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Nucleotide Sequence Variation in *Pneumocystis carinii* Strains That Infect Humans

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The nucleotide sequences of a portion of the large-subunit mitochondrial rRNA gene of *Pneumocystis carinii* derived from 12 patients were examined. Five sequences were found to be identical to the prototype sequence reported by Sinclair et al. (K. Sinclair, A. E. Wakefield, S. Banerji, and J. M. Hopkin, Mol. Biochem. Parasitol. 45:183–184, 1991). Six sequences differed from the prototype sequence at one to three positions. The remaining sequence was markedly different from the prototype sequence and appeared to be a hybrid of the human and rat *P. carinii* sequences. The results of this study indicate the existence of multiple *P. carinii* strains infecting humans and suggest that coinfections of animal and human *P. carinii* strains in the same host are possible.

Pneumocystis carinii causes a debilitating pneumonia in immunocompromised individuals, including those with AIDS and those receiving immunosuppressive therapy for transplantation or malignancy. *P. carinii* pneumonia is the major cause of death in AIDS patients (11, 16, 17), and it has been estimated that more than 100,000 AIDS patients have developed *P. carinii* pneumonia (9). *P. carinii* is also a common cause of pneumonia in immunodeficient, malnourished, and immunocompromised children (18).

Although strains infecting rats, mice, ferrets, and humans have been demonstrated to be different by examination of their reactivities to specific antisera (2, 10) and comparison of the nucleotide sequences of certain portions of the *P. carinii* genome (7, 22), it is unknown at present whether the *P. carinii* that infects humans represents a single organism or a group of organisms. In this study, we examine the nucleotide sequences of a portion of the large-subunit mitochondrial rRNA gene of *P. carinii* from several patients and detected sequence variations in the examined target. The results of this study suggest the existence of multiple *P. carinii* strains.

Bronchoalveolar lavage or open-lung biopsy specimens were used in this study. The organisms present in bronchoalveolar lavage specimens were pelleted and resuspended in 500 μl of proteinase K buffer (50 mM KCl, 15 mM Tris-HCl [pH 8.3], and 0.5% Nonidet P-40) containing 500 μg of proteinase K per ml. For tissue specimens, a portion weighing approximately 0.5 mg was homogenized in proteinase K buffer. Proteinase K was then added to a final concentration of 500 μg/ml. After incubation at 55°C for 45 min, the mixture was extracted with phenol and chloroform. The DNA present in the aqueous phase was precipitated with ethanol. The ethanol was then removed by vacuum drying, and the DNA was dissolved in 50 μl of TE buffer (Tris-EDTA). Ten microliters of this solution was used for polymerase chain reaction (PCR) with the primer pair published by Wakefield et al. (23). These primers amplify a fragment of approximately 340 bp of the large-mitochondrial rRNA subunit gene when rat- or human-derived *P. carinii* DNA was used as the template.

PCR was performed in a 100-μl mixture containing template DNA, PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin), 20 pmol each of the two PCR primers, 0.2 mM of the four deoxynucleoside triphosphates, and 2 U of *Taq*I DNA polymerase. The mixture was overlaid with 100 μl of mineral oil to prevent evaporation during thermal cycling. PCR was performed in three stages. The initial stage was 10 cycles, with each cycle consisting of 1.5 min at 94°C, 1.5 min at 50°C, and 3 min at 72°C. The second stage was 20 cycles with each cycle consisting of 1.5 min at 94°C, 1.5 min at 42°C, and 3 min at 72°C. The final stage was extension at 72°C for 10 min. The PCR products were electrophoresed in a 6% polyacrylamide gel to determine the sizes of the amplified products.

The PCR products were purified by using the Magic PCR Preps kit (Promega, Madison, Wis.). This kit removes primers and other components that are present in the PCR mixture. The purified products were then sequenced directly by using the *fmol* DNA sequence kit (Promega) by the manufacturer's protocol. The primer used for sequencing was labelled at its 5′ end with [γ-^{32}P]ATP.

The PCR products were sequenced from both ends with one of the PCR primers in each sequencing reaction. Some of the sequences were determined by sequencing the cloned PCR products, because direct sequencing failed to generate readable sequences. In these cases, multiple clones of the same PCR products were sequenced to verify the sequences. The PCR products were cloned into the TA-cloning vector pCRII (Invitrogen, San Diego, Calif.) after purification with the Magic PCR Preps kit (Promega), as described above. The cloned products were sequenced with the Sequenase kit (U.S. Biochemical Corp., Cleveland, Ohio) and appropriate primers.

We have sequenced PCR products of specimens from 12 different patients: one from Italy, one from Canada, and the others from Indiana. Five sequences are identical to the sequence reported by Sinclair et al. (22). The other seven sequences have several nucleotides that are different from those of the published sequence (Fig. 1). Within the 300 bp that we have sequenced, patient 1's sequence has a C-to-T change at position 85 and a G-to-A change at position 288. Patient 2's sequence has the same change at position 288.

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Hu Prt   TTGTGGTAAG TAGTGAAATA CAAATCGGAC TAGGATATAG CTGGTTTTCT   50
Pt 1     ********** ********** ********** ********** **********
Pt 2     ********** ********** ********** ********** **********
Pt 5     ********** ********** ********** ***-****** **********
Pt 6     ********** ********** ********** ********** **********
Pt 9     ********** ********** ********** ********** **********
Pt 11    ********** ********** ********** ********** **********
Pt 12    ********** ********** ********** ********** **********

Hu Prt   GCGAAAATTG TTTTGGCAAA TTGTTTATTC CTCTCAAAAA TAGTAGGTAT  100
Pt 1     ********** ********** ********** ****T***** **********
Pt 2     ********** ********** ********** ********** **********
Pt 5     ********** ********** ********** ********** **********
Pt 6     ********** ********** ********** ********** **********
Pt 9     ********** ********** ********** ********** **********
Pt 11    ********** ********** ********** ****-***** **********
Pt 12    ******TC*A ******T*G* *GAC**G**A T*ATTTGT*G *G---*****

Hu Prt   AGCACTGAAT ATCTCGAGGG AGTATGAAAA TATTTATCTC AGATATTTAA  150
Pt 1     ********** ********** ********** ********** **********
Pt 2     ********** ********** ********** ********** **********
Pt 5     ********** ********** ********** ********** **********
Pt 6     ********** ********** ********** ********** **********
Pt 9     ********** ********** ********** ********** **********
Pt 11    ********** ********** ********** ********** **********
Pt 12    ********** ****AA**** ********GG **C***CT*T G*********
                       CTTATGTTAGA

Hu Prt   TCTCAAAATA ACTATTTCTT AAAATAAATA ATCAGACTAT GTGCGATAAG  200
Pt 1     ********** ********** ********** ********** **********
Pt 2     ********** ********** ********** ********** **********
Pt 5     ********** ********** ********** ********** **********
Pt 6     ********** ********** ********** G********* **********
Pt 9     ********** ********** ********** ********** **********
Pt 11    ********** ********** ********** ********** **********
Pt 12    *****G**** G*****AA*A T*TGATG*GT ********TC T*********
                                          T

Hu Prt   GTAGATAGTC GAAAGGGAAA CAGCCCAGAA CAGTAATTAA AGCTCCCAAA  250
Pt 1     ********** ********** ********** ********** **********
Pt 2     ********** ********** ********** ********** **********
Pt 5     ********** ********** ********** ********** **********
Pt 6     ********** ********** ********** ********** ******T***
Pt 9     ********** ********** ********** ********** ******T***
Pt 12    **GAGG**** **G******* ********** ********** **********

Hu Prt   TTAATATTAA GTGAAATAAA AGTTGTTGGA TATCTAAGAC AGTTAAGAAG  300
Pt 1     ********** ********** ********** *******A** **********
Pt 2     ********** ********** ********** *******A** **********
Pt 5     ********** ********** ********** *******A** **********
Pt 6     ********** ********** ********** *******A** **********
Pt 9     ********** ********** ********** *******A** **********
Pt 11    ********** ********** ********** *******A** **********
Pt 12    ********** ********** ********** *******A** **********
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FIG. 1. Comparison of nucleotide sequences of a portion of the large-subunit mitochondrial rRNA gene of *P. carinii* from different patients. The top line is the human *P. carinii* sequence first reported by Sinclair et al. (22) and is designated Hu Prt for human *P. carinii* prototype. The other lines represent sequences derived from different patients. The bases that are identical to those of the prototype sequence are shown as asterisks. Missing bases (–) and bases that are different from those of the prototype sequence are indicated. Pt, patient.

Patient 5's sequence has a single-base deletion at position 34 and a G-to-A change at position 288. Patient 6's sequence has A-to-G, C-to-T, and G-to-A changes at positions 181, 248, and 288, respectively. Patient 9's sequence has a C-to-T change at position 248 and a G-to-A change at position 288. Patient 11's sequence has a base missing at position 85 and G-to-A change at position 288. Patient 12's sequence shows a marked polymorphism relative to other sequences; this patient was a 4-year-old girl who had acute lymphocytic leukemia. The specimen used in this study was a piece of tissue obtained from an open-lung biopsy in 1983. This sequence differs from the published sequence at 55 positions, including an insertion of the sequence CTTATGT TAGA at position 117 and a T at position 181. In addition, this sequence shows interesting characteristics. The first 99 nucleotides of the sequence are identical to the corresponding positions of that of rat *P. carinii*, the next 23 nucleotides are identical to that of human *P. carinii*, the following 113 nucleotides are identical to that of rat *P. carinii*, and the last 88 nucleotides are identical to that of human *P. carinii* (Fig. 2 and 3). Therefore, this sequence appears to be a hybrid of rat and human *P. carinii* sequences. It is unlikely that this hybrid sequence is a result of contamination of the specimen with rat *P. carinii*, since all of our human specimens were stored separately from the rat specimens and processed for PCR in a room in which no *P. carinii*-infected rats or tissue cultures were handled.

The results of this study indicate that nucleotide sequences of *P. carinii* from different patients may be different, implying the existence of multiple strains of *P. carinii*. The

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                |→ Rat
Pt 12   ********** ********** ********** ********** **********
Rat     TTGTGGTAAG GAGTGAAATA CAAATCGGAC TAGGATATAG CTGGTTTTCT   50
Hu Prt  ********** T********* ********** ********** **********
                                                        |→ Human
Pt 12   ********** ********** ********** *--*--**** *********--*
Rat     GCGAAATCTA TTTTGGTAGA TGACTTGTTA T--T--ATTT GTAGTGG--G  100
Hu Prt  ******AT*G ******C*A* --TTG*T*AT *CC*CTCAA- A-AA*A*TA*
                             |→ Rat
Pt 12   G***-----** *AC******* ********** ********** **********
Rat     -TATAGGCAG C--TGAATAT CTAACTTATG TTAGAAGGGA GTATGAAGGT  150
Hu Prt  G***------** *AC******* **C------- ----G***** *******AA*

Pt 12   *-********* ********** ***C****** *******--** **********
Rat     A-CTTACTTT GGATATTTAA TCTGAGAATA GCTATT--AA TATATGATGA  200
Hu Prt  *TT*AT**C- A********** ***C*A**** A*****TCTT A*-*AT*AAT
                                                |→ Human
Pt 12   ********** ********** ********** ********** **********
Rat     GTTATCAGAC TTCTTGCGAT AAGGTGAGGA GTCGAGAGGG AAACAGCCCA  250
Hu Prt  AA--******* *ATG****** *****AGAT* *****A**** **********

Pt 12   ***C*G**AT *****C***C C****AA*** *A******A* *****TT***
Rat     GAATAATATA TAAAGTTCCA AAATTGTTAT TGAGTGAATT AAAAGAAGTT  300
Hu Prt  ***C*G**AT *****C***C C****AA*** *A******A* *****TT***

Pt 12   GGA*A**TA* ******T*** ***
Rat     TTCTTTCGTA GACAGTCAAG AAG
Hu Prt  GGA*A**TA* ******T*** ***
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FIG. 2. Comparison of nucleotide sequence of a portion of the large-subunit mitochondrial rRNA gene of *P. carinii* from patient 12 with the prototype sequences of human and rat *P. carinii*. The top line represents the sequence derived from patient 12 (Pt 12). The sequence shown in the middle line is that of rat *P. carinii*. The bottom line is the prototype human *P. carinii* sequence (Hu Prt). Bases identical to those of the prototype sequence are shown as asterisks. Missing bases (–) and bases that are different from those of the prototype sequence are indicated. The beginnings of sequences that are homologous to the human or rat *P. carinii* sequence (| →) are shown.

observation that one sequence of the mitochondrial rRNA gene is a hybrid of the rat and human *P. carinii* sequences suggests that coinfection of rat and human strains of *P. carinii* in the same host is possible. Recombination may take place in rats or in humans. This recombination would imply that human *P. carinii* may infect animals and animal *P. carinii* may infect humans. This notion is also suggested by the study of Sethi (20), who showed that *P. carinii* from a human bronchoalveolar lavage specimen can proliferate in mice.

The finding that different strains of *P. carinii* have different nucleotide sequences may form a basis for studying strain variations and epidemiology of *P. carinii*. At present, little is known about *P. carinii* strains. Approximately 50% of AIDS patients have recurrent infections (6, 14, 21), but it is unknown whether a second episode of *P. carinii* infection represents a relapse caused by drug failure or a reinfection by a different strain of *P. carinii*. In addition, more than 60% of AIDS patients in the United States (1, 3, 12, 13, 15) develop *P. carinii* pneumonia some time during their illness in contrast to only 0 to 22% of AIDS patients in Africa. This observation may be due to differences in the virulence of strains prevalent in various geographic areas. Furthermore, transmission of *P. carinii* among patients has been suggested (4, 5, 8, 19) but has not been proven. The possibility that each *P. carinii* strain has a unique nucleotide sequence will allow us to answer these important questions.

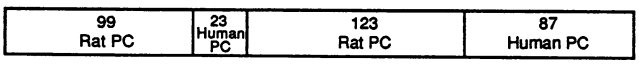

FIG. 3. Diagrammatic illustration of nucleotide sequence of a portion of the large-subunit mitochondrial rRNA gene of *P. carinii* from patient 12. Rat PC and Human PC represent the regions of the sequence that are identical to those of the rat and human *P. carinii* sequences, respectively. The numbers within the boxes indicate the numbers of nucleotides that are identical to those of the respective sequences.

Nucleotide sequence accession number. The hybrid sequence of patient 12 has been deposited in the GenBank data bank (accession no. L07891) and the EMBL data bank (accession no. Z19053).

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