Virulence profile of strains of *Cryptococcus neoformans* var. *grubii* evaluated by experimental infection in BALB/c mice and correlation with exoenzyme activity

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To evaluate the virulence profile of strains of *Cryptococcus neoformans* var. *grubii*, 62 strains of this yeast were inoculated into BALB/c mice. It was found that 69 % of the strains were significantly more lethal to the mice and were recovered from a higher percentage (60 %) of the organs compared with the other 31 % of the strains, which were recovered from 35 % of organs tested. Those strains that provoked higher death rates were also recovered from the central nervous system at a higher rate (84 %) than the less lethal strains (32 %). This finding led to an investigation of the factors that enhanced the capacity for neurological infection and death of the animals. The results of this study suggested that environmental strains present different degrees of virulence. The correlation of exoenzyme production before and after inoculation and between the groups of mice indicated that exoenzyme production had no influence on differences in virulence among the strains studied.

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INTRODUCTION

Cryptococcus neoformans is an opportunistic pathogenic yeast, commonly encountered in the excrement of pigeons and other birds (Montenegro & Paula, 2000; Baroni, 2001), which can be spread through the air and cause infection in humans, mainly in the lungs and central nervous system (Buchanan & Murphy, 1998; Casadevall & Perfect, 1998; Lacaz *et al.*, 2002). *C. neoformans* is the aetiological agent of cryptococcosis, a subacute and chronic systemic mycosis of cosmopolitan nature, with a tropism for the central nervous system (Casadevall & Perfect, 1998). In Brazil, according to Dias *et al.* (2003), *Cryptococcus* infection continues to be a lethal disease related to AIDS and, in the last few decades, cryptococcosis has assumed a prominent role at the public health level due to the growing number of AIDS cases (Pappalardo & Melhem, 2003). Most infections are caused by serotype A (Casadevall & Perfect, 1998). *C. neoformans* shows a remarkable genotypic diversity in Brazil (Barreto de Oliveira *et al.*, 2004).

Research has revealed various virulence factors inherent to *C. neoformans*, including melanin (Doering *et al.*, 1999),

production of exoenzymes (phospholipase and proteinase) (Brueske, 1986; Chen *et al.*, 1997; Vidotto *et al.*, 2000) and the presence of a capsule (Cox *et al.*, 2001; Steenbergen & Casadevall, 2003).

Recent studies have demonstrated that the production of phospholipase B, lysophospholipase and lysophospholipase transacylase is correlated with virulence of the yeast in mice and that these three enzymes are more active at temperatures ranging from 25 to 40 °C (Santangelo *et al.*, 1999).

As in other yeast-related diseases, proteinase production contributes to the virulence of *C. neoformans* (Casadevall & Perfect, 1998). Strains of *C. neoformans* isolated from patients with AIDS show good growth in yeast carbon base agar supplemented with 0.1 % BSA and 0.01 % polypeptide. Proteolytic activity has been detected in the exponential phase of yeast growth, where a decrease in the amount of BSA occurs (Aoki *et al.*, 1994).

The present study was aimed at verifying the virulence profile of strains of *C. neoformans* var. *grubii* isolated from the environment and correlation of virulence with the

production of phospholipase and proteinase by the strains before and after experimental infection.

METHODS

C. neoformans strains and serotypes. This study involved a total of 62 strains of *C. neoformans* isolated from pigeon excreta (Baroni, 2001) and one virulent strain of *C. neoformans* (ATCC 90112). Strains were identified as *C. neoformans* var. *grubii* serotype A (Franzot *et al.*, 1999) by previously described methods (Kurtzman & Fell, 1998) and serotyped by agglutination serotyping performed using eight factor-specific sera (Iatron) and interpreted according to the description of Ikeda *et al.* (1982).

Animals, experimental infection and recovery of strains. A total of 195 male BALB/c mice were used, weighing 20–25 g, supplied by and maintained at the Biotery of Isogenic Animals of ICB/USP. The animals were divided into 65 groups of three mice each, and 62 of the groups were inoculated with one of the 62 strains of *C. neoformans*. The three remaining groups were as follows: one was inoculated with strain ATCC 90112 as a positive control, one was inoculated with saline solution and one was kept without inoculation as a negative control. Soon after their isolation from the environment (5 days), strains were inoculated into mice through the intravenous route (0.1 ml) at a concentration of 10^6 c.f.u. ml⁻¹. One mouse from each group was subjected to necropsy. If more than one mouse died in a group, one of the dead mice was chosen at random for the necropsy; if no mice died, one mouse was sacrificed at random for the necropsy on day 50 post-inoculation.

Organs (heart, lungs, liver, kidneys, spleen, testicles, eyeballs and brain) were removed for evaluation. Each organ was washed three times with sterilized saline solution and then macerated and cultured on medium containing dopamine (Kurtzman & Fell, 1998). Incubation was carried out at 32 °C for 15 days. After growth of the brown to black colonies, one colony cultured from each organ was selected for serotype identification and evaluation of exoenzyme production. This experiment was conducted in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and was approved by the Ethical Committee for Animal Research of the Biomedical Sciences Institute/USP.

Identification of recovered strains and serotyping. Yeast colonies were identified by standard mycological techniques based on morphology (presence of a capsule assessed by staining with India ink), colour characteristics on DOPA medium, assimilation of carbohydrates and the production of urease (Kurtzman & Fell, 1998). Isolates were stored at -20 °C in 20 % glycerol until experiments were performed (Franzot *et al.*, 1998). Agglutination serotyping was performed using a standard serotyping kit (Iatron).

Exoenzyme production

Phospholipase. The randomly chosen isolates were subcultured on to fresh Sabouraud dextrose agar medium (Difco) and incubated at 32 °C for 48 h. After this period, they were cultured in triplicate on the surface of medium containing peptone, glucose, sodium chloride, calcium chloride and egg yolk for verification of phospholipase production. After 10 days of incubation, the diameter of the colony (a) and that of the colony plus its precipitation zone (b) were measured. Phospholipase activity was expressed as $Pz = a/b$. Thus, a high Pz value indicated low production of phospholipase. The mean Pz value was obtained from three separate samples of each strain (Price *et al.*, 1982).

Proteinase. Readings were taken daily for 10 days and enzyme activity was measured according to the technique described by Price

et al. (1982). Proteinase production (Ruchel *et al.*, 1982) was verified after previous cultivation of the samples on Sabouraud dextrose agar (Difco). Samples were cultured in medium containing yeast carbon base (Difco), BSA (fraction V; Sigma) and Protovit (Roche) and the mean Pz value was determined as for phospholipase.

Statistical analysis. Student's *t*-test was used to check for significant differences between the production of exoenzyme before and after inoculation, while Fisher's exact test was used to determine the significance of recovery of yeast from the organs, with statistical significance set at $P < 0.05$ (Altman, 1997).

RESULTS

Of the 62 strains isolated from the environment, 43 (69 %) led to the death of two or three animals in their group and were thus classified as highly virulent, while 19 (31 %) led to the death of one or no animals and were classified as low-virulence strains. The mean time from inoculation of the animals to their death was 24 days.

Recovery of *C. neoformans* from mouse organs post-inoculation

For the groups infected with strains classified as highly virulent, yeast was recovered from 60 % of the organs sampled, with a mean of 4.6 organs per animal. For the low-virulence strains, yeast was recovered from 35 % of the organs sampled, with a mean of 2.8 organs per animal. This difference was statistically significant ($P < 0.05$).

The rate of recovery of *C. neoformans* by organ is shown in Table 1, which revealed a statistically significant higher rate of recovery from the central nervous system (brain and eyeball) in animals inoculated with strains classified as highly virulent compared with those inoculated with low-virulence strains.

Production of exoenzymes

Table 2 shows the mean production of exoenzymes for the high- and low-virulence strains, before and after inoculation.

Table 1. Rates of recovery of *C. neoformans* from organs of animals experimentally inoculated with environmental strains of high and low virulence

Organ	High-virulence strains (%)	Low-virulence strains (%)
Heart	65	37
Liver	58	58
Lungs	65	47
Kidneys	63	58
Spleen	49	42
Testicles	37	21
Eyeball	51*	11*
Brain	84*	32*

*Statistically significant difference ($P < 0.05$).

Table 2. Mean production of the exoenzymes proteinase and phospholipase by 62 strains of *C. neoformans*

Results are expressed as the Pz value.

Strains	Proteinase		Phospholipase	
	Before	After	Before	After
High virulence	1.0	0.93	0.67	0.65
Low virulence	1.0	0.95	0.74	0.58

Of the strains classified as highly virulent, prior to inoculation 95 % produced phospholipase with a Pz value ranging from 0.45 to 1.0 and a mean of 0.67 ± 0.15 . After inoculation, 81 % of the strains produced this enzyme with a Pz value ranging from 0.32 to 1.0 and a mean of 0.65 ± 0.21 . Of the strains classified as low virulence, 80 % produced phospholipase before inoculation, with a Pz value ranging from 0.50 to 1.0 and a mean of 0.74 ± 0.16 . After inoculation, 81 % of the strains produced this enzyme, with a Pz value ranging from 0.26 to 1.0 and a mean of 0.58 ± 0.23 . The differences in the production rates of this enzyme before and after inoculation were not statistically significant, nor were the differences between the groups.

No strain produced proteinase before inoculation. After inoculation, 24 % of the highly virulent strains demonstrated proteolytic activity, with a Pz value ranging from 0.23 to 1.0 and a mean of 0.93 ± 0.16 . Of the strains classified as low virulence, 11 % demonstrated proteolytic activity, with a Pz value ranging from 0.26 to 1.0 and a mean of 0.95 ± 0.08 . Between the groups, the difference in the production rate of this enzyme was not statistically significant; however, the difference between production before and after inoculation was statistically significant.

DISCUSSION

There were significant differences in the virulence patterns associated with the *C. neoformans* environmental strains that were evaluated. These differences could be observed in their different abilities to cause death of the mice. Upon necropsy, the most virulent strains were more likely to be recovered from the central nervous system, suggesting that factors that promote neurological infection were associated with increased lethality.

Phospholipase is considered a potential factor related to the virulence of *C. neoformans* (Chen *et al.*, 1997) and has been directly linked to the pathogenicity of this yeast (Huerfano *et al.*, 2003; Noverr *et al.*, 2003). According to Vidotto *et al.* (2000), clinical samples isolated from AIDS patients are capable of producing more phospholipase than environmental ones, suggesting that enzyme production may be associated with host-invasion mechanisms.

In this study, we showed similar results for phospholipase production by environmental strains before and after

inoculation. Thus, it appears that production of this enzyme does not interfere with establishment of the yeast in the host, nor in the occurrence of central nervous system infection, in agreement with Santangelo *et al.* (2004). We propose that this enzyme acts as a helping factor in the infection process, but with no single predominant effect. Likewise, Buchanan & Murphy (1998), Cox *et al.* (2001) and Vidotto *et al.* (2005) have associated this enzyme with pathogenicity of the yeast.

The importance of phospholipase production in *C. neoformans* virulence is not yet known (Ghannoum, 2000). Additional studies will be essential to understand the importance of production of this enzyme and the correlation between enzyme production and pathogenicity and virulence.

None of the strains produced proteinase before inoculation of the mice, whereas all strains were pathogenic to the animals. Thus, proteinase production does not appear to be associated with *C. neoformans* virulence and its ability to invade the host. Production of the enzyme after inoculation suggests the importance of this enzyme as a survival factor of the yeast, as described by Aoki *et al.* (1994), who also observed this activity after a period of *in vivo* growth. It appears that the gene responsible for the production of this enzyme can be activated under certain conditions found only in the infection process. Possibly, proteinase can enable *C. neoformans* invasion into the host tissues during the infection process (Buchanan & Murphy, 1998; Vidotto *et al.*, 2005).

The activities of proteinase and phospholipase were studied as possible markers of virulence in the strains, as previous studies have indicated that these exoenzymes are important factors in virulence (Brueske, 1986; Chen *et al.*, 1997; Vidotto *et al.*, 2000). Despite earlier reports, in this study no association could be found between enzymic activities and virulence of the strains; there was, however, an association between proteolytic activity and growth of the strains *in vivo*.

Strains with neurotropic characteristics are considered to be the most virulent and have been found to be associated with the presence of other factors related to virulence such as urease (Olszewski *et al.*, 2004), synthesis of melanin-like pigments (Liu *et al.*, 1999; Zhu *et al.*, 2001; Noverr *et al.*, 2004) and capsule production (Cox *et al.*, 2001; Steenbergen & Casadevall, 2003). It is possible that these factors may have interfered with the occurrence of neurological and eyeball infections in our study.

In this study, central nervous system infection was the overriding factor in causing death of the mice and thus factors related to this type of infection need to be evaluated further to establish new and efficient virulence markers in *C. neoformans*.

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