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Karyotype Instability in *Cryptococcus neoformans* Infection

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The electrophoretic karyotypes of 32 clinical and 3 environmental *Cryptococcus neoformans* isolates from New York City were studied by contour clamped homogeneous electrophoresis. There was extensive variation among the electrophoretic karyotypes of isolates from different patients. Sequential *C. neoformans* isolates from patients with chronic or relapsing infection had very similar karyotypes. However, minor differences in electrophoretic karyotypes were detected among sequential isolates from 50% of the patients studied, suggesting the occurrence of chromosomal rearrangements or deletions in vivo. This hypothesis was tested by infecting mice, recovering isolates from mouse organs, and comparing the electrophoretic karyotypes before and after passage. Three clinical and three environmental strains were studied before and after passage in mice. Karyotype differences were detected after mouse passage for one clinical and two environmental strains. Our results indicate (i) extensive karyotype variation among isolates from a small geographic region, (ii) a high frequency of electrophoretic karyotype differences among sequential isolates from individual patients, and (iii) the occurrence of electrophoretic karyotype changes during experimental infection of mice. The implications of these observations are discussed.

Cryptococcus neoformans is a fungus which causes life-threatening meningoencephalitis in 6 to 8% of patients with AIDS (7). Cryptococcal meningitis is the most common fungal infection of the central nervous system, and there are more than 1,200 cases of cryptococcosis in New York City per year (7). In patients with AIDS, cryptococcal meningitis is usually incurable, and individuals who survive the initial infection are given lifelong antifungal therapy to reduce the likelihood of relapse (17). Analysis of initial and relapse isolates has shown that the majority of clinical recurrences are a result of the persistence of the initial infection, despite antifungal therapy (20).

Sequence analysis (3), restriction fragment length polymorphism analysis (8, 19, 21, 22), karyotype analysis of *C. neoformans* (9, 14), and random amplified polymorphic DNA analysis (13) have each shown extensive genetic variation among clinical and environmental isolates. Electrophoretic karyotypic analysis of *C. neoformans* revealed that isolates contain a variable number of chromosomes which also differ in their apparent molecular weights (9, 11, 14, 24). The overwhelming majority of clinical isolates can be distinguished by electrophoretic karyotype differences (9, 14). Genetic variation may translate into differences in virulence, clinical outcome, and response to therapy. However, relatively little is known about the biology of this pathogen in the environment (10) or the mechanism(s) responsible for the generation and maintenance of genetic variation.

In the course of studies analyzing the genetic relatedness between initial and relapse isolates, we identified one patient whose relapse isolates had minor differences in electrophoretic karyotype from the initial isolate, despite having identical restriction fragment length polymorphisms with a highly discriminatory DNA probe (20). This suggested either a mixed infection with very closely related strains having minor differences in karyotype or the occurrence of karyotype changes during human infection (20). To investigate this observation further, we have analyzed the electrophoretic karyotypes of multiple isolates from 10 patients. The results indicate that isolates from individual patients often exhibit karyotype differences. Electrophoretic karyotype differences were observed after experimentally infecting mice with three of six *C. neoformans* strains. The results have important implications for pathogenesis and epidemiological studies of *C. neoformans* infection by karyotype analysis.

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MATERIALS AND METHODS

***C. neoformans* strains.** *C. neoformans* B3, B5, and M1A are environmental strains isolated from pigeon excreta in New York City and have been previously described (8). Thirty-two clinical isolates were recovered from 10 patients in two Bronx, N.Y., hospitals which are approximately 3.5 km apart. J isolates originated from the Jacobi Hospital of the Bronx Municipal Hospital Center. MF isolates originated from Montefiore Hospital, Bronx, N.Y. Sequential isolates from individual patients were labelled A, B, etc. The clinical isolates were obtained from patients with AIDS suffering from cryptococcal meningitis. Figure 1 provides a schematic representation of the time between the sequential recovery of isolates from each patient. All clinical isolates were identified as *C. neoformans* by the hospital microbiology laboratories. All *C. neoformans* isolates used in the study originated from single colonies.

Electrophoretic karyotype. *C. neoformans* cultures were grown at 30°C in Sabouraud dextrose broth for 2 to 3 days at 30°C, and chromosomal DNA plugs were prepared from protoplasts by using minor modifications of previously published protocols (15, 16). Briefly, protoplasts were made by incubating cells in 1.1 M sorbitol–0.1 M sodium citrate (pH 6)–10 mg of NovoZym 234 (Novo Biolabs, Bagsvaerd, Denmark) per ml for 3 to 4 h at 30°C. Protoplast-containing agarose plugs were made by mixing protoplasts (4×10^8 to 6×10^8/ml) with 2% low-melt-temperature agarose (Bio-Rad, Richmond, Calif.) to yield a final agarose concentration of 0.66%. The plugs were then incubated overnight at 50°C in 1 mg of proteinase K (Boehringer, Mannheim, Germany) per ml–1% sarcosine–0.1 M EDTA–0.010 M sodium citrate (pH 8.0). The plugs were then washed four times in 50 ml of wash buffer (0.05 M EDTA, 0.020 M Tris-HCl [pH 8.0]) by incubating them at room temperature for 1 h per wash, given a final wash in 0.1× wash buffer for 1 h, and stored at 4°C until they were used. For electrophoretic karyotyping, the plugs were inserted into a 1.0% pulsed-field-certified agarose (Bio-Rad) gel (12 by 14 cm), and electrophoresis was performed in a CHEF DRIII variable-angle pulsed-field electrophoresis system (Bio-Rad) in 0.5× TBE (Tris-borate-EDTA) buffer at 14°C. The system was equipped with a cooling module (Bio-Rad) for constant temperature control. Electrophoretic conditions were switch times of 60 and 120 s at 6.0 V/cm for 24 h at an angle of 112°. The gels were stained with ethidium bromide and photographed.

Murine experiments. The B isolates were passaged in mice as described

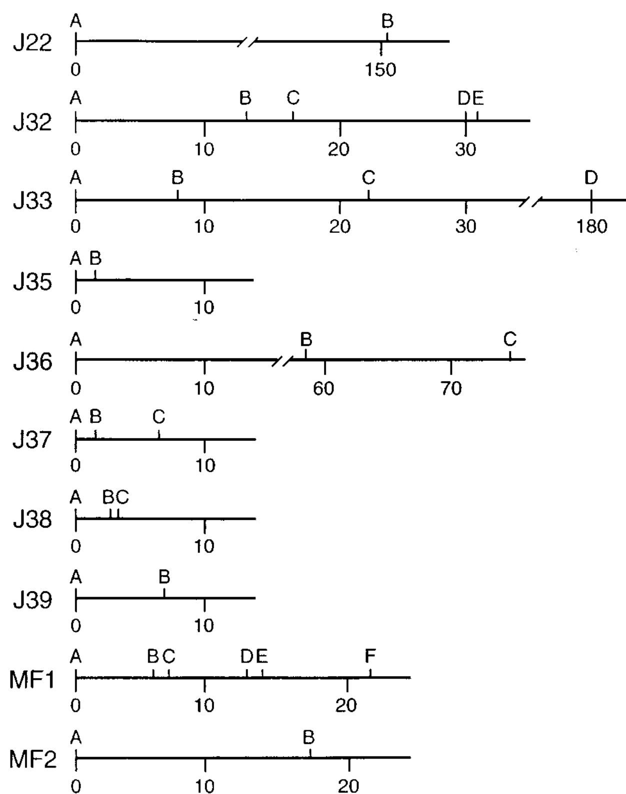

FIG. 1. Schematic representation of the temporal relationships of the clinical isolates from individual patients.

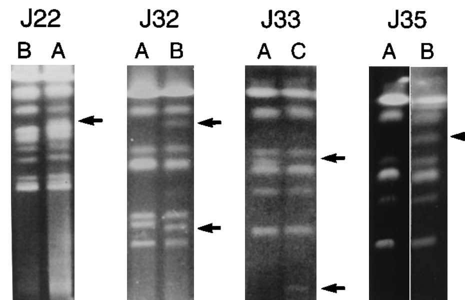

FIG. 2. Electrophoretic karyotype variants recovered from individual patients. Photographs of ethidium bromide-stained gels showing the electrophoretic karyotypes of sequential isolates from patients (isolates J22, J32, J33, and J35). The arrows denote the locations in the gel where changes in chromosome migration were observed. The letters correspond to the isolate designations in Fig. 1. For isolates from all patients, the largest chromosome and smallest chromosome bands are approximately 2,200 and 320 kb, respectively. The panel for J35 is a composite photograph of two lanes from the same gel.

previously (8). Three clinical isolates (isolates J22A, J32A, and J33A) were passaged in A/J mice (Jackson Laboratories). Briefly, *C. neoformans* isolates were grown in Sabouraud dextrose broth overnight, washed twice with 0.02 M phosphate-buffered saline (PBS; pH 7.2), suspended in PBS, and injected into the peritoneal cavity (10^7 yeast cells per mouse). At different times after infection the mice were killed by cervical dislocation and the lungs were removed and homogenized in PBS. Serial dilutions of the lung homogenates were plated onto Sabouraud dextrose agar to recover single colonies.

Data analysis. Isolates were compared in terms of chromosome number and apparent electrophoretic migration. Band similarity coefficients (S) for pairwise analysis between isolates were calculated by using $S = 2N_{AB}/(N_A + N_B)$, where N_A and N_B are the total number of bands in isolates A and B, respectively, and N_{AB} is the number of common bands (13). Student t-test analysis was used to compare the average S value calculated for multiple isolates from one patient with the S values for isolates from different patients.

RESULTS

Extensive karyotype variation for isolates from a small geographic area. Analysis of the electrophoretic karyotypes of 32 clinical isolates from patients in two closely located hospitals revealed 10 distinguishable and unique karyotype patterns. The average number of chromosomes was 7.7, and the chromosome number ranged from 6 to 11. The largest and smallest chromosomes were approximately 2,200 and 320 kb, respectively. The average ± standard deviation S value for 44 comparisons between isolates from different patients was 0.25 ± 0.06 (n = 44; range, 0.143 to 0.29).

Sequential isolates from individual patients reveal electrophoretic karyotype differences. The electrophoretic karyotypes of sequential isolates from 10 patients were determined and were analyzed for karyotype differences. The temporal separation for sequential isolates ranged from 1 day to 6 months (Fig. 1). Unlike isolates from different patients, sequential isolates recovered from an individual patient were either indistinguishable or differed at only one or two band positions. For sequential isolates from five patients (isolates J36, J37, J38, J39, and MF2), the karyotypes were indistinguishable (S = 1.0). There were electrophoretic karyotype differences among sequential isolates from five patients (isolates J22, J32, J33, J35, and MF1) (see Fig. 2 for representative electrophoretic karyotypes for isolates J22, J32, J33, and J35). The average ± standard deviation S value for comparisons between the five sequential isolates with karyotype differences from individual patients was 0.88 ± 0.06 (range, 0.8 to 0.95).

Electrophoretic karyotype changes during murine passage. The observation that sequential isolates from patients with chronic or recurrent infection had different electrophoretic karyotypes suggested that chromosomal rearrangements or deletions occurred during infection. To test this hypothesis, six *C. neoformans* strains (three environmental and three clinical strains) were passaged in mice, and individual colonies recovered from mouse organs were analyzed by pulsed-field gel electrophoresis. Karyotype analysis of 10 single colonies of strain B3 revealed two electrophoretic karyotypes: the original karyotype in 9 colonies and a new karyotype which differed at two band positions (data not shown). Karyotype analysis of four passaged colonies of strain B5 revealed two electrophoretic karyotypes: the original in three colonies and a new karyotype which had an extra chromosomal band in one colony (Fig. 3A). Analysis of 10 mouse-passaged colonies of strain M1A revealed no changes in karyotype (data not shown).

Clinical isolates from three patients (isolates J22, J32, and J33) whose sequential isolates had been found to exhibit changes in karyotype were also studied by murine passage of the original isolate. Strains J22A and J22B were recovered from a patient with AIDS who had a relapse 6 months after his initial presentation with cryptococcal meningitis, and the electrophoretic karyotypes of these strains differed (Fig. 2). Three mice were infected with a culture derived from a single colony of J22A. Analysis of 19 passaged colonies recovered from one mouse revealed two electrophoretic karyotypes: the original karyotype for 18 colonies and a new karyotype, with a deletion of one chromosome, which was different from the karyotype change observed in J22B (Fig. 3B). Analysis of 19 passaged colonies of strain J22A recovered from the second mouse and 10 colonies recovered from the third mouse revealed only the original karyotype (data not shown). Two mice were infected with a culture derived from a single colony of strain J32A, but analysis of 46 colonies recovered from the lungs of these mice

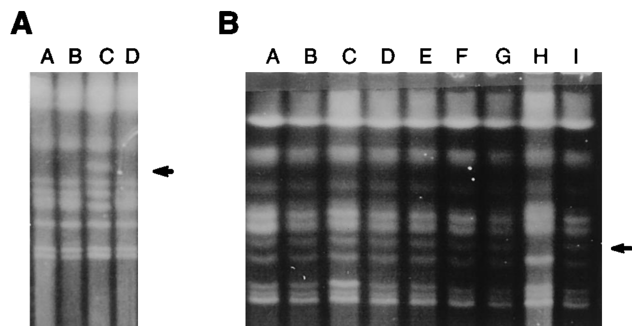

FIG. 3. Electrophoretic karyotype variants recovered from experimentally infected mice. (A) Karyotype of strain B5 before (lane A) and after (lanes B, C, D, and E) mouse passage. Lane C, a new electrophoretic karyotype variant. (B) Karyotype of strain J22A after mouse passage. Lanes A to G and I, karyotypes indistinguishable from that of the original isolate, isolate J22A; lane H, a new electrophoretic karyotype variant. The arrow denotes the location in the gel where changes in chromosome migration were observed. For both panels the largest chromosome and the smallest chromosome bands are approximately 2,200 and 320 kb, respectively.

revealed no change in electrophoretic karyotype (data not shown). Similarly, analysis of 15 colonies recovered from lung tissue of a mouse infected with strain J33A revealed no change in electrophoretic karyotype (data not shown). Thus, electrophoretic karyotype changes were noted in murine-passaged colonies from two environmental strains and one clinical strain.

DISCUSSION

Previous studies of the electrophoretic karyotype of *C. neoformans* isolates have established that clinical isolates exhibit great variation in chromosome number and size (9, 11, 14, 24). The high frequency of chromosome differences between strains makes electrophoretic karyotyping a highly discriminatory technique for distinguishing among isolates (9, 14). In the present study we confirmed these observations and demonstrated that extensive karyotype variation exists even among isolates recovered from patients in a geographically small area. In contrast, sequential isolates from individual patients had very similar or indistinguishable karyotypes. Karyotype differences between sequential isolates from individual patients did not exceed two chromosome positions. *S* values for inter- and intrapatient comparisons were 0.25 $\pm$ 0.06 (mean $\pm$ standard deviation; $n = 44$) and 0.94 $\pm$ 0.07 (mean $\pm$ standard deviation; $n = 10$), respectively ($P < 0.001$; Student *t* test). The high *S* value obtained for sequential isolates from an individual patient is consistent with and supportive of previous studies which have shown that sequential isolates from individual patients almost always belong to the same strain on the basis of restriction fragment length polymorphisms (20, 23), random amplified polymorphic DNA analysis (1, 2), karyotype (14), multilocus enzyme electrophoresis (1), and pyrolysis (12). Brandt et al. (2) have recently presented data showing that 21% of sequential isolates from patients with cryptococcal infection display altered karyotypes, despite identical random amplified polymorphic DNA profiles, a finding consistent with the results of our study.

The high frequency of electrophoretic karyotype differences among serial isolates from individual patients suggested either infection with mixed electrophoretic variants or the occurrence of karyotype changes during human infection. Since only one clinical isolate was available from each time point, we proceeded to study whether chromosome changes occurred in experimental murine infection. Electrophoretic karyotype changes after murine passage were found in three of six strains studied. The results of that experiment suggest that the karyotype differences observed among sequential isolates from some patients occurred as a result of human infection. For the patients whose serial isolates revealed karyotype differences, the differences in electrophoretic karyotype were generally found among isolates recovered earlier in infection, with later isolates generally exhibiting homogeneous karyotypes. Although the sample size is small and we did not examine multiple isolates from each time point, this observation suggests that some karyotype patterns predominated in later stages of infection, possibly as a result of selection during human infection.

Previous surveys of electrophoretic karyotypes among *C. neoformans* isolates have noted extensive variation among clinical isolates, and some investigators have speculated that the chromosomal variation may be the result of karyotype instability during infection (11, 14). Two prior studies have examined the karyotypes of *C. neoformans* isolates after passage in animal models of experimental infection (11, 14), but neither reported karyotype changes. In our study we may have increased the likelihood of detecting karyotype differences by testing a total of six strains, using several mice, and analyzing the karyotypes of multiple colonies recovered from the infected animals. An additional difference between our studies and prior studies is that we used three environmental strains and three clinical strains from patients, and karyotype changes had been demonstrated among serial isolates from these patients. If chromosome rearrangements or deletions are common during infection, then it is possible that the karyotypes of many clinical strains represent stable patterns which have already undergone rearrangements. Hence, we may have significantly increased the probability of detecting chromosome rearrangements by the use of environmental strains. Nevertheless, it is noteworthy that karyotype rearrangements were rare among the isolates passaged in mice and that no karyotype changes were observed for three strains.

Electrophoretic karyotype changes among isolates passaged in mice indicate that chromosomal changes can occur during infection. The occurrence of karyotype changes during murine infection contrasts with the fact that the electrophoretic karyotypes of *C. neoformans* isolates have been shown to be stable in vitro during passage for many years (11, 14). Changes in electrophoretic karyotype imply chromosomal breaks and/or rearrangements. The mechanism(s) responsible for the karyotype changes is unknown. There is precedent for this observation among other microorganisms. Chromosomal-length polymorphisms are common in fungi (25). Chromosomal deletions and rearrangements have been observed in *Candida albicans* (18) and *Plasmodium falciparum* (5), in which they have been associated with phenotypic switching phenomena and altered gametocyte production, respectively. Although most chromosomal breaks can be expected to be neutral or deleterious to the organism, it is possible that some chromosomal changes alter growth abilities and possibly enhance virulence. The persistence of infection in mammalian hosts implies continued replication and/or survival at a relatively high temperature (37°C) under conditions which are likely to include attack by host effector cells (i.e., macrophages, neutrophils, etc.). Exposure of *C. neoformans* isolates to reactive oxygen and nitrogen intermediates combined with replication at higher temperatures may produce an environment conducive to the generation and selection of new karyotype variants. In patients, antifungal therapy causes fungal cell damage, which could contribute to karyotype instability. However, the recovery of electrophoretic karyotype variants from mice that were not

treated with antifungal drugs suggests that this phenomenon can occur independently of antifungal therapy.

Electrophoretic karyotyping has been shown to be a sensitive tool for distinguishing among *C. neoformans* isolates (9, 14), and our results confirm this. However, the occurrence of chromosomal rearrangements in some strains suggests that one should use caution in interpreting minor differences in electrophoretic karyotypes. Our results provide evidence for the occurrence of genetic changes in some *C. neoformans* strains during infection. Chromosomal changes during human infection could contribute to the variability in the number and size of chromosomes among clinical *C. neoformans* isolates. Chromosomal changes combined with previous findings of polysaccharide structure variation among genetically related isolates from patients with recurrent meningitis (4) and changes in sterol content and antifungal susceptibility during passage of environmental strains in mice (6) provide evidence that *C. neoformans* isolates can undergo genotypic and phenotypic changes during infection. The implications of these observations for pathogenesis are unclear. However, these results strongly suggest that DNA typing techniques for *C. neoformans* isolates be validated for their stabilities during infection prior to interpretation of their results.

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