ANALYSIS OF PFCRT, PFMDR1, DHFR, AND DHPS MUTATIONS AND DRUG SENSITIVITIES IN PLASMODIUM FALCIPARUM ISOLATES FROM PATIENTS IN VIETNAM BEFORE AND AFTER TREATMENT WITH ARTEMISININ

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Abstract. We have analyzed artemisinin sensitivity in Plasmodium falciparum isolates obtained from patients in South Vietnam and show that artemisinin sensitivity does not differ before and after drug treatment. There was an increase in the level of mefloquine resistance in the isolates after drug treatment that was concomitant with a decrease in chloroquine resistance, suggesting that treatment with artemisinin has selected for increased mefloquine resistance. Mutations in the pfmdr1 gene, previously shown to be associated with sensitivity to mefloquine, were selected against. All isolates resistant to chloroquine encoded Thr-76 in the pfcr gene consistent with an essential role in the mechanism of chloroquine resistance. Mutations in pfmdr1 also were linked to chloroquine resistance. High levels of mutation in dhfr and dhps genes, which have previously been associated with Fansidar resistance, also were found, suggesting that this drug would not be useful for malaria control in this part of Vietnam.

INTRODUCTION

The development and spread of drug resistance in Plasmodium falciparum is a major problem for the treatment and control of malaria in many endemic countries. In Southeast Asia, 21.9 million cases of malaria were reported in 1995 alone. In Vietnam, chloroquine resistance was first reported in 1961 and reached a peak in 1990, ranging in incidence from 60–80% in some regions. Chloroquine resistance has now spread to most regions of Vietnam where malaria is endemic and has led to the widespread use of alternative antimalarial drugs (mefloquine, halofantrine, artemisinin derivatives, and pyrimethamine/sulfadoxine). Use of Fansidar (pyrimethamine + sulfadoxine) has increased rapidly over the last 30 years. As a consequence, reported levels of resistance to the drug have increased from approximately 20% to over 80%. Since 1991, artemisinin and its derivatives have been used to treat malarial patients in Vietnam. These drugs have become increasingly important as antimalarial therapy in this setting because of their rapid mode of action and efficacy against multidrug-resistant forms of P. falciparum malaria.

Resistance of P. falciparum to chloroquine has been associated with lower drug accumulation. Mutations in the pfcr gene have been strongly linked to the mechanism of chloroquine resistance. The presence of Tyr-76 residue within the Pfcr protein is linked to chloroquine resistance, suggesting that it plays an important role in the mechanism of resistance to this antimalarial. Additionally, mutations within the pfmdr1 gene have been shown to confer increased resistance to chloroquine, suggesting that they play a role in modulating higher levels of chloroquine resistance. The same mutations also have been shown to confer quinine resistance and alter the level of resistance and sensitivity to mefloquine and artemisinin.

The combination of pyrimethamine and sulfadoxine was effective when it was introduced in the late 1960s as a single-dose treatment of acute P. falciparum malaria. Over the years, however, resistance to Fansidar has been reported, mainly in areas of intense use, particularly areas of chloroquine resistance. Variant sequences of P. falciparum dihydrofolate reductase (DHFR), the target enzyme of pyrimethamine, were first described in 1988. Resistance to pyrimethamine has been shown to result from a mutation in the DHFR enzyme, changing Ser-108 to Asn-108, and subsequent mutations can greatly increase the level of resistance to this drug. Ten mutant genotypes for DHFR have been reported from a large number of field samples. Resistance to sulfonamide and sulfones has been shown to result from mutations within dihydropterote synthetase (DHPS). Distribution of mutations in the dhfr and dhps genes and their association with Fansidar resistance have been assessed in different geographic areas, and it has been found that a greater number of mutations in both dhfr and dhps is a good predictor of likely drug failure.

Vietnam has well-developed systems for malaria surveillance, with recent data showing that more than half the population lives in forests and mountainous areas where they may be at risk of malaria infection. Drug-resistant malaria has been an increasing problem in the last two decades, and although alternative drugs such as artemisinin and its derivatives have become widely used as first-line therapy for malaria, they have been associated with a high frequency of recrudescence. In this 1998 study, we have further categorized malaria drug resistance in Vietnam by analyzing mutations in the pfcr, pfmdr1, dhfr, and dhps genes in P. falciparum primary and recrudescent isolates collected from patients in an area of Binh Phuoc Province highly endemic for malaria.

MATERIALS AND METHODS

Study area and collection of blood samples. The subjects of the study lived on the Phu Rieng rubber plantation in southern Vietnam. The area is hyperendemic for malaria, which is aggravated by multidrug-resistant P. falciparum. The peak seasons of infection are March–June and September–December (annual main peak of transmission). The annual parasite rate/slide is 10–30% for both P. falciparum and P. vivax.

Blood samples were collected from all P. falciparum-positive cases before administering artemisinin (20 mg/kg on
day 0 and 10 mg/kg per day for days 1–4) and following re-appearance of parasites after treatment. Blood was drawn by venipuncture into a vacutainer tube containing K-oxalate/Na-fluoride. Thick and thin blood smears were examined after staining with Giemsa.

Parasites, DNA, and drug sensitivity assay. All parasites were cultivated in vitro by standard methods.25 Nonsynchronized parasites at an initial parasitemia of 0.8–1% were grown in 2% hematocrit in fresh erythrocytes. At the beginning of each experiment, a range of dilutions of each drug, in complete RPMI 1640-Hepes free hypoxanthine medium, 10% serum, and 5% albumax were prepared. Before testing for pyrimethamine susceptibility, parasites were grown in medium containing folate at 0.01 mg/L and p-aminobenzoic acid at 0.5 μg/L without hypoxanthine, supplemented with 8% NaHCO₃, 5% albumax, and 10% human serum for 6 days. Drug susceptibility tests were performed using [³H] hypoxanthine uptake as described previously.26 IC₅₀ values were determined from the drug concentration at which [³H] hypoxanthine uptake was reduced by 50% compared with controls. Each assay was performed in triplicate in two independent assays. In parallel with each isolate, standard controls (clones D10 and K1) were tested to establish that no undesired variation in media or drug solutions had occurred.

The following drug preparations were used: Chloroquine diphosphate salt (Sigma, Germany), mefloquine hydrochloride (gift from Hoffman LaRoche, Switzerland), quinine hydrochloride (Sigma, Germany), artemisinin (Institute of Quality Control, Hanoi, Vietnam) and pyrimethamine (Sigma).

Sequences of the primers used for detection of polymorphisms in pfmdr1,27,28 pfcrt,7 dhfr, dhps,27,28 and msp229 genes have been described previously.

The pfmdr1 gene was amplified by polymerase chain reaction (PCR) under the following conditions: 1 μL of DNA was used as template, 20–50 pmol of each primer, PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 mM MgCl₂), 200 μM dNTPs, 1 unit of Taq polymerase DNA (Perkin-Elmer Cetus, USA) in a final volume of 50 μL. The PCR was performed for 35 cycles at 94°C for 5 seconds, 48°C for 10 seconds, and 68°C for 30 seconds.

Mutations in pfcrt were detected as described previously.7 Amplification of the dhfr gene was by PCR using a reaction volume of 50 μL containing 5 μL of sample DNA, 25 pmoles of each primer, 200 μM dNTPs, 2.5 mM MgCl₂, 2.5 U of Taq polymerase (Perkin-Elmer Cetus), PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3). The PCR assay was performed for 35 cycles using thermocycling conditions (Perkin-Elmer Cetus), which include denaturation at 94°C for 1 min, annealing at 55°C for 1 minute, and extension at 72°C for 45 seconds. The dhps gene was amplified using PCR as described previously.27,28

PCR genotyping of the msp2 gene was performed as described previously.29 If an identified genotype pattern was found in both sample A (primary isolates) and sample B (recurrent isolate), the infection was most likely a recrudescence. In contrast, if the patterns of both samples differed, a reinfection was assumed although a minor population of the original infection may have been responsible.

Restriction fragment length polymorphisms were detected by digesting DNA fragments of pfcrt, pfmdr1, dhfr, and dhps obtained by PCR with the appropriate restriction endonu-
The primary and secondary isolates were analyzed to determine whether the parasites isolated after artemisinin treatment represented a breakthrough or a new infection (Table 3). The \textit{msp2} gene of each isolate was analyzed by PCR and the allele type classified as different (D) or the same size (S) as judged by mobility of the DNA fragment on agarose gels (Table 3). The parasites isolated after artemisinin treatment that had different \textit{msp2} alleles must result from either a new infection or, alternatively, a breakthrough of a minor parasite population from the original infection. The parasite isolates that had an \textit{msp2} allele of the same size are likely to represent a breakthrough of the original infection through treatment with artemisinin. Only six of the parasite isolates obtained after artemisinin had \textit{msp2} alleles of the same size as the original isolates, suggesting that these were recrudescent infections. Three isolates had mixed \textit{msp2} alleles. Nine of the recurrent isolates had different \textit{msp2} alleles, which must have originated from new infections or selection of a population from a primary infection with multiple parasites with different \textit{msp2} alleles.

The genotypes for \textit{pfcrt} and \textit{pfmdr1} for the primary and secondary isolates. Genomic DNA from the primary and secondary isolates was analyzed as to the genotype of the \textit{pfcrt} and \textit{pfmdr1} genes to determine whether they were linked to

\begin{table}[h]
\centering
\begin{tabular}{lccccccc}
\hline
Isolates & \text{IC}_{50} (nmol) & \text{IC}_{50} (nmol) & \text{IC}_{50} (nmol) & \text{IC}_{50} (nmol) & \text{IC}_{50} (nmol) & \text{IC}_{50} (nmol) & \text{IC}_{50} (nmol) \\
\hline
T65 & 31.3 ± 0.9 & 34.7 ± 4.3 & 15.4 ± 6.3 & 271.8 ± 20.7 & K & N & S & N & D \\
T17 & 14.7 ± 6.3 & 18.6 ± 6.3 & 21.4 ± 0.6 & 97.7 ± 11.3 & K & N & S & N & D \\
T44 & 12.0 ± 0.7 & 28.0 ± 0.2 & 28.1 ± 2.7 & 199.7 ± 52.0 & K & N & S & N & D \\
T60 & 31.2 ± 3.6 & 30.0 ± 5.3 & 28.5 ± 5.8 & 235.9 ± 3.2 & K & N & S & N & D \\
T4 & 16.0 ± 1.3 & 26.2 ± 3.3 & 36.1 ± 0.1 & 321.2 ± 68.8 & K & N & S & N & D \\
T43 & 31.2 ± 0.2 & 38.1 ± 0.1 & 40.0 ± 3.5 & 398.2 ± 67.3 & K & N & S & N & D \\
*T16 & 13.0 ± 0.1 & 23.7 ± 0.5 & 42.4 ± 5.0 & 150.1 ± 11.8 & K & N & S & N & D \\
T22 & 38.3 ± 2.7 & 15.5 ± 1.2 & 106.1 ± 7.3 & 348.3 ± 0.9 & T & N & S & N & D \\
T59 & 16.2 ± 2.1 & 26.2 ± 5.4 & 108.0 ± 1.7 & 338.0 ± 63.6 & T & N & S & N & D \\
T54 & 26.3 ± 0.4 & 10.6 ± 0.1 & 131.6 ± 47.4 & 291.8 ± 7.4 & T & Y & S & N & D \\
T40 & 35.6 ± 3.6 & 23.0 ± 0.6 & 133.4 ± 5.5 & 208.4 ± 4.6 & T & N & S & N & D \\
T29 & 36.1 ± 7.1 & 9.2 ± 0.3 & 134.2 ± 20.8 & 214.2 ± 5.7 & T & Y & S & N & D \\
T30 & 13.1 ± 2.7 & 11.0 ± 0.5 & 183.2 ± 21.5 & 426.2 ± 50.1 & T & N & S & D & D \\
T25 & 18.6 ± 6.8 & 32.3 ± 0.1 & 239.7 ± 12.3 & 289.7 ± 47.6 & T & Y & S & N & D \\
T9 & 15.4 ± 2.1 & 27.2 ± 3.3 & 292.2 ± 2.9 & 145.3 ± 24.8 & T & N & S & N & D \\
T64 & 36.9 ± 2.2 & 5.4 ± 0.1 & 335.6 ± 1.6 & 263.5 ± 10.2 & T & Y & S & N & D \\
T15 & 15.5 ± 3.6 & 5.5 ± 1.1 & 357.8 ± 27.8 & 532.3 ± 2.6 & T & N & S & N & D \\
T3 & 14.6 ± 0.3 & 19.9 ± 0.4 & 387.1 ± 8.9 & 465.9 ± 22.6 & T & N & S & D & D \\
T8 & 16.0 ± 0.1 & 33.1 ± 4.2 & 421.3 ± 14.9 & 236.8 ± 33.2 & T & N & S & N & D \\
TOR# & — & 20 & 100 & 450 & \\
\hline
\end{tabular}
\caption{The response of \textit{Plasmodium falciparum} in Vietnam to chloroquine, mefloquine, quinine, and artemisinin.}
\end{table}

\* Threshold of resistance.
\* Isolates below this line are defined as chloroquine-resistant.

The primary and secondary isolates were analyzed to determine whether the parasites isolated after artemisinin treatment represented a breakthrough or a new infection (Table 3). The \textit{msp2} gene of each isolate was analyzed by PCR and the allele type classified as different (D) or the same size (S) as judged by mobility of the DNA fragment on agarose gels (Table 3). The parasites isolated after artemisinin treatment that had different \textit{msp2} alleles must result from either a new infection or, alternatively, a breakthrough of a minor parasite population from the original infection. The parasite isolates that had an \textit{msp2} allele of the same size are likely to represent a breakthrough of the original infection through treatment with artemisinin. Only six of the parasite isolates obtained after artemisinin had \textit{msp2} alleles of the same size as the original isolates, suggesting that these were recrudescent infections. Three isolates had mixed \textit{msp2} alleles. Nine of the recurrent isolates had different \textit{msp2} alleles, which must have originated from new infections or selection of a population from a primary infection with multiple parasites with different \textit{msp2} alleles.

\* The genotypes for \textit{pfcrt} and \textit{pfmdr1} for the primary and secondary isolates. Genomic DNA from the primary and secondary isolates was analyzed as to the genotype of the \textit{pfcrt} and \textit{pfmdr1} genes to determine whether they were linked to
the drug resistance phenotypes. There was a perfect correlation with the presence of Tyr-76 encoded by the \textit{pfcrt} gene in both primary and secondary chloroquine-resistant isolates. Statistical analysis using Fisher’s exact test showed that \textit{pfcrt} was highly linked to the chloroquine resistance phenotype \((P = 0.00002)\). This is consistent with an important role for the protein encoded by the \textit{pfcrt} gene in the chloroquine resistance phenotype.\(^7\) Of the 12 chloroquine-resistant primary isolates, four encoded Tyr-86 and two had Asp-1042, which also were linked to chloroquine resistance. This is consistent with previous results, and most isolates that have mutations in \textit{pfcrt} showed an inverse correlation to chloroquine resistance. This is consistent with previous results, and most isolates that have mutations in \textit{pfcrt} showed an inverse correlation to chloroquine resistance.\(^8\) All the secondary isolates had a wild-type sequence for the \textit{pfcrt} gene.\(^7,8,34,35\) Statistical analysis of the presence of mutations in \textit{pfmdr1} showed that they also were linked to chloroquine resistance \((P = 0.043)\). The responses for the 12 chloroquine-resistant isolates are consistent with an important role in chloroquine resistance for \textit{pfcrt} mutations\(^7\) and involvement of \textit{pfmdr1} in modulation of the level of chloroquine resistance.\(^8\)

<table>
<thead>
<tr>
<th>Isolates</th>
<th>IC(_{50}) Artemisinin</th>
<th>IC(_{50}) Mefloquine</th>
<th>Pfcrt genotype</th>
<th>Pfmdr1 genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>T43-R25</td>
<td>23.9 ± 1.5</td>
<td>32.4 ± 2.6</td>
<td>21.0 ± 0.7</td>
<td>S</td>
</tr>
<tr>
<td>T64-R18</td>
<td>18.4 ± 1.0</td>
<td>49.3 ± 3.9</td>
<td>33.6 ± 4.6</td>
<td>S</td>
</tr>
<tr>
<td>T4-R19</td>
<td>11.7 ± 1.4</td>
<td>36.9 ± 4.5</td>
<td>21.2 ± 1.0</td>
<td>S</td>
</tr>
<tr>
<td>T6-R21</td>
<td>20.5 ± 1.7</td>
<td>25.0 ± 0.8</td>
<td>21.2 ± 0.7</td>
<td>D</td>
</tr>
<tr>
<td>T17-R14</td>
<td>9.57 ± 1.0</td>
<td>53.3 ± 5.9</td>
<td>40.6 ± 3.6</td>
<td>N</td>
</tr>
<tr>
<td>T61-R17</td>
<td>36.9 ± 2.2</td>
<td>53.3 ± 5.9</td>
<td>40.6 ± 3.6</td>
<td>S</td>
</tr>
<tr>
<td>T44-R14</td>
<td>24.0 ± 1.9</td>
<td>40.3 ± 3.7</td>
<td>45.7 ± 2.4</td>
<td>D</td>
</tr>
<tr>
<td>T29-R18</td>
<td>18.7 ± 1.5</td>
<td>36.4 ± 8.9</td>
<td>48.0 ± 7.5</td>
<td>S</td>
</tr>
</tbody>
</table>

\(^7\) The drug resistance phenotypes.\(^7,8,34,35\) There was a perfect correlation with the presence of Tyr-76 encoded by the \textit{pfcrt} gene in both primary and secondary chloroquine-resistant isolates. Statistical analysis using Fisher’s exact test showed that \textit{pfcrt} was highly linked to the chloroquine resistance phenotype \((P = 0.00002)\). This is consistent with an important role for the protein encoded by the \textit{pfcrt} gene in the chloroquine resistance phenotype.\(^7\) Of the 12 chloroquine-resistant primary isolates, four encoded Tyr-86 and two had Asp-1042, which also were linked to chloroquine resistance. This is consistent with previous results, and most isolates that have mutations in \textit{pfcrt} showed an inverse correlation to chloroquine resistance. This is consistent with previous results, and most isolates that have mutations in \textit{pfcrt} showed an inverse correlation to chloroquine resistance.\(^8\) All the secondary isolates had a wild-type sequence for the \textit{pfcrt} gene.\(^7,8,34,35\) Statistical analysis of the presence of mutations in \textit{pfmdr1} showed that they also were linked to chloroquine resistance \((P = 0.043)\). These results are consistent with an important role in chloroquine resistance for \textit{pfcrt} mutations\(^7\) and involvement of \textit{pfmdr1} in modulation of the level of chloroquine resistance.\(^8\)

### Table 4

**Response of \textit{Plasmodium falciparum} isolates to pyrimethamine compared with their \textit{dhfr} and \textit{dhps} genotypes**

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Pyrimethamine (IC_{50}) ((\mu)g/mL)</th>
<th>\textit{dhfr} genotype</th>
<th>\textit{dhps} genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>T43</td>
<td>0.006 A N C S I F G K A A</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>T65</td>
<td>0.008 A I R S I S G K A A</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>T65</td>
<td>0.013 A I C S I S G K A A</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>T65</td>
<td>0.022 A N C S I F G K A A</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>1.63 A N R N I S G E A S</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>T17</td>
<td>5.25 A I R N I S G K A A</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>T54</td>
<td>6.31 A I R N I S A K G A A</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>T22</td>
<td>6.33 A I R N I S G K A A</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>T40</td>
<td>7.50 A I R N L S G E A S</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>T30</td>
<td>8.61 A I R N L S G E A S</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>T9</td>
<td>9.33 A I R N L S G E A S</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>T15</td>
<td>9.57 A I R N L S G E A S</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>T16</td>
<td>10.92 A N R N L F G K A A</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>T54</td>
<td>15.95 A I R N I S G K A A</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>T64</td>
<td>16.29 A I R N I S G K A A</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>T25</td>
<td>18.61 A I R N I S G K A A</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>T59</td>
<td>21.76 A I R N L S G E A A</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>25.68 A I R N L S G E A S</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>T8</td>
<td>28.96 A I R N I F G E G A</td>
<td>A</td>
<td></td>
</tr>
</tbody>
</table>

\(^8\) The amino acid positions within \textit{dhfr} associated with pyrimethamine resistance.

\(^8\) The amino acid positions in \textit{dhps} associated with sulfadoxine resistance (Triglia et al., 1998).

\(^8\) Isolates above this line are defined as pyrimethamine sensitive while those below are resistant.
The **dhfr and dhps genotypes of *P. falciparum* isolates from Vietnam.** To test whether the *P. falciparum* isolates were multidrug-resistant, we obtained the sequence of the *dhfr* and *dhps* genes to determine if they encoded mutations involved in resistance to pyrimethamine and sulfadoxine (Fansidar). Additionally, we determined the sensitivity of the primary parasites for pyrimethamine. Four of the isolates were sensitive to pyrimethamine, and all had Ser-108 whereas all the pyrimethamine-resistant isolates had Asn-108 (Table 4). This is consistent with the essential role of Asn-108 in the mechanism of resistance to this antimalarial. Additionally, the pyrimethamine-resistant isolates had a number of other mutations within *dhfr* that have been shown previously to be involved in higher levels of resistance.

The sequence of the *dhps* gene also was obtained for all the isolates to determine whether they encoded mutations associated with resistance to sulfadoxine (Table 4). None of the isolates had a wild-type *dhps* gene, suggesting that there has been strong selection for mutations in this gene. This is consistent with the high levels of pyrimethamine resistance observed in these isolates.

**DISCUSSION**

Malaria is still a health concern in Vietnam, and the spread of drug-resistant *P. falciparum* has made the selection of appropriate antimalarials for control and treatment problematic. In 1996, the level of *P. falciparum* chloroquine resistance was approximately 60%, and sulfonamide resistance was 25–40%. The introduction of artemisinin and its widespread availability as a first-line treatment have been important changes in the clinical management of malaria in Vietnam, and the use of artemisinin for treatment and prophylaxis probably has reduced the number of deaths due to malaria. The development of decreased sensitivity to artemisinin would threaten its effectiveness. In this study, we have investigated the possibility of selection for decreased artemisinin sensitivity in *P. falciparum* parasites isolated after patients were treated with artemisinin. No detectable decrease in artemisinin sensitivity was observed. However, there was a statistically significant increase in mefloquine resistance of these isolates after artemisinin treatment failure.

Previous studies of artemisinin sensitivity have shown a wide variation in the level of sensitivity of *P. falciparum*. This suggests that there may be genetic alterations that can confer differential sensitivity to artemisinin. Transfection of mutations into the *pfmdr1* gene has altered the level of sensitivity to artemisinin, suggesting that the gene is a factor in determining the level of artemisinin sensitivity; this agrees with other studies showing that mutations in *pfmdr1* confer increased sensitivity to artemisinin in the progeny of a genetic cross of *P. falciparum*. An important finding of these studies was that the altered sensitivity observed with artemisinin also was seen with mefloquine, consistent with a role for *pfmdr1* in both of these phenotypes. The observation in this study that the *P. falciparum* isolates obtained after drug treatment were more mefloquine-resistant suggests that artemisinin treatment has directly selected for this increased drug resistance. This is consistent with the absence of *pfmdr1* mutations in all the parasite isolates obtained after artemisinin treatment, whereas six of the primary isolates had mutations in this gene.

If artemisinin treatment selected for increased mefloquine resistance and the absence of mutations in *pfmdr1*, then why was there no statistically significant difference seen for artemisinin? Previously, it has been shown that the measurement of artemisinin sensitivity is highly influenced by the inoculum effect in *P. falciparum*. The differences in artemisinin sensitivity that might be expected between the primary isolates and those isolated after artemisinin treatment would be quite small. Consequently, it may be difficult to quantitate the differences accurately enough to observe a slight decrease in artemisinin sensitivity. It will be interesting to follow artemisinin sensitivity in Vietnam over time to determine whether decreased sensitivity, and perhaps resistance, to this drug is developing.

It is clear from this study that T-76 in the *pfcr* gene is tightly linked to chloroquine resistance, as all chloroquine-resistant isolates had this mutation whereas all chloroquine-sensitive isolates had the wild-type sequence. This is consistent with an essential role for *pfcr* mutations in *P. falciparum* resistance to chloroquine. Mutations in the *pfmdr1* gene also were linked to chloroquine resistance. However, it is clear that they are not required for resistance, as some chloroquine-resistant isolates had wild-type sequence for this gene. Previous results have suggested that mutations in *pfmdr1* play a role in higher levels of chloroquine resistance, and the results presented here are consistent with this.

The increased mefloquine resistance of the secondary isolates was concomitant with a decrease in the level of chloroquine resistance, which has been previously observed in *in vitro* selected *P. falciparum* lines. Selection of parasites for increased mefloquine resistance resulted in a decrease in the level of chloroquine resistance as well as amplification of the *pfmdr1* gene. This was consistent with involvement in these phenotypes of increased expression of the protein encoded by *pfmdr1*. Transfection of different *pfmdr1* alleles into *P. falciparum* also had an inverse effect on resistance for chloroquine compared with that of mefloquine. The observations in this study, that the secondary parasites were more mefloquine- and less chloroquine-resistant and encoded the wild-type *pfmdr1* allele, are consistent with direct selection on this gene that alters sensitivity to these drugs. This selection must have occurred during artemisinin treatment.

Although Fansidar was not used for treatment in this study, it was of interest to determine whether these parasites were resistant to multiple drugs. We therefore tested the level of sensitivity to pyrimethamine and the genotype of the *dhfr* and *dhps* genes that have previously been shown to contain specific mutations that encode resistance to these drugs. We did not determine the sensitivity to sulfadoxine because of the difficulty of obtaining accurate measurements for this drug *in vitro*. The presence of Asn-108 in DHFR showed a complete linkage with pyrimethamine resistance, as expected from previous results. Only four isolates were sensitive to pyrimethamine, and all had mutations in the *dhps* gene, suggesting that there has been strong Fansidar pressure against *P. falciparum* in this area of Vietnam. The Gly-437 mutation in DHPS was observed in all isolates except one, consistent with this mutation’s being the first in this enzyme as a result of sulfa drug pressure.

The emergence of *P. falciparum* parasites that are resistant to multiple antimalarials has caused a major dilemma in the
selection of suitable drugs for the control and treatment of malaria caused by this parasite. Artemisinin is an important antimalarial that is effective against \textit{P. falciparum} parasites resistant to drugs such as chloroquine. The increasing identification of parasites that are resistant to many antimalarials, as well as the potential for the development of artemisinin resistance, is a major cause for concern. It is important that the level of artemisinin sensitivity of \textit{P. falciparum} be monitored in areas such as Vietnam where the drug is extensively used to allow early detection of the development of resistance.

Received May 2, 2002. Accepted for publication October 7, 2002.

Acknowledgments: We thank David Warhurst for providing information before publication that allowed us to genotype the \textit{pfmdr1}, \textit{dhfr}, and \textit{dhps} genes. We would like to acknowledge the Australian Red Cross Blood Bank in Melbourne for supplying human erythrocytes and serum, and the Australian Agency for International Development for support of the field work. AFC is an International Research Scholar of the Howard Hughes Medical Institute.

Financial support: This work is supported by a grant from the National Health and Medical Research Council of Australia.

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