**Pneumocystis carinii** Dihydropteroate Synthase but Not Dihydrofolate Reduction Gene Mutations Correlate with Prior Trimethoprim-Sulfamethoxazole or Dapsone Use

Liang Ma, Luciana Borio, Henry Masur, and Joseph A. Kovacs

Recent studies of the human *Pneumocystis carinii* dihydropteroate synthase (DHPS) gene suggest that *P. carinii* is developing resistance to sulfamethoxazole (SMX) and dapsone. To explore whether *P. carinii* is also developing resistance to trimethoprim (TMP), the human *P. carinii* dihydrofolate reductase (DHFR) gene was cloned, DHFR and DHPS genes in 37 *P. carinii* isolates from 35 patients were sequenced, and the relationship between TMP-SMX or dapsone use and gene mutations was analyzed. The DHFR gene sequences were identical in all isolates except 1 with a synonymous substitution. In contrast, the DHPS gene sequences showed mutations in 16 of the 37 isolates; prior sulfa/sulfone prophylaxis was associated with the presence of these mutations (*P* < .001). In addition to suggesting that there is less selective pressure on DHFR than on DHPS, this study reinforces the hypothesis that mutations in the DHPS gene are likely involved in the development of sulfa resistance in *P. carinii*.

The combination of trimethoprim (TMP) and sulfamethoxazole (SMX) serves as the first-line therapeutic and prophylactic regimen for pneumonia caused by *Pneumocystis carinii*, which remains a major opportunistic agent in patients infected with human immunodeficiency virus (HIV) and in other immunocompromised patients. This combination inhibits 2 key enzymes in folate metabolism. Dapsone is another commonly used prophylactic agent, and, like SMX, it targets the enzyme dihydropteroate synthase (DHPS), which catalyzes the condensation of para-aminobenzoic acid with 7,8-dihydropterin-pyrophosphate (DHPPP), forming 7,8-dihydropteroate. TMP acts on dihydrofolate reductase (DHFR), which catalyzes the reduction of dihydrofolate to tetrahydrofolate and is dependent on the reduced form of nicotinamide adenine dinucleotide phosphate.

The widespread use of DHFR and DHPS inhibitors in antimicrobial chemotherapy has resulted in the emergence of antifolate drug resistance in numerous bacteria and some protozoa [1–10], raising concerns that similar resistance would develop in *P. carinii*. The molecular mechanisms of antifolate resistance have been extensively studied, particularly in malaria. In *Plasmodium* species, it has been well established that DHFR point mutations are responsible for differential resistance to DHFR inhibitors [4–7], whereas DHPS point mutations are associated with sulfa resistance [8–10].

TMP-SMX has been used for the treatment and prevention of *P. carinii* pneumonia (PCP) for >20 years. Despite the proven efficacy of TMP-SMX in both AIDS and non-AIDS patients, studies have shown that failure to respond clinically to this combination is ultimately observed in 10%–40% of patients [11–13]; the factors leading to this failure are not well understood. Recently, mutations in the *P. carinii* DHPS gene have been identified [14], primarily in patients who had previously received TMP-SMX or dapsone for prophylaxis, which suggests that the *P. carinii* DHPS gene is evolving under selective pressure because of the use of sulfa drugs. Further studies from our group [15] and others [16] have indicated that point mutations of the *P. carinii* DHPS gene were associated with the failure of sulfa or sulfone prophylaxis in AIDS patients, suggesting that these mutations confer sulfa resistance in *P. carinii*. These mutations are at one of the active sites of the enzyme [17] and correlate with mutations that confer resistance to sulfa drugs in other organisms. Because TMP is used together with SMX and dapsone, it is of particular interest to ask whether *P. carinii* may simultaneously be developing mutations that suggest resistance to TMP.

Although the rat-derived *P. carinii* DHFR gene has been sequenced [18] and primers based on this sequence have been reported to amplify the human *P. carinii* DHFR gene [19–23], we could not successfully amplify a region of this gene with these primers. Given that homologous genes such as the DHPS gene [14] and those of major surface glycoproteins [24] are not available.
well conserved in *P. carinii* isolates from different hosts, it appeared likely that the DHFR gene was also poorly conserved.

In the present study, we have cloned and characterized the human *P. carinii* DHFR gene. We have also examined the entire coding sequences of the DHFR and DHPS genes from 37 *P. carinii* isolates obtained from 35 patients and have attempted to define the relationship between TMP-SMX or dapsone use and mutations in their therapeutic targets.

### Materials and Methods

**P. carinii organisms and DNA extraction.** Between 1985 and 1998, 37 human *P. carinii* samples were obtained from 35 patients, either during diagnostic procedures or at autopsy. Of the 35 patients, 26 had AIDS, and 6 had other diseases (i.e., lymphocytic m mia, metastatic neuroectodermal tumor, or rheumatoid arthritis); for 3 patients, no clinical data were available. The partial DHPS sequences for 2 patients have been reported elsewhere [15]. Categorization of prior exposure to specific anti-*Pneumocystis* medications was based on information available by chart review. Patients were classified as having had “prior prophylaxis” if the administration of drugs to prevent PCP was documented, regardless of duration, and as having had “prior therapy” if therapy for a prior episode of PCP was documented. The “prior exposure” category included patients with documented prior therapy or prophylaxis with specific drugs. The sources of samples and the number of samples from each source were as follows: bronchoalveolar lavage fluid, 18; induced sputum, 14; lung specimen at autopsy, 4; and oral wash, 1. Two patients had 2 samples obtained 1 month and 2.5 months apart, respectively. The presence of *P. carinii* in the samples was confirmed by microscopical detection of the organism (35 samples) or by diagnostic polymerase chain reaction (PCR) (2 samples). DNA was isolated from samples by treating cell pellets or lung homogenates with proteinase K and sodium dodecyl sulfate, followed by phenol/chloroform extraction and ethanol precipitation [25].

**Cloning of the DHFR gene.** Oligonucleotides based on regions of the rat-derived *P. carinii* DHFR gene [18] encoding amino acids conserved in the DHFR of other organisms (table 1) were used as primers in a conventional PCR procedure, to amplify the central coding region of the human *P. carinii* DHFR gene. The PCR mixture (100 μL) contained 0.2 μg of DNA extracted from an autopsy lung sample of a patient with PCP, 0.25 μM of each primer, 1× PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 2.5 mM MgCl₂), 0.2 mM dNTPs, and 2.5 U AmpliTaq Gold DNA polymerase (Perkin-Elmer, Foster City, CA). Amplification was performed in a PTC-100 programmable thermal controller (MJ Research, Inc., Watertown, MA) with cycling parameters as follows: 10 cycles of denaturation at 94°C for 9 min and then 35 cycles at 94°C for 1 min, 45°C for 2 min, and 72°C for 3 min, with a final extension at 72°C for 10 min. PCR products were subcloned into pCR 2.1 vector (Invitrogen) by use of the TA Cloning kit (Invitrogen). To amplify the remainder of the DHFR gene, inverse PCR [27, 28] was used. The template was prepared essentially as described by Ochman et al. [27]. In brief, human *P. carinii* genomic DNA (2.4 μg) was digested separately with restriction enzymes EcoRI, HindIII, SspI, and BstUI according to the manufacturer’s instructions (New England Biolabs, Beverly, MA). Digested products were ligated with T4 DNA ligase (New England Biolabs), extracted with phenol/chloroform extraction and ethanol precipitation [25].

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5′→3′)</th>
<th>Template</th>
</tr>
</thead>
<tbody>
<tr>
<td>FR01*</td>
<td>ATGAATCAGAAATGCTTATACATGTTGFE5</td>
<td>DHFR</td>
</tr>
<tr>
<td>FR634*</td>
<td>TATAAGCTTTGCTTACACATTGATTGT17</td>
<td>DHFR</td>
</tr>
<tr>
<td>Hu172</td>
<td>ACTTCCCCCATGCTTCAGACCC</td>
<td>DHFR</td>
</tr>
<tr>
<td>Hu528</td>
<td>TTCTCAGTCATGTGCTTCCTTG</td>
<td>DHFR</td>
</tr>
<tr>
<td>FR208</td>
<td>GCAGAAATGACGTATTATACATGAGACA</td>
<td>DHFR</td>
</tr>
<tr>
<td>FR242</td>
<td>GTCCTGATGCTTACGACCTTGATTC</td>
<td>DHFR</td>
</tr>
<tr>
<td>FR1018</td>
<td>GCTCTCAAAACTTGGTGTTACCGC</td>
<td>DHFR</td>
</tr>
<tr>
<td>FR1038</td>
<td>AACGATTACAATACTGCAACTAGTGC</td>
<td>DHFR</td>
</tr>
<tr>
<td>PK2*</td>
<td>CCGATCCCGACTTGAAGCTTCTTATTGTTTA</td>
<td>DHPS</td>
</tr>
<tr>
<td>PSB1*</td>
<td>TCTTGAACCTTATTACATTTCCATAACA</td>
<td>DHPS</td>
</tr>
<tr>
<td>PS634</td>
<td>GCAACCAGGCAAAACATTAAAGC</td>
<td>DHPS</td>
</tr>
<tr>
<td>PS747</td>
<td>GCACTTGTATGAGAAGCTTGTA</td>
<td>DHPS</td>
</tr>
<tr>
<td>PK95</td>
<td>GCGATGAGCTTACGTGGTTCAT</td>
<td>DHPS</td>
</tr>
<tr>
<td>PK160</td>
<td>GTTAATCTGTAGTATAAACCGTTTGGCATT</td>
<td>DHPS</td>
</tr>
<tr>
<td>PS876</td>
<td>ATAAATGCGAAAATACATTCGACCAAGT</td>
<td>DHPS</td>
</tr>
</tbody>
</table>

*NOTE.* DHFR, dihydrofolate reductase; DHPS, dihydropteroate synthase.

a FR01 and FR634 were based on the rat-derived *Pneumocystis carinii* DHFR gene [18]. PK2 and PSB1 were based on the rat-derived *P. carinii* fas gene [26] and the human-derived *P. carinii* DHPS gene (GenBank accession number U66282), respectively.

### Table 1. Oligonucleotide primers used for polymerase chain reaction analysis in the study.

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Rockland, ME), extracted with the Ultrafree-MC columns (Milipore, Boston, MA), and then subjected to direct sequencing, as described later.

Cloning of the DHPS gene. Because the reported human P. carinii DHPS gene [14] is incomplete in the 3' end of the coding region and because primers used in examining mutations in the DHPS gene are in the coding region, we sequenced the remaining coding regions as well as upstream and downstream regions. To amplify the 5' region, an upstream primer (PK2) was selected from a conserved region in the hydroxymethyldihydropterin pyrophosphokinase (PPPK) domain of the rat-derived P. carinii fas gene [26], and a downstream primer (PSB1) was taken from a previously obtained partial sequence of the human P. carinii DHPS gene [14]. PCR amplification was carried out with the touchdown procedure, as described previously. To amplify the 3' region of the DHPS gene, an inverse PCR was performed as described previously, by use of primers PS634 and PS747, which were designed from the 3' end of the reported human P. carinii DHPS sequence [14].

Amplification of the P. carinii DHFR and DHPS genes from clinical samples. Once the complete sequences of the DHFR and DHPS genes from human-derived P. carinii were determined, we designed PCR primers to allow amplification and sequencing of the entire DHFR and DHPS coding domains from clinical isolates. To obtain DNA in sufficient quality and quantity for sequencing, we performed a primary PCR for all samples and a secondary nested or heminested PCR for those samples for which the initial PCR products were not visible or were very faint on agarose gels. In the primary PCR, 2-5 μL of DNA were used in a final volume of 50 μL. In the secondary PCR, 1-5 μL of the primary PCR products were used in a final volume of 100 μL. To amplify the DHFR gene, the primers used in the primary PCR were FR208 and FR1038, which covered the DHFR domain from base -123 to base 735, and the primers used in the secondary PCR were FR242 and FR1018, which encompassed the DHFR domain from base -89 to base 709. To amplify the DHPS gene, the primers used in the primary PCR were PK95 and PS876, which spanned the DHPS domain from base 126 upstream of the first codon to base 79 beyond the stop codon TGA, and the primers used in the secondary PCR were PK160, which flanked the DHPS domain from base 61 upstream of the first codon, and PS876. The thermal cycling conditions for both the primary and secondary PCRs were as follows: 95°C for 9 min and then 35 cycles of 94°C for 1 min, 60°C for 2 min, and 72°C for 3 min, with a final cycle of 72°C for 10 min. PCR products were purified either on 1.2% agarose gels, as described previously, or by silica gel separation with the QIAquick PCR purification kit (QIAGEN, Santa Clarita, CA). The PCR products were then directly sequenced.

DNA sequencing and analysis. DNA sequencing with universal or sequence-specific primers was carried out by use of the dideoxy chain termination reaction and the ABI PRISM dye terminator cycle sequencing kit (Perkin-Elmer) and an ABI PRISM 377 automated DNA sequencer (Perkin-Elmer). Sequencing data were analyzed by use of Sequencher 3.0 software (Gene Codes Corp., Ann Arbor, MI). Amino acid sequences of both DHFR and DHPS were deduced from the nucleic acid sequences and analyzed with MacVector 6.0.1 software (Oxford Molecular Group, Oxford, UK).

Statistical analysis. Statistical analyses were performed with SSPI 6.1 software for Macintosh (SPSS Inc., Chicago) or the Primer of Biostatistics v3.0 software (McGraw-Hill, New York). Associations between DHPS mutations and sulfa/sulfone use were analyzed by use of Fisher’s exact test. P<.05 was considered significant.

Results

Cloning and sequencing of the DHFR gene. Conventional PCR amplification of human P. carinii genomic DNA by use of DHFR-specific primers FR01 and FR634 yielded a single fragment of ~600 bp, which was absent in normal human genomic DNA. Sequencing identified a 597-bp insert that appeared significantly homologous to the rat P. carinii DHFR gene as well as to other DHFR genes available in GenBank. Because no adequate genomic or cDNA library for human P. carinii is currently available, we used an inverse PCR to obtain the 5' and 3' ends of the gene. When SspI-digested and circularized human P. carinii DNA was used as a template, the PCR amplification produced a strong band of ~1.3 kb that was not present in the controls. Direct sequencing of this fragment revealed a 1281-bp sequence, which included the 5' and 3' ends of the DHFR coding sequence.

Combining the sequences of the conventional PCR and inverse PCR fragments produced a 1616-bp sequence (GenBank accession number AF090368). Alignment with the rat-derived P. carinii DHFR gene [18] revealed a 618-bp DHFR coding sequence interrupted by a 42-bp intron, an 846-bp 5' flanking sequence, and a 110-bp 3' flanking sequence. The A+T content is 67.2% in the entire 1616-bp sequence and 63% in the coding region, which is similar to that of other reported P. carinii genes. Although the 42-bp intron located at bases 267–308 is identical in location to that in the rat-derived P. carinii DHFR gene, it is 1 bp smaller than that intron and has a different sequence [18]. It contains a 5'-GT splice donor site and a 3'-AG splice acceptor site, which are similar to those in other introns identified in P. carinii [18, 29–31]. Inspection of the 5' noncoding region near the ATG start codon revealed the presence of a putative TATA box (TATAATT) at base ~46, and a putative CAAT box (GGCAATCG) at base ~17. Presumably, the sequence AATATA or ATAAAA, located beyond the stop codon at base 48 or 77, respectively, may serve as the polyadenylation signal.

Examination of the sequence upstream of the DHFR domain revealed a short open reading frame of 156 bp starting at base ~600; translation yields a 52-amino acid polypeptide with a molecular mass of 5774 Da. A GenBank search revealed that the deduced polypeptide was most similar (34% identity) to a hypothetical protein in Schizosaccharomyces pombe (GenBank accession number O14010).

The DHFR coding region was predictive of a 206-amino acid protein with a calculated molecular mass of 23,407 Da. Comparison with other DHFR sequences available in GenBank revealed that the human-derived P. carinii DHFR is most
Figure 1. Alignment of deduced sequence of human *Pneumocystis carinii* dihydrofolate reductase (DHFR) with that of DHFR from selected organisms. Boxes are drawn around identical (dark shading) and similar (light shading) residues. GenBank accession numbers for these sequences are rat *P. carinii*, M26495; *Schizosaccharomyces pombe* (residues 206–461), P36591; *Filobasidiella neoformans*, Q07801; *Candida albicans*, X78968; *Plasmodium falciparum*, J03772; *Toxoplasma gondii*, G729370; *Escherichia coli*, J00140; *Bacillus subtilis*, M20012; and *Homo sapiens*, J01609. Alignment was performed by use of ClustalW software (MacVector; Oxford Molecular Group, Oxford, UK).
closely related to the DHFR of rat-derived *P. carinii* (62% identity), *S. pombe* (39% identity), and *Filobasidiella neoformans* (35% identity). Figure 1 shows an alignment of 10 DHFR sequences from different organisms. In all the sequences, 16 residues are identical and 21 are similar. A putative DHFR signature for binding of the folate substrate [32], IGLkndLPW, is identified at residues 19–27.

Although the human *P. carinii* DHFR is most similar to the rat *P. carinii* DHFR, there are substantial differences in both the nucleotide and amino acid sequences. The 5′ and 3′ flanking sequences are 48% different in the overlapping regions, and the coding sequences are 30% (186 of 618) different. The deduced amino acid sequences are 38% (79 of 206) different.

Some investigators have reported that primers based on the rat *P. carinii* DHFR gene are useful in diagnosing human *P. carinii* infections [19–23]. To investigate this matter further, we used previously reported PCR conditions [20] to compare the previously used rat *P. carinii* DHFR primers [19, 20] with primers that were based on a homologous region of the human *P. carinii* DHFR gene in terms of their efficiency in amplifying the rat and human *P. carinii* DHFR genes (figure 2A). As shown in figure 2B, when PCR was performed by use of the rat *P. carinii* DHFR primers and rat *P. carinii* DNA, a single band with the predicted size of 273 bp was observed; when human *P. carinii* DNA was used, a strong band was also observed, but its size was smaller than expected. No product was seen when plasmid DNA containing the human *P. carinii* DHFR gene was amplified with these primers. In contrast, PCR with primers from the human *P. carinii* DHFR gene amplified a band of the expected size (273 bp) from human *P. carinii* genomic DNA and plasmid DNA containing the human *P. carinii* DHFR gene. This band was not seen in rat *P. carinii* DNA or in normal human DNA.

**Cloning and sequencing of the DHPS gene.** Conventional PCR amplification of human *P. carinii* genomic DNA by use of DHPS-specific primers PK2 and PSB1 produced a 987-bp fragment, including 183 bp of the 3′ end of the PPPK domain with 80% homology to the 3′ end of the PPPK domain of the rat-derived *P. carinii* fas gene [26], and 804 bp identical to the 5′ region of the published sequence of the partial human *P. carinii* DHPS gene (GenBank accession number U66282). The 3′ end of the gene was cloned by inverse PCR with primers PS634 and PS747. The 299-bp insert included the 3′ end of the gene and 79 bp of the 3′ untranslated region. The length of the nucleotide sequence assembled from the 2 PCR-generated fragments just mentioned is 1099 bp (GenBank accession number AF139132) and includes the entire DHPS domain (834 bp). The PPPK domain, which is interrupted by a 45-bp intron, deduces a 46-amino acid sequence that shares 71.7% identity with the C-terminal of the rat *P. carinii* PPPK domain [26].

**Polymorphisms of the P. carinii DHFR and DHPS genes in clinical isolates.** PCR was performed with primers from regions outside the coding region, to amplify the entire coding sequences of both the DHFR and DHPS genes in 37 *P. carinii* samples from 35 patients. The DHFR gene was amplified from 17 samples by use of a single round of PCR (858 bp) and from 20 samples by use of a nested PCR (798 bp). The DHPS gene was obtained from 14 samples by use of a single round of PCR (1030 bp) and from 23 samples by use of a nested PCR (965 bp). All the PCR products were directly sequenced on both strands.

All 37 samples showed identical DHFR sequences, except
Table 2. Polymorphisms of the human Pneumocystis carinii DHPS gene in 37 samples from 35 patients.

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>No. of samples</th>
<th>Codon*</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 (wild-type)b</td>
<td>17</td>
<td>Thr (ACA) Pro (CCT) Ser (TCA)</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>Thr (ACA) Pro (CCT) Ser (TCG)</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>Ala (GCA) Pro (CCT) Ser (TCG)</td>
</tr>
<tr>
<td>10b</td>
<td>9</td>
<td>Ala (GCA) Ser (TCT) Ser (TCA)</td>
</tr>
</tbody>
</table>

*NOTE: DHPS, dihydropteroate synthase.

a Nucleotides and amino acids that differ from the wild-type sequence are underlined.
b Including 2 samples from 1 patient.

for 1 sample, which had a single nucleotide change from T to C at position 312. This change is synonymous and does not result in an amino acid change.

In contrast, the DHPS sequences in the 37 samples exhibited polymorphisms at 3 nucleotide positions (table 2). Eighteen samples contained the wild-type sequence, which is characterized by the presence of Thr-55 and Pro-57. Three samples showed a single nucleotide change from A to G at position 513 (codon 171), which did not result in an amino acid change. In the remaining 16 samples, we observed nonsynonymous mutations leading to amino acid changes at codons 55 and 57. A single 55Thr→Ala mutation was observed in 6 samples, including 4 samples containing a mixture of Thr-55 and Ala-55. Mutations 55Thr→Ala coupled with 57Pro→Ser occurred in 10 samples, including 2 samples that had a mixture of Pro-57 and Ser-57 and 1 sample that had mixtures of Thr-55 and Ala-55 and of Pro-57 and Ser-57. Mutations 55Thr→Ala and 57Pro→Ser are identical to those identified in previous studies [14–16]. The nucleotide change at position 513 (codon 171) has not been previously reported. None of the polymorphisms at codons 23, 60, 111, or 248 that were reported by Lane et al. [14] were observed in the present study.

As noted previously, 7 samples had both the wild-type and mutant DHPS alleles present at codon 55 and/or codon 57, as determined by direct sequencing. To verify these mixed forms, we performed enzyme restriction analysis and subcloning of the PCR products. When the PCR products were subjected to digestion with AccI, which recognizes the wild-type sequence at codon 55, and HaeIII, which cleaves the wild-type sequence at codon 57, all 7 samples showed fragments that appeared in homozygous wild-type control samples and in homozygous mutant control samples (data not shown), indicating the presence of both wild-type and mutant sequences. We subcloned the PCR products of 1 sample with a clear mutation at codon 55 and a mixture of wild-type and mutant codons at 57 and of another sample with both wild-type and mutant codons at 55 and 57. In the first sample, we obtained 4 clones containing a mutant codon 55 and the wild-type codon 57 and 5 clones containing a mutant sequence at both codons 55 and 57. In the second sample, we obtained 6 clones containing the wild-type sequence and 2 clones containing a mutant sequence at both codons 55 and 57.

Correlation between prior sulfadiazine use and DHPS mutations at codon 55 and/or codon 57. Clinical data regarding prior therapy or prophylaxis were available for 30 and 32 patients, respectively (table 3). Two patients had a prior episode of PCP, but the therapy was not reported. Prior therapy with antifolate drugs was documented for 9 patients, of whom 8 had received TMP-SMX and 1 had received TMP-SMX followed by TMP-dapsone. Prior prophylaxis was documented for 13 patients, of whom 6 had received dapsone only and 7 had received TMP-SMX or TMP-dapsone. Six patients had received both prior prophylaxis and prior therapy. Of the 16 patients with prior therapy, 12 had prior episodes of PCP, and 3 of 16 patients with no documented prior exposure to any sulfa- or sulfone-containing agent had prior episodes of PCP.

Using results from the first isolate from the 2 patients who each had 2 isolates, we found a significant association between prior exposure to TMP-SMX or dapsone and the presence of mutations at codon 55 and/or codon 57. Mutations were found in 11 of 16 patients with documented prior exposure, compared with 3 of 15 patients without such exposure (P = .011). This association was related primarily to prior prophylaxis (P < .001); prior therapy was not associated with the presence of these mutations (P = .70). Of note, all 3 patients whose isolates showed mutations but who did not have prior exposure to SMX/dapsone had a mixed wild-type and mutant DHPS at codon 55, with a wild-type sequence at codon 57. For the 23 patients whose therapy regimens contained a sulfonamide or dapsone, no correlation was found between survival after initiation of the therapy and the presence of mutations at codon 55 and/or codon 57 (P = .36). Prior exposure to pentamidine was documented for 12 patients, 11 of whom also had prior exposure to TMP-SMX or dapsone. No correlation was found between prior exposure to pentamidine and the presence of DHPS mutations (data not shown).

Although no mutations in the DHFR gene were seen in the

Table 3. Correlation of the Pneumocystis carinii DHPS mutations at codon 55 and/or codon 57 with prior sulfadiazine use in 32 patients.

<table>
<thead>
<tr>
<th>DHPS mutations</th>
<th>Yes</th>
<th>No</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prior exposure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>11</td>
<td>5</td>
<td>.011</td>
</tr>
<tr>
<td>No</td>
<td>3</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Prior prophylaxis</td>
<td></td>
<td></td>
<td>.001</td>
</tr>
<tr>
<td>Yes</td>
<td>11</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>3</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Prior therapy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>5</td>
<td>5</td>
<td>.070</td>
</tr>
<tr>
<td>No</td>
<td>8</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: Two patients, one with wild-type and the other with mutant DHPS, had prior P carinii pneumonia, but the specific treatment was not recorded, and they were excluded from the prior therapy group. One of these patients, who did not receive prophylaxis, was also excluded from the prior exposure group. The other received dapsone as prophylaxis. DHPS, dihydropteroate synthase.
37 samples from 35 patients, 15 patients had been previously exposed to TMP, with 10 receiving prior therapy and 7 receiving prior prophylaxis with TMP.

Discussion

To better understand the relationship between TMP-SMX or dapsone use and mutations in their target enzymes, we cloned and sequenced the DHFR gene of human *P. carinii* and examined sequence polymorphisms of this gene, as well as those of the DHPS gene, in 37 clinical isolates of *P. carinii*. We found that the human *P. carinii* DHFR gene is closely related to but clearly different from the rat *P. carinii* DHFR gene. Thus, the DHFR gene, like all genes studied to date, is not well conserved in *P. carinii* isolates from different hosts.

To date, we have sequenced the entire DHFR and DHPS domains of 37 *P. carinii* isolates from 35 patients. Surprisingly, we found that despite prior exposure to TMP in 15 patients, the sequences of the DHFR gene in all the isolates were identical except for 1 isolate with a single synonymous substitution. By contrast, 16 (43%) of the 37 isolates (15 of 35 patients) had DHPS mutations at codon 55 or codon 57. Since the sequence polymorphism of *P. carinii* DHPS was first observed in 6 human isolates [14], efforts have been made to determine its clinical significance. Kazanjian et al. [16] examined partial *P. carinii* DHPS nucleotide sequences from 27 PCP patients and found that mutations at codons 55 and 57 were common in patients who had received sulfonamido prophylaxis, affecting 5 of 7 such patients, whereas only 2 of 13 patients who had not been receiving sulfonamide prophylaxis had mutant DHPS genes. Similarly, in a previous study [15], these mutations were detected in 2 patients with PCP (also included in this report) who had failed prophylaxis or treatment with TMP-SMX. The present study showed a significant association between TMP-SMX or dapsone prophylaxis and mutations at codon 55 or 57, supporting the hypothesis that DHPS mutations at these sites reflect the development of sulfanilamide resistance by human *P. carinii*. Prior therapy with TMP-SMX or dapsone for PCP was not associated with the development of these mutations, suggesting that long-term exposure to low levels of TMP-SMX or dapsone facilitates the development of these mutations.

The hypothesis regarding resistance is further supported by comparative structural and functional data of DHPS from other organisms. Figure 3 shows an alignment of the affected regions of the predicted *P. carinii* DHPS protein with the sequences of the predicted DHPS protein from other organisms, including both prokaryotic and eukaryotic species. The mutations (55Thr → Ala and 57Pro → Ser) are located in a highly conserved region, which is characterized by the sequence Thr-Arg-Pro, which is identical for all the organisms shown except *Plasmodium falciparum*, in which the sequence is Ser-Ala-Pro (codons 436–438). On the basis of the crystal structure of the *Escherichia coli* DHPS, the analogous 3 residues (codons 62–64 in *E. coli*) are involved in binding to the substrate DHPPP as well as to sulfonamide [17]. Mutations at or very near these positions are likely to alter the local structure and thus affect the binding of the substrate and sulfanilamide. Mutations at these positions have been shown to confer resistance to sulfanilamide in organisms such as *P. falciparum* [8, 9, 28], *E. coli* [3], and *Streptococcus pneumoniae* [1].

Including only the first isolate from the 2 patients who each had 2 isolates, 12 of 24 isolates obtained in the 1990s had DHPS mutations at codon 55 and/or codon 57, whereas 3 of 11 isolates obtained in the 1980s had these mutations. This finding suggests that the mutations are more likely to occur in recent patient isolates, which is in accord with a previous observation by Kazanjian et al. [16], who sequenced the DHPS gene in 27
human isolates obtained between 1977 and 1997 and detected 7 mutant isolates, all of which were obtained from 1995 through 1997.

On the basis of the data from the present study and previous studies [16], it appears evident that the 55Thr→Ala mutation is the most common mutation in the DHPS gene. This mutation can occur either alone or in combination with the 57Pro→Ser mutation, which is less common and almost always coupled with the 55Thr→Ala mutation, suggesting that the 55Thr→Ala mutation may be the first mutation to occur in response to drug pressure. The presence of a mixture of wild-type and mutant DHPS in 7 isolates suggests a transition from the wild-type to the mutant gene, although a mixed infection cannot be ruled out. Of note, 3 isolates from patients without prior exposure to SMX/dapsone showed a mixed wild-type and mutant DHPS at codon 55, with a wild-type sequence at codon 57. This finding may represent allelic variations or possible selective pressure from unidentified exposure to sulfonamide drugs.

One of the more striking findings of the study is the lack of mutations in the P. carinii DHFR gene, because mutations in the DHPS gene were found in 43% of isolates examined. Although further studies are needed because fewer patients were exposed to long-term TMP than to a sulfonamide, the discordant occurrence of mutations of the DHFR and DHPS genes in these isolates suggests that the selective pressures on the 2 genes are not comparable—that is, there might be less selective pressure on DHFR than on DHPS. Absence of mutations in the DHFR gene further suggests that the DHPS mutations are not random occurrences but rather the result of antibiotic pressure, and it supports the concept that TMP contributes little to the efficacy of the TMP-SMX combination against P. carinii. This particular fixed-combination product is used because it was shown to be effective in animal and human trials of PCP, but, in fact, it was initially chosen for trials because it had been formulated for bacterial indications and was readily available. Potent DHFR inhibitors such as trimetrexate and piritrexim are active as single agents against both human and murine PCP, whereas TMP is 2 logs less potent in vitro assays [18, 33–35]. Moreover, studies in animal models have found that TMP alone is ineffective in treating or preventing PCP [36, 37], that sulfonamides alone, including SMX, are highly effective [37, 38], and that the addition of TMP to sulfonamides does not enhance the efficacy of sulfonamides alone [37]. Thus, it is not clear whether TMP truly contributes to the efficacy of TMP-SMX therapy; in fact, it may add toxicity without substantial benefit.

The marked difference between the human- and rat-derived P. carinii DHFR genes explains the conflicting reports on the use of the rat P. carinii DHFR primers to diagnose human PCP. Schlager et al. [19, 20] first reported the successful use of primers based on the rat P. carinii DHFR gene, detecting both rat and human P. carinii in limited serum and respiratory specimens. Subsequent studies with these primers by other investigators [21, 22] showed low sensitivity (23%–71%) and specificity (45.5%). Ortinau et al. [23] reported that the amplified products from human P. carinii isolates were unrelated to P. carinii DHFR or any other form of DHFR. In the present study, we have demonstrated that the previously described primers do not amplify the human P. carinii DHFR gene (figure 2B). A comparison of the sequences of primers from a homologous region of the rat and human P. carinii DHFR genes shows that the sense and antisense primers differed by 25% (5 of 20) and 20% (4 of 20) of nucleotides, respectively (figure 2A). Of note, the 2 nucleotides at the 3′ ends of the antisense primers were different, which is likely to hamper the annealing of the primers with heterogeneous templates, thereby blocking PCR amplification. It is evident from these data that the previously reported P. carinii DHFR primers are specific for rat P. carinii but are not useful for the detection of human P. carinii.

Although definitive demonstration that P. carinii is developing resistance to sulfonamide drugs will require additional studies with native or recombinant enzyme or in vitro culture systems [39], the mounting molecular epidemiologic evidence reported here and elsewhere [14–16] supports the conclusion that P. carinii is indeed developing such resistance. At present, TMP-SMX remains a highly effective drug for the treatment of PCP. However, if resistance to sulfonamide drugs becomes more widespread and clinically significant in the future, combining sulfonamide drugs with inhibitors of P. carinii DHFR that are more potent than TMP would likely provide better anti-P. carinii activity.

Acknowledgments

We are indebted to Vivian Sorial and Ross E. Turner for technical assistance and to Dr. Qin Mei for support.

References


