CONCISE COMMUNICATION

Pneumocystis carinii Cytochrome b Mutations Are Associated with Atovaquone Exposure in Patients with AIDS

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This retrospective cohort study was conducted to determine whether Pneumocystis carinii cytochrome b gene mutations in patients with AIDS and P. carinii pneumonia (PCP) are associated with atovaquone exposure. Portions of the P. carinii cytochrome b genes that were obtained from 60 patients with AIDS and PCP from 6 medical centers between 1995 and 1999 were amplified and sequenced by using polymerase chain reaction. Fifteen patients with previous atovaquone prophylaxis or treatment exposure were matched with 45 patients with no atovaquone exposure. Cytochrome b coenzyme Q binding site mutations were observed in 33% of isolates from patients exposed to atovaquone, compared with 6% from those who were not (P = .018). There was no difference in survival 1 month after treatment between patients with or without cytochrome b mutations (P = .14). Thus, cytochrome b mutations are significantly more common in patients with AIDS and PCP with atovaquone exposure, but the clinical significance of these mutations remains unknown.

Atovaquone, a 2-hydroxynaphthoquinone, inhibits electron transport in the cytochrome bc1 complex, probably by binding to cytochrome b [1–3]. Atovaquone is an antipneumocystis agent that is principally reserved for human immunodeficiency virus (HIV)–infected patients who are intolerant to sulfa-containing drugs [4]. Atovaquone reduces the incidence of Pneumocystis carinii pneumonia (PCP) when used as prophylaxis [5–8] and is effective as treatment of mild-to-moderate severity PCP [4]. Nevertheless, PCP occurs in patients with AIDS who have been exposed to atovaquone prophylaxis [4], possibly because of nonadherence, inadequate drug tissue penetration, or atovaquone resistance.

Resistance to atovaquone develops in Plasmodium falciparum–infected patients [6] and may develop more quickly than for other drugs, because the cytochrome b gene is in the mitochondria where mutation rates are higher than in the nucleus [7]. We previously found cytochrome b mutations in 2 of 4 patients receiving atovaquone prophylaxis against PCP [8]. These mutations were found in the Qo region, 1 of 2 coenzyme Q binding sites on cytochrome b. Additional studies with atovaquone-resistant Plasmodium strains and Toxoplasma gondii also identified mutations in the Qo site [9, 10]. Thus, it appears that numerous organisms can become resistant to atovaquone via point mutations in the Qo site.

To determine whether atovaquone resistance in human P. carinii may occur, we conducted a multicenter study to determine whether cytochrome b mutations are associated with exposure to atovaquone in patients with AIDS and PCP. We also noted the incidence of treatment failure and mortality in patients with AIDS and PCP who had cytochrome b mutations.

Materials and Methods

We obtained patient specimens for diagnostic purposes from the following institutions: Indiana University Medical Center (Indianapolis); Wayne State University Medical Center (Detroit); Denver Health Medical Center; University of Washington Medical Center, Harborview Medical Center, Swedish Hospital Medical Center, Providence Medical Center, Virginia Mason Medical Center, and Group Health Cooperative Central and Eastside hospitals.
(Seattle); Grady Memorial Hospital (Atlanta); and San Francisco General Hospital. Preserved alcohol-fixed slides, paraffin-embedded cytopathology tissue, or frozen samples from bronchoalveolar lavages or sputum induction were sent to either the University of Michigan or the Centers for Disease Control and Prevention (CDC) for polymerase chain reaction (PCR) analysis.

A case patient was defined as a patient with AIDS and PCP who had significant exposure to atovaquone (≥5 days) or treatment of a previous episode of PCP with atovaquone. Atovaquone exposure was selected as a requirement for a case patient because of the infrequency of its use. Control patients, matched to case patients by year of PCP diagnosis, were patients with AIDS and PCP who had no record of atovaquone exposure. We selected 3 control patients for each case patient. Survival at 4 weeks of treatment was noted. Chart abstractions and assessments of treatment failure were done without knowledge of the *P. carinii* cytochrome b PCR results.

DNA was extracted from each sample and was PCR amplified and sequenced by modification of a procedure described elsewhere [11]. A single-round PCR was performed, using primers AH1 [8] and CBHB1 (5′-AGG TTC TTC AAC ATG TTG TGA ACC-3′). If this failed, a second nested reaction was done, using primers CBHBnest1 (5′-AGG AAT AGC TAA CAT AGC C-3′) and CBHAnest1 (5′-ATT GGA TCT TAT CGA ACT CCC-3′). For DNA samples extracted from fixed paraffin blocks, we performed 2 separate single-round PCR reactions, to obtain each of the 2 sequences coding for the Qo site. For the 5′ portion, we used CBHAnest1 and CBHB2 (5′-ACC CAG AGG ATT ACT ACT TCC-3′). For the 3′ portion, we used CBHBnest1 and AH4 (5′-GAT CGT CTG CCT TTC CAT CCC-3′). Each PCR reaction used the same touchdown protocol, as described elsewhere [11]. In some cases, a single-stage PCR was done using the 5′ primer CB56F (5′-CGG GTG TGA CTT TAG CTA TGC-3′) and the 3′ primer CB1008R (5′-CCA CCA GAG GAA TAA CAA CTA AG-3′) in a program that included 94°C for 5 min, followed by 35 cycles of 92°C for 30 s, 55°C for 30 s, 72°C for 1 min, and 72°C for 5 min.

PCR fragments were sequenced directly on an automated DNA sequencer (ABI 377; PE Biosystems) with Big Dye terminator chemistry (PE Biosystems), according to the manufacturer’s recommended protocol. Cycle sequencing of the mitochondrial cytochrome B locus was done by using the oligonucleotide primers described above plus CB696R (5′-AGT AGC CAT AGG ATT AGC C-3′) and CB457F (5′-AAT GTC ATT GTG GGG AGC GAC-3′).

Statistical analysis was done by using Epi Info software (version 6.02; CDC). Associations between atovaquone use and cytochrome b polymorphisms were analyzed by using 2-tailed Fisher’s exact test. *P < .05* was considered to be significant.

**Results**

We identified 253 patients with AIDS and PCP who had stored respiratory specimens from January 1991 to April 1999 and who had complete medical records available. DNA was successfully amplified by PCR from stored specimens of 208 patients who were in previous studies on *P. carinii* dihydropteroate synthase (DHPS) polymorphisms [11, 12]. Fifteen of the 208 patients had significant atovaquone exposure (1995–1999), and each was defined as a case.

The demographic and clinical characteristics of the 60 patients with PCP, according to whether there was atovaquone exposure or not, are shown in table 1. All samples were obtained between 1995 and 1999 from patients with AIDS. Mean CD4 cell counts in 53 patients was 61 cells/mm^3; mean HIV RNA virus load of 24 patients was 4.7 log_{10} copies/mm^3. Patients with atovaquone exposure had a higher incidence of previous PCP (*P = .02*); otherwise, there were no significant differences in any demographic or clinical characteristics between those who did or did not have atovaquone exposure. None of the 45 patients without significant atovaquone exposure had received atovaquone for other purposes.

Of the 15 patients with significant atovaquone exposure, 9 were given atovaquone as prophylaxis (mean duration, 4.8 ± 7.2 months; range, 1–13 months), and 6 were receiving atovaquone at the time of PCP diagnosis. Three patients discontinued atovaquone within 2 months of the PCP episode because
of intolerance. Six of the 15 patients with atovaquone exposure had received atovaquone for treatment of a prior episode of PCP. These episodes took place 13, 7, and 3 months before the present episode, in 3 of the 7 patients for whom prior PCP episode data were available. No patient who had received or was receiving atovaquone as therapy for toxoplasmosis before the PCP episode.

Forty-six samples were amplified and sequenced by either single-round or nested PCR, as described elsewhere [8]. For 12 samples, we only obtained sequence data for the Qo site. In 2 patients, we only obtained sequence data for the 5′ portion of the Qo site. In all, 49 of the 60 patients had wild-type sequences, including the latter 2 patients for whom sequence data was obtained only for the 5′ portion of the Qo site (GenBank accession no. AF320344). The sequences were identical to the sequence published elsewhere [8]. Three patients had point mutations that resulted in the substitution of a phenylalanine for a leucine in position 280 (GenBank AF321304). Since this amino acid is not involved in coenzyme Q or atovaquone binding, this mutation was probably due to strain variation. Eight patients had mutations in the Qo site, and none were identical to previously published mutations (T121I and L123F; [8]), but several mutations were in nearby codons—T100I, I120V, and S125A (GenBank AF320346, AF320342, and AF320343, respectively). Each was found in 1 patient. Two other mutations were found on the other Qo site peptide: P239L and L248F (GenBank AF320341 and AF320345, respectively). These were found in 3 and 2 patients, respectively.

Table 2 shows that patients with AIDS who had atovaquone exposure were significantly more likely to yield mutant P. carinii cytochrome b sequences. Overall, cytochrome b mutations were present in 8 (13%) of the 60 patients. Five of 15 patients with AIDS who had atovaquone exposure had mutant cytochrome b genes. In contrast, only 3 (6%) of 45 patients with AIDS who did not have atovaquone exposure had mutant cytochrome b genes. This difference is statistically significant (P = .018). Of the 9 patients who received atovaquone as prophylaxis against PCP, 5 (55%) had cytochrome b mutations. Of the 6 patients who received atovaquone for a prior episode of PCP, none had mutations. There were no statistically significant differences in demographic or clinical characteristics between patients with wild-type and those with mutant P. carinii strains (table 1).

Overall, 50 (84%) of the 60 patients survived the PCP episode: 7 (87%) of 8 with cytochrome b mutations survived, compared with 43 (83%) of 52 patients without a mutation. Thus, there was no difference in survival between patients with AIDS and PCP who did or did not have cytochrome b mutations. Nine of the 60 patients were treated with atovaquone for the PCP episode. Two of the 8 patients with cytochrome b mutations received atovaquone treatment; each patient survived. Seven of the 52 patients with wild-type strains received atovaquone treatment; each survived.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Atovaquone exposure</th>
<th>No atovaquone exposure</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′</td>
<td>8 (13)</td>
<td>3 (6)</td>
<td>11</td>
</tr>
<tr>
<td>Wild type</td>
<td>42</td>
<td>52</td>
<td>96</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>63</td>
<td>113</td>
</tr>
</tbody>
</table>

NOTE. Data are no. of patients. Nos. in parentheses are percentage of total patients in each column.

Discussion

Results of this multicenter study of 60 patients with AIDS with PCP suggest that mutations in the P. carinii cytochrome b gene, the atovaquone drug target, are associated with atovaquone exposure in patients with AIDS. In a previous study, we demonstrated sequence polymorphisms in the P. carinii cytochrome b gene in 2 patient isolates [8]. In the present study, we found a statistically significant association between the presence of point mutations in the Qo region and previous atovaquone exposure. When we combined the results of the present study with those of our prior study [8], 7 different cytochrome b Qo mutations were found in 10 patients. This contrasts with previous studies of sulfa resistance in P. carinii, in which the majority of mutant isolates had only 2 mutations [11–15]. There are 2 possible explanations for this difference. First, multiple mutations may result in a functional cytochrome b with reduced affinity for atovaquone, whereas only few mutations can allow for DHPS to function and still be resistant to sulfa drugs. Second, mutations to atovaquone may take place de novo in each treated patient, whereas a small number of sulfa-resistant strains are passed from person to person. Further studies that combine strain typing methods may help to differentiate between these explanations.

Unfortunately, a direct causal relationship between genetic mutations and phenotypic atovaquone resistance cannot be demonstrated by the measurement of the effects of atovaquone on the in vitro growth or cytochrome b enzymatic activity. Neither test can be performed on human-derived P. carinii, since the organism cannot be cultivated in media to permit susceptibility testing or be isolated in large enough quantities for enzyme assays.

This study shows that the overall incidence of cytochrome b mutations in P. carinii (16%) is lower than that of DHPS mutations seen in several studies (27%–48%). The lower incidence of atovaquone mutations is probably due to the more infrequent use of atovaquone for PCP prophylaxis or treatment of established episodes of PCP, compared with that of sulfa or sulfone drugs. Nevertheless, the results of this study parallel the association between the presence of point mutations in the DHPS gene and previous sulfa or sulfone prophylaxis [11–15]. Additional studies should be done to determine if cytochrome b mutations are associated with treatment failure or increased mortality.

In summary, our results show that mutations in the P. carinii atovaquone target gene are associated with prior exposure to
atovaquone. Further studies are needed to determine whether PCR determination of the presence of cytochrome b mutations could be useful in predicting response to atovaquone therapy. The presence of multiple mutations in patient specimens confirms studies that show atovaquone resistance in malaria [10]. Additional prospective trials that use this assay are needed to evaluate the clinical relevance of these findings and to determine the need for the development of new agents for the prophylaxis and therapy of PCP.

Acknowledgment

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References

2. Vaidya AB, Lashgari MS, Polege LG, Morrisey J. Structural features of Plasmodium cytochrome b that may underlie susceptibility to 8-aminoquinolines and hydroxynaphthoquinones. Mol Biochem Parasitol 1993;58:33–42.
ERRATUM

In an article in the 1 March 2001 issue of the Journal (Kazanjian P, Armstrong W, Hossler PA, et al. Pneumocystis carinii cytochrome b mutations are associated with atovaquone exposure in patients with AIDS. J Infect Dis 2001; 183:819–22), an author was omitted. Chao-Hung Lee, from Indiana University, should be added as the fourth author of this article.