What Makes *Cryptococcus neoformans* a Pathogen?

Kent L. Buchanan and Juneann W. Murphy
University of Oklahoma Health Sciences Center,
Oklahoma City, Oklahoma, USA

Life-threatening infections caused by the encapsulated fungal pathogen *Cryptococcus neoformans* have been increasing steadily over the past 10 years because of the onset of AIDS and the expanded use of immunosuppressive drugs. Intricate host-organism interactions make the full understanding of pathogenicity and virulence of *C. neoformans* difficult. We discuss the current knowledge of the characteristics *C. neoformans* must possess to enter the host and establish progressive disease: basic growth requirements and virulence factors, such as the polysaccharide capsule; shed products of the organism; melanin production; mannitol secretion; superoxide dismutase; proteases; and phospholipases.

Cryptococcus neoformans is an encapsulated fungal organism (Figure 1) that can cause disease in apparently immunocompetent, as well as immunocompromised, hosts (1,2). Most susceptible to infection are patients with T-cell deficiencies (1,2). *C. neoformans* var. *neoformans* causes most cryptococcal infections in humans, so this review will focus on information from the *neoformans* variety of this basidiomycetous fungus. *C. neoformans* var. *neoformans* is found worldwide; its main habitats are debris around pigeon roosts and soil contaminated with decaying pigeon or chicken droppings (1,3). Not part of the normal microbial flora of humans, *C. neoformans* is only transiently isolated from persons with no pathologic features (2,4). It is generally accepted that the organism enters the host by the respiratory route in the form of a dehydrated haploid yeast or as basidiospores. After some time in the lungs, the organism hematogenously spreads to extrapulmonary tissues; since it has a predilection for the brain, infected persons usually contract meningoencephalitis (1). If untreated, cryptococcal meningoencephalitis is 100% fatal, and even when treated with the most effective antifungal drugs, cryptococcal infections can be fatal if the host does not have adequate T-cell-dependent immune function (2).

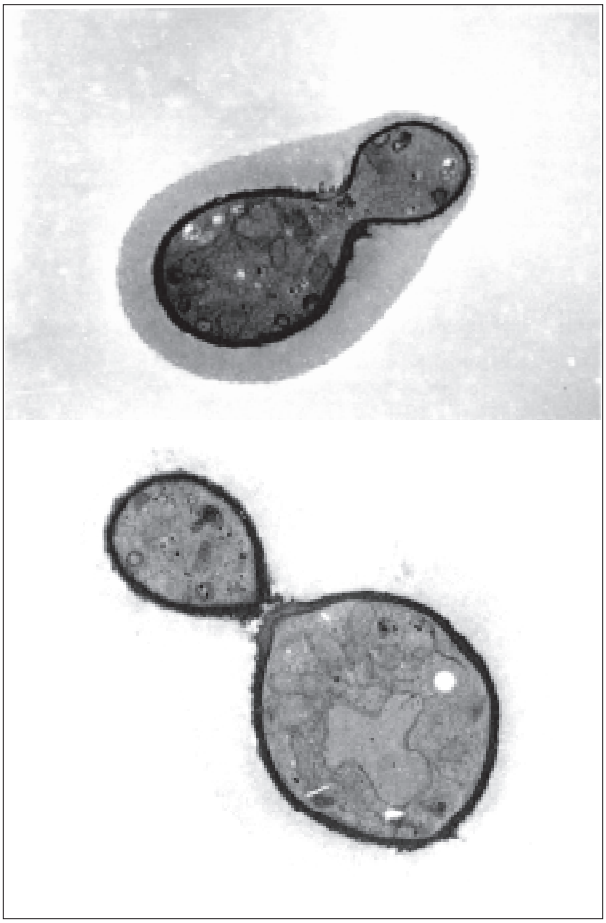

Figure 1. Transmission electron micrograph of budding *C. neoformans* showing the characteristic polysaccharide capsule.

Address for correspondence: Juneann W. Murphy, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA; fax: 405-271-3117; e-mail: kent-buchanan@ouhsc.edu or juneann-murphy@ouhsc.edu.

To be classified as a pathogen, an organism must be able to cause infection under certain conditions. By this definition, *C. neoformans* can certainly be classified as a pathogen. Because the immunodeficient are more susceptible than the immunocompetent to infection with this yeast-like organism, *C. neoformans* is frequently referred to as an opportunistic pathogen. The factors that make *C. neoformans* a pathogen can be divided into two major groups. The first comprises the basic characteristics needed to establish an infection and survive in the human host; the second comprises the virulence factors that affect the degree of pathogenicity.

Basic Characteristics for Pathogenicity

The Infectious Particle

To enter the alveolar spaces of the lungs and establish pulmonary infection, an organism must produce viable forms smaller than 4 µm in diameter. The typical vegetative form of *C. neoformans* is the yeast form with a cell diameter of 2.5 µm to 10 µm. The organism can also undergo sexual reproduction, and since it is a basidiomycete (*Filobasidiella neoformans*), it forms basidiospores. Sexual reproduction appears to occur much less frequently in nature than asexual or vegetative reproduction. The sexual spores (basidiospores) are approximately 1.8µm to 3µm in diameter and result from crosses of the α- and a-mating types on an appropriate medium (1). Although the exact nature of the infectious particles of *C. neoformans* is not known, they are presumed to be the dehydrated yeast cells or basidiospores of the appropriate size range to get into the lungs. Once inside the lungs, the yeast cells become rehydrated and acquire the characteristic polysaccharide capsule (Figure 2). In the case of basidiospores, they would convert to encapsulated blastoconidia.

Recently, Wickes et al. (5) reported that the α-mating type of *C. neoformans* can produce monokaryotic hyphae on a solid medium without a nitrogen source or water. The monokaryotic hyphae contain clamp connections and basidia with short chains of basidiospores and are similar in all respects except in nuclei number to the dikaryotic hyphae of the sexual forms (5). The haploid fruiting bodies formed by nitrogen-starved α-mating type isolates also produce abundant amounts of blastoconidia, which on the appropriate medium can produce haploid hyphae (5).

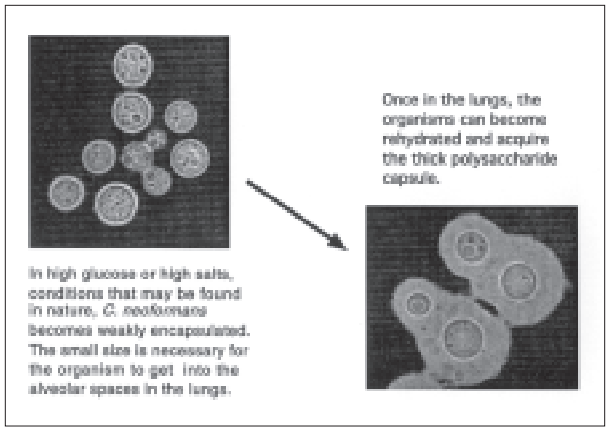

Figure 2. Proposed means of infection by *C. neoformans*.

None of the a-mating type strains tested produced the haploid fruiting bodies. Backcross studies indicated that the ability to undergo haploid fruiting lies within (or is tightly linked to) the *MAT*α locus, which is responsible for the α-mating type phenotype (5). These results suggest basidiospores from the monokaryotic hyphae may be another source of the infectious particle in nature. Regardless of the nature of the infectious particle (yeast cell or basidiospore), if a cryptococcal isolate is not able to produce small infectious particles, it cannot be pathogenic under the usual conditions for establishing the disease.

Mating Types

Kwon-Chung and Bennett (6) surveyed the mating types of *C. neoformans* isolates from environmental and clinical sources and found a 30- to 40-fold higher frequency of α-mating type than a-mating type. The skewed ratio of α-mating type to a-mating type was not thought to be due to a genetic preponderance towards α-mating type progeny because crosses between two test strains resulted in a 1:1 ratio of α-mating and a-mating type progeny (7). To examine the reason for the disparate ratio, Kwon-Chung and colleagues (8) constructed a pair of congenic strains of *C. neoformans* that differed only in their mating type. Survival studies with these congenic strains demonstrated that the α-mating type (B-4500) was significantly more virulent than the a-mating strain (B-4476) when injected intravenously into mice (8). Eighty percent of mice infected with 10^6 B-4500 (α-mating type) were

dead within 36 days; whereas, it took 93 days for 80% of mice infected with 10^6 B-4476 (a-mating type) to die (8). Although the mating type locus of *C. neoformans* has been cloned and partially characterized, the genes or gene products that contribute to the increased virulence of α-mating type isolates are not known.

The mating type locus (*MAT*α) cloned from an α-mating type *C. neoformans* isolate is 35 kb to 45 kb long (9). Moore and Edman (9) demonstrated that introducing a 2.1-kb fragment of the *MAT*α locus into an a-mating strain isolate stimulated filament formation on starvation medium. Basidia and basidiospores were not produced on the filaments. In contrast, electroporation of an α-mating strain with the same *MAT*α DNA fragment was ineffective in stimulating filament formation under the same growth conditions (9). Sequence analysis of the 2.1-kb fragment from the *MAT*α locus identified a 114-bp open reading frame that encodes a 38-amino acid propheromone peptide (9). The nature and the effects of the active pheromone peptide on mating and possibly virulence of *C. neoformans* are unknown; consequently, how the mating type relates to virulence at the genetic level is also unknown. However, the observation of haploid fruiting by α-mating type isolates of *C. neoformans* on nitrogen- and water-depleted medium (5) may help explain why the α-mating type isolates from clinical specimens are more frequent than the a-mating type strains (6).

Growth In Vivo

To cause infection in humans, a *C. neoformans* isolate must grow at 37°C in an atmosphere of approximately 5% CO_2 and at a pH of 7.3 to 7.4. To survive at 37°C, the organism must have an intact gene that encodes the *C. neoformans* calcineurin A catalytic subunit (10). Calcineurin is a serine-threonine specific phosphatase that is activated by Ca^{2+}-calmodulin and is involved in stress responses in yeasts (10). Although calcineurin A mutant strains of *C. neoformans* can grow at 24°C, they cannot survive in vitro at 37°C, in 5% CO_2, or at alkaline pH (10). Since these are similar to conditions in the host, one would predict that the calcineurin A mutant would not survive in the human host. In support of that prediction, Odom et al. have shown that such mutants are not pathogenic for immunosuppressed rabbits (10). Calcineurin A appears to be a basic requirement for *C. neoformans* survival in the host and consequently is a necessary factor for the pathogenicity of the organism.

Virulence Factors

Virulence factors increase the degree of pathogenicity of a microbe. *C. neoformans* has a number of virulence factors; generally, the virulence of an isolate cannot be attributed to any single factor, but rather it is attributed to many working in unison to cause progressive disease. As virulence factors go, those of *C. neoformans* would be considered low-grade. We will discuss each virulence factor separately; however, the severity of the host's disease results from a combination of several virulence factors superimposed on the host's innate and immune resistance status. The virulence factors that will be discussed are capsule, cryptococcal products, melanin production, mannitol production, and potential factors such as superoxide dismutase, proteases, phospholipase B, and lysophospholipase. The polysaccharide capsule and the soluble extracellular constituents of *C. neoformans* (referred to here as cryptococcal products) are probably the dominant virulence factors.

Capsule

C. neoformans has a capsule composed primarily of a high molecular weight polysaccharide that has a backbone of α-1,3-D-mannopyranose units with single residues of ß-D-xylopyranosyl and ß-D-glucuronopyranosyl attached (11-15). This polysaccharide is referred to as glucuronoxylomannan (GXM) (11) and has four serotypes: A and D, produced by *C. neoformans* var. *neoformans,* and B and C, produced by *C. neoformans* var. *gattii*. The evidence indicates that the capsule is a key virulence factor for *C. neoformans;* acapsular mutants are typically avirulent, whereas encapsulated isolates have varying degrees of virulence (16-19). Two capsular genes, *CAP59* and *CAP64,* have been described (20-22). Complementation of an acapsular, avirulent mutant with *CAP59* resulted in a virulent transformant with a capsular phenotype, and deletion of *CAP59* by homologous integration caused the encapsulated organism to become acapsular and avirulent (20). Similar observations have been made with the *CAP64* gene (21,22). Although their biochemical functions have not been defined, it appears that the two genes are essential for capsule formation and virulence.

Chemotactic Effects on Leukocytes

Some properties of the *C. neoformans* capsule enable the host to more effectively clear the organism from tissues; however, others protect the organism from host defenses. The capsules of serotype A and D isolates are chemotactic for neutrophils (23). Moreover, complement is fixed by cryptococcal capsules by the alternative pathway (24), and this process produces chemotactic peptides such as C5a (23). Chemotaxis of leukocytes induced by either of these mechanisms would be advantageous to the host.

Effects of Complement Interactions

Complement fixing by *C. neoformans* in tissue would result in chemotactic factor production and attraction of leukocytes into the infection site. Once in the infected tissue, the leukocytes would interact with and kill the organism. As complement is fixed, C3b and C3bi are deposited on the surface of the cryptococci (24). The capsule can mask the C3b and C3bi deposits (24-26); however, if they are not completely masked, the deposited complement components facilitate binding of the cryptococci to CR3 receptors on leukocytes (27). Such binding interactions are advantageous to the host; they enhance the opportunity for the leukocytes to kill the cryptococci either extracellularly or after phagocytosis. The organism can also be opsonized by antibodies to GXM, but the capsule may block the Fc portion of the antibody and prevent it from binding to Fc receptors on the phagocytic host cells (28). Some of these functions of the capsule favor the host; however, if the capsule is very large, the organism is protected (24-26). The cryptococci could deplete complement in the host, creating an environment that favors the cryptococci (29).

Effects on Phagocytosis

All considered, the capsule is more beneficial to the organism than to the host. Encapsulated *C. neoformans* cells are not phagocytized or killed by neutrophils, monocytes, or macrophages to the same degree as acapsular mutants (25,30-33). Encapsulated *C. neoformans* have a stronger negatively charged surface than acapsular cells or *Saccharomyces cerevisiae* cells (34). The high negative charge could cause electrostatic repulsion between the organism and the negatively charged host effector cells and reduce intimate cell-cell interactions required for clearance of the cryptococci (34).

Altered Antigen Presentation

The inability of macrophages to ingest the encapsulated organisms could also diminish antigen presentation to T cells and consequently reduce immune responses. This speculation has been experimentally demonstrated by Collins and Bancroft (35). Other studies with human alveolar macrophages have confirmed that antigen presentation is not as effective with encapsulated cryptococci as with acapsular strains (33,36). Unlike acapsular cells, encapsulated isolates cannot stimulate proliferative responses in T cells because of the reduced secretion of interleukin-1 (IL-1) by the antigen-presenting cells stimulated with the encapsulated yeasts (36).

Effects on Cytokine Production

In addition to being antiphagocytic, more resistant to killing, and less able to stimulate T-cell proliferation (25,30-33,35,36), highly encapsulated isolates of *C. neoformans* opsonized with a heat-labile serum component, presumably complement, do not stimulate monocytes and macrophages to produce proinflammatory cytokines such as TNFα, IL-1ß, and IL-6 as effectively as similarly opsonized nonencapsulated or weakly encapsulated isolates (37-41). The signal for induction of cytokine production can be a direct result of the attachment of the monocyte or macrophage to the acapsular cryptococci or can be an outcome of the phagocytic process, induced by acapsular cryptococci. Since the capsule blocks phagocytosis, any cytokines induced by the phagocytic process would not be induced by the encapsulated *C. neoformans* cells. If the cryptococcal cell wall materials must be exposed to induce cytokine production, the capsule would block the direct induction of cytokine production. Regardless of the mechanism of stimulation, the lack of production of proinflammatory cytokines could have a bearing on protection. TNFα is necessary for the induction of the protective immune response against *C. neoformans* (42). Consequently, the lack of or reduced production of TNFα in infections with highly encapsulated isolates of *C. neoformans* would prevent the induction of protective immunity, resulting in progressive disease. The roles of IL-1ß and IL-6 in protecting against *C. neoformans* have not been defined, but it is highly probable that the lack of these two cytokines could compromise the protective responses of the host and give cryptococci the advantage.

In contrast to the reduced TNFα, IL-1ß, and IL-6 produced by stimulating monocytes and macrophages with highly encapsulated cryptococci, IL-10 produced by these host cells increases after interaction with encapsulated strains (43). Neutralization of IL-10 with anti-IL-10 in cocultures of human peripheral blood mononuclear cells and encapsulated cryptococci increased the amounts of TNFα and IL-1ß produced, which indicates that the induction of IL-10 production by stimulating macrophages with encapsulated *C. neoformans* downregulates the production of the proinflammatory cytokines TNFα and IL-1ß (43). One might predict that the induction of high levels of IL-10 would also preferentially stimulate the induction of a T-helper 2 (Th2) response rather than a Th1 response in the T cells (44). Since the Th1 response is associated with protection against *C. neoformans* (45), increased levels of IL-10 would dampen induction of the protective immune response.

Encapsulated cryptococcal cells do not affect the different types of leukocytes in the same manner. Although encapsulated isolates do not stimulate macrophages to produce proinflammatory cytokines, they do stimulate neutrophils to produce proinflammatory cytokines and the chemotactic factor IL-8 more effectively than weakly encapsulated or acapsular organisms (46). As with the stimulation of macrophages by acapsular cryptococci to produce proinflammatory cytokines, serum is required for the encapsulated organisms to induce neutrophils to produce cytokines (46). In the case of cytokine production by neutrophils in response to encapsulated *C. neoformans,* it appears that the opsonization process releases a factor into the supernatant that induces the neutrophils to produce the cytokines (46). How these opposing in vitro observations with macrophages and neutrophils relate to the in vivo system or pathogenicity is yet to be determined.

Cryptococcal Products

In disseminated cryptococcosis, measurable levels of cryptococcal products are present in the body fluids of the patients (47). Although GXM is the major cryptococcal component in body fluids, it is highly probable that the organism also sheds galactoxylomannan (GalXM) and mannoproteins (MP) in vivo. This speculation is based on indirect evidence from in vivo studies and on the fact that GalXM and MP are in culture medium when the organism is grown in vitro (15,48). Cryptococcal antigens in serum or spinal fluid are diagnostic for cryptococcosis (47). Furthermore, if disseminated cryptococcosis patients have high cryptococcal antigen titers at the onset of therapy, they are less likely to respond to therapy or more likely to die before therapy is completed than patients with low cryptococcal antigen titers (49). The direct relationship of cryptococcal antigen levels in body fluids and the severity of disease suggests that the cryptococcal antigens in the host's circulatory system or spinal fluid may have adverse effects on host defenses.

Effects on Leukocyte Migration

It has been recently demonstrated in the mouse model that intravascular cryptococcal antigens inhibit the migration of leukocytes from the bloodstream into an inflammatory site (50). Intravascular cryptococcal antigens significantly reduce leukocyte infiltration into a site of acute inflammation induced by such stimuli as cryptococcal culture filtrate antigen, TNFa, or the chemotactic peptide FMLP (formylmethionyl leucyl phenylalanine) or into a delayed-type hypersensitivity reaction site induced by purified protein derivative of *Mycobacterium tuberculosis* or by *C. neoformans* antigen(s) (50). Each of the identified cryptococcal products, GXM, GalXM, and MP, when given intravenously to mice, can inhibit leukocyte migration (50). Considering that GXM is the dominant cryptococcal component in the host's circulation, one might predict that GXM is mainly responsible for the reduced leukocyte migration into inflammatory sites (50). These observations imply that pulmonary cryptococcosis patients, who have low to no cryptococcal antigen concentrations in their serum, would have a normal influx of leukocytes into pulmonary sites of infections. On the other hand, in severe pulmonary infections or in disseminated cryptococcosis patients who have high levels of circulating cryptococcal antigens, minimal inflammation would be expected in the infected tissues. In fact, for years investigators have commented on the minimal host tissue responses observed in patients with disseminated cryptococcosis (3). These recent data demonstrate that the circulating cryptococcal antigens are responsible, at least in part, for the lack of host tissue response.

Cryptococcal antigen(s) can potentially prevent leukocytes from migrating into an inflammatory site in two nonexclusive ways (51,52). First, GXM can stimulate neutrophils to shed L-selectin, a surface molecule necessary for the first step in neutrophil movement into tissues (51). Without L-selectin the neutrophils do not slow down and roll along the inflamed endothelial cells lining the blood vessels. With this first step in extravasation blocked, the numbers of neutrophils in infected tissues would be greatly reduced. Second, GXM and GalXM can bind to CD18, the beta chain of the adhesion molecule LFA-1, and prevent the binding of anti-CD18 to LFA-1 (52). Consequently, this binding of GXM and GalXM could prevent LFA-1 on the neutrophils from binding to the LFA-1 ligand, ICAM-1 on the inflamed endothelial cell surface. If leukocytes are inhibited from entering tissues by either or both of these mechanisms, the organism is not effectively eliminated, and the disease is more severe.

Induction of Immunomodulatory Cells

Cryptococcal antigens injected into the bloodstream of experimental animals can induce regulatory T cells, which dampen or ablate the anticryptococcal humoral as well as cell-mediated immune responses (53-71). Some clinical correlates support the concept that the antigenemia seen in disseminated cryptococcosis downmodulates the immune responses (72,73).

A long-lasting, specific immunologic unresponsiveness has been reported in persons cured of cryptococcal meningitis (72-74). Although they made antipneumococcal polysaccharide antibodies to the same degree as control volunteers after vaccination with pneumococcal polysaccharide, cured patients could not make antibodies to cryptococcal polysaccharides after vaccination with cryptococcal antigens (72,73). Henderson et al. (72,73) speculated that the intense, prolonged antigenemia associated with the cryptococcosis may account for the observed unresponsiveness. It is not unusual for patients with disseminated cryptococcosis to have depressed cell-mediated immune (CMI) responses to cryptococcal antigens (75,76). However, sufficient information is not available to determine whether the patients had a generalized defect in CMI function or had a specifically depressed CMI response because of the cryptococcal antigenemia.

Experimental animal models demonstrate convincingly that cryptococcal antigens given intravenously can induce immunoregulatory T cells that downmodulate the anticryptococcal CMI response (56-71). Serum from *C. neoformans*-infected mice with a cryptococcal antigen titer of 10^{-4} when transferred intravenously to naive mice induces regulatory T cells that specifically depress the anticryptococcal CMI response (67). Similar immunoregulatory T cells are induced by simulating the antigenemia with intravenous injection of cryptococcal culture filtrate antigen (56,57,60-63,66-69). The immunoregulatory $CD4^+$ T cells, which appear in the lymph nodes of the mice 7 days after antigen injection, diminish the induction of T cells responsible for the anticryptococcal delayed-type hypersensitivity response and reduce the ability of the mice to clear cryptococci from tissues (56,57,60,68). A second immunoregulatory T cell, a $CD8^+$ cell, has been found in spleens of mice (57). The $CD4^+$ immunoregulatory cells do not alter the numbers or types of leukocytes that infiltrate an anticryptococcal delayed-type hypersensitivity reaction; however, they do have a downregulating effect on IL-2 and IFNγ production at the site (77). In contrast, the $CD8^+$ immunoregulatory cells appear to affect the numbers of neutrophils that infiltrate the delayed-type hypersensitivity reaction site (Murphy, unpub. data). The details of the characteristics and functions of these immunoregulatory T cells have been reviewed (45). The available data strongly support the notion that cryptococcal products in the circulation induce an array of immunoregulatory T cells, which depress the anticryptococcal immune responses and protection.

More consideration should be given to the impact of high levels of cryptococcal antigen in the cerebrospinal fluid (CSF) on progression of disease. As suggested by Denning et al. (78), high cryptococcal antigen concentrations could change the osmolality of the CSF, thereby affecting its outflow and adsorption and increasing intracranial pressure. The increased pressure may cause headaches, visual loss, and early death (78). It is also possible that release of mannitol by *C. neoformans* contributes to increased intracranial pressure in cryptococcal meningitis patients (78).

Melanin Synthesis

One characteristic that differentiates pathogenic isolates of *C. neoformans* from nonpathogenic isolates and other *Cryptococcus* species is the organism's ability to form a brown to black

pigment on a medium (such as birdseed or caffeic acid agar) that contains diphenolic compounds (1). This pigment, first described by Staib (79), is a melaninlike compound produced by *C. neoformans* isolates with phenoloxidase activity (80). The importance of melanin production in *C. neoformans* virulence was first demonstrated in the early 1980s. Rhodes, Polacheck, and Kwon-Chung (81) reported that naturally occurring *C. neoformans* mutants lacking melanin (Mel^-) were less virulent in mice than melanin-producing strains. Others (82-84), using chemically induced mutants or isogenic pairs of *C. neoformans,* have confirmed and extended this observation.

Biochemical analyses suggest that in *C. neoformans* melanogenesis is accomplished by conversion of dihydroxyphenols such as 3,4-dihydroxyphenylalanine (DOPA) to dopaquinone (Figure 3). This conversion is catalyzed by a phenoloxidase and is the rate-limiting step, presumably because subsequent steps in the pathway, such as dopaquinone rearranging to dopachrome and ultimately autoxidation to melanin, are spontaneous (85). *C. neoformans* lacks a tyrosinase enzyme required for endogenous production of dihydroxyphenols (83); thus to produce melanin, a *C. neoformans* isolate must be able to acquire diphenolic compounds from its growth environment, and it must have the enzyme phenoloxidase to catalyze conversion of these compounds into the subsequent melanin intermediates. The brain is a tissue rich in catecholamines such as DOPA and is a favorite target for infection by *C. neoformans*. However, the organism cannot use catecholamines as a sole carbon source, which suggests that the brain is not a preferred nutritional niche for growth of

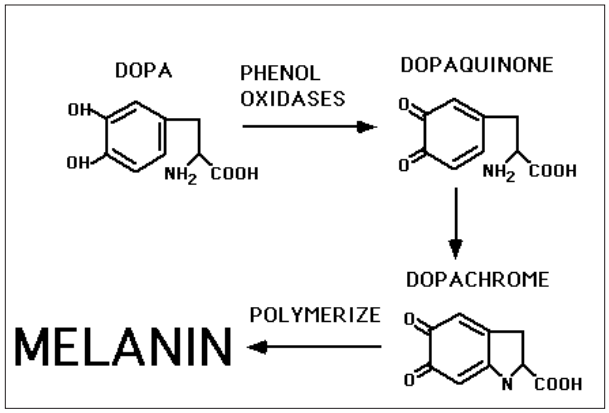

Figure 3. Proposed pathway for melanin synthesis by *C. neoformans* (85).

C. neoformans (86); rather, it may serve as a survival niche as described below.

Polacheck et al. (86) reported that melanin-producing isolates of *C. neoformans* were resistant to damage by an in vitro epinephrine oxidative system, whereas mutants lacking phenoloxidase activity were highly susceptible, as evidenced by decreased survival. Jacobson and Tinnell (87) found that melanin protected *C. neoformans* from damage by hypochlorite and permanganate but not by hydrogen peroxide. In these experiments, hypochlorite was 100 times more fungicidal than hydrogen peroxide, and *C. neoformans* could produce sufficient levels of melanin to effectively protect the organism from oxidative compounds produced by macrophages (87). Wang and Casadevall (88) extended these findings by examining survival of *C. neoformans* in the presence of nitric oxide and reactive nitrogen intermediates as well as in the epinephrine oxidative system described by Polacheck et al. (86). Wang and Casadevall cultured *C. neoformans* cells with L-DOPA to melanize the yeast cells (88). Melanized cryptococci survived damage by both nitrogen- and oxygen-derived oxidants significantly better than nonmelanized organisms of the same strain. These results support the hypothesis that *C. neoformans* uses catecholamines in the brain to make melanin, thereby protecting the organism from oxidative damage by scavenging free radicals (86).

Recently, cryptococcal diphenoloxidase has been purified, and the gene *CNLAC1* encoding this enzyme was cloned (89). The glycosylated protein has a molecular weight of 75 kDa and contains copper. The substrate specificity of the enzyme indicates that it is a laccase. *CNLAC1* contains 14 introns, a 624 amino acid open reading frame; transcriptional activity is derepressed in the absence of glucose (89), which confirms an earlier report of low glucose requirements for melanin formation (90). Disruption of *CNLAC1* resulted in loss of virulence of *C. neoformans*, whereas complementation with *CNLAC1* increased virulence of Mel^- mutants in mice (84); these results suggest that the laccase (phenoloxidase) encoded by *CNLAC1* is a potential virulence factor of *C. neoformans*. Furthermore, transcripts of the *CNLAC1* gene have been detected by reverse transcription-polymerase chain reaction (RT-PCR) in *C. neoformans* yeast cells isolated from CSF in a rabbit model of cryptococcal meningitis (84).

Besides acting as an antioxidant, melanin production may help *C. neoformans* survive in the host in other ways. Melanized yeast cells are less susceptible to amphotericin B than nonmelanized yeast cells, and this may contribute to the inability to effectively treat infections in immunocompromised hosts (91). Furthermore, phagocytosis of melanized *C. neoformans* by a macrophage cell line in the presence of an anti-GXM antibody was decreased, which suggests that melanin deposition in the cell wall may inhibit opsonization by specific antibodies (92). Recently, Huffnagle and coinvestigators (93) reported that melanized heat-killed *C. neoformans* strain 145 yeast cells stimulated less TNFα production by alveolar macrophages and less antigen-specific T-cell proliferation than nonmelanized heat-killed 145 strain cells. The authors suggest that melanin "cloaks" *C. neoformans* from recognition by host effector cells and inhibits induction of a protective T-cell-mediated immune response (93); however, it is possible that scavenging of host-produced oxidants and inhibition of phagocytosis by melanin may contribute to the decreased TNFα production and lymphoproliferation observed.

Although melanin production is important in the virulence of *C. neoformans*, there is very little evidence demonstrating the presence of melanin in vivo. One report indicates that phenoloxidase activity in *C. neoformans* is greatly diminished at 37°C compared with activity at 25°C, which suggests that melanin production may be limited in vivo (94). Detection of melanin in vivo is hampered by the lack of specific antibodies or stains. A modified Fontana-Masson stain has been used to detect a brown pigment in the cell wall of *C. neoformans* cells in histologic brain sections; however, the stain is not specific because *Cryptococcus laurentii*, which is Mel$^-$, also stains with Fontana-Masson (95).

In summary, accumulating evidence indicates that melanin production is an effective survival (virulence) factor of *C. neoformans,* and melanin may serve multiple roles in protecting this medically important fungus from host defenses. Two caveats should be noted in labeling melanin as a major virulence factor for *C. neoformans*. The *C. neoformans* isolate must be able to internalize the melanin precursors, and the phenoloxidase enzyme must make a sufficient amount of melanin precursor at 37°C. Consequently, much more work is needed to delineate the role of melanin production in virulence of *C. neoformans,* especially in light of the apparent temperature sensitivity of the cryptococcal phenoloxidase enzyme.

Mannitol Production

Accumulating evidence suggests that production of the hexitol D-mannitol may contribute to survival of *C. neoformans* in the host. Wong et al. (96) reported that of the 12 human isolates of *C. neoformans* examined, all produced and secreted D-mannitol into growth medium. Further, by using a rabbit cryptococcal meningitis model, they showed that *C. neoformans* can produce D-mannitol in vivo. CSF from rabbits treated with cortisone and infected intracisternally with *C. neoformans* contained more D-mannitol than CSF from controls, cortisone-treated uninfected rabbits, or rabbits infected with *C. neoformans* but not treated with cortisone (96). In the latter group, cryptococcal infection was limited. The levels of D-mannitol in infected rabbit CSF correlated well with both the numbers of culturable *C. neoformans* and the cryptococcal antigen titer of the CSF, which suggests that levels of D-mannitol in CSF may be prognostic (96); however, it is not known whether different isolates of *C. neoformans* vary in mannitol production. These authors suggested two means by which mannitol production may contribute to *C. neoformans* pathogenesis. First, high concentrations of D-mannitol in the CNS may contribute to brain edema. Second, mannitol is a potent scavenger of hydroxyl radicals, and cryptococcal-produced D-mannitol may help protect the organism from oxidative damage (96).

To investigate the role of mannitol in cryptococcosis, Chaturvedi and co-workers (97) isolated a low mannitol producing mutant after UV irradiation of *C. neoformans* strain H99. The mutant, mannitol low producer (MLP), was similar to the parent strain H99 in many growth and morphology characteristics and in known virulence factors such as capsule production and phenoloxidase activity (97). However, the mutant MLP strain was more susceptible to growth inhibition and killing by heat and high salt than the parent strain. In addition, the mutant MLP strain was significantly less virulent than the parent strain (MLP LD_{50} = 3.7 X 10^6 CFU, H99 LD_{50} = 6.9 X 10^2) (97). Further comparisons of H99 and MLP strains showed that polymorphonuclear leukocytes (PMNL) killed MLP significantly

better than H99 at several effector-to-target ratios (98). Addition of superoxide dismutase, mannitol, or dimethyl sulfoxide inhibited PMNL killing of both strains, but addition of catalase did not alter killing, which suggests that reactive oxygen intermediates such as the hydroxyl radical and hypochlorous acid are potent effector molecules against *C. neoformans* and mannitol may protect against oxidative killing by scavenging such compounds (98).

The results above indicate that mannitol production by *C. neoformans* correlates with increased resistance to heat stress, osmotic stress, and damage by reactive oxygen intermediates, as well as increased pathogenicity of this fungal agent. Additional studies are required to determine the role of mannitol production in virulence of *C. neoformans*. Isogenic strains lacking the enzyme required for mannitol production, namely mannitol dehydrogenase, are not available because the gene for this enzyme has not been found. However, a gene (*Mtl*) has been isolated from *C. neoformans* that can induce expression of the *S. cerevisiae* mannitol dehydrogenase gene (99). The 346 amino acid product encoded by the *Mtl* gene is thought to be involved in regulating mannitol production in *C. neoformans* and may be beneficial in future studies of mannitol production (99).

Other Potential Virulence Factors

Superoxide Dismutase

Jacobson and coinvestigators (100) examined superoxide dismutase (SOD) production by *C. neoformans* to determine whether SOD levels increased at 37°C to compensate for possible decreases in melanin production at this temperature. These investigators observed an increase in SOD levels at 37°C, which suggests that SOD may participate in free radical scavenging at this higher temperature in vivo (100); however, there is no evidence that SOD production serves as a virulence factor for *C. neoformans*.

Proteases

Muller and Sethi (101) reported that *C. neoformans* grown in culture produced a protease that could digest human plasma proteins, and Brueske (102) reported that *C. neoformans* culture supernatants contained a protease capable of digesting casein. However, neither of these studies determined whether the proteases were manufactured in vivo. Limited evidence indicating in vivo production of proteases has been presented by Salkowski and Balish (103). These investigators observed skin lesions on T-cell–deficient mice after intravenous infections with *C. neoformans* strain SLHA (103). Microscopic examination of the lesions suggested that the cryptococcal yeast cells were degrading collagen bundles in the dermis (103). Supernatants of SLHA strain cultures liquefied gelatin in vitro indicating that this cryptococcal strain secreted a collagenaselike protein (103). Thus, *C. neoformans* proteases possibly serve as virulence mechanisms by initiating invasion of host tissues; however, more studies with isogenic strains of *C. neoformans* are required before proteases can be listed as virulence factors.

Phospholipases

Recently, Chen and co-workers (104,105) reported phospholipase, lysophospholipase, and lysophospholipase-transacylase activity of *C. neoformans* grown on egg yolk agar. Of 50 strains tested, 49 had phospholipase activity (104), due to phospholipase B secreted into cultures (105). Analysis of four strains with varying levels of phospholipase activity indicated a correlation between phospholipase activity and virulence in BALB/c mice (104). The authors suggest that extracellular phospholipase activity produced by *C. neoformans* may disrupt mammalian cell membranes and allow the yeast cells to penetrate into host tissues (104,105); however, further investigations are necessary to establish the role, if any, of these types of products in the virulence of *C. neoformans*.

Regulation of Virulence

Regulation of virulence factors such as capsule production and melanin formation is not well understood. However, the gene GPA1, which encodes a G-protein α-subunit, is involved in the regulation of these virulence factors as well as in *C. neoformans* mating (106). Disruption of *GPA1* resulted in defects in mating in response to nitrogen starvation, capsule production in response to iron limitation, and melanin synthesis in response to glucose starvation (106). Furthermore, *gpa1* mutants were much less virulent in a rabbit model of cryptococcal meningitis (106). Reconstitution of the *gpa1* mutant with wild-type *GPA1* restored mating, capsule production, melanin synthesis, and virulence. Addition of cyclic AMP

also restored these phenotypes, which suggests that *C. neoformans GPA1* regulates these factors by sensing the nutritional signals of the environment and regulating cyclic AMP metabolism in the organism (106). These findings along with results of other molecular studies are intriguing and represent the initial steps in defining at the molecular level the factors controlling virulence in *C. neoformans.*

Conclusions

Virulent isolates of *C. neoformans* must be able to produce small particles that can get into the alveolar spaces, must be able to grow at 37°C at a pH of 7.3 to 7.4 in an atmosphere of approximately 5% CO_2, must have an intact calcineurin pathway, and (possibly) must be an α-mating type. The ability to produce a large capsule and shed great amounts of capsular material into the body fluids makes the organism highly virulent. Other factors, such as melanin, mannitol, superoxide dismutase, protease, and phospholipase production, may enhance the pathogenicity of *C. neoformans*. The effectiveness of many of these cryptococcal virulence factors depends on the status of the host's defensive mechanisms. Although we have learned much about the pathogenicity and virulence of *C. neoformans*, many gaps still remain in our knowledge.

Acknowledgments

We thank Drs. J.A. Alspaugh, J.R. Perfect, and J. Heitman for making their manuscript available before publication.

Dr. Buchanan is a research assistant professor in the Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA. Dr. Murphy is a George Lynn Cross Research Professor of Microbiology and Immunology in the same department.

References

1. Kwon-Chung KJ. Cryptococcosis. In: Kwon-Chung KJ, Bennett JE, editors. Medical mycology. Philadelphia: Lea & Febiger, 1992:397-446.
2. Mitchell TG, Perfect JR. Cryptococcosis in the era of AIDS—100 years after the discovery of *Cryptococcus neoformans*. Clin Microbiol Rev 1995;8:515-48.
3. Rippon JW. Cryptococcosis. In: Rippon JW, editor. Medical mycology. Philadelphia: W.B. Saunders Company; 1988, p. 582-609.
4. Duperval B, Hermans PE, Brewer NS, Roberts GD. Cryptococcosis, with emphasis on the significance of isolation of *Cryptococcus neoformans* from the respiratory tract. Chest 1977;72:13-9.
5. Wickes BL, Mayorga ME, Edman U, Edman JC. Dimorphism and haploid fruiting in *Cryptococcus neoformans*: association with the a-mating type. Proc Natl Acad Sci U S A 1996;93:7327-31.
6. Kwon-Chung KJ, Bennett JE. Distribution of and mating types of *Cryptococcus neoformans* among natural and clinical isolates. Am J Epidemiol 1978;108:337-40.
7. Kwon-Chung KJ, Hill WB. Sexuality and pathogenicity of *Filobasidiella neoformans* (*Cryptococcus neoformans*). In: Vanbreuseghem R, DeVroey C, Vanbreuseghem R, editors. Sexuality and pathogenicity of fungi. Paris: Masson; 1981. p. 243-50.
8. Kwon-Chung KJ, Edman JC, Wickes BL. Genetic association of mating types and virulence in *Cryptococcus neoformans*. Infect Immun 1992;60:602-5.
9. Moore TDE, Edman JC. The α-mating type locus of *Cryptococcus neoformans* contains a pheromone gene. Mol Cell Biol 1993;13:1962-70.
10. Odom A, Muir S, Lim E, Toffaletti DL, Perfect J, Heitman J. Calcineurin is required for virulence of *Cryptococcus neoformans*. EMBO J 1997;16:2576-89.
11. Cherniak R, Reiss E, Slodki ME, Plattner RD, Blumer SO. Structure and antigenic activity of the capsular polysaccharide of *Cryptococcus neoformans*. Mol Immunol 1980;17:1025-32.
12. Bhattacharjee AK, Kwon-Chung KJ, Glaudemans CP. Capsular polysaccharides from a parent strain and from a possible mutant strain of *Cryptococcus neoformans* serotype A. Carbohydr Res 1981;95:237-48.
13. Bhattacharjee AK, Bennett JE, Glaudemans CP. Capsular polysaccharides of *Cryptococcus neoformans*. Reviews of Infectious Diseases 1984;6:619-24.
14. Cherniak R. Soluble polysaccharides of *Cryptococcus neoformans*. Cur Top Med Mycol 1988;2:40-54.
15. Cherniak R, Sundstrom JB. Polysaccharide antigens of the capsule of *Cryptococcus neoformans*. Infect Immun 1994;62:1507-12.
16. Bulmer GS, Sans MD, Gunn CM. *Cryptococcus neoformans*. I. Nonencapsulated mutants. J Bacteriol 1967;94:1475-9.
17. Fromtling RA, Shadomy HJ, Jacobson ES. Decreased virulence in stable, acapsular mutants of *Cryptococcus neoformans*. Mycopathologia 1982;79:23-9.
18. Kozel TR, Cazin J Jr. Non-encapsulated variant of *Cryptococcus neoformans*. I. Virulence studies and characterization of soluble polysaccharide. Infect Immun 1971;3:287-94.
19. Kwon-Chung KJ, Rhodes JC. Encapsulation and melanin formation as indicators of virulence in *Cryptococcus neoformans*. Infect Immun 1986;51:218-23.
20. Chang YC, Kwon-Chung KJ. Complementation of a capsule-deficient mutation of *Cryptococcus neoformans* restores its virulence. Mol Cell Biol 1994;14:4912-9.
21. Chang YC, Penoyer LA, Kwon-Chung KJ. The second capsule gene of *Cryptococcus neoformans*, *CAP64*, is essential for virulence. Infect Immun 1996;64:1977-83.

22. Chang YC, Cherniak R, Kozel TR, Granger LC, Morris LC, Weinhold LC, Kwon-Chung KJ. Structure and biological activities of acapsular *Cryptococcus neoformans* 602 complemented with the *CAP64* gene. Infect Immun 1997;65:1584-92.
23. Dong ZM, Murphy JW. Mobility of human neutrophils in response to *Cryptococcus neoformans* cells, culture filtrate antigen, and individual components of the antigen. Infect Immun 1993;61:5067-77.
24. Kozel TR, Pfrommer GS. Activation of the complement system by *Cryptococcus neoformans* leads to binding of iC3b to the yeast. Infect Immun 1986;52:1-5.
25. Kozel TR, Mastroianni RP. Inhibition of phagocytosis by cryptococcal polysaccharide: dissociation of the attachment and ingestion phases of phagocytosis. Infect Immun 1976;14:62-7.
26. Kozel TR, Highison B, Stratton CJ. Localization on encapsulated *Cryptococcus neoformans* of serum components opsonic for phagocytosis by macrophages and neutrophils. Infect Immun 1984;43:574-9.
27. Griffin FMJ. Roles of macrophage Fc and C3b receptors in phagocytosis of immunologically coated *Cryptococcus neoformans*. Proc Natl Acad Sci U S A 1981;78:3853-7.
28. Kozel TR, Pfrommer GS, Guerlain AS, Highison BA, Highison GJ. Role of the capsule in phagocytosis of *Cryptococcus neoformans*. Reviews of Infectious Diseases 1988;10:S436-9.
29. Macher AM, Bennett JE, Gadek JE, Frank MM. Complement depletion in cryptococcal sepsis. J Immunol 1978;120:1686-90.
30. Bulmer GS, Sans MD. *Cryptococcus neoformans* II. Phagocytosis by human leukocytes. J Bacteriol 1967;94:1480-3.
31. Bulmer GS, Sans MD. *Cryptococcus neoformans.* III. Inhibition of phagocytosis. J Bacteriol 1968;95:5-8.
32. Kozel TR, Gotschlich EC. The capsule of *Cryptococcus neoformans* passively inhibits phagocytosis of the yeast by macrophages. J Immunol 1982;129:1675-80.
33. Vecchiarelli A, Pietrella D, Dottorini M, Monari C, Retini C, Todisco T, Bistoni F. Encapsulation of *Cryptococcus neoformans* regulates fungicidal activity and the antigen presentation process in human alveolar macrophages. Clin Exp Immunol 1994;98:217-23.
34. Nosanchuk JD, Casadevall A. Cellular charge of *Cryptococcus neoformans*: Contributions from the capsular polysaccharide, melanin, and monoclonal antibody binding. Infect Immun 1997;65:1836-41.
35. Collins HL, Bancroft GJ. Encapsulation of *Cryptococcus neoformans* impairs antigen-specific T-cell responses. Infect Immun 1991;59:3883-8.
36. Vecchiarelli A, Dottorini M, Pietrella D, Vecchiarelli A, Dottorini M, Pietrella D, et al. Role of human alveolar macrophages as antigen-presenting cells in *Cryptococcus neoformans* infection. Am J Respir Cell Mol Biol 1994;11:130-7.
37. Levitz SM, Tabuni A, Kornfeld H, Reardon CC, Golenbock DT. Production of tumor necrosis factor alpha in human leukocytes stimulated by *Cryptococcus neoformans*. Infect Immun 1994;62:1975-81.
38. Naslund PK, Miller WC, Granger DL. *Cryptococcus neoformans* fails to induce nitric oxide synthase in primed murine macrophage-like cells. Infect Immun 1995;63:1298-1304.
39. Vecchiarelli A, Retini C, Pietrella D, Monari C, Tascini T, Beccari T, Kozel TR. Downregulation by cryptococcal polysaccharide of tumor necrosis factor alpha and interleukin-1 beta secretion from human monocytes. Infect Immun 1995;63:2919-23.
40. Delfino D, Cianci L, Migliardo M, Mancuso G, Cusumano V, Corradini C, Teti G. Tumor necrosis factor-inducing activities of *Cryptococcus neoformans* components. Infect Immun 1996;64:5199-204.
41. Delfino D, Cianci L, Lupis E, Celese A, Petrelli ML, Curro F, et al. Interleukin-6 production by human monocytes stimulated with *Cryptococcus neoformans* components. Infect Immun 1997;65:2454-6.
42. Huffnagle GB, Toews GB, Burdick MD, Boyd MB, McAllister KS, McDonald RA, et al. Afferent phase production of TNF-α required for the development of protective T cell immunity to *Cryptococcus neoformans*. J Immunol 1996;157:4529-36.
43. Vecchiarelli A, Retini C, Monari C, Tascini C, Bistoni F, Kozel TR. Purified capsular polysaccharide of *Cryptococcus neoformans* induces interleukin-10 secretion by human monocytes. Infect Immun 1996;64:2846-9.
44. Moore KW, O'Garra A, de Waal Malefyt R, Vieira P, Mosmann TR. Interleukin-10. Annu Rev Immunol 1993;11:165-90.
45. Murphy JW. Cell-mediated immunity. In: Howard DH, Miller JD, editors. The Mycota VI. Human and animal relationships. Berlin: Springer-Verlag; 1996. p. 67-97.
46. Retini C, Vecchiarelli A, Monari C, Tascini C, Bistoni F, Kozel TR. Capsular polysaccharide of *Cryptococcus neoformans* induces proinflammatory cytokine release by human neutrophils. Infect Immun 1996;64:2897-903.
47. Gordon MA, Vedder DK. Serologic tests in diagnosis and prognosis of cryptococcosis. JAMA 1966;197:961-7.
48. Reiss E, Cherniak R, Eby E, Kaufman L. Enzyme immunoassay detection of IgM to galactoxylomannan of *Cryptococcus neoformans*. Diagnostic Immunology 1984;2:109-15.
49. Diamond RD, Bennett JE. Prognostic factors in cryptococcal meningitis. A study in 111 cases. Ann Intern Med 1974;80:176-81.
50. Dong ZM, Murphy JW. Intravascular cryptococcal culture filtrate (CneF) and its major component glucuronoxylomannan (GXM) are potent inhibitors of leukocyte accumulation. Infect Immun 1995;63:770-8.
51. Dong ZM, Murphy JW. Cryptococcal polysaccharides induce L-selectin shedding and tumor necrosis factor receptor loss from the surface of human neutrophils. J Clin Invest 1996;97:689-98.
52. Dong ZM, Murphy JW. Cryptococcal polysaccharides bind to CD18 on human neutrophils. Infect Immun 1997;65:557-63.
53. Murphy JW, Cozad GC. Immunological unresponsiveness induced by cryptococcal capsular polysaccharide assayed by the hemolytic plaque technique. Infect Immun 1972;5:896-901.
54. Kozel TR, Gulley WF, Cazin J Jr. Immune response to *Cryptococcus neoformans* soluble polysaccharide: immunological unresponsiveness. Infect Immun 1977;18:701-7.
55. Breen JF, Lee IC, Vogel FR, Friedman H. Cryptococcal capsular polysaccharide-induced modulation of murine immune responses. Infect Immun 1982;36:47-51.

56. Murphy JW, Moorhead JW. Regulation of cell-mediated immunity in cryptococcosis. I. Induction of specific afferent T suppressor cells by cryptococcal antigen. J Immunol 1982;128:276-83.
57. Murphy JW, Mosley RL, Moorhead JW. Regulation of cell-mediated immunity in cryptococcosis. II. Characterization of first-order T suppressor cells (Ts1) and induction of second-order suppressor cell. J Immunol 1983;130:2876-81.
58. Morgan MA, Blackstock R, Bulmer GS, Hall NK. Modification of macrophage phagocytosis in murine cryptococcosis. Infect Immun 1983;40:493-500.
59. Blackstock R, Hall NK. Nonspecific immune suppression by *Cryptococcus neoformans* infection. Mycopathologia 1984;86:35-43.
60. Murphy JW. Effects of first-order *Cryptococcus*-specific T-suppressor cells on induction of cells responsible for delayed-type hypersensitivity. Infect Immun 1985;48:439-45.
61. Murphy JW, Mosley RL. Regulation of cell-mediated immunity in cryptococcosis. III. Characterization of second-order T suppressor cells (Ts2). J Immunol 1985;134:577-84.
62. Mosley RL, Murphy JW, Cox RA. Immunoadsorption of *Cryptococcus*-specific suppressor T-cell factors. Infect Immun 1986;51:844-50.
63. Khakpour FR, Murphy JW. Characterization of a third-order suppressor T cell (Ts3) induced by cryptococcal antigen(s). Infect Immun 1987;55:1657-62.
64. Blackstock R, McCormack JM, Hall NK. Induction of a macrophage-suppressive lymphokine by soluble cryptococcal antigens and its association with models of immunological tolerance. Infect Immun 1987;55:233-9.
65. Blackstock R, Hernandez NC. Inhibition of phagocytosis in cryptococcosis: phenotypic analysis of the suppressor cell. Cell Immunol 1988;114:174-87.
66. Murphy JW, Cox RA. Induction of antigen-specific suppression by circulating *Cryptococcus neoformans* antigen. Clin Exp Immunol 1988;73:174-80.
67. Murphy JW. Influence of cryptococcal antigens on cell-mediated immunity (CMI). Reviews of Infectious Diseases 1988;10:5432-5.
68. Murphy JW. Clearance of *Cryptococcus neoformans* from immunologically suppressed mice. Infect Immun 1989;57:1946-52.
69. Murphy JW. Immunoregulation in cryptococcosis. In: Kurstak E, editor. Immunology of fungal diseases. New York: Marcel Dekker; 1989. p. 319-45.
70. Blackstock R, Hall NK, Hernandez NC. Characterization of a suppressor factor that regulates macrophage phagocytosis in murine cryptococcosis. Infect Immun 1989;57:1773-9.
71. Blackstock R, Zembala M, Asherson GL. Functional equivalence of cryptococcal and haptene-specific T suppressor factor (TsF). I. Picryl and oxazolone-specific TsF, which inhibit transfer of contact sensitivity also inhibit phagocytosis by a subset of macrophages. Cell Immunol 1991;136:435-47.
72. Henderson DK, Bennett JE, Huber MA. Long-lasting, specific immunologic unresponsiveness associated with cryptococcal meningitis. J Clin Invest 1982;69:1185-90.
73. Henderson DK, Kan VL, Bennett JE. Tolerance to cryptococcal polysaccharide in cured cryptococcosis patients: failure of antibody secretion *in vitro*. Clin Exp Immunol 1986;65:639-46.
74. Salvin SB, Smith RF. An antigen for detection of hypersensitivity to *Cryptococcus neoformans*. Proc Soc Exp Biol Med 1961;108:498-501.
75. Diamond RD, Bennett JE. Disseminated cryptococcosis in man: decreased lymphocyte transformation in response to *Cryptococcus neoformans*. J Infect Dis 1973;127:694-7.
76. Graybill JR, Alford RH. Cell-mediated immunity in cryptococcosis. Cell Immunol 1974;14:12-21.
77. Buchanan KL, Murphy JW. Regulation of cytokine production during the expression phase of the anticryptococcal delayed-type hypersensitivity response. Infect Immun 1994;62:2930-9.
78. Denning DW, Armstrong RW, Lewis BH, Stevens DA. Elevated cerebrospinal fluid pressures in patients with cryptococcal meningitis and acquired immunodeficiency syndrome. Am J Med 1991;91:267-72.
79. Staib F. *Cryptococcus neoformans* und *Guizotia abyssinica*. Zeitschrift für Hygiene 1962;148:466-75.
80. Shaw CE, Kapica L. Production of diagnostic pigment by phenoloxidase activity of *Cryptococcus neoformans*. Applied Microbiology 1972;24:824-30.
81. Rhodes JC, Polacheck I, Kwon-Chung KJ. Phenoloxidase activity and virulence in isogenic strains of *Cryptococcus neoformans*. Infect Immun 1982;36:1175-84.
82. Kwon-Chung KJ, Polacheck I, Popkin TJ. Melanin-lacking mutants of *Cryptococcus neoformans* and their virulence for mice. J Bacteriol 1982;150:1414-21.
83. Torres-Guerrero H, Edman JC. Melanin-deficient mutants of *Cryptococcus neoformans*. J Med Vet Mycol 1994;32:303-13.
84. Salas SD, Bennett JE, Kwon-Chung KJ, Perfect JR, Williamson PR. Effect of the laccase gene, *CNLAC1*, on virulence of *Cryptococcus neoformans*. J Exp Med 1996;184:377-86.
85. Polacheck I, Kwon-Chung KJ. Melanogenesis in *Cryptococcus neoformans*. Journal of General Microbiology 1988;134:1037-41.
86. Polacheck I, Platt Y, Aronovitch J. Catecholamines and virulence of *Cryptococcus neoformans*. Infect Immun 1990;58:2919-22.
87. Jacobson ES, Tinnell SB. Antioxidant function of fungal melanin. J Bacteriol 1993;175:7102-4.
88. Wang Y, Casadevall A. Susceptibility of melanized and nonmelanized *Cryptococcus neoformans* to nitrogen- and oxygen-derived oxidants. Infect Immun 1994;62:3004-7.
89. Williamson PR. Biochemical and molecular characterization of the diphenol oxidase of *Cryptococcus neoformans*: identification as a laccase. J Bacteriol 1994;176:656-64.
90. Polacheck I, Hearing VJ, Kwon-Chung KJ. Biochemical studies of phenoloxidase and utilization of catecholamines in *Cryptococcus neoformans*. J Bacteriol 1982;150:1212-20.
91. Wang Y, Casadevall A. Growth of *Cryptococcus neoformans* in presence of L-dopa decreases its susceptibility to amphotericin B. Antimicrob Agents Chemother 1994;38:2648-50.

92. Wang Y, Aisen P, Casadevall A. *Cryptococcus neoformans* melanin and virulence: mechanism of action. Infect Immun 1995;63:3131-6.
93. Huffnagle GB, Chen GH, Curtis JL, McDonald RA, Strieter RM, Toews GB. Down-regulation of the afferent phase of T cell-mediated pulmonary inflammation and immunity by a high melanin-producing strain of *Cryptococcus neoformans*. J Immunol 1995;155:3507-16.
94. Jacobson ES, Emery HS. Temperature regulation of the cryptococcal phenoloxidase. J Med Vet Mycol 1991;29:121-4.
95. Kwon-Chung KJ, Hill WB, Bennett JE. New, special stain for histopathological diagnosis of cryptococcosis. J Clin Microbiol 1981;13:383-7.
96. Wong B, Perfect JR, Beggs S, Wright KA. Production of the hexitol D-mannitol by *Cryptococcus neoformans* in vitro and in rabbits with experimental meningitis. Infect Immun 1990;58:1664-70.
97. Chaturvedi V, Flynn T, Niehaus WG, Wong B. Stress tolerance and pathogenic potential of a mannitol mutant of *Cryptococcus neoformans*. Microbiology 1996;142:937-43.
98. Chaturvedi V, Wong B, Newman SL. Oxidative killing of *Cryptococcus neoformans* by human neutrophils. Evidence that fungal mannitol protects by scavenging reactive oxygen intermediates. J Immunol 1996;156:3836-40.
99. Perfect JR, Rude TH, Wong B, Flynn T, Chaturvedi V, Niehaus W. Identification of a *Cryptococcus neoformans* gene that directs expression of the cryptic *Saccharomyces cerevisiae* mannitol dehydrogenase gene. J Bacteriol 1996;178:5257-62.
100. Jacobson ES, Jenkins ND, Todd JM. Relationship between superoxide dismutase and melanin in a pathogenic fungus. Infect Immun 1994;62:4085-6.
101. Muller HE, Sethi KK. Proteolytic activity of *Cryptococcus neoformans* against human plasma proteins. Med Microbiol Immunol (Berl) 1972;158:129-34.
102. Brueske CH. Proteolytic activity of a clinical isolate of *Cryptococcus neoformans*. J Clin Microbiol 1986;23:631-3.
103. Salkowski CA, Balish E. Cutaneous cryptococcosis in athymic and beige-athymic mice. Infect Immun 1991;59:1785-9.
104. Chen SCA, Muller M, Zhou JZ, Wright LC, Sorrell TC. Phospholipase activity in *Cryptococcus neoformans*: a new virulence factor? J Infect Dis 1997;175:414-20.
105. Chen SCA, Wright LC, Santangelo RT, Muller M, Moran VR, Kuchel PW, Sorrell TC. Identification of extracellular phospholipase B, lysophospholipase, and acyltransferase produced by *Cryptococcus neoformans*. Infect Immun 1997;65:405-11.
106. Alspaugh JA, Perfect JR, Heitman J. *Cryptococcus neoformans* mating and virulence are regulated by the G-protein α subunit GPA1 and cAMP. Genes Dev 1997;11:3206-17.