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)

DAVID S. PETERTSON, DAVID WALLIKER*, AND THOMAS E. WELLEMS

Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

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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-methylene tetrahydrofolate: 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.

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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 Thaihong and Beale (15). Parasites were cultured in microtiters 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 NaClO₄ 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-

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.

*Permanent address: Department of Genetics, University of Edinburgh, Edinburgh, Scotland.
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 HindIII 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 DHFR-TS 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. 1A). 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. 1B). Relative intensities of hybridization to HRP-II and DHFR-TS indicated no differences in the DHFR-TS gene copy number regardless of pyrimethamine resistance.

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.

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

<table>
<thead>
<tr>
<th>Clone</th>
<th>Phenotype*</th>
<th>RFLP†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>D21</td>
</tr>
<tr>
<td>Parents</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>3D7</td>
<td>S</td>
<td>2</td>
</tr>
<tr>
<td>HB3</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>Progeny</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X2</td>
<td>R</td>
<td>2</td>
</tr>
<tr>
<td>X6</td>
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</tr>
<tr>
<td>X8</td>
<td>S</td>
<td>1</td>
</tr>
<tr>
<td>X10</td>
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<td>XP9</td>
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<td>2</td>
</tr>
<tr>
<td>XP10</td>
<td>R</td>
<td>2</td>
</tr>
</tbody>
</table>

*Drug sensitivities of the parents and progeny: R, pyrimethamine-resistant; S, pyrimethamine-sensitive (ID₅₀ values of 3D7 and HB3 are given in Table 2).
†RFLPs were detected by Southern blot analysis of HindIII-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.
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***

<table>
<thead>
<tr>
<th>Clone or isolate*</th>
<th>Origin</th>
<th>Susceptibility1, ng/ml</th>
<th>Amino acid residue2</th>
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<tr>
<td>3D7</td>
<td>The Netherlands†</td>
<td>2, 2</td>
<td>Ser</td>
</tr>
<tr>
<td>SL/D6</td>
<td>Sierra Leone</td>
<td>1, 1</td>
<td>Ser</td>
</tr>
<tr>
<td>L.E5</td>
<td>Liberia</td>
<td>1, 1</td>
<td>Ser</td>
</tr>
<tr>
<td>It.G2,F6</td>
<td>Brazil</td>
<td>1, 2</td>
<td>Thr</td>
</tr>
<tr>
<td>FCR3†</td>
<td>Gambia</td>
<td>4, 4</td>
<td>Thr</td>
</tr>
<tr>
<td>HB3</td>
<td>Honduras</td>
<td>256, 256</td>
<td>Asn</td>
</tr>
<tr>
<td>Camp</td>
<td>Malaysia</td>
<td>128, 256</td>
<td>Asn</td>
</tr>
<tr>
<td>It.D12</td>
<td>Brazil</td>
<td>1024, 1024</td>
<td>Asn</td>
</tr>
<tr>
<td>7G8</td>
<td>Brazil</td>
<td>2048, 2048</td>
<td>Asn</td>
</tr>
<tr>
<td>Dd2</td>
<td>Indochina</td>
<td>4096, 8192</td>
<td>Asn</td>
</tr>
</tbody>
</table>

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. 3A). 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.

†Microtitre 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).

§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.
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 population 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). Voltz 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. 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 ItD12 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 \textit{P. falciparum} strains.

We thank Drs. Mary Morry and George Cross for providing the \textit{P. falciparum} TS gene probe and Dr. Louis H. Miller for his advice and encouragement.