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Aging as an emergent factor that contributes to phenotypic variation in *Cryptococcus neoformans*

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Abstract

Cryptococcus neoformans, similar to other eukaryotes, undergoes replicative aging. Replicative life spans have been determined for clinical *C. neoformans* strains, and although they are a reproducible trait, life spans vary considerably among strains. *C. neoformans* has been proposed as an ideal model organism to investigate the contribution of replicative aging in a fungal pathogen population to emerging phenotypic variation during chronic cryptococcal infections. *C. neoformans* cells of advanced generational age manifest a distinct phenotype; specifically, a larger cell size, a thicker cell wall, drug resistance, as well as resistance to hydrogen peroxide-mediated killing. Consequently, old cells are selected in the host environment during chronic infection and aging could be an unanticipated mechanism of pathogen adaptation that contributes to persistent disease. Aging as a natural process of phenotypic variation should be further studied as it likely is also relevant for other eukaryotic pathogen populations that undergo asymmetric replicative aging.

Keywords

C. neoformans; aging; pathogen; phenotypic variation

INTRODUCTION

Studies on aging have traditionally focused on fungi, such as *Saccharomyces cerevisiae* (Kaeberlein, 2010; Kennedy and Guarente, 1996; Smith et al., 2007) and *Schizosaccharomyces pombe* (Roux et al., 2010b; Roux et al., 2006) with the goal to improve human longevity. However, recent studies in pathogenic fungi, such as *Candida albicans* (Fu et al., 2008) and *Cryptococcus neoformans* (Bouklas et al., 2013; Cordero et

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al., 2011; Jain et al., 2009a) have focused on other aspects and revealed effects of aging on phenotypic variation in these pathogens. Specifically, studies have investigated how age-related phenotypic changes alter virulence-associated traits and change host pathogen interactions. Importantly, this work indicated that selection of older generation cells within the infecting pathogen population occurs throughout chronic infection. The resulting phenotypic variation and biological advantage conferred on old cells could affect the outcome and promote persistent disease. Thus, aging of eukaryotic pathogen populations may contribute to phenotypic variation, which is an unanticipated mechanism of pathoadaptation and should be further studied in relation to virulence and host pathogen interaction.

Phenotypic variation in *C. neoformans*

Over the years, several studies indicated that during the process of infection, *C. neoformans* can undergo "microevolution," which produces phenotypic variants with altered virulence (Guerrero and Fries, 2008; Gupta and Fries, 2010; Jain et al., 2006a; McFadden et al., 2007; Ormerod et al., 2013; Pietrella et al., 2003). Initially, evidence for this phenomenon was derived from investigations with serial isolates from patients with chronic cryptococcosis. Comparison of virulence of these strains demonstrated significant differences in experimental murine infections (Fries and Casadevall, 1998) and also changes in the polysaccharide capsule (Cherniak et al., 1995). Extensive variability of capsule volume and induction was noted among clinical strains and found to correlate with phagocytosis indices (Zaragoza et al., 2003a). In addition, *in vivo* analysis of infecting fungal population in the murine host demonstrated dynamic capsule sizes and antibody binding patterns (Garcia-Hermoso et al., 2004). Furthermore, more pronounced capsule induction was observed in the lung environment when compared to capsules on *C. neoformans* cells in the brain environment (Rivera et al., 1998; Vartivarian et al., 1993). Capsular polysaccharide changes were shown to affect many aspects of this anti-opsonic shield. Capsular changes have been, for instance, associated with differing binding patterns of capsular antibodies, complement (Charlier et al., 2005), as well as eliciting different immune responses (Cheng et al., 2009), and importantly altering phagocytosis by macrophages (Zaragoza et al., 2003b). The latter is of particular importance because phagocytosis is important for the transmigration of *C. neoformans* across the blood-brain barrier, and therefore these studies predicted that capsule changes would affect dissemination (Charlier et al., 2009; Shi et al., 2010). It is important to emphasize that cell size variation can also occur independent of capsule induction. Increasing numbers of cells with larger body sizes were observed in tissue of mice chronically infected with *C. neoformans* (Feldmesser et al., 2001). Giant *C. neoformans* cells have been further investigated and are now referred to as titan cells (Okagaki et al., 2010; Zaragoza et al., 2010; Zaragoza and Nielsen, 2013). These cells are 30 – 50 μm large and polyploid. A less dramatic, but nonetheless significant and consistent cell size enlargement, is observed in the course of replicative aging of *C. neoformans* cells (Bouklas et al., 2013).

Replicative aging creates phenotypic variants within a fungal population

Cell size increase has been a hallmark of generational aging in all eukaryotic yeast cells examined to date (Bilinski and Bartosz, 2006; Yang et al., 2011), but certainly not all cell

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size differences reflect generational differences. The cell size increase in aging *C. neoformans* cells is much less impressive (6 – 15 μm) compared to that of titan cells. However, the ensuing size increase is observed consistently in all clinical *C. neoformans* strains examined to date (Figure 1). Analogous to older *S. cerevisiae* mother cells, the cell body size of replicating *C. neoformans* mother cells increases throughout the process of replicative aging (Jain et al., 2009a). *C. neoformans* strain cell size increase was found to be more gradual than described for *Candida albicans* (Jain et al., 2009a) and variable among strains (Bouklas et al., 2013). In *S. cerevisiae,* birth size has been shown to dictate the maximal size that is reached by cells at death (Yang et al., 2011). Specifically, a small birth size predicted a greater number of divisions, and hence a longer replicative life span in a systematic study of *S. cerevisae* mutants. However, in *C. neoformans*, birth size and replicative life span were uncorrelated in 360 examined cells derived from clinical and environmental *C. neoformans* strains (Figure 1). Similarly, in the prokaryotic pathogen, *Mycobacterium smegmatis*, birth and elongation lengths were uncorrelated (Aldridge et al., 2012). It is conveivable that birth size as a limitation to life span may be lost in pathogens, especially those that also exploit intracellular growth niches. In pathogens, complex selection mechanisms may affect cell size, which appears to be an emerging rather than a predetermined trait. More studies need to be done to shed light on the molecular mechanisms that regulate cell size in *C. neoformans*.

Another distinct finding of *C. neoformans* cells is that their bud scars (Woyke et al., 2002) heal during replication, such as during melanin-defect repairs (Nosanchuk and Casadevall, 2003), or chitooligosaccharide production (Rodrigues et al., 2008). Consequently, the cell wall of older *C. neoformans* cells does not become weak as in older *S. cerevisiae* cells, in which it can be successfully stained for bud scars (Powell et al., 2003). Instead, the *C. neoformans* cell wall grows thicker with age (Figure 2), which likely contributes to the resilience of older cells. Cell size increase of older cells proportionally affects both capsule and cell body, and thus is not the result of an over-induced capsule. With increase of cell size, the ability of older *C. neoformans* cells to resist phagocytosis by macrophages increases when compared to the younger and smaller cells of that strain; dramatic differences were observed within 10 generations (Bouklas et al., 2013). In addition, more pronounced inhibition of killing by macrophages was observed for older cells, and was most likely the result of enhanced resistance to hydrogen peroxide, which was also documented for older cells. Doubling times only change dramatically in the last third of the life span, and are not greatly affected in very young cells or in middle aged cells.

Phenotypic switching in older cells and its implications for phenotypic variation

C. neoformans strains also undergo phenotypic switching (Fries et al., 1999; Fries et al., 2001; Goldman et al., 1998; Jain et al., 2005), which is defined as the spontaneous emergence of colonies with an altered colony morphology at a rate that is higher than that of somatic mutation (Slutsky et al., 1985). These phenotypic switch variants show enhanced virulence (Fries et al., 2001; Goldman et al., 1998; Guerrero et al., 2010; Jain et al., 2006a; Jain et al., 2009b; Jain et al., 2006b), a finding corroborated by similar observations in other fungal pathogens, such as *C. glabrata* (Lachke et al., 2002; Srikantha et al., 2005). Interestingly, a recent study (Bouklas et al., 2013) showed that phenotypic switching to a

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hypervirulent switch variant was also accompanied by a significant loss of replicative life span in the hypervirulent variant compared to the parent, which was at least partially reverted. Thus, life span and phenotypic switching may be regulated by overlapping complex epigenetic regulation, which ultimately could be activated *in vivo*. Furthermore, studies have also shown that aging of *C. neoformans* cells is accompanied by an increased phenotypic switching rate, which most likely is the result of age-related genomic instability (Jain et al., 2009a). These findings indicate that the emergence of older cells by itself or in concert with phenotypic switching could increase the heterogeneity of the pathogen population during infection. Sophisticated mathematical modeling has been done (Bouklas et al., 2013; Jain et al., 2009a) to assess the impact of this process.

Drug resistance in older cells and its implications for phenotypic variation

Time killing curves of young and old cells with different drug concentrations have shown that old *C. neoformans* cells resist killing by antifungals, such as amphotericin B (AMB) and fluconazole, better than young cells (Jain et al., 2009a). These data have also been confirmed in more recent studies (Bouklas et al., 2013) with clinical *C. neoformans* strains that were directly isolated from cerebrospinal fluid (CSF) of patients. Dramatic differences in killing were observed even with cells that were aged for 10 generations (0 – 50% killing of old cells compared to 100% killing of young cells with sub-therapeutic levels of AMB). Especially, resistance to AMB could be clinically relevant as this drug has poor central nervous system (CNS) penetration, and the fungus is potentially exposed to sub-therapeutic concentrations. Future studies will need to determine whether increased resistance of old *C. neoformans* cells to antifungal agents is the result of phenotypic drug tolerance, genetic drug resistance, or both. Electron microscopy images of old *C. neoformans* cells have documented a thickened cell wall in older cells (Figure 2), which could lead to insufficient drug penetration, a possible mechanism by which increased resistance is conferred upon older cells. It would also be reasonable to study the cell wall composition of older cells, which could have an altered sugar composition (Reese et al., 2007) or even lipid deposition (Rodrigues et al., 2007), and potentially serve as a trapping ground for antifungals before they can reach the cell membrane. Increased age could also lead to genomic instability (Jain et al., 2009a; McMurray and Gottschling, 2003), which in return may promote heteroresistance (Sionov et al., 2010) and chromosomal loss (Hu et al., 2011; Li et al., 2012; Ormerod and Fraser, 2013).

Comparison of "old cells" and titan cells

Of the described morphological forms of *C. neoformans*, old cells could be mistaken for titan cells. Titan cells, however, occur as early as 24 hours after inoculation in murine pulmonary infection, are 5 to 10 times larger than infecting *C. neoformans* cells, and are therefore not phagocytosed by macrophages (Okagaki and Nielsen, 2012). In contrast, old cells, which rise to a size approximately 30 – 63% larger than infecting cells are smaller than titan cells. They emerge late after weeks of infection in spinal fluids of rats because they are rare and require selection. Titan cells manifest an altered capsule, cell wall, ploidy, and resistance to nitrosative and oxidative stress (Zaragoza and Nielsen, 2013). Old cells are larger, but their size still permits uptake by macrophages, even though less easily with increasing age (Bouklas et al., 2013). Similar to titan cells, they demonstrate an altered cell

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wall (Figure 2), and resistance to oxidative stress (Bouklas et al., 2013). Whether these cells have increased ploidy or nitrosative resistance needs to be determined. Genome duplication, for instance, can occur in the absence of cell division during endoreplication in *S. cerevisiae*, where large cells with ploidy have also been observed (Kondorosi et al., 2000). Both titan and old cell morphologies may demonstrate a strategy for *C. neoformans* to generate phenotypic variation at different points and sites of infection. Titan cells, which account for 20% of cryptococcal cell population during pulmonary infection may be more relevant to earlier infection; whereas old cells, for which proportion is modeled to be high in meningitis may be more relevant to later CNS infection.

Selection of phenotypic variants within a cell population

Phenotypic variation can affect the entire fungal population, a subset of the fungal population, or only individual cells; the latter two require selection in order to dominate within a fungal population. Phenotypic changes, such as universal capsule enlargement commonly constitute a global response to an environmental signal like low iron, glucose, or carbon dioxide levels, and therefore this change manifests in all cells of the fungal population. As long as the environmental signal is operative, selection is not required. In contrast, epigenetically-regulated changes of the polysaccharide capsule that occur in the setting of phenotypic switching affect a smaller proportion of the fungal population. However, these capsular changes are stable and inherited by the progenies, and because they confer a biological advantage *in vivo*, the switch variants with a large mucoid capsule are selected and accumulate. Although significant differences in doubling times of *C. neoformans* variants are observed (Jain et al., 2006a; Jain et al., 2009b), host selection still contributes to their emergence because the slower growing mucoid variants persist and outgrow the faster growing parent smooth cells (Fries et al., 2001; Jain et al., 2009a).

One aspect that is unique about emerging older cells within a cryptococcal pathogen population is the fact that the trait being of “old age” is a global natural change that occurs eventually in all cells, but is not inherited by the next generation (Figure 3). Inheritance of “old age” trait occurs in *S. cerevisiae* cells only in the last 30% of their life span (Kennedy et al., 1994), or in long lived mutants (Delaney et al., 2011), and likely does not matter for the middle aged *C. neoformans* population that persists in the host during chronic infection (Figure 4). Therefore, the probability of emerging “old cells” is actually low unless significant selection occurs and young generations are preferentially killed, whereas older generations survive. Hence, high selection pressures that include the host immune cells and antifungal treatment contribute to the persistence of old cells, and shift the generational pyramid of pathogen population. In other fungi, there appears to be a trade-off between reproduction and longevity, as evidenced by *S. cerevisiae* mutants that extend life span, but reduce reproduction and fitness (Delaney et al., 2011). Mathematical modeling was used to show that these mutants had significant defects in their fitness that resulted from reduced maximal growth rate and a cell-cycle delay. Whether this is true in *C. neoformans* will need to be examined.

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Phenotypic variation of *C. neoformans* in the host environment

Ideally relevance of phenotypic variations observed *in vitro* should be investigated in the human host environment. In patients with chronic cryptococcosis, high cryptococcal burden, neurocognitive impairment, and poor fungal clearance are markers of death and adverse outcome (Bicanic et al., 2009; Dromer et al., 2007). The factors responsible for these surrogate markers are still poorly understood. It is conceivable that the ability of *C. neoformans* to undergo phenotypic variation contributes to differences in clinical outcomes. The capsule has been established to be an important determinant of phenotypic variation (McFadden et al., 2007). Raised intracranial pressure (ICP) during chronic infection has been attributed to capsule shedding and/or the capsule on the organism itself (Fries et al., 2005; Robertson et al., 2014). Recent investigations have examined phenotype variation for the first time in *C. neoformans* cells derived directly from the spinal fluid (*ex vivo*) of infected patients enrolled in a standardized treatment trial in an attempt to correlate capsule size with ICP, fungal clearance, and CSF inflammation (Robertson et al., 2014). These data confirmed that the *C. neoformans* population in the spinal fluid is more heterogeneous than *in vitro* propagated *C. neoformans* populations. Thus variation of cell and capsule size is more consistent with an evolved population exposed to diverse selection pressures during chronic infection. Noteworthy, despite extensive size variation, titan cells (> 30 μm) were only observed in a small percentage (3.6%) of samples and seemed to occur in a subfraction of patients. Larger *ex vivo* capsule size always remained associated with ICP and decreased inflammatory response. In contrast, the degree of capsular polysaccharide shedding did not correlate with ICP, and most importantly *ex vivo* and *in vitro* capsule size did not correlate.

Another highly relevant finding with respect to phenotypic variation in clinical strains is that high fungal uptake into macrophages *in vitro* was associated with high CSF fungal burden in the patient who was infected with that strain and ultimately the patient's survival (Sabiiti et al., 2014). As predicted by *in vitro* data (Zaragoza et al., 2008), high fungal uptake strains in this study had smaller capsules. In clinical strains "high fungal uptake strains" also manifested enhanced laccase activity, which would likely confer a higher degree of melanization. To date, laccase activity in old *C. neoformans* cells has not been compared to that in young cells. Interestingly, intracellular proliferation rate (IPR) was inversely correlated with uptake. This finding may also explain why younger smaller cells are more easily phagocytosed compared to older cells, but do not have a higher IPR, unless compared to extremely old cells. In the host, high fungal uptake may be important to improve transmigration across the BBB or dissemination to various organs, and the low IPR may result in a lower inflammatory response. Last, it is noteworthy that phenotypic switching has been observed in serotype A, D, and C strains, and in all cases was shown to result in variants with altered virulence (Guerrero et al., 2010; Jain and Fries, 2008). Recent analysis of clinical strains (Robertson et al., 2014) yielded predominantly smooth colony morphologies and established switched colonies were not detected on the plates (n = 200 to 1000 colonies plated) (T. Bicanic, personal communication).

Experimental challenges

Unfortunately to this date, technical methods are not available to establish the precise generational age of *C. neoformans* cells in human specimens. However recent data from our

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laboratory (Bouklas et al., 2013) addressed that question by determining the age of individual *C. neoformans* cells recovered from the spinal fluid and brains of chronically-infected rats. As discussed, generational age of *C. neoformans* cells cannot be determined by bud scar count alone as done in *S. cerevisiae* (Bouklas and Fries, 2013; Powell et al., 2003), but has to be determined indirectly through correlation of remaining life span with the overall life span potential of an individual *C. neoformans* strain. In addition many traits, such as mating and melanization are investigated in growing populations, which by default generates young cells and creates a generationally mixed yeast population. Consequently a causative relation cannot be established between the investigated trait and old phenotype. The above study established that *ex vivo* fungal populations derived from rats with chronic CNS cryptococcosis contain old cells as they accumulated through selection over the course of infection. Such a shift in generational distribution could also be demonstrated in two *C. neoformans* populations derived from spinal fluid of treated and untreated humans. While the study controlled for *in vivo* stresses that may result in adaptation to a shortened RLS, we acknowledge that epigenetic or genetic re-programming could have lead to a shortened RLS. An independent mechanism to identify older cells would be most beneficial. Additionally, demonstrating this in other pathogens, as well as further investigations on the transcriptional signature of old cells may shed some light on this issue, and help circumvent the challenge of more precise markers for the generational age of *C. neoformans* cells.

Relevance of replicative aging for phenotypic variation in other pathogens

Aging research in *C. neoformans* (Bouklas et al., 2013; Jain et al., 2009a) has demonstrated considerable variation in replicative life span among clinical strains and highlighted the importance of aging for the outcome of infection. Phenotypic variation is commonly described in encapsulated organisms, including *Klebsiella*, *Neisseria,* and *Staphylococcus*, which are easy to detect as they commonly change colony morphologies (Poolman et al., 1985; Proctor et al., 2006; Randall, 1939); however, this is certainly not limited to encapsulated organisms. Phenotypic changes as a consequence of aging have also been described for other eukaryotic organisms that are not encapsulated, such as *Candida albicans* (Fu et al., 2008) and *Schizosaccharomyces pombe* (Roux et al., 2010a). Now the relevance for pathogenesis has to be further investigated in other pathogenic fungi, such as *Candida* spp. It is conceivable that this unanticipated mechanism of generating phenotypic variation may even be relevant for prokaryotes, and may be exclusive of capsular or cell wall changes. It appears that cells of *Mycobacterium* species undergo acentric division and asymmetrical polar growth to give rise to unequal daughters (Singh et al., 2013), leading to differences in elongation lengths and growth rates (Joyce et al., 2012) that differentially affect the antibiotic susceptibilities, at least for *M. smegmatis* (Aldridge et al., 2012). The relevance of asymmetric division to other bacteria remains to be studied, but has been suggested in *Caulobacter crescentus*, where each division leads to one flagellated “swarmer” cell and one immotile “stalked” cell (Tsokos and Laub, 2012). Thus, in both eukaryotic and prokaryotic pathogens that undergo some form of asymmetric division, there is a potential for generation of phenotypic variants.

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CONCLUSIONS

Fungal pathogens, such as *C. neoformans*, continue to evade the host immune system by successfully undergoing phenotypic variation during chronic infection. Hence, despite the use of widespread antifungal therapy, they continue to persist. In order to treat such persistent infections, it may be time to reassess our approaches to studying the pathogenesis of this fungal pathogen. The ability of the pathogen to replicate and age in the host poses a new challenge and adds another layer of complexity to the pathogenesis of *C. neoformans*. Thus, a reductionist approach to study variations that are inherited by the progeny may not be sufficient in light of the growing evidence, which indicates that not all variations are inherited. The distinct phenotype of old cells could confer an unanticipated virulence trait that could be relevant to the pathogenesis of other eukaryotic, and even some prokaryotic pathogens. Thus, aging should be further investigated in relation to host pathogen interactions.

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Highlights

- *Cryptococcus neoformans* is capable of generating phenotypic variants during chronic infection.
- *C. neoformans* undergoes replicative aging, which results in old cells with a distinct and conceivably advantageous phenotype.
- Old cells have been shown to be selected *in vivo*.
- Aging could generate phenotypic variation and contribute to cryptococcal persistence.

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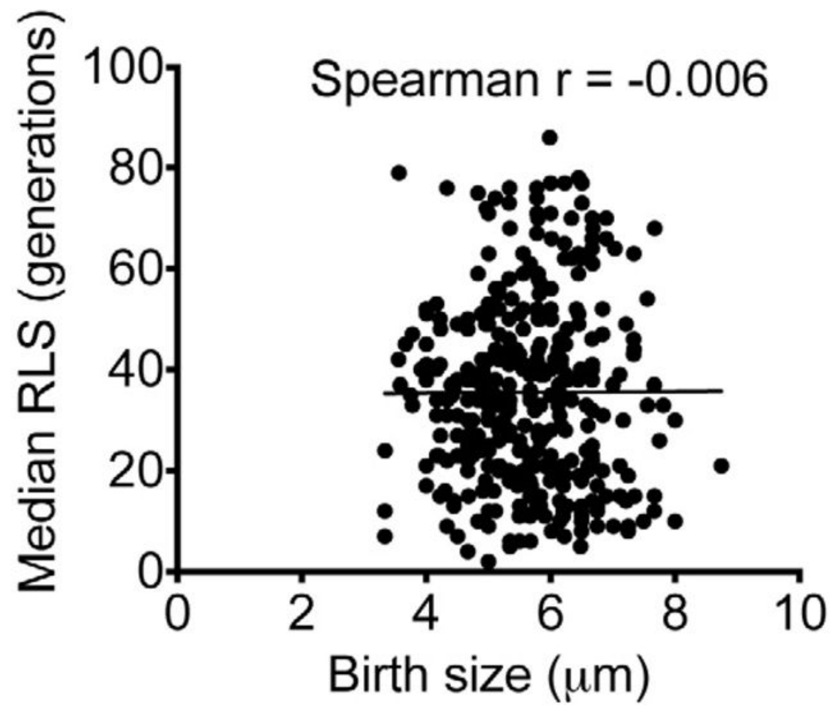

Figure 1. Birth size of clinical *C. neoformans* cells does not correlate with the strain's replicative life span (RLS)

Birth cell body size of *C. neoformans* cells (n = 360) did not correlate with the median RLS of the strain (Spearman r = –0.006).

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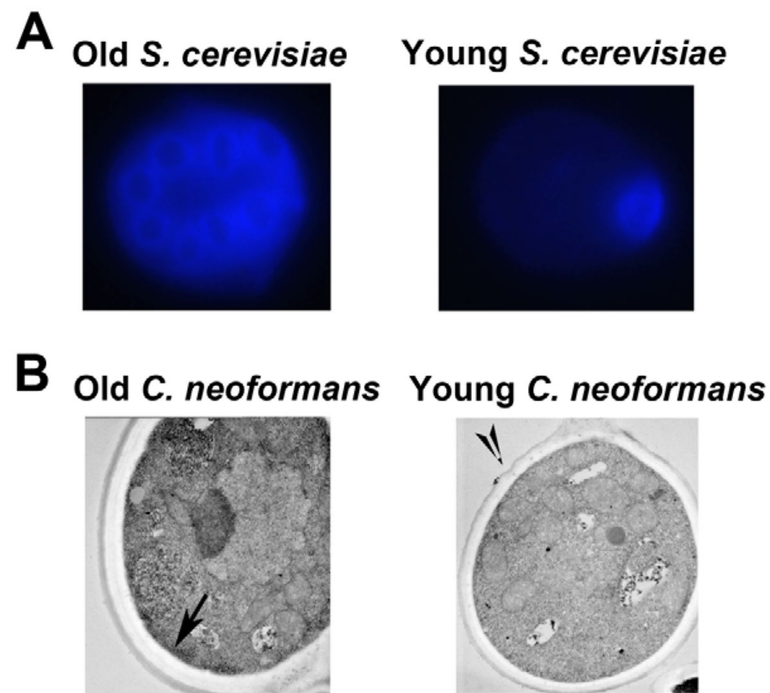

Figure 2. The cell walls of old *C. neoformans* cells are not weakened with age unlike those of old *S. cerevisiae* cells

(**A**) Old *S. cerevisiae* cells can be stained with calcofluor for bud scars, which weaken the cell wall with each division. (**B**) Old *C. neoformans* cells cannot be accurately stained for bud scars, and the cell wall appears to thicken with each division.

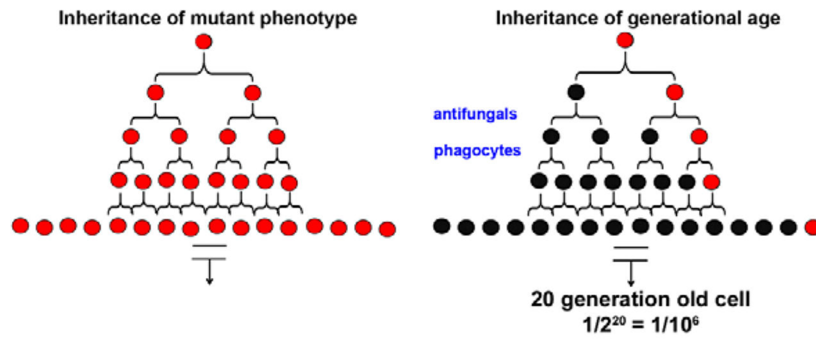

Figure 3. Schemata demonstrating the rare probability of finding old cells

The probability of finding a mutant phenotype (red) in a clonally expanding population is 1/1 regardless of the number of replications because the phenotype is inherited by all progeny. However, the probability of finding a cell with a specific age (20-generation-old cell in red) is rare, and is $1/10^6$ in a population that has replicated 20 times.

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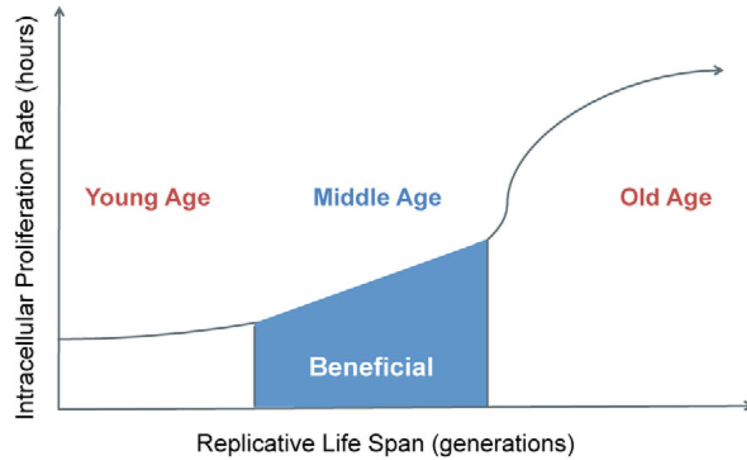

Figure 4. A model that depicts the relationship between replicative age and intracellular proliferation rate of *C. neoformans*

The pathogen is most likely to benefit during middle age, when it has acquired the old age phenotype and still proliferates at a rate comparable to that of young cells.