Does titan cell morphology confer innate fluconazole resistance to *Cryptococcus neoformans*?

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Introduction

Cryptococcus neoformans is an opportunistic pathogen that infects primarily immunocompromised persons. Although AIDS patients are the population most frequently afflicted with these infections, but other immunocompromised populations are susceptible. Approximately a million cases of cryptococcal meningitis occur worldwide with an estimated 600,000 people succumbing to this disease (Park et. Al 2009). Infection primarily occurs by contact with soil that has been contaminated with bird excrement (Sorrel and Ellis 1997).

Treatment of Cryptococcal meningitis can be arduous. Protocol for the treatment of AIDS patients usually starts with a course of amphotericin b administered intravenously, and flucytosine may be included in the treatment as well (Van der Horst et al. 1997). In order to prevent infection relapse, orally administered azoles are typically prescribed. Without the use of maintenance therapy, relapse rates of infection can be as high as 60% (Powderly et al. 1992).

Fluconazole is a first generation triazole based compound used in the treatment of a wide variety of fungal infections. The antifungal activity of these agents comes from the way they target and disrupt the ergosterol synthesis pathway in the fungus. Ergosterol is a critical component of the fungal plasma membrane and is functionally analogous to human cholesterol, maintaining fluidity and structure of the membrane (Bowman and Free 2006). Triazoles inhibit cytochrome P-450-dependant 14α-demethylase activity, a key step in the biosynthesis of ergosterol. This activity leads to a distortion of the cell membrane, and the accumulation of ergosterol precursors within the cell (Ghannoum et al. 1994). While this activity is primarily fungistatic against *C. neoformans*, at higher concentrations the activity is fungicidal (Torres-Rodríguez et al. 2008).

Perhaps the most interesting aspect of *C. neoformans* is its ability to adopt a morphology known as the titan cell. These cells are remarkably different from the standard *C. neoformans* cell type in that they can be much larger, being up to a hundred micrometers in diameter. The cell wall can be 20-30 times thicker and their capsules are more robust as they are highly crosslinked. Titan cells are also always polyploid. *C. neoformans* achieves this morphology by undergoing endoreplication, where the chromosomal material is copied, but the nucleus does not divide. Tetraploid and octoploid titan cells are common, though titan cells bearing as many as 64 chromosomal copies have been documented, a dramatic difference considering the standard cells are usually haploid or diploid (Zaragoza and Nielson 2013). Endoreplication likely evolved in *C. neoformans* as a response to predation from amoebas (Casedevall et al. 2003). Due to their large size, they cannot be engulfed by amoebas such as *Acanthamoeba castellani*. This evolutionary trait has implications for infection in animals, because like the predatory ameobas, the macrophages of the host immune system will engulf foreign entities. Titan cells have in fact been found to resist engulfment by alveolar macrophages, and thusly, have been implicated in establishing infection. The titan cells have been observed to appear as early as 24hrs of infection in the mammalian lung environment and have been shown to persist throughout the course of infection (Okagaki et al. 2010).

The question explored within this study is that the properties of the titan cell morphology confer fluconazole resistance to *Cryptococcus neoformans*. Both the thicker cell wall, the larger and highly crosslinked capsule could prevent compounds from penetrating the yeast cell, and the greater ploidy could mean that there is a greater amount of protective transcription afforded to the cell. If titan cells are indeed drug resistant, this would provide one plausible mechanism by which relapse can happen in infected patients as this cell type could persist in the body following primary treatment.

Methods

The strain selected for this experiment was strain WM628 supplied by ATCC. WM628 is a hybrid strain of both A and D serotypes, with this sample being originally isolated from an Australian AIDS patient. This strain of *C. neoformans* was selected because it contained both a and α mating types. In cases where both mating types are present, the proportion of titan cells doubles, rising from 20% to 40% of the total cell population (Okagaki et al 2011). This selection increases the probability of obtaining a large population of titan cells.

Both potato-dextrose agar (PDA) and yeast-peptone agar (YPD) were used for growth of solid cultures of the fungus. To create a stock suspension, 20mL of RPMI 1640 was inoculated with the fungus and placed in the shaker for 24hrs at 130rpm. All growth was done at 25^{o}C to avoid the formation of desiccation of yeast cells as these cells are the means by which the majority of infections occur.

The drug used to test this organism was Fluconazole. The sample was obtained from Sigma-Aldrich, catalogue number F-031. To establish a baseline to which the resistance of the titan cells could be compared to, the susceptibility of the standard cell type to Fluconazole had to be examined. Before testing the susceptibility of the stock fungus to Fluconazole, the amount of colony forming units in the original suspension had to be determined. Five serial dilutions were performed, starting with a 1/20 dilution, followed by 1/10 dilutions performed for the subsequent four solutions. 100µL from each dilution was then plated and incubated for 48hrs. The number of colonies formed was then were then counted, and from there, the concentration of colony forming units in the original suspension was calculated. For the susceptibility series, a master suspension of fungi had to be prepared. The desired concentration of cells for this experiment was 2.5x10^{3}CFU/mL. Seven conditions were tested for the susceptibility of the fungus with triplicates. The conditions were 0, 10, 20, 40, 60, 80, and 100µg/mL of Fluconazole. Once the wells had all been filled, the fungus was incubated for 48hrs. These were then plated and incubated for another 48hrs, after which the number of colonies formed was scored.

In order to induce the titan cell morphology, the fungus was incubated inside larvae of the greater wax moth *Galleria mellonella*. These waxworms were obtained from Petsmart. Prior to infection, the abdomen of the worm was cleaned with 70% ethanol. Eleven waxworms in total were each injected with s10µL of fungal suspension using a 28ga syringe. Following injection, the worms were placed in the incubator at 25^{o}C and left for a week. The worms were then examined.

Results

Examination of the stock *Cryptococcus neoformans* under a microscope revealed that the cells had very small capsules. Cells varied in size, ranging from 5µm to 10µm at the largest. No titan cells were observed in the standard cell population.

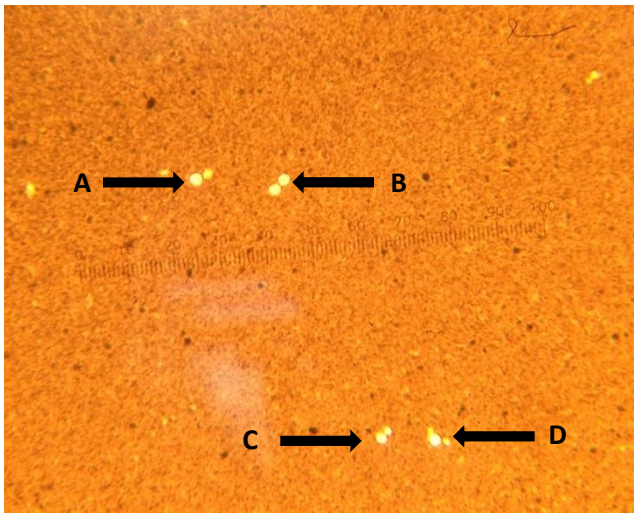

Fig. 1. India ink stain of *Cryprtococcus neoformans* from stock fungal suspension. Image was taken at 400x magnification. Cell groups are indicated with arrows. Larger cells seen in annotations A, C, and D are of the standard cell type while the smaller cells are young cells that have recently formed by budding.

With the particular range of concentrations of Fluconazole examined, the MLC of this strain of *C. neoformans* could was not determined. The MLC was designated as where there would be fewer than 3

CFU appearing on the plate. This cut-off point never observed however. It appeared not to matter what concentration the Fluconazole the fungus was treated with, as the amount of colony forming units was effectively the same regardless of the concentration of Fluconazole present.

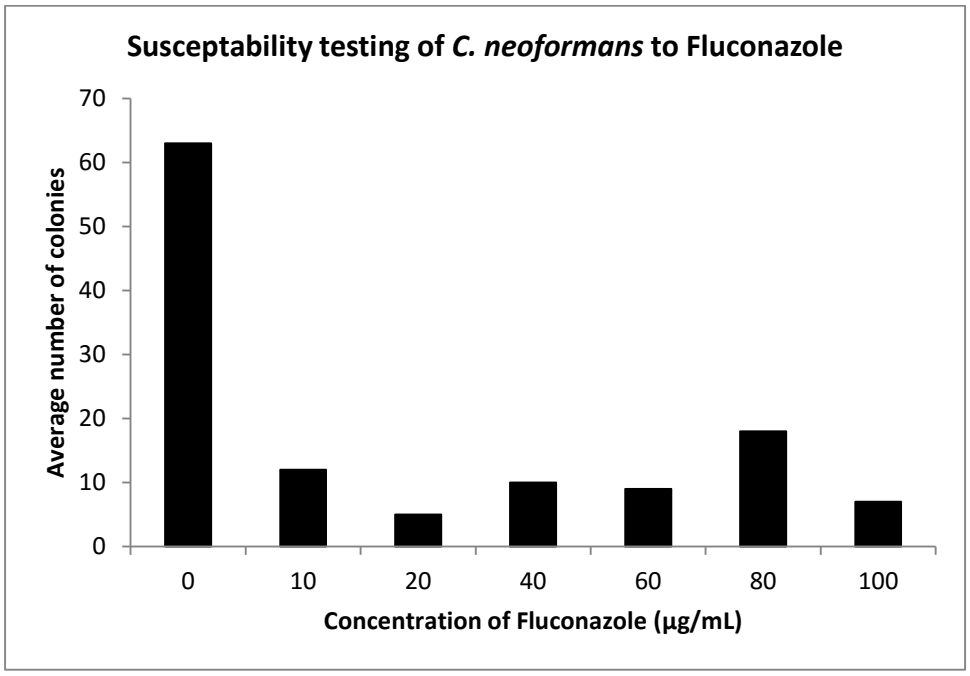

Fig. 1. Average number of *C. neoformans* colonies formed on YPD after incubation for 48hrs with different concentrations of Fluconazole. Concentrations measured in µg/mL.

After a week in the incubator, the waxworms were pulled out and examined. All of the worms were dead and necrotized, and, being slightly shrivelled, the worms had the consistency of hard raisins. A proportion of the lymph within their body had leaked out, forming a sticky film that glued the worms to the inside of the tube. The worms were deemed unusable and extraction of the *Cryptococcus* from their lymph was not pursued.

Discussion

The results from the initial susceptibility testing appear demonstrate some background level of resistance to the Fluconazole in this strain of cells. Previous research has the found the MLC of *C. neoformans* to Fluconazole to be around 80µg/mL (Casadevall et al. 1993). This value was the benchmark for what was expected to be observed. There is a clear killing effect as the introduction of as little as 10µg/mL dramatically reduced the number of colonies formed when compared to the control. Therefore, considering the response of this strain to the Fluconazole, the selection of the particular strain and drug makes for an ill control. It would have been a better to either use a different strain of the fungus, or to use a different drug. Amphotericin B is one possible alternative drug that could have been used as this compound is the first line drug used in the treatment of cryptococcal meningitis. It has a more pronounced killing effect on the fungus, with a MLC of around 0.58µg/mL (Casadevall et al. 1993). Amphotericin B was not used in this study due to its extreme toxicity in humans. Accidental exposure to the Amphotericin could lead to damage to multiple organ systems in the body, being neurotoxic, nephrotoxic and can lead to cardiopulmonary damage (Laniado-Laborín and Cabrales-Vargas 2009). In speculating as to why strain W628 was resistant to the drug, it is a pretty safe inference that this resistance cannot be attributed to the formation of titan cells. Cells were not found to be any larger than 10µm so something else might be happening here.

Incubating *C. neoformans* inside waxworms was not a good method to induce titan cell formation. Though literature previously published by García-Rodas and others in 2011 had shown that cell enlargement does occur following injection of *G. mellonella* with the fungus, it was unrealistic to expect that a pure population of *Cryptococcus* cells could be extracted from the worm, and that this population could be segregated into both titan and standard cell types. The approach is simply too messy with all the cellular detritus and bacterium that would be present following pulverisation and filtration of the waxworms. Furthermore, the volume of fungal inoculum that can be injected into the worms is rather limited. Even if the purification of the fungus from were to be achieved, how would the

proper concentrations of titan cells be determined? These cells can vary widely in size, depending on the number on endoreplications they've done so a photo spectrometer would not be able to be used.

Were this question to be pursued again in a future study, a very different strategy would be pursued. While *Galleria mellonella* does has its place as a model for studying the pathogenesis of the fungus, however, the course of infection in a mouse model is a far better representation of the progression of the disease that occurs in human cases. All ethical considerations aside for the wellbeing of the animal, the titan cell question would be better pursued in this organism. Strains of *C. neoformans* have been found that produce a much greater frequency of titan cells during infection in comparison to the type strain. Non-titan cell forming strains have also been discovered. In a revised approach to this study, immunocompromised mice would be infected with one of these three strains. One strain that could be used is designated *otc 1* Δ and is an overproducer of titan cells. *otc 1* Δ has been found to commit over 50% of its total population in the mammalian lung environment (Okagaki, Nielson 2012). Okagaki and Nielson state that the enhancement in titan cell production is attributable to a mutation in a single gene. The exact gene where this mutation has occurred is unknown. In a future mouse study examining the whether or not titan cells confer some drug resistance to the organisms, and excellent test could be performed if this mutation was identified. With the identification of this mutation, a standard strain of the *C. neoformans* could be given the mutation using CRISPR technology. CRISPR could also be used to delete that same strain for the titan cell phenotype. Exploring this approach to an in-vivo experiment would make for a much better test as it would allow to control for any differences in virulence factors that may be present between the otherwise different strains.

Conclusions

The design of this study was inadequate to answer the questions posed within. Over the course of the study however, aspects about the behaviour of *C. neoformans* were learned that give clues as to ways in which the question of drug resistance in titan cells could be studied. With a larger budget, and access to vertebrates, mouse studies could be pursued looking at the course of treatment following infection with strains of *C. neoformans* that commit a different percentage of their cell population to the titan cell morphology. Ultimately, the question of whether or not the titan cell morphology confers drug resistance to the fungus, thereby providing a plausible mechanism by which relapse can occur following primary treatment remains unknown.

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