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

Correspondence

Cornelius J. Clancy
clancyn@medicine.ufl.edu

^{1,2}Department 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/–*) infected with $3 \times \text{LD}_{50}$ 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.

Received 22 December 2005

Revised 22 March 2006

Accepted 29 March 2006

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 5×10^6 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 NaHCO_3 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 mg 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 (P_z). *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% NaHCO_3 , 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% NaHCO_3 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 CO_2 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, 1×10^4 A549 cells in 200 μl ; high control, 1×10^4 A549 cells containing 1% Triton X-1000 in 200 μl ; test sample, 1×10^4 A549 cells and 5×10^5 yeast in 200 μl . Cells were incubated at 37°C in a humidified CO_2 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: $[(\text{Test sample} - \text{Low control}) \times (\text{High control} - \text{Low control})^{-1}] \times 100$.

Statistical analysis. The LD_{50} 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 LD_{50} 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, Iatron) and mating type α by PCR with specific primers that amplify portions of the *STE20a* or *STE20x* allele (Nielsen *et al.*, 2003). We determined the LD_{50} for each of the isolates at 15 days following the intracranial (i.c.) inoculation of ICR mice (Table 1). The median LD_{50} was 996 c.f.u. (range 51 to $>20\,000$). The LD_{50} 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 LD_{50} (i.e. $\text{LD}_{50} > 20\,000$ 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 LD_{50} 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 $3 \times \text{LD}_{50}$ (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

Isolate	LD ₅₀ (c.f.u.)	Tissue burden [c.f.u. (g brain) ⁻¹]	Doubling time (h)	<i>In vitro</i> capsule (μm)	<i>In vivo</i> capsule (μm)	Phospholipase (P ₂)*	Phenoloxidase [U (mg protein) ⁻¹]	Proteinase [U (mg protein) ⁻¹]	Flu MIC (μg ml ⁻¹)
V18	51	1.38 ± 0.35 × 10 ⁸	2.13 ± 0.46	2.31 ± 0.36	2.07 ± 0.8	0.64 ± 0.08	5 026 ± 889	ND	1
V3	67	3.21 ± 0.24 × 10 ⁶	4.10 ± 0.16	2.51 ± 0.37	5.07 ± 0.3	0.63 ± 0.03	796 ± 132	206.7 ± 18.8	2
V5	94	1.01 ± 0.12 × 10 ¹¹	4.08 ± 0.57	2.30 ± 0.14	5.34 ± 4.3	0.64 ± 0.09	11 300 ± 1276	181.6 ± 12.6	4
V21	99	6.02 ± 0.12 × 10 ⁶	4.09 ± 0.25	2.53 ± 0.08	6.84 ± 3.4	0.63 ± 0.07	3 381 ± 444	5.1 ± 0.9	4
V7	119	7.80 ± 0.81 × 10 ⁹	3.66 ± 0.23	2.73 ± 0.07	4.43 ± 2.5	0.70 ± 0.09	7 272 ± 618	51.7 ± 6.3	2
V10	194	5.20 ± 0.83 × 10 ⁹	4.18 ± 0.13	3.32 ± 0.18	4.53 ± 1.1	0.68 ± 0.06	4 833 ± 515	21.7 ± 3.7	8
V8	250	1.43 ± 0.16 × 10 ⁸	4.20 ± 0.58	2.62 ± 0.28	5.87 ± 1.8	0.62 ± 0.11	6 134 ± 666	ND	4
V20	836	4.40 ± 0.66 × 10 ⁸	4.80 ± 0.18	2.48 ± 0.27	6.10 ± 1.2	0.66 ± 0.11	28 333 ± 3986	12.7 ± 2.0	8
V13	973	5.01 ± 0.77 × 10 ¹¹	5.47 ± 0.16	2.13 ± 0.38	2.08 ± 1.7	0.62 ± 0.08	1 173 ± 211	32.4 ± 5.5	4
V15	1019	3.26 ± 0.29 × 10 ⁹	5.13 ± 0.24	2.18 ± 0.40	4.63 ± 1.4	0.60 ± 0.12	1 040 ± 147	ND	8
V14	8270	1.17 ± 0.97 × 10 ⁹	5.85 ± 0.26	2.28 ± 0.09	2.28 ± 0.9	0.66 ± 0.08	5 712 ± 369	6.2 ± 1.1	4
H12	>20 000	1.14 ± 0.34 × 10 ⁸	6.27 ± 0.29	2.42 ± 0.09	4.10 ± 3.1	0.69 ± 0.07	29 500 ± 3874	4.9 ± 1.2	16
H16	>20 000	1.74 ± 0.46 × 10 ⁷	4.42 ± 0.21	2.56 ± 0.17	2.43 ± 1.9	0.68 ± 0.14	3 285 ± 300	6.3 ± 0.8	2
H19	>20 000	2.71 ± 0.21 × 10 ⁷	7.14 ± 1.18	2.31 ± 0.22	1.73 ± 0.5	0.59 ± 0.07	20 352 ± 1677	11.9 ± 2.2	0.5
H17	>20 000	2.30 ± 0.16 × 10 ⁴	8.36 ± 0.66	2.49 ± 0.08	2.49 ± 0.9	0.71 ± 0.12	4 410 ± 452	ND	4
H2	>20 000	2.27 ± 0.10 × 10 ⁵	5.68 ± 0.21	3.10 ± 0.11	2.21 ± 2.0	0.73 ± 0.16	10 667 ± 1221	184.6 ± 19.5	2
H6	>20 000	2.50 ± 0.24 × 10 ²	4.26 ± 1.10	2.27 ± 0.13	2.27 ± 0.1	0.70 ± 0.13	2 722 ± 103	6.5 ± 0.9	2
H11	>20 000	5.98 ± 0.45 × 10 ²	5.53 ± 0.10	2.55 ± 0.24	1.09 ± 0.3	0.70 ± 0.14	1 500 ± 198	108.0 ± 13.3	4

ND, None detected.

*P₂, precipitation zone ratio (see Methods).

Table 2. Pathogenicity of three virulent and three hypovirulent *C. neoformans* isolates in *nu/–* and *nu/nu* mice

Eight mice were infected with each of the virulent isolates, and ten mice with the hypovirulent isolates.

Virulent isolates	Survival rate in <i>nu/–</i> mice (%)	Mediandays to death in <i>nu/–</i> mice (range)	Survival rate in <i>nu/nu</i> mice	Days to death in <i>nu/nu</i> mice
V15	0	14 (8–23)	Not done*	Not done*
V18	0	10 (7–15)	Not done*	Not done*
V21	0	10 (6–14)	Not done*	Not done*
Hypovirulent isolates	Survival rate in <i>nu/–</i> mice (60 days) (%)	Days to death in <i>nu/–</i> mice	Survival rate in <i>nu/nu</i> mice (60 days) (%)	Days to death in <i>nu/nu</i> mice
H2	100	No deaths	100	No deaths
H6	90	Day 15 (1 death)	70	Days 9, 28, 29 (3 deaths)
H11	90	Day 16 (1 death)	50	Days 5, 8, 23, 24, 42 (5 deaths)

*The virulent isolates were not tested in *nu/nu* mice because they caused 100 % mortality within 23 days in immunocompetent mice (*nu/–*).

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 ($3 \times \text{LD}_{50}$ 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 ($250\text{--}1.01 \times 10^{11}$ c.f.u. g^{-1}) (Table 1); the median burden was 1.26×10^8 c.f.u. g^{-1} . Overall, we demonstrated that there was an inverse correlation between tissue burden and LD_{50} (i.e. higher tissue burden correlated with lower LD_{50}) ($P=0.038$; 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 P_z 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 LD_{50} and doubling time (i.e. lower LD_{50} was associated with shorter doubling time) ($P=0.0001$; non-parametric Spearman rank correlation test), and an inverse association between LD_{50} and capsule size *in vivo* ($P=0.028$; non-parametric Spearman rank correlation test). There was no correlation between LD_{50} and phospholipase, phenoloxidase and proteinase activities, capsule size measured *in vitro*, and fluconazole MIC.

Multivariate analysis including the factors found to be associated with LD_{50} by univariate analyses identified both the doubling time and capsule size *in vivo* as significantly

associated with LD₅₀ ($P=0.017$ 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 (1×10^7 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 sublethal inoculum of each isolate (5×10^6 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

Table 3. Tissue burdens following intranasal inoculation of mice

Tissue burden	Mean [log (c.f.u. g ⁻¹)]		P-value
	Isolate V7	Isolate H16	
Lung burden at 8 h	7.22 ± 0.16	7.19 ± 0.23	NS
Lung burden at day 3	7.81 ± 0.23	7.20 ± 0.34	0.002
Lung burden at day 7	8.20 ± 0.06	7.15 ± 0.17	<0.0001
Lung burden at day 14	8.36 ± 0.14	7.41 ± 0.16	<0.0001
Lung burden at day 21	8.57 ± 0.25	7.32 ± 0.73	<0.0001
Kidney burden at day 14	5.33 ± 0.60	4.18 ± 0.82	0.005
Kidney burden at day 21	5.62 ± 0.63	4.08 ± 1.44	0.0005
Brain burden at day 21	6.37 ± 0.50	5.60 ± 0.37	0.01

NS, Not significant.

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 LD₅₀ (range 51 to $>20\,000$ c.f.u.) and the recovery of viable organisms from infected brains (range $250\text{--}1.01 \times 10^{11}$ 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 LD₅₀. 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 (■).

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 LD₅₀ 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 vivo* correlated with LD₅₀ 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; LD₅₀ 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 LD₅₀ 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 vivo* (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 LD₅₀. 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 LD₅₀ (e.g. isolate V18; LD₅₀ 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 LD₅₀ 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 meningoencephalitis, 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.*, 2005). 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

- Andrade, V. S., Sarubbo, L. A., Fukushima, K., Miyaji, M., Nishimura, K. & de Campos-Takaki, G. M. (2002). Production of extracellular proteases by *Mucor circinelloides* using D-glucose as carbon source/substrate. *Braz J Microbiol* **33**, 106–110.
- Barchiesi, F., Cogliati, M., Esposto, M. C., Spreghini, E., Schimizzi, A. M., Wickes, B. L., Scalise, G. & Viviani, M. A. (2005). Comparative analysis of pathogenicity of *Cryptococcus neoformans* serotypes A, D and AD in murine cryptococcosis. *J Infect* **51**, 10–16.
- Blackstock, R. & Murphy, J. W. (1997). Secretion of the C3 component of complement by peritoneal cells cultured with encapsulated *Cryptococcus neoformans*. *Infect Immun* **65**, 4114–4121.
- Blackstock, R., Buchanan, K. L., Cherniak, R., Mitchell, T. G., Wong, B., Bartiss, A., Jackson, L. & Murphy, J. W. (1999). Pathogenesis of *Cryptococcus neoformans* is associated with quantitative differences in multiple virulence factors. *Mycopathologia* **147**, 1–11.
- Blasi, E., Barluzzi, R., Mazzolla, R., Mosci, P. & Bistoni, F. (1992). Experimental model of intracerebral infection with *Cryptococcus neoformans*: roles of phagocytes and opsonization. *Infect Immun* **60**, 3682–3688.
- Blasi, E., Barluzzi, R., Mazzolla, R., Tancini, B., Saleppico, S., Puliti, L., Pitzurra, M. & Bistoni, F. (1995). Role of nitric oxide and melanogenesis in the accomplishment of anticryptococcal activity by the BV-2 microglial cell line. *J Neuroimmunol* **58**, 111–116.
- Casadevall, A. & Perfect, J. R. (1998). *Cryptococcus neoformans*. Washington, DC: American Society for Microbiology.
- Casadevall, A., Rosas, A. L. & Nosanchuk, J. D. (2000). Melanin and virulence in *Cryptococcus neoformans*. *Curr Opin Microbiol* **3**, 354–358.
- Chang, Y. C. & Kwon-Chung, J. K. (1994). Complementation of a capsule-deficiency mutation of *Cryptococcus neoformans* restores its virulence. *Mol Cell Biol* **14**, 4912–4919.
- Chang, Y. C. & Kwon-Chung, J. K. (1998). Isolation of the third capsule-associated gene, *CAP60*, required for virulence in *Cryptococcus neoformans*. *Infect Immun* **66**, 2230–2236.
- Chang, Y. C. & Kwon-Chung, J. K. (1999). Isolation, characterization, and localization of a capsule-associated gene, *CAP10*, of *Cryptococcus neoformans*. *J Bacteriol* **181**, 5636–43.
- Chang, Y. C., Penoyer, L. A. & Kwon-Chung, K. J. (1996). The second capsule gene of *Cryptococcus neoformans*, *Cap64*, is essential for virulence. *Infect Immun* **64**, 1977–1983.
- Chen, L. C., Blank, E. S. & Casadevall, A. (1996). Extracellular proteinase activity of *Cryptococcus neoformans*. *Clin Diagn Lab Immunol* **3**, 570–574.
- Chen, S. C. A., Muller, M., Zhou, J. Z., Wright, L. C. & Sorrell, T. C. (1997). Phospholipase activity in *Cryptococcus neoformans*: a new virulence factor? *J Infect Dis* **175**, 414–420.
- Collins, H. L. & Bancroft, G. J. (1991). Encapsulation of *Cryptococcus neoformans* impairs antigen-specific T-cell responses. *Infect Immun* **59**, 3883–3888.
- Cox, G. M., McDade, H. C., Chen, S. C. A. & 8 other authors (2001). Extracellular phospholipase activity is a virulence factor for *Cryptococcus neoformans*. *Mol Microbiol* **39**, 166–175.
- Curtis, J. L., Huffnagle, G. B., Chen, G. H., Warnock, M. L., Gyetko, M. R., McDonald, R. A., Scott, P. J. & Toews, G. B. (1994). Experimental murine pulmonary cryptococcosis: differences in pulmonary inflammation and lymphocyte recruitment induced by two encapsulated strains of *Cryptococcus neoformans*. *Lab Invest* **71**, 113–126.
- Cutler, J. E. (1991). Putative virulence factors of *Candida albicans*. *Annu Rev Microbiol* **45**, 187–218.
- Dixon, D. M. & Polak, A. (1986). *In vivo* and *in vitro* studies with an atypical, rhinotrophic isolate of *Cryptococcus neoformans*. *Mycopathologia* **96**, 33–96, 40.
- Dong, Z. M. & Murphy, J. W. (1995). Effects of the two varieties of *Cryptococcus neoformans* cells and culture filtrate antigens on neutrophil locomotion. *Infect Immun* **63**, 2632–2644.
- Dykstra, M. A., Friedman, L. & Seed, B. (1977). Capsule size of *Cryptococcus neoformans*: control and relationship to virulence. *Infect Immun* **16**, 129–135.
- Feldmesser, M., Kress, Y. & Casadevall, A. (2001). Dynamic changes in the morphology of *Cryptococcus neoformans* during murine pulmonary infection. *Microbiology* **147**, 2355–65.
- Franzot, S. P., Mukherjee, J., Cherniak, R., Chen, L., Hamdan, J. S. & Casadevall, A. (1998). Microevolution of a standard strain of *Cryptococcus neoformans* resulting in differences in virulence and other phenotypes. *Infect Immun* **66**, 89–97.
- Fries, B. C. & Casadevall, A. (1998). Serial isolates of *Cryptococcus neoformans* from patients with AIDS differ in virulence for mice. *J Infect Dis* **178**, 1761–1766.
- Fromtling, R. A., Abruzzo, G. K. & Ruiz, A. (1988). *Cryptococcus neoformans*: a central nervous system isolate from an AIDS patient that is rhinotropic in a normal mouse model. *Mycopathologia* **102**, 72–86.
- Garcia-Hermoso, D., Dromer, F. & Janbon, G. (2004). *Cryptococcus neoformans* capsule structure evolution *in vitro* and during murine infection. *Infect Immun* **72**, 3359–3365.
- Grosse, G., Mishra, S. K. & Staib, F. (1975). Selective involvement of the brain in experimental murine cryptococcosis. II. Histopathological observations. *Zentralbl Bakteriol* **230**, 518–533.
- Hasenclever, H. F. & Mitchell, W. O. (1960). Virulence and growth rates of *Cryptococcus neoformans* in mice. *Ann N Y Acad Sci* **89**, 156–62.

- Iwata, K., Yamashita, T., Ohsumi, M., Baba, M., Naito, N., Taki, A. & Yamada, N. (1990). Comparative morphological and biological studies on the itraconazole- and ketoconazole-resistant mutants of *Cryptococcus neoformans*. *J Med Vet Mycol* **28**, 77–90.
- Jacobson, E. S. & Emery, H. S. (1991). Catecholamine uptake, melanization, and oxygen toxicity in *Cryptococcus neoformans*. *J Bacteriol* **173**, 401–403.
- Janbon, G. (2004). *Cryptococcus neoformans* capsule biosynthesis and regulation. *FEMS Yeast Res* **4**, 765–771.
- Kagaya, K., Yamada, T., Miyakawa, Y., Fukazawa, Y. & Saito, S. (1985). Characterization of pathogenic constituents of *Cryptococcus neoformans* strains. *Microbiol Immunol* **29**, 517–532.
- Kozel, T. R. (1995). Virulence factors of *Cryptococcus neoformans*. *Trends Microbiol* **3**, 295–299.
- Kwon-Chung, K. J. & Rhodes, J. C. (1986). Encapsulation and melanin formation as indicators of virulence in *Cryptococcus neoformans*. *Infect Immun* **51**, 218–223.
- Kwon-Chung, K. J., Polacheck, I. & Popkin, T. J. (1982). Melanin-lacking mutants of *Cryptococcus neoformans* and their virulence for mice. *J Bacteriol* **150**, 1414–1421.
- Kwon-Chung, K. J., Wickes, B. L., Stockman, L., Roberts, G. D., Ellis, D. & Howard, D. H. (1992). Virulence, serotype, and molecular characteristics of environmental strains of *Cryptococcus neoformans* var. *gattii*. *Infect Immun* **60**, 1869–1874.
- Leighton, T. J., Doi, R. H., Warren, R. A. J. & Kelln, R. A. (1973). The relationship of serine protease activity to RNA polymerase modification and sporulation in *Bacillus subtilis*. *J Mol Biol* **76**, 103–122.
- Littman, M. L. & Tsubura, E. (1959). Effect of degree of encapsulation upon virulence of *Cryptococcus neoformans*. *Proc Soc Exp Biol Med* **101**, 773–776.
- Macher, A. M., Bennett, E., Gadek, J. E. & Frank, M. M. (1978). Complement depletion in cryptococcal sepsis. *J Immunol* **120**, 1686–1690.
- Mitchell, T. G. & Perfect, J. R. (1995). Cryptococcosis in the era of AIDS – 100 years after the discovery of *Cryptococcus neoformans*. *Clin Microbiol Rev* **8**, 515–548.
- National Committee for Clinical Laboratory Standards (1997). *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts*. Approved standard. Document M-27A. Wayne, PA: National Committee for Clinical Laboratory Standards.
- Nielsen, K., Cox, G., Wang, P., Toffaletti, D., Perfect, J. R. & Heitman, J. (2003). Sexual cycle of *Cryptococcus neoformans* var. *grubii* and virulence of congenic α and α isolates. *Infect Immun* **71**, 4831–4841.
- Nosanchuk, J. D. & Casadevall, A. (2003). Budding of melanized *Cryptococcus neoformans* in the presence or absence of L-dopa. *Microbiology* **149**, 1945–1951.
- Noverr, M. C., Williamson, P. R., Fajardo, R. S. & Huffnagle, G. B. (2004). *CNLAC1* is required for extrapulmonary dissemination of *Cryptococcus neoformans* but not pulmonary persistence. *Infect Immun* **72**, 1693–1699.
- Polacheck, I., Hearing, V. J. & Kwon-Chung, K. J. (1982). Biochemical studies of phenoloxidase and utilization of catecholamines in *Cryptococcus neoformans*. *J Bacteriol* **150**, 1212–1220.
- Polak-Wyss, A. (1991). Protective effect of human granulocyte colony-stimulating factor (hG-CSF) on *Cryptococcus* and *Aspergillus* infections in normal and immunosuppressed mice. *Mycoses* **34**, 205–215.
- Retini, C., Vecchiarelli, A., Monari, C., Tascini, C., Bistoni, F. & Kozel, T. R. (1996). Capsular polysaccharide of *Cryptococcus neoformans* induces proinflammatory cytokine release by human neutrophils. *Infect Immun* **64**, 2897–2903.
- Rhodes, J. C., Polacheck, I. & Kwon-Chung, K. J. (1982). Phenol-oxidase activity and virulence in isogenic strains of *Cryptococcus neoformans*. *Infect Immun* **36**, 1175–1184.
- Rivera, J., Feldmesser, M., Cammer, M. & Casadevall, A. (1998). Organ-dependent variation of capsule thickness in *Cryptococcus neoformans* during experimental murine infection. *Infect Immun* **66**, 5027–5030.
- Salas, S. D., Bennett, J. E., Kwon-Chung, K. L., Perfect, J. R. & Williamson, P. R. (1996). Effect of the laccase gene *CNLAC1*, on virulence of *Cryptococcus neoformans*. *J Exp Med* **184**, 377–86.
- Vanbreuseghem, R. (1967). White mice sensitivity to *Cryptococcus neoformans*. Production of resistance against a cerebral inoculation. *Ann Soc Belg Med Trop* **47**, 281–294.
- Van Cutsem, J., Fransen, J., Van Gerven, F. & Janssen, P. A. J. (1986). Experimental cryptococcosis: dissemination of *Cryptococcus neoformans* and dermatropism in guinea pigs. *Mykosen* **29**, 561–575.
- Vecchiarelli, A., Retini, C., Pietrella, D., Monari, C., Tascini, C., Beccari, T. & Kozel, T. T. (1995). Down-regulation by cryptococcal polysaccharide of tumor necrosis factor alpha and interleukin-1 beta secretion from human monocytes. *Infect Immun* **63**, 2919–2923.
- Vecchiarelli, A., Retini, C., Monari, C., Tascini, C., Bistoni, F. & Kozel, T. R. (1996). Purified capsular polysaccharide of *Cryptococcus neoformans* induces interleukin-10 secretion by human monocytes. *Infect Immun* **64**, 2846–2849.
- Velez, J. D., Allendoerfer, R. R., Luther, M., Rinaldi, M. G. & Graybill, J. R. (1993). Correlation of in vitro azole susceptibility with in vivo response in a murine model of cryptococcal meningitis. *J Infect Dis* **168**, 508–510.
- Williamson, P. R. (1997). Laccase and melanin in the pathogenesis of *Cryptococcus neoformans*. *Front Biosci* **2**, e99–e107.