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Genetics

Evidence that a point mutation in dihydrofolate reductase–thymidylate synthase confers resistance to pyrimethamine in falciparum malaria

(*Plasmodium falciparum*/drug resistance/folic acid antagonists/genetic linkage analysis/polymerase chain reaction)

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ABSTRACT **Analysis of a genetic cross of *Plasmodium falciparum* and of independent parasite isolates from Southeast Asia, Africa, and South America indicates that resistance to pyrimethamine, an antifolate used in the treatment of malaria, results from point mutations in the gene encoding dihydrofolate reductase–thymidylate synthase (EC 1.5.1.3 and EC 2.1.1.45, respectively). Parasites having a mutation from Thr-108/Ser-108 to Asn-108 in DHFR–TS are resistant to the drug. The Asn-108 mutation occurs in a region analogous to the C α-helix bordering the active site cavity of bacterial, avian, and mammalian enzymes. Additional point mutations (Asn-51 to Ile-51 and Cys-59 to Arg-59) are associated with increased pyrimethamine resistance and also occur at sites expected to border the active site cavity. Analogies with known inhibitor/enzyme structures from other organisms suggest that the point mutations occur where pyrimethamine contacts the enzyme and may act by inhibiting binding of the drug.**

Dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3) catalyzes the conversion of dihydrofolate to tetrahydrofolate, a cofactor required for the biosynthesis of thymidylate, purine nucleotides, and certain amino acids. Differences in the structure of the vertebrate and microbial enzymes have made DHFR an excellent target for chemotherapeutic agents. In malaria, drugs such as proguanil and pyrimethamine are potent inhibitors of the *Plasmodium* enzyme and have been widely used in chemotherapy for nearly 40 years (1–3). The effectiveness of these agents, however, has been blunted by the appearance of drug-resistant parasite strains. To determine the nature of pyrimethamine resistance, investigators have directed studies to two mechanisms by which changes in drug susceptibility might occur. Several reports have implicated overproduction of the enzyme (4, 5) or overproduction of a structurally altered enzyme (6, 7). Other investigators have correlated resistance with altered binding of the inhibitor as a result of a structural change in the enzyme (8–12).

In this work we examine the molecular basis of pyrimethamine resistance in isolates and clones of *Plasmodium falciparum* from geographically distant sources. We show through a genetic cross that resistance in a Honduran clone, HB3, is linked to a point mutation to Asn-108 in the DHFR–thymidylate synthase (TS; 5,10-methylenetetrahydrofolate: dUMP *C*-methyltransferase, EC 2.1.1.45) gene. Sequence analysis of other *P. falciparum* isolates and clones confirms this mutation in resistant parasites and, in addition, identifies at least two other point mutations that are associated with increased resistance.

MATERIALS AND METHODS

Parasite Cultures and Pyrimethamine Susceptibility Assays. Parent and progeny clones from a *P. falciparum* HB3×3D7 cross (13) were cultivated *in vitro* by standard methods (14). Pyrimethamine susceptibilities were determined by the method of Thaithong and Beale (15). Parasites were cultured in microtiter plates and exposed to pyrimethamine at serial dilutions of 0.5 to 32,768 ng/ml, with one change of medium at 48 hr. Drug tests of all clones were performed concurrently with identical lots of serum and cells. Giemsa-stained blood smears were examined to determine drug susceptibility.

DNA Extraction. Parasites were obtained from infected erythrocytes by lysis in TSE (100 mM NaCl/50 mM EDTA/20 mM Tris, pH 8.0) containing 0.15% saponin. After centrifugation at 5000 × *g*, the parasites were resuspended in 2% (vol/vol) Triton X-100 in TSE. Nuclei were collected by centrifugation at 2000 × *g*, resuspended in TSE, and lysed by addition of sodium dodecyl sulfate (SDS) to 2% and $NaClO_4$ to 0.5 M. The lysate was gently mixed at room temperature for 1 hr, extracted twice with phenol equilibrated in TE, and finally extracted twice with phenol/chloroform. The solution was brought to 0.2 M in sodium acetate, one and one-half volumes of 95% EtOH were added, and the DNA was spooled out of solution. Southern blotting was performed by standard methods (16).

Pulsed-Field Gradient (PFG) Electrophoresis. Parasites were embedded in agarose blocks for PFG electrophoresis as described (17). PFG gel electrophoresis (18) was performed by using the apparatus described by Carle and Olson (19). Electrophoresis conditions and the methods for isolation and restriction of DNA are reported elsewhere (20).

DNA Probes. Chromosome 4-specific probes were isolated from genomic libraries constructed in pUC vectors (20). The *P. falciparum* histidine-rich protein II (HRP-II) gene was detected with pDL4.1 (21). The *P. falciparum* TS gene probe was kindly provided by Mary Morry and George Cross, The Rockefeller University, New York.

Polymerase Chain Reactions (PCRs) and Nucleotide Sequencing. The DHFR domain of the *P. falciparum* DHFR–TS gene was amplified from 50 ng of genomic DNA by the PCR method (22). *Thermus aquaticus* (*Taq*) polymerase was obtained from Perkin–Elmer/Cetus and used according to the recommended protocol. Sequencing of the amplified DNA was performed by two methods. In the first, the PCR amplification was performed by using a primer with an attached T7 phage promoter, and RNA transcripts from the amplified product were sequenced by using reverse transcrip-

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Abbreviations: DHFR, dihydrofolate reductase; TS, thymidylate synthase; HRP-II, histidine-rich protein II; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; PFG, pulsed-field gradient.

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tase (23). In the second method, direct sequencing of the amplified DNA was performed by the method of Higuchi *et al.* (24). Primers used for the PCR and for sequencing are described in the legend to Fig. 3.

RESULTS

Linkage of Pyrimethamine Resistance to the DHFR–TS Gene. In studies of chromosome size polymorphisms among the progeny clones from the *P. falciparum* HB3×3D7 cross (13, 17, 20), we observed that progeny having a 3D7-size chromosome 4 had inherited the same pyrimethamine sensitivity as the 3D7 parent. Progeny having an HB3-size chromosome 4, or one of intermediate size, exhibited pyrimethamine resistance at the level of the HB3 parent. Restriction fragment length polymorphism (RFLP) analysis with chromosome 4-specific probes was therefore performed (Table 1). Two probes, pC4.H42 and pC4.H31, showed linkage to resistance in all progeny. A third probe, pC4.D21, was linked in all progeny except for clone XP5, indicating that the gene involved in resistance was located opposite of the crossover mapped in XP5 chromosome 4 (20).

To confirm the role of the DHFR–TS gene in pyrimethamine resistance, we examined the inheritance of a RFLP of the gene and determined its chromosomal location. Hybridization with a probe specific for the TS domain of the *Plasmodium* DHFR–TS gene revealed a *Hin*fI fragment of 4.3 kb from the pyrimethamine-sensitive 3D7 parent and a 4.2-kb fragment from the pyrimethamine-resistant HB3 parent. As indicated in Fig. 1 and Table 1, all pyrimethamine-resistant progeny inherited the HB3 4.2-kb RFLP, whereas all sensitive progeny have the 3D7 4.3-kb RFLP.

The chromosomal location of the DHFR–TS gene was determined by hybridizing the TS gene probe to restriction fragments of *P. falciparum* chromosome 4. Fig. 2 shows that the DHFR–TS gene is positioned in a 380-kb *Bgl* I fragment from the central region of the chromosome.

Identification of Point Mutations in the DHFR–TS Active Site. Resistance to DHFR inhibitors in various organisms has been shown to arise by diverse mechanisms, including gene amplification (25), point mutations at the active site of the gene (26, 27), and alteration of drug uptake (28). To determine if gene amplification could account for pyrimethamine resistance in parasites from the *P. falciparum* cross, we compared hybridization intensities of the TS probe to genomic DNA from the parent and progeny clones (Fig. 1*A*). Hybridization signals from control experiments with the HRP-II gene, known to be single copy in the 3D7 and HB3 parents (17), were used to confirm the amounts of genomic DNA from the different clones (Fig. 1*B*). Relative intensities of hybridization to HRP-II and DHFR–TS indicated no differences in the DHFR–TS gene copy number regardless of pyrimethamine resistance.

Table 1. Linkage of pyrimethamine resistance to the DHFR–TS locus on *P. falciparum* chromosome 4

		RFLP†			
Clone	Phenotype*	D21	H31	H42	DHFR
Parents					
3D7	S	1	1	1	1
HB3	R	2	2	2	2
Progeny					
X2	R	2	2	2	2
X6	R	2	2	2	2
X8	S	1	1	1	1
X10	S	1	1	1	1
XP1	R	2	2	2	2
XP2	R	2	2	2	2
XP3	R	2	2	2	2
XP4	R	2	2	2	2
XP5	R	1	2	2	2
XP8	R	2	2	2	2
XP9	R	2	2	2	2
XP10	R	2	2	2	2

*Drug sensitivities of the parents and progeny: R, pyrimethamine-resistant; S, pyrimethamine-sensitive (ID_{50} values of 3D7 and HB3 are given in Table 2).

†RFLPs were detected by Southern blot analysis of *Hin*fI-digested DNA. Chromosome 4-specific probes pC4.D21 (D21), pC4.H31 (H31), and pC4.H42 (H42) were hybridized and washed as described (20). DHFR–TS inheritance was determined as noted in Fig. 1.

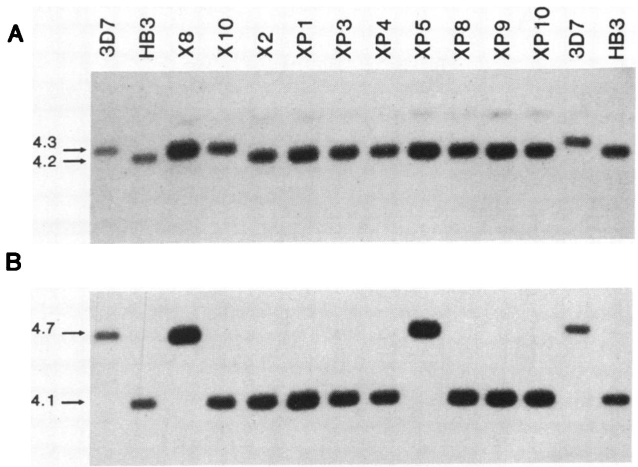

FIG. 1. RFLP analysis and copy number comparison of the DHFR–TS gene in pyrimethamine-resistant and -sensitive clones. (*A*) The *Plasmodium* TS gene probe detects a 4.2-kb *Hin*fI fragment in pyrimethamine-resistant clones and a 4.3-kb fragment in sensitive clones (compare with Table 1). (*B*) Signals from the HRP-II probe pDL4.1 show the same relative intensities as those from the TS probe. Comparable signals indicate that the DHFR–TS gene and the HRP-II gene do not vary in copy number among the clones. The nitrocellulose blot was stripped with 0.2 M NaOH/0.1% SDS between hybridizations as described (17).

The nucleotide sequences of the HB3 and 3D7 DHFR domains were compared by direct sequencing of DNA obtained by the PCR method (22). Fig. 3 shows the PCR strategy. The nucleotide sequences of the DHFR domains were found to be identical except for a point mutation in the HB3 gene, which encodes Asn-108 (AAC) instead of Ser-108 (AGC). Two progeny of the 3D7×HB3 cross were also examined. The DHFR domain of clone XP5 (pyrimethamine-resistant) encodes Asn-108, whereas that of clone X10 (pyrimethamine-sensitive) encodes Ser-108.

To determine whether other pyrimethamine-resistant *P. falciparum* parasites also have point mutations, we examined the pyrimethamine susceptibilities and DHFR domains of eight additional isolates and clones from geographically distant locations (Table 2). All of the pyrimethamine-sensitive parasites (3D7, SL/D6, L.E5, It.G2.F6, and FCR3) have Thr-108 (ACC) or Ser-108 (AGC). A mutation to Val-16 (GCA → GTA) also occurs in the FCR3 isolate. In contrast, all the resistant parasites exhibit the point mutation to Asn-108. The HB3 clone and Camp isolate tolerate a 100- to 200-fold increase in pyrimethamine. In the It.D12 and 7G8 clones, an additional mutation to Ile-51 (AAT → ATT) is associated with a 4- to 8-fold increase in resistance. Even higher levels of resistance occur in the Dd2 clone, which contains Asn-108, Ile-51, and a third mutation from Cys-59 to Arg-59 (TGT → CGT). Remarkably, full sequence data from the DHFR domains of the 3D7, SL/D6, FCR3, HB3, 7G8, and Dd2 genes and partial sequence data spanning mutations of the L.E5, It.G2.F6, Camp, and It.D12 genes revealed no other nucleotide variations.

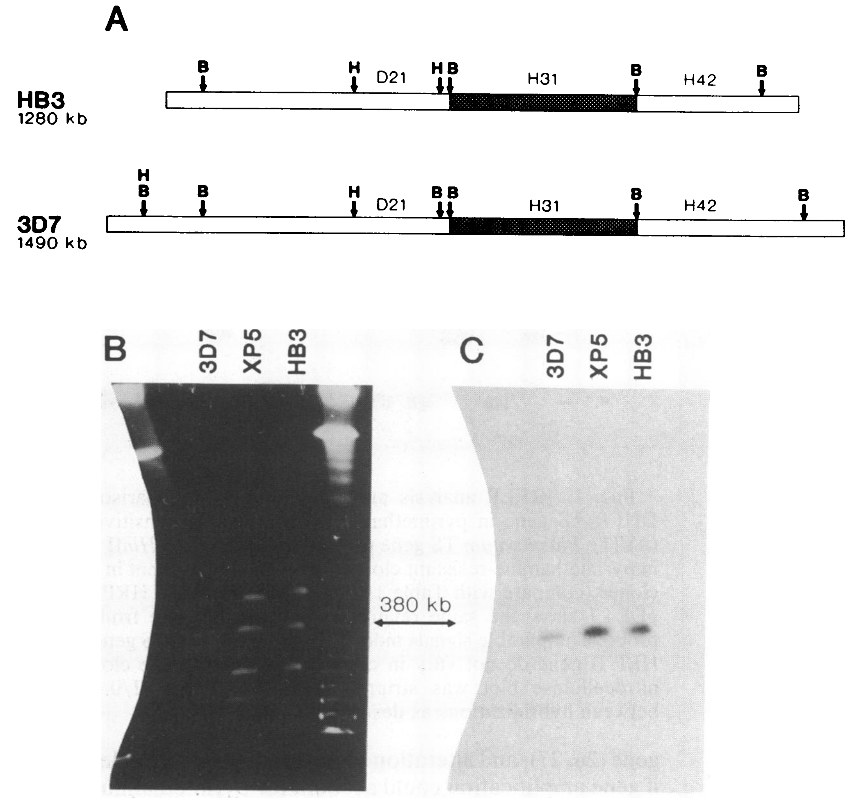

FIG. 2. Localization of the DHFR–TS gene to a 380-kb *Bgl* I restriction fragment from the central region of chromosome 4. (*A*) Full-length restriction maps of the 3D7 and HB3 chromosomes show the positions of restriction fragments containing the pC4.D21 (D21), pC4.H31 (H31), and pC4.H42 (H42) sequences. Size differences between the chromosomes map to subtelomeric regions while central regions are largely conserved (20). The 380-kb *Bgl* I fragment containing the DHFR–TS gene is marked by the crosshatched pattern. B, *Bgl* I; H, *Bss*HII. (*B*) PFG electrophoresis of chromosome 4 *Bgl* I restriction fragments from the HB3, 3D7, and XP5 clones. Bacteriophage λ (48.5 kb) concatemers provide size standards. (*C*) Hybridization of the *P. falciparum* TS probe to the 380-kb *Bgl* I fragments from the chromosome restriction digests.

DISCUSSION

The results of this work provide strong evidence that point mutations in the DHFR–TS gene confer pyrimethamine resistance to *P. falciparum*. In a cross of a pyrimethamine-resistant clone (HB3) with a pyrimethamine-sensitive clone (3D7), the resistant phenotype was inherited with the HB3 linkage group containing the DHFR–TS gene. Sequence data show that the HB3 DHFR domain differs from that of 3D7 by a single mutation from Ser-108 to Asn-108. Evidence that it is the DHFR–TS gene itself that governs pyrimethamine resistance (and not another closely linked gene affecting a process such as drug transport or metabolism) is provided by the sequence data from eight other *P. falciparum* isolates and clones obtained from Southeast Asia, Africa, and South America. All sensitive parasites we examined have Thr-108 or Ser-108 in the DHFR domain, whereas all resistant parasites have Asn-108. Considering the innumerable recombination events separating the different *P. falciparum* samples, the Asn-108 mutation is implicated strongly as the major mechanism of pyrimethamine resistance in these parasites.

Other workers (29) have reported that the sequence of the pyrimethamine-sensitive isolate FCR3 contains Asn-108 and Ala-16. Since this conflicted with our finding that the Asn-108

Table 2. Point mutations and pyrimethamine susceptibility in *P. falciparum*

Clone or isolate*	Origin	Susceptibility†, ng/ml	Amino acid residue‡ 108	51	59	16
3D7	The Netherlands§	2, 2	Ser	Asn	Cys	Ala
SL/D6	Sierra Leone	1, 1	Ser	Asn	Cys	Ala
L.E5	Liberia	1, 1	Ser	Asn	Cys	ND
It.G2.F6	Brazil	1, 2	Thr	Asn	Cys	Ala
FCR3¶	Gambia	4, 4	Thr	Asn	Cys	Val
HB3	Honduras	256, 256	Asn	Asn	Cys	Ala
Camp	Malaysia	128, 256	Asn	Asn	Cys	Ala
It.D12	Brazil	1024, 1024	Asn	Ile	Cys	Ala
7G8	Brazil	2048, 2048	Asn	Ile	Cys	Ala
Dd2	Indochina	4096, 8192	Asn	Ile	Arg	Ala

ND, not determined.

*3D7, SL/D6, L.E5, It.G2.F6, HB3, It.D12, 7G8, and Dd2 are cloned parasites. FCR3 and Camp are uncloned isolates. Full sequence data from the DHFR domains of the 3D7, SL/D6, FCR3, HB3, 7G8, and Dd2 genes were obtained and confirmed in both directions (Fig. 3*A*). Nucleotide sequences were found to be identical except for the identified mutations. Partial sequence data spanning mutations were obtained from the L.E5, It.G2.F6, Camp, and It.D12 genes.

†Microtiter plate assay, 2.5% hematocrit. Values are separate determinations of the pyrimethamine concentration at which parasitemia was reduced by >50% compared to control.

‡Residues are numbered according to the assignment of Bzik *et al.* (29).

§Derived from Amsterdam Airport isolate NF54, thought to be of African origin.

¶Sequences of the original FCR3 isolate and of the FCR3-A2 and FCR3-D4 clones were independently determined and were found to be identical. The sequences differ from that of Bzik *et al.* (29) in that a threonine occurs at position 108 and valine occurs at position 16.

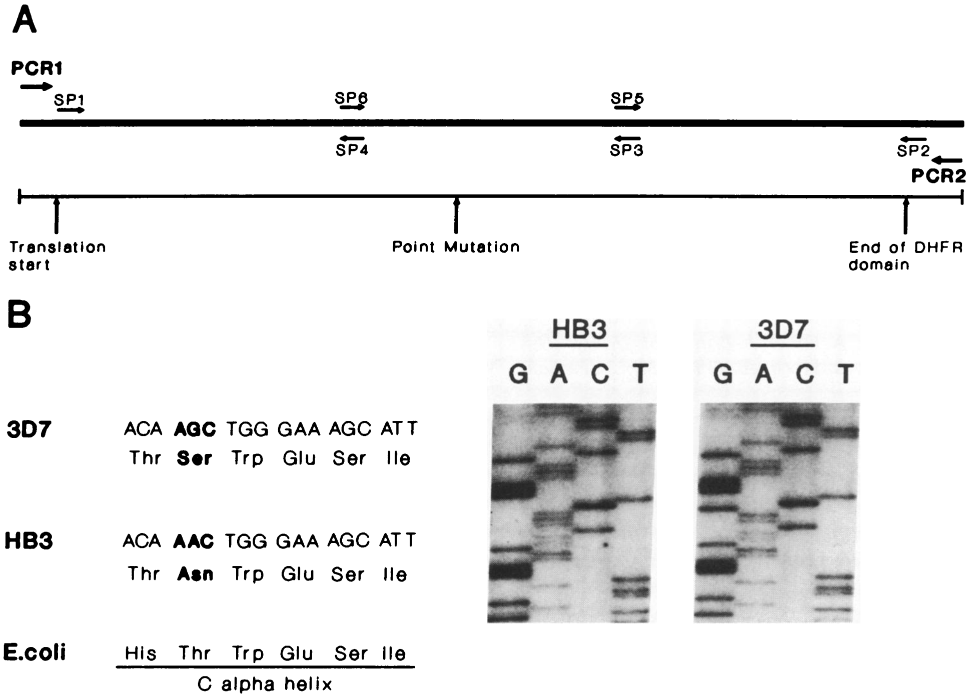

FIG. 3. PCR strategy for amplification and nucleotide sequencing of the *P. falciparum* DHFR domain. (*A*) Primers PCR1 and PCR2 immediately flanking the DHFR domain were used for DNA amplification. Fifty nanograms of *P. falciparum* DNA (representing ≈1.5×10^6 copies of the genome) were used as starting material, enough to ensure that even early replicative errors would not appear in the sequence data (22). Sequencing of both strands was completed from RNA transcripts or denatured DNA by using the indicated primers. RNA transcripts were obtained from DNA amplified with a PCR primer with an attached T7 phage promoter. Primers were derived from the *P. falciparum* sequence of Bzik *et al.* (29): PCR1, TTATTTATATATTTATATTTTTCTCC; PCR2, CTTCTCCTTTTATACAATTTTGTTC; SP1, ATGATGGAACAAGTCTGCGAC; SP2, ACATTTTATTATTCGTTTTC; SP3, TTTAATTTCCCAAGTAAAAC; SP4, TGTTTAAATATTTACATCTC; SP5, GTTTTACTTGGGAAATTAAA; SP6, GAGATGTAAATATTTAAACA. (*B*) Comparison of the 3D7 and HB3 sequences in the region of the point mutation. A nucleotide change from guanosine to adenosine produces a change in the amino acid sequence from Ser-108 in the pyrimethamine-sensitive 3D7 clone to Asn-108 in the pyrimethamine-resistant HB3 clone. The nucleotide sequences of the 3D7 and HB3 DHFR domains are otherwise identical. The homologous sequence of the C α-helix bordering the active site cavity of the *Escherichia coli* DHFR gene is shown for comparison.

mutation confers resistance, we examined the DHFR–TS sequences of three different FCR3 lines: the original uncloned FCR3 isolate and the FCR3 clones A2 and D4 (obtained from William Trager, The Rockefeller University, New York). In all three sequences, Thr-108 was found instead of Asn-108, and Val-16 was found instead of Ala-16. Our data therefore indicate that the reported sequence (29) is not that of the pyrimethamine-sensitive organism. It is possible that a subpopulation of pyrimethamine-resistant organisms was the origin of the reported sequence. We note that FCR3 is an uncloned isolate. Mixtures of parasites differing in their susceptibility to drugs are commonly found in natural infections of *P. falciparum* (30–32).

The mutation to Asn-108 occurs at a site analogous to that of the threonine residue in the C α-helix of bacterial, avian, and mammalian enzymes (33). Volz *et al.* (34) have determined the crystal structure of avian DHFR in complex with 2,4-diamino-5,6-dihydro-6,6-dimethyl-5-(4′-methoxyphenyl)-*s*-triazine, a phenyltriazine inhibitor structurally similar to pyrimethamine. In this structure, Thr-56 in the C α-helix makes van der Waals contact with the methoxyphenyl group of the inhibitor. This close analogy suggests that comparable contacts may occur between amino acid 108 of the *P. falciparum* enzyme and the chlorophenyl group of pyrimethamine. The Asn-108 mutation would be expected to affect these contacts and thereby may inhibit binding of pyrimethamine. Indeed, Chen *et al.* (12) have compared the kinetic properties of partially purified DHFR from the 3D7, HB3, and 7G8 clones. Affinity for pyrimethamine was shown to be reduced when tested against the partially purified HB3 and 7G8 preparations.

In addition to the Asn-108 mutation, *P. falciparum* clones It.D12 and 7G8 have a mutation that encodes Ile-51 instead of Asn-51. These parasites are 4- to 8-fold more resistant to pyrimethamine than HB3. The Dd2 clone, which has both of these mutations plus an additional mutation of cys-59 to Arg-59, is the most resistant of all; it has 16 to 32 times the resistance of HB3. Alignment of amino acids 51 and 59 with the sequences of known bacterial and vertebrate sources (33) places both of these mutations near, or in, the B α-helix at the back of the active site cavity. Two analogies suggest that these mutations may affect inhibitor binding. First, x-ray crystallographic data show that in avian DHFR amino acids at similar positions make hydrophobic contact with a phenyltriazine inhibitor structurally related to pyrimethamine (34). Second, point mutations in the B α-helix of bacterial and mammalian DHFRs have been found to lower binding affinity to inhibitors (26, 27).

It is interesting to note that the point mutation to Asn-108 has been found in pyrimethamine-resistant parasites from widely distant malarious regions throughout the world. Foci of resistance have been noted to arise in different countries after mass administration of antifolates (35). We presume that many, if not all, of the point mutations observed in this study arose independently. The mutation to Asn-108 in particular may provide a major mechanism of pyrimethamine resistance in natural strains of *P. falciparum*, and mutations at other sites may further increase resistance. It is hoped that clarification of the structural and functional features of these mutations will aid the development of alternative drugs against pyrimethamine-resistant malaria.

In an independent study, Cowman *et al.* (36) have also identified point mutations in the DHFR of pyrimethamine-resistant *P. falciparum* strains.

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