

## Persistence, Replacement, and Microevolution of *Cryptococcus neoformans* Strains in Recurrent Meningitis in AIDS Patients

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Six separate human immunodeficiency virus-positive patients with cryptococcal meningitis were each found to have been infected with a unique strain of *Cryptococcus neoformans* on the basis of genomic DNA fingerprinting analysis with the microsatellite sequence-containing oligonucleotide probe (GGAT)<sub>4</sub> and by random amplification of polymorphic DNA. Two patients (A and B) experienced a recurrent episode of infection. Between 12 and 16 single-colony isolates recovered from primary isolation media (>50% of *C. neoformans* colonies recovered) from cerebrospinal fluid specimens were fingerprinted from both patients during each episode. The fingerprints of both isolate collections from patient B were very similar, although minor polymorphisms were evident in both sets of profiles. The fingerprints of the isolate collection from the initial episode of infection in patient A were also identical to each other, apart from minor polymorphisms, but they were clearly different from the corresponding profiles of the isolate collection from the recurrent episode, the latter of which were completely identical, apart from minor polymorphisms in a single isolate. Furthermore, prolonged storage and in vitro subculture of the isolates did not alter the fingerprint profiles. These results provided convincing evidence that patients A and B were each infected with a single *C. neoformans* strain during each episode of infection and that in patient B, the same strain persisted and caused both episodes, while in patient A, a different strain was responsible for each episode. The prevalence of polymorphisms in multiple single-colony isolates from both patients also suggested that *C. neoformans* populations may undergo microevolution.

*Cryptococcus neoformans* is an encapsulated basidiomycetous yeast species which occurs naturally in the environment and is frequently associated with pigeon droppings and soil contaminated with avian guano (11). Under most circumstances, inhalation of this organism fails to cause symptomatic infection (14). However, in 5 to 10% of individuals with AIDS, severe life-threatening disease in the form of meningoencephalitis can occur (6). The vast majority of the isolates responsible for these infections are *C. neoformans* var. *neoformans* serotype A (10).

Because of the perceived importance of these organisms as human pathogens, the species has been subjected to intense study during the last decade. In this regard, the recent development and application of techniques designed to differentiate between individual isolates are of particular relevance because of the high incidence of recurrent cryptococcal infections in AIDS patients once antifungal therapy has ceased (20). In order to design effective antifungal drug treatment regimens, it is important to determine if recurrent disease is the result of reinfection with the original strain or infection with a novel strain. Unfortunately, individual strains of *C. neoformans* are morphologically and physiologically indistinguishable, and isolates of *C. neoformans* var. *neoformans* can only be divided into three serotypes (serotypes A, D, and A-D) (9). Consequently, techniques used in the epidemiological analysis of these organisms have concentrated on detecting genetic differences between individual isolates. To date, a wide variety of molecular typing systems have been applied to *C. neoformans* epidemiol-

ogy, including electrophoretic karyotyping (17, 19), PCR fingerprinting (13), random amplified polymorphic DNA (RAPD) analysis (2, 8), multilocus enzyme typing (1, 2), allelic variation of the *URA5* locus (3, 5), and DNA fingerprinting with (i) genomic DNA probes (5, 18, 22, 23, 26, 27), (ii) mitochondrial DNA probes (25), and (iii) oligonucleotide probes homologous to microsatellite sequences (8). The general consensus from many of these studies is that *C. neoformans* strains exhibit considerable genetic heterogeneity and that recurrent infections are apparently due to the persistence of the original infecting strain (17, 22, 23). However, a recent study by Haynes et al. (8) indicated, on the basis of oligonucleotide and RAPD fingerprint analysis of genomic DNA, that in two patients (from five examined in total), recurrent infections may have been due to reinfection with a novel strain. In addition, this study indicated that one patient was coinfecting with more than one strain during a single episode of infection. These data have aroused some controversy (4), and their clinical implications are of sufficient importance to warrant the analysis of additional cases of recurrent *C. neoformans* infection in order to confirm unequivocally that reinfection with novel strains may occur. The purpose of this study was to corroborate these earlier findings by analyzing multiple single-colony isolates recovered from patients during two recurrent episodes of meningitis and to determine the extent of genetic diversity within phenotypically homogeneous populations of *C. neoformans* recovered from the same clinical specimen.

### MATERIALS AND METHODS

***C. neoformans* isolates.** *C. neoformans* isolates were recovered from six human immunodeficiency virus-infected individuals with cryptococcal meningitis at The Chelsea and Westminster Hospital, London, United Kingdom, between January 1994 and April 1995. In the case of four of these patients, *C. neoformans* isolates were recovered from a single episode of infection. The remaining two patients,

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both of whom were homosexual males with AIDS, experienced two successive episodes of meningitis. These two patients, termed A and B, respectively, presented with the classical symptoms of cryptococcal meningitis for the first time in November 1994, and in both cases, *C. neoformans* was isolated by plating of aliquots of cerebrospinal fluid (CSF) on Sabouraud's agar. After treatment with 0.6 to 0.7 mg of amphotericin B  $\text{kg}^{-1} \text{day}^{-1}$  for 2 to 4 weeks, both patients made a complete clinical recovery. Thereafter, each patient received 200 to 400 mg of fluconazole  $\text{day}^{-1}$  on an ongoing basis. However, clinical symptoms of meningitis reappeared in patient A in April 1995 and in patient B in March 1995, and in both cases, *C. neoformans* was again isolated from CSF samples. The time intervals between the initial and recurrent isolation of *C. neoformans* from patients A and B were 158 and 112 days, respectively. No additional specimens were taken from either patient during the symptom-free period between episodes of meningitis. Isolates were recovered on Sabouraud's agar after incubation at 37°C for 48 h and subcultured on fresh media prior to identification with the API ID 32C Yeast Identification System (bioMérieux, Marcy l'Etoile, France) and urea assimilation. Each isolate was stored in Protect cryo-storage vials (STC, Heywood, Lancashire, United Kingdom) at -20°C prior to detailed analysis. The CSF specimens from the initial and recurrent episodes of meningitis for both patients A and B each yielded between 10 and 30 *C. neoformans* CFU on primary isolation media. In the case of both episodes of infection in patient A and in the recurrent episode of infection in patient B, 20 individual well-separated *C. neoformans* colonies from the primary isolation plates were selected at random and stored; 16 of these from each isolate collection were subjected to further detailed analysis. In the case of the initial episode of infection in patient B, only 12 *C. neoformans* CFU were recovered on primary isolation media, all of which were stored and subjected to detailed analysis. The isolate collections from the initial episodes of infection in patients A and B were labelled A<sub>1</sub>1-20 and B<sub>1</sub>1-12, respectively, while those from the recurrent episodes were labelled A<sub>11</sub>1-20 and B<sub>11</sub>1-20, respectively.

**Fluconazole susceptibility testing.** The susceptibility of *C. neoformans* clinical isolates to fluconazole was determined by broth microdilution in RPMI 1640 medium (15). Isolates were grown in 96-well microtiter plates (Corning) incubated at 35°C for 72 h with agitation (16). An end point of 80% growth inhibition ( $\text{IC}_{80}$ ) was determined for each isolate by measuring the  $A_{405}$  with an automated microplate reader (Spectra I; SLT-Labinstruments, Salzburg, Austria).

**DNA fingerprinting.** *C. neoformans* total cellular DNA was purified as described previously (22). Restriction fragments generated by digesting total cellular DNA samples to completion with the restriction enzyme *EcoRI* (Promega Corp., Madison, Wis.) were separated by electrophoresis through 0.8% (wt/vol) agarose gels and transferred onto nylon membrane filters (MSI, Westboro, Mass.) according to the method of Southern (21). The oligonucleotide probe (GGAT)<sub>4</sub> was end labelled with [ $\gamma$ -<sup>32</sup>P]dATP (Amersham International Plc., Little Chalfont, Buckinghamshire, United Kingdom [ $>5,000 \text{ Ci mmol}^{-1}$ ]) and T4 polynucleotide kinase (Promega Corp.) according to the manufacturer's instructions. Labelled (GGAT)<sub>4</sub> was hybridized to the digested DNA as described previously (24).

**RAPD.** PCRs were performed in a final volume of 25  $\mu\text{l}$  containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 3.0 mM MgCl<sub>2</sub>; 200  $\mu\text{M}$  (each) dATP, dCTP, dTTP, and dGTP (Promega Corp.); 20 pM primer; 0.5 U of *Taq* DNA polymerase (Promega Corp.); and approximately 10 ng of *C. neoformans* total genomic DNA. The following oligonucleotide primers were used: 1, 5'-GCGATCCCCA3'; 2, 5'-(GATA)<sub>3</sub>3'; and 3, 5'-AACGCGCAAC3'. Amplification reactions were performed in a Perkin-Elmer Cetus DNA thermal cycler under the following conditions: 94°C for 5 min, 36°C for 5 min, and 72°C for 5 min for 4 cycles followed by 30 cycles at 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min. This was followed by an incubation period of 72°C for 10 min. After amplification, 20- $\mu\text{l}$  aliquots of the reaction mixtures were electrophoresed in 1.5% (wt/vol) agarose gels, and the amplified products were visualized under UV light after being stained with ethidium bromide.

## RESULTS

**Oligonucleotide fingerprinting of *C. neoformans* isolates.** Total cellular DNA was purified from a single isolate of *C. neoformans* recovered in each case from CSF, skin, and blood samples obtained from two patients with meningitis and from CSF cultures recovered from another four patients suffering from meningitis. Aliquots of DNA from these isolates were digested to completion with *EcoRI*, and the resulting fragments were separated by agarose gel electrophoresis and transferred to nylon membrane filters prior to hybridization analysis with the <sup>32</sup>P-labelled oligonucleotide probe (GGAT)<sub>4</sub>. The fingerprints generated from isolates recovered from different anatomical sites in the same patient were found to be indistinguishable. However, the fingerprints generated from isolates recovered from separate patients were each found to be very

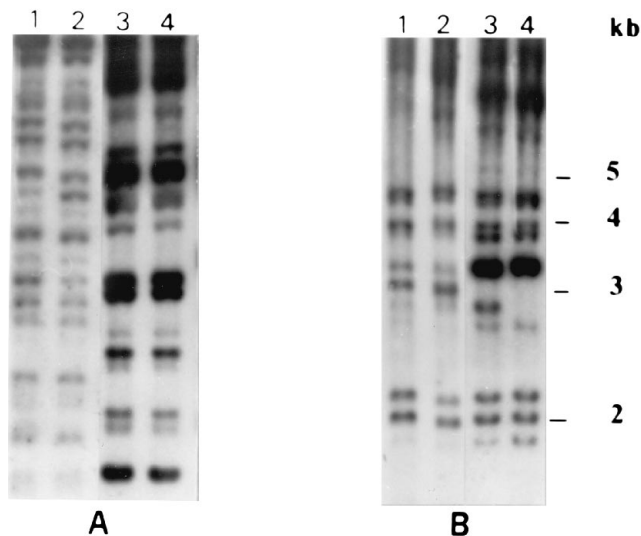


FIG. 1. Autoradiogram of *EcoRI*-digested total cellular DNA from single-colony isolates of *C. neoformans* recovered from CSF specimens from patients A and B during two successive episodes of meningitis and hybridized with the oligonucleotide probe (GGAT)<sub>4</sub>. Lanes 1 and 2 and 3 and 4 show fingerprint profiles generated from two single-colony isolates recovered from the same CSF sample during the initial and recurrent episodes of infection, respectively. (A) Lanes 1 to 4 show profiles generated from isolates A<sub>1</sub>1, A<sub>1</sub>2, A<sub>11</sub>1, and A<sub>11</sub>2, respectively. (B) Lanes 1 to 4 show profiles generated from isolates B<sub>1</sub>1, B<sub>1</sub>2, B<sub>11</sub>1, and B<sub>11</sub>2, respectively. The relative positions of molecular size standards are indicated to the right.

different (data not shown), indicating that each patient was infected with a unique strain of *C. neoformans*.

Total cellular DNA was purified from three single-colony isolates from the initial and recurrent isolate collections recovered from two AIDS patients (A and B), each of whom experienced two successive episodes of meningitis, and DNA fingerprints were generated as described above. In the case of patient A, the hybridization profiles obtained with the three isolates recovered during the initial episode of meningitis (data for isolates A<sub>1</sub>1 and A<sub>1</sub>2 are shown in Fig. 1A, lanes 1 and 2) were identical to each other but were clearly significantly different from the corresponding profiles obtained with the three isolates (also identical to each other) recovered during the recurrent episode of infection (data for isolates A<sub>11</sub>1 and A<sub>11</sub>2 are shown in Fig. 1A, lanes 3 and 4). In contrast, the hybridization patterns obtained with both sets of isolates recovered from patient B were found to be very similar, but some band differences were evident. For example, there was a hybridization band with a size of approximately 3 kb, which was present in the profiles of the three isolates from the initial episode of infection (data for isolates B<sub>1</sub>1 and B<sub>1</sub>2 are shown in Fig. 1B, lanes 1 and 2) but was absent in the corresponding profiles of the three isolates from the recurrent episode (data for isolates B<sub>11</sub>1 and B<sub>11</sub>2 are shown in Fig. 1B, lanes 3 and 4). Direct visual analysis of ethidium bromide-stained agarose gels containing separated *EcoRI*-generated fragments of genomic DNA from the isolates concerned showed that the restriction fragment length polymorphism patterns of the recurrent isolates from patient B lacked a heavily stained band, also approximately 3 kb in size, that was present in the restriction fragment length polymorphism patterns of the three isolates from the initial episode of infection (data not shown). Furthermore, an additional hybridization band with a size of approximately 2.8 kb was present in the profile of isolate B<sub>11</sub>1 which was not present in the profile of isolate B<sub>11</sub>2 (Fig. 1B, lanes 3 and 4).

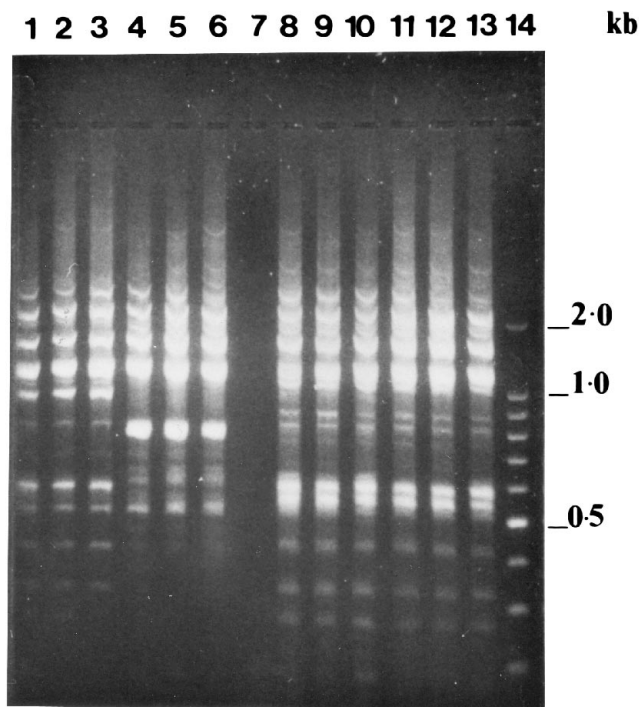


FIG. 2. RAPD products from single-colony isolates of *C. neoformans* recovered from CSF specimens from patients A and B during two successive episodes of meningitis and amplified with oligonucleotide primer 1 (5'GCGATCCCC A3'). Lanes 1 to 3 (isolates A<sub>I</sub>1 to 3) and 8 to 10 (isolates B<sub>I</sub>1 to 3) show RAPD profiles generated from three single-colony isolates recovered from the same CSF sample from patients A and B, respectively, during the initial episodes of infection. Lanes 4 to 6 (isolates A<sub>II</sub>1 to 3) and 11 to 13 (isolates B<sub>II</sub>1 to 3) show RAPD profiles generated from three single-colony isolates recovered from the same CSF sample from patients A and B, respectively, during recurrent episodes of infection. Lane 14, 100-bp DNA ladder size standards (Promega).

The fingerprint profiles of the isolates described above were found to be stable after repeated subculture (minimum of eight times) of the isolates over a 6-month period. During this time, total cellular DNA was prepared from each isolate on three separate occasions, and in each instance, fingerprint profiles identical to those shown in Fig. 1 were obtained with (GGAT)<sub>4</sub>.

**RAPD analysis of recurrent isolates.** The RAPD profiles generated with primer 1 and target DNA from three single-colony isolates from the same clinical specimen examined from the initial episode of infection in patient A (A<sub>I</sub>1, A<sub>I</sub>2, and A<sub>I</sub>3) were identical to each other (Fig. 2, lanes 1 to 3). Similarly, the corresponding profiles obtained with the target DNA from an additional three single-colony isolates from the same clinical specimen recovered during the recurrent episode of infection (A<sub>II</sub>1, A<sub>II</sub>2, and A<sub>II</sub>3 [Fig. 2, lanes 4 to 6]) were also found to be identical to each other. However, the patterns obtained from both sets of isolates were totally distinct. In contrast, the RAPD patterns generated with the three isolates examined from both the initial and recurrent episodes of meningitis in patient B (B<sub>I</sub>1, B<sub>I</sub>2, B<sub>I</sub>3, B<sub>II</sub>1, B<sub>II</sub>2, and B<sub>II</sub>3) were all found to be identical (Fig. 2, lanes 8 to 13). Additional RAPD experiments with the same target DNA as that used in the experiments described above and primers 2 and 3 also generated distinct profiles for the three A<sub>I</sub> and the three A<sub>II</sub> isolates and were unable to discriminate between the three B<sub>I</sub> and the three B<sub>II</sub> isolates. RAPD fingerprint profiles were also found to be reproducible after repeated subculture of the isolates with target DNA prepared on three separate occasions.

**Analysis of multiple single-colony isolates.** In order to investigate the possibility that patients A and B may have been infected with more than one strain of *C. neoformans* during both episodes of meningitis, total cellular DNA was prepared from an additional 13 single-colony isolates (9 in the case of the initial episode of infection in patient B) recovered, in each case, from the same clinical specimens as the isolates used in the experiments described above, and then hybridization fingerprints were generated with (GGAT)<sub>4</sub>. For both patients, only a single fingerprint pattern was found for each group of single-colony isolates examined. As in the results described above, the patterns of the 13 additional A<sub>I</sub> isolates differed considerably from those of the 13 additional A<sub>II</sub> isolates, while those of the 9 additional B<sub>I</sub> isolates were almost identical to those of the 13 additional B<sub>II</sub> isolates. However, whereas the A<sub>II</sub> isolates all yielded identical fingerprint patterns (fingerprints from 14 isolates are shown in Fig. 3a), the corresponding profiles of the A<sub>I</sub>, B<sub>I</sub>, and B<sub>II</sub> isolate collections, respectively, although essentially homogeneous, showed numerous subtle but distinct minor polymorphisms. An example of the profiles obtained with the B<sub>II</sub> isolate collection is shown in Fig. 3b.

RAPD fingerprints of the additional single-colony isolates from each isolate collection from patients A and B were also generated with primer 2 and primer 3, respectively. The results confirmed the findings obtained in the experiments described above with three single-colony isolates from each isolate collection. However, minor polymorphisms were evident in some of the RAPD profiles of all four single-colony isolate collections, including one single-colony isolate from the recurrent episode of infection in patient A (Fig. 4).

**Fluconazole susceptibility of *C. neoformans* isolates.** The fluconazole susceptibilities of isolates 1 to 3 from the initial and recurrent isolate collections for both patients A and B were determined by broth microdilution. Isolates A<sub>I</sub>1 to 3, B<sub>I</sub>1 to 3, and B<sub>II</sub>1 to 3 yielded an IC<sub>80</sub> of 32 μg ml<sup>-1</sup>, whereas isolates A<sub>II</sub>1 to 3 yielded an IC<sub>80</sub> of 8 μg ml<sup>-1</sup>.

## DISCUSSION

Only a small number of studies have focused on the relationship between isolates of *C. neoformans* from successive episodes of meningitis in individual patients (8, 17, 22, 23). Some of these studies have indicated that relapse of cryptococcal meningitis is due to the persistence of the originally infecting strain (17, 22, 23), whereas another study has shown that in two separate individuals, recurrence of infection was apparently due to reinfection with a novel strain (8). In addition, the latter study provided evidence that one patient was infected with more than one *C. neoformans* strain during a single episode of meningitis. The present study was undertaken to confirm these findings by analysis of multiple *C. neoformans* single-colony isolates recovered from the same clinical specimen obtained from two patients during each of two successive episodes of disease. Fingerprint analysis of genomic DNA with the microsatellite sequence-containing oligonucleotide probe (GGAT)<sub>4</sub> and RAPD analysis with three separate oligonucleotide primers showed that the six patients included in the study were each infected by different *C. neoformans* strains. This is in agreement with earlier studies which described the genetic heterogeneity of serotype A *C. neoformans* strains (3, 7, 8, 17, 26, 27). In addition, in the case of the two patients from which *C. neoformans* isolates were recovered from specimens taken from different anatomical sites during the same episode of meningitis, all of the isolates from the same individual yielded the same fingerprint pattern.

Two of the six patients under study (A and B) suffered

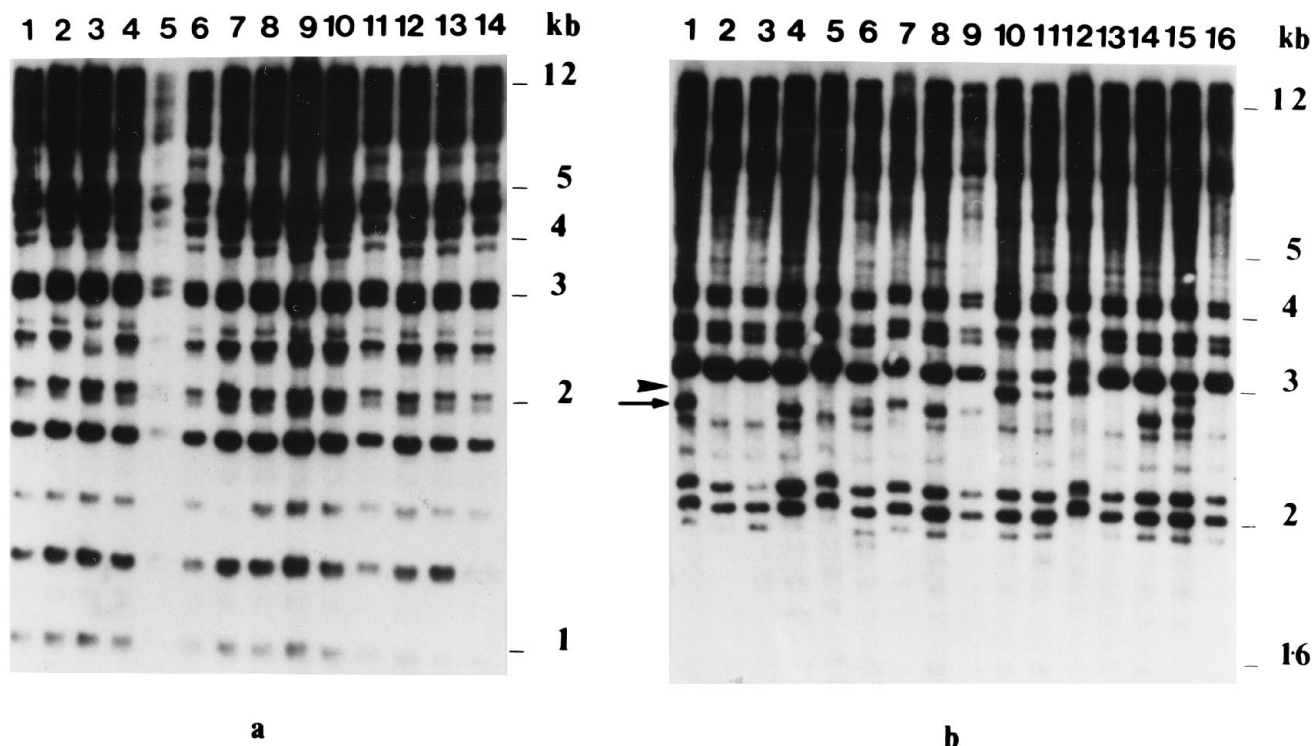


FIG. 3. Autoradiograms of *Eco*RI-digested total cellular DNA from *C. neoformans* single-colony isolates recovered from the same CSF specimen in the case of patients A and B, respectively, during the recurrent episode of meningitis after hybridization analysis with the oligonucleotide probe (GGAT)<sub>4</sub>. (a) Profiles shown in lanes 1 to 14 were from single-colony isolates A<sub>11</sub>1 to A<sub>11</sub>14, respectively. (b) Profiles shown in lanes 1 to 16 were from single-colony isolates B<sub>11</sub>1 to B<sub>11</sub>16, respectively. The arrow and arrowhead shown to the left of panel b indicate the relative positions of polymorphic hybridization bands with sizes of approximately 2.6 and 2.9 kb present in isolate profiles shown in lanes 1, 4, 6, 7, 8, 9, 14, and 15 and lanes 10, 11, 12, and 15, respectively.

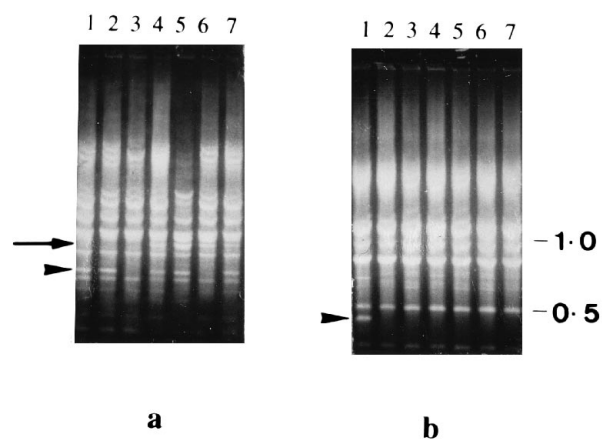


FIG. 4. Amplified RAPD products from single-colony isolates of *C. neoformans* recovered from CSF specimens from patient A during the initial and recurrent episodes of infection. (a) Profiles shown in lanes 1 to 7 were from single-colony isolates (A<sub>11</sub>5 to A<sub>11</sub>11) from the same specimen recovered during the initial episode of infection. (b) Profiles shown in lanes 1 to 7 were from single-colony isolates (A<sub>11</sub>1 to A<sub>11</sub>7) from the same specimen recovered during the recurrent episode of infection. The positions of the molecular size reference markers indicated to the right are in kilobase pairs. The arrowhead and arrow shown to the left of panel a indicate the relative positions of polymorphic bands with sizes of approximately 0.7 and 0.9 kb present in isolate profiles shown in lanes 1, 2, 4, 5, and 7 and 4, 5, 6, and 7, respectively. The arrowhead to the left of panel b indicates the position of a single polymorphic band with a size of approximately 0.45 kb present only in lane 1.

relapses in infection within 6 months of clinical resolution of the symptoms after therapy. Preliminary experiments were performed with three single-colony isolates recovered from CSF specimens from patients A and B during each episode of disease by both DNA fingerprinting techniques (Fig. 1 and 2). The results showed that the (GGAT)<sub>4</sub>-generated hybridization patterns obtained with the three single-colony isolates from the initial episode of meningitis in patient A, although identical to each other, were significantly different from the corresponding profiles of the three single-colony isolates recovered during the recurrent episode of disease in the same patient. In fact, the two sets of profiles, each of which contained  $\geq 15$  clearly resolved hybridization bands, shared no bands in common, strongly suggesting that patient A was infected with unrelated *C. neoformans* strains during each episode of meningitis. In contrast, the hybridization patterns obtained with the three single-colony isolates from both the initial and recurrent episodes of infection in patient B were very similar, with the majority ( $\sim 75\%$ ) of bands shared in common. These results suggested that the same strain of *C. neoformans* was responsible for both episodes of disease. This also suggested that the recurrent episode of infection was due to persistence of the strain responsible for the initial episode in patient B. However, the possibility that patient B was reinfected with the same strain from an environmental source cannot be discounted.

Fluconazole susceptibility data obtained from three single-colony isolates from each episode of meningitis in both patients A and B showed that isolates from the recurrent episode in patient A had a fourfold lower ( $8 \mu\text{g ml}^{-1}$ ) fluconazole IC<sub>80</sub> than that obtained with isolates from the initial episode ( $32 \mu\text{g ml}^{-1}$ ). In contrast, all six isolates tested from patient B yielded

the same fluconazole  $IC_{80}$  ( $32 \mu\text{g ml}^{-1}$ ). These results strengthen the conclusions derived by fingerprinting analysis that the recurrent episode of meningitis in patient A was caused by a novel strain, while both episodes in patient B were caused by the same strain.

On the basis of the results described above, it was still possible that both patients A and B were infected with two or more strains of *C. neoformans* during each episode of disease and that this was not reflected in the single-colony isolates tested from primary isolation plates. If two strains were present during a particular episode of infection in different relative abundance, the chance of detecting a particular strain would directly reflect the number of single-colony isolates tested from the primary isolation plates. A strain present in low abundance relative to a second strain during a particular episode of disease in a given patient might not be detected if only a few colonies were sampled. A change in the relative abundance of the two strains during a subsequent episode of disease, assuming that both strains persisted between episodes, could lead to the conclusion that the recurrent episode was due to a novel strain. To unequivocally determine the presence of multiple strains during a specific episode of infection would require analysis of all of the *C. neoformans* colonies recovered on primary isolation. However, from a routine perspective, this would clearly not be practical for logistical reasons. To improve the chances of detecting the presence of more than one *C. neoformans* strain from each of the specimens obtained during both episodes of infection in patient A and from the recurrent episode of infection in patient B, a total of 16 single-colony isolates from the primary isolation plates were analyzed (i.e., an additional 13 single-colony isolates from each specimen). This represented >50% of the total number of *C. neoformans* colonies recovered from the original specimens in each case. In the case of the initial episode of infection in patient B, all 12 (i.e., 100%) of the *C. neoformans* CFU recovered on primary isolation were analyzed. Fingerprinting analysis of the additional single-colony isolates from the initial and recurrent specimens from patients A and B confirmed the preliminary findings described above with each of three single-colony isolates from each episode of infection. The very close similarity of the fingerprint patterns obtained with the initial and recurrent isolate collections from patient B makes it unlikely that this patient was infected by more than one strain of *C. neoformans* during each episode of meningitis and suggests that both disease episodes were caused by the same strain. Furthermore, although the fingerprint patterns obtained with the single-colony isolate collections from the initial and recurrent episodes of disease in patient A were totally different from each other, the profiles obtained with the 16 isolates comprising each collection were remarkably homogeneous, apart from minor polymorphisms evident in patterns from isolates from the initial episode. These findings and the confirmatory data obtained by RAPD analysis provided strong evidence that patient A was infected with a single strain of *C. neoformans* during each episode of disease and that a different strain was responsible for each episode.

As far as we are aware, the present work and another recent report from our laboratories (8) are the only studies which have indicated that recurrent episodes of meningitis in the same patient can be caused by different strains of *C. neoformans*. The only other published studies on this subject indicate that recurrent episodes of meningitis in the same patient are due to persistence of the same *C. neoformans* strain responsible for the initial episode of disease (17, 22, 23). There are several possible reasons for the disparity between our results and those of previous studies, including different patient man-

agement and treatment regimens. However, the use of different fingerprinting techniques in the various studies to distinguish between isolates and their relative discriminatory powers is probably more important. Each of the fingerprinting probes employed to date recognizes different genetic markers and is likely to have a different discriminatory ability.

Failure to detect minor polymorphisms in the single-colony isolate collection from the recurrent episode of infection in patient A by fingerprinting with the (GGAT)<sub>4</sub> probe was surprising, given that they were readily detectable in both isolate collections from patient B and in the initial isolate collection from patient A (Fig. 4a, lane 1). However, minor polymorphisms were evident in the RAPD profiles of one of the 16 single-colony isolates from the recurrent episode of meningitis in patient A. It is possible that some strains of *C. neoformans* are inherently more unstable or genetically pleomorphic and thus are more prone to generate polymorphisms than others. In order to investigate whether the polymorphisms were due to genetic instability in vitro, three of the single-colony isolates from each isolate collection recovered from patients A and B were subcultured at least eight times over a 6-month period, and fingerprinting experiments were repeated on three separate occasions with freshly prepared total cellular DNA. The results demonstrated that the fingerprint profiles of the single-colony isolates were reproducible. Furthermore, storage of isolates for a period of at least 6 months at  $-20^{\circ}\text{C}$  did not detectably affect the fingerprint profiles. All of these results demonstrated that the generation of polymorphisms was not due to genetic instability in vitro; the generation of polymorphisms is more likely to be a response to unfavorable environmental conditions in vivo. Similar polymorphisms in *Candida albicans* DNA fingerprint profiles have recently been described and have been attributed to the occurrence of microevolution (12).

It has been suggested that *C. neoformans* strains only rarely reproduce sexually and that clinical populations of the organism are usually clonal in origin (5, 14). In the absence of genetic exchange through sexual reproduction, microevolution could conceivably confer a selective advantage under unfavorable environmental conditions.

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