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Involvement of Multiple *Cryptococcus neoformans* Strains in a Single Episode of Cryptococcosis and Reinfection with Novel Strains in Recurrent Infection Demonstrated by Random Amplification of Polymorphic DNA and DNA Fingerprinting

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We compared the abilities of random amplification of polymorphic DNA and DNA fingerprinting, with oligonucleotide probes, to type five pairs of *Cryptococcus neoformans* clinical isolates recovered from five separate human immunodeficiency virus-positive patients in London, England. The two techniques had comparable discriminatory abilities when applied to these isolates. A total of eight different isolate types were demonstrated in these patients. No isolate type was observed in more than one patient. Two of the isolate pairs recovered from single episodes of cryptococcosis within 1 day of each other were genotypically indistinguishable by both methods. The other three pairs of isolates were all distinguishable. One of these isolate pairs was obtained from a single episode of cryptococcosis, while the other two were obtained from recurrent infections. These results indicate that multiple strains of *C. neoformans* may be responsible for a single episode of cryptococcosis and that recurrent infection may occur as a result of reinfection with a novel strain.

The encapsulated basidiomycetous yeast *Cryptococcus neoformans* is present in the environment worldwide, particularly in association with avian guano and tree debris (21). Inhalation of the infectious propagule of this organism usually results in a self-limiting, asymptomatic pulmonary infection in an immunocompetent host (8). Although the absolute incidence of symptomatic cryptococcosis is low, patients with depressed cell-mediated immunity are at increased risk. This is particularly true for patients with AIDS, in whom an incidence of cryptococcosis between 5 and 10% has been reported (5). The most common site of extrapulmonary infection is the meninges, and recurrent cryptococcal meningoencephalitis is the major cause of life-threatening fungal infection in this patient population (15).

It is important to establish whether isolates of *C. neoformans* recovered from cases of recurrent cryptococcosis arise from persistence of the initial organism or reinfection with a novel strain. This has major implications for patient management. If relapse occurs, then therapies which effectively eradicate all infecting organisms need to be developed or adapted. However, if patient reinfection is common, then efforts should be made to reduce the exposure of individuals at risk.

Serological techniques have been used to distinguish two varieties and five serotypes of *C. neoformans*, including *C. neoformans* var. *neoformans* (serotypes A, D, and A-D) and *C. neoformans* var. *gattii* (serotypes B and C). *C. neoformans* var. *neoformans* predominates in patients infected with human immunodeficiency virus type 1 (HIV-1) (2). Although some antigenic variation has been demonstrated within the capsular polysaccharide, this conventional typing system is not sufficiently sensitive to discriminate between individual strains (4, 18).

Molecular typing methods have been applied successfully to the typing of a number of pathogenic fungi, e.g., *Histoplasma capsulatum* (11), *Aspergillus fumigatus* (12), and *Candida albicans* (9). When similar methods were first applied to the typing of *C. neoformans*, discrimination at the strain level remained elusive (16, 24, 26). However, several recent reports have established that genotypic variation in *C. neoformans* can be identified by molecular techniques. Random amplification of polymorphic DNA (RAPD) by PCR, which requires no previous knowledge of the target DNA, has been used to generate 12 distinct profiles from 12 *C. neoformans* var. *neoformans* isolates obtained from disparate sources (6). Similarly, pulsed-field gel electrophoresis has been used to demonstrate polymorphisms in the chromosome-like band pattern of 13 *C. neoformans* var. *neoformans* strains (27). Furthermore, DNA fingerprinting of restriction endonuclease-digested DNA with dispersed repetitive DNA sequences cloned from *C. neoformans*, including CNRE-1 (19) and UT-4p (25), has demonstrated that this technique can be used successfully to discriminate between *C. neoformans* isolates. However, all of these typing systems have been used singly and the isolate discrimination data obtained have not been substantiated by a second confirmatory technique.

In the present study, we compared the abilities of RAPD-PCR and DNA fingerprinting, with synthetic oligonucleotide probes, to type five pairs of *C. neoformans* var. *neoformans* isolates recovered from five separate HIV-infected patients in

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TABLE 1. *C. neoformans* isolates recovered from five separate HIV-positive patients with cryptococcosis at The Westminster Hospital from 1989 to 1991

Patient no.	Isolate no.	Isolate source	Time (days) between recoveries of isolates
1	1a	Blood	1
	1b	Blood	
2	2a	Blood	194
	2b	CSF	
3	3a	Blood	103
	3b	Blood	
4	4a	Blood	8
	4b	CSF	
5	5a	CSF	0
	5b	Blood	

West London, England. This was performed to determine if recurrence of cryptococcosis in these patients was due to relapse or new infection.

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MATERIALS AND METHODS

***C. neoformans* isolates.** Ten isolates of *C. neoformans* var. *neoformans* were recovered from five HIV-infected patients with cryptococcosis at The Westminster Hospital from 1989 to 1991. Isolates were recovered from blood culture or lumbar puncture cerebrospinal fluid (CSF) specimens collected for microbiological examination (Table 1). Isolates from patients 2 and 3 were obtained 194 and 103 days apart, respectively. During these periods, patient 2 had two negative blood cultures and one negative CSF culture while patient 3 had a single negative culture of each type of specimen. Therefore, isolates 2a and 2b and isolates 3a and 3b were obtained from recurrent episodes of disease whereas isolates from patients 1, 4, and 5 were obtained from a single episode of cryptococcosis. All isolates were stored at −20°C in 10% (wt/vol) glycerol in 1-ml aliquots.

RAPD. A minimal extraction procedure was developed to obtain sufficient *C. neoformans* DNA for RAPD-PCR analysis. Two 1-ml aliquots of each isolate were thawed, washed twice with phosphate-buffered saline, and resuspended in 500 μl of 50 mM Tris-HCl (pH 8.0)–10 mM EDTA–150 mM NaCl–2% (wt/vol) sodium dodecyl sulfate. These were incubated at 65°C for 1 h and then at 100°C for 5 min. Cell debris was removed by centrifugation at 9,500 × *g* for 5 min, and the supernatant was extracted once with aqueous phenol (pH 8.0). DNA was precipitated by using 0.6 volume of isopropanol, washed with 70% ethanol, vacuum dried, and resuspended in 50 μl of sterile distilled water. Aliquots (1 μl) of the DNA extracts were then used as the template DNA in a 50-μl PCR containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 200 μM each dATP, dCTP, dGTP, and dTTP; 2 μM oligonucleotide primer; and 0.5 U of *Taq* DNA polymerase. The oligonucleotide primers used were 5′-CAGGCCCTTC-3′ (primer 1), 5′-AACGGCAAC-3′ (primer 2), and 5′-$(GATA)_4$-3′ (primer 3). Reactions were cycled as described by Williams et al. (28). Following thermal cycling, 15-μl aliquots of the amplification reaction mixtures were electrophoresed on 1.2% (wt/vol) agarose gels and the amplification products were visualized with UV light following staining with ethidium bromide.

DNA fingerprinting. *C. neoformans* isolate DNA for fingerprinting was prepared essentially as described by Spitzer and Spitzer (19) with the following modifications. (i) *C. neoformans* isolates were grown for 18 h in 2% (wt/vol) glucose and 1% (wt/vol) peptone at 37°C in a shaking incubator set at 250 rpm. (ii) Mureinase (USB) was used instead of Novozym 234 for generation of spheroplasts. (iii) Spheroplasting was performed overnight at 30°C. (iv) Cetyltrimethylammonium bromide precipitation of carbohydrates was not performed. (v) The isolated DNA was resuspended in distilled water.

For fingerprinting analysis, DNA prepared as described above was digested with *Eco*RI and the resulting fragments were separated by agarose gel electrophoresis and transferred onto nylon membrane filters by the method of Southern (17). Individual oligonucleotides were end labelled with [γ-^{32}P]dATP by using T4 polynucleotide kinase (Promega Corp.) in accordance with the manufacturer's instructions. *Eco*RI-digested *C. neoformans* DNA was probed with [γ-^{32}P] dATP-labelled oligonucleotides 5′-$(GT)_8$-3′, 5′-$(GTG)_5$-3′, 5′-$(GATA)_4$-3′, 5′-$(GACA)_4$-3′, and 5′-$(GGAT)_4$-3′ under conditions described previously (22).

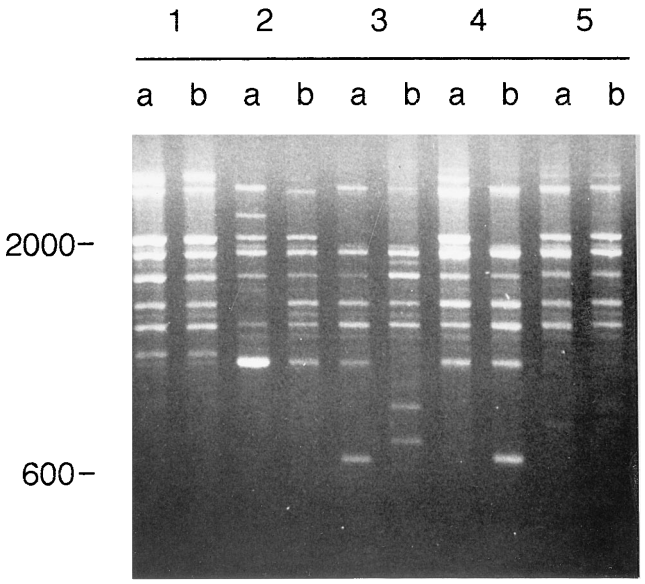

FIG. 1. Amplified RAPD products obtained with primer 3 [5′-$(GATA)_4$-3′] from five pairs of *C. neoformans* isolates from five separate patients. The numbers above the line are patient numbers, and those below the line are isolate numbers. Positions of molecular size reference markers (100-bp ladder; BRL, Uxbridge, United Kingdom) are given on the left in base pairs.

RESULTS

Random amplification of polymorphic DNA. The abilities of the three separate oligonucleotide primers to discriminate between clinical isolates of *C. neoformans* were assessed by RAPD analysis. To ensure reproducibility of the technique, RAPD experiments were performed with separate DNA preparations made from stored glycerol stock cultures, for each isolate respectively, and from isolates which had been subcultured three times. For each individual isolate, indistinguishable profiles were obtained with the separate DNA preparations. Each oligonucleotide primer yielded arrays of amplified DNA products ranging in size from 200 to 1,500 bp for each of the 10 clinical isolates examined. However, the three primers possessed different discriminatory abilities. Primer 1 (5′-CAGGC CCTTC-3′) was the least discriminatory, yielding RAPD profiles which allowed only isolate pair 3a and 3b to be discriminated from each other (data not shown). Primer 2 (5′-AAC GGCAAC-3′) was marginally more discriminatory in that it yielded RAPD profiles which allowed isolate pair 2a and 2b and pair 3a and 3b to be distinguished (data not shown). Primer 3 [5′-$(GATA)_4$-3′] possessed the greatest discriminatory ability, being able to distinguish between isolate pairs 2a and 2b, 3a and 3b, and 4a and 4b (Fig. 1). None of the primers were able to discriminate between isolate pair 1a and 1b or 5a and 5b. Interestingly, primer 3 was unable to differentiate between isolates 3a and 4b although these isolates were recovered from separate patients. However, these two isolates were distinguished on the basis of RAPD profiles generated with primer 1 (data not shown). Furthermore, discrimination between these isolates by DNA fingerprinting was possible.

DNA fingerprinting. Only very faint hybridization fingerprints were obtained when *Eco*RI-digested *C. neoformans* DNA was hybridized with oligonucleotide probes 5′-$(GT)_8$-3′, 5′-$(GTG)_5$-3′, 5′-$(GATA)_4$-3′, and 5′-$(GACA)_4$-3′. However, oligonucleotide 5′-$(GGAT)_4$-3′ yielded consistent and reproducible strong hybridization fingerprints (Fig. 2).

Fingerprints obtained with isolate pairs 1a and 1b and 5a

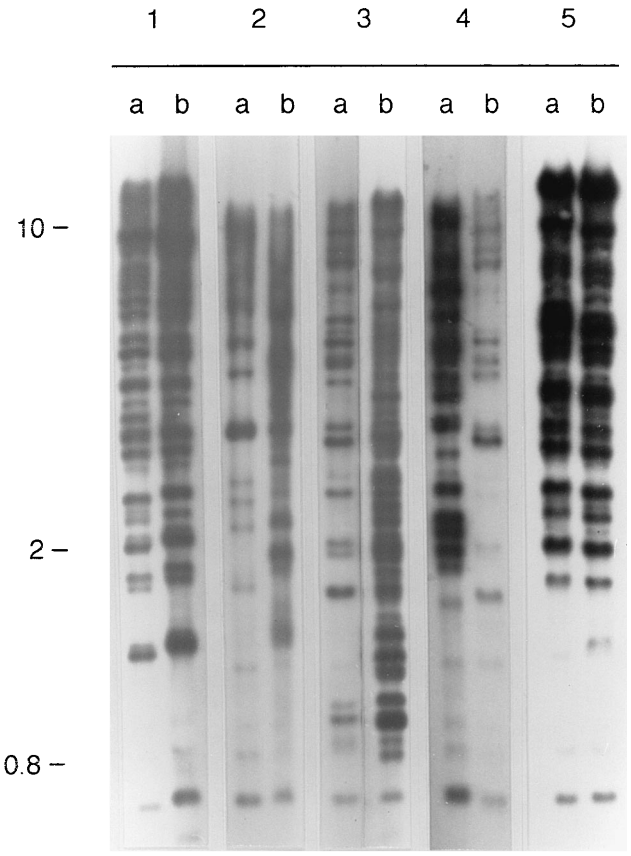

FIG. 2. Autoradiograms of *Eco*RI-digested genomic DNAs from five pairs of *C. neoformans* isolates from five separate patients following hybridization with [γ-^{32}P]dATP-labelled primer 5′-$(GGAT)_4$-3′. The numbers above the line are patient numbers, and those below the line are isolate numbers. Positions of molecular size reference markers (1-kb DNA ladder; BRL) are given in kilobase pairs on the left. All of the hybridization fingerprints shown here were generated in the same experiment. The lane order has been adjusted for consistency with Fig. 1.

and 5b were indistinguishable, apart from a single-band difference in each case, respectively. Isolate pairs 2a and 2b, 3a and 3b, and 4a and 4b each yielded grossly different DNA fingerprints (Fig. 2). DNA fingerprints from isolates obtained from different patients were all distinguishable.

DISCUSSION

Strain discrimination, reproducibility, and typeability are essential requirements for the development of any typing system. A suitable system for *C. neoformans* isolate discrimination would greatly facilitate studies of the epidemiology and virulence of this pathogenic yeast. Many systems have been proposed, but they often lack the necessary reproducibility and discriminatory power to be useful in differentiating between individual strains (3, 16, 24, 26). However, recent reports have demonstrated that molecular typing techniques are able to discriminate *C. neoformans* at the strain level (6, 19, 25, 27). Our results have confirmed that molecular methods do have sufficient discriminatory power, reproducibility, and typeability to be useful as *C. neoformans* typing systems. However, caution must be exercised when using RAPD-PCR to define identity between strains: the 10 isolates in this study were typed differently by all three primers. Similarly, Aufauvre-Brown et al. (1) were only able to discriminate among three isolates of *A. fumigatus* with 7 of 44 oligonucleotide decamers. Optimization of RAPD-PCR for typing of *C. neoformans* strains would have several advantages over other molecular methods, including speed, cost, ease of performance, sample volume throughput, and a requirement for little specialized equipment.

The finding that four of five oligonucleotide probes used in this study did not produce informative DNA hybridization fingerprints was unexpected. All eukaryotic genomes contain short repetitive microsatellite sequences which are excellent for detection of polymorphisms (23). Probes complementary to these short repetitive elements have been used successfully to fingerprint genomic DNA digests from a large number of fungal genera, including *Penicillium*, *Aspergillus*, *Trichoderma*, and *Candida* (13, 14, 22). The observation that only 5′-$(GGAT)_4$-3′ bound efficiently to *C. neoformans* DNA suggests that the organization and nucleotide sequences of these microsatellites in this fungus differ from those already examined.

Many studies have shown that clinical and environmental isolates of *C. neoformans* are highly polymorphic (6, 7, 16, 25, 27). Our results reinforce these findings. We have demonstrated that each of the five HIV-positive patients was infected with at least one *C. neoformans* strain which was genetically distinct from those infecting the other four patients. A total of eight different strains were isolated from these patients, who were all from the London area and were infected over a 24-month period. Recent work in our laboratories has extended these observations. We have analyzed 26 isolates recovered from 21 patients, including 6 HIV-negative patients. 5′-$(GGAT)_4$-3′ fingerprint patterns indicate that none of the strains were shared between patients (10). In a recent survey of *C. neoformans* isolates from New York City using restriction fragment length polymorphism analysis as a typing system, 6 types were detected among eight environmental strains and 12 types were found among 17 clinical isolates. Two of these types were observed in both the environmental and clinical isolates (7). We are currently sampling bird guano to determine if the polymorphic nature of our clinical isolates is reflected in the environmental strains prevalent in London.

In the present study, isolate pairs 1a and 1b and 5a and 5b were indistinguishable by both typing methods. These isolates were obtained either on the same day (from blood culture and CSF) or 1 day apart (both blood culture isolates) during the same episode of cryptococcosis. No further isolates were available from these two patients, so we were unable to determine if persistence of the originally infecting strain occurred. In contrast, the RAPD profiles and DNA fingerprints obtained from isolates 4a and 4b, which were recovered 8 days apart, were found to be substantially different, suggesting that more than one strain was responsible for the infection in this patient. This is the first report which demonstrates that multiple strains of *C. neoformans* can be isolated from an individual patient with cryptococcosis. Similarly, the pairs of isolates recovered from patients 2 and 3 were also readily distinguishable from each other, although the isolates in each pair were recovered 194 and 103 days apart, respectively, and the patients were culture negative for *C. neoformans* within these periods. These data strongly suggest that reinfection with a novel *C. neoformans* strain occurred in both of these patients. However, an alternative explanation for the recovery of distinguishable isolate pairs from these two patients is the persistence of one of multiple strains responsible for the primary infection.

Only one previous study aimed at determining if recurrent cryptococcosis is due to persistence of the original strain or reinfection with a second, different strain has been reported (20). Spitzer and coworkers studied 11 isolates recovered from

four patients (three HIV positive and one HIV negative). Indistinguishable DNA fingerprints were demonstrated in sequential isolates recovered from the same patients with a probe (termed CNRE-1) consisting of dispersed, repetitive DNA cloned from *C. neoformans* on *Sst*I-digested total cellular DNA. This was interpreted as strong evidence for the persistence of the original strain in each of the patients despite antifungal therapy. However, in the same report, karyotyping of the same strains revealed two large polymorphic bands in an individual isolate termed J9A which were not present in three other isolates recovered from the same patient 4, 5, and 6 months later. Given our finding that a single typing method can give results which may be refuted by other techniques, we suggest that these observations do not exclude the possibility that recurrent cryptococcosis may occur as a result of reinfection with a novel strain.

During the course of these studies, we also employed the technique of pulsed-field gel electrophoresis to ascertain its usefulness in discriminating between our strains. However, although the technique was found to be as discriminatory as RAPD-PCR and DNA fingerprinting, it was also very time consuming and expensive. Therefore, to analyze a large number of colonies recovered from primary isolation plates of patients with recurrent cryptococcal disease to determine the extent of reinfection and persistence, techniques such as RAPD-PCR and DNA fingerprinting will prove invaluable. The findings of such a study would have obvious implications for the management of patients with recurrent cryptococcosis. While persistence does occur, more aggressive antifungal regimens have to be developed to eradicate the original strain. Reinfection with a different strain demands that policies which reduce exposure of the at-risk patient population be developed. Developing such policies will be difficult, given the largely urban nature of HIV disease; in such urban locations, environmental cryptococci abound.

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