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MINIREVIEW

Role of Phagocytosis in the Virulence of *Cryptococcus neoformans*†

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INTRACELLULAR VERSUS EXTRACELLULAR: IMPORTANCE OF ADAPTATION

Phagocytosis is a receptor-mediated process that leads to the internalization of foreign particles into phagocytic cells. Phagocytic cells, such as macrophages, dendritic cells, and neutrophils, are characterized by their ability to express a series of phagocytic receptors designed to recognize, bind, and trigger the ingestion of pathogens as well as cellular debris and apoptotic cells. After phagocytic uptake, ingested particles are destroyed as they progress along the degradative endocytic pathway that leads to the formation of the mature phagolysosome. Thus, macrophages, dendritic cells, and neutrophils represent the first line of defense against microorganisms, including fungal pathogens, by providing a method for their removal and destruction. Two recent and comprehensive reviews, by Mansour and Levitz and by Roeder et al., discuss the mechanisms by which fungal pathogens interact with receptors on the surfaces of macrophages, dendritic cells, and neutrophils (70, 87).

Defining the mechanisms by which the host immune response controls infections remains a poorly understood area because of the complex nature of multistep host-pathogen interactions, particularly for facultative intracellular pathogens. These microorganisms are able to survive and replicate both intra- and extracellularly, increasing the dynamic cross talk with all arms of the host immune system. In this respect, phagocytosis can be considered either an opportunity or an obstacle for microbial pathogens. Viruses, many bacteria, and protozoa that are obligate intracellular parasites can only replicate inside their host cells. If these microorganisms avoid phagocytosis, they will not survive and grow in the extracellular environment. Other pathogens, including some bacteria and many fungi, can replicate and survive both extra- and intracellularly. The choice of lifestyle depends on the production of a specific pathogen's factors and/or on conditions that these pathogens find in the host.

Facultative intracellular pathogens have evolved the ability to avoid phagocytosis by blocking adhesion to and/or internalization by phagocytic cells. To survive and proliferate intracellularly, this class of microbes has developed mechanisms to avoid destruction by the degradative pathway, including escape from the phagosome into the host cytosol, the avoidance of phagolysosome fusion, and survival within the phagolysosome. Of the facultative intracellular pathogens, *Cryptococcus neoformans* has developed unique factors that regulate pathogen invasion and dissemination based on its ability to choose between the intra- and extracellular compartments. The identification of genes or factors of the pathogen (or the host) that contribute to one lifestyle (intracellular) with respect to the other (extracellular) can be exploited for the development of novel prevention and treatment strategies for not only cryptococcosis but also, potentially, other fungal infections.

ESTABLISHMENT OF CRYPTOCOCCAL INFECTION AND IMPORTANCE OF FUNGAL MORPHOLOGY

Fungal pathogens infect the host by using diverse morphologies, such as spores, hyphae, and yeasts, which are found to elicit diverse responses by the host due to the versatile activation of phagocytic cells. Thus, the determination of the role of a specific form (e.g., yeast or hypha) in the development of fungal infections is a difficult task. An insightful review by Gow et al. argues that no simple or universal relationship can be drawn between morphology and host invasion (43). It is believed that, except for *Aspergillus* spp. and *C. neoformans*, other fungal pathogens, such as *Blastomyces dermatitidis*, *Candida tropicalis*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Penicillium marneffei*, *Sporothrix schenckii*, and *Wangiella dermatitidis*, are found as yeast forms during human infections, although they enter the body as spores. *C. neoformans* infects humans as a spore and/or yeast form. Once inhaled, the fungal particles (basidiospores or yeast cells) do not necessarily cause a symptomatic infection (36–38). Perhaps the most comprehensive studies addressing this hypothesis are from the work of Garcia-Hermoso et al. showing that individuals can harbor dormant organisms for as long as 13 years before infection is activated (38). These studies raised additional questions, such as where the organism resides during its dormant state, how it survives, and what allows its reactivation—the pathogen, the host, or a combination of both. Now that the *C. neoformans* and human genome projects are completed and molecular and genetic tools have been developed for studying the pathobiology of interactions between *C. neoformans* and human cells, researchers are beginning to address these questions. Results from studies conducted with *C. neoformans* as a molecular model can be applied to other life-threatening fungal pathogens for which these tools have not yet been developed.

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Other pathogenic yeasts, such as *Candida albicans*, can be found as a yeast form and/or hyphae in infected tissues, and the formation of hyphae is considered a virulence factor since early studies of this fungus suggested that hyphae are important for disease establishment (90, 95). Another *Candida* species, *Candida glabrata*, has only a limited ability to produce hyphae (22), but it is still pathogenic and represents the second most common cause of disseminated *Candida* infection (93). Although changes in morphology may represent an important factor for tissue invasion, once the disease state is established, a unicellular morphology is favored for most fungi. Another example is given by infections due to *Aspergillus fumigatus*, whose conidia (or spores) establish invasive disease in the lung exclusively by hyphal development. The development of hyphae may be advantageous for the pathogen considering that it provides increased resistance to phagocytosis by macrophages and other leukocytes (23, 65). However, the molecular mechanisms by which hyphae of *C. albicans* or *A. fumigatus* inhibit phagocytosis in vivo are largely unknown.

Evidence supporting the importance of fungal morphology in the regulation of the host response comes from the ability of dendritic cells to distinguish between different cell types (e.g., conidia and hyphae of *C. albicans* or *A. fumigatus*) and to polarize a T-cell response accordingly; phagocytosis of conidia induces a Th1 response whereas phagocytosis of hyphae induces a Th2 response (7, 29). Interestingly, mice receiving dendritic cells challenged with *Aspergillus* conidia are resistant to infection, whereas mice receiving dendritic cells challenged with hyphae are more sensitive to infection by *Aspergillus* (8). These studies suggest that the administration of dendritic cells that are activated in vitro toward a Th1 response against fungal pathogens could be used as an effective immune enhancer or vaccine. Therefore, changes in fungal morphology are advantageous for fungal evasion of the host immune response, for their dissemination, and for disease development.

In cases in which an infection develops with the same cell type, the occurrence of a phenotype switch has been proposed. Phenotypic switching is a phenomenon defined as the spontaneous emergence of colonies with an altered morphology at different frequencies. These colonies with an altered morphology, e.g., opaque instead of white or mucoid instead of smooth yeast colonies, result from the differential expression of specific genes or factors which in turn may affect the course of infection. Phenotypic switching occurs in many microorganisms, including bacteria (*Mycobacterium avium* [14] and *Mycoplasma* [110]), viruses (human immunodeficiency virus [HIV] [12]), protozoa (*Trypanosoma brucei* [104] and *Plasmodium falciparum* [79]), and fungi (*C. albicans* [96, 97, 105], *Candida glabrata* [10], and *C. neoformans* [34, 101, 112]). In *C. neoformans*, phenotypic switching occurs in vivo and influences the outcome of infection (35). Changes in morphology are clearly associated with virulence, but until now it has been difficult to establish the extent of this phenomenon.

PRODUCTION OF ANTIPHAGOCYTIC FACTORS BY *C. NEOFORMANS*

Capsule and phagocytosis. More direct evidence of cell morphology's effect on virulence is provided by the production of a capsule surrounding the cell wall of microorganisms. Indeed, many bacteria (e.g., *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) (109), *Cryptococcus* spp., and many other fungi (e.g., *Malassezia furfur* [74], *Rhinosporidium seeberi* [51], *Tricosporon beigelii* [24, 53, 72], *Blastocystis hominis* [59] *Exophilia* spp. [111], *Sporothrix schenckii* [39, 69], and *Tremella mesenterica* [25, 84]) change cell morphology by producing a polysaccharide capsule. Among fungi, the capsule of *C. neoformans* is perhaps the most studied since it is a characteristic and well-established virulence factor for this fungus. For a comprehensive analysis of the structure, synthesis, assembly, degradation, and regulation of the *C. neoformans* capsule, an outstanding review is available by Bose et al. (6). Studies have identified many genes and pathways involved in capsule production by *C. neoformans*, such as the *CAP* and *CAS* gene families and the *MAN1*, *UXS1*, *AGS1*, *GPA1*, *PKA1*, *PKR1*, and *STE12* genes (reviewed in references 6 and 27). However, the role and mechanisms by which many of these genes and pathways regulate capsule production during infection remain to be elucidated.

The *C. neoformans* capsule is the most well-studied fungal antiphagocytic factor. The capsule inhibits the phagocytosis of the fungus by macrophages (5, 44, 63), dendritic cells (107), and neutrophils (26, 28, 55, 114) and also inhibits the internalization of fungal cells by endothelial cells (49). Alveolar macrophages may be considered the first host defense against *C. neoformans*. Studies on the role of the capsule in the interaction between *C. neoformans* and macrophages resulted in compelling findings, which were described recently by Harrison and Levitz (47). Once it is internalized by macrophages, *C. neoformans* may (i) grow and eventually lyse the macrophage, (ii) be killed by the macrophage, or (iii) live within the macrophage in an equilibrium for an undetermined amount of time. In the lungs, all three outcomes are possible. Interestingly, the capsule plays an important role not only in the inhibition of phagocytosis but also in the inhibition of killing by macrophages.

More than 30 years ago, it was shown that acapsular cryptococci are readily ingested by neutrophils and macrophages, whereas encapsulated cryptococci are resistant to phagocytosis (11, 54). These observations have been corroborated by many investigators (reviewed in references 15 and 56). However, the fact that encapsulated cells can be phagocytosed in the lung (31, 32) suggests that the capsule promotes virulence by mechanisms other than simply preventing phagocytosis. Furthermore, the modulation of this phagocytic process by the capsule is regulated not only by its size but also by its structure and composition, by the presence or absence of serum, and by the source of phagocytic cells. For instance, cryptococci grown in vitro under conditions that induce small capsules are somewhat resistant to phagocytosis, but the resistance can be overcome by the addition of normal serum. Cryptococci grown in vitro to produce large capsules are also resistant to phagocytosis, and the resistance cannot be overcome by the addition of normal serum (44, 77, 113). The incubation of encapsulated cryptococci (with small or big capsules) in normal human serum leads to activation of the alternative complement cascade and the deposition of opsonic C3 (iC3b) fragments at the capsular surface and in its interior (57, 58). Thus, complement opsonization and capsule size inversely regulate phagocytosis: if the capsule size increases (in vitro), the efficacy of complement-mediated phagocytosis decreases. The effect of capsule

size on phagocytosis is not affected when yeast cells are opsonized with an antibody. Additionally, if iC3b is localized at the capsule surface (outer edge), yeast cells are readily phagocytosed, whereas when iC3b is localized inside the capsule, yeast cells are poorly phagocytosed (113). Recent elegant studies by Gates et al. indicated that complement deposition in the capsule differs depending on whether the encapsulated yeast cells are obtained in vivo (from the brain) or in vitro (from the growth medium for capsule induction) (40). The density of the capsular matrix between cells grown in vitro under capsule induction and cells harvested from infected tissue also differs. In vivo, the density of the capsular matrix (high glucuronoxylomannan [GXM] concentration) is much higher than that in vitro, without a significant change in the capsule's size. As a consequence, the increased density in GXM in the capsule from cells obtained in vivo decreases the deposition of both complement and antibodies compared to the deposition of complement or antibodies in cells with the same sized capsule but obtained from an in vitro culture. Although the pathobiological effects and the molecular mechanism(s) by which changes in the capsule's density occur are not known, it is exciting to speculate that this phenomenon may have important implications in the regulation of phagocytosis and virulence.

The type of phagocytic cells may also have an important role on the outcome of infection by *C. neoformans*. In murine models, rat and mouse alveolar macrophages will kill *C. neoformans*, regardless of the presence of serum or gamma interferon (IFN-γ) or other cytokines (4, 63). Murine resident peritoneal macrophages can kill acapsular cells without macrophage activation or yeast opsonization, but they can kill encapsulated cells only if IFN-γ is provided or if yeast cells are opsonized with either fresh serum or an anticapsular antibody (62). Human alveolar macrophages have anticryptococcal activity but are incapable of fungicidal activity unless fresh serum is added (108). In contrast to murine macrophages, human macrophages kill acapsular strains more easily than encapsulated strains (106). Surprisingly, the addition of IFN-γ to human alveolar macrophages not only fails to increase anticryptococcal activity but actually has a detrimental effect (13, 64, 83). Freshly isolated human blood monocytes or neutrophils kill *C. neoformans* (26, 73). However, if they are induced to differentiate into macrophages during in vitro culturing, their killing activity is lost, although they can still inhibit cryptococcal growth. It has been suggested that the loss of killing activity may be due to a loss of myeloperoxidase activity during differentiation (47), but this hypothesis is not supported by previous studies in which mice lacking myeloperoxidase activity were not hypersensitive to *C. neoformans* infection (1). Although differences in the killing of *C. neoformans* by human and murine macrophages have been observed, the relative importance of the murine model is supported by the similarity between the human and mouse pathologies of cryptococcosis (15, 19, 48, 78, 82).

Rabbit macrophages behave differently than human or murine macrophages, at least when isolated from the cerebrospinal fluid, and they are incapable of killing *C. neoformans* in vitro (81). This is intriguing considering that rabbits are naturally resistant to infection by *C. neoformans* introduced directly into the cisterna magna. Thus, one would expect a strong anticryptococcal activity by their cerebrospinal macrophages. Perhaps rabbit cerebrospinal macrophages need to be in their natural host environment to kill *C. neoformans*, or maybe the killing of *C. neoformans* in this model is exerted mainly by macrophages or other phagocytic cells that are localized in other compartments. The rabbit is a robust model for cryptococcosis because this animal is particularly resistant to cryptococcal infection unless it is immunocompromised, mimicking the clinical course of cryptococcal infection in humans. Studies on the role of rabbit macrophages (cerebrospinal, alveolar, or peritoneal) in the killing activity against *C. neoformans* should be encouraged. Collectively, these studies stress the complexity of interactions between *C. neoformans* and phagocytic cells. Phagocytic cells are required for fungal clearance, but their functions should be investigated, with consideration given to their environment, their interconnection with other arms of the immune response (cellular and humoral), and the specific factor(s) produced by the microorganism(s). Through the modulation of its size, structure, and composition, the *C. neoformans* capsule regulates this interface, and studies addressing this issue are warranted because they may reveal a key fungal and/or host factor(s) that is important for the development of cryptococcosis.

Capsule and virulence. Acapsular cryptococcal strains are not pathogenic (17). On the other hand, although other cryptococcal species produce a similar capsule, they also are not pathogenic (25). This suggests that the capsule is necessary but not sufficient for fungal cells to cause disease and that its ability to aid infection is enhanced by other virulence factors. This hypothesis is supported by reports showing that during human cryptococcosis, acapsular or poorly encapsulated strains can be isolated. Kimura et al. reported a case of pulmonary cryptococcosis due to an acapsular or poorly encapsulated strain in a patient suffering from hepatocarcinoma (52). Laurenson et al. reported a case of cryptococcal meningitis that was also due to an acapsular or poorly encapsulated strain in a patient infected by HIV (60). Moser et al. isolated an acapsular or poorly encapsulated strain from a patient with epidermoid carcinoma and pulmonary blastomycosis (75). Other cases of isolation of *C. neoformans* acapsular or small-capsule strains from patients with cryptococcosis have been reported (46, 86, 92). More reports have been observed in Brazil and were cited by a recent review (80), but overall it is difficult to establish whether the human cases noted above are due to acapsular or poorly encapsulated strains. Nevertheless, studies of severely immunocompromised animal models also proved interesting. Salkowski and Balish showed that a capsule may not always be necessary for *C. neoformans* to cause disease in severely immunodeficient mice (92). In their study, the fate of an acapsular strain of *C. neoformans* was studied in immunocompetent versus immunocompromised mice, and it was shown that in immunocompetent mice the acapsular strain was not able to cause infection and was readily cleared. In contrast, mice deficient in cell-mediated immunity or in both innate and cell-mediated immunity were susceptible to infection by this acapsular strain, and the infection was not due to reversion to an encapsulated state. Although these observations were derived from a single study, the results are intriguing and raise the following question: if cryptococcosis mainly afflicts patients

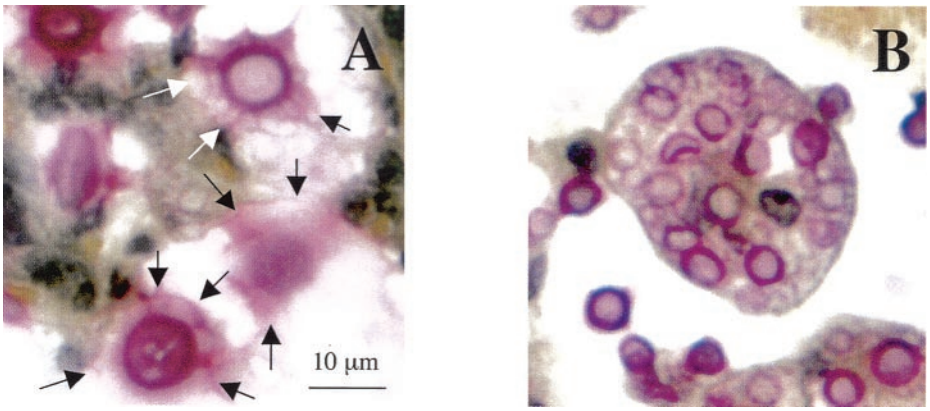

FIG. 1. Histopathology of tissue from CBA/J mouse lungs, stained with mucicarmine at 8 days (A) and 60 days (B) postinfection with *C. neoformans* strain H99 and viewed by light microscopy. At early stages of the infection, *C. neoformans* produces a large capsule, which was partially collapsed during tissue handling (arrows in panel A indicate putative capsule edges), whereas at late days the fungus is mainly poorly encapsulated and engulfed within giant cells (B). Magnification, ×40.

with severe deficiencies in cell-mediated immunity, then why does *C. neoformans* produce the capsule during infection?

C. neoformans produces a capsule during infection, but not always to the same extent. For instance, this fungus produces a larger capsule in one organ (brain) than in another (lung) (85). In the lungs of CBA/J mice, the capsule size of *C. neoformans* wild-type strain H99 changes during the infection, being significantly larger during the early (1 to 8 days) phase than during the late phase (30 to 60 days). Early in infection, yeast cells are found both intra- and extracellularly, and the extracellular yeast cells are bigger than intracellular cells and are characterized by the presence of a large capsule (Fig. 1A). Later in infection, *C. neoformans* cells are small and poorly encapsulated and are predominantly found inside giant cells localized in the lung (Fig. 1B). These observations are corroborated by studies in which multinuclear giant cells containing engulfed *C. neoformans* cells are found in the lungs, lymphoid tissue, and the brain (30, 48, 61). It appears that the presence of giant cells containing *C. neoformans* is related to the activation of a Th1 response (94). Indeed, these giant cells containing yeast cells were apparently absent during infection in a Tgε26 mouse model, which lacks T and NK cells (M. Del Poeta, unpublished observation). Thus, it is possible that during a late phase of the infection, *C. neoformans* develops new mechanisms to evade the immune response, such as those that will allow it to survive and grow inside host cells. As a consequence, the capsule might not be essential for fungal virulence during the proliferation stage, but it is essential preferentially at early stages of infection (primary infection-reactivation-dissemination). In addition, modification of the capsule size may be the consequence of an effect of the host immune response on genes or proteins involved in its synthesis. In this respect, it is possible that when the capsule is not needed for the survival of fungal cells in the host, its production is down-regulated. Although a few reports suggest that acapsular or poorly encapsulated strains can cause disease, the majority of cases of cryptococcosis are caused by encapsulated strains. In addition, the current view that this virulence effect is due to capsular antiphagocytic properties is based on in vitro studies and has not yet been demonstrated in vivo. These observations indicate that the capsule plays a key role in the regulation of virulence, although the molecular mechanisms by which it exerts its virulence effect are not completely understood.

As mentioned above, in addition to the capsule size, *C. neoformans* can regulate the overall organization of the capsule, which is mainly composed of two polysaccharides, GXM and galactoxylomannan, which make up ~90 and 7%, respectively, of the capsular mass. The organization of GXM is highly variable among varieties of *C. neoformans* and in the same strain during growth in vitro (76). Importantly, it has been suggested that the capsular organization in a strain isolated from a relapse case of cryptococcosis differed from that in an isolate from a previous episode (18). These findings suggest that changes in capsule structure may be related to responses to a specific environment, with important implications for the host immune response. Indeed, it was recently demonstrated that when complement component 3 (C3) is deposited inside the capsule, *C. neoformans* is poorly ingested by macrophages. In contrast, when C3 is deposited on the outer capsule edge, fungal cells are readily phagocytosed (113). The observation that capsule densities, in terms of GXM composition, differ in vivo from those observed in vitro (40) may also suggest a different effect of these cells on phagocytosis. Interestingly, it has been observed that the capsule's structural heterogeneity changes during infection, and these changes are opposite to those observed when yeast cells are grown in vitro. Garcia-Hermoso et al. showed that during infection there is selection of a subpopulation of yeast cells containing capsules with a similar structure. In contrast, during in vitro growth, they observed an increased diversity in capsule structure over time. They proposed an interesting model of adaptation in which the selection of a more homogenous capsule is due to in vivo selective pressure, which is absent in vitro (36). Taken together, these results imply that changes in capsule structure, density, or organization may alter opsonin deposition, with an immediate effect on the phagocytosis of fungal cells and the outcome of infection. Studies addressing capsular functions during infection should also take into consideration its molecular structure in relation to the status of the host immune

response, which might regulate the fungal response, resulting in the production of capsules that differ in size or organization.

Current animal models for studying the impact of cell morphology, including capsule formation, on the virulence of *C. neoformans* could be misleading. For instance, studies of *C. neoformans* pathogenesis in mice or rabbits focus on primary infection because these studies use animals which do not carry the fungus in the lungs in a latent state. Using rats, Goldman et al. developed a model of persistent *C. neoformans* infection (42), although a rat model in which a latent infection can be reactivated by immunosuppression is still under investigation. In humans, primary cryptococcal infection occurs when the fungus is inhaled into the alveolar spaces. After inhalation, it is widely accepted that most immunocompetent subjects control the infection, although the fungus remains in a dormant state. When immunosuppression subsequently occurs, reactivation of the latent infection then leads to fungal dissemination and the development of meningoencephalitis. Perhaps an animal model most similar to human cryptococcosis would involve infection of an immunocompetent animal followed by the induction of immunosuppression so that the fungus can reactivate and disseminate. In this respect, perhaps the change in morphology and capsule production would only be required for the reactivation and dissemination, but not the proliferation, of the pathogen at the infection site. Thus, the differences in fungal cell morphology and capsule size, structure, or composition should be considered in relation to different stages of the natural infection, such as primary infection versus colonization or reactivation, dissemination, and proliferation. This type of study is particularly challenging with currently available animal models.

PRODUCTION OF APP1 PROTECTS *C. NEOFORMANS* FROM PHAGOCYTOSIS

Another example illustrating how the plasticity of *C. neoformans* regulates virulence factors in response to the host is the production of specific proteins that directly alter the fungus-host interaction. One of these proteins was recently characterized as antiphagocytic protein 1 (App1) (66). During the 1980s and 1990s, studies in B. Bolaños's laboratory at the University of Puerto Rico focused on the identification and purification of a cytoplasmic factor(s) of *C. neoformans* involved in the regulation of phagocytosis of yeast cells by alveolar macrophages (AMs). Graduate students and postdoctoral fellows in his laboratory partially isolated and purified a 20- to 23-kDa protein from a crude cytoplasmic extract as a cryptococcal factor that specifically inhibited phagocytosis. Rabbit polyclonal antibodies were raised against this cytoplasmic factor and used in 1997 by Martinez-Mariño to screen a *C. neoformans* cDNA library in J. Perfect's laboratory at Duke University Medical Center (71). A putative gene encoding a 20- to 23-kDa protein was identified and sequenced from both serotype A and D strains of *C. neoformans* (GenBank accession no. AF180107, AF180108, and AY101600). Interestingly, it was found by the use of polyclonal antibodies that this cytoplasmic factor is also present in the extracellular culture medium (66, 71). These results were further corroborated by the fact that this protein was found in the sera of AIDS patients with disseminated cryptococcosis (66, 91). Martinez-Mariño produced a recombinant protein by using the isolated cDNA clone and found that the recombinant protein was recognized by the rabbit polyclonal antibody made against the native protein. In 2002, the same gene was independently isolated by differential display reverse transcription-PCR in my laboratory at the Medical University of South Carolina as a gene whose expression is regulated by the level of inositol phosphoryl ceramide synthase 1 (Ipc1) activity (66, 68). This gene was named *APP1* for antiphagocytic protein 1.

Through a biochemical and a genetic approach, it was demonstrated that App1 inhibits the phagocytosis of *C. neoformans* by AMs through a complement-mediated mechanism. As a consequence, a *C. neoformans app1* mutant is more easily internalized by AMs than the wild-type strain. This observation was confirmed both in vitro and ex vivo. An *app1*Δ strain showed no difference in capsule size, melanin formation, or growth at 30 or 37°C compared to the parent wild-type strain. Furthermore, the *app1*Δ mutant strain was less virulent than the wild-type strain in an A/Jcr mouse model, which is deficient for complement C5 (66). This decrease in virulence of a strain without App1 was also observed in models of both CBA/J and C57BL/6J mice, which are immunocompetent (M. Del Poeta, unpublished data). These results suggest that without App1, *C. neoformans* is more easily ingested by macrophages in these mouse models and is killed by activated professional phagocytic cells. However, *C. neoformans* causes infection mainly in immunodeficient subjects, such as those with HIV, lacking T and natural killer (NK) cells. A mouse model resembling this condition is represented by Tgε26 mice, which are deficient in T and NK cells. In this model, a mutant lacking App1 was also more readily ingested by lung AMs, but this mutation exacerbated the infection compared to the infection produced by a *C. neoformans* wild-type strain. Since macrophages need to be activated by an efficient and coordinated cellular response to control the fungus, these results suggest that if the cellular immune response is impaired, then the phagocytosis of *C. neoformans* by macrophages may be detrimental for the host because *C. neoformans* grows intracellularly at a higher rate than it does extracellularly and also because it can disseminate to organs and tissues within host macrophages.

On the other hand, the observation that App1 is found in the sera of AIDS patients infected with cryptococcosis raises the question of the purpose of this secretion and whether this secretion is regulated by an active or passive mechanism. It is possible that App1 is found extracellularly as a result of yeast cell death or disruption and not because it is actively secreted. If this is this case, its level in serum can be monitored and may represent an important prognostic factor and indicator of yeast cell death due to the administration of antifungal therapy. As a consequence, an increased level of App1 in the serum might predict recovery, since antibodies detected against the cytosolic antigen during the course of cryptococcosis have been associated with an improved prognosis (78). If the production of App1 occurs through an active mechanism and if App1 inhibits phagocytosis, then why would *C. neoformans* produce App1 when the cellular immune response is impaired? Perhaps in the same AIDS patients the host immune responses were not completely deficient and/or the expression of App1 was actually at its lowest and not a physiological level. It is also possible that App1 is differentially expressed during different stages of

C. neoformans infection. The latter hypothesis may be supported by preliminary observations that anti-App1 antibodies are produced during infection by *C. neoformans* in an immunocompetent murine model, suggesting that the host responds to App1 production, although the scope of this host response has yet to be elucidated (67). If anti-App1 antibody production mainly occurs during the early stage of infection, this may have implications for prognosis and diagnosis.

A different and exciting scenario is also possible considering that *C. neoformans* interacts not only with alveolar macrophages but also with other phagocytic cells, such as dendritic cells. This interaction is not as well understood as the interaction with macrophages. For instance, the *C. neoformans* capsule interferes with the maturation and activation of human dendritic cells (107). Dendritic cells are actively involved in the host cellular response against *C. neoformans* (2, 3, 102), but their role in phagocytosis and killing has only recently begun to be explored (41, 103, 107). For *C. albicans*, the understanding of the interaction with dendritic cells is more developed. Once inside dendritic cells, the majority of *C. albicans* cells are eventually degraded, although single yeast cells can survive inside the phagosome provided that they entered via complement receptor 3 (CR3) (88, 89). This finding suggests that the fungus may exploit CR3 on dendritic cells as a niche to avoid degradation and allow intracellular persistence. Intriguingly, CR3 engagement by the fungus on host cells other than dendritic cells results in the activation of an antiphagocytic response and the destruction of the fungus (33, 45). These results suggest that the signaling events following phagocytosis through CR3 among phagocytic cells are different. Whether these different signaling mechanisms also occur for the interaction of *C. neoformans* with phagocytic cells is not known. However, it is tempting to speculate that dendritic cells, not alveolar macrophages, are the phagocytic cells in which *C. neoformans* resides during its dormant state. Since App1 acts through a complement receptor, its action may specifically target a subspecies of phagocytic cells, such as those that would degrade the fungus and not those that would allow the fungus to survive intracellularly. In this case, App1 production during primary infection would favor the persistence of cryptococcal cells within an immunocompetent host in a specific host cell compartment. Clearly, further investigations are warranted to determine the physiological and pathobiological roles of App1 during infections of both immunocompetent and immunodeficient hosts by *C. neoformans*.

SURVIVAL IN THE ENVIRONMENT AND SURVIVAL IN THE HOST: IS THERE A CONNECTION?

As discussed above, *C. neoformans* is a facultative intracellular pathogen that can use host cells to disseminate more efficiently, evade the host immune response, and under certain circumstances survive in a dormant state until reactivation occurs upon sustained immunodeficiency (19, 35, 66). Interestingly, it has been suggested that the intracellular replication and dissemination of *C. neoformans* may have evolved from its ability to survive and replicate inside amoebae (99, 100). Amoebae feed on microorganisms similar to phagocytosis by macrophages, and a fascinating hypothesis suggests that amoebae could be the earliest form of macrophages and perhaps gave rise, by an unknown evolutionary pathway, to the modern macrophage (50). Innate immunity in eukaryotes can be considered as arising from the need of a unicellular microorganism such as an amoeba to discriminate between food and other amoebae. Indeed, if an amoeba could not make this distinction, it would have led to its extinction. Therefore, the nature of this discrimination represents the basis of the recognition between self and nonself, which is one of the most basic functions of the immune system. In this scenario, *C. neoformans* may have acquired the ability to cause disease in humans because of the defense mechanisms that it developed to evade environmental predators such as amoebae (99). This hypothesis has been suggested for other facultative intracellular pathogens, such as *Legionella pneumophila* and *Mycobacterium avium*, for which passage through amoebae increases their virulence composite (9, 20, 21). The production of antiphagocytic factors, such as the polysaccharide capsule and App1 of *C. neoformans*, could be the evolutionary result of this interaction. Thus, studies addressing the cross talk between environmental fungi and protozoa may provide significant insights into the molecular mechanisms by which fungi adapted and became virulent to mammalian hosts (16, 98).

CONCLUSIONS

Pathogenic fungi represent an increasing cause of morbidity and mortality worldwide. Chronic infection by *C. neoformans* results from the production of virulence factors and from altered host immune responses. By adapting to the host, this microorganism appears to have mastered the intricacies of cellular biology, physiology, immunology, and pathobiology. Regarding the course of this host-pathogen adaptation, there is much to be learned about the possibility of decreasing its ability to grow and disseminate within the host. Thus, modulation of the expression level of antiphagocytic factors may play a key role in the outcome of the infection, and these factors may represent targets for novel therapeutic strategies to control the development of cryptococcal disease. Results from studies of this model organism can serve as a paradigm for other facultative intracellular pathogens, providing the basis not only for antimicrobial discovery but also for the development of vaccination and immunotherapies to combat microorganisms that affect mainly immunocompromised hosts.

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