Sequences of *Pneumocystis carinii* f. sp. *hominis* Strains Associated with Recurrent Pneumonia Vary at Multiple Loci

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The sequences of the internal transcribed spacer (ITS) of *Pneumocystis carinii* f. sp. *hominis* strains from 7 of 15 AIDS patients were found to vary during discrete episodes of *P. carinii* pneumonia. Changes in the ITS sequence correlated with changes in the mitochondrial large-subunit rRNA sequence. The coincidence of changes in the sequences of the ITS, which is located in the nucleus, with changes in a mitochondrial gene excludes mutation as the cause of the genetic differences between *P. carinii* f. sp. *hominis* strains isolated during different episodes of *P. carinii* pneumonia and supports the hypothesis that recurrent *P. carinii* pneumonia is caused by reinfection rather than by reactivation of latent organisms. Thus, limiting the exposure of immunocompromised patients to *P. carinii* f. sp. *hominis* should help prevent *P. carinii* pneumonia.

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**Materials and Methods**

Bronchoalveolar lavage (BAL) fluids were collected and processed as described previously (1). Briefly, BAL fluids were obtained from patients followed at the University of Cincinnati AIDS Treatment Center and were stored at 4°C. Portions of each BAL fluid were examined for the presence of bacterial, legionella, fungal, mycobacterial, and viral organisms. *P. carinii* f. sp. *hominis* organisms were visualized and counted by using Diff-Quik stain (Baxter Scientific Products, McGaw Park, Ill.) and methenamine silver stain. One-microliter aliquots of the BAL fluids were used as templates in PCRs.

For the PCR, oligonucleotides NITSF and NITSR (14) were used under the following conditions: a 95°C hot start for 5 min and 30 cycles of incubation at 95°C for 60 s, 42°C for 60 s, and 72°C for 60 s. Reaction volumes were 25 μl containing 200 μM (each) dATP, dCTP, dGTP, and dTTP, 1.25 U of *Tfl* polymerase (Epiconcentre, Madison, Wis.), 1.5 mM MgCl₂, and 20 ng of each primer. One microliter of the amplification product was used for the nested PCR with primers ITS3F/RI and ITS3R/RI (14) by using the same PCR conditions described above, except that the annealing temperature was 50°C. Sample 3.2 could not be amplified by the nested ITS PCR. Therefore, the PCR was performed with a new set of ITS2 primers, ITS2U (5'-GTGGCGAAAATGCGATAAGTA-3') and ITS2L (5'-TATGCCTTAAGTTACGCGGT-3'), which are located at nucleotide positions 2013 (9) (within the 5.8S rRNA gene) and 2352 (9) (within the 26S rRNA gene), respectively. PCR conditions were as follows: a 95°C hot start for 5 min and 40 cycles of incubation at 95°C for 60 s, 50°C for 120 s, and 72°C for 60 s. The DNA produced by the PCR was gel purified and directly sequenced (12). To show that sequences were not influenced by PCR error, the ITS1 region from 9 samples and the ITS2 region from 20 samples were amplified twice by using BAL fluid as the source of template DNA. The sequences of the PCR products were identical for all duplicate samples.

**Results**

ITS sequences of *P. carinii* f. sp. *hominis* from initial and recurrent disease episodes. ITS1 and ITS2 genotypes were determined for isolates in samples from 15 patients. For each sample, 100 bp of the ITS1 sequence and 177 bp of the ITS2 sequence were determined. Figure 1 presents two examples of
products because it is part of a homopolymeric tract that was susceptible to artifactual variation (14). However, our control experiments, in which samples were each amplified twice (samples 2.1, 2.2, 4.2, 4.3, 5.1, 5.2, 8.1, and 8.2 in Table 3) or four times (sample 7.1) indicated that such artifacts were not in evidence when PCR products were sequenced directly. Hence, all 20 polymorphic nucleotide positions of the ITS1 and ITS2 sequences, including positions in homopolymeric tracts, were used to score the genotypes of isolates from patients, which are listed in Table 3.

Table 4 indicates that the 31 patient samples contained five combinations of ITS1 and ITS2 genotypes, B 1-1a1-1, B1-1b2-1, A2-1c1, B1-2a1-1, and B1-3b2-1 (which was mixed with B1-1a1-1). Three ITS genotypes that had been described previously (Bc, B1d, and Cα3) (14) were not observed in samples from the 15 patients.

Major and minor genotypes were also observed in the mtrRNA, in which genotype CC was predominant (23 samples) (Table 4). The frequencies of combined ITS-mtrRNA genotypes are also presented in Table 4. Two-thirds of the patient samples were B 1-1a1-1CC.

**Concurrent genotype switching of ITS and mtrRNA sequences in episodes of *P. carinii* f. sp. *hominis* pneumonia.** Analysis of the ITS1 and ITS2 together showed that 7 of 15 patients were infected with *P. carinii* f. sp. *hominis* of a different genotype.

**TABLE 3. ITS and mtrRNA genotypes of AIDS patients**

<table>
<thead>
<tr>
<th>Patient sample&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ITS genotype&lt;sup&gt;b&lt;/sup&gt;</th>
<th>mtrRNA genotype&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Patient sample</th>
<th>ITS genotype</th>
<th>mtrRNA genotype</th>
</tr>
</thead>
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<tr>
<td>1.1</td>
<td>A2-1c1</td>
<td>AC+AT&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.2</td>
<td>B1-2a1-1</td>
<td>CC</td>
</tr>
<tr>
<td>2.1</td>
<td>B1-1a1-1</td>
<td>CC</td>
<td>6.3</td>
<td>B1-1a1-1</td>
<td>CC</td>
</tr>
<tr>
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<td>B1-1a1-1</td>
<td>AC</td>
<td>9.1</td>
<td>B1-1a1-1</td>
<td>CC</td>
</tr>
<tr>
<td>3.1</td>
<td>A2-1c1</td>
<td>AC</td>
<td>9.2</td>
<td>B1-1a1-1</td>
<td>CC</td>
</tr>
<tr>
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<td>CC</td>
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<td>B1-1a1-1</td>
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<tr>
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<tr>
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<td>CC</td>
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<td>CC</td>
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<td>14.1</td>
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<td>CC</td>
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<td>B1-1a1-1</td>
<td>CC</td>
</tr>
</tbody>
</table>

<sup>a</sup> Numbers and decimals are patient and episode numbers, respectively.

<sup>b</sup> Capital and lowercase letters are genotypes for ITS1 and ITS2, respectively (see Tables 1 and 2).

<sup>c</sup> The first and second letters represent nucleotides at mtrRNA sites 85 and 248, respectively. The mtrRNA genotypes of isolates from patients 1 through 14 were described previously (7). That for patient 15 was also described previously (8).

<sup>d</sup> Primers pITS2<sup>U</sup> and pITS2<sup>L</sup> were used for the PCR. No data for ITS1 were obtainable.
type in each of two successive episodes of disease (patients 1, 2, 3, 4, 5, 7, and 8) (Table 3). Isolates from different PCP episodes in these same seven patients had different mtrRNA genotypes (Table 3). Conversely, isolates from the initial and subsequent bouts of PCP in eight patients (patients 6, 9, 10, 11, 12, 13, 14, and 15) had the same ITS (B1, a1, b1, 1) and mtrRNA (CC) genotypes (Table 3). ITS genotype B1, a1, b1, 1 and mtrRNA genotype CC are the prevalent genotypes in our database of samples from humans (data not shown); therefore, distinguishing reinfection from reactivation in the eight patients is not possible with the current multilocus genotyping system.

**DISCUSSION**

The concurrence of genotype switching at loci in two different cellular compartments argues that genotype switching was not due to mutation during the first disease episode but, rather, was due to infection with two different strains of *P. carinii* f. sp. *hominis*. The different strains were presumably acquired independently in separate infection events.

Two plausible alternatives to reinfection as the cause of genotype switching have been examined in previous studies (7). One such alternative would be selection of a preexisting mtrRNA genotype (7). Another, there was no correlation between recurrence of disease and a particular mtrRNA genotype (7).

A second way to reconcile genotype switching with reactivation would be to assume that sampling of the lung was inadequate to detect in the sample taken at the first disease episode a minority population of organisms with the genotype seen during the second disease episode. However, previous studies by allele-specific PCR indicated that this was not the case (7).

In addition to genotype switching, four other lines of evidence support the reinfection hypothesis. First, PCR of homogenized lung tissue from 15 healthy individuals failed to detect *P. carinii* f. sp. *hominis* DNA (11). Second, immunocompetent rats can eliminate *P. carinii* if they are kept free of exogenous *P. carinii* for at least 1 year (13, 15). Third, infected severe combined immunodeficient mice whose immune systems were transiently reconstituted with normal lymphocytes did not develop *P. carinii* pneumonia after the normal lymphocytes were depleted (3). Fourth, epidemiological studies have indicated that *P. carinii* pneumonia outbreaks occur in AIDS patients and transplant recipients, suggesting the de novo acquisition of organisms just prior to the presentation of disease (2, 6, 16). We conclude that the genotype switching observed in isolates from AIDS patients experiencing recurrent episodes of PCP was not due to mutations occurring in some of the organisms present in the first disease episode and that reinfection remains the most likely explanation for this phenomenon. If reinfection occurs in AIDS patients, limiting the exposure of immunocompromised patients to *P. carinii* f. sp. *hominis* should help to prevent PCP.

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**REFERENCES**


