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"Virulence Mechanisms and *Cryptococcus neoformans* pathogenesis"

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Abstract

The human fungal pathogen *Cryptococcus neoformans* is able to rapidly and effectively adapt to varying conditions, favoring its survival in the environment and in the infected host. Many microbial phenotypes have been specifically correlated with virulence in this opportunistic pathogen, such as capsule production, melanin formation, and the secretion of various proteins. Additionally, cellular features such as the cell wall and morphogenesis play important roles in the interaction of this fungus with host immune recognition and response pathways. Survival in the face of host stress also requires maintaining RNA/DNA integrity. Additionally, aging and senescence of the fungal cells determines resistance to host-derived stresses. New mechanisms regulating the expression of these virulence-associated phenotypes have been recently explored. Importantly, human clinical studies are now confirming the roles of specific microbial factors in human infections.

Pathogenic microorganisms live in a complex relationship with the infected host. During the initial encounter, the pathogen must be able to sense the host environment and to respond with adaptive cellular changes. This response often includes the induction of specific phenotypes that makes the microorganism better able to survive and proliferate within this new environment. Some of these attributes may allow the microbe to evade host immune recognition. Alternatively, some microorganisms induce significant host damage, either through the elaboration of host-damaging toxins, or often through induction of the host inflammatory process itself.

The human fungal pathogen *Cryptococcus neoformans* is a very frequent cause of human disease. However, in contrast to microorganisms living in continuous contact with humans, *C. neoformans* is not an especially well-adapted human pathogen. In fact, it typically causes serious, symptomatic infections only in highly immunocompromised patients, or in those patients with specific defects in immunity (reviewed in (Kozel, 1995) (Rodrigues, *et al.*,

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1999)). Predominantly growing in the environment, *C. neoformans* interacts relatively infrequently with a mammalian host. Therefore, many of its virulence-associated phenotypes likely serve roles in general environmental survival, or in protection during encounters with non-human hosts (Steenbergen, *et al.*, 2001).

Classical virulence-associated phenotypes

Capsule

The polysaccharide capsule is one of the most striking characteristics of *C. neoformans*. This structure is induced in response to several conditions associated with the host, such as neutral/alkaline pH, high CO_2 levels, and iron deprivation (Granger, *et al*., 1985, Vartivarian, *et al*., 1993). Also, it requires a complex rewiring of metabolic pathways to provide the cellular energy and carbohydrate precursors necessary to rapidly create this complex structure (O'Meara & Alspaugh, 2012).

Tamara Doering and colleagues have begun to develop an intricate model of the transcriptional networks required for cryptococcal capsule formation. Beginning with strains with mutations in specific transcription factor genes required for full capsule expression, they performed detailed comparative transcriptional profiling experiments using deep RNA sequencing. The specific gene expression profile for each strain was determined, and these altered patterns were analyzed by GeneProphet, a computer algorithm developed by the laboratory of Michael Brent. This analysis allowed detailed mapping of the transcriptional “hubs” among these transcription factors, or central mediators of capsule regulation (Haynes, *et al*., 2011). These studies also establish detailed and testable models for the interaction/intersection of diverse signaling pathways controlling different aspects of capsule formation. In this way, new capsule genes may be identified among the many *C. neoformans* “genes of unknown function” if their expression patterns mimic those of known capsule genes.

Oscar Zaragoza from the Instituto de Salud Carlos III in Madrid described detailed kinetic studies of cryptococcal capsule formation. This “energy costly” biosynthesis process is highly dependent on intact mitochondrial function. Capsule enlargement also occurs primarily during the G1 phase of the cell cycle, in which the cells are relatively arrested in many cell growth processes.

Phospholipase activity

Phospholipases cleave phospholipids to produce various biologically active compounds. These enzymes alter the microenvironment of infection and can favor *C. neoformans* survival in the host (Santangelo, *et al*., 2004). Julie Djordjevic from Westmead Millenium Institute in Sydney described a detailed analysis of inositol polyphosphate kinases as mediators of phospholipase C (PLC) signaling. Although two of these kinases play similar roles in other yeast-like species, their specific metabolic and regulatory roles would not have been predicted from non-pathogenic model yeasts, suggesting novel roles in host interaction.

Investigators from the May laboratory at the University of Birmingham described their recent work studying phospholipase B activity. Strains with mutations in the *PLB1* gene

were less able to survive in macrophages in cell culture. The *plb1* knockout strain had a 50% decrease in intracellular proliferation compared to wild type. Additionally the mutant strain underwent a profound morphological change, producing enlarged cells approaching the size of titan cells.

Extracellular vesicles

Many factors associated with *C. neoformans* pathogenesis must be transported from intracellular sites of synthesis to locations on the cell surface or outside of the plasma membrane (Rodrigues, *et al*., 2008). Often, these factors lack the signal peptide motifs common to many eukaryotic secreted proteins. The Rodrigues laboratory in Rio de Janeiro reported that several elements of unconventional secretion mechanisms were involved in the export of capsule polysaccharide and other virulence– associated molecules. Several of these molecules direct Golgi assembly and membrane structure, suggesting that unconventional secretory pathways involve membrane– bound structures moving from inside to outside the cell. Such structures have been visualized as microvesicles, present inside the cell as well as in the extracellular space.

Numerous investigators described the role of these microvesicles in *C. neoformans* pathogenesis, especially in the transport of substances associated with classical virulence factor expression. Microvesicles contain many substances, including capsule precursors, melanin, and secreted enzymes (Rodrigues, *et al*., 2008). These “virulence bags” are quite stable, and they appear to be able to act locally on either the extracellular surface of the microorganism or to influence the interaction with host cells (Oliveira, *et al*., 2010). Marcio Rodrigues used electron microscopy to define ways in which *C. neoformans*-derived microvesicles might traverse the cell wall and be released to the extracellular space. Ambrose Jong from the University of California – Los Angeles demonstrated how these stable, *Cryptococcus*-derived microvesicles might favor alterations of the blood brain barrier to allow *C. neoformans* to better enter the central nervous system. This work complements other studies from this research group that have defined specific fungal-endothelial cell interactions that favor *C. neoformans* entry into the brain parenchyma (Jong, *et al*., 2008). Such studies offer new insights into the mechanisms of neurotropism for microorganisms such as *C. neoformans*.

Cell wall changes and adaptation to the host

In addition to the production of specific enzymes and structures that favor pathogen survival, *C. neoformans* also actively modulates its cell wall in response to host-specific signals (Baker, *et al*., 2007). This remodeling results in immune evasion by shielding more immunogenic surface features (Bose, *et al*., 2003, O'Meara & Alspaugh, 2012). Additionally, the cell wall serves as the point of capsule attachment, and wild-type cells maintain the cell wall in manner that favors capsule binding (Reese & Doering, 2003).

Jennifer Lodge and Maureen Donlin from Washington University and St. Louis University, respectively, discussed the role of chitin and chitosan biosynthesis in *C. neoformans* pathogenesis. *C. neoformans* is unique among human fungal pathogens in the levels of various cell wall carbohydrates, including chitin, chitosan, and glucans (Banks, *et al*., 2005,

Baker, *et al*., 2007). Therefore, the roles of chitin and chitosan in *C. neoformans* cell wall integrity, immune recognition, and induction of protective immunity cannot likely be directly extrapolated from experiments in other systems. In previous work, this collaborative group demonstrated the importance of chitosan, the acetylated form of chitin, in *C. neoformans* cell wall integrity (Baker, *et al*., 2007). Using a large collection of mutant strains defective in various aspects of the formation of these cell wall polymers, they have now demonstrated how altered chito-oligomer composition in the cell wall influences host immune activation. In fact, mutant strains with deficient chito-oligomer composition were able to induce protective immunity in mice, protecting inoculated animals during a subsequent challenge with a wild type strain. This observation is striking because the natural infection does not typically induce this type of protective immunity.

Alterations in cell wall chitin content were discussed by other groups as well. The Alspaugh laboratory from Duke University described the role of the Rim101 signaling pathway in pH sensing and host response to infection (O'Meara, *et al*., 2013). They demonstrated that the Rim pathway is required to maintain cell wall structure during infection. Without this pathway, the cell wall undergoes massive disorganization under host-derived stresses, leading to impaired capsule attachment, excessive exposure of immunogenic chito-oligomers, and failed immune evasion.

Marcio Rodrigues' research group determined that host-derived chitinases are required for chemical modification of the cell wall leading to effective capsule attachment. Because the *C. neoformans* cell wall is comparatively rich in chitin and chitosan compared to other pathogenic fungi, chitin exposure likely plays a significant role in cryptococcal pathogenesis and immune recognition (Banks, *et al*., 2005, Baker, *et al*., 2007).

Increased chitin deposition in the cell wall was also noted in titan cells by Kirsten Nielsen at the University of Minnesota. Together with Oscar Zaragoza from Madrid, this research team was one of the first to recognize the importance of the titan/giant cell morphotype in infection (Okagaki, *et al*., 2010, Zaragoza, *et al*., 2010). These enlarged yeast-like cells are primarily present during pulmonary infection, growing 15 to 100 μm in size with polyploid genomes. Titan cells are resistant to many host-derived stresses, thus potentially acting as a mechanism for pathogen dormancy and reactivation. Furthermore, the polyploid titan parent cells can give rise to haploid and aneuploid progeny, potentially allowing rapid generation of genetic diversity. Dr. Nielsen demonstrated that these cells possess increased chitin in their cell surface, inducing a non-protective Th-2 immune response, further favoring pathogen survival.

This observation adds to the growing body of knowledge about the complex roles of various cell wall components on cell viability, stress response, and immune activation. In this instance, increased chitin exposure in titan cells is associated with a late, adaptive immune response characterized by increased Th-2 type cytokines. Other investigators have demonstrated that *C. neoformans* strains with excessive chitooligomer exposure induce a hyperactivation of innate immune responses very early during a pulmonary infection, despite reduced viability in vivo (O'Meara, *et al*., 2013). Moreover, as described above, the Lodge laboratory demonstrated that infections with a chitosan-deficient strain induced a

protective composite immune response, preventing death during subsequent experimental infection challenges with wild-type strains. Further studies of the cryptococcal cell wall will likely define important aspects of the interface between fungal and host cells, and the ways in which this interaction determines the nature of the immune response to infection.

Cells under stress – a common theme in microbial pathogenesis

As fungal cells enter the mammalian host, they encounter numerous host-derived stresses. As a successful pathogen, *C. neoformans* has adapted numerous cellular mechanisms to survive these challenges. For example, Sarah González-Hilarion from the Janbon laboratory at the Pasteur Institute described their work studying nonsense-mediated mRNA decay (NMD). During detailed studies of the *C. neoformans* transcriptome, they noted the presence of introns in most cryptococcal genes, as well as alternatively spliced mRNA variants. Many of these alternate transcripts are thought to be nonproductive events, targeted for nonsense-mediated mRNA decay. This group mutated the principal NMD factors, noting altered transcript profiles by deep mRNA sequencing, as well as reduced stress tolerance in the mutant strains.

The theme of modifying mRNA pools as a means of rapid adaptation to the host was also addressed by John Panepinto from the University of Buffalo. He described post-transcriptional mechanisms by which *C. neoformans* might rapidly adapt to changing environments. This laboratory group used micro-arrays to measure mRNA abundance after various stresses. In this way they were able to directly measure mRNA kinetics, demonstrating the roles of various RNA polymerase subunits in mediating rapid changes in the abundance of specific mRNA species. Mutations in the *CCR4* and *RPB4* genes, both of which are involved in mRNA stability and decay, resulted in decreased cryptococcal virulence. These studies suggest an important role in mRNA regulation in stress adaptation in this fungal pathogen.

In addition to dynamic changes in RNA stability, Alex Idnurm from the University of Missouri at Kansas City has begun to investigate DNA repair and its role *in C. neoformans* pathogenesis. All pathogens must be able to repair DNA damage that occurs during infection. This damage often accumulates through multiple stress-associated processes. Indeed, basic DNA repair mechanisms are required for fungal pathogenesis. However Dr. Idnurm has proposed that lower-level DNA damage might be beneficial to the pathogen in the generation of increased genetic diversity and adaptation to new stresses associated with infection.

Bettina Fries from the Albert Einstein College of Medicine described her ongoing studies in replicative aging and stress tolerance. Cells that have undergone repeated replications, considered “old cells”, were more resistant to host-derived stresses than younger cells. Moreover, these in vitro observations were corroborated by in vivo studies in which replicatively older cells accumulated in a rat model of cryptococcosis.

Regulation of virulence-associated phenotypes

One of the interesting questions posed during the scientific presentations was how the microorganism senses the host and regulates the vast arrays of cell protective mechanisms. Jim Kronstad from the University of British Columbia described his ongoing work in iron sensing an acquisition by *Cryptococcus neoformans*. The host efficiently sequesters iron and other micronutrients from the microorganism, creating a nutrient-poor environment, inhospitable for fungal growth. In response, *C. neoformans* possesses high affinity mechanisms to acquire iron from mammalian sources such as heme (Hu, *et al*., 2013). Through a series of targeted mutations in iron reductases and siderophore transporters, the Kronstad laboratory has demonstrated the importance of iron acquisition in infection. Also the low-iron signal, acting through the Cir1 regulator, ESCRT pathway, and HapX/Rim101 transcription factors, likely acts as a major activator of protective phenotypes.

Another host signal that likely results in pathogen adaptation is the alkaline pH of the mammalian environment. Kyla Selvig from Duke University demonstrated that the fungal alkaline pH-responsive pathway, activating the Rim101 transcription factor, is only partially conserved in basidiomycetes such as *C. neoformans*. Through random insertional mutagenesis studies, she identified a novel surface protein required for Rim101 activation. This protein may act as a sensor/activator of the alkaline pH response in this group of fungi.

In addition to responding to host-derived signals, *C. neoformans* may also activate protective mechanisms through quorum sensing, or determination of the density of microorganisms in the general environment. The laboratory group of Hiten Madhani from the University of California-San Francisco identified the Qsp1 protein as a possible factor in microbial quorum sensing. The *QSP1* gene is a direct target of three transcription factors with previously described roles in cryptococcal virulence. The Qsp1 gene product accumulates during high-density growth conditions. It also activates a number of genes itself, potentially allowing the microorganism to respond to microbial conditions associated with infection. This work builds upon previous investigations in the Kwon-Chung laboratory at the National Institutes of Health. This research team identified that deletion of the *TUP1* gene resulted in a mutant strain that could not grow at low cell densities, and this phenotype was suppressed by dense cell cultures. Their identification of an 11-mer peptide that contributed to quorum sensing suggested that fungi might have many different mechanisms to sense and respond to population growth signals (Lee, *et al*., 2007).

Translational visions for studies in microbial virulence attributes

Many of the studies discussed during the course of this conference focused on the molecular analysis of cellular phenotypes associated with stress response and pathogenesis. Several of these factors, such as the production of surface capsule and the induction of melanin pigments, have been supported extensively in animal models of infection (Kozel, 1995). However, the importance of many of these microbial phenotypes in human infections has never been directly tested. Wilbur Sabiiti described new clinical studies in patients with cryptococcal meningitis, assessing a collection of clinical *C. neoformans* isolates for in vitro phenotypes associated with patient prognosis and clinical outcome. By profiling a large set

of clinical strains, this research team identified virulence-associated phenotypes in the microorganism that have a direct effect on patient outcome. Cells that were efficiently engulfed by macrophages in vitro were associated with a high CSF fungal burden in the patients from whom these samples were collected. Also, *C. neoformans* clinical isolates that demonstrated higher laccase activity, associated with melanin production, were less effectively cleared by antifungal therapy. Therefore, these important clinical studies have begun to establish the relevance of many microbial factors, such as melanin formation and pathogen–macrophage interactions, that have been studied in the research laboratory for several years. These types of translational and corroborative studies create an important foundation, supporting the relevance of extensive basic investigations in this human fungal pathogen.

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References

Baker LG, Specht CA, Donlin MJ, Lodge JK. Chitosan, the deacetylated form of chitin, is necessary for cell wall integrity in Cryptococcus neoformans. Eukaryotic Cell. 2007; 6:855–867. [PubMed: 17400891]

Banks IR, Specht CA, Donlin MJ, Gerik KJ, Levitz SM, Lodge JK. A chitin synthase and its regulator protein are critical for chitosan production and growth of the fungal pathogen Cryptococcus neoformans. Eukaryot Cell. 2005; 4:1902–1912. [PubMed: 16278457]

Bose I, Reese AJ, Ory JJ, Janbon G, Doering TL. A yeast under cover: the capsule of Cryptococcus neoformans. Eukaryot Cell. 2003; 2:655–663. [PubMed: 12912884]

Granger DL, Perfect JR, Durack DT. Virulence of Cryptococcus neoformans: regulation of capsule synthesis by carbon dioxide. J. Clin. Invest. 1985; 76:508–516. [PubMed: 3928681]

Haynes BC, Skowyra ML, Spencer SJ, et al. Toward an integrated model of capsule regulation in Cryptococcus neoformans. PLoS Pathog. 2011; 7:e1002411. [PubMed: 22174677]

Hu G, Caza M, Cadieux B, Chan V, Liu V, Kronstad J. Cryptococcus neoformans requires the ESCRT protein Vps23 for iron acquisition from heme, for capsule formation, and for virulence. Infect Immun. 2013; 81:292–302. [PubMed: 23132495]

Jong A, Wu CH, Shackleford GM, et al. Involvement of human CD44 during Cryptococcus neoformans infection of brain microvascular endothelial cells. Cell Microbiol. 2008; 10:1313–1326. [PubMed: 18248627]

Kozel TR. Virulence factors of Cryptococcus neoformans. Trends Microbiol. 1995; 3:295–299. [PubMed: 8528612]

Lee H, Chang YC, Nardone G, Kwon-Chung KJ. TUP1 disruption in Cryptococcus neoformans uncovers a peptide-mediated density-dependent growth phenomenon that mimics quorum sensing. Mol Microbiol. 2007; 64:591–601. [PubMed: 17462010]

O'Meara TR, Alspaugh JA. The Cryptococcus neoformans capsule: a sword and a shield. Clin Microbiol Rev. 2012; 25:387–408. [PubMed: 22763631]

O'Meara TR, Holmer SM, Selvig K, Dietrich F, Alspaugh JA. Cryptococcus neoformans Rim101 is associated with cell wall remodeling and evasion of the host immune responses. MBio. 2013; 4

Okagaki LH, Strain AK, Nielsen JN, et al. Cryptococcal cell morphology affects host cell interactions and pathogenicity. PLoS Pathog. 2010; 6:e1000953. [PubMed: 20585559]

Oliveira DL, Freire-de-Lima CG, Nosanchuk JD, Casadevall A, Rodrigues ML, Nimrichter L. Extracellular vesicles from Cryptococcus neoformans modulate macrophage functions. Infect Immun. 2010; 78:1601–1609. [PubMed: 20145096]

Reese AJ, Doering TL. Cell wall alpha-1,3-glucan is required to anchor the Cryptococcus neoformans capsule. Mol Microbiol. 2003; 50:1401–1409. [PubMed: 14622425]

Rodrigues ML, Alviano CS, Travassos LR. Pathogenicity of Cryptococcus neoformans: virulence factors and immunological mechanisms. Microbes Infect. 1999; 1:293–301. [PubMed: 10602663]

Rodrigues ML, Nakayasu ES, Oliveira DL, Nimrichter L, Nosanchuk JD, Almeida IC, Casadevall A. Extracellular vesicles produced by Cryptococcus neoformans contain protein components associated with virulence. Eukaryot Cell. 2008; 7:58–67. [PubMed: 18039940]

Santangelo R, Zoellner H, Sorrell T, et al. Role of extracellular phospholipases and mononuclear phagocytes in dissemination of cryptococcosis in a murine model. Infect Immun. 2004; 72:2229–2239. [PubMed: 15039347]

Steenbergen JN, Shuman HA, Casadevall A. Cryptococcus neoformans interactions with amoebae suggest an explanation for its virulence and intracellular pathogenic strategy in macrophages. Proc Natl Acad Sci U S A. 2001; 98:15245–15250. [PubMed: 11742090]

Vartivarian SE, Anaissie EJ, Cowart RE, Sprigg HA, Tingler MJ, Jacobson ES. Regulation of cryptococcal capsular polysaccharide by iron. J Infect Dis. 1993; 167:186–190. [PubMed: 8418165]

Zaragoza O, Garcia-Rodas R, Nosanchuk JD, Cuenca-Estrella M, Rodriguez-Tudela JL, Casadevall A. Fungal cell gigantism during mammalian infection. PLoS Pathog. 2010; 6:e1000945. [PubMed: 20585557]

Highlights for review

1. *C. neoformans* virulence-associated phenotypes include capsule, phospholipase activity and extracellular vesicles
2. Cell wall compositional changes mediate adaptation to the host
3. Cellular stress responses include DNA/RNA stability, titan cell formation, and replicative aging
4. Virulence-associated phenotypes are regulated by host conditions (nutrient availability, pH, etc.) and quorum sensing
5. New clinical studies are confirming the importance of virulence-associated phenotypes in human infections