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***C. neoformans* constitutes an ideal model organism to unravel the contribution of cellular aging to the virulence of chronic infections**

Tejas Bouklas¹ and Bettina C. Fries^{1,2}

¹Department of Microbiology and Immunology, Albert Einstein College of Medicine of Yeshiva University, 1300 Morris Park Avenue, Bronx, New York 10461

²Department of Medicine (Division of Infectious Diseases), Albert Einstein College of Medicine of Yeshiva University, 1300 Morris Park Avenue, Bronx, New York 10461

Abstract

Aging affects all organisms, from unicellular yeasts to multicellular humans. Studies in model organisms demonstrate that the pathways that mediate the two forms of aging, replicative and chronological, are highly conserved. Most studies are focused on the effect of aging on an individual cell rather than a whole population. Complex longevity regulation, however, makes aging a highly adaptive trait that is subject to natural selection. Recent studies have shed light on the potential relevance of aging in fungal pathogens, which undergo replicative aging when they expand in the host environment. Hence, pathogens causing chronic infections can constitute ideal model organisms in unraveling the contribution of selection to aging within a population and help elucidate the contribution of aging itself to the virulence of infections.

Keywords

C. neoformans; aging; pathogen

INTRODUCTION

Aging affects the very existence of humans, and hence the majority of aging research has focused on deciphering molecular mechanisms to improve human lifespan [1]. Large genetic screens in model organisms, such as *Saccharomyces cerevisiae* [2,3], *Schizosaccharomyces pombe* [4], and *Caenorhabditis elegans* [3] have demonstrated that many pathways of longevity are conserved in higher order organisms, including mammals [5]. Limited studies have also been done in *Candida albicans* [6], *Kluyveromces lactis* [7] and most recently *Cryptococcus neoformans* [8,9]. Most aging studies in fungi have focused on investigating the mechanisms and regulation of aging in individual cells, rather than its effect on the fungal population itself. Emerging work in the pathogenic fungi, *C. albicans* and *C. neoformans* indicates that studies on replicative aging should be expanded to study the effect

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Corresponding author mailing address: Bettina C. Fries, MD, Department of Medicine, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Ullmann 1203, Bronx, New York 10461, Phone: (718) 430-2365 Fax: (718) 430-8968
bettina.fries@einstein.yu.edu.

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of aging on phenotypic variation within a pathogen population. This review will outline why aging could matter for virulence in eukaryotic pathogens.

Replicative versus chronological aging

The term aging has to be defined precisely and for the purpose of this review definitions employed in *Saccharomyces cerevisiae* research will be used [10,11]. This yeast has been used as a model for aging since the 1950's when Mortimer and Johnston first described its finite lifespan [12]. In that study, replicative lifespan (RLS) was defined as the number of mitotic divisions a cell undergoes before death. RLS is quantified in generations and is different from chronological lifespan (CLS), which measures the time in days that starving yeast cells survive non-replicating after transitioning through the stationary growth phase [13,14]. These two forms of aging have now become well established in other fungi, including the fission yeast *Schizosaccharomyces pombe* [15,16].

High throughput screening has been devised to study chronological aging [17]. In contrast, investigations on replicative aging are predominately done by manual separation of daughter cells from mother cells using a dissection microscope. From genetically manipulated *S. cerevisiae* strains, large numbers of senescent cells can also be isolated [18–20]. From several large scale studies, it has been concluded that replicative and chronological aging are linked [21,22], but other studies in *S. cerevisiae* involving mutants also suggest that the two are controlled independently [2,23–25].

Chronological aging and its potential role in virulence

CLS is likely to contribute to virulence because microbial persistence is highly relevant in chronic infections. Activation of regulatory pathways controlling chronological aging during chronic infection may allow fungal pathogens to persist in nutritionally deprived host environments. Dense biofilms [26] in candida pseudo membranes, or cryptococcomas [27,28] constitute niches where yeast cells neither grow nor die. One *in vitro* study on chronological aged *C. neoformans* cells found that the capsule of the fungus, a major virulence factor, is dramatically altered under such conditions [9]. Phenotypic and antigenic variation in the capsule was proposed to contribute to the ability of *C. neoformans* to persist in host tissue. Recent advances in understanding the complex role of autophagy in fungal infections are also relevant for chronological aging (reviewed in [29]). Autophagy, a lysosome-mediated catabolic process of eukaryotic cells to digest their own constituents, is required for chronological longevity in *S. cerevisiae* [30]. In non-dividing cells, autophagic breakdown is thought to contribute to long-term survival and longevity extension because it is an important mechanism to remove damaged organelles and proteins, which accumulate during aging [31]. For *C. neoformans* a facultative intracellular pathogen [32], autophagy-mediated nutrient recycling is necessary to propagate within the phagolysosome of macrophages [33], whereas ingested *C. albicans* yeast cells undergo hyphal transition, thereby escaping from the phagolysosome [34]. In addition, elegant molecular typing studies in *C. neoformans* have in fact demonstrated that active disease originates from fungal cells that infected the host decades earlier and remained dormant in granulomas before the patient acquired HIV [35] when the latent infection was reactivated. In these cases, chronological aging and its regulation could also be highly relevant to maintaining persistence in latent disease.

Replicative aging in pathogenic yeasts

Human pathogenic fungi predominantly reside in the environment and become accidental pathogens when their spores invade the human host [36,37]. Most pathogen populations in the host establish themselves through clonal expansion even though some parasites also require sexual replication in order to maintain their life cycle (reviewed in [38]). Even if

many fungi retained the ability to replicate sexually [39], this appears to occur infrequently and only in the environment. The life cycle of *Pneumocystis* is thought to include both an asexual phase and a sexual phase [40], but studies on aging would be too challenging without the ability to grow it *in vitro*. While *C. albicans* mating has been observed on the skin of mice [41], it is still unclear where or when mating might take place in humans. Hence, given that most fungi undergo only asexual reproduction in the host, replicative aging is likely to occur during infection and thus could be relevant to the establishment of a pathogen population in the host.

To date, replicative aging has been investigated in the two pathogenic fungi, *C. neoformans* [8] and *C. albicans* [6], both of which replicate by budding every 1–3 hours. For *C. neoformans*, cells of advanced generational age can be isolated by elutriation in combination with streptavidin-conjugated magnetic beads that bind to biotin labeled cell walls [42,43]; however, in *C. albicans*, hyphae formation in older cells interferes with magnetic bead selection. Therefore, elegant methods were devised that took advantage of the fact that *C. albicans* has both a hyphal and a yeast morphology that can be easily separated by sucrose gradient centrifugation [6]. Growth of hyphal morphology is induced by serum *in vitro* in replicating mothers that are then switched to a non-inducing medium and bud off yeast cells. This method isolates large amounts of relatively pure old mothers with hyphal cell morphology. While budding on solid medium, both forms can be dissected under the microscope, which permits precise determination of an RLS in individual yeast cells of clinical strains.

Analogous to older *S. cerevisiae* mother cells, the cell body size of replicating *C. neoformans* and *C. albicans* mother cells increases throughout the process of replicative aging [8]. Although in that study, *C. neoformans* cell size increase was more gradual (7.3 ± 0.71 to 9.1 ± 1.2 μm at 24 generations) than that of aging *C. albicans* cells, which almost doubled in size in only 12 generations (4.3 ± 0.4 μm to 7.6 ± 1.7 μm at 12 generations). Of note is that bud scar counts did not reliably identify the generational age of *C. albicans* and *C. neoformans* cells (Figure 1).

Relevance of replicative aging for the pathogenesis of infectious diseases

A clonally expanding pathogen population that invades the host can cause damage to the host, and to circumvent this, the host mounts a defense that serves as a powerful force capable of selecting for genotypic and phenotypic variants within the original pathogen population. Consequently, this host-pathogen interaction promotes co-evolution of host and pathogen populations, likely altering the course of this confrontation.

Recent investigations on aging in prokaryotic and eukaryotic pathogens have shed light on how aging may impact virulence of a pathogen population in the setting of chronic disease [6,8,44]. Similar to *S. cerevisiae* [45], *C. neoformans*, and *C. albicans* divide asymmetrically, as part of which the mother cell acquires an increasing cell size and cell wall thickness. Interestingly, size measurements on electron microscopy images of *C. neoformans* cells in infected murine tissue demonstrated gradually increasing cell size, as well as cell wall thickness in the course of a chronic murine infection [46]. Recent data from our laboratory showed that *in vitro* aged *C. neoformans* cells have a selective advantage over younger cells and are more resistant to phagocytic uptake and antifungal killing [8]. In particular, they were more resistant to phagocytic uptake and to killing by the antifungal drugs, amphotericin B and fluconazole (Figure 2A–B). At the same time, older cells were able to establish a central nervous system (CNS) infection in mice. Their larger size did not interfere with transmigration of the blood brain barrier, or alternatively older cells were more resistant in circulation and thus able to bud off younger smaller daughter cells that then crossed the blood brain barrier, which is essential to establish CNS infection [47,48]. Similar

resistance to killing by caspofungin of older *C. albicans* cells (Figure 2C) was observed. Although aging in prokaryotic and eukaryotic organisms is fundamentally different and cannot be compared, recent studies in *Mycobacterium smegmatis* indicate that older bacterial cells are similarly more resistant to antimicrobial agents [44]. Furthermore, opposed to passaged laboratory strains, natural yeast populations typically manifest heterogeneous lifespans, which means that within one population, some cells live long and others die early (Figure 3). This natural heterogeneity in RLS also allows for selection of variants with either long or short RLS. Recent investigations on long-lived *S. cerevisiae* mutants indicate that prolonged longevity comes at the cost of fitness early in life [49], which could be detrimental for a pathogen that has to establish an infection in a defensive host environment. In summary, experimental data indicates that old age-associated phenotypic changes convey a biological advantage and in human hosts treated with antimicrobials, this could be highly relevant to the course of chronic infection, where pathogens, such as *C. neoformans* reproduce asexually.

Challenges in the investigation of aging in pathogenic fungi

The above findings raise important questions; for example, can the long or short RLS of a strain affect the virulence of a pathogen and would cells of advanced replicative age even accumulate during chronic infection in the host?

A straightforward reductionist approach would not allow conclusions on the effect of RLS on virulence. *S. cerevisiae* data demonstrates that RLS is regulated by global regulators [50,51] and although many of them share homologs in *C. neoformans* and *C. albicans* (Table 1), their loss also commonly compromises growth, or stress resistance; hence, their virulence cannot be compared to the wild type strain in animal models. For example, in *C. neoformans*, *tor1Δ* [52] mutants are not viable, the *ras1Δ* mutants exhibit growth defects at 37 °C [53], and in *C. albicans* these mutants lack serum induced hyphal differentiation [54]. Also *sch9Δ* mutants exhibit reduced mating in *C. neoformans* or hyphal formation in *C. albicans* [55,56]. Therefore, lifespan related effects could not be independently evaluated in these mutants.

Studying the function of these genes in “naturally” aged cells is also inherently difficult because they are exceedingly rare. In a logarithmically growing yeast strain with an average RLS of 20 replications, 50% of the population is composed of virgin cells, 25% is one generation old, and only 1 in 2 million cells is 20 generations old. Even after prolonged growth, they transition into stationary growth phase and then arrest, and most cells are only a few generations old [21]. This raises also the question if old cells would even accumulate during chronic infection. Ideally, one would establish a reproducible experimental system that allows for the study of “mature” pathogen populations, which have been actively dividing over many generations and therefore have yielded a wide generational distribution within the emerging pathogen population. Chronic infection models in susceptible hosts may constitute a good system to investigate if aging occurs in expanding pathogen populations. Furthermore, selection forces on regulatory pathways of aging could be studied because the pathogen population continues to replicate over weeks and at the same time, an effective host response constantly kills off microbes and prevents early saturation of the population. Selection of specific generations would be reflected in an altered generational distribution in the emerging population. Both for *C. neoformans* and *C. albicans*, chronic infection models have been established in rodents. In *C. neoformans* the chronic meningoencephalitis (CME) model in rats accurately mimics the human disease [57]. Inocula as low as 100 cells are sufficient to establish chronic infection over weeks in spinal fluid. The cisterna magna can be tapped to retrieve small amounts of spinal fluid, and the yeast can be easily recovered and visualized in the fluid without sacrificing the animal. For *C. albicans*, catheter related urinary tract infection models [58] similar to the CME model allow access to the pathogen in

urine for visualization and culture of pathogens. However, *C. albicans* is not a commensal in mice, and therefore this model does not mimic many aspects of the human host response to candiduria.

Despite the advantages, major challenges are still encountered when studying the relevance of microbial aging for virulence and outcome of infection. The caveat to a chronic infection model is that doubling times and resulting generations are difficult to estimate as we lack precise methods to gauge the degree of killing of a pathogen by the host. At the same time, as outlined above, bud scar stains and quantification as a direct measurement of generational age is not reliable in these pathogenic yeasts. In addition, the heterogeneity of RLS [59], as well as the fact that age is an emerging trait that is not inherited [60] within a pathogen population requires sophisticated modeling strategies that incorporate both fecundity and fitness. Lastly, aging may vary based on location since certain host niches, such as the spinal fluid may be low in glucose, which may have an independent effect on replicative aging [61,62].

CONCLUSIONS

In humans, dramatic changes are seen within one generation, most likely because humans are multi-cellular organisms, and aging represents the sum of all individual aging events. For resilience of human populations, the distribution of young, midlife, and old members matters for overall fitness. In a unicellular organism like yeast, measurable phenotypic changes of old age occur only after many generations. Phenotypic changes that are associated with old age could potentially be beneficial for the pathogen. The effect on the overall resilience of the pathogen population may be small as the progeny does not inherit the phenotype of old age. It may, however, be significant if the young progenies are preferentially killed and old cells are left behind.

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HIGHLIGHTS

- Aging is evolutionarily conserved among organisms from all orders.
- Replicative and chronological aging are two distinct forms of aging.
- Nutrition and genetic background variably affect both forms of aging.
- Aging affects whole populations and may matter for virulence in chronic infection.
- Several challenges exist in the study of aging, especially in pathogen populations.

***S. cerevisiae* (strain Fy86)**

0-2 generation old

8-10 generation old

***C. neoformans* (strain RC-2)**

0-2 generation old

8-10 generation old

Figure 1. Bud scar stains can be used to quantitate age of a cell in *S. cerevisiae*, but are unreliable biomarkers in other fungi

(A) Calcofluor reliably stained for budscars of 0–2 generation and 8–10 generation old *S. cerevisiae* cells. (B) Bud scar staining with Calcofluor (data not shown), and wheat germ agglutinin lectin did not reliably distinguish 0–2 generation and 8–10 generation old *C. neoformans* cells.

Figure 2. Older *C. neoformans* and *C. albicans* cells had increased resistance to antifungals in time-kill assays

(A) Old (18 generations old) *C. neoformans* cells were more resistant to killing by therapeutic doses of amphotericin B and (B) fluconazole compared to young (0–2 generation old) cells. (C) Old (10 generation old) *C. albicans* cells were more resistant to killing by caspofungin compared to young (0–2 generation old) cells (* $p < 0.02$). Copyright © American Society for Microbiology, [Eukaryotic Cell, 6, 2009, 858-66, DOI: 10.1128/EC.00017-09]

Figure 3. Replicative lifespan (RLS) was different in cells of one strain and among different strains

The number of replications was determined by microdissection of individual *C. neoformans* cells from a lab passaged strain (H99), which had a heterogeneous RLS, but whose median RLS was reproducible. RLS of this strain was less variable than a low passaged strain (I58).

Table 1Major longevity regulating genes from *S. cerevisiae* have homologs in pathogenic fungi.

<i>S. cerevisiae</i>	RLS	<i>C. neoformans</i>	<i>C. albicans</i>
SIR2	↑	Histone deacetylase (CNAG_04866.2)	SIR2
CDC25	↑	CDC25 (CNAG_07942.2)	CSC25
RAS1	↑	RAS1 (CNAG_01672.2)	RAS1
RAS2	↓	RAS2 (CNAG_04761.2)	RAS2
SCH9	↑	AGC/RSK protein kinase (CNAG_04040.2)	SCH9
CYR1	↑	ATP-dependent RNA helicase ded1 (CNAG_00809.2)	CYR1
GPA2	↑	G-protein alpha subunit (CNAG_04505.2)	GPA2
DBP3	↑	ATP-dependent RNA helicase (CNAG_02502.2)	DBP3
TOR1	↑	TOR1 (CNAG_06642.2)	TOR1
LAG2	↓	Longevity assurance protein (CNAG_06717.2)	no homolog
RPD3	↑	Histone deacetylase 1-1 (CNAG_05276.2)	RPD3
SNF4	↑	Nuclear protein (CNAG_00360.2)	SNF4
ZDS1	↑	Hypothetical protein (CNAG_05791.2)	ZDS1
SOD1	↑	Cu/Zn superoxide dismutase (CNAG_01019.2)	SOD1
SOD2	↓	SOD2 (CNAG_04388.2)	SOD2
AFG3	↓	ATPase (CNAG_04032.2)	AFG3
IDH1	↓	Isocitrate dehydrogenase (CNAG_07851.2)	IDH1
IDH2	↓	Isocitrate dehydrogenase (CNAG_07363.2)	IDH2
RIM1	↓	Transcription factor PacC (CNAG_05431.2)	RIM1
RPL7a	↓	RPL4 (CNAG_05555.2)	RPL7a
RPL13a	↓	RPL13a (CNAG_06231.2)	RPL13a
RPL23a	↓	RPL23a (CNAG_03053.2)	RPL23b
SAM1	↓	Methionine adenosyltransferase (CNAG_00418.2)	SAM1
SAS2	↓	Myst4 protein (CNAG_03583.2)	SAS2