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HISTOPLASMA CAPSULATUM AT THE HOST-PATHOGEN INTERFACE

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Abstract

Histoplasma capsulatum is the most common cause of invasive fungal pulmonary disease worldwide. The interaction of *H. capsulatum* with a host is a complex, dynamic process. Severe disease most commonly occurs in individuals with compromised immunity, and the increasing utilization of immunomodulators in medicine has revealed significant risks for reactivation disease in patients with latent histoplasmosis. Fortunately, there are well developed molecular tools and excellent animal models for studying *H. capsulatum* virulence and numerous recent advances have been made regarding the pathogenesis of this fungus that will improve our capacity to combat disease.

Keywords

Histoplasma capsulatum; pathogenesis; genetics; vaccine; antibody

1. Introduction

Fungal pathogens continue to gain clinical importance largely due to the increasing number of immunocompromised individuals worldwide. The dimorphic fungus *Histoplasma capsulatum* var. *capsulatum* is a model pathogen for the study of invasive mycotic disease. *H. capsulatum* is primarily acquired via aerosol exposure with the inhalation of microconidia or hyphal fragments. It has been estimated that *H. capasulatum* is responsible for ~500,000 infections in the USA each year, making it the most prevalent pulmonary fungal pathogen [1]. *H. capsulatum* produces a broad spectrum of disease ranging from a mild influenza-like illness to a disseminated form that may involve virtually any tissue. The fungus is endemic worldwide, but there are regions with notably high incidences of infection [2], such as areas along the Ohio and Mississippi River Valleys in the USA and in Rio de Janeiro State in Southeastern Brazil.

Although the majority of symptomatic infections follow primary exposures to *H. capsulatum*, reactivation of latent infection can result in significant disease, particularly in the setting of immunosuppression such as with individuals chronically receiving steroids or

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patients on chemotherapy [3]. Individuals with advanced HIV disease are also at significant risk for severe infection due to reactivation of latent lesions or primary disease, and disseminated disease occurs in 95% of individuals with AIDS [4]. Also, initiation of HAART in patients with prior infection with *H. capsulatum* can result in an immune reconstitution inflammatory syndrome [5]. Furthermore, reactivation disease can develop in liver transplant recipients with disease originating from latent infections in the transplanted organs [6]. Additionally, reactivation histoplasmosis has increasingly occurred in patients receiving anti-cytokine therapies, especially inhibitors of TNF- α [reviewed in [7]]. Hence, disease severity and the manifestations of histoplasmosis are significantly impacted by the competence of the host immune response.

2 The host-fungal interface

The outcome of infection with *H. capsulatum* is dependent on dynamic interactions between innate immunity, adaptive immunity and fungal virulence factors [8]. Control of *H. capsulatum* infection is largely based on activation of cellular immunity in concert with innate responses, as progressive disease with dissemination predominantly occurs in the absence of intact cellular immunity [9]. Interestingly, there is recent experimental evidence demonstrating that susceptibility to *H. capsulatum* strongly depends on genetic predisposition [10].

T cells and phagocytes are essential to host resistance against *H. capsulatum* [9,11]. Protective immunity is characterized by the induction of cytokine production by T cells, particularly IFN-γ and TNF-α, which subsequently activate phagocytic cells. The primary effector cells in host resistance to *H. capsulatum* are macrophages. However, the role of macrophages in histoplasmosis is complex, since these cells also provide a protective environment for *H. capsulatum* as the fungus survives and replicates in the phagolysosomes of macrophages. In contrast, dendritic cells can kill ingested *H. capsulatum* yeast cells [12] and dendritic cells presenting *H. capsulatum* antigens can stimulate specific $CD8^+$ T cells to effectively control fungal infection [13].

TNF-α production is induced rapidly after primary infection and neutralization of TNF-α increases the fungal burden and mortality of mice infected with *H. capsulatum* [14,15]. Inhibition of TNF-α results in the generation of antigen specific CD4+CD25+ T cells that interfere with effective immunity in mice [16]. The experimental findings correlate with the clinical findings that inhibitors of TNF-α greatly increase the risk for reactivation of latent histoplasmosis resulting in severe diseases [7].

Mice deficient in IFN-γ have accelerated mortality [17]. Similarly, patients with defects in IFN-γ signaling are at risk for severe histoplasmosis [18]. Adjuvant therapy with IFN-γ can improve the outcome of murine histoplasmosis [19] and has been used successfully in a child with a defect in his IFN-γ receptor [18].

H. capsulatum induces the production of antibodies, which historically has provided a means for non-culture based methods of diagnosis [20]. Notably, antibody has been shown to affect *H. capsulatum* pathogenesis in an animal model [21]. Supporting a role of antibody in histoplasmosis, mice lacking B cells have accelerated mortality after experimental reactivation histoplasmosis [22].

3.*H. capsulatum* and our molecular toolbox

H. capsulatum is mycelial in the environment, whereas the organism exists as a yeast-like, unicellular fungus that reproduces by budding at human physiological temperatures. The mechanisms controlling the switch from the mycelial to the yeast form of *H. capsulatum* are complex, but are largely dependent on the shift in temperature and availability of nutrients

[reviewed in [23]]. The fungus is a prototypical intracellular pathogen that survives within phagolysomes by regulating the intracellular milieu of macrophages [reviewed in [24]].

In addition to dimorphism, several virulence determinants of *H. capsulatum* have been characterized. Perhaps the best studied is the heat shock protein 60 that serves as the ligand for *H. capsulatum* binding to CR3 on macrophage, which initiates the fungus' intracellular parasitism of these cells [25]. *H. capsulatum* heat shock proteins are upregulated during the mycelia-yeast transition and are broadly involved in chaperoning of proteins. The M and H antigens of *H. capsulatum* have long been utilized as serological markers of histoplasmosis [20]. The M antigen, also known as Catalase B, is a constitutively expressed protein posited to play a role in counteracting the oxidative defense reaction mechanism of host phagocytic cells [26]. The H antigen is a secreted beta-glucosidases purportedly involved in remodeling of the cell wall and nutrient acquisition [27]. Initially identified in a differential hybridization screen, yeast phase specific protein 3 (YPS3) is a cell surface and secreted protein of uncertain function that has been associated with virulence since silencing of the YPS3 gene significantly attenuates virulence in vitro and during murine infection [28]. *H. capsulatum* secretes a calcium binding protein (CBP) during yeast-phase growth that is essential for growth in calcium limiting conditions, such as encountered in vivo, and required for virulence during murine infection [29]. Although certain strains of *H. capsulatum* have lost alpha-(1,3)- glucans from their cell surface during microevolution events and have maintained virulence, strains that display alpha-(1,3)-glucans on their cell surface are severely attenuated if the production of this glucan is disrupted. Also, deletion or silencing of alpha-(1,3)-glucan synthase [30] or alpha-(1,4)-amylase [31] interferes with alpha-(1,3)-glucans and the resulting mutants have significantly reduced virulence. Interestingly, alpha-(1,3)-glucans inhibit the recognition of *H. capsulatum* from host effector cells by blocking the beta-glucan receptor dectin-1 [32]. *H. capsulatum* produces melanin in its cell wall [33] that protects the fungus from antifungal drugs [34] and the pigment is also thought to inhibit damage from host defenses, including host derived free radicals and microbicidal peptides [35].

Several studies have described genetic and/or genomic heterogeneity among *H. capsulatum* isolates based on restriction fragment length polymorphisms (RFLP), arbitrary-primer PCR analysis, ribosomal DNA sequencing and other gene sequencing comparisons (reviewed in [36]). Molecular characterization studies have identified seven genetically distinguishable phylogenetic species that diverged as much as 13 million years ago. For example, there are two discrete genetic lineages in North America, *Histoplasma* class I (NAm I) and *Histoplasma* class II (NAm II) that also differ significantly in virulence [37]. Currently, the sequencing of the genome of *H. capsulatum* is underway at Washington University for NAm II (http://genome.wustl.edu/genome.cgi?GENOME=Histoplasma%20capsulatum) and at the Broad Institute for NAm I (http://www.broad.mit.edu/annotation/genome/histoplasma_capsulatum/Home.html). The sequence data may clarify the genomic basis for the difference in virulence and interactions with the host immune system, and serves as a rich resource for molecular work on this pathogen.

The development of molecular genetic tools in *H. capsulatum* is essential for elucidating important questions, such as the mechanisms for mycelia to yeast phase transition, survival in macrophages, and regulation of virulence associated factors. Although, *H. capsulatum* is a perfect fungus with a characterized sexual cycle, laboratory strains cultured in vitro rapidly lose the ability to mate [38], significantly complicating the application of classic recombination studies. Also, the sexual mould form requires BSLIII conditions. The first *H. capsulatum* mutants were *URA5* auxotrophs created using UV radiation [39]. Subsequent transformation experiments for genetic complementation showed that the bacterial plasmid constructs containing the *Podospora anserina URA5* gene usually integrated randomly, and often tandem amplifications or rearrangements were present. Analysis of introduced foreign DNA revealed

that *H. capsulatum* actively modifies transforming plasmids, adding guanosine rich hexanucleotide repeats to the ends of the linear DNA fragments [40]. Subsequent developments exploited the autonomous replication potential of the presented foreign DNA and telomeric shuttle vectors were constructed for genetic complementation in uracil auxotrophs [41]. The first gene targeting experiment deleted the *URA5* gene in *H. capsulatum* [42]. The bacterial hygromycin resistance gene (*hph*) was used to delete the target gene, allowing positive selection. However, the transformation experiments showed a very low (1.4×10^{-3}) homologous recombination frequency. To prevent the high frequency of illegitimate integration, a two-step gene knock-out strategy has been develop using telomeric linear plasmids that enable the creation of homologous recombinant mutants [29]. More recently, double stranded RNA induced RNA interference has been successfully used for target gene expression silencing in numerous eukaryotes [30]. In addition to introducing foreign DNA by electroporation and biolistic procedures, an *Agrobacterium tumefaciens* mediated transfection method has proven to be the most efficient method for DNA transformation in *H. capsulatum* [43]. In particular, the *A. tumefaciens* T-DNA technique readily allows for forward genetic screens and can be a powerful approach to identify virulence associated genes.

4. Potential for a vaccine

There is a general consensus among medical mycologist that there is sufficient disease due to *H. capsulatum* to merit the development of a vaccine. The knowledge that immunization of mice with sublethal inocula of *H. capsulatum* induces protective immunity to subsequent lethal challenge suggests that an effective human vaccine can be achieved. Since the 1970's we have known that immunization with *H. capsulatum* ribosomes confers protective responses [44, 45]. However, major advances in vaccinology for histoplasmosis came with the finding that recombinant heat shock protein 60 from *H. capsulatum* induces vigorous protective immune responses that primarily depend on Vβ 8.1/8.2$^+$ CD4$^+$ T cells [46,47]. Interestingly, immunization with a second heat shock protein of 70 kDa does not result protective cellular responses [48]. More recently, it has been shown that CD8$^+$ T cells can confer protection following immunization with heat shock protein 60 in the absence of CD4$^+$ cells, and that CD8$^+$ T cells can be efficiently stimulated by dendritic cells [13]. The findings are significant since they indicate that it may be possible to induce protective responses in individuals with altered immunity, including individuals with advanced HIV infection.

Although heat shock protein 60 has been the major focus of vaccine development for *H. capsulatum*, additional targets have been identified. For example, protection from pulmonary infection can also be achieved in mice with immunization with recombinant H antigen [49]. Immunization with purified cell free antigen mixtures from *H. capsulatum* can protect mice from systemic infection [50]. Protective immunity is also achievable with recombinant *H. capsulatum* Sec31 [51]. Futhermore, efforts are underway to define pan-fungal vaccine targets. A hybrid histidine kinase that is a global regulator of mycelia-yeast morphogenesis has been identified in the major dimorphic fungi, including *H. capsulatum*, and gene deleted strains may be used for vaccines [52]. A second potential method for a universal fungal vaccine is the utilization of beta-glucans [53]. Hence, there are several exciting avenues for the pursuit of a safe and effective vaccine for histoplasmosis.

5. Antibody therapy

We have shown that antibody can modify the pathogenesis of experimental histoplasmosis [21]. Although the protective effects of IgM isotype monoclonal antibodies (mAbs) that target histone 2B on the fungal cell wall were modest, survival significantly improved when antibody was used concomitantly with amphotericin B. We have more recently identified protective

mAbs to the M antigen and heat shock protein 60 and the IgG isotype mAbs that target these proteins appear to be more effective therapeutics for histoplasmosis [54].

Recently, we found that negative costimulation pathways play a critical role in *H. capsulatum* pathogenesis [55]. The PD-1/PD-L interaction inhibits T cell activation and it has been exploited by a variety of viruses, parasites, and bacteria to attenuate antimicrobial immunity and enhance survival in the host [56]. Using our experimental murine infection model with *H. capsulatum*, we determined that PD-L1 is upregulated on alveolar and peritoneal macrophages as well as on all mononuclear cells in the lungs and splenocytes. The absence of negative co-stimulation significantly alters the fate of mice challenged with *H. capsulatum*, where all mice deficient in PD-1 and 70% of mice receiving antibody to block PD-1 survived an otherwise lethal infection [55].

6. Summary

H. capsulatum is a fungus with a worldwide distribution that is one of the most common systemic mycoses of humans. Despite the availability of broad spectrum antifungal agents and intensive care units, the mortality rate from this fungus continues to be unacceptably high. Recent developments including the availability of genomic information, methods for generating gene deficient strains, and increased understanding of host responses to infection and vaccination are providing important insights that will lead to improved care of patients with histoplasmosis.

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TABLE 1

MOLECULAR TOOL BOX BY DATE DEVELOPED FOR USE IN *H. CAPSULATUM*

Selectable markers
1988 URA5 [39]
1998 Hygromycin [42]
Transformation methods
1990 PEG-LiAc [57]
1998 Electroporation [42]
2002 *Agrobacterium tumerfaciens* [43]
Episomal plasmids
1993 Telomeric plasmid shuttle vector pWU44 [40]
Reporter genes
1998 *lacZ* [42]
2000 *gfp* [58]
2006 *CRP1* [59]
Gene disruption
2000 *CBP1* [29]
Microarray
2003 Partial genomic microarray [60]
RNA interference
2004 *AGS1* [30]
2006 *AMY1* [31]
2007 *YPS3* [28]
Single chain variable antibody fragments (scFv)
2007 nonimmune library of scFv of immunoglobulin human genes displayed on the surface of M13 filamentous phages [61]