JOURNAL OF CLINICAL MICROBIOLOGY, May 1993, p. 1217–1223
0095-1137/93/051217-07$02.00/0

Vol. 31, No. 5

Evidence for Two Genetic Variants of *Pneumocystis carinii* Coinfecting Laboratory Rats

MELANIE T. CUSHION,[1] JIANXIONG ZHANG,[2] MICHAEL KASELIS,[1] DAVID GIUNTOLI,[2] SAUNDRA L. STRINGER,[2] AND JAMES R. STRINGER[2]*

Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, Ohio 45267-0524,[2] and Department of Internal Medicine, Division of Infectious Diseases, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0560[1]

Received 22 September 1992/Accepted 20 February 1993

***Pneumocystis carinii* pneumonia is an oftentimes fatal infection for hosts in an immunocompromised state. The disease occurs in a wide variety of mammals, but the etiologic agent of this disease has been referred to as *P. carinii* regardless of the host species. However, even within a single host species, such as laboratory rats, distinct varieties of *P. carinii* have been identified from differences in the electrophoretic migration of chromosomes in agarose gels. Here we present evidence indicating that some laboratory rats can contain two different genetic variants of *P. carinii* that differ not only in electrophoretic karyotype but also in the presence of a particular repeated DNA sequence, in the presence of an intron in the 18S ribosomal RNA gene, and in the sequence of part of the 18S rRNA gene. Most of the rat colonies studied were infected with *P. carinii* that contained the repeated DNA and the 18S rRNA gene intron. The other type of rat-derived *P. carinii*, which lacked the repeated DNA and the intron in the 18S rRNA gene, was found as a coinfection with the first. Parasite populations from different coinfected rats contained the two variants in different proportions.**

Pneumocystis carinii is the etiologic agent of a lethal pneumonia in patients with AIDS and in other immunocompromised individuals (21, 29). The organism also occurs in a wide variety of other mammalian hosts as an infection or apparent colonization (14). Little is known about genetic variation of *P. carinii* preparations, owing to the lack of a continuous in vitro culture system and the consequent unavailability of clonal stocks. Morphological analyses by both light microscopy and electron microscopy have found few features to be distinctively different among *P. carinii* isolates prepared from different mammalian hosts, rat- and human-derived organisms being the most studied (2, 4, 12, 20). Antigens of *P. carinii* from rats, ferrets, and humans show cross-reactivity, but the cross-reacting antigens from different *P. carinii* isolates can migrate differently upon electrophoresis through sodium dodecyl sulfate (SDS)-polyacrylamide gels, suggesting that *P. carinii* is not a single genetic entity (9, 10, 17, 18).

Direct evidence for genetic diversity among *P. carinii* isolates from rats has been provided by examining *P. carinii* chromosomes via pulsed-field gel electrophoresis. Various pulsed-field gel electrophoresis techniques have shown that different electrophoretic karyotypes can be obtained from *P. carinii* organisms isolated from different immunosuppressed rat strains (13, 19, 30, 31). Our previous electrophoretic karyotyping studies of rat-derived *P. carinii* showed that in most infected rat colonies each rat contained the same karyotypic type of *P. carinii* but that different rat colonies contained different types of *P. carinii* (13). The typical electrophoretic karyotype observed for rat-derived *P. carinii* contained 13 to 15 bands, which migrated between 250 and 700 kbp, and contained the repeated DNA Rp3-1 (27). However, some rat colonies produced *P. carinii* that did not exhibit the typical 13- to 15-band karyotype but instead exhibited a 22-band karyotype.

Two hypotheses to explain the nature and origin of the 22-band karyotype seemed plausible. One possibility was that the 22 bands were produced by two separate types of *P. carinii* that both infected the same rat. Alternatively, all of the chromosomes seen in the 22-band karyotype could have been present in the nucleus of a single *P. carinii* variety.

In the present study, DNA hybridization, the polymerase chain reaction (PCR), and DNA sequencing produced evidence indicating that the 22-band pattern resulted from coinfection with two types of *P. carinii* that may be different species. This is the first report that *P. carinii* pneumonia may result from coinfection with two genetically distinct varieties of the pathogen. The existence of *P. carinii* variants and coinfection in the immunosuppressed-rat model raises the possibility of unrecognized variation and complexity in human *P. carinii* pneumonia.

MATERIALS AND METHODS

Rat colonies. Three strains of rats were used: Long Evans and Brown Norway from room 083, Charles River Breeding Laboratories (Wilmington, Mass.), and Fischer 344 from colony 24, Hilltop Lab Animals Inc. (Scottsdale, Pa.). Fifteen to 27 viral antibody-positive (Vab^+) male rats (125 to 149 g) of each strain were received in filtered containers. Rats in groups of three were kept in polycarbonate shoebox cages, each fitted with a microisolator top (3-μm exclusion; Lab Products, Inc., Federalsburg, Md.). The cages were placed in horizontal-flow hoods (Germ Free Industries, Miami, Fla.). All bedding (Anderson Bed O' Cobs Combo; Industrial Products, Maumee, Ohio) and water was autoclaved prior to entry into the animal room. Animals were fed irradiated food (rodent chow 20; Picolab, St. Louis, Mo.). Water was supplemented with Cephradine (E. R. Squibb and Sons, Princeton, N.J.) by injection into each water bottle to a final concentration of 250 mg/ml. All manipulations of the animals (e.g., cage change-out, injections) were performed in a horizontal-flow changing station by personnel outfitted

* Corresponding author.

with sterile gowns, gloves, masks, shoe covers, and hair caps. Cages were changed on a weekly basis, and water bottles (600-ml total volume) were changed twice a week. After 7 days of acclimation, rats received weekly subcutaneous injections of methylprednisolone acetate (DepoMedrol; The Upjohn Co., Kalamazoo, Mich.) at 4 mg/week.

Isolation of *P. carinii* organisms. After 6 to 12 weeks of immunosuppression, rats were sacrificed by carbon dioxide narcosis, the lungs were removed, and impression smears of cross sections of the lungs were examined to determine the severity of infection (16). The lungs were homogenized and prepared for pulsed-field gel electrophoresis as previously described (13), with the following modifications. After homogenization in a laboratory blender (Stomacher; Tekmar Inc., Cincinnati, Ohio), the preparations were filtered through gauze to remove large particulate host material, treated with 0.85% ammonium chloride, and then passed at least twice through 10-μm-pore-size filters (Mitex; Millipore Corp., Bedford, Mass.). Each pair of lungs was processed separately, and each preparation of *P. carinii* was kept separate. The *P. carinii* preparations were monitored for other microbes by using microscopic and microbiological methods as previously described (13, 28). No other eukaryotic microbes were detected by culture or tinctorial staining techniques.

Preparation of organisms for pulsed-field gel electrophoresis. *P. carinii* organisms were suspended in 150 mM NaCl–10 mM $MgCl_2$–10 mM Tris (pH 7.2) and treated with pancreatic DNase I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) at 10 μg/ml for 30 min at 37°C to digest extracellular DNA. Control experiments showed that DNase I treatment did not alter the *P. carinii* band pattern but did remove heterogeneous-size DNA that migrates as a smear that can obscure *P. carinii* bands. The organisms were washed successively with 0.250 and 0.125 M EDTA and collected by centrifugation at 2,400 × *g*. Organisms were embedded at a concentration between 4×10^8 and 4×10^9 organisms per ml in agarose by reconstituting organism pellets in 0.125 M EDTA in 0.8% low-melting-point agarose (Boehringer Mannheim Biochemicals). Between 1 and 5 ml of molten agarose containing *P. carinii* was transferred into a disposable spectrophotometer cuvette (Fisher Scientific, Cincinnati, Ohio). The embedded organisms produced in this manner, rather than in petri dishes as previously described (13), allowed us to load standard-size blocks (approximately 3 by 5 by 10 mm) into slots on a gel. Gel-embedded organisms were treated with 0.25 mg of proteinase K per ml in a solution of 1% *n*-lauroylsarcosine (Sigma Chemical Co., St. Louis, Mo.)–0.45 M EDTA–0.01 M Tris (pH 8.0) and digested at 55°C for 18 to 48 h, depending on the size of the plug. Digested embedded organisms were stored in 0.5 M EDTA (pH 8.0) at 4°C.

FIGE. Gels for field inversion gel electrophoresis (FIGE) contained 1% FMC SeaKem GTG agarose (FMC Bioproducts, Rockland, Maine) prepared in 0.5× TBE (45 mM Tris hydrochloride, 45 mM boric acid, 1 mM EDTA) for a total volume of 200 ml. Final gel dimensions were 20 by 25 cm. FIGE was performed with a PC 750 Pulse Controller (Hoefer Scientific Instruments, San Francisco, Calif.) and a horizontal gel electrophoresis system. The buffer system was 0.5× TBE supplemented with 0.1 M glycine. FIGE gels were run in a cold room; the buffer temperature was maintained at 6 to 8°C by circulation through an ice bath. *P. carinii* chromosomes were separated by using a two-step run. For the first 48 h, the gels were run at 160 V with 50-s forward and 25-s reverse pulsing. The buffer was changed, and the gel was run for an additional 96 h at 120 V with the same pulse times. The buffer was again changed after the first 48 h of the 96-h run. Gels were stained with ethidium bromide and photographed. Lambda multimers (Hoefer Instruments) were used to produce standard curves from which the *P. carinii* band sizes were estimated. FIGE gels were prepared for transfer by treatment with 0.25 M HCl for 20 min and then denaturation with 0.5 M NaOH for 60 min. DNA was transferred by capillary flow onto nylon membranes (Hybond N^+; Amersham, Amersham, United Kingdom) with 0.4 M NaOH, as recommended by the vendor. Prehybridization was done as described previously (13). Hybridization and washing conditions were as described in the figure legends.

Hybridization probes. Two probes, Rp3-1 and 19c, were isolated from a library of rat-derived *P. carinii* genomic DNA constructed in Lambda Fix (Stratagene Inc., La Jolla, Calif.) (13). Rp3-1 is a copy of a DNA sequence that is present on every chromosome of the rat-derived *P. carinii* from which it was isolated. 19c contains a copy of the rRNA locus from rat-derived *P. carinii*. On the basis of nucleotide sequence information, two oligonucleotide probes were designed to be used as hybridization probes that would be specific for either the prototype 18S rRNA gene or the variant 18S rRNA gene. The sequence of probe 1, which was designed to be specific for the prototype, was 5′ *CT*T ATC GTT *G*CC AAT AAC C*C*A TC*A* 3′. The sequence of probe 2, which was designed to be specific for the variant, was 5′ TAT ATC GTT CCC AAT AAC CTA TCG 3′. Probes 1 and 2 differed at the five positions in italics in probe 1. Oligonucleotides were labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P]dATP (23). Other probes were prepared by random priming (23).

DNA amplification. *P. carinii* DNA samples were obtained from the same agarose blocks used for karyotyping. A 1-mm^3 sample was melted at 72°C and then diluted 1:10 in water. A 1.0-μl aliquot was added to a 50-μl reaction mixture that contained 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 200 μM deoxynucleoside triphosphates, 2.5 U of *Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, Conn.), and 20 pmol of each primer. Primer A was 5′ TTG ATT ATG TCC CTG CCC TTT G 3′. Primer B was 5′ ATC CTT CCG CAG GTT CAC CTA 3′. This primer pair allowed amplification of sequences that spanned the 390-bp self-splicing intron in the 18S ribosomal gene (26). The samples were amplified in a DNA thermal cycler (Perkin Elmer Cetus) for 30 cycles (94°C, 1 min; 54°C, 1 min; 72°C, 2 min). A 20-μl sample from each reaction was electrophoresed in 1.4% agarose gels run in 0.5× TBE at 100 to 120 V for 1 h. After the gel was stained with ethidium bromide, it was inspected with UV light and the products were sized by comparison with size markers. PCR products were sequenced with a PCR sequencing kit from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.).

Nucleotide sequence accession number. The sequence of the variant has been entered into the GenBank data base under accession number L11279.

RESULTS

Electrophoretic karyotype analysis of *P. carinii* prepared from rats. Two distinct electrophoretic patterns were produced from *P. carinii* harvested from the lungs of Long Evans rats received from a single room (083) at the Charles River Wilmington facilities. Of the seven *P. carinii* preparations from Long Evans rats that produced clear karyotypic patterns, four exhibited 13 bands (Fig. 1, lane 1). The same

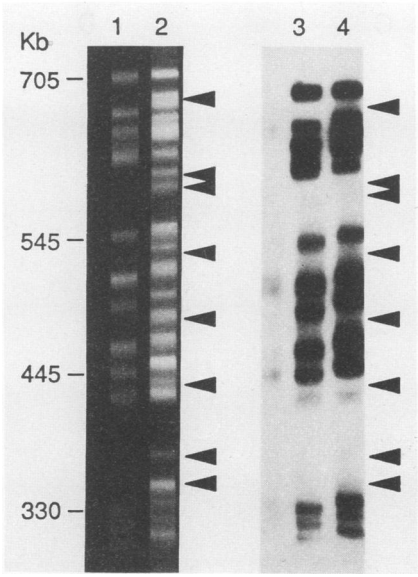

FIG. 1. Two electrophoretic karyotypes from rat-derived *P. carinii*. *P. carinii* organisms were isolated from lungs of Brown Norway rat 1159 (lanes 1 and 3) and Long Evans rat 1145 (lanes 2 and 4). Organisms were subjected to FIGE. Lanes 1 and 2 show the DNA bands visualized by staining with ethidium bromide. Lanes 3 and 4 show the radiographic image obtained when the samples in lanes 1 and 2 were hybridized to a radioactive DNA made from the repeated sequence Rp3-1. Hybridization was done in 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 0.015 M sodium citrate)–0.1% SDS–50 μg of salmon sperm DNA per ml–10× Denhardt's solution (23) for 12 to 18 h at 65°C. After hybridization, washing was done in 2× SSC–0.4% SDS at 60°C twice for 30 min each time. Arrowheads indicate bands present in the 22-band karyotype only. Apparent sizes of bands are indicted on the left. Kb, kilobase pairs.

13-band pattern was also produced by *P. carinii* prepared from Brown Norway rats that were housed in the same room with the Long Evans rats. The remaining three preparations of *P. carinii* prepared from Long Evans rats exhibited 22 bands (Fig. 1, lane 2). The 22-band patterns appeared to contain all of the bands present in the 13-band patterns plus 9 additional bands, 8 of which are indicated by arrowheads in Fig. 1.

Analysis of electrophoretic karyotypes by hybridization. Previous work had yielded a cloned copy of a repetitive sequence from rat-derived *P. carinii*. This repeat, named Rp3-1, hybridized to all of the bands present in 13-band electrophoretic karyotypes characterized at the time (27). To determine whether the Rp3-1 repeat was present in the *P. carinii* under analysis here, *P. carinii* chromosomes separated by FIGE were hybridized to an Rp3-1 probe. Figure 1 shows that all of the bands in the 13-band karyotype hybridized to the repeated sequence Rp3-1, and all but one band gave a very strong radioactive signal (lane 3) (the smallest band appears relatively weak in Fig. 1, but other experiments [data not shown] showed that this was due to a problem in transferring the DNA from the gel to the nylon membrane.) By contrast, in the 22-band karyotype, only 13 bands hybridized to Rp3-1 (lane 4). These bands comigrated with the bands seen in a 13-band preparation of *P. carinii*. This suggested that the organism preparations that exhibited the 22-band karyotype contained two kinds of organisms, one being *P. carinii* similar to that which produces a 13-band karyotype and the second being perhaps a different type of *P. carinii*. From the intensity of ethidium bromide staining, it appeared that if two types of *P. carinii* were present, they were in roughly equal numbers in this particular rat. An alternative explanation, that the *P. carinii* organisms were contaminated by another microbe unrelated to *P. carinii*, seemed less likely because microbiological surveillance cultures were negative for the presence of other eukaryotic microbes, and microscopic analyses of stained slides and phase-contrast microscopy of *P. carinii* preparations did not reveal the presence of any other microbe. It would be expected that an organism present in numbers large enough to produce visible bands upon ethidium bromide staining of a FIGE gel would be detected by these methods.

To explore the origin of the Rp3-1-negative FIGE bands in the 22-band karyotype further, a FIGE blot containing one 22-band karyotype (Fig. 2A, lane 4) and three 13-band karyotypes (lanes 3, 5, and 6) was probed with an rRNA gene probe isolated from a genomic library prepared from rat-derived *P. carinii* (13). This clone, called 19c, contained a 12-kbp fragment of the *P. carinii* genome that included the 18S rRNA gene, the intergenic transcribed sequence, the 5.8S rRNA gene, and the 26S rRNA gene. The 19c probe was expected to hybridize to rat-derived *P. carinii* and to cross-hybridize to other varieties of *P. carinii*, as well as to other fungi, such as *Saccharomyces cerevisiae* and *Cryptococcus neoformans*. Figure 2B shows strong hybridization to a band in both *S. cerevisiae* and *C. neoformans* (lanes 2, 7, and 8). Note that the *S. cerevisiae* rRNA band, which is known to be over 1,000 kbp, migrated at an apparent molecular size of 465 kbp under these FIGE conditions. Such aberrantly fast migration during FIGE has been described before (5). In the four *P. carinii* samples, a single band hybridized to the rRNA gene probe in each of the 13-band karyotypes (lanes 3, 5, and 6), while two bands appeared in the 22-band preparation (lane 4). The hybridization signal in the *P. carinii* lanes was strikingly weak compared with those of the other two fungi. This is due to the low number of genes that encode rRNA in *P. carinii*, where there may be as few as two rRNA genes, compared with more than 100 rRNA genes in other fungi (9a). The band patterns of the three 13-band karyotypes were somewhat different. This was expected on the basis of our previous findings (13).

DNA sequence analysis of 18S rRNA genes. To test further the hypothesis that the 22-band karyotype was due to infection with two different *P. carinii* forms, a portion of the 18S rRNA gene was selected for comparative sequence analysis. The complete sequence of a rat-derived *P. carinii* 18S rRNA gene had been previously reported (8). The last 600 bases of the 18S rRNA gene were selected for analysis because this region had been previously reported to contain an intron (26) and to be a region that exhibits a high degree of variation among known 18S rRNA sequences (3, 8).

To determine the sequence of the two rRNA loci present in the 22-band karyotype, the PCR was used to amplify the desired DNA segments from genomic DNA. Amplification of DNA from a 13-band karyotype produced a single product of the size expected for an 18S rRNA gene containing the intron previously described. By contrast, amplification of DNA from a 22-band karyotype produced two products of different sizes. One PCR product was of the size expected for *P. carinii* containing the intron. The second PCR product was smaller and appeared to be the size expected for a gene lacking the intron. The identities of the PCR products were confirmed by DNA sequencing. The sequence of the larger PCR product was found to be identical to the previously

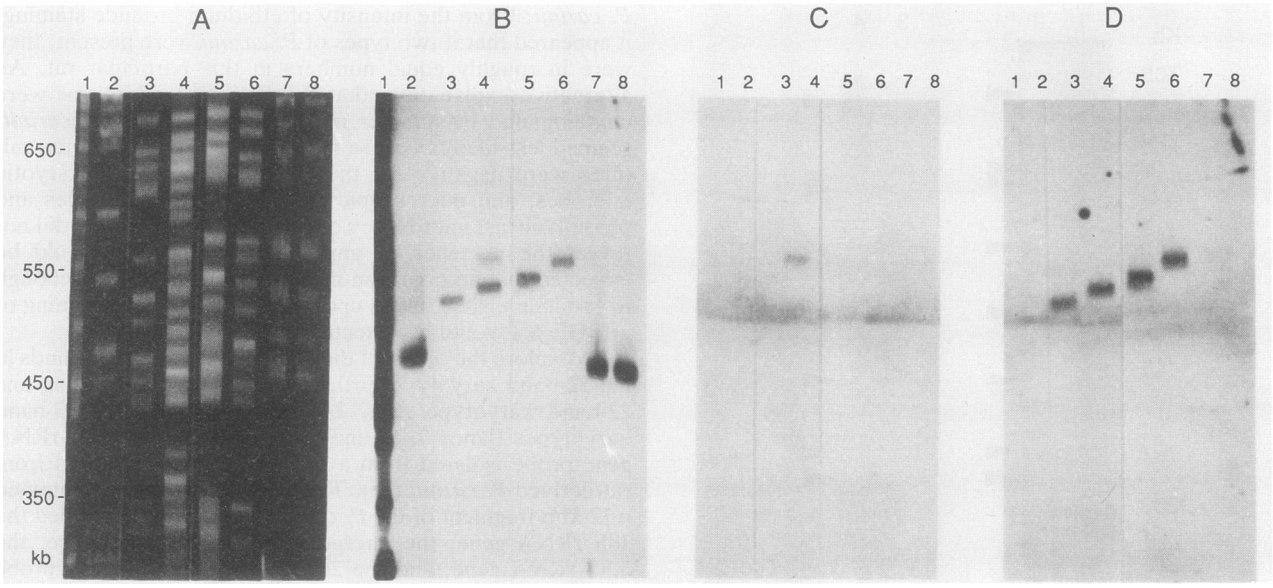

FIG. 2. Hybridization of *P. carinii* karyotypes to 18S rRNA gene probes. Four samples of *P. carinii* subjected to FIGE produced the band patterns shown in panel A. The DNA was transferred from the gel to a nylon membrane, which was hybridized to a radioactive probe containing the rRNA genes of *P. carinii* (B). Hybridization was done as described in the legend to Fig. 1. The blot was stripped and rehybridized to an oligonucleotide that could base pair perfectly with a segment of the variant 18S rRNA gene (C). The blot was again stripped and hybridized to an oligonucleotide that could base pair perfectly with a segment of the prototype 18S rRNA gene (D). Hybridization to oligonucleotides was done in 6× SSPE–1% SDS at 50°C overnight. After hybridization, washing was done in 2× SSC–0.4% SDS at 50°C twice for 30 min each time. Lanes: 1, lambda markers; 2, *S. cerevisiae* AB972; 3, *P. carinii* from Long Evans rat 1129; 4, *P. carinii* from Long Evans rat 1140B; 5, *P. carinii* from Fischer rat 1083; 6, *P. carinii* from Fischer rat 1088; 7 and 8, *C. neoformans* H99. Molecular sizes are indicated on the left in kilobase pairs.

reported sequence of a rat-derived *P. carinii* isolate (8, 26). This sequence is referred to hereafter as the prototype rat-derived *P. carinii* 18S rRNA gene sequence. The sequence of the smaller PCR product showed that it lacked the intron, as was suspected from its size. In addition to lacking the intron, the small PCR fragment also differed in sequence from the prototype. This sequence is referred to hereafter as the variant rat-derived *P. carinii* 18S rRNA gene sequence. Figure 3 shows a comparison of 121-bp segments of the sequences of the variant and the prototype. The two sequences differed at 8 of 121 positions, showing 6.6% divergence. These differences were not artifacts generated by the PCR. The experiment was done twice, and the sequence was obtained directly from the PCR product, which limits the chance of obtaining artifactual results due to cloning and sequencing of the product of an erroneous round of polymerization. In addition, seven of the eight divergent nucleotides were clustered within a 36-base region that is known to be hypervariable compared with adjacent sequences (3, 8). The sequence differences found were validated by the hybridization experiments described below.

To assess the significance of the sequence divergence observed, corresponding sequences from other species were analyzed (Table 1). The variant sequence was most similar to that of the *P. carinii* prototype. The sequence least similar to either the variant or the prototype *P. carinii* sequence was from a rat, proving that the variant sequence could not have been due to contamination of the *P. carinii* preparation with host DNA. The two sequences derived from the 22-band karyotype *P. carinii* were as similar to one another as sequences from two *Candida* species (*Candida albicans* ATCC 18804 and *C. tropicalis* ATCC 750) were to each other. By contrast, both the prototype and variant sequences

Prototype: GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTTAATGAGGT
Variant: GTACACACCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGT

Prototype: CTTCGGACTGGTGATGGGTTATTGGCAACGATAAGCCTATTACTGG
Variant: CTTCGGACTGGCGATAGGTTATTGGGAACGATATACCTATTGCTGG

Prototype: AAAGTTGATCAAATTTGGTCATTTAGAGGA
Variant: GAAGTTGATCAAATTTGGTCATTTAGAGGA

FIG. 3. Comparison of sequences from 18S rRNA genes from prototype and variant rat-derived *Pneumocystis* forms. Sequences were derived directly from PCR products. Each experiment was done twice. The sequences shown were aligned by eye and start at base, 1631 in accordance with Edman et al. (8). Bases underlined in the variant sequence differ from the corresponding bases in the prototype.

TABLE 1. Percent divergence of 18S rRNA genes

Organism	% Divergence[a] from:				
	P. carinii variant	*C. albicans*[b]	*C. tropicalis*[b]	*S. cerevisiae*[c]	Rat[d]
P. carinii prototype	6.6	21.5	21.9	24.8	32.5
P. carinii variant		21.5	21.9	25.7	32.5
C. albicans			5.8	14.9	27.8
C. tropicalis				16.6	29.4
S. cerevisiae					30.2

[a] Percent divergence = (number of mismatched nucleotides/total number of nucleotides) × 100.
[b] Sequence from reference 1.
[c] Sequence from reference 22.
[d] Sequence from reference 6.

were much less similar to those of other fungi. The two *P. carinii* sequences were approximately 22% divergent from the two *Candida* species and 25% divergent from the *S. cerevisiae* sequence.

Validation of 18S rRNA sequences via hybridization to *P. carinii* chromosomes. A pair of oligonucleotides were synthesized that were designed to hybridize specifically to either the prototype 18S gene or to the variant 18S gene. The two oligonucleotides were each 24 bases long and differed in sequence at five positions (see Materials and Methods). The oligonucleotides were made radioactive and hybridized to FIGE blots. The variant probe hybridized to a 555-kbp band in the 22-band preparation and did not hybridize to a band in the 13-band karyotypes (Fig. 2C). The prototype oligonucleotide hybridized to a band in each of the 13- and 22-band preparations (Fig. 2D). These bands corresponded to those previously found to hybridize to the probe containing the entire rRNA gene locus (Fig. 2B).

A karyotype composed primarily of the variant form of *P. carinii*. The results of DNA sequencing and oligonucleotide hybridization experiments showed the presence of two closely related 18S rRNA genes in the *P. carinii* isolate showing a 22-band karyotype. This suggested that the 22-band karyotypes were the product of mixtures of two types of *P. carinii*, i.e., (i) the prototype, which contains Rp3-1 and an intron in the 18S rRNA gene, and (ii) the variant, which lacks Rp3-1 and the 18S rRNA gene intron. If this were the case, one might expect the two types of *P. carinii* to exist separately from one another. To address this question, 24 13-band karyotypes were analyzed by hybridization to an Rp3-1 probe and to the 18S rRNA oligonucleotides described above. Twenty-three karyotypes were strongly positive for Rp3-1 and hybridized to the oligonucleotide designed to be specific for the prototype rat-derived *P. carinii* but did not hybridize to the variant-specific oligonucleotide (data not shown). These data identified these 23 samples of *P. carinii* as prototypes.

One of the 24 karyotypes, 1087, appeared to be the variant type of rat-derived *P. carinii*. The first indication that 1087 might be the variant type of *P. carinii* was the FIGE band pattern. As can be seen in Fig. 4B, lane 1, the 1087 karyotype contained bands that migrated between 350 and 400 kbp and at 600 kbp. Bands of these sizes had previously been seen only in 22-band karyotypes. Figure 4B also shows a 22-band karyotype (lane 2) in which the pattern of bands matched the pattern that would be produced by mixing the 1087 karyotype (lane 1) with the 13-band karyotype shown in lane 3.

The karyotype from *P. carinii* sample 1087 was further analyzed by hybridization to Rp3-1 and to 18S rRNA oligonucleotides. Figure 4A shows that Rp3-1 hybridized to the 1087 karyotype, but there was much less Rp3-1 hybridization to the 1087 karyotype than to the 13-band karyotype containing prototype rat-derived *P. carinii* (compare lanes 1 and 2 with lanes 3 and 4). Many of the Rp3-1-positive bands did not comigrate with the bands that stained well with ethidium bromide but, instead, comigrated with bands from a 13-band karyotype run in a neighboring lane of the same gel (compare lanes 1 and 4). This suggested that the 1087 sample of *P. carinii* was not a pure variant *P. carinii* but rather that it was a mixture whose principal component was the variant type of *P. carinii*. Results obtained by hybridization of FIGE blots to rRNA gene oligonucleotide probes and by sequencing of 18S rRNA genes were consistent with this idea. Figure 4A shows that one of the variant-specific oligonucleotides hybridized strongly to a 555-kbp band in the ethidium bromide-stained gel (lane 6). By contrast, hybridization to the prototype-specific oligonucleotide produced a relatively faint band (lane 7) that could not be aligned with a bright band in the ethidium bromide-stained gel. Both the variant and prototype rRNA gene bands appeared following hybridization to an 18S rRNA gene (lane 5). Again, the variant band was darker than the prototype band.

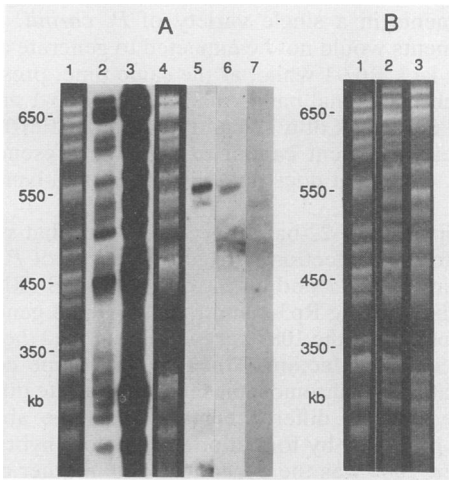

FIG. 4. Electrophoretic karyotype composed primarily of the variant *P. carinii*. (A) Electrophoretic karyotypes stained with ethidium bromide or hybridized to 18S rRNA gene probes. Lanes: 1, *P. carinii* preparation 1087 FIGE bands stained with ethidium bromide; 2, 1087 FIGE blot hybridized to Rp3-1; 3, *P. carinii* preparation 1080B FIGE blot hybridized to Rp3-1; 4, 1080B FIGE bands stained with ethidium bromide; 5 to 7, 1087 hybridized to the 18S rRNA gene, the variant-specific oligonucleotide, and the prototype-specific oligonucleotide, respectively. Hybridization to the 18S rRNA probe was done as described in the legend to Fig. 1. Hybridization to oligonucleotides was done as described in the legend to Fig. 2. (B) Electrophoretic karyotype from 1087 (lane 1) compared with a 22-band karyotype (lane 2) and a 13-band karyotype (lane 3). Molecular sizes are indicated on the left in kilobase pairs.

These data showed that the 1087 karyotype contained two types of 18S rRNA genes, which were on different chromosomes that were present in different amounts. The chromosome bearing the variant 18S rRNA gene was much more abundant than the chromosome bearing the prototype 18S rRNA gene. This was in agreement with the data obtained with Rp3-1, which indicated that the sample contained two populations of chromosomes that differed in abundance. The chromosomes of the majority population appeared to lack Rp3-1, and the chromosomes of the minority population contained the repeated DNA.

PCR was used to determine the sequence of 18S rRNA genes from 1087 DNA. With the primers designed to amplify the 3′ end of the 18S rRNA gene, two products of different sizes were produced. These two PCR products were the same sizes as those previously produced from the 22-band karyotype. The PCR products from 1087 DNA were sequenced, and the large PCR product matched that of the prototype and the small PCR product matched that of the variant.

DISCUSSION

The data described here are incompatible with the idea that the 22-band karyotypes were produced by genome

rearrangements in a single variety of *P. carinii*. Genome rearrangements would not be expected to generate a set of 9 bands that lack Rp3-1 while, at the same time, preserving a set of 13 chromosomal bands that contain Rp3-1 and comigrate with the bands of a 13-band karyotype. Furthermore, genome rearrangement cannot explain the presence of an 18S rRNA allele that does not exist in most 13-band karyotypes.

The properties of 22-band karyotypes fit what would be expected for a coinfection with two varieties of *P. carinii*, one containing Rp3-1 and an intron in the 18S rRNA gene and the other lacking Rp3-1 and the 18S rRNA gene intron. The composition of the 1087 karyotype provided the clearest indication of a coinfection. The 1087 karyotype contained two populations of chromosomes that were quite different in abundance. Such a difference in chromosome abundance cannot be produced by formation of a diploid hybrid.

However, 1087 was the exception, and in other cases the variant form of the 18S rRNA gene was seen in 22-band karyotypes in which the abundance of chromosomes containing Rp3-1 was approximately equal to the abundance of chromosomes lacking Rp3-1. In these cases, we cannot rule out the possibility that the karyotype observed represented the karyotype of a single organism formed by mating between prototype and variant *P. carinii* organisms, but this possibility seems unlikely on the basis of genetic arguments. If mating between the prototype and variant forms of rat-derived *P. carinii* were to occur, it would produce hybrids, but these would not be expected to be static. They would presumably also be able to undergo sexual reproduction. In this process, genetic recombination would be expected to occur, which would transfer Rp3-1 sequences to chromosomes lacking it. In addition, the chromosomes in the hybrid would segregate independently. Several rounds of this process would be expected to spread Rp3-1 among all chromosomes in each of the varieties of *P. carinii* capable of mating. However, *P. carinii* preparation 1087 did not contain Rp3-1 on the chromosomes visible by ethidium bromide staining and none of the 22-band karyotypes analyzed contained Rp3-1 on every chromosomal band. These considerations led us to conclude that mating between prototype and variant *P. carinii* forms occurs infrequently, if ever, and that 22-band karyotypes are most likely due to coinfections.

While the prototype and variant rat-derived *P. carinii* forms appear to be reproductively incompatible, which is consistent with the idea that these organisms are separate species, reproductive incompatibility cannot be proved in these uncultivatable organisms. However, reproductive incompatibility is not the only criterion recognized as a basis for species classification. Another way to assess whether the prototype and variant rat-derived *P. carinii* forms might be separate species is to compare the degree of genetic divergence between the prototype and variant rat-derived *P. carinii* forms with that exhibited by *C. albicans* and *C. tropicalis*, two closely related but distinct fungi that do not have a sexual cycle but have been deemed to be separate species on the basis of morphology, physiology, and molecular genetic markers (1, 24). The prototype and variant *P. carinii* forms were as divergent from one another as *C. albicans* was from *C. tropicalis*, as assessed by sequence analysis of part of the 18S rRNA gene. In addition, the variant *P. carinii* lacked prototype repeated DNA, which is analogous to the situation found in the two *Candida* species, which also do not share the same repeated DNA (24). Another interesting comparison relating genetic divergence to biological and clinical distinctiveness has recently been reported. In the 121-bp region of the 18S rRNA gene that we analyzed in our study, *Histoplasma capsulatum* was found to be only 1.6% divergent from *Coccidioides immitis* and only 3.2% divergent from *Trichophyton rubrum* (3). These three fungal human pathogens are very diverse morphologically, ecologically, and clinically (3), yet their 18S rRNA genes are less divergent from one another than the two rat-derived *Pneumocystis* 18S rRNA genes are from each other. All things considered, it does not seem inappropriate to consider the two *Pneumocystis* variants to be different species.

The discovery of two genetic variants of rat-derived *P. carinii* adds to the impetus to formulate a more specific nomenclature for these organisms. In addition to differences among rat-derived *P. carinii* isolates, evidence is accumulating which indicates that *P. carinii* isolates from different host species are also different enough to consider renaming them (10, 13, 16, 18, 25). It was previously suggested (on the basis of serological differences and apparent differences in host range) that *Pneumocystis* nomenclature be modified to indicate the host species, whereby rat-derived *P. carinii* would be called *P. carinii* Rattus and human-derived *P. carinii* would be called *P. carinii* Hominis (15). This suggestion has merit in that it conserves the universally recognized two-part name *P. carinii* and the appended name of the host species makes the source of the organisms clear in a succinct way, but it is not in accordance with the *International Code of Botanical Nomenclature* (11). In addition, retention of the single species name *carinii* may not be appropriate given the degree of genetic difference seen even among *Pneumocystis* populations found in the same host species, as in the variants described in this report. Accepted criteria for a new nomenclature are lacking, but assignment of definitive new names to the new kinds of *Pneumocystis* forms that are emerging from molecular genetic analyses is needed and justified and the field would benefit by formulation of criteria for this purpose.

In addition to focusing attention on the need to refine *P. carinii* classification and nomenclature, the existence of distinct genetic variants of rat-derived *P. carinii* as different as the prototype and variant forms raises a number of questions. It is not clear what other differences between these two organisms might be found. Are they antigenically identical? Do they behave the same way when placed into culture in vitro? Are drug target enzymes, such as dihydrofolate reductase (7), identical in the two organisms? While the existence of two variants of rat-derived *P. carinii* presents a number of new questions, it also presents new opportunities to analyze *P. carinii* genes and proteins at the functional level via comparison of DNA and protein sequences derived from these close relatives.

From these and other data, it is reasonable to infer that many genetically distinct varieties of *P. carinii* may exist, both within a host species and among different host species. Hence, it seems likely not only that human-derived *P. carinii* is distinct from *P. carinii* isolates from rats and other species, as has been suggested on the basis of analysis of antigens and mitochondrial DNA (10, 16, 18, 25), but that there are multiple varieties of human *P. carinii* capable of causing *P. carinii* pneumonia, either individually, or in combinations. Variation in human *P. carinii* could have other ramifications, as well. An example is development of a new diagnostic test, such as the PCR. Such a test must be capable of detecting all varieties of the pathogen. At the same time, finding variants of human-derived *P. carinii* would allow exploration of clinical questions, such as

whether the *P. carinii* seen in recurrent episodes of infection are the same or not and whether there may be types of *P. carinii* that are more resistant to chemotherapy.

ACKNOWLEDGMENTS

This work was supported by grants R01 AI 29839 and R01 AI 28471, in conjunction with a Public Health Service program project grant in mycology (P01 AI 28932) from the National Institutes of Health.

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