

Genetic Diversity in Human-Derived *Pneumocystis carinii* Isolates from Four Geographical Locations Shown by Analysis of Mitochondrial rRNA Gene Sequences

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The opportunistic fungal pathogen *Pneumocystis carinii* is a frequent cause of pneumonia in immunocompromised hosts. In this study, we have compared the DNA sequences of a portion of the mitochondrial large-subunit rRNA gene of *P. carinii* (an informative locus showing up to 27% differences among isolates of *P. carinii* from human-, rat-, mouse-, ferret-, rabbit-, and horse-infected lungs) obtained from human-derived isolates from widely disparate geographical areas, including Britain, the United States, Brazil, and Zimbabwe. A single-base polymorphism which varied among samples was identified. Apart from this nucleotide, the DNA sequences of all samples were identical. The sequences of the British samples were shown to be stable over a period of 4 years. These data suggest that there is relatively low genetic diversity among isolates of human-derived *P. carinii* from different global regions.

The opportunistic fungus *Pneumocystis carinii* is an important cause of potentially fatal pneumonia in immunocompromised individuals, particularly AIDS patients. *P. carinii* has been identified in the lungs of a variety of different mammalian hosts (8), and although the parasites appear to be morphologically indistinguishable, immunological and genetic studies indicate that these organisms are not identical. The inability to continuously culture the organism in vitro has prevented the establishment of clonal stocks of the organism and impeded research into the genetic relationship between different isolates. Analysis of the chromosomes of rat- and human-derived *P. carinii* by pulsed-field gel electrophoresis has shown that the karyotypes of these organisms are similar but distinct (18). DNA sequences of *P. carinii* from a number of different genetic loci have been examined. A portion of the gene encoding the mitochondrial large-subunit rRNA has been investigated in *P. carinii* derived from six different mammalian hosts: the infected lungs of rats, humans, ferrets, rabbits, mice, and horses (13, 15, 21). Chromosomal loci have also been examined, including the *P. carinii* β -tubulin gene from infected human and rat lungs (3, 4), the major surface glycoprotein (msg) from rat- and human-derived *P. carinii* (9, 19) and ferret-derived organisms (gpA) (5), the nuclear rRNA operon (11, 12), and the AROM protein, involved in the aromatic amino acid biosynthetic pathway (unpublished data). All cumulative DNA

sequence data demonstrate that *P. carinii* organisms isolated from different mammalian hosts are genetically distinct.

Genetic divergence has also been shown in *P. carinii* infecting the same host species. Electrophoretic karyotype analysis and DNA sequence data have identified different types of *P. carinii* infecting rat lungs (2, 11). In this paper, we examine genetic diversity in *P. carinii* infecting humans in samples obtained from widely dispersed geographical locations in order to examine the possibility that differences in the incidence and manifestation of *P. carinii* pneumonia in different regions of the world are due to infection by different strains of the organism. We have obtained samples from four different sources: Britain, the United States, Brazil, and Zimbabwe. We have examined the sequence of a portion of the *P. carinii* mitochondrial large-subunit rRNA gene since this locus has been shown to be highly informative, displaying up to 27% differences among *P. carinii* organisms isolated from the infected lungs of six different mammalian hosts (13, 15, 21). We report the comparison of sequence data from these isolates of human-derived *P. carinii*.

MATERIALS AND METHODS

Samples. Samples of bronchoscopic alveolar lavage were collected from human immunodeficiency virus (HIV)-infected patients with *P. carinii* pneumonia, confirmed by positive histochemical staining with Grocott's methenamine silver stain, typical clinical presentations, and response to therapy. Samples were obtained from Britain (Middlesex Hospital, London; 12 samples); the United States (St. Jude Children's Research Hospital, Memphis, Tenn.; 4 samples); South America (Hospital de Clinicas de Porto Alegre, Porto Alegre, Brazil; 8 samples), and Africa (Parienyatwa Hospital, Harare, Zimbabwe; 6 samples). The British samples spanned a 4.5-year period, from January 1989 to August 1993. Samples from

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Britain, the United States, and Africa were frozen immediately after bronchoscopy, stored at -20°C , and transported to the Institute of Molecular Medicine prior to DNA extraction. Samples from Brazil were frozen following bronchoscopy and subsequently thawed, and DNA was extracted in Brazil. All DNA amplification reactions were carried out at the Institute of Molecular Medicine.

DNA extraction. The samples were treated with proteinase K (Boehringer Mannheim) at a final concentration of 1 mg/ml in the presence of 0.5% sodium dodecyl sulfate–10 mM EDTA (pH 8.0) at 50°C for 18 h. One phenol-chloroform extraction was performed on the samples, and the DNA was then purified and concentrated with a DNA binding resin (Magic DNA Clean-up System; Promega).

DNA amplification. Amplification was performed on the samples by using the oligonucleotide primers pAZ102-H and pAZ102-E, with one biotinylated and one unmodified oligonucleotide primer in each reaction (21). The final concentration of the reaction mix was 50 mM KCl, 10 mM Tris (pH 8.0), 0.1% Triton X-100, 3 mM MgCl_2 , 400 μM (each) deoxynucleoside triphosphates, 1 μM oligonucleotide primer, and 0.025 U of *Taq* polymerase (Promega) per μl . Forty cycles of amplification were performed, with denaturation at 94°C for 1.5 min, annealing at 55°C for 1.5 min, and extension at 72°C for 2 min. Negative controls with no added template DNA were included after each sample to monitor for cross-contamination. All experimental stages were carried out in duplicate.

Direct sequencing of DNA amplification product. Single-stranded DNA sequencing template was generated from the amplification product, using streptavidin-coated magnetic particles (Dynal), and sequenced by the dideoxy chain termination method (14).

RESULTS

Amplification was performed on extracted DNA from each sample, using the *P. carinii*-specific oligonucleotide primers pAZ102-H and pAZ102-E (20, 22, 23). *P. carinii*-specific amplification product (344 bp) was obtained from all of the samples. The products of amplification were sequenced directly, without cloning, by the generation of a single-stranded DNA sequencing template, using a biotin-streptavidin magnetic bead separation method, and the sequences of a 196-bp portion of the PCR product, equivalent to the fragment spanning 34 to 229 bp of the Sinclair et al. sequence (15), were compared. The possibility of any cross-contamination between samples was continually monitored at every stage of the experimental procedure.

All 12 British samples had very similar DNA sequences (Fig. 1), a sequence that was the same as the sequence we previously reported (13, 15, 21). We have identified a single-base polymorphism at base 85 (Sinclair et al. nomenclature [15]). We have found adenine, thymine, and cytosine, but not guanine, at this residue in different samples.

Eighteen samples from the United States, Brazil, and Zimbabwe were examined, all from patients with *P. carinii* pneumonia, confirmed by histochemical staining with methenamine silver. When the sequence of the amplification products from these samples was compared with those from the British samples, no differences were observed. The residue at base position 85 was also variable in these samples.

DISCUSSION

Data from molecular studies suggest that genetic diversity exists among isolates of *P. carinii* infecting a particular mam-

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      40              60              80
GATATAGCTGGT*TTTCTGCGAAAATGTTTGGCAAATGTTTATTCCTCTAAAAATAG
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      100             120             140
TAGGTATAGCACTGAATATCTCGAGGGAGTATGAAAATTTTATCTCAGATATTTAATCT
-----
      160             180             200
CAAAATAACTATTTCTTAAATAAATAATCAGACTATGTGCGATAAGGTAGATAGTCGAA
-----
      220
AGGAAACAGCCAGA
-----

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FIG. 1. DNA sequence of a 196-bp portion of the PCR product from the amplification of a part of the gene encoding the mitochondrial large-subunit rRNA from human-derived *P. carinii*. The polymorphic base is marked (*). Base numbering nomenclature is as in Sinclair et al. (15).

malian host (17). The karyotype of rat-derived *P. carinii* has been examined, using pulsed-field gel electrophoresis, and differences in the karyotype of the organism isolated from different colonies of rats have been observed (1). Analysis of the *P. carinii* nuclear major rRNA operon has also demonstrated differences among isolates from rats (11, 12). The electrophoretic karyotype of isolates from the same rat colony, however, has been shown to be stable over a period of 2 to 3 years (1, 7). DNA sequence data and electrophoretic karyotyping have suggested the possibility of coinfection with two variant strains of *P. carinii* within a single rat host (2).

Genetic heterogeneity has also been observed in human-derived *P. carinii* (10). However, studies on the major rRNA operon from human-derived *P. carinii* from five HIV-infected patients in the United States showed that, although the sequence of this gene was different from that of the rat-derived *P. carinii*, each of the human-derived sequences was identical (11).

In this study, we have studied the DNA sequence of a portion of the mitochondrial large-subunit rRNA gene, part of which spans a phylogenetically nonconserved region of this molecule (6). This locus has been shown to be informative in a comparison of *P. carinii* isolated from human-, rat-, mouse-, ferret-, rabbit-, and horse-infected lungs, demonstrating up to 27% differences among these isolates (13, 15, 21).

The results of this study indicate that genetic diversity in *P. carinii* organisms isolated from different samples of infected human lung does occur, as demonstrated by the nucleotide polymorphism at base 85. This base was found to be polymorphic in samples from all four geographical locations and is in accord with the findings of Lee et al., who also reported variation at this residue (10). No significant trend in the relative frequency of each base at this residue in the samples from the different geographical locations was observed, possibly due to the relatively small number of samples analyzed. However, in some samples the data indicated the presence of two different bases at this residue, providing further evidence in support of the premise that mixed infections of different types of *P. carinii* can occur and that *P. carinii* infection may not necessarily be clonal.

Apart from this variable base, however, the sequences of all samples were identical, both those from widely distant geographical locations and the British samples obtained over a 4-year period. The genetic locus used in this study has provided an informative tool with which to analyze the diversity among *P. carinii* organisms isolated from different host species. In comparison to the high levels of diversity found at this locus in these organisms, data from this study suggest that there is

relatively low genetic diversity among isolates of human-derived *P. carinii* from different global regions. Sequences which appeared to be a hybrid of rat- and human-derived *P. carinii*, as reported by Lee et al. (10), were not found in any of the samples in this study. In contrast to the sample from which the hybrid was isolated, an open lung biopsy in 1983 of a 4-year-old girl with acute lymphocytic leukemia, all samples in this study were obtained from adults who were immunocompromised as a consequence of HIV infection, the earliest case being from 1988.

These data do not in any way preclude the possibility of variability among the isolates at other genetic loci, such as those encoding antigenically important epitopes. Analysis of patterns of antibody response to specific *P. carinii* antigens has suggested that there are regional differences in the frequency of antibody responses to certain *P. carinii* antigens (16). Our data, however, suggest that genetic differences of the type found at this locus among isolates of *P. carinii* infecting different mammalian hosts are not present in strains of *P. carinii* infecting humans from widely dispersed geographical locations. Further analysis of DNA sequences from these samples of *P. carinii* at other genetic loci will help to elucidate the extent of relatedness among these isolates.

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