JOURNAL OF CLINICAL MICROBIOLOGY, July 1997, p. 1687–1690 Vol. 35, No. 7
0095-1137/97/$04.00+0

Study of Internal Transcribed Spacer and Mitochondrial Large-Subunit Genes of *Pneumocystis carinii hominis* Isolated by Repeated Bronchoalveolar Lavage from Human Immunodeficiency Virus-Infected Patients during One or Several Episodes of Pneumonia

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Received 27 December 1996/Returned for modification 17 February 1997/Accepted 4 April 1997

The objective of this study was to type, analyze, and compare *Pneumocystis carinii hominis* strains obtained from different samples during a given or recurrent episodes of *P. carinii* pneumonia (PCP) for epidemiologic purposes. We studied 36 bronchoalveolar lavage (BAL) or induced sputum (IS) samples from 16 human immunodeficiency virus-infected patients with one or several episodes of PCP. PCR amplification and direct sequencing were performed on the two internal transcribed spacers (ITS1 and ITS2) of *P. carinii hominis* rRNA genes by using DNA extracted from BAL or IS samples, and the sequences were compared to the mitochondrial large-subunit (mt LSU) gene sequence determined in a previous study in our laboratory. The studies of the mt LSU and ITS sequences showed that some patients ($n = 10$) were infected with the same strains of *P. carinii hominis* during a given episode of PCP. In one patient infected with strains with identical sequences in several episodes, the recurrence could have been due to reactivation of organisms not eliminated by treatment during the first episode or to de novo infection by an identical strain. In five patients infected with strains with different sequences in each episode, recurrence was due to de novo infection. Sequence analysis of these two *P. carinii hominis* gene regions showed that de novo infection can occur in AIDS patients with recurrent PCP.

Pneumocystis carinii, a fungus, causes pneumonia in immunocompromised patients, especially those with AIDS. The epidemiology of *P. carinii* pneumonia (PCP) is poorly documented. Airborne transmission has been established, but the infective form and its source causing human infections remain unknown. Given the inability to culture *P. carinii* in vitro, molecular biology-based methods have been used to study *P. carinii* epidemiology. Karyotyping techniques such as pulsed-field gel electrophoresis have shown strong host species specificity among *P. carinii* strains infecting different hosts (humans, ferrets, and rats) (16, 21, 25). Biodiversity in *P. carinii hominis* has also been demonstrated by restriction fragment length polymorphism analysis (22) and analysis of the nucleotide sequences of 5.8S, 26S, and the mitochondrial large-subunit (mt LSU) rRNA genes showing that *P. carinii hominis* forms a group of different organisms (10, 12, 13, 27).

Infection can be due to reactivation or de novo infection. Serological observations have suggested that *P. carinii hominis* infection is due to reactivation of latent organisms acquired early in childhood (2, 19, 28). In previous work, we examined the mt LSU gene and found that recurrent episodes of PCP could be due to de novo infection (8). In the present study, we sequenced the DNA region containing both internal transcribed spacers (ITSs; ITS1 and ITS2) located between the 18S and 5.8S rRNA genes and between the 5.8S and 26S rRNA genes and compared those sequences with those obtained for the mt LSU gene.

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MATERIALS AND METHODS

Samples. Samples were obtained from patients in two Paris hospitals (Hôpital Saint-Antoine and Hôpital Tenon) between April 1992 and March 1995. Bronchoscopic alveolar lavage (BAL) and induced sputum (IS) samples were collected from human immunodeficiency virus-infected patients with PCP diagnosed by direct examination with standard stains (Giemsa and toluidine blue O) and indirect immunofluorescence. We examined 32 BAL and 4 IS samples from 16 human immunodeficiency virus-seropositive patients obtained during one or several episodes of PCP (one episode, 10 patients [20 BAL and 2 IS samples]; two episodes, 5 patients [9 BAL and 2 IS samples]; and three episodes, 1 patient [3 BAL samples]). The first episodes lasted approximately 2 months. The second and third episodes occurred more than 4 months after the first one(s).

DNA extraction. BAL and IS specimens were pelleted and treated with proteinase K (Boehringer Mannheim, Meylan, France) at a final concentration of 0.4 mg/ml in the presence of 500 μl of proteinase K buffer (10 mM Tris [pH 8], 0.5% sodium dodecyl sulfate, 25 mM EDTA, 0.1 M NaCl) at 56°C for 1 h (7). After inactivation of proteinase K at 94°C for 10 min, the mixture was extracted with phenol and chloroform. The DNA present in the aqueous phase was precipitated with ethanol (17).

DNA amplification. PCR of the mt LSU rRNA gene was performed with specific primer pairs pAZ102-E (5′-GAACCGGTCGATAGTGCAC-3′) and pAZ102-H (5′-GTGTACGTTGCAAAGTACTC-3′) as described in our previous work (8). The nested PCR with the *P. carinii*-ITS-PCR mixture was performed in a total volume of 100 μl containing PCR buffer (12), 5 μl of template, 0.2 μM (each) PCR primer, 0.2 μM (each) deoxynucleoside triphosphate, and 2.5 U of *Taq* DNA polymerase (Pharmacia Biotech, Orsay, France), with 10 μl of mineral oil used to prevent evaporation during thermal cycling (7). PCR was done as described by Lu et al. (14). The first step was done with primers 1724F (5′-AAGTTGATCAAATTTGGTC-3′) and 3454R (5′-GAACCGGTCGATAG TGCAC-3′), and the second step was done with primers ITS1F (5′-CGTAGG TGAACCTGCGGAAGGATC-3′) and ITS2R1 (5′-GTTCAGCGGGTGATCC TGCCTG-3′). Reactions were run in a Perkin-Elmer thermocycler. For PCR with the primer set 1724F-3454R, the initial step was 10 min of denaturation at 94°C; the second step was 35 cycles at 94°C for 1 min, 47°C for 1 min, and 72°C for 3 min; and the final step was 10 min of extension at 72°C. With the ITS1F-ITS2R1 primer set, after initial DNA denaturation at 94°C for 10 min, 35 cycles were run, as follows: 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with 10 min of extension at 72°C after the 35 cycles (14). A negative control (without DNA), a positive control (with *P. carinii hominis* DNA), and a positive PCR control (amplification of the β-globin gene) were included.

TABLE 1. Alignment of ITS1 sequence types of *P. carinii hominis* isolates from different patients

ITS1 type	Nucleotide sequence at the following position(s) (bp)[a]:					
	6	14–15	21–26	50–51	67	76–77
A1	C	−A	TTCCCTA	TC	−	−−
A2	C	TA	TTCCCTA	TC	−	−−
B1	T	−A	TTCCCTA	TC	−	AG
B2	T	−A	ATCCCTA	TC	−	AG
B3	T	−C	ATCCCCA	TATC	−	AG
B4	T	−A	ATTTCCCCA	TC	T	AG
B5	T	−A	ATCCCTA	TC	T	AG
B6	T	−A	TTCCCTA	TC	T	AG

[a] Only the variable positions are represented, and the different types have been described previously (7). −, missing bases.

Purification of PCR products. PCR products were electrophoresed in a 2% agarose gel (type II; Medium EEO; Sigma Saint Quentin Fallavier, France) with 1× Tris-borate-EDTA (0.089 M Tris-borate, 0.0089 M boric acid, 0.002 M EDTA) and ethidium bromide (0.5 μg/ml; Sigma). The bands were visualized with UV light (17). The amplified fragments were purified by using the Wizards PCR Preps DNA purification system kit (Promega, Coger, France) and the Geneclean II kit (Bio 101, Ozyme, France), following the manufacturers' instructions and as described previously (7, 8, 10).

Sequencing of PCR products. The purified products were sequenced directly from both ends, without cloning, on an automated sequencer (model 373A; Applied Biosystems, Perkin-Elmer, Saint Quentin en Yvelines, France) by using the Prism Ready Reaction Dye Deoxy Terminator Cycle Sequencing kit FS (Applied Biosystems, Perkin-Elmer). Each purified fragment was sequenced at least twice with each primer (the ITS1 region with ITS1F and ITS4 [5′-GCGT TCAAAAATTCGATGAT-3′] and the ITS2 region with ITS2R1 and ITS2F1 [5′-CGATAAGTAGTGTGAATTGC-3′]).

RESULTS

The mt LSU sequences have been analyzed in a previous work (8), and several strains have been identified according to the presence of nucleotides at different positions (10). On the basis of the ITS1 and ITS2 sequences, four types of *P. carinii hominis* have previously been described previously (14). These are types Ac, Ba, Bb, and Bc, in which the uppercase letters represent the ITS1 type and the lowercase letters represent the ITS2 type (14). In the present study, a total of 72 nucleotide sequences were compared with each other and with the prototype sequences chosen by Lu et al. (14).

P. carinii hominis ITS1 sequences (157-bp fragment) fell into two major types (types A and B) which differed in sequence at several positions (Table 1). There were some sporadic variations within the two types at positions 14, 21, and 67, and mutations not reported by Lu et al. (14) were observed, notably in two type B strains. The first one (type B3) had an A-to-C change at position 15, a T-to-C change at position 26, and a 2-bp insertion (AT) between positions 50 and 51. The second one (type B4) had two T insertions between positions 22 and 23 and a T-to-C change at position 26 (Table 1). This was used to subclassify the sequences into two types A (types A1 and A2) and six types B (types B1, B2, B3, B4, B5, and B6) strains and showed the presence of eight different strains of ITS1 *P. carinii hominis*. Sequences identical to the prototype sequence were also found (type A2).

The *P. carinii hominis* ITS2 sequences (177-bp fragment) were also aligned (Table 2). The comparison revealed three types (types a, b, and c). As with the ITS1 sequences, there were sporadic sequence variations within the three types at positions 67 and 162. New mutations were also observed, such as a T insertion between positions 70 and 71, a T and an A insertion between positions 160 and 161, and a T deletion at position 163 (Table 2). We subclassified the ITS2 sequences into four a types (types a1, a2, a3, and a4), two b types (types b1 and b5), and 1 c type (type c1), for a total of seven different strains of ITS2 *P. carinii hominis*. Sequences identical to the ITS2 prototype sequence were also identified and corresponded to type a1.

The comparison between the mt LSU and ITSs sequences showed that *P. carinii* strains with identical mt LSU rRNA gene sequences can have different ITS genes.

The mt LSU rRNA gene, ITS1, and ITS2 sequences in strains recovered from a given patient during a given episode of PCP were identical. (Table 3).

Among the six patients with several episodes of PCP, each isolate recovered from patient 13 had identical mt LSU rRNA gene, ITS1, and ITS2 sequences, and isolates recovered from five patients had different mt LSU rRNA gene and ITS sequences (Table 4).

DISCUSSION

In this study, we analyzed sequence variations in the ITS1 and ITS2 regions of clinical *P. carinii hominis* isolates. By using combinations of ITS1 and ITS2 sequences, a total of 11 types (types A1c1, A2c1, B1a1, B2a1, B3a4, B4a3, B5a1, B6a1, B6a2, B6b1, and B6b5) were identified among the 36 samples, a proportion similar to that reported by Lu et al. (14), who found four types in 12 samples (14).

Samples from patients 9 and 14 contained two mt LSU rRNA gene sequences in the same sample, showing that these patients were simultaneously infected with two genetically distinct strains of *P. carinii hominis* during the first or second episode. Hong et al. (4) have reported mixed infections in rats (4), and we and others have reported mixed infections in humans (6–8, 14, 23). This coinfection was not confirmed by the ITS region sequences in samples from patient 14. In patient 9, the encountered difficulty in reading ITS sequences (probably caused by the different *P. carinii hominis* DNAs competing in the sequencing reaction) did not confirm the coinfection. To test for mixed infection, it could be useful to clone the amplified fragment into a vector prior to sequencing. Contrary to direct sequencing, this approach would imply that several recombinant clones might be sequenced in the same sample (e.g., 11 recombinant clones in one sample) (23). Furthermore, if the different *P. carinii hominis* DNAs are not present in equal amounts, the clone with the major strain will probably be sequenced more often.

The mt LSU rRNA gene and ITS sequences in each sample from 10 patients in whom repeat BAL was performed during a single episode because no improvement was observed after receiving specific therapy were identical. The base changes were not randomly distributed, but all occurred at the same

TABLE 2. Alignment of ITS2 sequence types of *P. carinii hominis* strains from different patients

ITS2 type	Nucleotide sequence at the following position(s) (bp)[a]:					
	50–53	59	63–67	70–71	160–163	165
a1	TAA	−	AATAT	AT	−−AT	G
a2	TAA	−	AATAA	AT	−−AT	A
a3	TAA	−	AATAT	AT	−−−−	A
a4	TAA	−	AATAT	ATT	−−AT	A
b1	TAA	−	AATAT	AT	ATAT	G
b5	TAA	−	AATAT	AT	ATATAT	G
c1	−−−	A	−−−−−	AT	−−AT	A

[a] Only the variable positions are represented, and the different types have been described previously (7). −, missing bases.

TABLE 3. Typing of *P. carinii hominis* strains from human immunodeficiency virus-infected patients with one episode of PCP

Patient no. and specimen	Date of BAL	ITS1 type	ITS2 type	mt LSU type[a]
1				
BAL1	21 April 1993	B2	a1	3
BAL2	29 April 1993	B2	a1	3
2				
BAL1	20 April 1993	A2	c1	3
BAL2	10 May 1993	A2	c1	3
3				
BAL1	15 Sept 1993	B3	a4	3
BAL2	15 Oct 1993	B3	a4	3
4				
BAL1	18 Oct 1993	B4	a3	1
BAL2	25 Oct 1993	B4	a3	1
5				
BAL1	4 Jan 1994	B5	a1	3
BAL2	14 Jan 1994	B5	a1	3
BAL3	25 Jan 1994	B5	a1	3
6				
BAL1	18 Feb 1994	B6	b1	3
BAL2	14 April 1994	B6	b1	3
7				
BAL1	7 April 1994	B6	b5	3
BAL2	5 May 1994	ND[b]	ND	3
8				
BAL1	18 May 1994	B6	a2	1
BAL2	6 June 1994	B6	a2	1
BAL3	30 June 1994	B6	a2	1
9				
IS	15 Dec 1994	B6	NR[c]	2 + 3
BAL	21 Dec 1994	NR	NR	2 + 3
10				
IS	1 March 1995	B5	a1	3
BAL	14 March 1995	B5	a1	3

[a] mt LSU rRNA gene types were described by Latouche et al. (8).
[b] ND, not done (not enough DNA).
[c] NR, sequence not readable.

variable positions. This indicated that the *P. carinii hominis* populations collected in BAL specimens over a short period during the same episode of pneumonia were homogeneous. The same strain of *P. carinii hominis* thus persisted under therapy, which did not lead to genetic changes. These identical results indicate that these typing procedures are reproducible. In samples from four patients (patients 1, 2, 3, and 5), the ITS1 and ITS2 gene sequences were variable, whereas the mt LSU rRNA gene sequences were identical. In addition, samples from three patients (patients 6, 7, and 8) with identical ITS1 gene sequences had different ITS2 gene sequences. Relative to the mt LSU rRNA gene, in which five allelic types have been described (6), only three were observed in our study, and the ITS genes appeared to have a higher levels of variation, with 11 types identified. Therefore, the ITS regions demonstrate the utility of this genetic locus in epidemiology studies and are more informative when trying to detect biodiversity (7, 11, 15).

With regard to the patients with several episodes of PCP, samples from patient 13 appeared to have identical mt LSU rRNA gene and ITS sequences during both episodes, indicating that the recurrence was due to reactivation of remnant organisms not eliminated by treatment during the first episode or to de novo infection by an identical strain of *P. carinii hominis*. In the other five patients (patients 11, 12, 14, 15, and 16), whose samples had different sequences in each episode, the infection was clearly due to de novo infection by a genetically distinct strain of *P. carinii hominis.*

Recently, Keely et al. (5) obtained similar results and showed that genotype switching was not an artifact of the lung site sampled and that reinfection was the most plausible explanation for the genetic variation seen in the organisms associated with recurrent PCP episodes in AIDS patients. Furthermore, latency in humans has been called into question by the failure to detect *P. carinii hominis* DNA in healthy subjects (18). Experiments in mice have shown that latency is not an inevitable outcome of *P. carinii* infection (3). Nested PCR experiments with rats indicate a sequential and gradual decrease in *P. carinii*, suggesting that *P. carinii* is cleared from the lungs and that the persistence of latent *P. carinii* organisms is limited (24). This has also been shown for humans by Roux et al. (20), who reported that patients with a first positive BAL specimen had negative results about 3 weeks later, suggesting that *P. carinii hominis* had been cleared.

We conclude that sequencing of two *P. carinii hominis* gene regions (mt LSU rRNA and ITS) is complementary and throws light on the epidemiology of PCP. De novo infection appears to account for some cases of recurrent PCP in AIDS patients. The infection was acquired from an exogenous source, either from *P. carinii* organisms present in the environment or from patient-to-patient spread.

Recently, DNA sequences identical to those of *P. carinii hominis* have been detected not only in a hospital environment

TABLE 4. Typing of *P. carinii hominis* strains from human immunodeficiency virus-infected patients with two or three episodes of PCP

Patient no. and specimen	Date of BAL	ITS1 type	ITS2 type	mt LSU type[a]
11				
BAL1	17 April 1992	B1	a1	3
BAL2	14 March 1995	B2	a1	3
12				
BAL1	7 April 1994	B5	a1	2
BAL2	13 Oct 1994	B5	a1	3
13				
BAL1	13 May 1994	B6	b5	3
BAL2	13 Oct 1994	B6	b5	3
14				
BAL1	1 April 1994	B6	a2	3
BAL2	1 March 1995	B6	a1	2 + 3
15				
IS1	21 Oct 1994	B2	a1	3
IS2	23 Jan 1995	B5	a1	3
BAL	14 Feb 1995	B5	a1	3
16				
BAL1	2 Sept 1993	A1	c1	1
BAL2	21 April 1994	B6	a2	1
BAL3	28 Oct 1994	B6	a2	3

[a] mt LSU rRNA gene types were described by Latouche et al. (8).

(1) but also in a rural location (26). Person-to-person transmission is not yet proven, and the study of three human immunodeficiency virus-infected couples by typing of *P. carinii hominis* strains from each member of the couple ruled out this mode of transmission (9). These results need to be confirmed with a larger number of samples. At present without this information, it seems more important to offer therapeutic prophylaxis to immunosuppressed patients with a high risk of PCP rather than protection from environmental exposure.

ACKNOWLEDGMENTS

This work was supported by l'Agence Nationale de la Recherche sur le SIDA (ANRS) (grant Rin 329), France.

We thank David Young.

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