Serial isolates of *Cryptococcus neoformans* from Patients with AIDS Differ in Virulence for Mice

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Serial isolates of *Cryptococcus neoformans* from patients with chronic infection can exhibit minor karyotype changes as a result of chromosome length polymorphism (CLP). This study investigated whether serial *C. neoformans* isolates with CLP from 4 patients with AIDS exhibited biologic and phenotypic differences. CLP permits the identification of serial isolates in murine mixed infection. The parameters studied were virulence in mice, capsule size, colony morphology, melanization, protease production, MICs of antifungal drugs, and growth rates in vitro. Two parameters of virulence in mice were studied: persistence in tissue and survival time after lethal infection. Serial *C. neoformans* isolates were shown to differ in ability to persist in vivo, virulence in a murine infection model, in vitro growth rates at 37°C, and capsule size. Melanin and protease production and MICs of antifungal drugs were comparable for serial isolates. These observations suggest microevolution of *C. neoformans* during human infection. This process may allow the fungal population to change, escape eradication by the immune system, and thus cause chronic infections.

_Cryptococcus neoformans_ is a fungus that causes life-threatening meningoencephalitis in 6%-8% of patients with AIDS. The mortality of cryptococcal infection remains high despite therapy, and relapses are common unless the patient receives lifelong suppressive therapy with antifungal drugs [1, 2]. Previous studies have demonstrated extensive karyotype variation among *C. neoformans* isolates from various parts of the world. Furthermore, even isolates from a relatively small geographic area, such as New York City, exhibited extensive chromosomal length polymorphism (CLP) [3–7]. In addition, analysis of serial *C. neoformans* isolates from patients with chronic infection has shown minor karyotype differences among serial isolates in up to 40% of patients. This phenomenon was reproduced in the laboratory by demonstrating that karyotype changes occur for some strains during chronic infection in mice [5].

*C. neoformans* is notorious for causing chronic infections that are difficult to eradicate, even after prolonged fungicidal therapy. The mechanism of persistence in *C. neoformans* infection is not understood. Studies of antifungal drug susceptibility of serial and relapse isolates have not demonstrated significant differences [6, 8], suggesting that drug resistance is probably not a frequent cause of persistent infection. Other biologic properties have not been investigated. Recent work by Franzot et al. [9] showed that laboratory strain 24067 can undergo changes in vitro leading to altered virulence and growth characteristics. Some of the 24067 variants exhibited karyotype changes involving one or two chromosomes. This observation demonstrated that this fungus can undergo frequent phenotypic and genotypic changes. This led to the suggestion that these changes are part of a dynamic process of microevolution, which could contribute to the ability of *C. neoformans* to cause chronic infection [9].

Other evidence for microevolution in *C. neoformans* comes from studies of serial isolates from patients and mouse-passaged isolates. Currie et al. [10] demonstrated stable changes in sterol composition for isolates passaged in mice in relation to the parent strain. Cherniak et al. [11] showed that the chemical structure of the polysaccharide capsule of serial *C. neoformans* isolates from individual patients could differ. Furthermore, Cleare et al. [12] and Mukherjee et al. [13] demonstrated that the epitope distribution on the polysaccharide capsule of *C. neoformans* can change during in vitro passaging, as evidenced by an altered staining pattern with epitope-specific antibodies. More recently, Goldman et al. [14] demonstrated high-frequency phenotypic switching in 2 strains of *C. neoformans*, resulting in two or more colony morphologies.

On the basis of these results, we hypothesized that *C. neoformans* strains can undergo changes during chronic human infection that affect virulence. To test this hypothesis, we investigated the biologic characteristics of serial isolates of *C. neoformans* from 4 patients with AIDS.
Material and Methods

*C. neoforms isates.* Isolates J22 (a and b), J32 (a1, a2, and b), J33 (a and b), and J35 (a and b) were recovered from patients with AIDS at Jacobi Hospital of the Bronx Municipal Center. The clinical isolates were identified as *C. neoforms* by the hospital microbiology laboratory and were streaked to single colonies prior to analysis. Minor differences in karyotypes, involving one or three chromosome bands, among sequential isolates have been described [5]. These strains were selected for analysis because the “a” and “b” isolates could be distinguished by a change in electrophoretic karyotype. In this study, the term “strains” refers to the *C. neoforms* recovered from individual patients that differ by DNA typing. “Isolate” refers to serial *C. neoforms* isolates recovered from an individual patient infected with 1 strain over a period of time. The “a” isolate represents the first isolate recovered from the patient and the time of recovery and is referred to as day 0. The “b” isolate was recovered any time after day 0. For J22, the “b” isolate was recovered on day 154, in association with a clinical relapse of infection. J32b, J33b, and J35b are from days 14, 8, and 1, respectively. All strains were maintained on Sabouraud slants at 4°C. The J32a1 and J32a2 isolates were both recovered on day 0 from the same primary culture and have “mucoid” and “smooth” phenotypes, respectively. The J32b isolate is also smooth. The phenotypes were scored visually after 72 h of growth at 30°C on agar plates. They were stable during in vitro and in vivo passages. The karyotypes of the 2 isolates differed from each other and from J32b and are shown in figure 1.

*In vivo persistence and virulence studies.* *C. neoforms* cells were grown in Sabouraud dextrose broth overnight and washed twice with 0.02 M PBS (pH 7.2). A 1:1 mixture of 10⁶ yeast cells of the early (a) and late (b) isolate were injected intraperitoneally into 2 6- to 8-week old male A/J mice (Jackson Laboratory, Bar Harbor, ME). For each experiment, the number of live cells of each isolate injected into mice was verified by plating the inoculum on Sabouraud agar and counting colonies. Mice were killed after 2 weeks, and the lungs and brains were removed and homogenized. The homogenate was plated on agar to recover single colonies for karyotype analysis. For survival studies, A/J mice were infected intraperitoneally with either 5 × 10⁶ or 10⁷ yeast cells in PBS. Each group contained 5 mice. Mice were monitored daily for signs of disease.

*Karyotype analysis.* Chromosomal DNA plugs were prepared from cultures derived from single colonies. Briefly, protoplasts were made by incubating yeast cells at 30°C with 10 mg/mL Novozym 234 (Novo Biolabs, Bagsvaerd, Denmark) in 1.1 M sorbitol and 0.1 M sodium citrate (pH 5.5). Plugs were made with low-melt agarose (Bio-Rad, Richmond, CA) to yield a final concentration of 0.66% agarose and 5-8 × 10⁶ yeast cells per milliliter. The plugs were incubated overnight at 50°C in 1 mg/mL proteinase K (Boehringer Mannheim, Mannheim, Germany) in 1% sarcosine, 0.1 M EDTA, and 0.010 M sodium citrate buffer (pH 8.0). After washing, the plugs were inserted into a 1% pulse-field certified agarose gel (Bio-Rad), and electrophoresis was done in a CHEF DR III variable-angle pulse-field electrophoresis system (Bio-Rad) in 0.5% Tris-boric acid-EDTA at 12°C. The electrophoresis conditions were a switch-time of 60 and 120 s at 6.0 V/cm for 24 h at an angle of 112°. Gels were stained with ethidium bromide and photographed.

Restriction fragment length polymorphism (RFLP) analysis. DNA was isolated according to standard protocols, and isolates were typed by Southern blot with the *C. neoforms* repetitive element-1 (CNRE-1) (provided by S. G. and E. D. Spitzer, SUNY at Stony Brook, NY) [15–17]. Genomic DNA was digested with ScaI (Promega, Madison, WI), and the resulting fragments were resolved on a 1% agarose gel and transferred to positively charged nylon membranes (Boehringer Mannheim) by using a 10× SSC solution. After UV cross-linking, the membranes were probed with CNRE-1 labeled with [α-³²P]dCTP. Hybridization was done in a solution of denatured salmon sperm (0.75 mg/mL) in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 7% SDS, and 10× Denhardt’s reagent in 0.0020 M sodium phosphate buffer (pH 7.2) at 65°C. Filters were washed with 3× SSC, 5% SDS, 10× Denhardt’s reagent, and 1× SSC–1% SDS at 65°C. Bands were visualized by autoradiography.

Growth rates. For growth studies, 50 mL of Sabouraud dextrose broth was inoculated with 10⁶ yeast cells from an overnight starter culture, and the culture was grown overnight at 37°C with moderate shaking at 150 rpm. Absorbance at 600 nm was measured with a spectrophotometer (Pharmacia Biotech, Cambridge, UK) after different times in culture. For the logarithmic growth phase, the data can be represented by the simple model of an exponential growth function of the form \[ N(t) = N_0 e^{at}, \] where *N(t)* = absorption at time *t*, *a* = growth factor. Least-square fits of the data with the help of a statistical program (SOLVE) to the two-parameter formula yields the doubling time \( t_d = \ln(2)/a \), which represents the logarithm of the exponential function. Growth differences established by absorbance were confirmed by comparing counts of colony-forming units (cfu). *C. neoforms* isolates were grown in 96-well microtiter plates in Sabouraud dextrose broth at 37°C, and aliquots were plated in triplicate on Sabouraud agar plates at 4, 8, 12, and 24 h.

Melanization. Melanin production was assessed visually by...
Results

Persistence of serial isolates from patients in mice. Two mice were infected with equal cell numbers of the “a” and “b” isolates from strains J22, J32, J33, and J35. We were able to inject comparable numbers of cfu for the individual pairs by establishing the number through multiple hemocytometer counts of the inoculum. The distance from the edge of the capsule to the cell was measured for C. neoformans cells with the help of a grid. Capsule size was measured for 20 cells after growth in Sabouraud broth for 48 h, and a mean value was determined.

Antifungal drug susceptibility assays. MICs of amphotericin B (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT) and fluconazole (Roerig Pfizer, New York, NY) were determined as per the National Committee for Clinical Laboratory Standards protocol [19]. Final drug concentrations ranged from 125 to 0.03 µg/mL for fluconazole and from 2 to 0.03 µg/mL for amphotericin B.

Statistical analysis. The statistical tests used were χ², t, Fisher’s exact, and log-rank. The true Epistat version 2.1 (Epistat Services, Round Rock, TX) software was used to calculate survival differences applying log-rank survival analysis. P values < .05 were considered significant.

Table 1. Summary of in vivo persistence of serial isolates during mixed infection with 2 serial isolates from same strain of C. neoformans.

<table>
<thead>
<tr>
<th>Strain</th>
<th>n”</th>
<th>“a”/“b”-injected</th>
<th>“a” recovered</th>
<th>“b” recovered</th>
<th>Pd</th>
</tr>
</thead>
<tbody>
<tr>
<td>J22 a/b</td>
<td>18</td>
<td>90/77</td>
<td>9</td>
<td>9</td>
<td>.78</td>
</tr>
<tr>
<td>J32 a1/b</td>
<td>276</td>
<td>105/110</td>
<td>38</td>
<td>23</td>
<td>.0001</td>
</tr>
<tr>
<td>J32 a2/b</td>
<td>44</td>
<td>153/157</td>
<td>23</td>
<td>1.0</td>
<td>.016</td>
</tr>
<tr>
<td>J33 a/b</td>
<td>20</td>
<td>105/127</td>
<td>18</td>
<td>2</td>
<td>.0016</td>
</tr>
<tr>
<td>J35 a/b</td>
<td>24</td>
<td>71/72</td>
<td>0</td>
<td>24</td>
<td>.0002</td>
</tr>
</tbody>
</table>

a No. of colonies (from 2 mice) analyzed by CHEF DRIII variable-angle pulse-field electrophoresis system (CHEF).
b Refers to ratio of “a” and “b” isolates in inoculum as determined by no. of colony-forming units.
c Refers to no. of colonies of “a” or “b” karyotype, as determined by CHEF.
d By χ².

Discussion

In vivo growth rates and phenotypic characteristics of serial C. neoformans isolates. Colony morphology, in vitro growth, melanin production, protease activity, and antifungal drug susceptibility were compared for the serial isolates of strains J22, J32, J33, and J35 (table 3). Differences in phenotypic characteristics were noted for J32 and J33. J32a1 colonies had a mucoid colony phenotype, while J32b colonies were smooth. The relative virulence of the “a” and “b” isolates for J22, J32, J33, and J35 strains were studied. The measurement for virulence was average survival time after infection with equal inocula of the “a” and “b” isolate. For each strain, two survival experiments were done using inocula of 5×10⁶ and 10⁷ yeast cells. Comparable inoculum size was documented by establishing cfu counts of the inoculum. Table 2 summarizes the results of these experiments. Median survival times of mice injected with 5×10⁶ and 10⁷ cells of the “a” and “b” isolates differed significantly for all strains. Median survival times of mice injected with J22a, J32b, J33a, and J35b were significantly shorter than for mice injected with the corresponding “a” or “b” isolate (P = .015, .019, .013, and .013, respectively). These differences in survival time between serial isolates were also found when the mice were injected with a higher inoculum (10⁸). To assure that equal amounts of live Cryptococcus cells were injected per mouse, we diluted the inoculum solution and plated them on Sabouraud agar plates. These control cfu counts were comparable for the injected “a” and “b” isolates of the individual strains (table 2, column 4). Hence, the observed survival differences could not be explained by differences in inoculum size.

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Table 2. Summary of survival differences of mice injected with serial isolates of C. neoformans.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Exp.</th>
<th>Inoculum size</th>
<th>Inoculum ratio “a/b”</th>
<th>Survival time in days (range)b</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>J22</td>
<td>1</td>
<td>5 x 10^6</td>
<td>63/65</td>
<td>17 (7)</td>
<td>.007</td>
</tr>
<tr>
<td>J22</td>
<td>2</td>
<td>1 x 10^7</td>
<td>100/110</td>
<td>26 (13)</td>
<td>.02</td>
</tr>
<tr>
<td>J32</td>
<td>1</td>
<td>5 x 10^6</td>
<td>100/115</td>
<td>20 (5)</td>
<td>.019</td>
</tr>
<tr>
<td>J32</td>
<td>2</td>
<td>1 x 10^7</td>
<td>140/120</td>
<td>17 (5)</td>
<td>.023</td>
</tr>
<tr>
<td>J33</td>
<td>1</td>
<td>5 x 10^6</td>
<td>75/69</td>
<td>42 (14)</td>
<td>.013</td>
</tr>
<tr>
<td>J33</td>
<td>2</td>
<td>1 x 10^7</td>
<td>66/60</td>
<td>6 (50)</td>
<td>.031</td>
</tr>
<tr>
<td>J35</td>
<td>1</td>
<td>5 x 10^6</td>
<td>84/72</td>
<td>21 (15)</td>
<td>.013</td>
</tr>
<tr>
<td>J35</td>
<td>2</td>
<td>1 x 10^7</td>
<td>82/75</td>
<td>16 (7)</td>
<td>.007</td>
</tr>
</tbody>
</table>

NOTE. Exp., experiment.

a No. of colony-forming units of diluted inoculum solution after plating on agar.
b Median (range) in days.

c By log-rank test.
d For both J35 strains, 4 mice were still alive at day 60.

In this study, we demonstrate that serial isolates from individual patients with chronic infections can differ in their ability to persist in mouse tissues. We selected serial isolates with CLPs to be able to distinguish them after mixed infection in mice. For isolates J22a and b and J32a2 and b, there was comparable persistence in murine tissues after mixed infection. For J33, the “a” isolate was more prevalent in tissue than the “b” isolate, whereas for J32 and for J35, the “b” isolate was more prevalent. Hence, there was no consistency regarding the relative ability of “a” and “b” isolates to persist in vivo. Since the J35 “a” and “b” isolates were recovered only 1 day apart, this observation suggests biologic differences among C. neoformans isolates recovered within a short period of time. The J32 strain was found to have extensive karyotype variation. We found two distinct karyotypes in the original J32a isolate. Both karyotypes differed from that of the J32b isolate recovered 14 days after the “a” isolate. Of interest, the “b” isolate persisted in the patient infected with J32 and was found in 3 subsequent serial C. neoformans isolates of this patient recovered from the J32 patient on days 16, 29, and 30. CNRE-1 RFLPs were identical for the J32a1, a2, and b isolate. Previous studies have shown that strains from different patients can have the same CNRE pattern, however CNRE-1 DNA typing methods were shown to be highly discriminatory, and these results correlate with other DNA typing methods [6, 15, 16]. In addition, infection with multiple strains of C. neoformans appears to be rare [8, 20]. Hence, the J32 isolates most likely represent variants of 1 strain. In vivo persistence, phenotype, and karyotype differed between J32a1 and J32b. However, there was no difference in persistence and phenotype between J32a2 and J32b. It is not known whether the recently described phenomenon of phenotypic switching is involved in the changes observed for serial isolates [14].

The exact relevance of virulence differences in mice to human infection is uncertain, since the milieu experienced by C. neoformans in chronic human and murine infections may be different. In this regard, significant differences have been reported for antimicrobial mechanisms of human and murine immune effector cells. For example, mouse neutrophils lack defensins, which are found in human neutrophils, and nitric oxide appears to be an important antimicrobial effector molecule in murine infection.

Table 3. Summary of phenotypic characteristics of C. neoformans isolates.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Doubling time (h)</th>
<th>Colony morphology</th>
<th>Size (μm)</th>
<th>Production of</th>
<th>MIC (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cell</td>
<td>Capsule</td>
<td>Melanin</td>
</tr>
<tr>
<td>J22a</td>
<td>2.35 ± 0.09</td>
<td>Smooth</td>
<td>6.1 ± 0.93</td>
<td>1.25 ± 0.09</td>
<td>Yes</td>
</tr>
<tr>
<td>J22b</td>
<td>2.36 ± 0.08</td>
<td>Smooth</td>
<td>6.3 ± 0.70</td>
<td>1.25 ± 0.70</td>
<td>Yes</td>
</tr>
<tr>
<td>J32a1</td>
<td>3.65 ± 0.14</td>
<td>Mucoid</td>
<td>4.5 ± 0.51</td>
<td>0.9 ± 0.12</td>
<td>Yes</td>
</tr>
<tr>
<td>J32b</td>
<td>3.52 ± 0.17</td>
<td>Smooth</td>
<td>4.2 ± 0.44</td>
<td>0.81 ± 0.11</td>
<td>Yes</td>
</tr>
<tr>
<td>J33a</td>
<td>4.1 ± 0.13</td>
<td>Smooth</td>
<td>4.9 ± 0.65</td>
<td>0.9 ± 0.13</td>
<td>Yes</td>
</tr>
<tr>
<td>J33b</td>
<td>9.5 ± 0.30</td>
<td>Smooth</td>
<td>7.7 ± 0.92</td>
<td>1.35 ± 0.16</td>
<td>Yes</td>
</tr>
<tr>
<td>J35a</td>
<td>3.92 ± 0.17</td>
<td>Smooth</td>
<td>4.2 ± 0.77</td>
<td>1.0 ± 0.0</td>
<td>Yes</td>
</tr>
<tr>
<td>J35b</td>
<td>4.21 ± 0.02</td>
<td>Smooth</td>
<td>3.8 ± 0.58</td>
<td>1.0 ± 0.0</td>
<td>Yes</td>
</tr>
</tbody>
</table>

NOTE. Amb, amphotericin B; Flu, fluconazole.
but not human macrophages. Hence, one cannot necessarily conclude that differences in virulence in mice correspond to differences in humans [21–23]. Nevertheless, the finding that serial isolates from chronic human infection can differ in mouse virulence implies biologic differences consistent with the concept of microevolution.

The relative virulence of serial isolates was studied by determining both the average survival time of mice infected with the same inoculum and the tissue persistence, as measured by organ cfu. We observed significant differences in the median survival time of mice infected with the “a” and “b” isolates for each of the 4 strains. The magnitude of the survival differences varied with the strain and inoculum dose. There was correlation between higher organ cfu and reduced survival time for J32, J33, and J35 but not J22. Isolates J33a, J32b, and J35b were more predominant in tissues and were associated with shorter survival times. However, for J22, the “a” and “b” isolates had comparable organ cfu yet differed significantly in their ability to shorten the survival time of infected mice.

Dissociation between tissue persistence and mean survival has been described for both C. neoformans and Candida albicans. For C. neoformans, antibody-treated mice have been shown to have significantly prolonged survival compared with untreated mice despite comparable organ cfu burdens [24]. Survival differences were also described in C. neoformans-infected CD8-depleted mice that had no differences in organ cfu [25]. Conversely, monoclonal antibody administration has been shown to reduce organ cfu for some C. neoformans strains without affecting survival [26]. For C. albicans, significant differences in survival time were noted for chitin-deficient variants despite comparable organ cfu [27]. The mechanism by which some strains persist in mouse tissues without killing mice whereas others lead to rapid death is not understood. Our results and those from previous studies suggest that organ persistence and survival time after infection may measure different aspects of virulence.

Growth rates at 37°C were comparable for J22, J32, and J35 and differed only for J33b, which grew significantly slower than all other C. neoformans strains. The slower replication time of J33b correlated with its reduced virulence, and this is consistent with reports showing that slower growth at 37°C is associated with reduced virulence [28]. However, growth rate differences cannot account for the differences in virulence observed for J22 and J32, and for J35 “a” and “b” isolates. Although the capsule of C. neoformans constitutes an important virulence factor [29], capsule size differences, as seen in the “a” and “b” isolates of J33, have not been consistently associated with virulence. The J33 strain is derived from a patient who relapsed about a year after his initial presentation; of interest, the karyotype of the relapse isolate was identical with that of the “a” isolate. Melanization [30] and extracellular proteolytic activity [18] did not differ among the serial isolates. Strain J22 lacked proteolytic activity and was still virulent during murine infection. This was also documented for 24067 variants by Franzot et al. [9], who showed that the presence or absence of proteolytic activity did not correlate with virulence in a murine infection model. Our results suggest that other virulence traits are responsible for the observed difference.

Despite the association of several traits with virulence in laboratory strains, the clinical relevance of the established virulence factors has not been established to date. Most clinical strains exhibit a polysaccharide capsule, can melanize, and contain a mating locus. In this respect, some investigators have proposed that a combination of virulence traits might be responsible for differences in virulence of clinical C. neoformans strains as opposed to single genes [31, 32]. One intriguing hypothesis is that an underlying genomic variability allows C. neoformans to express a subset of genes that encode for virulence traits. An association between chromosomal rearrangement and phenotypic changes has been described for some Candida strains [33]. Further studies to prove a causative relationship between the observed CLP and the phenotypic changes are needed for the described C. neoformans strains.

In summary, we have shown that serial clinical isolates of C. neoformans strains differ in virulence. Our study suggests that C. neoformans undergoes microevolution, resulting in variants with significant biologic differences. These results are important because previous studies have shown that relapse of cryptococcal meningitis is the result of persistent infection rather than reinfection with a new strain. The ability of C. neoformans to undergo changes, possibly as a result of DNA rearrangements and selection, could be a mechanism for evasion of host immune response, which may contribute to persistent infection.

Acknowledgment

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