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Review Series

Cryptococcus neoformans is an opportunistic yeast that is present worldwide and interacts with various organisms. In humans, it is responsible for cryptococcosis, a deadly invasive fungal infection that represents around 220,000 cases per year worldwide. Starting from the natural history of the disease in humans, there is accumulating evidence on the capacity of this organism to enter dormancy. In response to the harsh host environment, the yeast is able to adapt dramatically and escape the vigilance of the host's immune cells to survive. Indeed, the yeast exposed to the host takes on pleiotropic phenotypes, enabling the generation of populations in heterogeneous states, including dormancy, to eventually survive at low metabolic cost and revive in favorable conditions. The concept of dormancy has been validated in *C. neoformans* from both epidemiological and genotyping data, and more recently from the biological point of view with the characterization of dormancy through the description of viable but nonculturable cells.

Dormancy in *Cryptococcus neoformans*: 60 years of accumulating evidence

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Cryptococcus neoformans **is an opportunistic yeast that is present worldwide and interacts with various organisms. In humans, it is responsible for cryptococcosis, a deadly invasive fungal infection that represents around 220,000 cases per year worldwide. Starting from the natural history of the disease in humans, there is accumulating evidence on the capacity of this organism to enter dormancy. In response to the harsh host environment, the yeast is able to adapt dramatically and escape the vigilance of the host's immune cells to survive. Indeed, the yeast exposed to the host takes on pleiotropic phenotypes, enabling the generation of populations in heterogeneous states, including dormancy, to eventually survive at low metabolic cost and revive in favorable conditions. The concept of dormancy has been validated in** ***C. neoformans*** **from both epidemiological and genotyping data, and more recently from the biological point of view with the characterization of dormancy through the description of viable but nonculturable cells.**

Cryptococcus neoformans is a basidiomycetous opportunistic yeast that is widely present in the environment. It causes human cryptococcosis, which mainly affects immunocompromised patients and presents as a meningoencephalitis (1) that is lethal without treatment. Clinical presentation is often diagnosed late because clinical symptoms are initially mild with a subacute to chronic evolution (2).

Humans are exposed to *C. neoformans* from the environment. In nature, this fungus can survive the predation of various organisms ranging from protozoans to metazoans through ready-made virulence traits (3). *C. neoformans* interacts closely with unicellular or multicellular organisms (2–4) and with cells dedicated to innate immune responses in metazoans (macrophages, dendritic cells, natural killer lymphocytes) with various propensity to be phagocytosed and killed (4–6). *C. neoformans* is a facultative intracellular pathogen (7). Interaction of *C. neoformans* with host cells can lead to phagocytosis, yeast replication within the phagolysosome, and is sometimes associated with host cell lysis or with nonlytic exocytosis or cell-to-cell transfer and eventually killing of the yeast (8–13). These phases have been well studied in different models of interaction with host cells but mainly within macrophages. Indeed, intracellular persistence and multiplication in immune cells provide advantages to the fungus by allowing escape from the immune response and later dissemination through epithelial barriers (14, 15).

Characteristics of the infection depend on both hosts and microbial factors. Fungal factors described as virulence factors influence the outcome of infection, according to data obtained in the mouse model of cryptococcosis (16), but also in vitro (17) and in humans (8, 18). Microbial adaptation to the hosts is complex and has been studied globally in lungs using histopathology (7) and global transcriptome analysis upon ameba (19) and macrophage ingestion (20) or upon early infection of mice and rabbits (21, 22). Quiescence or dormancy is one such adaptation that appears successful for enhancing the fungus's ability to survive, persist, reactivate, and then disseminate (23).

About 60 years of research focusing on how *C. neoformans* causes infection in humans is available in the literature, leading to the recent biological demonstration of dormancy in this organism. This Review aims to summarize this 60 years of research, starting from the knowledge of human infection and ending with the characterization of dormancy biologically. The Review is assembled to elucidate how this knowledge has been integrated to lead to more recent findings on the biology of *C. neoformans* characterizing dormancy, focusing on (a) the description of the natural history of *C. neoformans* infection in humans; (b) the concept of dormancy in fungi; and dormancy in *C. neoformans* (c) in vivo and (d) in vitro. To finish, a section is dedicated to (e) the relevance of the biological findings to human infection and a discussion of unsolved questions that can provide the bases of future work in the field.

Natural history of cryptococcosis in humans

Cryptococcosis is one of the most frequent invasive fungal infections in humans worldwide (24). The vast majority of patients with cryptococcosis are HIV$^+$, mostly those with fewer than 100 CD4$^+$ T cells per microliter. Nevertheless, in Western countries, the number of cryptococcosis cases recorded in HIV$^-$ individuals becomes higher than the number in HIV$^+$ patients (25). Immunocompromised HIV$^-$ patients at risk of cryptococcosis are mainly solid organ transplant recipients, patients with systemic autoimmune disease, and those with hematological malignancies (26).

The natural history of cryptococcosis is described as following two main routes. The first, although rare, occurs after exposure

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to *C. neoformans* while immunocompromised, leading to rapidly progressive cryptococcosis; the second is reminiscent of tuberculosis, with a phase of latency followed by reactivation and dissemination. This second route appears to be the main mechanism of infection and so will be further developed in this Review.

First route of infection: ready-made for disease

Confronted with the need to survive in nature and to survive different hosts in different environments, *C. neoformans* has selected ready-made virulence traits (3). From a deterministic point of view, the *C. neoformans* population also needs diversity to survive predators harboring different killing propensities. The plasticity of the *C. neoformans* genome could lead to this diversity (27). *C. neoformans* and *C. gattii* are haploid organisms that can be found as diploid organisms both in nature and in hosts (28, 29). Generation of hybrids is possible between the varieties *grubii* (serotype A) and *neoformans* (serotype D) (29, 30) but also between *C. neoformans* and *C. gattii*, again illustrating this plasticity (31).

C. neoformans has long been associated with pigeon droppings (32). Indeed, pigeon fanciers are known to have higher levels of anti–*C. neoformans* antibodies than control individuals (33). The presence of *C. neoformans* in human dwellings was a risk factor (odds ratio 2.05) for the development of cryptococcosis in HIV^+ patients from Brazil (34). *C. gattii* has also been found in indoor environments in Brazil, although links with human cryptococcosis have not been demonstrated (35). Several cases of cryptococcosis have been reported in immunosuppressed patients in contact with birds (pigeons, parrots, cockatoos, cockatiels) (36–39). The presence of *C. neoformans* has also been observed in the excreta of some zoo animals (tawny frogmouths, palm cockatoos, military macaws, gray parrots) (40). Nosocomial cases of cryptococcosis acquired in various hospital settings have also been suspected (41, 42). Transmission of *C. neoformans* through transplanted deep organs from a contaminated donor has occurred (43, 44), with, in some cases, the demonstration of the same strain in different patients with transplanted organs from the same donor (45).

Primary cryptococcosis initiates with lung involvement and then disseminates from the lung in immunocompromised hosts. Primary pulmonary cryptococcosis is observed in immunocompetent and immunocompromised hosts. It can be recognized within a broad range of presentation, from isolated asymptomatic nodules that can mimic cancer lesions to more disseminated lesions of the lung with respiratory failure (46–48). Primary cutaneous cryptococcosis is also a clinical entity that happens after environmental inoculation in immunocompetent or immunocompromised hosts (49, 50).

Second route of infection: ready-made for latency

The majority of cases of cryptococcosis arise from a natural history of infection following three steps: primary infection in childhood and immune control, followed by a silent phase of latency that can last for years, and finally reactivation and dissemination that are responsible for the symptoms of the disease mainly occurring upon immunosuppression.

Early environmental exposure. Inhalation of aerosolized particles from soil (desiccated yeasts or basidiospores) is thought to be the major route of infection in humans (51). Primary infection with *C. neoformans* occurs mainly in immunocompetent children as demonstrated by serologic studies with unrecognized (asymptomatic) infection as the main clinical presentation. The proportion of children immunized against *C. neoformans* increases with age. Acquisition of cryptococcal antibodies begins very early (1 year) with minimal reactivity of the sera. After 5 years, 70% of children react with *C. neoformans* antigens (52). However, the acquisition of anticryptococcal humoral immunity varies among geographic areas. Cryptococcal antibodies are very common in Bronx children but not in another New York area (Dutchess County), nor are they common in Manila, the Philippines, another densely populated urban area (53). Environmental exposure may depend on climatic and environmental factors (temperature, humidity, pigeon density), but also on human sociological factors (habitat conditions, financial resources). These findings support epidemiological data revealing that cryptococcosis in immunocompromised individuals is more prevalent in some areas of the world, especially in Africa (24, 54).

Notably, *C. gattii* exposure and primoinfection does not follow the same epidemiological trends as *C. neoformans*, based on studies realized in endemic areas in animals and humans (55, 56).

Latency. Serologic evidence of early cryptococcal immunity in immunocompetent hosts without recognized infection seems paradoxical considering the very low frequency of cryptococcosis in immunocompetent hosts. However, immune control of yeasts by immunocompetent hosts following primoinfection is possible, with latency of the disease or complete clearance of the fungus as a consequence. Immunocompetent adults frequently exposed to *C. neoformans* had positive skin test but did not develop clinical disease (57). Autopsy studies have raised the hypothesis that pulmonary granulomas could be the site of persistence, because *C. neoformans* is observed in subpleural nodules and draining lymph nodes in immunocompetent and immunocompromised hosts (58). Indeed, there have been several reports of *C. neoformans* lymphadenitis being exclusively found in and isolated from lymph nodes, arguing that initial immune control of the yeast operates in lymph nodes (59–65). From recent and old reports, the lymph nodes associated with lymphadenitis correspond to granuloma composed of epithelioid cells, giant cells, and necrosis surrounded by a T cell infiltrate together with yeasts (64, 66, 67).

Analysis of clinical isolates of *C. neoformans* var. *grubii* recovered in France from patients born in Africa (who moved to France with a median of 110 months elapsing before isolation of the yeast in France) revealed that yeast genotypes from these patients clustered together, distinct from the yeast genotypes recovered from patients born in Europe (68). This study is the main epidemiological evidence for this latency stage of the disease. This latency can be translated into the capacity of dormancy of the yeasts, which appears to be the more plausible explanation from the point of view of the biology of the organism. The same conclusion is also drawn from a serologic survey of solid organ transplant recipients (immunocompromised hosts). Interestingly, sera obtained before and after transplantation from transplanted patients with cryptococcosis were compared with sera from control transplanted patients without history of cryptococcosis. Among patients with cryptococcosis, half exhibited antibody reactivity against *C. neoformans* only after transplantation, suggesting that they were exposed and developed the disease after transplantation during immunosuppression.

But for the other half of the patients, antibody reactivity against *C. neoformans* was found before transplantation; these patients' early development of cryptococcosis after transplantation suggests that reactivation and dissemination occur rapidly after transplantation from a preexisting isolate in transplant recipients, thus validating again the latency phenomenon (69). Additionally, a report of *C. gattii* infections in patients who travelled to endemic areas years or months before the Vancouver Island *C. gattii* outbreak provides more evidence for latency (70).

Reactivation. The earliest manifestation of reactivation is observed in individuals in whom asymptomatic cryptococcal antigenemia is detected (71–73). Viable yeasts are not recovered from clinical sample at this step, but treatment is mandatory to prevent symptoms and dissemination (74, 75). Pulmonary cryptococcosis is a well-described clinical entity that can evolve differentially depending on the immune status of the hosts. In immunocompetent hosts, *C. neoformans* does not usually disseminate, whereas the possibility of dissemination in immunocompromised patients is high. It is likely that dissemination occurs after reactivation of lung-persistent yeasts, crossing the lung epithelial barrier and disseminating through capillary blood (76, 77). However, abnormal chest x-ray or CT scan was observed in 39% of HIV^+ patients and 55% of HIV^- patients at diagnosis, although dissemination represented 60.6% and 38.5% of the cases, respectively (1). However, pulmonary symptoms are not the main clinical manifestation of cryptococcosis in immunocompromised patients. Indeed, most are diagnosed at the stage of dissemination or meningoencephalitis (1). Cryptococcosis is characterized by a high frequency of central nervous system involvement with positive cerebrospinal fluid and dissemination through blood. Cryptococcosis is more severe in male HIV^+ patients and those infected with *C. neoformans* serotype A (1). Acute cryptococcal meningoencephalitis (CM) is always fatal without antifungal therapy (78). Treatment of CM requires an antifungal therapy induction based on amphotericin B and flucytosine (79). Based on recent large clinical trials in African settings, 1 week of amphotericin B combined with oral flucytosine followed by high-dose fluconazole is now recognized as the reference therapy (75, 80). Mycological failure after 2 weeks of induction is recognized as a factor of bad prognosis, which requires continuation of the induction therapy (79). Mycological failure is independently associated with initial dissemination, high serum antigenemia (>1:512), and lack of initial flucytosine treatment (1, 80, 81). The 3-month mortality rate during the management of acute CM approximates 15% to 20% in Western countries despite adequate treatment and management. It is still not clear whether this mortality rate is due to individuals' immune status, genetic factors (82), fungal determinants, or a combination of these. Nevertheless, two reports clearly identified that fungal determinants specific to the strain are responsible for a given phenotype of interaction with host cells (high phagocytosis, high intracellular proliferation) that is associated with mortality in patients (8, 83).

The concept of dormancy in fungi

All microorganisms are exposed to periodic constraint conditions and react by inhibiting their growth, entering into a nonreplicative state called quiescence or dormancy (84, 85). Three main strategies can be delineated in these conditions. The first is the "bust and bloom" strategy (85), by which the microorganism population will grow rapidly with growth maximization, but upon nutrient exhaustion, the majority of the individuals will die, with only few cells surviving. These residual cells will resume growth rapidly upon exposure to nutrients (86). The second strategy is quiescence, in which the bulk of the population exposed to a nutrient-limited environment will arrest or slow growth to enter a viable, nonreplicating state for a long time. This can last months or years for *Mycobacterium tuberculosis* (87). These cells keep a baseline and specific metabolic capacity, maintain their membrane potential, and do not undergo major morphological change (88). The third strategy is called true dormancy, with sporulation as the purest form, in which an asymmetrical replication leads to the formation of a metabolically inactive spore (89). The spore harbors specific morphology but shares some biological features with quiescent cells.

Quiescence in *Saccharomyces cerevisiae* has been studied for a long time. Recently, a strain of *S. cerevisiae* was found "alive" in bottles of beer and champagne from the 18th century found in a shipwreck in the Baltic Sea, suggesting that this phenomenon can last for years in specific conditions. Quiescent yeasts are mainly obtained from cultures grown to saturation in glucose-rich media (stationary phase) where all nutrients have been consumed. Different phases have been described, including (a) a first phase of glycogen production upon rarefaction of glucose (at about 50%) (90), followed by (b) the regulation of trehalose before and after glucose exhaustion. Then, (c) the yeasts undergo a phase of diauxic shift (following glucose depletion) in which growth is slow and metabolism is adapted to limitation of nutrients, relying on respiratory growth of nonfermentable sugars such as ethanol or acetate with a switch toward respiration, fatty acid pathway, and glyoxylate cycle pathway and, as a consequence, increased formation of antioxidant defenses (scavenging of ROS) (91). The yeast population obtained in the stationary phase is described as a heterogeneous population including quiescent cells (composed of daughter and young mother cells) but also nonquiescent cells, which lose their ability to accumulate ROS, exhibit genomic instability, and become senescent or apoptotic (92).

In *C. neoformans*, growth arrest in the G_1 or G_2 period has been demonstrated in the stationary phase (93). No specific morphological differences in the mitochondrial apparatus were observed in the logarithmic versus the stationary phase (Figure 1 and ref. 94). No comprehensive analysis of the metabolism of *C. neoformans* in the stationary compared with the logarithmic phase existed until recently, as part of the investigation of a specific phenotype observed upon exposure to drastic conditions (95).

Dormancy in *C. neoformans* in vivo

The body of evidence for dormancy comprises various parameters, primarily viability, which should not be based on culturability, reactivation upon specific stimuli, or specific biological activity. Measurement of viability requires the use and adaptation of tools available to test viability versus death in mammalian cells (23). For a long time, viability and its mirror, killing or death of *C. neoformans*, were investigated using CFU counting (96). Other means to assess the viability or death of yeast have now been developed, including the use of intercalating dyes such as propidium iodide that are able to diffuse and stain the DNA of the yeast only if the extracellular

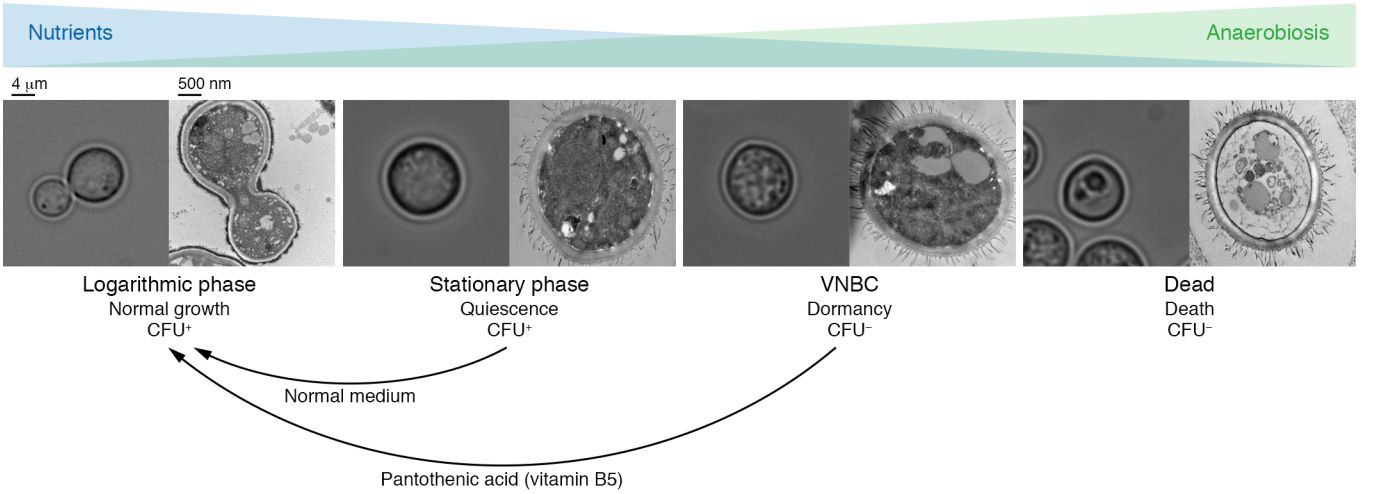

Figure 1. Schematic representation of the evolution of *C. neoformans* phenotypes and morphology upon incubation under progressive nutrient deprivation and anaerobiosis. The reference strain H99 was used in all conditions. STAT: stationary phase; yeast peptone dextrose (YPD), 22 hours with agitation at 150 rpm. VBNC: after incubation for 8 days in anaerobiosis and nutrient deprivation. DEAD: morphology of dead cells called Drop Cn, including one or two large vacuole-like structures. Each panel shows optical microscopy (left) and electron microscopy (right) images. Yeast cells under agitation and in glucose-rich medium (YPD) are actively multiplying in logarithmic phase (LOG). Quiescent yeasts are culturable (STAT) and do not need specific stimuli to grow in normal glucose-rich medium (YPD). Dormant yeasts (VBNC) are not spontaneously culturable and need a trigger stimulus for reactivation (addition of pantothenic acid). Dead yeasts (DEAD) are irreversibly unable to grow again (refs. 23, 95).

membrane loses integrity (97). Another means is based on Live/Dead stains that are not based on DNA staining but on the presence of intracellular esterase released if the cytoplasm is no longer intact (95). These allow assessment of viability or death using flow cytometry. Other dyes can be used with the same principle (23, 98). These methods assume that a dead yeast cell will lose membrane integrity, which may not be necessary at first. Apoptosis should also be checked in this context to determine whether the cell is oriented toward cell death or will remain viable. The existence of apoptosis in fungi is debated (99), but there is evidence for the presence of caspase-like proteins in *C. neoformans* (100) that could act as effectors of mechanisms related to caspase-dependent cell death. Nevertheless, apoptosis in fungi cannot be directly equated with what is known in mammalian cells (99, 101).

In *C. neoformans*' stationary phase, it was shown that only a small proportion of the population was unable to grow and was considered dead (23). From in vivo experiments and interaction with macrophages, it has been shown that yeast cells were able to keep their round shape and capsule although dead as shown with different means (23). These dead *C. neoformans* yeast cells have been called Drop Cn owing to the presence of a large central vesicle inside the cell. The cell wall was shown to be thicker than that in stationary-phase yeasts (Figure 1). In these dead cells, the intracellular content is collapsed around vesicles including remaining membranes (stained with MDY64) and nucleic acids (stained with SYTO85) but with no organized nucleus (negative DAPI staining) and no mitochondria (negative MitoTracker staining) (23). These cells were able to retain CMFDA staining (glutathione staining) in their remaining capsule and cell wall, which was supposed to be intracellular, producing fluorescence artifacts, allowing detection despite being dead. To prevent such bias, multispectral imaging flow cytometry was used, allowing observation of the fluorescence within the cells to assess location (23). Apart from those dead cells, this study highlighted that a heterogeneous population of yeasts was generated in the lungs of infected mice and upon macrophage interaction. Indeed, the view of the existence of a homogeneous population of yeasts in specific conditions turns out to be inadequate, raising the question of the accuracy of studies dealing with analysis of the global population of yeasts recovered in specific settings. Nevertheless, global transcription analyses supported the idea of fungal adaptation to hostile environments such as the macrophage phagolysosome (19, 20, 102), inside amebae (19), in the lung during murine infection (21), in the central nervous system of rabbits (22), or in human cerebrospinal fluid (103).

Heterogeneous populations generated during murine infection included (a) active yeasts able to bud and multiply, (b) dead yeasts, and also (c) a population of more dormant yeasts. These dormant yeasts were less prone to grow in comparison with the stationary phase, which is already considered as a state in which almost all yeasts are quiescent. This explains why these cells have been called dormant instead of quiescent cells (23). These cells also had a decreased response to stress (low glutathione production), increased mitochondrial expression, increased autophagy, and decreased gluconeogenesis-associated transcriptional activity (23).

Dormancy in *C. neoformans* studied in vitro

In the previous study using the mouse model, the authors were able to generate as few as 10^4 dormant yeast cells even after pooling several mouse lungs, which is obviously insufficient to study basic biological processes that would allow characterization of dormancy. Therefore, the authors worked on an in vitro model to enable them to generate a high number of dormant yeast cells. Recently, they released their study of the standardized conditions allowing the generation of yeast cells harboring a phenotype close to that of dormant cells generated in the lungs of infected mice (95). These conditions are based on a combination of low oxygen and

limited nutrients inspired by the Wayne and the Loebel models, two well-documented models of conditions that allow generation of quiescent *M. tuberculosis* (104). After stationary phase in yeast peptone dextrose (YPD) and exposure to anaerobiosis and nutrient starvation during 8 days, the authors observed that 95% of yeast cells were viable, with few dead cells. They demonstrated that cells were not apoptotic upon TUNEL staining. Over time, these yeast cells showed a decreased culturability on YPD agar plates, ending with about 1% of the cells still able to grow on agar at day 8 of incubation. The phenotype observed in the in vivo subpopulation was resumed with delayed growth (increased latency) and low stress response (95). In total, the population obtained was homogeneously composed of cells characterized as viable but nonculturable cells (VBNCs) (Figure 1), a phenotype well known in many bacteria and first described in 1982 in *E. coli* (105). Among fungi, this phenotype has been described in *S. cerevisiae* (106), in *Candida stellata* (107), and in *Brettanomyces bruxellensis* grown in wine synthetic medium and induced by sulfur dioxide (108). *C. neoformans* VBNCs were induced by hypoxia and nutrient deprivation, and a proportion of them were able to be reactivated by vitamin B_5 (pantothenic acid) with a doubling number of culturable cells (Figure 1). Notably, it has been shown in a specific model in *E. coli* that the VBNCs were potentially unable to reactivate (109). Pantothenic acid is known to play a role in the process of division (cell cycle) and in the quorum-sensing phenomenon (110). The use of diluted medium (which is poor in nutrients) to try to reactivate VBNCs was attempted, reflecting the observation that rich medium can be deleterious and induce death (111). Diluted medium did not lead to reactivation of more cells than rich medium, but rather, the cells that did reactivate exhibited faster growth and an increased doubling time in comparison with rich medium. This cell phenotype induced by diluted medium has been called rewiring (95). Finally, drawing on large omics methods, the authors of this study were able to show that *C. neoformans* VBNCs harbored a decreased and specific metabolic activity on the basis of phenotypic microarray, transcriptome, secretome, and proteome analyses (95). Specifically, the fatty acid pathway was required for the maintenance and the viability of the VBNCs, and quorum sensing and mTOR pathways seemed to play an important role in generating and/or maintaining the phenotype. Interestingly, acetyl-CoA is a key precursor for both fatty acids and pantothenic acid, suggesting that regulation of acetyl-CoA is a major factor for the generation of VBNCs (112). Based on these findings, a basic model of the evolution of *C. neoformans* yeast cells from logarithmic phase to dormancy and dead cells can be summarized as depicted in Figure 1.

An analysis of the bulk population of yeasts maintained 8 days in nutrient deprivation and anaerobiosis to generate VBNCs identified another subpopulation of yeasts that are still able and ready to grow on agar-rich medium. This population can be considered as persister cells. Persister cells have been described in a population of bacteria exposed to fungicidal antibiotics as the small proportion of bacteria able to tolerate spontaneously and stochastically lytic drugs via different mechanisms (113, 114). It has been shown that persisters and VBNCs can coexist in a specific model in the bacterium *Vibrio vulnificus* (115). Persister cells have been described in *Candida albicans* biofilm (116) and seem to play a role in recurrent infection in human oral candidiasis associated with natural biofilm (117). In VBNC-inducing conditions in *C. neoformans*, remaining cells able to grow rapidly after long exposure to harsh conditions could be related to such persister cells, since a particular metabolism seems to occur distinct from that of the VBNCs, which need a specific stimulus to grow again. This needs to be studied in detail in future research.

Relevance and unsolved questions

The recent study highlighting the capacity of *C. neoformans* to switch to VBNCs can be viewed as a model to explore dormancy and metabolism in this organism and in pathogenic fungi in general. Indeed, this phenotype per se has not yet been observed biologically in human infection, but experimental conditions and the number of yeast cells needed to obtain the demonstration are clearly not compatible with what can be recovered from the cerebrospinal fluid of a patient with CM. The observation of yeast cells in the cerebrospinal fluid of patients after 7 days of induction therapy with a negative culture on regular medium obviously raises the question of whether these yeasts are VBNCs. Being involved in clinical diagnosis, I have observed that the morphology of these nonculturable yeasts is abnormal and close to that observed in murine infection and called Drop Cn (see above) (8). Indeed, dead yeasts are known to persist and keep their intact shape, although different stainings can help differentiate them from regular and living yeasts (8). VBNCs, or at least part of the VBNC population, have proven to be reactivatable by pantothenic acid, part of the demonstration that these cells are VBNCs. The mechanism behind the specific reactivation has yet to be elucidated. Pantothenic acid (vitamin B_5) is a precursor of coenzyme A, an essential compound that participates in the metabolism of fatty acids, carbohydrates, and proteins through the formation of various active thioesters and promotes virulence and growth (118). Indeed, fatty acids have been shown to be critical in VBNCs (95). VBNCs obtained in *C. neoformans* can be considered as similar to those obtained from bacteria or parasites, as the definition relies on viability, culturability, and reactivation upon specific stimuli. Nevertheless, the conditions allowing the generation of VBNCs and the stimuli that reactivate the population are different in different organisms. Among organisms, many common conditions of induction rely on stresses including starvation, low oxygen, low temperature, desiccation, or a combination of these. On the other hand, resuscitation conditions are extremely variable depending on the organism, such as increased nutrient availability, temperature modifications, addition of chemicals, or addition of host factors (119).

The biology of dormancy in *C. neoformans* is a budding field, and yet there are many more questions than answers. We still lack data on the effect of antifungal drugs on dormant yeast cells, because an experimental setting that allows demonstration of the effect or absence of effect is not easy to implement in dormant cells that are intrinsically not cultivable. Indeed, turning on dormancy with some VBNC inductors that remain to be discovered would definitely aid in treating acute infection. On the other hand, inducing VBNCs could also cause relapse by producing insensitivity to current antifungal strategies. These factors need to be addressed. Moreover, we have no data yet on the possible extension of the VBNC phenotype to clinical isolates of *C. neoformans* type VNI and to other phylogenetic lineages or species. There is

a chance that all clinical isolates could have varying propensities to generate VBNCs, and so the impact on infection could be variable, as already shown for phenotypes of interaction with macrophages (8, 83). We are currently exploring the effect of the host on the induction and maintenance of VBNCs with regard to the level of activation of primary monocytes. Another important subject we are currently exploring is the impact of VBNC metabolism on the physiology of macrophages. Both studies aim to understand the interplay between host and fungal metabolisms, opening the way to discovery of specific pathways that could be modulated to push the system in one or the other direction (more killing or less proliferation of yeasts).

Conclusions

In summary, *C. neoformans* can adapt fantastically to various environments, even very drastic ones, such as 8 days of complete anaerobiosis without extracellular nutrients. *C. neoformans* uses strategies to resist these conditions. It is first perfectly able to enter quiescence in nutrient starvation conditions (stationary phase) or to be pushed into dormancy under additional anaerobiosis exposure. In vivo, one can imagine that VBNCs/dormant yeasts are most likely hidden in the innate immune cells for years before being able to reactivate and multiply in the body of immunocompromised patients but also in the environment. This makes *C. neoformans* the first relevant pathogenic organism in which to study fungal dormancy and its role in pathogenesis in humans.

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