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Ten Challenges on *Cryptococcus* and Cryptococcosis

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Abstract

Cryptococcosis has become a significant public global health problem worldwide. Caused by two species, *Cryptococcus neoformans* or *Cryptococcus gattii*, this life-threatening infection afflicts not only immunocompromised individuals but also apparently immunocompetent subjects. Hence, cryptococcosis should no longer be considered merely an opportunistic infection. In this article, we focus on ten unanswered questions/topics in this field with the hope to stimulate discussion and research on these topics that would lead not only to a better understanding of the physiopathology of this disease but also to a better diagnosis and prognosis.

Keywords

Cryptococcus neoformans; *Cryptococcus gattii*; Cryptococcosis; Meningoencephalitis;
Environment; Infectious particles; Mating; Virulence; Host immunity; Systems biology

Infections have probably shaped human history more than war and famine combined, and the evolution of human society has had an important effect on human infectious diseases. Indeed, the emergence of industrialization followed by the development of high population densities in association with industrialized areas gave rise to health and sanitation problems

that proved to be major catalysts for the spread of pathogens. Thus, human action is currently the single most important driver of infectious epidemiology.

Fungi, such as *Cryptococcus* spp., are one such group of increasingly important pathogens. First, isolated from the environment (peach juice) by the Italian Francesco Sanfelice in 1894, and subsequently isolated from a female patient by a pathologist, Busse, and a physician, Buschke, *Cryptococcus* spp. has become a major threat to a growing number of immunocompromised patients and, intriguingly, also apparently immunocompetent subjects. The Centers for Disease Control estimate over 1 million new cases/year of cryptococcosis worldwide in patients with AIDS, with *Cryptococcus*-related deaths over 650,000/year, making HIV-associated deaths caused by cryptococcosis more frequent than those caused by tuberculosis [1, 2]. This is a drastic increase considering that prior to the mid-1950s, fewer than 300 cases of cryptococcosis had been reported in the medical literature (Reviewed in [3]).

Perhaps the most alarming news in the cryptococcal field is the emergence of *Cryptococcus* as a pathogen for immunocompetent individuals. The epidemic of human cryptococcosis that was initially clustered in Vancouver Island, Canada [4, 5], is now spreading on the Pacific Northwest of the United States [6–9], targeting almost exclusively immunocompetent subjects. This epidemic is caused by *Cryptococcus gattii*, a different species from *Cryptococcus neoformans*. *C. gattii* is closely associated with trees and can become hypervirulent and pathogenic to immunocompetent humans and animals [6, 7]. Thus, *Cryptococcus* afflicts also immunocompetent subjects and it should not be considered merely an opportunistic pathogen [10]. It is possible however that our perception of immunocompetent may be an artifact of our inability to detect immunodeficiency and perhaps even some common factor(s) in such individuals. Nonetheless, these epidemiological data underscore the potential for this fungus to continue to emerge in unexpected geographic and clinical settings and currently cryptococcosis is a significant public health problem globally, including in the United States.

The increasing medical importance of *Cryptococcus* spp. has been paralleled by increased research on this organism and cryptococcosis. The most recent 8th International Conference on *Cryptococcus* and Cryptococcosis (ICCC) held in Charleston, SC, May 1–5, 2011, brought together over 261 scientists from around the world, discussing epidemiology, pathogenesis, genomics, metabolomics, immunology, clinical manifestations, diagnosis, prognosis, and current and future therapeutic strategies. Since its inception in 1989, the 8th ICCC was the most attended meeting, indicating the interest that this field has generated not only among basic researchers but also among clinicians and epidemiologists.

In this article, we would like to highlight some important unanswered questions/topics in this field with the hope to stimulate discussion and research on these topics that would lead not only to a better understanding of the physiopathology of cryptococcosis but also to a better diagnosis and prognosis. The inspiration for this approach was from the mathematician David Hilbert who in 1900 proposed ten unsolved problems in mathematics that were highly influential in affecting the development of the discipline in the twentieth century [11]. We hope that by just formulating ten questions that our colleagues in the field

will consider them in their work. We note that these are just ten important questions and do not mean to imply that these are the only questions of importance in the field.

How Does an Environmental Microbe Suddenly Become a Major Human Pathogen?

Part of this question can be answered by the exponential increase of immunosuppressed patients in the second half of the twentieth century, which made cryptococcosis a relatively common disease as a result of increased vulnerability. However, the part of the question that deals with development of human pathogenic potential in the environment is more enigmatic. Recent studies have discussed the role of the environment in the virulence attributes of *Cryptococcus* spp. [12–14] but the mechanisms underlying this phenomenon are largely unknown. Also unknown is if and how the fungus interacts with natural non-vertebrate hosts, such as certain plants, and if, which, and how human activities influence this interaction. For example, it is not understood why the epidemic of *C. gattii* originated and how it is spreading. Specific interaction of different mating strains between themselves and with the local flora and fauna may have produced a hypervirulent genotype able to cause infection in some apparently immunologically intact hosts that come in contact with the fungus. On the other hand, some human activities may have contributed to the onset and spreading of the epidemic. For instance, the introduction of a large number of eucalyptus trees in the United States could have provided a suitable habitat for *C. gattii*. Outdoor activities such as recreational activities and wood chipping, which is then used for landscaping, may have promoted the anthropogenic dispersion of the fungus [15]. The large use of pesticide may help in controlling the spread of certain microbes but may also favor the replication of others by changing the ecosystem in which they live. For example, most pesticides are antifungals [16–20] and studies have shown that when *C. neoformans* is exposed to antifungals (e.g., fluconazole), this not only upregulates genes involved in the ergosterol pathway, thus promoting resistance, but also those controlling cell wall maintenance, lipid and fatty acid metabolism, stress and virulence [21]. Thus, it is possible that the acquisition of drug resistance and increases in microbial virulence may be parallel events following exposure of this fungus to natural or synthetic compounds used by humans to improve crop, fishery, and livestock quality and yield. Thus, studies addressing the dynamics of the interaction of *Cryptococcus* with the environment should also take into consideration specific human activities, in particular the ecosystem.

What are the Infectious Particles that Cause the Cryptococcal Infection and How Do People Acquire the Infection?

Because of uncertainty as to whether human cryptococcosis is the result of acute infection or reactivation of a latent infection, or both, it is difficult to link cases to point sources in the environment, although certain such associations have been made [22]. It is generally accepted that the primary infection is acquired by inhalation of desiccated cells and/or basidiospores from environmental niches contaminated by pigeon droppings or other bird's excreta (almost exclusively for *C. neoformans*) or by decaying wood and other plant materials (mostly for *C. gattii*). However, although mating and haploid fruiting (two

mechanisms to generate spores) have been demonstrated experimentally, spores have never been observed in nature. On the other hand, recent studies were able to devise a methodology to isolate spores in the laboratory and show their infectious properties [23, 24]. Thus, future studies in this field can be expected to decipher the gene/factor required for spore infectivity. In addition, unequivocal evidence that infections occur only by inhalation are lacking. Gastrointestinal cryptococcosis has been documented in the literature [25–30] and meningoencephalitis occurs in mice upon ingestion of *C. neoformans* [31–33]. Thus, taking into consideration that *C. neoformans* has been isolated from a variety of foods, epidemiological studies should take into consideration that contaminated food/water could be a source of infection.

What is the Role of Sexual Reproduction of *C. neoformans*?

The overwhelming majority of *C. neoformans* isolates are clonal such that this microbe has a predominantly clonal population structure [34, 35]. However, detailed genetic analysis has shown that a proportion of isolates trace their ancestry to sexual replication events. This raises the question of whether sexual reproduction is simply used to generate species diversity and/or to better adapt to the ever changing natural environment. Recent population genetic studies have shown that both α - α opposite sex mating and α - α unisexual reproduction occur in nature for both *C. neoformans* and *C. gattii* [36–40], such that these mechanisms could lead to increased fitness. These reproductive mechanisms are clearly important for maintaining cryptococcal life in the natural environments and they can be exploited by the organism to survive in harsh ecosystems by generating more robust strains [41]. These events may occur in many niches in the environment but whether they can also occur during the course of human infection is still controversial. In fact, the temperature of 37°C of the human body does not allow *C. neoformans* to transition from yeast to hypha, thus preventing both mating and unisex reproduction. Understanding how sex and reproduction are regulated not only will shed light on the mechanisms of cryptococcal transmission (e.g., spore formation) but, importantly, will elucidate how virulence is gained from and regulated by the environment.

Why are Certain Individuals Susceptible to Cryptococcosis While Others are Not?

Even during the height of the HIV epidemic when no antiretroviral drugs were available only about 10% of all patients with AIDS and profound CD4 lymphopenia developed cryptococcosis despite near universal serological evidence for infection. Consequently, there must be factors that affect susceptibility to disease apart from defective cell-mediated immunity. The problem of host susceptibility to the infection by *C. neoformans* has acquired much attention in recent years, mainly due to the epidemic of *C. gattii* infection in immunocompetent individuals. Host susceptibility is particularly important for the infection caused by *C. neoformans* in immunocompromised patients. However, given the widespread distribution of *C. gattii* and *C. neoformans* in the environment, cryptococcosis remains relatively rare. Thus, there must be some host factors that predispose to disease, such as genetic backgrounds, specific immunological status and/or a particular lifestyle (e.g., specific drug intake, food consumption, outdoor activities, or the specific ecosystem in

which the patient lives). In this regard, the recent observation that susceptibility to *C. neoformans* was associated with deficits in humoral immunity [42] suggests that it may be possible to develop diagnostic criteria that will identify those at risk for disease. These factors should be carefully considered to finally predict host susceptibility although it is recognized that the difficulty may lie on the collection of information related to the patient. Clinicians, immunologists, geneticists, epidemiologists, basic scientists and bioinformaticists must work together to tackle each aspect of possible vulnerability for every patient. This could be coordinated by the creation of a “*Cryptococcus* portal” where such information could be uploaded, integrated, analyzed and shared in real time with other investigators.

Can We Predict the Likelihood for Bloodstream Dissemination Before the Fungus Reaches the Brain?

Regardless of whether the lung involvement is a result of a primary infection or a secondary site of infection the fungus will escape the lung and reach the brain in certain individuals. No matter how many cells reach the brain, once there, *Cryptococcus* quickly replicates eventually destroying the surrounding tissue. In the absence of therapy, meningoencephalitis is uniformly lethal and even with antifungal therapy brain infection often becomes chronic and cannot be eradicated. Thus, studies on fungal and/or host factors that are predictors of an active but focal (e.g., lung) disease and/or that dissemination into the blood stream is imminent are warranted. Once the fungus reaches the bloodstream, entrance into the brain will, most likely, occur rapidly, within minutes or a few hours (reviewed in [43]). Thus, the question of whether certain fungal and/or host components can be detected in body fluids (e.g., serum) that can be used to infer the severity of the focal disease and the likelihood of extra-pulmonary dissemination is an important area for future investigation. The identification of such markers and the prompt administration of antifungal therapy may significantly delay or totally prevent complications due to dissemination to the brain.

How Does *C. neoformans* Invade the Brain?

Among the organs that *C. neoformans* could access while traveling in the bloodstream, it has a remarkable neurotropism that makes it distinct from other fungal diseases. The mechanism responsible for this neurotropism is not understood. It is now generally accepted that *Cryptococcus* traverses the blood brain barrier (BBB) at the capillary vasculature [44–47], either directly or riding inside a macrophage Trojan horse, or both, and reaching either the Virchow–Robin spaces and/or the subarachnoidal space. Several mechanisms have been proposed to explain how cryptococcal cells traverse the BBB and the fungal factors promoting it, but which mechanism(s) will most likely occur during infection in humans is still unknown (reviewed in [43]).

What are the Fungal Factors Regulating Pathogenicity?

One key factor regulating cryptococcal virulence is the polysaccharide capsule. Yet little is known on how the capsule components are assembled and organized. Chitins, chitosans, beta-glucans and glucosylceramides are each components of the cell wall but, again, how

this architecture is organized is not known. Several additional factors have been identified to promote cryptococcal growth in the human body (37°C, 5% CO₂, pH from 5 to 7.4) but how they are stimulated is largely unknown. An analysis of the relative contribution of the known virulence factors revealed that the capsule, melanin and other known virulence factors accounted for less than half of the total virulence complex [48]. Hence, most fungal factors contributing to virulence remain unaccounted for. Among over 100 species of *Cryptococcus* only a few cause infection in humans because the majority cannot grow at 37°C. Thus, genomic, proteomic and metabolomic studies should also include those strain(s) that are not capable of causing disease in humans to identify similarities and differences that will promote an infectious versus a noninfectious outcome.

Can Systems Biology Help to Understand Cryptococcal Pathogenicity?

In recent years, we have used computational analysis to model antibody-mediated phagocytosis [49], melanin formation [50] and the adaptation to acidic versus alkaline environments [51, 52] with the intent to integrate certain phenotypes and metabolic pathways with pathogenicity. Systems biology is an extremely powerful tool that should complement studies of physiopathology and epidemiology of *Cryptococcus*. Consider, for example, the non-lytic exocytosis phenomenon [53, 54]. It might be impossible to fully understand the mechanisms of this phenomenon by only applying a reductionist approach because of the continual changes in the synthesis and degradation of cryptococcal (and host) components every time a fungal cell moves in or out of a host cell. If this movement was to occur several times during an experimental observation, it would severely complicate the analysis. Systems biology can provide a simultaneous investigation of stimuli and responses in terms of space, time and context. The spatial aspect accounts for the compartmentalization and the topographic relationships among the components; the time will allow for the study of the dynamic aspects of the process; and the context will account for the interdependencies among all components partaking in the adaptation process. This approach can help to elucidate the ultimate effect of the non-lytic exocytosis in an immunodeficient versus an immunocompetent subject taking into consideration the total number of “ins” and “outs” with certain host parameters (e.g., number of macrophages, their activation level) throughout the infection. Thus, the application of systems biology may provide significant insights into the complex interplay between the fungus and the host immune system.

The Cross-Talk of *Cryptococcus* with the Host Immune System

Defense against microbial pathogens is critical for human survival. Such a function makes the host more robust against external and internal microbes. In comparison to bacterial infections, fungal diseases in animals and humans are relatively rare. This suggests that either the human immune system is able to efficiently control fungal infections and/or that, in being mostly saprophytic instead of parasitic, fungi prefer to derive their nourishment from dead or decaying organic matter. In general, mammals are relatively resistant to fungal diseases, a fact that may be related to their high basal temperatures, which create a thermal restriction zone for the overwhelming majority of environmental fungi [55]. Indeed, fungi commonly inhabit areas of decaying vegetation and, by doing so, they learnt to coexist with

plants (e.g., ectomycorrhiza and vascular arbuscular mycorrhiza) [56, 57]. However, during the last three decades, the increase of immunocompromised subjects has led to a dramatic rise of fungal infections. Consequently, there is a need for enhanced understanding of human natural defense mechanisms against fungi to provide insights into the nature of this interaction and on how we can manipulate it to prevent disease. Issues such as the association of B and T cell epitopes with protective immunity against cryptococcosis, the role of immunological memory in host defense against *C. neoformans*, and the contribution of immunosuppression induced by the fungus are still unresolved. Considering that infection with *C. neoformans* could contribute to the development of allergy [58], the interaction of this fungus with the immune system is more complex than anticipated.

Cryptococcus Species and Clinical Perspectives

To date, cryptococcosis is caused by two species: *C. neoformans* and *C. gattii*. There are differences in clinical manifestations between these two species, which are mostly due to the differences in ecology, epidemiology, biology and host association (reviewed in [59]). Despite great biological differences between the two species the clinical syndromes are very similar. Hence, the approach to induction, consolidation and suppressive treatment for CNS, disseminated disease or pulmonary cryptococcosis caused by *C. gattii* are the same as for those caused by *C. neoformans* [60]. However, infection by *C. gattii* may require additional follow up and, in some cases, a longer induction therapy may be required for *C. gattii* infection. In addition, *C. gattii* has a propensity of inducing massive inflammation that can result in lung cryptococcomas that can require surgical removal. Hence, making a differential diagnosis between *C. neoformans* and *C. gattii* infection is increasingly important.

In recent years, there have been a number of papers proposing the introduction of new *Cryptococcus* species based mainly on the genomic differences of the sequenced strains. Further studies should elucidate the effect of such new species on clinical manifestations and prognosis as well as the impact of additional nomenclature on the overall management of cryptococcosis. Lastly, the recent finding that a significant proportion of human cases of cryptococcosis are caused by mixed infections [61] suggests a need for rethinking how biological variability could contribute to persistence and therapeutic failure.

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