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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**Pneumocystis carinii** f. sp. *hominis* is the form of *P. carinii* that causes pneumonia in AIDS patients. *P. carinii* pneumonia (PCP) is commonly thought to be the result of the reactivation of latent organisms from work done with rats. An early study indicated that laboratory rats treated with corticosteroids for several weeks and maintained under barrier-housed conditions produced fulminant *P. carinii* pneumonia (5). However, more recent data have indicated that latency is not always maintained in experimental animals (3), and our previous studies with patients experiencing multiple episodes of PCP have suggested that recurrent disease may be due to the de novo acquisition of *P. carinii* f. sp. *hominis* organisms (7, 8). Our previous studies indicated that the sequence of the mitochondrial gene encoding the RNA in the large ribosomal subunit (mtrRNA) changed with disease episode in half of the patients studied (genotype switching). These sequence changes occurred at two nucleotide positions, positions 85 (A, C, or T) and 248 (C or T) (7). A simple explanation of these changes is the postulation that recurrent PCP is caused by the de novo acquisition of a new strain of *P. carinii* f. sp. *hominis* proximal to the time of PCP symptoms. If this were true, then it might be expected that the sequences of other variable loci, such as the internal transcribed spacer (ITS) region of the nuclear gene encoding cytoplasmic rRNAs (10) would also differ with disease episode. On the other hand, if a change in the mtrRNA gene sequence were due to a mutation that occurred during the first disease episode, then the probability of a concurrent change at another locus could be expected to be low, assuming that mutations at different loci occur independently. To investigate this issue, we determined the sequences of the ITSs of *P. carinii* f. sp. *hominis* isolates from 15 AIDS patients, isolates from 7 of whom had previously exhibited mtrRNA genotype switches accompanying a recurrence of PCP (7, 8). We found that ITS genotype switching occurred in isolates from all seven of these patients, indicating that reinfection, not mutation, was responsible for these genetic changes.

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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 *Taq* polymerase (Epicentre, 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/R1 and ITS3R/R1 (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, ITS2L (5′-GTGGGAAAATGCGATAAGTA-3′) and ITS2U (5′-TATGCTTAAGTTCAACGCAGGT-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
the ITS sequences that were observed. The ITS1 sequences exhibited variations at 6 nucleotide positions (nucleotides 6, 14, 23, 68, 77, and 78), and the ITS2 sequences exhibited variations at 15 nucleotide positions (nucleotides 2, 50 to 52, 59, 63 to 67, 116, 156, 162, 163, and 165). These data are summarized in Tables 1 and 2, respectively. There were four different ITS1 sequences (A2-1, B1-1, B1-2, and B1-3) and three different ITS2 sequences (a1-1, b1-1, and c1). The predominant ITS1 and ITS2 genotypes were B1-1 (23 samples) and a1-1 (28 samples), respectively. B1-1 corresponds to the genotype called genotype B1 by Tsolaki et al. (14). Genotype a1-1 corres-
ponds to the genotype called genotype a by Lu et al. (10) and is similar to the genotype called genotype a1 by Tsolaki et al. (14). Genotype a1-1 corresponds to the genotype called genotype a by Lu et al. (10) and is similar to the genotype called genotype a1 by Tsolaki et al. (14).

Most of the variable nucleotide positions that we observed have been described previously; however, a study by Tsolaki et al. (14) did not detect variability at sites 14 and 23 of the ITS1 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, B1-1/a1-1, B1-2/a1-1, A2/c1, B1/b2-1, and B1/b2-1 (which was mixed with B1/a1-1). Three ITS1 genotypes that had been described previously (Be, B1, and Ca) (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 P. carinii f. sp. hominis isolates were B1/a1-1, CC.

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 geno-

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.

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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 sequences (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-a1-1) and mtrRNA (CC) genotypes (Table 3). ITS genotype B1-a1-1 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 minority strain of *P. carinii f. sp. hominis* that happened to be resistant to the anti-*Pneumocystis* drugs used to treat the initial episode of disease. It is not known if drug resistance occurs in *P. carinii*. However, in a previous study, we identified three patients (patients 2, 3, and 5, Table 3) who exhibited genotype switching and were successfully treated with the same drug (sulfamethoxazole, pentamide, or atovaquone) during initial and subsequent episodes of *P. carinii* pneumonia. The success of treatment in both disease episodes suggests that drug resistance was not involved in these genotype switches (7). In addition, 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 second disease episode a majority 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.

**ACKNOWLEDGMENTS**

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**REFERENCES**


**TABLE 4. Frequencies of ITS and mtrRNA genotypes**

<table>
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<th>ITS genotype (no. of samples)</th>
<th>mtrRNA genotype (no. of samples)</th>
<th>Combined ITS-mtrRNA genotype (no. of samples)</th>
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<td>B1-a1-1 CC (21)</td>
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<td>AC (6)</td>
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<td>a2-1 CC (1)</td>
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* a See footnote b of Table 3.

* b See footnote c of Table 3.

* c See footnote e of Table 3.