CONCISE COMMUNICATION

Rapid Selection of \textit{Plasmodium falciparum} Dihydrofolate Reductase Mutants by Pyrimethamine Prophylaxis

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A prospective study was conducted to measure the selective effect of pyrimethamine prophylaxis on point mutations in \textit{Plasmodium falciparum} dihydrofolate reductase (DHFR). A total of 109 Malian children were given pyrimethamine weekly for 5 weeks. \textit{P. falciparum} infections were analyzed by polymerase chain reaction for DHFR mutations, which were dramatically more frequent among prophylaxis-breakthrough infections than at baseline: the prevalence of Asn-108 rose from 13% to 100%, Ile-51 from 4% to 50%, and Arg-59 from 11% to 90%. Eight persistent infections lacking detectable DHFR mutations at baseline developed multiple mutations within 1 week of the patients' starting pyrimethamine prophylaxis. Microsatellite analysis found no evidence of clonal identity among baseline and breakthrough infections. Analysis of these data demonstrates that under prophylaxis conditions, pyrimethamine is strongly selective for DHFR mutations, which arise extremely rapidly under drug pressure, even when undetectable in the initial infection. These findings have implications for prophylaxis regimens with other antifolate drugs.

As chloroquine-resistant \textit{Plasmodium falciparum} malaria spreads across Africa, pyrimethamine-sulfadoxine is the only available and affordable alternative for the treatment of uncomplicated malaria. Pyrimethamine alone has been used for chemoprophylaxis of malaria, although it is less effective than other available drugs [1]. In vitro resistance of \textit{P. falciparum} to pyrimethamine and the other dihydrofolate reductase (DHFR) inhibitors is caused by specific point mutations in \textit{P. falciparum} DHFR [2]. These mutations alter the shape of the active site cavity where the DHFR inhibitors bind the enzyme, reducing the binding affinities for drugs. A single point mutation causing a Ser$\rightarrow$Asn change at codon 108 causes moderate pyrimethamine resistance, and the addition of Asn$\rightarrow$Ile-51 mutations or Cys$\rightarrow$Arg-59 mutations or both confers higher levels of resistance. Ile$\rightarrow$Leu-164, when combined with Asn-108 and Ile-51, with Asn-108 and Arg-59, or with all 3 confers high-level pyrimethamine resistance, but this mutation has not yet been detected in Africa. A 5-amino acid repetitive insert near the active site pocket, termed the Bolivia repeat, is common in South America [3] but does not appear to play a role in resistance and may be a compensatory mutation [4]. Neither this insert nor a Cys$\rightarrow$Arg-50 mutation associated with pyrimethamine resistance in South America has been found in Africa. Point mutations in the \textit{P. falciparum} gene encoding dihydropteroate synthase (DHPS), the target enzyme of sulfadoxine, are similarly associated with sulfadoxine resistance in vitro and in epidemiological surveys [3, 5].

Studies in Tanzania by Clyde and Shute [6] in the 1950s found that in vivo parasitological resistance to pyrimethamine arose within months under prophylaxis conditions, and drug pressure is generally assumed to be responsible for the development and spread of antifolate-resistant malaria. Several studies have shown that DHFR mutations are selected after a single episode of pyrimethamine-sulfadoxine treatment [7–10]. These studies
have not addressed the rapidity with which mutations arise, nor have studies been done on the selective effect of antifolate drugs under prophylaxis conditions. Although pyrimethamine is now rarely used for malaria prophylaxis, another antifolate combination, trimethoprim-sulfamethoxazole, is being recommended for prophylaxis of opportunistic infections in persons with human immunodeficiency virus (HIV) infection or AIDS in Africa, where HIV and malaria share common distributions [11, 12]. A better understanding of the impact of prophylaxis regimens on drug-resistant genotypes will help to inform antifolate prophylaxis policies.

To elucidate the specific role of DHFR mutations on pyrimethamine resistance in vivo and the effect of pyrimethamine drug pressure under prophylaxis conditions, we conducted a prospective study of weekly pyrimethamine prophylaxis on \textit{P. falciparum} DHFR genotypes in a rural Malian village where antifolates were not in use. We hypothesized that the presence of DHFR mutations before beginning prophylaxis would be predictive of persistent infection and prophylaxis failure and that pyrimethamine would select for DHFR mutations during a short prophylaxis period.

\section*{Materials and Methods}

\textbf{Ethical review.} The study protocol was reviewed and approved by Institutional Review Boards at the University of Maryland and the University of Mali.

\textbf{Study site and inclusion criteria.} Tieneguebougou is a rural village of \textasciitilde \textasciitilde 200 residents 30 km northeast of Bamako, Mali. \textit{P. falciparum} malaria is holoendemic, with intense seasonal peaks of infection and disease. Parasitemia prevalence rates are 40\%–50\% in the dry season (November to May) and 70\%–85\% in the rainy season (June to October). Most residents of Tieneguebougou are subsistence farmers, and there is little cash-based economic activity. A village dispensary uses chloroquine to treat uncomplicated malaria and quinine to treat severe malaria. No use of pyrimethamine-sulfadoxine or other antifolates was reported by villagers or village health workers, nor was pyrimethamine-sulfadoxine found in the village dispensary at the time of the study.

The study was conducted during the 1996 rainy season. Children 2–12 years old who had no significant health problems or contraindications to taking pyrimethamine and whose parents gave informed consent were considered for inclusion in the study.

\textbf{Pyrimethamine prophylaxis and follow-up.} Standard prophylaxis doses of pyrimethamine were given weekly for 5 weeks (a quarter tablet for patients 2–3 years old, a half tablet for those 4–10 years old, and a whole tablet for those 11–12 years old; 1 tablet contained 25 mg pyrimethamine). Subjects were followed actively on days 7, 14, 21, 28, 35, and 42 and passively by 24-h availability of a study clinician to evaluate and treat study subjects who presented with medical complaints. At all active and passive follow-up times, malaria smears and filter-paper samples were collected from patients’ fingers. Filter papers were air dried and stored for later polymerase chain reaction (PCR) analysis or restriction digestion analysis of DHFR or both, and we also analyzed DHPS mutations by use of primers and conditions as described elsewhere [3].

\textbf{Microsatellite analysis.} Microsatellite analyses were performed to determine whether clonal or strain identity existed among pre- and postprophylaxis infections. The microsatellite markers ta99, CAL, ta87, ta21, and “DHFR,” located on chromosomes 3, 14, 6, and 4, respectively [13], were amplified by a nested PCR by use of primers described on the Internet at http://www.ncbi.nlm.nih.gov/Malaria/Mapsmarkers/PfGMap/PfGMap.html, with modifications as described at http://medschool.umaryland.edu/CVD/plowe.html.

\textbf{Statistical analysis.} Prevalence rates of mutations were compared by use of $\chi^2$ or Fisher’s exact tests for 2-tailed significance at $P = .05$.

\section*{Results}

The villagewide survey found a low overall prevalence of DHFR mutations before starting the prophylaxis study: 12.8\% Asn-108, 4.2\% Ile-51, and 10.9\% Arg-59 ($n = 119$, figure 1). The Leu-164 mutation was not seen in any pre- or postprophylaxis infections. The prevalence of DHFR mutations at day 0 among infections that cleared was not different from the prevalence among those that did not clear after prophylaxis

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Prevalence of dihydrofolate reductase mutations in \textit{Plasmodium falciparum} infections before pyrimethamine prophylaxis and in breakthrough infections occurring during prophylaxis in a Malian village.}
\end{figure}
was started (data not shown), indicating that DHFR mutations were not predictive of prophylaxis failure. At day 0, 52 subjects had asymptomatic *P. falciparum* infections, and 57 had no parasitemia. Fifty-two breakthrough infections (new infection after initial absence or clearance of parasitemia) occurred in 46 subjects. The prevalence of DHFR mutations in the breakthrough infections rose to 100% Asn-108, 50% Ile-51, and 90% Arg-59 (n = 52; figure 1). The increase in prevalence at each allele was significant (P < .05).

Eight subjects (15% of those with parasitemia at day 0) had persistent parasitemia, failing to clear their asymptomatic infections for at least 1 week after starting pyrimethamine prophylaxis. All 8 had only wild-type DHFR detected at day 0, and all 8 had Asn-108 mutations and Ile-51 mutations or Arg-59 mutations or all 3 at day 7. The striking finding that multiple DHFR mutations arose after a single week of pyrimethamine pressure raised the question of whether the mutations arose de novo in just 1 week or were present on day 0 at levels below the detection threshold of the PCR assay. To increase the sensitivity of the PCR, we digested the amplified DHFR domain of these samples with the restriction enzyme *Alu*I, which cuts the wild-type sequence at codon 108, so that only DHFR containing a mutation at codon 108 would remain intact. We then again attempted to amplify the digestion product by use of primers specific for the Asn-108 mutation; however, we saw no amplification, suggesting that the mutations could have occurred spontaneously under pressure of the drug. Nevertheless, it remains possible that a minuscule number of mutant parasites, too few to detect even with these techniques, were present at day 0 and expanded under drug pressure.

To assess this possibility, we analyzed the pre- and postprophylaxis samples with microsatellite markers to provide a genetic fingerprint of the infections. PCR primers chosen on the basis of conserved-sequence flanking polymorphic noncoding repeat regions throughout the genome are used to amplify these variable regions as neutral markers to identify genetic identity or nonidentity among field isolates. The use of several markers enabled us to distinguish genetically distinct infections. An identical microsatellite pattern that used several markers strongly suggests genetically identical infections; different sized products from ≥1 microsatellite amplification signifies genetic nonidentity. One of the markers is in the promoter region of the gene encoding DHFR. Identical microsatellite patterns at days 0 and 7 (particularly with the marker in the DHFR promoter region) would have supported spontaneous mutation in otherwise genetically identical infections. However, we found a different microsatellite pattern between day 0 and day 7 in all 8 persistent infections. Three subjects had persistent asymptomatic infection at weekly intervals for 4 weeks after starting pyrimethamine prophylaxis. Microsatellite analysis of those infections, as shown in figure 2, indicates that each individual’s infections were genetically distinct at each time, with the exception of subject B, whose infections on days 7 and 14 were identical with respect to the microsatellite markers used.

DHPS mutations were also analyzed in pre- and postprophylaxis infections. Only the Ser-436 and Gly-437 mutations were detected in this setting, and there was no evidence of selection of these 2 mutations by pyrimethamine (data not shown), confirming that pyrimethamine does not select for DHPS mutations.

**Discussion**

Whether DHFR mutations arise de novo under drug pressure or exist at a very low background rate, this study demonstrates that even where there is little use of antifolates and baseline prevalence of DHFR mutations is low, DHFR mutations arise very rapidly under the sustained drug pressure of prophylaxis. The lack of association between DHFR genotype at day 0 and prophylaxis failure may result from the low baseline prevalence of the highly pyrimethamine-resistant triple DHFR mutant or from the importance of host immunity and other factors unrelated to intrinsic resistance that affect prophylaxis efficacy.

We could document no pyrimethamine-sulfadoxine use in this small rural village. Trimethoprim is cross-resistant with pyrimethamine in vitro [14], and the intermittent use of trimethoprim-sulfamethoxazole (Bactrim, Septra, Septin, or Cotrimoxazole) for treating bacterial infections could account for a low background rate of DHFR mutations. One study reported that trimethoprim-sulfamethoxazole treatment of *falciparum* malaria selected a previously unreported DHFR genotype with wild-type Ser-108 and the Ile-51 and Arg-59 mutations [15]. However, this novel genotype has not been found in several global surveys of DHFR genotypes, even in...
areas where trimethoprim-sulfamethoxazole is widely used for bacterial infections [3, 7], nor was the identification of this unusual genotype by restriction digestion confirmed by direct DNA sequencing. The hypothesis that trimethoprim and pyrimethamine resistance are caused by different DHFR mutations is at odds with the results molecular epidemiological surveys and in vitro studies [14] and requires independent confirmation.

Although pyrimethamine is now rarely used for prophylaxis, recent studies have supported the use of long-term trimethoprim-sulfamethoxazole prophylaxis for persons with HIV infection or AIDS in Africa [11, 12]. The potential impact of widespread trimethoprim-sulfamethoxazole prophylaxis on antifolate-resistant malaria in areas where both malaria and HIV are prevalent should be carefully assessed in light of this evidence of rapid selection for DHFR mutants under prophylaxis conditions.

Acknowledgments

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References