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Dual Infections with Pigmented and Albino Strains of *Cryptococcus neoformans* in Patients with or without Human Immunodeficiency Virus Infection in India

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***Cryptococcus neoformans* is an encapsulated yeast-like fungus of worldwide distribution. Melanin production is an important virulence factor of *C. neoformans*. We report the identification of distinct cryptococcal isolates with either pigmented or white colony phenotypes on L-dihydroxyphenylalanine agar plates in three patients who presented with meningitis to the All India Institute of Medical Sciences in India. Two of the patients were also infected with human immunodeficiency virus. Biochemical studies, India ink analysis, immunofluorescence with antibodies specific to capsular antigen, and serotyping confirmed that the melanotic and albino strains were *C. neoformans* serotypes A and D, respectively. Genotyping with M13 and [GACA]4 primers revealed that all the *C. neoformans* isolates were genetically different. The CNLAC1 gene associated with melanin production was identified in all the strains by PCR. Standard MIC testing revealed that the strains had similar susceptibilities to amphotericin B, but time-kill assays with the antifungal showed reduced susceptibility in melanin-producing strains. Infection studies with A/Jcr mice showed that the melanin-lacking yeast were less virulent than melanin-producing isolates. These findings indicate that these patients had dual infections with pigmented and albino strains of *C. neoformans* that were phenotypically and biologically different. Continued surveillance of primary isolates from patients with cryptococcosis by analyzing phenotypic differences and by molecular methods may reveal that mixed infections occur more commonly than is currently realized.**

Cryptococcus neoformans is an opportunistic fungal pathogen that primarily causes life-threatening infection in immunocompromised individuals. Cryptococcosis with central nervous system involvement is generally fatal unless specifically treated (1). In human immunodeficiency virus (HIV)-infected individuals, cryptococcosis is an AIDS-defining infection (3) and, in the absence of immune reconstitution, the patients who survive the initial presentation require life-long suppressive therapy to reduce the likelihood of relapse (33). The major characteristics of *C. neoformans* that promote virulence are its ability to grow at 37°C (32), the production of polysaccharide capsule, and the expression of laccase (17, 46).

C. neoformans laccase catalyzes the formation of melanin in the presence of a variety of phenolic precursors (46, 47). Melanins are complex polymers with covalently linked aromatic subunits, including indoles, phenols, and hydroxynaphthalenes (30). In *C. neoformans*, melanin forms an electron-dense layer in the cell wall (29, 44) and comprises approximately 15% of the dry weight of late-stationary-phase melanized *C. neoformans* (45). Melanin synthesis in *C. neoformans* is associated with protection against oxidants, extremes in temperature, enzymatic degradation, UV light, microbicidal peptides, antifungals, and macrophages in vitro (reviewed in reference 30). Pigmentation also induces the production of antimelanin antibodies (31) and interferes with protective T-cell responses (15). Furthermore, cryptococcal laccase has been demonstrated to confer significant protection against murine alveolar macrophages independent of dopamine (44).

We detected distinct pigmented and albino phenotypes of *C. neoformans* from cerebral fluid samples from three patients at the All India Institute of Medical Sciences (AIIMS), New Delhi, India. This study was undertaken to determine whether these isolates were the result of dual infections with *C. neoformans* or a result of phenotypic variation. *C. neoformans* can undergo phenotypic variation and microevolution after in vitro or in vivo passage (9–13, 38). Phenotypic switching results in reversible phenotypic changes in microorganisms. Phenotypic switching in *C. neoformans* has been shown to result in significant differences in capsule size, melanin production, growth rates, virulence in mice (9), and susceptibility to antifungal drugs (13). Microevolution of *C. neoformans* occurs in vivo and in vitro and results in phenotypic as well as genotypic changes in the organism (12). The results of our investigation revealed that the patients were infected with distinct strains of *C. neoformans* and that the melanin-producing and albino strains were biologically different.

(The data in this paper are from a thesis to be submitted by Piyali Mandal in partial fulfillment of the requirements for the degree of doctor of philosophy in the Department of Microbiology, AIIMS, New Delhi, India.)

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MATERIALS AND METHODS

Cultures. From April to September 2003, colonies from primary isolates from 25 patients with cryptococcal meningitis were screened for melanin production on bird seed agar (Difco, Sparks, MD) at AIIMS. In three patients (identified as

numbers 500, 716, and 764), melanized and white colonies were isolated and named according to the patient identifier followed by b for black (500b, 716b, and 764b) or w for albino (500w, 716w, and 764w). The available clinical information regarding the patients was limited to data indicating that two (500 and 764) of the three patients were infected with HIV. The isolates were maintained on Sabouraud dextrose agar (SAB; Difco).

C. neoformans 500w and -b, 716w and -b, and 764w and -b were also plated on chemically defined agar medium (60 mM glucose, 117.6 mM KH_2PO_4, 40 mM $MgSO_4 \cdot 7H_2O$, 52 mM glycine, 12 μM thiamine, 2% agar) with or without 1 mM L-dihydroxyphenylalanine (L-DOPA) and incubated at 30°C. The resulting colonies were collected, washed in phosphate-buffered saline (PBS), and subjected to a melanin isolation procedure as described elsewhere (36).

Identification of the isolates as *C. neoformans*. The pigmented (500b, 716b, and 764b) and albino (500w, 716w, and 764w) strains were subjected to the following biochemical tests according to the instructions of the manufacturer (bioMérieux, Durham, NC): (i) API 20, (ii) ID 32, and (iii) VITEK. Serotyping of the strains was determined by slide agglutination test using a crypto check kit (IATRON Laboratories, Tokyo, Japan). Immunofluorescence was performed with monoclonal antibodies (MAb) 12A1 and 13F1, antibodies specific for the capsular polysaccharide (GXM) of *C. neoformans* as described previously (7). These MAbs have been used for identifying serotypes A and D of *C. neoformans* (4).

Molecular typing of the isolates. High-molecular-weight DNA was extracted from the *C. neoformans* strains. Yeast cells were grown at 30°C in SAB broth with shaking for 48 h, collected by centrifugation, and suspended in SCB (1.1 M D-sorbitol and 0.1 M sodium citrate). The cells were again collected and incubated in SCB with 10 mg/ml lysing enzyme (Sigma Chemical Corp., Cleveland, OH) for 3 h at 30°C. The resulting protoplasts were collected, suspended in lysis buffer (10 mM sodium citrate, 1% sarcosine, 5 mM EDTA) with 0.1 mg/ml RNase A, and incubated at 65°C for 2 h followed by phenol-chloroform extraction. PCR amplification and analysis were done using the oligonucleotide minisatellite core sequence of wild-type M13 phage (5′-GAGGGTGGCGGTTCT-3′) and [GACA]4 primers. The reactions were performed in a final volume of 50 μl with 30 ng of high-molecular-weight genomic cryptococcal DNA, 0.01 ng primers, 5 U *Taq* polymerase, and 45 μl of PCR Supermix (Invitrogen, Carlsbad, CA). The amplification was performed for 40 cycles in an Eppendorf thermal cycler (94°C for 5 min, 94°C for 1 min, 52°C for 1 min, 72°C for 1 min, and 72°C for 10 min). The amplification products were separated on a 1.4% agarose gel in 1× Tris-borate-EDTA for 7 h at 3 V/cm and observed under UV light.

Determination of the presence and expression of CNLAC1. The presence of the CNLAC1 gene in each strain was confirmed using the specific primers for the CNLAC1 gene (8). PCR was done with the primers 5′-GGCGATACTATTAT CGTA-3′ and 5′-TTCTGGAGTGGCTAGAGC-3′. The reaction was performed and the amplification products separated and observed as described above.

The expression of the CNLAC1 gene was determined by Western blot analysis. Cells were grown in SAB for 48 h and were centrifuged at 2,000 rpm for 10 min. The cells were suspended in 0.1 M Na_2HPO_4 with protease inhibitor and treated for 6 min in a bead beater at 2-min intervals alternating with 5 min on ice. Supernatants were separated from cellular debris by centrifugation at 8,000 rpm for 10 min. Western blot analysis was performed using the supernatants in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis with the laccase-binding MAb G3P4D3 (48) at 5.0 μg/ml followed by horseradish peroxide-labeled anti-mouse antibody at a dilution of 1:5,000 (Southern Biotechnology Associates). Labeling was visualized using Pico luminescent developer (Pierce, Rockford, IL) according to the manufacturer's instructions.

Growth characteristics. Several characteristics of growth were studied to determine differences between the melanized and albino isolates. Cultures of *C. neoformans* were grown in SAB at 37°C to confirm their capacity to grow at mammalian body temperatures. The growth rates of the isolates with and without L-DOPA was determined at 30°C by measuring cell density at different time intervals by hemacytometer. After 10 days of growth in the presence or absence of L-DOPA, India ink preparations were made and measurements were obtained for cell and capsular size by light microscopy.

Susceptibility of *C. neoformans* to AMB. Antifungal susceptibility testing was performed for amphotericin B (AMB; Sigma) according to the National Committee for Clinical Laboratory Standards M-27A protocol (28), except that cultures were grown in SAB with and without L-DOPA at 30°C for 15 days in a rotary shaker at 125 rpm prior to use. This allowed the cells capable of melanin production to become pigmented. The cells were collected by centrifugation at 2,000 rpm for 15 min and suspended in their respective medium with or without L-DOPA for 48 h to induce log-phase growth for testing, per the National Committee for Clinical Laboratory Standards M-27A protocol (28). Final concentrations of AMB were 0.06 to 4 μg/ml. The minimum concentration at which the suspension was optically clear was determined to be the MIC.

Time-kill assay. The cells used for MIC testing were also subjected to a time-kill assay as described previously (41). Briefly, cell suspensions were made in PBS at concentrations of 3×10^4 cells/ml as determined by hemacytometer counts. Cell suspensions of 900 μl were added to 100-μl aliquots of different concentrations of AMB and incubated at 30°C for 2 and 4 h. The final concentration of AMB in the tubes ranged from 0 to 2.0 μg/ml. The cells were plated for CFU (1 colony = 1 CFU) on SAB agar at 30°C. The percent survival was determined by dividing the CFU of the fungal cells by the CFU of the cells incubated in PBS (drug-free control).

Fungal burden and survival studies. The animal experiments were performed with *C. neoformans* strains 500b and 500w. The yeast cells were grown in SAB without L-DOPA for 48 h. The cells were then collected by centrifugation, washed with PBS, and suspended in PBS. For fungal burden studies, A/JCr mice (6- to 8-week-old females; National Cancer Institute, Rockville, MD) were inoculated intraperitoneally with 2×10^7 yeast cells. After 3 or 7 days, the mice were sacrificed to determine the fungal load in the lung. Lungs were homogenized in 3 ml PBS, and 50 μl of the homogenized tissue was plated on SAB agar and incubated at 30°C for 72 h. For survival studies, A/JCr mice were inoculated intraperitoneally with 1×10^8 yeast cells. The mice were observed twice daily, and moribund mice were sacrificed prior to death. All surviving mice were sacrificed at 69 days after infection. The animal studies were performed in compliance with institutional guidelines for animal experimentation at the Albert Einstein College of Medicine.

Determination of phenotypic switching. In addition to the SAB agar plates made from homogenized lung tissue described above, aliquots of the homogenate were also plated on chemically defined agar medium with 1 mM L-DOPA and incubated at 30°C for 10 days to observe whether any in vivo switching from melanin-producing to melanin-lacking *C. neoformans* occurred, or vice versa. Similarly, melanin-producing and albino isolates were grown in SAB broth, plated onto chemically defined agar medium with 1 mM L-DOPA, and incubated at 30°C 15 days to observe whether phenotypic changes occurred with these strains in vitro.

Statistical analysis. Statistical significance was determined using Student's *t* test or the Kruskal-Wallis nonparametric analysis of variance test (Primer; McGraw-Hill, New York, N.Y.), depending on the data.

RESULTS

Identification of isolates as *C. neoformans*. Isolates from the three patients with albino and melanotic colonies on bird seed agar were selected for further study (patient 500, isolates 500b and 500w; patient 716, isolates 716b and 716w; patient 764, isolates 764b and 764w). In the presence of L-DOPA, the melanin-producing colonies (500b, 716b, and 764b) became gray-brown by 5 days and black after 10 days of incubation. The albino cells (500w, 716w, and 764w) remained white, even after 15 days of incubation. As previously demonstrated with melanized *C. neoformans* yeast cells (36), treatment of the pigmented cells with enzymes, denaturant, and hot acid resulted in the isolation of black particles similar in size and shape to their propagules, as demonstrated by light microscopy (data not shown). Albino cells subjected to this treatment were completely solubilized, demonstrating an absence of melanin formation.

The biochemical tests with API 20, ID32, and VITEK indicated that the albino (500w, 716w, and 764w) and pigmented (500b, 716b, and 764b) strains were *C. neoformans*. Serotyping of the isolates with the IATRON system and the MAbs 13F1 and 12A1 showed that the albino isolates were each serotype D and the pigmented isolates were serotype A. Hence, the pigmented and albino isolates are *C. neoformans* and the serotypes of the pigmented and albino isolates are serotypes A and D, respectively.

PCR fingerprinting. The PCR fingerprinting analysis with M13 minisatellite core sequence and [GACA]4 showed differences in the band pattern of melanin-producing and white *C.*

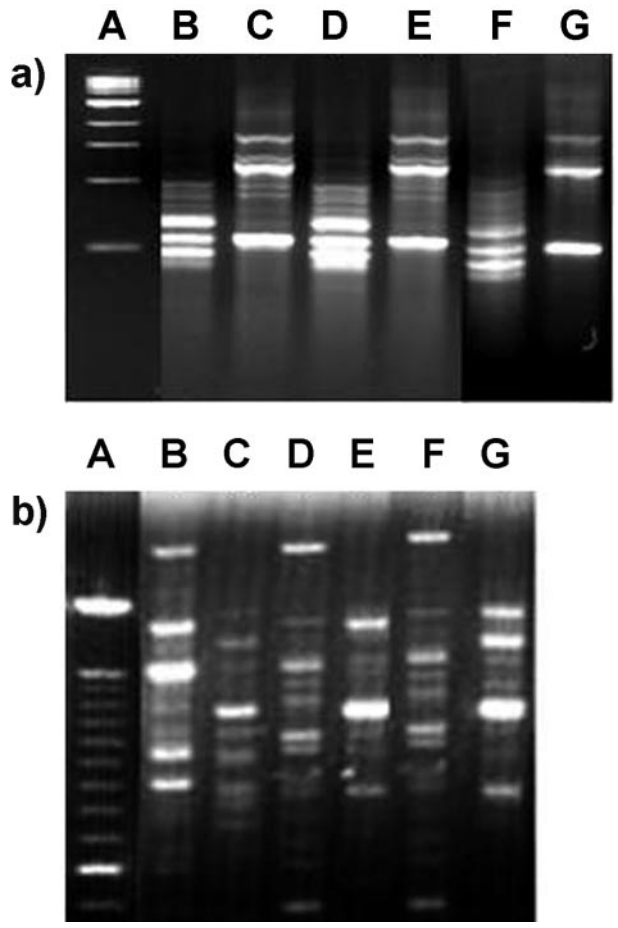

FIG. 1. Random amplified polymorphic DNA analysis with M13 (a) and [GACA]4 (b) showing different band patterns for 500w (B), 500b (C), 716w (D), 716b (E), 764w (F), and 764b (G). Lane A represents the molecular marker.

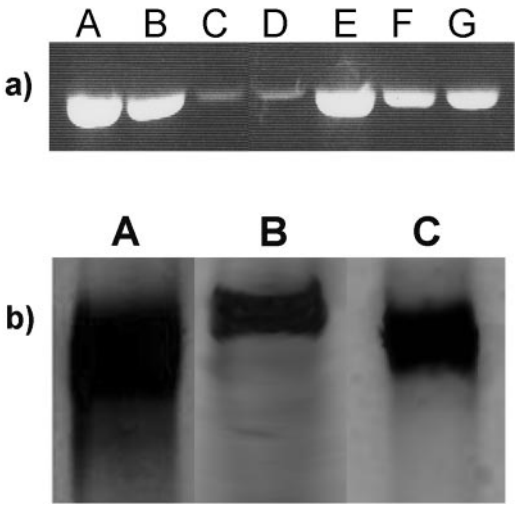

FIG. 2. The albino and melanotic isolates contained the CNLAC1 gene and expressed laccase. (a) PCR with specific primers for the CNLAC1 gene in *C. neoformans* ATCC 24067 (A), 500w (B), 500b (C), 716w (D), 716b (E), 764w (F), and 764b (G). (b) Western blot analysis of whole-cell suspensions of albino and melanotic strains of *C. neoformans*: recombinant laccase (A), 500w (B), or 500b (C). The cells were derepressed in the absence of glucose, and the blots were developed with G3P4D3 clone monoclonal antibody specific for laccase (48).

neoformans isolates (Fig. 1). The main bands for the melanotic strains were in accordance with VN I and the albino strains were in accordance with VN IV as described elsewhere (26). This establishes that the two types of *C. neoformans* isolates obtained from single patients were different and had distinct serotypes.

Determination of the presence and expression of CNLAC1. The presence of the CNLAC1 gene was examined using specific primers for CNLAC1, and both the melanin-producing and albino isolates contained the gene (Fig. 2). The sequences of bases of the identified isolates (preliminary GenBank accession numbers: 500b, bankit718434; 500w, bankit705707; 716b, bankit718444; 716w, bankit718448; 764b, bankit718458; 764w, bankit705711) were compared with nucleotides 2285 to 2846 of the standard serotype D strain ATCC 3501 CNLAC1 sequence (GenBank accession number L22866) (46). Analysis revealed that all the melanotic strain CNLAC1 sequences were the same and all the albino strains were also identical. The nucleotide sequences translated to 112 amino acids. The albino serotype D strains were homologous with the standard ATCC 3501 strain CNLAC1 sequence, except for a histidine-to-tyrosine replacement at position 62 effected by the single substitution of an adenine for cytidine. This substitution affects one of the potential copper binding sites (46). In contrast, there were several different amino acids encoded by the serotype A melanotic strains compared to the standard ATCC strain, but the metal binding sites were intact. The isoleucine at position 14 in the pigmented isolates is similarly found in several other laccase sequences, where it does not affect copper binding (46). Additionally, Western blot analysis showed that CNLAC1 was expressed in both the albino and pigmented number 500 isolates (Fig. 2).

Growth characteristics. The albino and pigmented strains grew at 37°C on SAB agar. Additionally, the growth rate of the albino and pigmented *C. neoformans* isolates grown in SAB with and without L-DOPA were similar (data not shown).

C. neoformans isolates 500b and 500w were used for cell size comparisons. More than 30 cells were analyzed per group and the experiment was repeated twice with similar results. The diameter of albino 500w *C. neoformans* cells was significantly smaller than 500b yeast cells when grown with L-DOPA (5.6 ± 0.7 versus 6.6 ± 0.9 μm; $P = 0.03$) or without L-DOPA (5.6 ± 1.0 versus 5.9 ± 1.1 μm; $P < 0.001$). However, the total cell size (cell body and capsule) of the albino 500w yeast cells was significantly larger than the 500b cells under either growth condition (with L-DOPA, 15.9 ± 5.1 versus 12.7 ± 2.1 μm; without L-DOPA, 15.4 ± 2.3 versus 11.8 ± 2.6 μm; $P < 0.001$). Hence, the cells capable of producing melanin were found to have smaller capsule-to-cell ratios compared to the albino *C. neoformans* regardless of the presence or absence of phenolic substrate. Interestingly, the cell size of 500b yeast cells was significantly smaller when the isolate was grown with L-DOPA compared to without L-DOPA (6.6 ± 0.9 versus 5.9 ± 1.1 μm; $P = 0.009$).

Susceptibility to amphotericin B. The in vitro susceptibilities of the *C. neoformans* cells were the same for the three albino

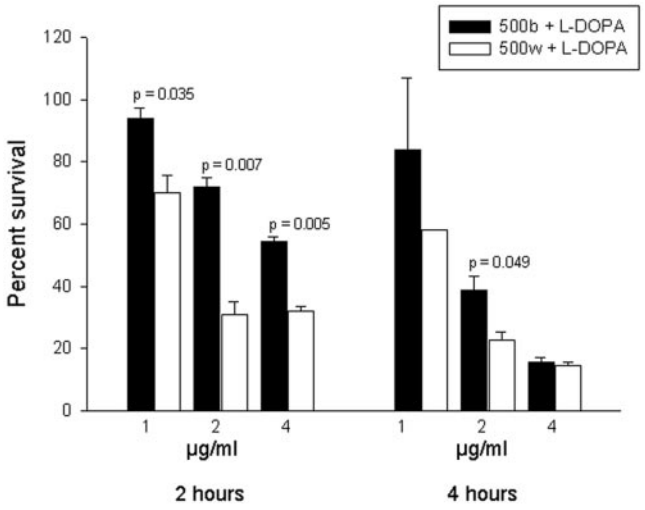

FIG. 3. Comparison of survival rates of melanotic strains with albino strains of *C. neoformans* after exposure to different concentrations of amphotericin B for 2 and 4 h in comparison to the *C. neoformans* cells incubated in PBS. Similar time-kill results were obtained with *C. neoformans* strains 716 (b and w) and 764 (b and w). The antifungal susceptibility test and the time-kill assay were repeated once, and the results were reproduced.

and three pigmented isolates tested. The MICs were 0.5 μg/ml. The susceptibility testing was repeated twice with similar results. When the albino and the melanin-producing *C. neoformans* isolates were grown with L-DOPA, the pigmented cells were significantly less susceptible to killing by AMB after 2-h incubations than the albino yeast (Fig. 3). The protective effect was more pronounced after 2 h compared to 4 h, when increased cell death occurred under all conditions examined. Consistent with previous reports (18, 41), the melanin-producing isolates were significantly less susceptible to antifungal killing when grown in the presence of L-DOPA than in its absence at 1, 2, and 4 μg/ml AMB. However, there were no differences in survival when albino yeast cells were grown without L-DOPA (data not shown).

Virulence of *C. neoformans* in mice. *C. neoformans* isolates 500b and 500w were used for the in vivo studies. There were significant differences in the mean number of *C. neoformans* cells in the lungs of mice after 3 days of infection with 2×10^7 albino 500w or pigmented 500b yeast cells (500w, $5.3 \times 10^3 \pm 1.1 \times 10^3$; 500b, $2.7 \times 10^4 \pm 9.7 \times 10^3$; $P = 0.003$). Although at day 7 of infection three of the five mice infected with 500w *C. neoformans* cleared the fungus and yeast were recovered from all mice infected with 500b yeast, the mean number of CFU between the groups was not significantly different (500w, 7.6×10^2; 500b, 9.2×10^2). This indicated that the melanin-producing and albino *C. neoformans* isolates had different rates of clearance in vivo. The inflammatory response was also more intense in mice infected with the melanin-producing cells compared to the albino yeast, particularly early in infection (Fig. 4). Interestingly, when the mice were simultaneously inoculated with a mixture of 1×10^7 pigmented 500b cells and 1×10^7 albino 500w yeast cells, the albino cells were cleared at day 7 but the melanotic 500b cells were found at a concentration of 10^3 cells/ml of lung tissue by CFU determinations on L-DOPA agar. The CFU experiments were performed twice with similar results.

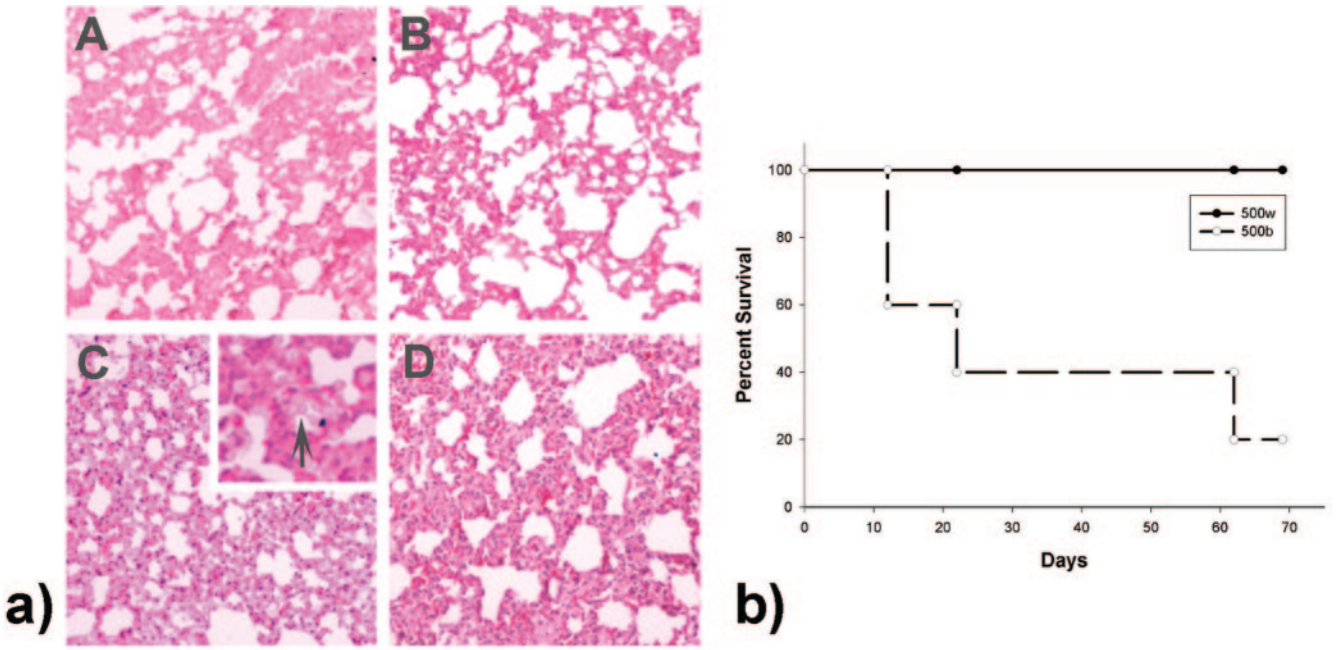

FIG. 4. Albino isolates are less virulent than melanotic *C. neoformans.* (a) Light microscopy of eosin- and hematoxylin-stained lung tissue of A/Jcr mice after 3 and 7 days of infection with 2×10^7 melanin-producing 500b (A and C) or albino 500w (B and D) *C. neoformans* cells. Original magnification, ×20. The arrow in the insert in panel C indicates the presence of numerous cryptococcal yeast cells (original magnification, ×100). (b) Survival experiment of mice infected with 1×10^8 albino 500w or pigmented 500b *C. neoformans* cells. $n = 5$ mice. The experiment was repeated, and similar results occurred.

Analysis of the L-DOPA plates inoculated with lung homogenates showed that the melanin-producing *C. neoformans* 500b cells all retained their ability to produce melanin in the presence of L-DOPA. The *C. neoformans* 500w cells remained white after recovery from the mice onto L-DOPA plates. Thus, there was no in vivo phenotypic alteration in melanin production. Similarly, there was no switching of melanization observed using cells grown in vitro on testing approximately 12,000 colonies each for the three pairs of *C. neoformans* isolates studied.

The melanin-producing *C. neoformans* 500b isolate was significantly more lethal than the albino 500w strain ($P = 0.013$) (Fig. 4). Whereas no deaths occurred in mice infected with the albino strain, death occurred as early as 12 days after infection with the pigmented strain.

DISCUSSION

This study was initiated to determine whether pigmented and albino yeast colonies isolated from individual patients represented phenotypic variation of a single strain or a dual infection. First, it was essential to demonstrate that the isolates were *C. neoformans* rather than other yeasts. In this regard, other *Cryptococcus* species, such as *C. albidus*, *C. laurentii*, and *C. curvatus*, have been reported to rarely cause human infection (reviewed in reference 3), and these species have the capacity to express laccase and synthesize melanin (19). Spontaneous melanin-deficient mutants can occur in the laboratory (35), and melanin-deficient *C. neoformans* has been reported in human cryptococcosis (40). Interestingly, spontaneous melanin-deficient mutants can revert to their melanin-producing phenotype during murine infection, which restores virulence (35). No phenotypic switching occurred in vitro or in vivo with our isolates. Using biochemical and serological tests, we demonstrated that our pigmented isolates were all *C. neoformans* serotype A (*C. neoformans* var. *grubii*) and the albino isolates were serotype D (*C. neoformans* var. *neoformans*). Further testing by two DNA analysis techniques confirmed that the pairs of isolates were distinct from each other. The genotyping data were also in concordance with the serotyping data, as they demonstrated VN I and VN IV patterns for the serotype A and D isolates, respectively (25). The genetic patterns also showed that the strains are not diploid and are genetically distinct (5). Although it is impossible to completely rule out that the albino isolates were environmental contaminants, the DNA fingerprints of each of the albino isolates was different and the likelihood of three different cryptococcal isolates contaminating our plates is extremely low. Hence, the three individuals studied appeared to have dual infections with melanin-producing and melanin-lacking isolates.

Mixed infections with *C. neoformans* have been previously reported. In 1995, Haynes et al. reported that paired isolates were genetically distinct in three of five individuals with HIV and cryptococcosis and suggested that this represented dual infection (14). Subsequently, a larger study of serial isolates from 33 individuals with HIV documented the persistence of a single *C. neoformans* isolate and failed to find evidence for dual infection or reinfection with a second strain (2). Nevertheless, additional instances of dual infections and acquisition of a second strain of *C. neoformans* have been described (16, 21, 37, 38). Since routine typing of isolates is performed on one or two selected colonies, a strain present in low numbers relative to a second strain might not be detected. There also remains the question as to whether individuals with dual infections acquire the organism concomitantly or sequentially. Since environmental sites can harbor more than a single strain of *C. neoformans* (6), it would be possible to have an initial mixed infection. It is significant that infection with *C. neoformans* can result in the down-regulation of diverse host immune responses, primarily through interactions of the capsular polysaccharide with host immune cells (reviewed in reference 43). The suppression of protective responses would facilitate the persistence of less-virulent coinfecting strains of *C. neoformans*. Supporting this possibility are reports describing saprobic nonneoformans varieties of *Cryptococcus*, such as *C. laurentii*, in mixed infections with *C. neoformans* (22).

In *C. neoformans*, CNLAC1 is primarily responsible for melanin production (8). The albino isolates described here expressed CNLAC1 in vitro. The partial CNLAC1 gene sequences of the albino isolates were identical to the sequence of the standard ATCC 3501 strain (GenBank accession number L22866) (46) except at position 62, where a tyrosine replaced histidine. This position represents a potentially important copper binding site, and the change from an aliphatic to aromatic may affect the capacity for metal binding by the albino strains. The copper binding sites are intact in the pigmented isolates. Interestingly, a recent report of the analysis of CNLAC1 sequences of spontaneous melanin-deficient mutants that occurred during routine maintenance of laboratory strains also found that the deficient isolates were homologous to their parental strains (39). It is possible that there are mutations outside of the partially sequenced area of the CNLAC1 gene in the albino isolates that alters its activity or in other genes that may affect melanin production, such as through the cyclic AMP cascade (34). Recently it has been shown that there are two genes responsible for the melanin production (27, 34). LAC1 codes for the laccase enzyme that catalyzes the rate-limiting step in diphenol oxidation and melanin production. The second gene is LAC2, which is adjacent to LAC1 and encodes a second laccase that shares 75% nucleotide identity with LAC1. Hence, the fact that melanization does not occur in our albino isolates may suggest a defect outside of the laccase system. Also, since laccase is normally transported to the cell wall and secreted by *C. neoformans* (48), the albino isolates could have a defect in transportation and/or secretion of the enzyme that would interfere with its ability to polymerize melanin on the cell wall. Isolates of *C. neoformans* have been shown to vary in their capacity to utilize different indole compounds to produce melanin (24). It is possible that the albino isolates could melanize in the presence of a substrate other than the L-DOPA indolic compound used in this study. Though unlikely, the albino strains may have defects in catecholamine transport.

In contrast to other reported melanin-deficient mutants (23), the albino isolates had the capacity for survival at human body temperatures. The cell sizes in the study varied from 5.5 to 10.5 μm and the size of capsules from 7.0 to 13.9 μm, which clearly demonstrates that there is substantial strain-to-strain variation in these characteristics. Since the albino and pigmented strains were different, it is therefore not surprising that there are differences in their sizes. Furthermore, melanin and

capsule are discordantly regulated (20). Although standard MIC susceptibility testing did not show any differences between the strains, the strains that produced melanin in the presence of L-DOPA were significantly more resistant to killing by AMB than the albino isolates. This finding is consistent with prior publications, reporting that time-kill experiments reveal that *C. neoformans* melanization is protective against AMB (18, 41, 42). Melanotic *C. neoformans* strains have been reported to be more virulent in animal models than melanin-lacking strains (23). We similarly have shown that the melanin-producing strains isolated from our patients were more virulent than the albino strains. Additionally, the fungal burden and pulmonary inflammation were significantly reduced early after infection with the albino strain compared to the melanin-producing isolate. In mixed infections, the albino isolate was cleared more rapidly than the pigment-producing strain.

The findings in this study are in agreement with prior work showing that melanin production is protective in *C. neoformans* yeast cells during infection. As demonstrated by genetic analysis, the isolates from the three individuals were all distinct. The clinical importance of albino strains of *C. neoformans* alone or in mixed cryptococcal infections is not clear. The actual incidence of mixed infections is also unknown. Future studies that critically examine large numbers of primary cultures of *C. neoformans* from patients for phenotypic differences may clarify this issue. Continued surveillance of primary isolates from patients with cryptococcosis by analyzing phenotypic differences and by molecular methods may reveal that mixed infections occur more commonly than is currently realized.

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