$_{108}$ in the S group. Twelve percent belonged to the R group. Three percent belonged to the NR group (Table 1). Asn$_{108}$ was found in all patient groups, but to a lesser extent in the S group (52%) and in higher proportions in the R and NR groups (i.e., 34 and 14%, respectively). Mixed populations were found in equal numbers in R and S individuals.

The unequal distribution of genotypes between the groups is of statistical significance ($P < 0.001$ for position 108). The mutant genotypes Ile$_{51}$ and Arg$_{59}$ were significantly associated with the R and NR groups ($P = 0.002$ for position 51 and $P = 0.007$ for position 59, respectively).

There is a strong linkage between positions 51, 59, and 108: 32% (44 parasite samples) of the patients had the combination Asn$_{51}$-Cys$_{59}$-Ser$_{108}$. The combination Asn$_{53}$-Cys$_{59}$-Ser$_{108}$ was found in 40% of the patients (56 samples).

For the analysis of parasite strains before and after treatment, we collected 40 pairs, each consisting of the parasite strain before treatment and that from the recrudescent infection. This group contained 9 NR and 31 R individuals. Before treatment, 5 patients harbored a mixed population (1 NR and 4 R), 10 carried a sensitive population (2 NR and 8 R), and 25 had resistant parasites (7 NR and 18 R) as defined by the presence of Asn$_{108}$. The analysis of the nonresponder and recrudescent parasites showed that although a low dose of Mef-Pyr-Sdx or Pyr-Sdx was given, all strains were found to be Pyr$^+$. In this comparison, the statistical significance is less pronounced if mixed isolates were calculated as resistant strains ($P = 0.007$). If the mixed populations were calculated as sensitive strains, the difference in the distribution is highly significant ($P < 0.001$).

Patients with a sensitive DHFR genotype on day 0 had a second attack after 22 ± 3 days. Patients harboring resistant strains were parasitemic again after 18 ± 2 days. Although this difference is statistically not significant, it might indicate that in patients with a sensitive DHFR genotype on day 0, resistant parasites were present but below the detection limit of the DNA sequence analysis.

Parasite strain typing of the 10 isolates with a sensitive DHFR genotype revealed that 4 of the 10 contained different parasites at the time of the second sampling. This might be due to a true reinfection, or this parasite strain was not picked up in the first sample because the parasitemia of this particular strain was below the detection limit.

### TABLE 1. Distribution of amino acid changes of the *P. falciparum* DHFR at positions 51, 59, and 108 within patient groups on admission*

<table>
<thead>
<tr>
<th>Amino acid position</th>
<th>No. (%) of patients with amino acid change</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>Position 51</td>
<td></td>
</tr>
<tr>
<td>NR</td>
<td>10 (7)</td>
</tr>
<tr>
<td>R</td>
<td>33 (24)</td>
</tr>
<tr>
<td>S</td>
<td>96 (69)</td>
</tr>
<tr>
<td>Total</td>
<td>139 (100)</td>
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<tr>
<td>Position 59</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>NR</td>
<td>10 (7)</td>
</tr>
<tr>
<td>R</td>
<td>33 (24)</td>
</tr>
<tr>
<td>S</td>
<td>96 (69)</td>
</tr>
<tr>
<td>Total</td>
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<tr>
<td>Position 108</td>
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</tr>
<tr>
<td>Total</td>
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<tr>
<td>NR</td>
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<tr>
<td>R</td>
<td>33 (24)</td>
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<tr>
<td>S</td>
<td>96 (69)</td>
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<td>Total</td>
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* $P < 0.001$ for position 108, $P = 0.002$ for position 51, and $P = 0.007$ for position 59. The percentages of parasites with wild-type or mutated genotypes are 47% for Asn$_{51}$ versus 40% for Ile$_{51}$, 51% for Cys$_{59}$ versus 38% for Arg$_{59}$, and 50% for Ser$_{108}$ versus 42% for Asn$_{108}$.

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Patients with an Ala437 mutation came down with malaria after drug treatment. Although statistically not significant, Gly at position 437 increased in a statistically significant way in 50% of the samples. In the Gabonese cross-sectional investigation presented here, we also detected Pyr resistant parasite genotypes after drug treatment with a low-dose treatment.

DHPS genotypes. For the determination of the DHPS genotype, we investigated 160 samples by DNA sequence analysis. We focused on the region around codon 437, since this position seems to be most responsible for mediation of drug resistance. No other mutation could be detected at positions 540, 581, and 613 when 30 isolates were analyzed by DNA sequence. Most of the parasites had Ala at positions 436 and 437. We found that 65% of the parasites had this genotype. Twenty-four percent had Ser436. The resistant genotype Gly437 was found in 26% of the patients. Different from the DHFR genotyping, no significant correlation between the resistant genotype on day 0 and the response to the drugs could be seen. However, when 36 pairs of parasites—isolated from the same patients before and after drug treatment—were compared, a significant difference in the distribution of genotypes could be seen. A higher prevalence of any mutation in recrudescent or nonresponding parasites could not be seen (P = 0.069) (Table 2). When we calculated the mutations separately, we observed that the distribution of position 436 was not significantly different (P = 0.584). Only the frequency of Gly at position 437 increased in a statistically significant way (P = 0.029).

The times of appearance of resistant parasites after treatment are different—although statistically not significant—whether parasites had a sensitive or resistant DHPS genotype. Patients with an Ala437 mutation came down with malaria after 21 ± 6 days, whereas patients with the Gly437 mutation had a second attack after 16 ± 6 days. This again could be due to the fact that the samples typed as sensitive contain resistant populations below the detection limit. This population needs more time to recrudesce up than the resistant-typed population that is just slowed down by the drug.

**DISCUSSION**

The parasite genotypes linked to Pyr are highly frequent in various parasite populations around the world. In Vietnam, Pakistan, and numerous African areas, Pyr can be observed in up to 50% of the samples. In the Gabonese cross-sectional investigation presented here, we also detected Pyr in 50% of the samples.