Cryptococcus neoformans var. grubii isolates recovered from persons with AIDS demonstrate a wide range of virulence during murine meningoencephalitis that correlates with the expression of certain virulence factors

Cornelius J. Clancy,1,3† M. Hong Nguyen,1,3† Ruth Alandoerffer,4 Shaoji Cheng,1 Kenneth Iczkowski,2,3 Malcolm Richardson4 and John R. Graybill4

1,2Department of Medicine, Division of Infectious Diseases1 and Department of Pathology2, University of Florida College of Medicine, FL, USA
3VA Medical Center, Gainesville, FL, USA
4University of Texas Health Science Center, and the Audie Murphy Memorial Hospital, San Antonio, TX, USA

Cryptococcus neoformans is a common cause of meningoencephalitis among AIDS patients. Several C. neoformans virulence factors have been identified, but the relative importance of particular factors is unknown. This study examined the correlation of the virulence of 18 C. neoformans var. grubii isolates from AIDS patients with the expression of several well-described virulence factors. The LD50 at 15 days after intracranial inoculation of ICR mice was <100 c.f.u. for 22 % of isolates, 100–1000 for 28 %, 1000–10 000 for 11 % and >20 000 for 39 %. Higher cryptococcal concentrations in brains were noted for isolates with lower LD50 (P<0.002). In survival studies, no immunocompetent BALB/c mice (nu/nu) infected with 3×LD50 of three virulent isolates (LD50 = 62, 99, 1280) survived beyond 23 days, whereas 100 %, 90 % and 90 % of mice infected with 20 000 c.f.u. of three hypovirulent isolates (LD50 >20 000) survived for 60 days (P<0.0001). Even among BALB/c nude (nu/nu) mice, survival rates over 60 days were 100 %, 70 % and 50 %, respectively, for the hypovirulent isolates. Growth rate at 37 °C and capsule size within brains correlated with LD50 by univariate (P<0.0001 and 0.028, respectively) and multivariate (P=0.017 and 0.016, respectively) analyses. There was no correlation between LD50 and capsule size in vitro, phospholipase activity, melanin formation, proteinase activity and fluconazole MIC. In conclusion, AIDS patients are susceptible to infection by C. neoformans isolates of wide-ranging virulence, including isolates that are markedly hypovirulent. The virulence of a given isolate reflects a composite of factors rather than the contribution of a dominant factor. Growth at 37 °C and capsule size in vivo make particularly important contributions.

INTRODUCTION

Cryptococcus neoformans is a yeast-like fungus that is generally found in soil with a high content of avian guano or vegetative debris (Casadevall & Perfect, 1998; Mitchell & Perfect, 1995). Infections of humans, believed to be acquired by inhalation of small yeast cells or basidiospores, are often clinically silent. In fact, the most commonly recognized manifestation of cryptococcal disease (cryptococcosis) is not pneumonia but rather meningitis or meningoencephalitis. Cryptococcal central nervous system (CNS) infections and other forms of cryptococcosis are largely, but not exclusively, diseases of persons with AIDS or other immunosuppressed conditions. The pathogenesis of cryptococcosis reflects the interaction between host susceptibility, immune response to the infecting organism, and the virulence potential of the given C. neoformans strain. In North America, over 90 % of cases are caused by strains of serotype A, C. neoformans var. grubii, which are believed to be more pathogenic than strains of serotype D, C. neoformans var. neoformans (Mitchell & Perfect, 1995; Casadevall & Perfect, 1998).

†These authors contributed equally to this work.

Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; i.c., intracranial; LDH, lactate dehydrogenase.
Several factors have been identified that contribute to virulence of *C. neoformans* strains during animal models of cryptococcosis. Among the best characterized are the ability to grow at 37 °C, the presence of a protective polysaccharide capsule, being of the α-mating type, the ability to synthesize the antioxidant pigment melanin, and extracellular phospholipase and proteinase activities. In general, investigators have implicated these factors in virulence by studying them individually, either by using isogenic gene disruption and reconstitution strains or by comparing strains that differ in expression of a phenotype of interest. As for other fungal pathogens, however, it is likely that the virulence of *C. neoformans* depends upon the coordinated expression of multiple factors (Kozel, 1995; Blackstock et al., 1999). Along these lines, it has been suggested that *C. neoformans* and other medically relevant fungi contain a set of genes that express a subset of traits constituting a composite virulence phenotype (Cutler, 1991; Casadevall & Perfect, 1998). Under given conditions, selected factors in the so-called virulence set might not be absolutely necessary to cause disease; rather, a critical number of factors might need to act in concert. The fact that some *C. neoformans* isolates demonstrate tissue tropism suggests that their composite virulence phenotypes are adapted to particular sites of infection (Grosse et al., 1975; Dixon & Polak, 1986; Van Cutsem et al., 1986; Fromtling et al., 1988; Barchiesi et al., 2005). It is likely that other isolates are more generally equipped to cause disease at diverse sites, although this hypothesis has not been widely tested.

It is clear from animal models of cryptococcosis that individual clinical and environmental *C. neoformans* strains differ in relative virulence (Vanbreuseghem, 1967; Hasenclever & Mitchell, 1960; Kwon-Chung et al., 1992; Blasi et al., 1992, 1995; Blackstock & Murphy, 1997; Curtis et al., 1994; Blackstock et al., 1999; Fries & Casadevall, 1998). Comparing two *C. neoformans* var. *grubii* strains of variable virulence in the lungs of mice, Blackstock et al. (1999) showed that the more virulent isolate had higher expression of several virulence factors. However, since the expression of multiple factors by a larger number of well-characterized strains recovered from unique patients has not been studied, the relative importance of particular virulence factors is unknown. The primary objective of the present study was to correlate the virulence of 18 *C. neoformans* var. *grubii* isolates recovered from the cerebrospinal fluid (CSF) of AIDS patients with the expression of several well-characterized virulence factors. We assessed virulence by measuring mortality rates during murine meningoencephalitis and tissue burdens within infected brains. In addition, we studied tissue tropism for two isolates by comparing their levels of virulence in the CNS with their abilities to cause disease in the lungs after intranasal inoculation.

**METHODS**

**Organisms.** Eighteen isolates of *C. neoformans* var. *grubii* were obtained from the Fungus Testing Laboratory at the University of Texas Health Sciences Center at San Antonio, Texas. These isolates were recovered from the CSF of HIV-infected patients. Upon arrival at the Fungus Testing Laboratory, the isolates were streaked onto Sabouraud-dextrose agar (SDA) plates and incubated at 35 °C for 24–48 h. They were then streaked onto SDA slants and grown overnight at 35 °C, prior to storage at −80 °C. For this study, all animal experiments were performed using isolates obtained from the −80 °C frozen stock. For each isolate, *in vitro* studies were conducted as the animal experiments proceeded, using isolates maintained on SDA slants at 4 °C. Five of the eighteen isolates were previously included in a study correlating fluconazole susceptibility *in vitro* to the response to fluconazole among mice with cryptococcal meningoencephalitis (Velez et al., 1993). These isolates are labelled V5, V15, H12, H19 and H11 in the present study, corresponding to isolates 6, 3, 17, 4 and 8, respectively, in the earlier study. In the present study, fresh cultures of the five isolates were obtained from the frozen stock and LD₅₀ values during murine meningoencephalitis were redetermined using the methods described below. As the data indicate, there were no significant differences in LD₅₀ between the earlier study and this.

**Murine models of cryptococcosis.** Male ICR outbred mice were obtained from Harlan Sprague–Dawley. *nu/nu* and *nu/nu* mice on a BALB/c background were obtained from a breeding colony at the University of Texas Health Sciences Center at San Antonio, Texas. Yeast cells grown overnight in brain heart infusion (BHI) broth were washed, counted and adjusted to the desired concentration in sterile saline. Viability of cells was confirmed by quantitative culture for each experiment.

**Cryptococcal meningoencephalitis.** Prior to infection, the mice were anaesthetized with inhaled methoxyflurane. The cranium was swabbed with 70% alcohol and the inoculum was injected in the midline of the cranium with a 26-gauge needle in a volume of 0.06 ml.

**Assessment of LD₅₀.** For each isolate, eight ICR mice per group were initially inoculated with cryptococcal cells over a broad range of concentrations (10–400 c.f.u. per mouse). For those isolates for which an LD₅₀ could not be determined, inocula were increased to the range of 400–20 000 c.f.u. per mouse. The LD₅₀ was calculated by Probit analysis on day 15 of infection.

**Assessment of tissue burdens.** Mice were sacrificed by cervical dislocation on day 9 of infection. The brains were removed, weighed and homogenized in 0.9% saline containing amikacin and piperacillin (60 µg ml⁻¹ for both). The homogenates were serially diluted and plated onto SDA plates for burden quantification.

**Assessment of capsule size within infected brains.** The capsule width was measured in cells obtained directly from homogenized brain tissue on day 9 using an eyepiece micrometer mounted on a phase-contrast microscope (resolution 0.12 µm). The width of the capsule was determined at a magnification of 1000 × by measuring the distance between the outer edge of the yeast cell wall and the outer edge of the capsule. For each isolate, at least 10 cells in each of 20 fields (≥200 total cells) were examined in two mice.

**Pulmonary cryptococcosis.** ICR mice received intraperitoneal injections of cortisone acetate (125 mg kg⁻¹ per mouse) 36 h prior to and 36 h after challenge with *C. neoformans* (Polak-Wyss, 1991). Prior to infection, mice (≥10 mice per group) were anaesthetized with inhaled methoxyflurane. The inocula were instilled slowly in equally divided aliquots (25 µl) to each nare using a micropipette and sterile tip, and mice were held upright until complete inhalation and resumption of normal breathing. The outcomes assessed were: (1) mortality over 21 days among mice infected with 1 × 10⁷ c.f.u.
per mouse; (2) tissue burden in lungs, kidneys and brains of mice infected with a sublethal inoculum of 5 × 10⁶ c.f.u. per mouse; and (3) histopathology.

**Measurements of phenotypes in vitro.** Growth rate was determined in yeast-extract-glucose (YEG) broth at 37 °C in a shaker incubator (150 r.p.m.) (Fries & Casadevall, 1998). An aliquot of the culture was obtained hourly for colony determination by plate count as well as by optical density. Capsule size in vitro was measured for yeast cells incubated overnight at 30 °C in induction medium (1.7 g yeast nutrient base without amino acids and without ammonium sulfate supplemented with 1:5 g asparagine and 20 g glucose per litre of buffer containing 12 mM NaHCO₃ and 35 mM MOPS, pH 7.1; Garcia-Hermoso et al., 2004). Yeast cells were then suspended in India ink and capsule sizes of 200 cells were measured as within murine brains. Melanin production was measured indirectly using a standardized determination of phenoloxidase activity (Polacheck et al., 1982). One unit of enzyme activity was defined as the amount which caused a change in absorbance at 480 and 300 nm of 0-001. Protein concentration was measured by reference to a standard curve. Extracellular proteinase activity was measured as previously described (Andrade et al., 2002; Leighton et al., 1973). Units of proteolytic activity were expressed as ng azoalbumin hydrolysed per hour. Specific activity was calculated as the ratio between total protease activity and total protein concentration in 1 ml sample (U mg⁻¹). The extracellular protein content was determined using bovine serum albumin (Sigma) as the standard. Extracellular phospholipase activity was determined using a modified egg yolk agar method (Chen et al., 1997). Each isolate was tested in duplicate on at least six separate occasions. Cultures were incubated at 30 °C, and the diameter of the zone of precipitation around the isolate was measured at 48, 72 and 96 h. The index of phospholipase activity was the ratio of the diameter of the colony to the total diameter of the colony plus precipitation zone (Pᵣ). In vitro susceptibility to fluconazole was determined using a macrodilution method approved by the National Committee for Clinical Laboratory Standards (1997).

**Adherence to A549 cells.** Cells of the A549 human lung epithelial line (ATCC CAL-185) were grown to confluence in 24-well plates (Costar) in Dulbecco’s modified Eagle medium (Gibco) supplemented with 0.37% NaHCO₃, 200 U penicillin ml⁻¹, 200 μg streptomycin ml⁻¹ and 10% heat-inactivated fetal bovine serum (FBS) (A549 medium). A549 monolayers were washed with Earle’s balanced salts (EBS) (Sigma) containing 0.22% NaHCO₃ and 5% FBS (EBS-FBS). Yeast cells were grown overnight in YPD medium at 37 °C, washed with phosphate-buffered saline (PBS) and resuspended in EBS-FBS. After co-incubation at 37°C in a humidified CO₂ incubator for 4.5 h (the time determined to be optimal in preliminary experiments), A549 cells were washed with EBS-FBS, and 500 μl warm SDA added to the well. At the same time, A549 cells in duplicated wells were lysed with 0.5% sodium deoxycholate. Then 500 μl warm SDA was added to the wells. Colonies were counted from lysed and non-lysed wells after incubation for 48–72 h at 37 °C. There were no significant differences in the numbers of yeast cells for a given inoculum in the lysed and non-lysed wells. Adherence was calculated as percentage of the original inoculum recovered.

**Damage to A549 cells.** Damage to A549 cells was measured using a standard lactose dehydrogenase (LDH)-cytotoxicity assay kit (BioVision). Confluent A549 cells were washed three times with PBS and resuspended in A549 medium. The following samples were prepared individually in triplicate wells of a 96-well plate: background control, 200 μl medium alone; low control, 1 × 10⁴ A549 cells in 200 μl; high control, 1 × 10⁵ A549 cells containing 1% Triton X-1000 in 200 μl; test sample, 1 × 10⁴ A549 cells and 5 × 10⁵ yeast in 200 μl. Cells were incubated at 37°C in a humidified CO₂ incubator for 5 h (the time determined to be optimal in preliminary experiments). Following centrifugation, 100 μl supernatant per well was transferred into corresponding wells of an optically clear 96-well plate and 100 μl kit reaction mixture added. The plate was incubated at room temperature in the dark for 30 min and absorbance read at 500 nm. The percentage cytotoxicity was calculated as: ([Test sample−Low control] × [High control−Low control]⁻¹) × 100.

**Statistical analysis.** The LD₅₀ was exponentially transformed to approach a normal distribution prior to statistical analysis. Non-parametric correlation using the Spearman rank correlation test was used to correlate LD₅₀ and specific virulence factors. A multivariate analysis to assess independent factors of virulence was performed using multiple linear regression.

**RESULTS**

*C. neoformans* isolates recovered from the CSF of persons with AIDS exhibit a wide range of virulence during murine cryptococcal meningoencephalitis

The 18 isolates were demonstrated to be of serotype A (*C. neoformans* var. *grubii*) by a slide agglutination test (Crypto-check kit, latex) and mating type *x* by PCR with specific primers that amplify portions of the STE20α or STE20z allele (Nielsen et al., 2003). We determined the LD₅₀ for each of the isolates at 15 days following the intracranial (i.c.) inoculation of ICR mice (Table 1). The median LD₅₀ was 996 c.f.u. (range 51 to >20,000). The LD₅₀ was <100 c.f.u. for 22% (4/18) of isolates, 100–1000 c.f.u. for 28% (5/18), and 1000–10,000 c.f.u. for 11% (2/18). For 39% (7/18) of isolates, mortality rates following inoculation of up to 20,000 c.f.u. were insufficient for calculation of an LD₅₀ (i.e. LD₅₀ >20,000 c.f.u.). Indeed, four isolates (H2, H6, H11, H19) failed to kill any mice during the 30 days following inoculation of 20,000 c.f.u.. Three additional isolates resulted in the deaths of 43%, 29% and 29% of mice over 30 days (isolates H12, H16 and H17, respectively). The seven isolates for which LD₅₀ exceeded 20,000 c.f.u. were classified as hypovirulent. The others were considered virulent. We denoted the hypovirulent isolates with an ‘H’ preceding the isolate number, and the virulent isolates with a ‘V’ preceding their number.

To further assess relative virulence, we performed survival studies in which we infected immunocompetent BALB/c mice that were heterozygous for the recessive *nu* gene (*nu/−*) with one of three virulent (V18, V21 and V15; 8 mice each) or three hypovirulent isolates (H2, H6 and H11; 10 mice each). Mice infected with virulent isolates received i.c. inoculations of approximately 3 × LD₅₀ (180, 300 and 3850 c.f.u., respectively), whereas mice infected with hypovirulent isolates received 20,000 c.f.u. each. As shown in Table 2, none of the mice infected with the virulent isolates survived past day 23. Survival over 60 days among mice infected with hypovirulent isolates H2, H6 and H11, on the other hand, was 100%, 90% and 90%, respectively. Survival was significantly greater for mice infected with a hypovirulent isolate than a virulent isolate (P<0.0001, Fisher’s exact test).
Table 1. Characterization of 18 C. neoformans isolates recovered from the CNS of different HIV-infected patients

<table>
<thead>
<tr>
<th>Isolate</th>
<th>LD_{50} (c.f.u.)</th>
<th>Tissue burden [c.f.u. (g brain)^{-1}]</th>
<th>Doubling time (h)</th>
<th>In vitro capsule (μm)</th>
<th>In vivo capsule (μm)</th>
<th>Phospholipase ( (P_v)^* )</th>
<th>Phenoloxidase [U (mg protein)^{-1}]</th>
<th>Proteinase [U (mg protein)^{-1}]</th>
<th>Flu MIC (μg ml^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>V18</td>
<td>51</td>
<td>1.38 ± 0.35 × 10^{6}</td>
<td>2.13 ± 0.46</td>
<td>2.31 ± 0.36</td>
<td>2.07 ± 0.8</td>
<td>0.64 ± 0.08</td>
<td>5.026 ± 889</td>
<td>ND</td>
<td>1</td>
</tr>
<tr>
<td>V3</td>
<td>67</td>
<td>3.21 ± 0.24 × 10^{6}</td>
<td>4.10 ± 0.16</td>
<td>2.51 ± 0.37</td>
<td>5.07 ± 0.3</td>
<td>0.63 ± 0.03</td>
<td>381 ± 1444</td>
<td>5.1 ± 0.9</td>
<td>4</td>
</tr>
<tr>
<td>V5</td>
<td>94</td>
<td>1.01 ± 0.12 × 10^{11}</td>
<td>4.08 ± 0.57</td>
<td>2.30 ± 0.14</td>
<td>5.34 ± 4.3</td>
<td>0.64 ± 0.09</td>
<td>11 300 ± 1276</td>
<td>51 ± 6.3</td>
<td>2</td>
</tr>
<tr>
<td>V21</td>
<td>99</td>
<td>6.02 ± 0.12 × 10^{6}</td>
<td>4.09 ± 0.25</td>
<td>2.53 ± 0.08</td>
<td>6.84 ± 3.4</td>
<td>0.63 ± 0.07</td>
<td>7 381 ± 444</td>
<td>21 ± 7.3</td>
<td>8</td>
</tr>
<tr>
<td>V7</td>
<td>119</td>
<td>7.80 ± 0.81 × 10^{9}</td>
<td>3.66 ± 0.23</td>
<td>2.73 ± 0.07</td>
<td>4.43 ± 2.5</td>
<td>0.70 ± 0.09</td>
<td>7 272 ± 618</td>
<td>51 ± 6.3</td>
<td>2</td>
</tr>
<tr>
<td>V10</td>
<td>194</td>
<td>5.20 ± 0.83 × 10^{9}</td>
<td>4.18 ± 0.13</td>
<td>3.32 ± 0.18</td>
<td>4.53 ± 1.1</td>
<td>0.68 ± 0.06</td>
<td>4 833 ± 515</td>
<td>21 ± 7.3</td>
<td>8</td>
</tr>
<tr>
<td>V8</td>
<td>250</td>
<td>1.43 ± 0.16 × 10^{9}</td>
<td>4.20 ± 0.58</td>
<td>2.62 ± 0.28</td>
<td>5.87 ± 1.8</td>
<td>0.62 ± 0.11</td>
<td>6 134 ± 666</td>
<td>ND</td>
<td>4</td>
</tr>
<tr>
<td>V20</td>
<td>836</td>
<td>4.40 ± 0.66 × 10^{4}</td>
<td>4.80 ± 0.18</td>
<td>2.48 ± 0.27</td>
<td>6.10 ± 1.2</td>
<td>0.66 ± 0.11</td>
<td>28 333 ± 3986</td>
<td>12 ± 7 ± 2.0</td>
<td>8</td>
</tr>
<tr>
<td>V13</td>
<td>973</td>
<td>5.01 ± 0.77 × 10^{11}</td>
<td>5.47 ± 0.16</td>
<td>2.13 ± 0.38</td>
<td>2.08 ± 1.7</td>
<td>0.62 ± 0.08</td>
<td>1 173 ± 211</td>
<td>32 ± 4 ± 5.5</td>
<td>4</td>
</tr>
<tr>
<td>V15</td>
<td>1019</td>
<td>3.26 ± 0.29 × 10^{9}</td>
<td>5.13 ± 0.24</td>
<td>2.18 ± 0.40</td>
<td>4.63 ± 1.4</td>
<td>0.60 ± 0.12</td>
<td>1 040 ± 147</td>
<td>ND</td>
<td>8</td>
</tr>
<tr>
<td>V14</td>
<td>8270</td>
<td>1.17 ± 0.97 × 10^{9}</td>
<td>5.85 ± 0.26</td>
<td>2.28 ± 0.09</td>
<td>2.28 ± 0.09</td>
<td>0.66 ± 0.08</td>
<td>5 712 ± 369</td>
<td>6 ± 2 ± 1.1</td>
<td>4</td>
</tr>
<tr>
<td>H12</td>
<td>&gt; 20 000</td>
<td>1.40 ± 0.34 × 10^{4}</td>
<td>6.27 ± 0.29</td>
<td>2.42 ± 0.09</td>
<td>4.10 ± 3.1</td>
<td>0.69 ± 0.07</td>
<td>29 500 ± 3874</td>
<td>4 ± 9 ± 1.2</td>
<td>16</td>
</tr>
<tr>
<td>H16</td>
<td>&gt; 20 000</td>
<td>1.74 ± 0.46 × 10^{7}</td>
<td>4.42 ± 0.21</td>
<td>2.56 ± 0.17</td>
<td>2.43 ± 1.9</td>
<td>0.68 ± 0.14</td>
<td>3 285 ± 300</td>
<td>6 ± 3 ± 0.8</td>
<td>2</td>
</tr>
<tr>
<td>H19</td>
<td>&gt; 20 000</td>
<td>2.71 ± 0.21 × 10^{7}</td>
<td>7.14 ± 1.8</td>
<td>2.31 ± 0.22</td>
<td>1.73 ± 0.5</td>
<td>0.59 ± 0.07</td>
<td>20 352 ± 1677</td>
<td>11 ± 9 ± 2.2</td>
<td>0.5</td>
</tr>
<tr>
<td>H17</td>
<td>&gt; 20 000</td>
<td>2.30 ± 0.16 × 10^{4}</td>
<td>8.36 ± 0.66</td>
<td>2.49 ± 0.08</td>
<td>0.71 ± 0.12</td>
<td>4.410 ± 452</td>
<td>ND</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>H2</td>
<td>&gt; 20 000</td>
<td>2.27 ± 0.10 × 10^{5}</td>
<td>5.68 ± 0.21</td>
<td>3.10 ± 0.11</td>
<td>2.21 ± 2.0</td>
<td>0.73 ± 0.16</td>
<td>18 ± 4 ± 19 ± 5</td>
<td>6 ± 5 ± 0.9</td>
<td>2</td>
</tr>
<tr>
<td>H6</td>
<td>&gt; 20 000</td>
<td>2.50 ± 0.24 × 10^{2}</td>
<td>4.26 ± 1.10</td>
<td>2.27 ± 0.13</td>
<td>2.27 ± 0.1</td>
<td>0.70 ± 0.13</td>
<td>272 ± 103</td>
<td>6 ± 5 ± 0.9</td>
<td>2</td>
</tr>
<tr>
<td>H11</td>
<td>&gt; 20 000</td>
<td>5.98 ± 0.45 × 10^{2}</td>
<td>5.53 ± 0.10</td>
<td>2.55 ± 0.24</td>
<td>1.09 ± 0.3</td>
<td>0.70 ± 0.14</td>
<td>1 500 ± 198</td>
<td>108 ± 0 ± 13.3</td>
<td>4</td>
</tr>
</tbody>
</table>

ND, None detected.

*\( P_v \) precipitation zone ratio (see Methods).
Since cryptococcal meningoencephalitis is largely a disease associated with impaired cell-mediated immunity, we also tested the hypovirulent isolates in T-cell-deficient mice. We infected ten BALB/c nude mice (nu/nu) that were littermates of the immunocompetent nu/– mice with 20 000 c.f.u. of isolates H2, H6 and H11. Similar to the findings among nu/– mice, survival among nu/nu mice infected with isolate H2 was 100 % over 60 days. Among nu/nu mice infected with H6 and H11, survival rates over 60 days were 70 % and 50 %, respectively (Table 2). The median time to death for nu/nu mice infected with isolate H11 was shorter than that for their nu/– littermates (P=0·02, Student’s t-test).

As a final assessment of each isolate’s virulence in the CNS, we measured cryptococcal tissue burdens within the brains of ICR mice 9 days after i.c. inoculation (3 × LD50 for all virulent isolates except isolate V14, and 20 000 c.f.u. for isolate V14 and all hypovirulent isolates). There was a remarkable range of tissue burdens (250–1·01 × 1011 c.f.u. gm−1) (Table 1); the median burden was 1·26 × 108 c.f.u. gm−1. Overall, we demonstrated that there was an inverse correlation between tissue burden and LD50 (i.e. higher tissue burden correlated with lower LD50) (P=0·038; Spearman rank correlation).

The expression of certain virulence factors correlates with the virulence demonstrated during murine cryptococcal meningoencephalitis

Next, we characterized the expression of several well-described virulence factors by each of the isolates to determine if they correlated with the virulence observed in mice with cryptococcal meningoencephalitis (Table 1). Since reports have suggested that fluconazole resistance might influence the virulence of individual strains (Iwata et al., 1990), we also determined fluconazole MICs.

The growth rate at 37 °C in vitro was measured as doubling time in YEG liquid medium. The median doubling time for the 18 isolates was 4·61 h (range 2·13–8·36 h). The median capsule size after overnight growth in vitro was 2·48 μm (range 2·13–3·32 μm). The median capsule size was similar within homogenized brain tissues (3·29 μm), but the range of sizes was greater (range 1·09–6·84 μm). Extracellular phospholipase activity, measured as a zone of precipitation using an egg yolk agar assay (Chen et al., 1997), was detected for all isolates; the median Pz value (i.e. ratio of the diameter of the colony to the total diameter of the colony plus the precipitation zone) was 0·66 (range 0·59–0·73). Melanin production, assessed by measuring phenoloxidase activity, was also detected for each of the isolates. The median activity was 4930 U mg−1 (range 796–29 500 U mg−1). We did not detect extracellular proteinase activity for four isolates (V18, V8, V15 and V17). Among the remaining isolates, the median activity was 17·2 U (mg protein)−1 (range 4·9–206·7 U (mg protein)−1). The median fluconazole MIC for the isolates was 4 μg ml−1 (range 0·5–16 μg ml−1) and the MIC90 was 8 μg ml−1. No isolates were resistant in vitro to fluconazole (i.e. MIC ≥ 64 μg ml−1).

There was a positive association between LD50 and doubling time (i.e. lower LD50 was associated with shorter doubling time) (P=0·0001; non-parametric Spearman rank correlation test), and an inverse association between LD50 and capsule size in vivo (P=0·028; non-parametric Spearman rank correlation test). There was no correlation between LD50 and phospholipase, phenoloxidase and proteinase activities, capsule size measured in vitro, and fluconazole MIC.

Multivariate analysis including the factors found to be associated with LD50 by univariate analyses identified both the doubling time and capsule size in vivo as significantly
associated with LD$_{50}$ ($P=0.017$ and $0.016$, respectively; multiple regression analysis).

**The relative virulence of two isolates within murine lungs following inhalation is similar to that observed during meningoencephalitis**

We compared the abilities of a virulent and a hypovirulent isolate (V7 and H16, respectively) to cause disease following intranasal inoculations ($1 \times 10^7$ c.f.u.) of mice immunosuppressed with cortisone acetate. Mice infected with isolate V7 suffered significantly greater mortality during pulmonary cryptococcosis than mice infected with strain H16 ($P<0.001$; log rank test) (Fig. 1).

To evaluate the burden of the *C. neoformans* isolates within infected tissues, we infected mice intranasally with a sub-lethal inoculum of each isolate ($5 \times 10^6$ c.f.u.). The burden of organisms within the lungs did not differ 8 h after inhalation (Table 3). At 3, 7, 14 and 21 days, however, lungs of mice infected with isolate V7 exhibited significantly greater tissue burdens than mice infected with isolate H16. Histopathological findings corroborated the tissue burden data. At 8 h, lungs infected with the two isolates were indistinguishable, with a few organisms visible (the majority of which were found within the airspaces) and minimal parenchymal invasion. At 7 days, lungs infected with isolate V7 revealed much more extensive inflammation than the lungs infected with isolate H16 (data not shown).

Organisms were recovered from the kidneys of all mice on day 14, and from the brains and kidneys of all mice on day 21, consistent with the establishment of disseminated cryptococcosis. Tissue burdens of isolate V7 were significantly higher than isolate H16 in each instance (Table 3).

**The more virulent isolate exhibits greater adherence to human pneumocytes in vitro and causes greater cellular damage**

Next, we studied the interactions between the two isolates and A549 human lung carcinoma epithelial cells (ATCC CAL-185) *in vitro*. We found that isolate V7 exhibited significantly greater adherence to A549 cells than isolate H16 ($12.7 \pm 6.5\%$ versus $4.1 \pm 2.7\%$; $P=0.0003$). Finally, the more virulent isolate also caused greater damage to the A549 cells, as measured by an LDH release assay of cytotoxicity at 5 h ($20 \pm 6.2\%$ cytotoxicity versus $6.6 \pm 2.9\%$; $P=0.02$).

**DISCUSSION**

To our knowledge, this is the largest study to correlate the CNS virulence of *C. neoformans* var. *grubii* isolates from different patients with the expression of several well-characterized virulence factors. The most notable finding of the study was the wide range of virulence exhibited by the isolates, as measured by both LD$_{50}$ (range 51 to >20 000 c.f.u.) and the recovery of viable organisms from infected brains (range 250–1·01 $\times 10^7$ c.f.u. g$^{-1}$). In particular, we found that 39% (7/18) of the isolates were markedly hypovirulent, causing significantly lower tissue burdens and unable to kill enough immunocompetent mice to permit the calculation of an LD$_{50}$. Moreover, one of three hypovirulent isolates tested in T-cell-deficient nude (nu/nu) mice did not cause any mortality over 60 days (isolate H2), and the other two isolates were associated with mortality rates of only 30 and 50% (isolates H6 and H11, respectively). At the same time, the overall survival times among nude mice infected with isolate H11 were significantly shorter than among immunocompetent littermates, as would be predicted for an opportunistic pathogen that causes disease principally among patients with impaired cell-mediated immunity. Taken together, our data highlight that an organism’s disease-causing potential in humans is shaped, in large part, by immune status and other host factors such as genetic characteristics. Clearly, significant numbers of AIDS patients and others with profound suppression of cell-mediated immunity are unable to defend themselves from CNS infection by organisms of even minimal virulence.

The ranges in the expression of selected virulence factors by *C. neoformans* isolates were also remarkable. Doubling time, for example, ranged from 2·13 to 8·36 h (isolates V18 and H17, respectively), and phenoloxidase activity from 796

![Fig. 1. Kaplan–Meier survival curve of mice infected intranasally with *C. neoformans* isolates V7 (●) and H16 (■).](image-url)
to 29,500 U (mg protein)$^{-1}$ (isolates V3 and H12, respectively). The inclusion of a large number of isolates that demonstrated wide ranges of LD$_{50}$ and virulence factor expression enabled us to evaluate the relationships between specific factors and virulence. There was no association between virulence and capsule size in vitro, extracellular phospholipase activity, the amount of melanin formation (measured as phenoloxidase activity), proteinase activity and fluconazole MIC. However, we found that growth rate at 37°C (measured as doubling time) and capsule size in vitro correlated with LD$_{50}$ by univariate and multivariate analyses. Despite the significance of these factors, neither was indispensable or dominant. For example, one of the most virulent isolates (V3; LD$_{50}$ 67 c.f.u.) exhibited the smallest capsule within the brain. As such, our findings support a composite virulence phenotype (Cutler, 1991; Casadevall & Perfect, 1998), in which a variety of factors contribute to the overall pathogenicity of a C. neoformans isolate and a limited contribution by one factor can be compensated by the expression of others.

The finding that growth rate at 37°C correlated with LD$_{50}$ is consistent with previous studies. The ability to grow at human body temperatures of 37°C and above is essential for pathogenic fungi to cause disease. In a previous study of seven C. neoformans strains of various serotypes, the three fastest growing strains at 37°C were the most virulent following the intravenous inoculation of mice (Kagaya et al., 1985). The finding of an association between capsule size within infected brains and virulence, on the other hand, is more notable. Generally taken to be the most important cryptococcal virulence factor (Janbon, 2004), the capsule inhibits (but does not prevent) phagocytosis in vitro (Feldmesser et al., 2001), facilitates survival and replication within macrophages (Feldmesser et al., 2001), inhibits migration of leukocytes (Dong & Murphy, 1995), alters antigen presentation (Collins & Bancroft, 1991), dysregulates cytokine secretion (Retini et al., 1996; Vecchiarelli et al., 1995, 1996), and can deplete complement (Macher et al., 1978). Disruption of multiple genes that are individually necessary for capsule synthesis results in acapsular strains that are avirulent in murine cryptococcosis (Chang & Kwon-Chung, 1994, 1998, 1999; Chang et al., 1996). Although it has long been recognized that the capsule thickness of C. neoformans increases during infection (Littman & Tsubura, 1959), a correlation between capsule size in vivo and relative virulence has not to our knowledge been reported previously.

Our findings are particularly noteworthy since capsule size in vitro did not correlate with virulence, an observation consistent with previous studies (Littman & Tsubura, 1959; Dykstra et al., 1977). Taken together, the data have important implications. Clearly, the regulation of capsule formation varies depending on the local environment, as previously suggested by studies showing organ-dependent variation of capsule size (Rivera et al., 1998). More generally, results obtained under in vitro conditions cannot necessarily be extrapolated to settings in vivo. As such, we must acknowledge that factors like melanin formation and proteinase activity that did not correlate with virulence in this study might nevertheless be important within infected brains.

It is not surprising that we did not find correlations between phospholipase, phenoloxidase and proteinase activities in vitro and LD$_{50}$. Although phospholipase and phenoloxidase activity are clearly linked to virulence, and melanin can be detected within human brains and other tissues infected with C. neoformans (Casadevall et al., 2000; Chen et al., 1997; Cox et al., 2001; Jacobson & Emery, 1991; Kwon-Chung et al., 1982; Kwon-Chung & Rhodes, 1986; Nosanchuk & Casadevall, 2003; Noverr et al., 2004; Rhodes et al., 1982; Salas et al., 1996; Williamson, 1997), the levels of phospholipase and phenoloxidase activity have not been shown to be associated with virulence. Like the overwhelming majority of clinical isolates, all our isolates exhibited phospholipase and phenoloxidase activity (Kwon-Chung et al., 1982; Chen et al., 1997). The data, therefore, suggest that some minimum levels of phospholipase and melanin formation are likely to play roles in pathogenesis, but there are no gains in virulence beyond these levels. Along similar lines, previous studies have failed to find a correlation between the amount of proteinase activity and virulence (Chen et al., 1996; Franzot et al., 1998; Fries & Casadevall, 1998). In fact, we found that even strains lacking detectable proteinase activity were associated with low LD$_{50}$ (e.g. isolate V18; LD$_{50}$ 51 c.f.u.), suggesting that this property might be dispensable for the pathogenesis of disease (Chen et al., 1996; Franzot et al., 1998; Fries & Casadevall, 1998). Since we did not detect any resistance to fluconazole, it is not possible to assess a previous report that azole-resistant C. neoformans isolates demonstrate attenuated virulence (Iwata et al., 1990). Nevertheless, we did not find any correlation between LD$_{50}$ and fluconazole MIC.

We demonstrated that the level of virulence for two isolates in the CNS also corresponded with virulence in the lungs following intranasal inoculation. Isolate V7, which was virulent during meningoecephalitis, caused greater mortality, higher tissue burdens within homogenized lungs, and a more profound inflammatory response than the hypovirulent isolate H16. These findings are consistent with our observations that isolate V7 demonstrated greater adherence to pulmonary epithelial cells in vitro and caused greater damage to these cells. Clearly, the relative virulence of at least some C. neoformans isolates is conserved within diverse organs (Barchiesi et al., 2003). We also noted that both isolates were able to disseminate from the lungs to brains and kidneys. Consistent with the findings in the lungs, the tissue burdens of isolate V7 were greater in the brains and kidneys after 21 days than those of isolate H16. We cannot conclude from these experiments, however, whether this stems from differences among the isolates in tropism for these organs or merely reflects increased numbers of isolate V7 gaining access to the bloodstream from the lungs.
In conclusion, we have characterized the CNS virulence of 18 C. neoformans var. grubii isolates recovered from the CSF of AIDS patients, and correlated this with the expression of several virulence factors in vitro. In addition to the insights into the pathogenesis of cryptococcosis provided by our data, this study is important because our collection of isolates now represents a valuable resource for future research. For example, we are planning to characterize a number of the isolates for virulence within murine lungs, as well as more thoroughly investigate interactions with pneumocytes. Other future investigations will include comparisons of transcriptional profiles under conditions relevant to pathogenesis, and studies of the interactions between isolates and host cells important to the pathogenic process such as alveolar macrophages.

ACKNOWLEDGEMENTS

Drs Clancy and Nguyen’s research is conducted in laboratories at the North Florida/South Georgia VA Medical Center, Gainesville, FL, USA. Their participation in the project was supported by the Medical Research Service of the Department of Veterans Affairs. Dr Clancy was supported by a research grant from the American Lung Association of Florida. The project was conducted as part of the University of Florida Mycology Research Unit.

REFERENCES


