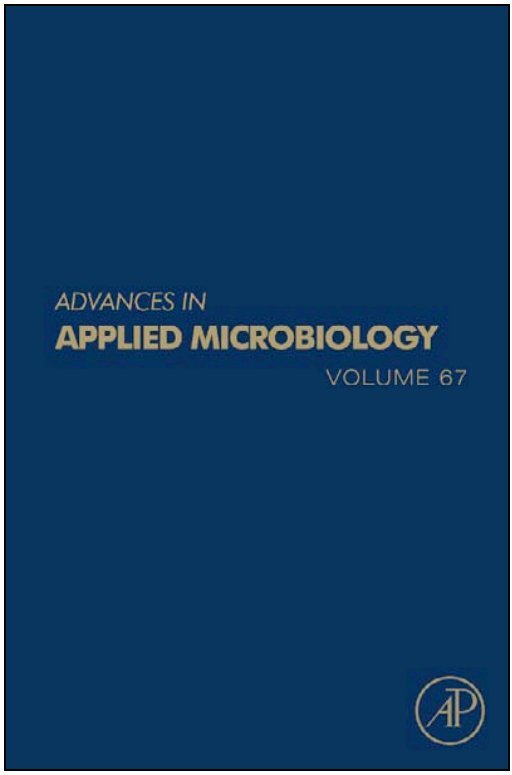

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CHAPTER 5

Virulence in *Cryptococcus* Species

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Contents

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Abstract *Cryptococcus neoformans* and *Cryptococcus gattii* are the cause of life-threatening meningoencephalitis in immunocompromised and immunocompetent individuals respectively. The increasing incidence of cryptococcal infection as a result of the AIDS epidemic, the recent emergence of a hypervirulent cryptococcal strain in Canada and the fact that mortality from cryptococcal disease remains high have stimulated intensive research into this organism. Here we outline recent advances in our understanding of *C. neoformans* and *C. gattii*, including intraspecific complexity, virulence factors, and key signaling pathways. We discuss the molecular basis of cryptococcal virulence and the interaction between these pathogens and the host immune system. Finally, we discuss future challenges in the study and treatment of cryptococcosis.

I. *CRYPTOCOCCUS* AND CRYPTOCOCCOSIS

The genus *Cryptococcus* contains 39 heterobasidiomycetous fungal species characterized as variously encapsulated budding yeasts, of which only *Cryptococcus neoformans* and *Cryptococcus gattii* are commonly considered as the causative agents of cryptococcosis (Casadevall and Perfect, 1998). *C. neoformans* was first identified as a human pathogen in the 1890s (Buschke, 1895; Busse, 1894). It exists predominantly as a vegetative haploid form and is heterothallic with each cell existing as one of two distinct mating types: MATa or MATα. In response to nutrient limitation, cells of opposite mating type mate to form the filamentous teleomorph (Kwon-Chung, 1975, 1976). Under the microscope, most clinical isolates of *C. neoformans* appear as encapsulated spherical yeasts in both tissue and culture (Mitchell and Perfect, 1995). The capsule size varies according to the strain and culture conditions with most isolates having a medium-sized capsule resulting in a total diameter of 4–10 μm (Fig. 5.1A). Poorly encapsulated strains have diameters of only 2–5 μm whereas heavily encapsulated isolates can have a cell diameter of up to 80 μm (Casadevall and Perfect, 1998).

C. neoformans can cause human infections following inhalation of the small airborne propagule (believed to be either basidiospores or poorly encapsulated yeast cells) originating from certain environments such as soil and avian habitats. Therefore, the lung is invariably the portal of entry and initial site of infection (Casadevall and Perfect, 1998). However,

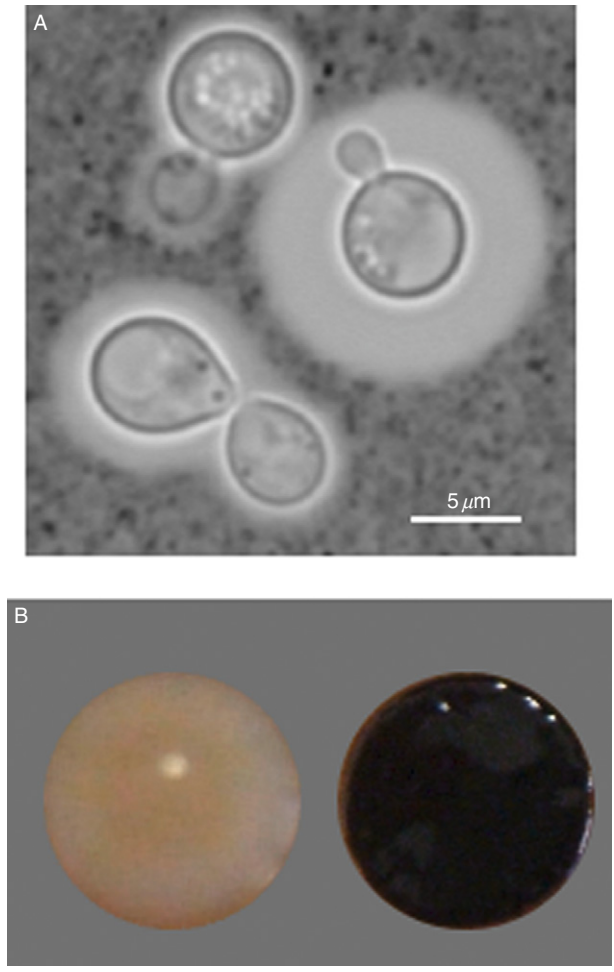

FIGURE 5.1 (A) India ink staining reveals the capsule (of various sizes) around budding *C. neoformans* cells; (B) Melanin and nonmelanin forming colonies of *C. gattii* serotype B on L-DOPA medium after 7 days at 25 °C.

C. neoformans not only has the ability to simply colonize the host's respiratory tract without causing disease (latency) in immunocompetent individuals (Garcia-Hermoso *et al.*, 1999), but is also capable of disseminating to any organ of the human body, with a predilection for the central nervous system (CNS). The resulting meningoencephalitis represents the most severe form of the disease and is uniformly fatal if untreated (Casadevall and Perfect, 1998).

Conventional nomenclature classified *C. neoformans* into five serotypes (A, B, C, D, and AD) and three varieties: *C. neoformans* var. *neoformans* (serotype D), *C. neoformans* var. *grubii* (serotype A), and *C. neoformans* var.

gattii (serotype B and C) (Franzot *et al.*, 1999; Kwon-Chung *et al.*, 1982). Each serotype is characterized by a specific structure of glucuronxylomannan (GXM), the main capsule component (Cherniak *et al.*, 1995). In the last decade, a number of DNA genetic typing techniques have been used to genotype and study the epidemiology of *C. neoformans* species. These techniques include electrophoretic karyotyping by pulsed field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), DNA hybridization studies, amplified fragment length polymorphism (AFLP), polymerase chain reaction (PCR) fingerprinting, and multi locus sequence typing (MLST) (Boekhout *et al.*, 1997, 2001; Brandt *et al.*, 1995; Currie *et al.*, 1994; Litvintseva *et al.*, 2006; Meyer *et al.*, 1999; Ruma *et al.*, 1996; Varma and Kwon-Chung, 1992). These techniques resulted in the elevation of *C. neoformans* var. *gattii* to the species level, based on genetic variability and lack of evidence for genetic recombination between *C. neoformans* and *C. gattii* (Kwon-Chung *et al.*, 2002). Moreover, *C. gattii* differs from *C. neoformans* in phenotypic characters, natural habitat, epidemiology, clinical manifestations of disease, and response to antifungal treatment (Casadevall and Perfect, 1998; Chen *et al.*, 2000; Sorrell, 2001; Speed and Dunt, 1995). The *C. neoformans*–*C. gattii* species complex is further divided into nine major molecular types or genotypes (Fig. 5.2).

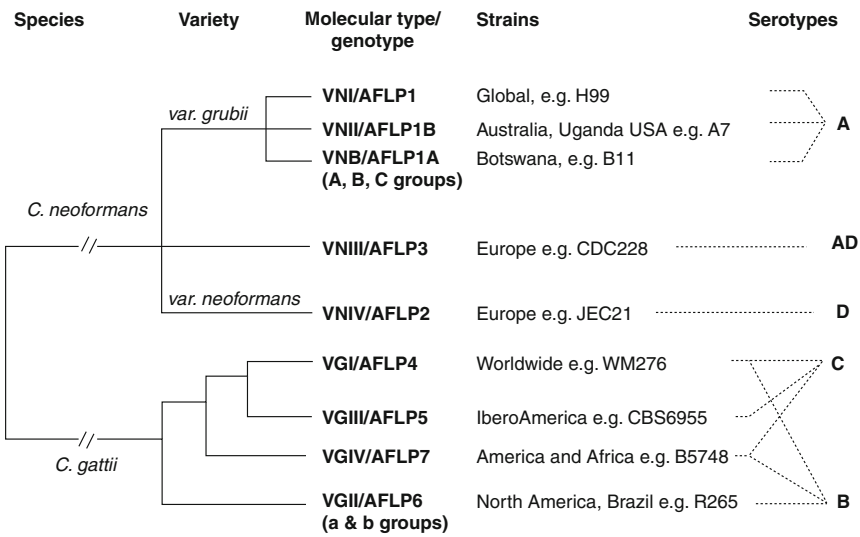

FIGURE 5.2 A schematic phylogeny of the *C. neoformans*–*C. gattii* species complex. For *C. neoformans*, two monophyletic lineages, corresponding to the varieties *grubii* and *neoformans*, are clearly present along with the hybrid population. Within *C. neoformans*, serotypes correspond to genotypes. For *C. gattii*, four monophyletic lineages corresponding to the previously described genotypic groups are consistently found but in this group serotypes and genotypes do not necessary correlate with each other.

C. neoformans var. *grubii* isolates correspond to molecular types VNI, VNII, and VNB; *C. neoformans* var. *neoformans* corresponds to VNIV; and serotype AD isolates correspond to molecular type VNIII. *C. gattii* corresponds to four molecular types: VGI, VGII, VGIII, and VGIV, and a recent study by Bovers *et al.* has proposed to treat these four molecular types as different taxa (varieties), just like var. *neoformans* and var. *grubii* (Bovers *et al.*, 2008).

A. *C. neoformans*

C. neoformans usually infects immunocompromised patients (although some exceptions have recently been reported, e.g., (Chen *et al.*, 2008b)). It can be found in the environment worldwide, and is commonly associated with pigeon guano or soil (Casadevall and Perfect, 1998). Most *C. neoformans* isolates are serotype A or serotype D. A and D serotypes diverged about 18 million years ago and have always been described as varieties, not as separate species (Fan *et al.*, 1994; Xu *et al.*, 2000). Nevertheless, a recent proposal is that these two varieties of *C. neoformans* should be described as different species (Bovers *et al.*, 2008), because they have diverged to such an extent that normal mating is no longer possible (Sun and Xu, 2007), and comparison of their genomes shows that there has not been any recent DNA exchange between these two varieties (Kavanaugh *et al.*, 2006). Further detailed studies are required to analyze the ongoing speciation events within this clade.

Serotype A is the predominant serotype of *C. neoformans* isolated from infected patients, responsible for 95% of all *C. neoformans* infections (Hull and Heitman, 2002). It is subdivided into three molecular types: VNI (AFLP1), VNII (AFLP1B), and VNB (AFLP1A) according to MLST and AFLP analysis (Boekhout *et al.*, 2001; Bovers *et al.*, 2008; Litvintseva *et al.*, 2006) (Fig. 5.2). Such sub-classification is confirmed by recent comparative genome hybridization (CGH) data (Hu *et al.*, 2008). VNI is the most common molecule type, contributing 78% of *C. neoformans* isolates (Meyer *et al.*, 1999). The VNB cluster can be further separated into three groups: VNB-A, VNB-B, and VNB-C (Litvintseva *et al.*, 2006). Initially, VNB strains were found only in Botswana (Litvintseva *et al.*, 2006), but more recently they have also been recovered from Brazilian pigeon droppings and patients in Rwanda, Portugal, and Brazil (Bovers *et al.*, 2008).

Serotype D strains are found globally, but they are more prevalent in areas with temperate climates, such as Europe, where 30% of isolates are serotype D (Dromer *et al.*, 1996). This restricted distribution may be due to the fact that serotype D strains are more susceptible to killing by high temperature than cells of serotype A (Martinez *et al.*, 2001). The clinical manifestations of human infections caused by serotype A or D are similar,

although differences in virulence potential in animal models have been reported (Barchiesi *et al.*, 2005; Lin *et al.*, 2008)

Serotype AD is the result of a fusion event between a serotype A strain and serotype D strain followed by impaired meiosis due to genomic incompatibilities (Boekhout *et al.*, 2001; Cogliati *et al.*, 2001; Lengeler *et al.*, 2001; Xu *et al.*, 2002). AD strains are therefore diploid (or aneuploid), containing two sets of chromosomes, and possessing two mating type alleles. Serotype AD strains are relatively common: a recent analysis of environmental and clinical populations of *C. neoformans* in North America revealed that ~7.5% of strains isolated from the environment are AD hybrids (Litvintseva *et al.*, 2005a). Thus far, the majority of the globally isolated serotype AD strains originate in Africa (Litvintseva *et al.*, 2007).

B. *C. gattii*

C. gattii was first described after being isolated from a leukemic patient in 1970 (Vanbreuseghem and Takashio, 1970). It mainly infects individuals with no immunological defects, although AIDS-associated *C. gattii* infections have also been reported (Chen *et al.*, 2000, 2008b; Litvintseva *et al.*, 2005b). It has been consistently isolated from decaying wood of several tree species, especially the red gum group of eucalyptus trees (*Eucalyptus* ser. *Exsertae Blakely*) (Ellis and Pfeiffer, 1990, 1992; Fortes *et al.*, 2001; Krockenberger *et al.*, 2002; Lazera *et al.*, 2000). The geographic distribution of *C. gattii* was originally thought to be limited to tropical and subtropical regions of the world (Kwon-Chung and Bennett, 1984). However, recent studies have revealed its worldwide distribution. For instance, VGI (AFLP4) strains were found to be the most widely distributed (Campbell *et al.*, 2005; Chen *et al.*, 2008b; Meyer *et al.*, 2003); Strains of the VGII (AFLP6) type are found in areas like Australia and America (Fraser *et al.*, 2005; Kidd *et al.*, 2004, 2005; Meyer *et al.*, 2003); The VGIII (AFLP5) type predominates IberoAmerican countries (Meyer *et al.*, 2003) and can also be found in India (Bartlett *et al.*, 2007), whilst the VGIV (AFLP7) type, which has been associated with infections in HIV-positive patients (Bovers *et al.*, 2006; Litvintseva *et al.*, 2005b), is found in South Africa (Meyer *et al.*, 2003) and Central America (Bartlett *et al.*, 2007) etc.

Until recently, *C. gattii* has been under-studied because *C. gattii* infections comprise only 1% of cryptococcosis cases worldwide. Even in areas like Australia, where *C. gattii* is endemic, the rate of infection is 0.94 cases per million residents per year (Chen *et al.*, 2000; Sorrell, 2001). However, a recent outbreak of cryptococcosis caused by *C. gattii* has stimulated detailed investigation of this organism. This ongoing outbreak was first noted in 1999 on Vancouver Island, British Columbia (BC), Canada. Between 2002 and 2006, the average annual cryptococcosis incidence rate was 6.5 cases/million in BC and 27.9 cases/million on Vancouver

Island (Control, 2007). In addition to human infections, cryptococcal disease has been diagnosed in animals such as dogs, cats, horses, and even porpoises. In fact, veterinary cases have been diagnosed two to three times more frequently than human cases (Lester *et al.*, 2004). So far, the fungus has infected more than 176 individuals and spread from Vancouver Island to other regions of Canada and the Pacific Northwest (MacDougall *et al.*, 2007).

Interestingly, the majority (>97%) of cryptococcal isolates from the island have been found to belong to the VGII molecular type, with the rest being VGI (Kidd *et al.*, 2004). These VGII isolates have been further separated into two discrete subtypes: a major form common in environmental and clinical isolates (VGIIa/AFLP6a, hypervirulent, e.g., CDCR265), and a rare minor form presented by one clinical and several environmental samples (VGIIb/AFLP6b, with attenuated virulence, e.g., CDCR272) (Fraser *et al.*, 2005; Kidd *et al.*, 2004, 2005). So far, the VGIIa genotype has accounted for 78% of the examined veterinary cases and 87% of the human cases on Vancouver Island (Bartlett *et al.*, 2007). Surprisingly, MLST (Kidd *et al.*, 2005) and gene genealogy analysis (Fraser *et al.*, 2005) revealed that VGIIa and VGIIb strains found on Vancouver Island share similar or identical genotypes with isolates from other parts of world. For example, the VGIIa genotype was also shared by the NIH444 strain (from a patient in Seattle, 1971, which is considered as the potential origin of the VGIIa subtype), CBS7750 (from a *Eucalyptus* tree in San Francisco, 1992), and isolates from other parts of the North America (e.g., KB10455). The VGIIb genotype was also observed among environmental and clinical isolates from Australia (e.g., Ram005, NT-13), as well as a clinical isolate from Thailand (MC-S-115) (Fraser *et al.*, 2005; Kidd *et al.*, 2005). A recent study confirmed the global distribution of the outbreak genotypes (Meyer *et al.*, 2007). The wide distribution of Vancouver genotypes in other geographical areas makes it difficult to accurately determine a specific origin. Current hypotheses are that the species is either a long-term resident of BC (ancient population), or represents a particularly virulent genotype that may be well adapted to the local conditions and has been recently introduced to BC. For instance, Fraser *et al*. reported that the VGIIa and VGIIb strains from Vancouver Island shared 14 identical loci after examining 30 alleles, and hypothesized that VGIIa isolates might be the result of same-sex mating (α/α) between a VGIIb isolate and a second unknown VGII isolate in Australia, in transit or in the Pacific Northwest (Fraser *et al.*, 2005). However, Meyer *et al.* revealed that there were VGIIa and VGIIb isolates recovered as early as in 1986 in South America, suggesting that these genotypes may have been present for a long time in the Americas rather than being a result of a recent recombination event as suggested by Fraser *et al.* (Meyer *et al.*, 2007).

C. Other species

Besides *C. neoformans* and *C. gattii*, there are at least 37 other cryptococcal species found in a wide variety of environmental locations, such as Antarctica, the Himalayas, and saline water (Casadevall and Perfect, 1998). However, since most of them are not able to survive in mammalian tissue due to the relatively high body temperature and host immune system, infection caused by these species is rare (Kordossis *et al.*, 1998; Krajden *et al.*, 1991; Kunova and Krcmery, 1999; Loison *et al.*, 1996). Among those causing non-*neoformans/gattii* cryptococcosis, *Cryptococcus laurentii* (20 cases) and *C. albidus* (18 cases) are responsible for most (80%) of such infections (Khawcharoenporn *et al.*, 2007). The transmission, virulence factors and host immune response to these species resembles that of *C. neoformans* (Ikeda *et al.*, 2000; McCurdy and Morrow, 2003), although the level of laccase activity is lower than that seen in *C. neoformans* (Ikeda *et al.*, 2002). A systematic review of non-*neoformans* infection can be found in Khawcharoenporn *et al.*, 2007.

D. Cryptococcosis

Following inhalation of the infectious particle, a primary pulmonary lymph-node complex is formed. In most cases, symptoms do not develop, indicating that most immunocompetent people either clear or control the infection before widespread symptomatic dissemination occurs (Casadevall and Perfect, 1998). Yet frequently the yeast will reside in a dormant state, probably within the lymph-node complex (Baker, 1976). Among patients with significant alterations of immunity, including patients with prolonged corticosteroid administration, hematological malignancies, or HIV infection, however, disseminated disease is often seen. *Cryptococcus* can cause localized infections in any organ involving the skin, eyes, myocardium, bones, joints, lungs, prostate gland, urinary tract, or CNS (Perfect, 1989). Dissemination may occur from a primary infection. For example, it was reported that acute infection could occur when immunocompromised individuals are exposed to large numbers of cryptococcal cells (Nosanchuk *et al.*, 2000b). However, there is increasing evidence indicating that dissemination is the result of reactivation of dormant disease (Perfect, 1989). For instance, it has been reported that patients coming from tropical areas can be diagnosed with *C. gattii* cryptococcosis long after they have left their countries of origin (Dromer *et al.*, 1992). Similarly, Garcia-Hermoso and colleagues analyzed cryptococcal clinical isolates recovered from patients diagnosed with cryptococcosis in France but born in Africa. The RAPD profiles of these isolates were significantly different from that of those from 17 European patients, suggesting that *Cryptococcus* can be acquired long before the infection

develops, as these patients had been living in France for approximately 10 years and had not been in contact with an African environment for as long as 13 years (Garcia-Hermoso *et al.*, 1999).

Cryptococcosis occurs in both animals and humans, but animal-to-human or human-to-human transmission has not been documented, other than rare examples of iatrogenic transmission (Lin and Heitman, 2006) and a mother-to-child transmission (Sirinavin *et al.*, 2004). The clinical presentation of cryptococcosis can be acute or chronic, and manifestation varies depending on stage of the disease. Typical symptoms associated with meningoencephalitis are significantly raised cerebrospinal fluid (CSF) opening pressure (>25 cm H_2O) (occurs in more than 50% of patients with HIV-associated cryptococcal meningitis) (Graybill *et al.*, 2000), resulting in headache, fever, altered mental status, visual loss, dementia, or even coma (Casadevall and Perfect, 1998). For pulmonary cryptococcosis, symptoms range from asymptomatic pulmonary nodules to acute respiratory distress syndrome (Casadevall and Perfect, 1998; Saag *et al.*, 2000). According to a recent study in 166 patients, symptoms including cough (58%), dyspnea (46%), and fever (38%) are the most frequent manifestations of infection (Baddley *et al.*, 2008). Both *C. neoformans* and *C. gattii* affect the lung and CNS. However, the infections caused by the two species have important differences in epidemiology, clinical presentation, and therapeutic outcome (Kwon-Chung and Bennett, 1984; Sorrell, 2001). For instance, *C. gattii* appears to invade the brain parenchyma more commonly than *C. neoformans*, and in *C. gattii* infected patients, pulmonary infections and pulmonary mass-like lesions are more common (Mitchell and Perfect, 1995; Speed and Dunt, 1995).

Since 1981, infections due to *Cryptococcus* have been a major cause of morbidity and mortality in individuals with depressed immune system as a consequence of the AIDS epidemic, as 5–10% of all individuals with CD4+ lymphopenia develop life-threatening cryptococcosis (Steenbergen and Casadevall, 2003). Nowadays, cryptococcosis ranks as one of the three common life-threatening opportunistic infections in people with AIDS worldwide (Levitz and Boekhout, 2006). Even though the prevalence of cryptococcosis in HIV-infected individuals has declined because of highly active antiretroviral therapy, it remains epidemic in Africa and Southeast Asia, where up to 30% of AIDS patients are affected (Bicanic and Harrison, 2004; Idnurm *et al.*, 2005). In fact, cryptococcosis has been recognized as an AIDS-defining illness in areas like Zimbabwe, where 91% of AIDS patients are infected (Mwaba *et al.*, 2001). Although less common, cryptococcosis in HIV-negative patients also has a high mortality rate (Kiertiburanakul *et al.*, 2006), particularly in areas such as northern Brazil, where *C. gattii* is endemic and accounts for 62.7% of all cryptococcosis cases (Nishikawa *et al.*, 2003).

1. Antifungal therapy

Untreated cryptococcal meningitis is uniformly fatal, although survival can range from years to only a few weeks (Mwaba *et al.*, 2001). There are several well-established antimicrobial reagents for treatment, and amphotericin B, a polyene introduced in the mid-1950s, was the first effective therapy developed. Amphotericin B binds to ergosterol in the fungal plasma membrane to cause increased permeability to protons and monovalent cations such as potassium (Brajtburg *et al.*, 1990). It was also found to stimulate inflammatory cytokine production from innate immune cells through CD14 and Toll-like receptors (TLRs) (Sau *et al.*, 2003). In many resource-poor areas where amphotericin B is not available, fluconazole, a triazole that inhibits fungal ergosterol synthesis is widely used (Jarvis and Harrison, 2007). It has excellent absorption and CSF penetration and is widely available at low cost in generic form. However, the slow response to therapy with fluconazole means that it is better suited to long-term maintenance therapy than initial therapy (Bozzette *et al.*, 1991; Powderly *et al.*, 1992). Flucytosine (5-Fc) is another commonly used anticryptococcal drug. It is a synthetic antimycotic compound and was initially developed as an anticancer drug in the 1970s. It has no intrinsic antifungal capacity, but after it has been taken up by *Cryptococcus*, it is converted into 5-fluorouracil (5-Fu), a pyrimidine analogue that inhibits fungal RNA and DNA synthesis (Vermes *et al.*, 2000). Flucytosine is commonly prescribed in combination with amphotericin B, because such combination has been shown to have higher efficiency compared to amphotericin B alone in both non-HIV-associated and HIV-associated infection (Bennett *et al.*, 1979; Brouwer *et al.*, 2004; van der Horst *et al.*, 1997). The optimal current therapy is with amphotericin B 0.7–1 mg/kg/day plus flucytosine 100 mg/kg/day for two weeks, followed by fluconazole 400 mg/day for 8 weeks and 200 mg/day thereafter (Bicanic and Harrison, 2004).

The emergence of antifungal drug resistance has not been a major problem to date in areas like Australia and New Zealand (Chen *et al.*, 2000). However, in sub-Saharan Africa, resistance can be very high. For instance, in Nairobi Kenya, flucytosine resistance was observed in 21% of cryptococcal strains and only 23.8% of these strains were susceptible to fluconazole (65% susceptible in a dose-dependent manner and 11.2% resistant) (Bii *et al.*, 2007). Differences in the antifungal susceptibilities of the two species of *Cryptococcus* have also been reported. A study conducted by Trilles *et al.* found that *in vitro*, *C. gattii* was less susceptible to seven antifungal compounds as compared with *C. neoformans*, although both showed equal susceptibility to amphotericin B and flucytosine (Trilles *et al.*, 2004).

2. Immunotherapy

Immunotherapeutic strategies, mainly based on introducing antibodies and cytokines, have been developed to restore and boost host defense mechanisms to *Cryptococcus*. Antibodies against capsular and cell wall have been demonstrated to provide protection in animal models of cryptococcal infection (Casadevall *et al.*, 1998; Dromer *et al.*, 1987; Mukherjee *et al.*, 1992; Rachini *et al.*, 2007; Sanford and Stollar, 1990). However, adjunctive use of antibody therapy in mice with established cryptococcal infection was also reported to cause cardiovascular collapse and death in some strains of mice due to the release of platelet-activating factor (Lendvai *et al.*, 2000; Savoy *et al.*, 1997). Nevertheless, a murine IgG1 (Mab 18B7) has reached phase I trial in patients recovering from HIV-associated cryptococcal meningitis (Larsen *et al.*, 2005) and radioimmunotherapy (radiation was delivered by specific radio-labeled antibodies leading to antibody-specific killing of *Cryptococcus*) is under evaluation in the murine model (Dadachova *et al.*, 2004).

Several cytokines (Th1 type) have been shown to augment the antifungal activity of effectors cells against cryptococcal infection. In a murine cryptococcal infection, administration of IL-12 resulted in up to 10-fold decreases in the cryptococcal burden in the CNS. Significantly, the combination of fluconazole with IL-12 showed synergistic effects on reducing organism burden (Clemons *et al.*, 1994). Similarly, the importance of interferon-γ (IFNγ) in the clearance of cryptococci, especially from the CSF, has been demonstrated by several groups (Kawakami *et al.*, 1996; Siddiqui *et al.*, 2005; Zhou *et al.*, 2007), and IFNγ can potentiate amphotericin B mediated reduction of infection in the brain (Lutz *et al.*, 2000). A recent phase II study to evaluate the safety and antifungal activity of adjuvant recombinant interferon (rIFN)-γ1b in HIV patients with acute cryptococcal meningitis showed a trend towards improved mycological and clinical success without adverse effects on CD4 count or HIV viral load (Pappas *et al.*, 2004).

With the use of molecular biology, several genes and their encoded proteins have now been identified which may help elicit a protective immune response. One such group is the mannoproteins. Mannoproteins are a group of glycoproteins present in the capsule (discussed in detail in Section II). They are recognized by the mannose receptor and presented to T cells by dendritic cells (Levitz and Specht, 2006; Mansour *et al.*, 2006). Recent *in vivo* and *in vitro* studies have reported that mannoproteins were the major T cell antigenic determinants from *C. neoformans* and both CBA/J and C57BL/6 mice benefited from immunization with mannoproteins (Mansour *et al.*, 2004; Specht *et al.*, 2007). Another molecule with therapeutic potential is a synthetic oligodeoxynucleotide containing an unmethylated CpG motif (CpG-ODN). CpG-ODN is a TLR ligand, which

was found to protect mice from infection with *C. neoformans* by altering the Th1-Th2 cytokine balance toward a Th1-biased immune response (Edwards *et al.*, 2005; Miyagi *et al.*, 2005). Combination of CpG-ODN with antifungal chemotherapy or with mannoproteins seems to provide a beneficial effect in a murine model of pulmonary and disseminated infection (Dan *et al.*, 2008; Kinjo *et al.*, 2007), suggesting a rationale for vaccination strategies that combine mannosylated antigens with TLR ligands to achieve synergistic promotion of host defense against *C. neoformans* infection.

3. Outcomes

The mortality from cryptococcosis remains unacceptably high. The last US Mycoses Study Group treatment trial of HIV-associated cryptococcal meningitis showed the lowest mortality to date, which is still 9.4% at 10 weeks (Bicanic and Harrison, 2004). In France, Dromer *et al.* observed an overall mortality rate of 6.5% in the first 2 weeks and 11.5% over the next 10 weeks (Dromer *et al.*, 2007). In Southeast Asia, even in the context of amphotericin B based therapy, acute mortality has ranged from 22% to more than 40% (Brouwer *et al.*, 2004; Imwidthaya and Poungvarin, 2000). For instance, with amphotericin B plus flucytosine, 34% of patients with *C. gattii* meningitis in Papua New Guinea died during their first admission, at a median of 8 days (Seaton *et al.*, 1996). In African areas where amphotericin B is not available, results with fluconazole monotherapy at 200 mg/day or fluconazole plus flucytosine in combination showed 44% mortality at 8 weeks (Mayanja-Kizza *et al.*, 1998; Mwaba *et al.*, 2001). The main reasons for the ongoing high mortality of cryptococcal disease include the inadequacy of current antifungal therapy, restricted access to some drugs in many areas and the problem of raised CSF pressure (Antinori, 2006; Bicanic and Harrison, 2004; Jarvis and Harrison, 2007; Perfect, 2007).

E. Genome sequencing project

The genome sequence of five cryptococcal strains (JEC21, B3501A, H99, WM276, and R265) has been completed (Hu *et al.*, 2008). The JEC21 genome (sequenced at TIGR) comprises a total of 20 Mb of DNA, containing approximately 6572 genes (Loftus *et al.*, 2005), 10% of which are unique to *C. neoformans* (Idnurm *et al.*, 2005). The intron-rich genome encodes a transcriptome abundant in alternatively spliced (4.2% of transcriptome) and antisense messages (53 genes). The genome is also rich in transposons (~5%), many of which cluster at centromeric regions. The presence of these transposons results in genetic plasticity and may be responsible for karyotype instability and phenotypic variation (Loftus *et al.*, 2005). The sequence difference between JEC21 and B3501A (another

serotype D isolate, sequenced at Stanford University) is restricted to 50% of their genomes, which overall are 99.5% identical at the sequence level (Loftus *et al.*, 2005). The genome sequencing project has been reviewed recently by Idnurm *et al.* (Idnurm *et al.*, 2005).

As a basidiomycete fungus, *C. neoformans* is evolutionarily distinct from ascomycete fungi such as *Saccharomyces cerevisiae*, the fission yeast *Schizosaccharomyces pombe*, and many common human fungal pathogens including *Candida albicans* and *Aspergillus fumigatus* (Hull and Heitman, 2002). The completed *C. neoformans* and *C. gattii* genome sequences permit comparative genomics with fungi from other phyla, although a detailed comparison based on all five cryptococcal genomes has not yet been undertaken. In addition, the availability of these sequences has made the construction of tiling microarrays and CGH studies feasible. CGH in combination with physical mapping and sequencing has already been used to study the genome variability within *C. neoformans* species and potentially allows for detailed characterization of the genome of emerging clinically significant strains (e.g., isolates from the Vancouver Outbreak) in the future (Hu *et al.*, 2008). These studies will provide important information on the mechanisms of genome microevolution in these pathogens.

II. VIRULENCE FACTORS

C. neoformans and *C. gattii* have a number of well-defined virulence factors, which strongly influence the degree of pathogenicity of individual isolates. A recent study by Rodrigues *et al.* demonstrated that *C. neoformans* was able to secrete vesicles containing many of its virulence factors, including GXM, laccase, urease, and phospholipase B (Rodrigues *et al.*, 2008). The extracellular vesicles manifested various sizes and morphologies, including electron-lucid membrane bodies and electron-dense vesicles. During disseminated cryptococcosis, measurable levels of cryptococcal products are detected in the body fluid of patients (Gordon and Vedder, 1966), suggesting that these ''virulence factor delivery bags'' may represent an efficient and general way of delivering pathogenesis-related molecules to the extracellular environment by *C. neoformans* (Rodrigues *et al.*, 2008). Below several well-characterized virulence factors are discussed in detail.

A. Capsule

The importance of capsule as a virulence factor was demonstrated by the observation that acapsular variants of *C. neoformans* very rarely cause human disease (Alspaugh *et al.*, 1998). The capsule is composed of 90–95%

GXM and 5% galactoxylomannan (GalXM) (Rakesh *et al.*, 2008). GXM is a large polymer with a repeating structure of α-1,3-mannose with β-D-xylopyranosyl, β-D-glucuronosyl and 6-*o*-acetyl branching. This structure determines the serotype of *C. neoformans* and *C. gattii*, because different capsule structures can be distinguished by antibodies. GalXM is an α-1,6 galactan that contains branches of β-1,3-galactose-α-1,4-mannose-α-1,3 mannose (Vaishnav *et al.*, 1998). It has a much smaller mass than GXM: $1.01 \times 10^5 \text{gmol}^{-1}$ versus $1.7–7.4 \times 10^6 \text{gmol}^{-1}$ (McFadden *et al.*, 2006). In addition to GXM and GalXM, several mannoproteins (<1%) such as MP-98 and MP-99 have been identified within the cryptococcal capsule (Huang *et al.*, 2002; Levitz *et al.*, 2001). So far, a total of 53 mannoproteins are predicted by genomic databases (Levitz and Specht, 2006). These mannoproteins share several structural features, including N-terminal signal sequences, serine/threonine (S/T)–rich C-terminal regions, and glycosylphosphatidylinositol (GPI) anchor motifs. When mannosylated and glycosylated, they act as critical cryptococcal antigens responsible for stimulating T-cell responses by promoting dendritic cell maturation and activation (Mansour *et al.*, 2004; Pietrella *et al.*, 2005; Specht *et al.*, 2007).

Many *C. neoformans* genes in capsular synthesis and formation have been identified. Chang *et al.* cloned and sequenced four genes (*CAP10, CAP59, CAP60*, and *CAP64* genes) responsible for capsule synthesis in serotype D isolates. Each of these capsule genes is required for virulence in a murine model (Chang and Kwon-Chung, 1994, 1998, 1999; Chang *et al.*, 1996). *CAP59*, the first capsule-associated gene isolated, encodes a transmembrane protein (Chang and Kwon-Chung, 1994; Chang *et al.*, 1995), which is involved in the process of GXM export (Garcia-Rivera *et al.*, 2004). *CAP64*, the second capsule-associated gene identified, was used to complement an acapsular strain (602), resulting in capsule production and a fatal infection in mice (Chang *et al.*, 1996, 1997). *CAP60* and *CAP10* are the other two characterized capsule genes, which encode proteins localized to the nuclear membrane and cytoplasm respectively (Chang and Kwon-Chung, 1998, 1999). All four *CAP* genes have been shown to be essential in capsule synthesis, but the biochemical function of their products is ill defined. There are many other genes involved in, but not essential to, capsule formation. For instance *CAS1* and *CAS3* are involved in the acetylation of GXM (Janbon *et al.*, 2001; Moyrand *et al.*, 2004), whilst *UXS1* and *UGD1* along with *CAS31*, *CAS32*, *CAS33*, *CAS34*, and *CAS35* are important for proper xylosylation of GXM (Bar-Peled *et al.*, 2001; Moyrand *et al.*, 2004). Genome analysis identified more than 30 new genes that are likely to be involved in capsule biosynthesis, including a family containing seven members of the capsule-associated (*CAP64*) gene and a second family of six capsule-associated (*CAP10*) genes (Loftus *et al.*, 2005).

The capsule is important for *C. neoformans* survival in its host, where it increases the fitness of *C. neoformans* by providing direct protection for the yeast. For instance, the capsule inhibits phagocytosis of *C. neoformans* by professional phagocytes in the absence of opsonins (Kozel and Gotschlich, 1982) and resists phagosome digestion (Tucker and Casadevall, 2002). Capsular material also acts directly against the host. In macrophages, *C. neoformans* releases polysaccharide from its capsule into vesicles around the phagosome and accumulation of these vesicles in the cytoplasm of the host cell results in macrophage dysfunction and lysis (Feldmesser *et al.*, 2000; Tucker and Casadevall, 2002). High levels of capsular polysaccharide antigens in the CSF can change the osmolarity of the CSF, thereby affecting its outflow and leading to increased intracranial pressure, headaches, and visual disturbance (Denning *et al.*, 1991). In addition, capsular material was reported to repress the migration of host phagocytes (e.g., neutrophils) (Dong and Murphy, 1995, 1997; Ellerbroek *et al.*, 2004), interfere with cytokine secretion (Retini *et al.*, 1996; Villena *et al.*, 2008), directly inhibit T-cell proliferation (Yauch *et al.*, 2006), induce macrophage apoptosis mediated by Fas ligand (Villena *et al.*, 2008), and delay maturation and activation of human dendritic cells (Lupo *et al.*, 2008; Vecchiarelli *et al.*, 1994).

The cryptococcal capsule size varies depending on the environmental conditions and seems to be tightly regulated (Fig. 5.1A). In nature, cryptococcal cells rarely display the large capsule seen in clinical isolates. The infectious particles, in order to be inhaled and penetrate the small airway, have to be smaller than 4 μm in diameter with little or no capsule (Casadevall and Perfect, 1998). However, during infection, the capsule is dynamically enlarged and the size varies depending on the affected organ. For instance, the lung and brain environment appears to act as an active inducer of capsule growth (Rivera *et al.*, 1998). Capsule size can also be experimentally modulated by growing *C. neoformans* in diluted Sabouraud broth in the presence of serum, or in a CO_2 rich atmosphere in DMEM media with low iron concentration (Vartivarian *et al.*, 1993; Zaragoza and Casadevall, 2004). These conditions are present in host environment and may thus promote capsule production during infection.

Although the presence of capsule significantly contributes to the virulence of *C. neoformans*, it is not the only requirement. Many non-*neoformans* cryptococcal species possess a capsule, but are not pathogenic. Also in one study acapsular *C. neoformans* was found to cause persistent infections in the brains of nude mice, but not in mice with defects only in innate immunity (Casadevall and Perfect, 1998), suggesting that when mammalian immunity is sufficiently impaired, even noncapsular strains retain their virulence potential.

B. Melanin

The ability of *C. neoformans* to produce melanin was discovered by Staib in the 1960s (Polacheck, 1991) (Fig. 5.1B). Melanin is a negatively charged, hydrophobic pigment of high molecular weight that is formed by the oxidative polymerization of phenolic compounds (Casadevall *et al.*, 2000). Melanin synthesis in *C. neoformans* is catalyzed by laccase in the presence of certain o-diphenolic compounds, such as 3,4-dihydroxyphenylalanine (L-Dopa) (Williamson, 1997). In the environment, melanin protects yeast from UV light, high temperatures, freezing and thawing (Rosas and Casadevall, 1997; Wang and Casadevall, 1994). Nosanchuk and colleagues have demonstrated that *C. neoformans* cells recovered from human brain tissue are melanized (Nosanchuk *et al.*, 2000a) and gene disruption studies indicate that wild type melanin-producing *C. neoformans* are more virulent (Casadevall *et al.*, 2000). Compared to nonmelanized *C. neoformans* cells, melanized cells are less susceptible to oxidants (Emery *et al.*, 1994), and killing by antifungal drugs (e.g., caspofungin and amphotericin B) (van Duin *et al.*, 2002). Since production of an oxidative burst after phagocytosis is an important mechanism by which immune effector cells mediate antimicrobial action, these results suggest that melanin may enhance virulence by protecting fungal cells against attack by the immune system. This is further supported by the observation that melanized cells were more resistant to phagocytosis and cell death caused by phagocytic effector cells (Huffnagle *et al.*, 1995). It is important to note that some non-*neoformans* cryptococci are able to form melanin as well, such as *Cryptococcus podzolicus* (Petter *et al.*, 2001), although they are not pathogenic.

The importance of melanin production to the virulence has motivated studies to define components of this pathway. Two laccase genes: *LAC1* (Torres-Guererro and Edman, 1994) and *LAC2* (Missall *et al.*, 2005; Zhu and Williamson, 2004) were identified as central enzymes in melanin biosynthesis. Other genes including *VPH1*, *CLC1*, *CCC2*, *ATX1*, and *MBF1* have also been found to be essential (Erickson *et al.*, 2001; Walton *et al.*, 2005; Zhu and Williamson, 2003), although in most cases the mode of action of these components is not well characterized.

C. Ability to grow at physiological temperature

The ability to grow at physiological temperatures is essential for the virulence of *C. neoformans* and *C. gattii*. Although some cryptococcal species also possess capsules and/or produce melanin (e.g., *C. podzolicus*), only rarely are they capable of *in vitro* growth at 37 °C, and thus none of them cause consistent infection in mammals (Perfect, 2005). *C. neoformans* is enriched in bird guano, but birds do not become infected, probably because *C. neoformans* does not live well at the avian body temperature

of 40–42 °C (Mitchell and Perfect, 1995). Therefore, this temperature restriction is an important determinant of *C. neoformans* pathogenicity.

Early studies identified over a dozen genes as being necessary for high-temperature growth (summarized in (Perfect, 2005)). One such gene, CNA1, encodes the *C. neoformans* calcineurin A (CNA1). When CNA1 was disrupted in H99, the resulting mutant strain was found to be viable at 24 °C but not at mammalian physiological temperature. Correspondingly, the mutant strain was avirulent in an immunocompromised rabbit model of cryptococcal meningitis (Odom *et al.*, 1997). Therefore, a role for the regulation of growth at elevated temperatures by signaling cascades involving calcineurin has been proposed. Many cryptococcal genes are known to be regulated by temperature, although they are not necessarily required for high-temperature growth. A microarray transcriptional profiling of *C. neoformans* genes showing altered expression at 37 °C versus 25 °C described 49 genes induced at 37 °C, including *MGA2*, which showed significantly higher expression during growth at 37 °C, and was also important for normal growth at high temperature (Kraus *et al.*, 2004). Similarly, a recent study using an alternative approach called representational difference analysis (RDA) has revealed 29 genes that are upregulated at 37 °C, with some overlaps with the genes identified by Kraus *et al.* (Rosa *et al.*, 2008). These newly defined genes seem to have a variety of functions, ranging from stress signaling, cell wall assembly, membrane integrity, and basic metabolism (Kraus *et al.*, 2004; Rosa *et al.*, 2008; Steen *et al.*, 2002). Functional studies of genes identified in these work by targeted gene disruption followed by validation in animal models may contribute to a better understanding of their role in virulence and pathogen-host interactions.

D. Degradative enzymes

Proteinase

Both environmental and clinical isolates of *C. neoformans* have proteinase activity (Casadevall and Perfect, 1998). They have been shown to degrade host proteins including collagen, elastin, fibrinogen, immunoglobulins, and complement factors (Chen *et al.*, 1996). Tucker and Casadevall also proposed that replication of *C. neoformans* inside macrophages is accompanied by the production of enzymes including proteinases and phospholipases to damage the phagosomal membrane (Tucker and Casadevall, 2002). Therefore, cryptococcal proteinases can cause tissue damage, providing nutrients to the pathogen and protection from the host.

Phospholipases

Phospholipases are a heterogeneous group of enzymes that are able to hydrolyze one or more ester linkages in glycerophospholipids. The action of phospholipases can result in the destabilization of membranes,

cell lysis, and release of lipid second messengers (Ghannoum, 2000; Santangelo *et al.*, 1999). *C. neoformans* secrets a phospholipase enzyme that demonstrates phospholipase B (PLB), lysophospholipase hydrolase, and lysophospholipase transacylase activities. As with proteinases, phospholipases contribute to the degradation of host cell membrane and thus cell lysis. There is a correlation between phospholipase expression and virulence in a dose-dependent manner among the strains used to infect mice (Chen *et al.*, 1997; Ghannoum, 2000). Disruption of *PLB1* gene led to reduced virulence *in vivo* and growth inhibition in a macrophage like cell line (Cox *et al.*, 2001). Phospholipase can also cleave dipalmitoyl phosphatidylcholine, one of the main components of lung surfactant, and thus assists fungal spread (Steenbergen and Casadevall, 2003). Furthermore, recent studies demonstrated that phospholipase B of *C. neoformans* enhances adhesion of *C. neoformans* to a human lung epithelial cell line (Ganendren *et al.*, 2006) and dissemination of cryptococcosis in a murine model (Santangelo *et al.*, 2004).

Urease

Urease catalyzes the hydrolysis of urea to ammonia and carbamate and is an important pathogenic factor for certain bacteria (Steenbergen and Casadevall, 2003). The cryptococcal urease, Ure1, is an important virulence factor and mice infected with a *ure1* mutant strain live longer than mice infected with the wild type strain H99 (Cox *et al.*, 2000). Although urease was not required for growth in the brain, the dissemination patterns in the brain, spleen, and other organs after intravenous inoculation differed from the wild type strain, leading to the proposal that Ure1 is important for CNS invasion by enhancing yeast sequestration within microcapillary beds (such as within the brain) during hematogenous spread, thereby facilitating blood-to-brain transmission (Olszewski *et al.*, 2004).

E. Mating type

Most clinical and environmental cryptococcal isolates have been observed predominantly as vegetative haploid yeast. Like other basidomycetes, traditional mating can occur when opposite mating types (a and α) recognize and fuse with one another to produce a filamentous dikaryon, resulting in a transient a/α diploid state that immediately undergoes meiosis and sporulation producing a and α haploid progeny (Kwon-Chung, 1975, 1976). *Cryptococcus* can also undergo same-sex mating (monokaryotic fruiting), especially between two α cells to form stable α/α diploids and also α haploid progeny (Lin *et al.*, 2005). Mating without a partner of the opposite mating type might provide a survival advantage, particular under harsh or changing conditions (Lin *et al.*, 2007).

Several interesting observations implicate mating type as a virulence factor. Firstly, MATα cells are much more prevalent than MATa cells. For instance, in a survey of natural and clinical isolates, the *MAT*α mating type was 40-fold more abundant in environmental isolates and 30-fold more abundant in clinical isolates than its MATa counterpart (Kwon-Chung and Bennett, 1978). In addition, most of the Vancouver isolates are α mating type (Fraser *et al.*, 2003). Secondly, when congenic α and a strains (JEC21) of serotype D (genetically identical except at the mating type locus) were studied in a murine model of cryptococcosis, the MATα strain was found to be significantly more virulent than the MATa strain (Kwon-Chung *et al.*, 1992). Congenic α and a cells in the serotype A H99 background show the same pathogenicity level in various mammalian models (Nielsen *et al.*, 2003), but α cells have an enhanced predilection to penetrate the CNS during coinfection with a cells, which provides an explanation for the prevalence of α stains in clinical isolates (Nielsen *et al.*, 2005).

The finding that MATα cells are more prevalent and virulent than MATa cells has promoted molecular analysis of the *MAT*α mating type locus. Initially, an ~50 kb region presented only in MATα strains was defined as the *MAT*α locus, and it contains many α-specific genes including STE12α (Karos *et al.*, 2000). However, the actual size of the *MAT* locus appears to be much larger than that. It is more than 100 kb in length for both *C. neoformans* and *C. gattii*, containing>20 genes, including those involved in pheromone production and sensing, establishing cell type identity, components of a MAP kinase pathway, and those do not seem to have a function in mating (Fraser and Heitman, 2004; Lengeler *et al.*, 2002). There is still much to be learned about the linkage of sex and pathogenesis, especially at the genetic level. Detailed reviews on life cycle and mating type locus can be found in (Hull and Heitman, 2002; Idnurm *et al.*, 2005).

F. Phenotypic switching

Phenotypic switching has been observed in both prokaryotes and eukaryotes and involves stochastic switching between two or more alternative and heritable phenotypes. It occurs by spontaneous tuning in gene expression in order to escape recognition by the immune system and to adapt to a new host environment. Phenotypic switching is reversible and readily detectable in a fraction of cell population (D'Souza and Heitman, 2001).

The first detection of phenotypic switching in *C. neoformans* was reported by Fries and Casadevall in 1998 (Fries and Casadevall, 1998), in which they demonstrated that *C. neoformans* was able to undergo microevolution during chronic infection. Subsequently, in 2001, Fries

et al. showed for the first time that *C. neoformans* was able to undergo phenotypic switching *in vivo* during serial passage in mice (Fries *et al.*, 2001). So far, phenotypic switching has been observed in serotype A, B, and D strains (Guerrero *et al.*, 2006; Jain *et al.*, 2006), and always leads to changes in virulence by causing changes in capsule or cell wall morphology. For example, a *C. gattii* strain was found to switch reversibly between two colony morphologies. Switching to mucoid colonies (with a thicker layer of capsule) was observed during pulmonary infection and resulted in enhanced intracellular survival due to a larger capsule. However, only smooth colonies (with a thin layer of capsule) could be grown from brain homogenates in infected mice, probably because the thin capsule permits better crossing of the blood–brain barrier (Jain *et al.*, 2006). Phenotypic switching of *C. neoformans* was also shown to influence the outcome of the human immune response. For example, the mucoid colony phenotype elicits a macrophage- and neutrophil-dominated immune response, while the smooth colony phenotype elicits a lymphocyte-dominated immune response (Pietrella *et al.*, 2003). The ability of this organism to cause chronic infections even after prolonged antifungal therapy may be, in part, attributable to phenotypic switching (Guerrero *et al.*, 2006).

G. The origin and maintenance of virulence factors

C. neoformans and *C. gattii* are environmental saprophytes, mainly found in soil and trees, so humans probably represent an inadvertent host species rather than a primary niche. There is much evidence supporting the hypothesis that cryptococcal virulence originated due to environmental selective pressure. Firstly, many environmental isolates of *C. neoformans* are virulent in animals, indicating that these virulence factors have been developed without previous interaction with host animals. Secondly, a broad range of animals are susceptible to this organism and these hosts are not required for replication or viability of the pathogen (Casadevall *et al.*, 2003). Thirdly, many virulence factors appear to have ''dual use'' capacities that allow survival advantages in both animal hosts and in the environment. For instance, in bird excreta, the primary role of urease may be to enable *C. neoformans* to convert urea to the usable nitrogen source ammonia (Levitz, 2001). Decaying wood contains large amount of the aromatic polymer lignin, a substrate of laccases. Thus, it has been hypothesized that cryptococcal laccase helps the organism establish an ecological niche in rotting wood (Lazera *et al.*, 2000). The capsule can protect the fungus against dehydration and thus provide a survival advantage in conditions of low humidity (Aksenov *et al.*, 1973). Melanized *C. neoformans* cells, as mentioned earlier, are more resistant to UV

radiation, temperature extremes, and heavy metals (Rosas and Casadevall, 1997). In addition, phospholipase and protease can serve important nutritional roles (Chen *et al.*, 1996). Hence these virulence factors are not solely developed for survival inside mammalian hosts.

Finally, *C. neoformans* is a facultative intracellular parasite, surviving both inside and outside of phagocytes. Infection of macrophages and amoebae by *C. neoformans* was found to be very similar, and it has therefore been postulated that mammalian virulence factors in *C. neoformans* evolved as a defense mechanism against environmental predators (Malliaris *et al.*, 2004; Steenbergen and Casadevall, 2003; Steenbergen *et al.*, 2001). The observation that *C. neoformans* can be ingested by living amoebae was first reported by Bunting and colleagues nearly 30 years ago (Bunting *et al.*, 1979). Steenbergen *et al*. then demonstrated that incubation of *C. neoformans* and the amoeba *Acanthamoeba castellanii* results in phagocytosis of yeast cells and intracellular proliferation in a phagocytic vacuole followed by killing of amoebae; a process that is identical to that seen to occur in mammalian macrophages infected with this pathogen (Steenbergen *et al.*, 2001). Another amoeba, *Dictyostelium discoideum*, is also susceptible to infection with *C. neoformans* and the interactions are similar to those described previously for this fungus with macrophages. In addition, *C. neoformans* virulence was enhanced after growth in *D. discoideum*, and this enhancement correlated with increased capsule size and melanization (Steenbergen *et al.*, 2003). Both studies support the idea that pathogenicity of *C. neoformans* towards macrophages and vertebrate hosts may result from evolutionary pressure exerted by environmental predators. Similarly, Mylonakis *et al.* have demonstrated that soil-dwelling nematodes may also exert strong selective pressure on *Cryptococcus* species (Mylonakis *et al.*, 2002). Whilst nonpathogenic cryptococcal species (*C. laurentii* and *Cryptococcus kuetzingii*) are killed by the nematode *Caenorhabditis elegans*, wild type strains of *C. neoformans* are lethal to the worms. Furthermore, the interaction involves a number of genes that are also important during the host pathogen interaction in mammals, including *GPA1*, *PKA1*, *RAS1*, and *PKR1* (Mylonakis *et al.*, 2002). Virulence might also be maintained through infection of small rodents or other mammals that, after death, reintroduce virulent strains back to the environment (Idnurm *et al.*, 2005).

In conclusion, it appears increasingly likely that many virulence factors in *C. neoformans* and *C. gattii* are ''ready made'' (Casadevall *et al.*, 2003) due to environmental selective pressure rather than ''specially made'' in order to colonize mammalian hosts. There are many existing environmental reservoirs that are expected to affect the fitness of fungal cells in that environment and to provide selective pressures for virulence attributes leading to differences in fitness during mammalian infection.

III. SIGNALING PATHWAYS REGULATING PATHOGENICITY

Six major signaling pathways have been demonstrated to modulate morphological differentiation, virulence, and stress responses. They are the cAMP-PKA pathway, three MAP kinase pathways involving Cpk1, Hog1, and Mpk1, the Ras specific pathway and the Ca^{2+}-calcineurin pathway. These pathways are also responsible for regulating differentiation and pathogenicity in other fungi and are largely structurally and functionally conserved in serotype A and D strains, although there are serotype-specific differences.

A. cAMP-PKA

There is conservation of function in cAMP signaling pathways in fungi since a large and diverse group of fungi (including *C. albicans* and *A. fumigatus*) employ similar signaling elements (Alspaugh *et al.*, 1998; Liebmann *et al.*, 2003; Rocha *et al.*, 2001). In *C. neoformans*, cAMP signaling is triggered by environmental stimuli (mainly nutritional, such as starvation) through a G-protein-coupled receptor (e.g., Gpr4 (Xue *et al.*, 2006)) and the Gα protein called Gpa1 (Alspaugh *et al.*, 1997). Gpa1 activates a conserved cAMP pathway through the enzyme adenylyl cyclase (Cac1), which generates cAMP and leads to activation of Protein Kinase A (PKA) by causing the release of the regulatory subunits (Pkr1) from the two catalytic units of PKA (Pka1 and Pka2) (Pukkila-Worley and Alspaugh, 2004). In serotype A strains, Pka1 plays a major regulatory role, while in serotype D strains, Pka2 does so (Hicks *et al.*, 2004).

The cAMP-PKA pathway regulates several important processes in *C. neoformans*, including capsule production, melanin formation, and mating. *gpa1* mutants, *cac1* mutants, and *pka1* mutants all display similar defects in mating, capsule, and melanin production (Alspaugh *et al.*, 1997, 2002; D'Souza *et al.*, 2001). For instance, *C. neoformans gpa1* mutant strains could not produce melanin, showed markedly attenuated capsule production in response to the normal inducing condition of severe iron starvation, and were sterile. Correspondingly, in a rabbit model of cryptococcal meningitis, the mutant strain was markedly impaired in the ability to maintain CNS infection compared to the isogenic *wild type* strain (Alspaugh *et al.*, 1997). Disruption of *PKR1* suppresses the capsule and melanin defects of the *gpa1* mutant, causes cells to display an enlarged capsule phenotype, and results in hypervirulence (D'Souza *et al.*, 2001). In addition, a recent microarray study comparing the transcriptome of mutants (*pka1* and *pkr1*) to a *wild type* strain revealed a novel relationship between cAMP signaling and the secretory pathway in *C. neoformans* (Hu *et al.*, 2007). In the *pka1* and *pkr1* mutants, transcriptional

changes occur to many key components important for the secretory pathway, such as those responsible for translocation (Sec61 and Hsp70/Kar2), vesicle formation and fusion (Bet1, syntaxin), Golgi transport (α-1,6-mannosyltransferase), and vesicle delivery to the plasma membrane (e.g., Ypt3). This study along with the observation that *C. neoformans* secretes vesicles containing many of its well-defined virulence factors, suggests a model in which PKA regulates the expression of secretory pathway components to control the elaboration of virulence factors at the cell surface (Hu *et al.*, 2007; Rodrigues *et al.*, 2008).

B. MAP kinase pathway

The pheromone-activated MAP kinase pathway is another conserved pathway, in which the G protein β subunit (Gpb1) activates the transcriptional regulator Ste12α, whose downstream targets include *STE20*, *STE11*, and *STE7* (Lengeler *et al.*, 2000). *gpb1* mutants are sterile, defective in haploid fruiting and exhibit a severe defect in cell fusion assays (Wang *et al.*, 2000). Although studies disrupting the *STE12α* gene found that in both serotype A and D, Ste12α is absolutely required for monokaryotic fruiting, it seems only to augment virulence in serotype D, but not serotype A strains. In serotype D strains, Ste12α was found to control the expression of many virulence-associated genes, and disruption of the *STE12α* gene resulted in a significant reduction in virulence in a mouse model (Chang *et al.*, 2000), whereas earlier studies by Yue *et al.* demonstrated that the *STE12α* homolog is largely dispensable for virulence in a number of serotype A strains (Yue *et al.*, 1999).

More recently, the Pbs2-Hog1 MAP kinase pathway has been shown to have a significant impact on virulence of serotype A and some serotype D strains (Bahn *et al.*, 2005). The fungal Hog1 MAPK mediates responses to a plethora of environmental cues, including osmotic shock, UV irradiation, oxidative damage, and high temperature. Intriguingly, Hog1 is regulated in an opposite fashion in a majority of *C. neoformans* strains (especially highly pathogenic isolates, e.g., H99), compared to some of the serotype D strains and other model yeasts. In *S. cerevisiae,* MAPK Hog1 is dephosphorylated in normal conditions and following osmotic shock, a two component system can activate MAPK kinase Pbs2 through activation of Ssk1, which subsequently phosphorylates MAPK Hog1 (Bahn *et al.*, 2006). Phosphorylated Hog1 then translocates to the nucleus where it activates expression of target genes (Hohmann, 2002). A similar pathway has been observed for some *C. neoformans* serotype D strains, such as JEC21. However, in most *C. neoformans* strains, the Hog1 MAPK is constitutively phosphorylated by Pbs2 MAPK kinase under normal *in vitro* growth conditions, and upon osmotic shock, Hog1 is rapidly dephosphorylated (Bahn *et al.*, 2005). It was proposed that phosphorylated

Hog1 under normal conditions is mainly responsible for negatively regulating virulence factors, including capsule and melanin, and sexual development. In addition, the phosphorylated Hog1 concentrates in the nucleus, where it can interact with other transcription factors resulting in cross-talk with signaling cascades that regulate virulence factor expression in *C. neoformans* (Bahn *et al.*, 2006). For example, experimental data demonstrated that Hog1 negatively regulates melanin production by acting on PKA downstream targets for melanin synthesis, whilst Hog1 also negatively regulate capsule production by acting on upstream of Gpa1 or PKA itself. Furthermore, it is possible that Hog1 represses factors such as Ste12 that modulate melanin and capsule production (Bahn *et al.*, 2005). Under stress conditions, Hog1 is rapidly dephosphorylated, which could be the active form of the MAPK to induce stress defense genes in *C. neoformans*. For instance, it was reported that fludioxonil treatment can activate the HOG pathway by rapid dephosphorylation of the Hog1 MAPK in the majority of *C. neoformans* strains (Kojima *et al.*, 2006).

The Mpk1 MAP kinase pathway regulates cell-wall integrity and growth at high temperature. It is well studied in *S. cerevisiae*, and the function of Mpk1 in promoting growth at 37 °C in *S. cerevisiae* is conserved in *C. neoformans*. In this pathway, upstream components such as membrane sensors that detect stresses to the cell wall (Gray *et al.*, 1997; Verna *et al.*, 1997) and the Rho1 GTPase are responsible for activating protein kinase C (PKC), which in turn activates the Mpk1/Slt2 MAPK cascade (Kamada *et al.*, 1996; Lee *et al.*, 1993). *C. neoformans* mutants lacking Mpk1 are attenuated for virulence in the mouse model of cryptococcosis (Kraus *et al.*, 2003) and become more sensitive to antifungal drugs like fludioxonil (Kojima *et al.*, 2006).

C. Ras pathway and the Ca^{2+}-calcineurin pathway

The Ras-Cdc24 pathway and Ca^{2+}-calcineurin pathway independently control *C. neoformans* growth at high temperature. *C. neoformans ras1* mutant strains are viable, but they are unable to grow at 37 °C and thus less virulent in rabbit and murine models of cryptococcosis (Waugh *et al.*, 2002). The high temperature growth defect of *C. neoformans ras1* mutant strains was associated with a failure of actin polarization at elevated temperature (Waugh *et al.*, 2002). Similarly, calcineurin mutant strains are found to be viable, but do not survive *in vitro* conditions that mimic the host environment and are no longer pathogenic in a murine model of cryptococcal meningitis (Odom *et al.*, 1997).

Ras also plays a dual role to activate a MAP kinase cascade and to regulate cAMP production in *C. neoformans*. Initial experiments defining the Ras pathway in a serotype A strain indicated that Ras1 mediates MAP kinase, cAMP, and Ras-specific signal transduction pathways (Alspaugh

et al., 2000). By northern blot analysis, Ras1 was demonstrated to play a major role in the transcriptional regulation of genes in the pheromone response pathway. It also controls pheromone-independent signaling mechanisms which are essential for filamentation, development, and pathogenicity (Waugh *et al.*, 2003). Ras2 is expressed at a very low level compared to Ras1, and a *ras2* mutant showed no differences in vegetative growth rate, differentiation, or virulence factor expression, nor was it attenuated in the murine inhalational model of cryptococcosis. However, when overexpressed, Ras2 was able to restore mating and high temperature growth of a *ras1* mutant, indicating Ras1 and Ras2 may share overlapping functions (Waugh *et al.*, 2002).

The calcineurin pathway is well characterized. Besides its importance for growth at high temperature, it is also essential for cell integrity, monokaryotic fruiting, and mating (Cruz *et al.*, 2001; Fox *et al.*, 2001; Kraus *et al.*, 2003, 2005; Odom *et al.*, 1997). In this pathway, both calcineurin A and B subunits were found to be essential for virulence (Fox *et al.*, 2001), by binding to Cbp1 (calcineurin binding protein 1) (Gorlach *et al.*, 2000), and activating as-yet unidentified downstream transcription factors.

IV. *CRYPTOCOCCUS* AND THE HOST RESPONSE

Exposure to *C. neoformans* is thought to be common, but in a normal host the infection is usually self-limiting. In contrast, in immunocompromised individuals, the infection is not restricted to the primary site of infection, but frequently disseminates to the CNS. This suggests that phagocytes *in vivo* are able to dispose of *C. neoformans* effectively (or at least maintain the pathogen in a latent stage), only when T-cell defenses are intact. This probably involves activation of macrophages by T-cell derived cytokines (mainly Th1 type, including TNFα, IFNγ, IL-2, and IL-12) and granuloma formation to contain replicating organisms. In other words, phagocytes are ''temporary protectors'' until the acquired immune response is established. This part of review will focus on the interaction between phagocytic effector cells and *C. neoformans* in the presence and absence of a secondary immune response.

A. Immunocompromised host

In immunocompromised individuals, the innate immune response is the major barrier to cryptococcal infection. Although many studies have identified several innate factors such as serum, complement, and saliva that discourage infections (Baum and Artis, 1961, 1963; Hendry and Bakerspigel, 1969; Igel and Bolande, 1966; Nassar *et al.*, 1995; Szilagyi

et al., 1966), the outcome of the infection is largely dependent on the interaction between the pathogen and phagocytic effector cells (Shao *et al.*, 2005).

1. Neutrophils

An *in vivo* study on cryptococcal infection in mice by Feldmesser *et al.* (2000) noted that macrophages and neutrophils are the only inflammatory cells in contact with *C. neoformans* in the lung. Many *in vitro* studies also demonstrated that neutrophils could phagocytose and kill *C. neoformans* (Chaturvedi *et al.*, 1996; Kozel *et al.*, 1984; Mambula *et al.*, 2000; Miller and Mitchell, 1991). However, *in vivo*, neutrophils were only found to occasionally ingest *C. neoformans* for the first few days after infection, indicating that they predominate only in the early stage of an experimental infection (Feldmesser *et al.*, 2000). Furthermore, neutrophil-depleted mice had significantly higher levels of IL-4/IL-10 (Th2 cytokines) and IL-12/TNFα (Th1 cytokines), and they lived longer than control mice, suggesting neutrophil depletion is protective against *C. neoformans* pulmonary infection. The enhanced survival observed in neutrophil-depleted mice may be a result of a more effective killing of the pathogen triggered by IL-12 and TNFα, and reduced damage to the host moderated by IL-4 and IL-10 (Mednick *et al.*, 2003). Therefore, neutrophils probably do not contribute significantly to direct killing of invading *C. neoformans*, but rather play an important role in balancing the Th1/Th2 cytokine profile in the late stage of infection.

2. Dendritic cells

Recent studies also show that dendritic cells phagocyte *C. neoformans in vitro* (Kelly *et al.*, 2005; Syme *et al.*, 2002) and *in vivo* (Wozniak *et al.*, 2006). Dendritic cells are antigen-presenting cells that act as sentinels in the peripheral tissues, constantly sampling the antigens in their environment. During cryptococcal infection, dendritic cells are thought to be more important in the initial presentation of antigens to the naive T cells to induce an adaptive immune response. Indeed, they induce a stronger T-cell response to *C. neoformans* than alveolar macrophage or monocyte-derived macrophage cells (Mansour *et al.*, 2006; Syme *et al.*, 2002). Several major antigens (e.g., mannoproteins) known to drive T cell responses to *C. neoformans* were also found to be mainly presented by dendritic cells (Levitz and Specht, 2006).

3. Macrophages

Macrophages, also involved in antigen presentation and cytokine production (Casadevall and Perfect, 1998), have long been regarded as the phagocyte that initially encounters inhaled *C. neoformans* and act as the primary phagocytic cell at all times of infection in both murine and rat

models of infection (Bolanos and Mitchell, 1989; Feldmesser *et al.*, 1998, 2000; Goldman *et al.*, 2000; Levitz, 1994). Phagocytosis of *C. neoformans* by macrophages can be mediated by receptors such as the mannose receptor, β-glutan receptor, antibody receptors, and complement receptors. Phagocytosis via the latter two receptors is efficient (Casadevall and Perfect, 1998). Depending on the environment they adapt to, *C. neoformans* cells can actively ''choose'' to avoid being phagocytosed to a certain extent by regulating their antiphagocytic factors. For instance, *C. neoformans* was found to switch reversibly between two colony morphologies which were associated with changes in capsule (Jain *et al.*, 2006). Capsule is a major anti-phagocytic factor in the absence of opsonins (Kozel and Gotschlich, 1982; Kozel and Mastroianni, 1976). It inhibits phagocytosis partly by lessening presentation of phagocytic ligands to alveolar macrophages (Vecchiarelli *et al.*, 1994). In addition, encapsulated *C. neoformans* have a more negatively charged surface than acapsular cells, which causes electrostatic repulsion between the cryptococci and negatively charged phagocytic cells and thus reduces cell-cell interaction (Nosanchuk and Casadevall, 1997). However, in the presence of opsonins including antibody and complement components (*in vivo*), the antiphagocytic property of the capsule is usually diminished (Feldmesser *et al.*, 2000). App1 (antiphagocytic protein 1) is another factor found to regulate phagocytosis. It was first identified as a regulator of complement-mediated phagocytosis (Luberto *et al.*, 2003). App1 inhibits phagocytosis through a specific and novel mechanism without affecting other cryptococcal anti-phagocytosis factors, such as capsule and melanin. Without App1, *C. neoformans* is more likely to be ingested by macrophages. Interestingly, it was found that the App1 mutant strain is less virulent than the wild type strain in A/J, CBA/J and C57BL/J mouse models, which are immunocompetent, whereas in a T-cell and natural killer (NK) cell deficient mouse model, the *app1* mutant strain exacerbated the infection as compared with the infection caused by a wild type strain. These results indicate that when the cellular immune response is impaired, phagocytosis can be an advantage for *C. neoformans* infection because *C. neoformans* grows faster intracellularly than it does extracellularly (Feldmesser *et al.*, 2000) and also it can be transported more efficiently by macrophages from organ to organ (Del Poeta, 2004; Luberto *et al.*, 2003). Therefore, modulation of the expression of antiphagocytic factors by *C. neoformans* may play a key role in the outcome of infection.

Following particle internalization by macrophages, the resulting intracellular vacuole (known as the phagosome) is subsequently fused with lysosomes to form the phagolysosome. This process is called phagosome maturation and the newly formed phagolysosome possesses a number of complementary degradative properties including a very low pH, hydrolytic enzymes for particle digestion, bactericidal peptides, and the ability

to generate toxic oxidative compounds (Vieira *et al.*, 2002). Usually the phagolysosome is very efficient at digesting internalized microorganisms. However, for *C. neoformans*, four outcomes have been observed after phagocytosis. They are: (1) The yeast is killed by the macrophage (Brummer and Stevens, 1994; Casadevall and Perfect, 1998); (2) the yeast remains latent inside the macrophage (Alvarez and Casadevall, 2007; Del Poeta, 2004; Ma *et al.*, 2007); (3) the yeast grows within the phagosome, eventually causing macrophage lysis (Feldmesser *et al.*, 2000, 2001; Tucker and Casadevall, 2002); and (4) the yeast exits the macrophage by a novel expulsive process without causing death of either the yeast or the host macrophage (Alvarez and Casadevall, 2006; Ma *et al.*, 2006) (Fig. 5.3). Therefore, *Cryptococcus* can manipulate macrophages in various ways. Currently it is unclear as to what decides the outcome of the intracellular yeast/macrophage interaction, but it is generally established that *in vitro*,

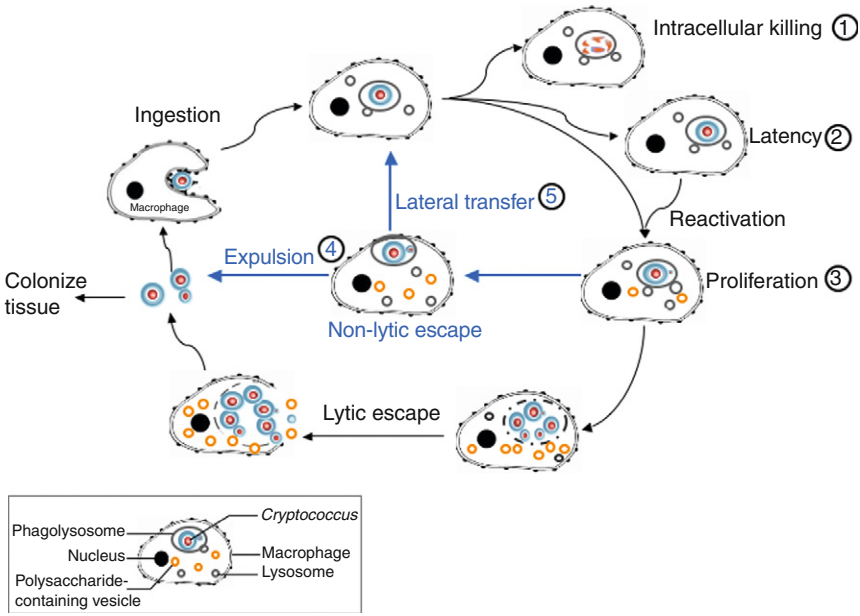

FIGURE 5.3 Macrophage parasitism by *C. neoformans*. Following phagocytosis, the internalized cryptococci can be killed by macrophages (1) or remain latent (2). When the host becomes immunocompromised, some of the cryptococci or latent population can reactivate and proliferate intracellularly (3), followed by the lytic burst of the host cells and release of the intracellular yeast cells into the extracellular environment. The released yeast cells can then carry on infecting more macrophages or establish extracellular dominance. Nonlytic escape pathways were also observed for *C. neoformans*, during which the yeast cells are expelled by macrophages without causing death of either party (4) or the intracellular yeast cells are directly delivered to a neighboring macrophage via lateral transfer (5).

macrophages activated with Th1 cytokines (secreted by CD4+ T cells) are more efficient at eradicating intracellular cryptococci than those activated with Th2 cytokines (Brodie *et al.*, 1994; Chen *et al.*, 1994; Kawakami *et al.*, 1995; Mody *et al.*, 1991; Weinberg *et al.*, 1987). In addition, the fate of intracellular cryptococci varies with strain ((Zaragoza *et al.*, 2007); (Ma *et al.*, unpublished data)) and local environment (e.g., oxygen availability) (Voelz *et al.*, unpublished data).

In the absence of a T-cell mediated immune response, intracellular survival and proliferation of *Cryptococcus* is very common. This intracellular behavior is important for pathogenicity, because it provides a basis for dissemination and latency: intracellular cryptococci are carried by infected macrophages to different parts of the body without being exposed to any extracellular hazards, such as complement components or antifungal agents present in the blood. This so-called ''Trojan horse'' mechanism of dissemination (Chretien *et al.*, 2002; Santangelo *et al.*, 2004) is supported by the observation that *C. neoformans* was found almost exclusively in macrophages in chronic and latent infection (Feldmesser *et al.*, 2000; Goldman *et al.*, 2000). Intracellular parasitism of macrophages by *C. neoformans* was reported in the early 1970s, when most ingested *C. neoformans* were found to be resistant to intracellular killing by either peritoneal exudate cells from Lewis rats or monocyte-derived macrophages (Diamond and Bennett, 1973; Mitchell and Friedman, 1972).

Unlike many other intracellular pathogens which persist within the phagosome by either affecting phagolysosome maturation (e.g., *Legionella pneumophila*) (Nguyen and Pieters, 2005) or by escaping from the phagosome and then proliferating in the host cytosol (e.g., *Listeria monocytogenes*) (Cossart *et al.*, 2003), *C. neoformans* has been demonstrated to persist inside apparently normal mature phagosomes in human monocyte-derived macrophage (MDM) *in vitro* (Levitz *et al.*, 1999). The pH of *C. neoformans*-containing phagosomes was similar to that observed following uptake of dead fungi over 24 h, and these phagosomes also colocalized with LAMP-1, a highly glycosylated protein found in endosomal and lysosomal compartments that is commonly used as a late mature phagosome marker, indicating that *C. neoformans* does not interfere with phagosome-lysosome fusion. In fact, *C. neoformans* grows more rapidly in acidic media than in neutral or alkaline media and appears to be able to resist the action of the macrophage lysosomal enzymes, which function optimally at acid pH (Levitz *et al.*, 1999). *In vivo*, intracellular persistence was associated with replication and residence in a membrane bound phagosome (Feldmesser *et al.*, 2000, 2001). Recent electron microscopy studies by Tucker and Casadevall revealed that intracellular residence by *C. neoformans* is accompanied by the accumulation of polysaccharide-containing vesicles, which originated from the phagosome, followed by macrophage dysfunction and lysis (Tucker and Casadevall, 2002).

Many virulence factors required for cryptococcal intracellular survival have already been identified, including capsule and melanin synthesis proteins, proteinases, and phospholipases, an alternative oxidase (*AOX1*) (Akhter *et al.*, 2003), inositol phosphosphingolipid-phospholipase C1 (*ISC1*) (Shea *et al.*, 2006), *SKN7* (Coenjaerts *et al.*, 2006), and vacuole protein *VPS41* (Liu *et al.*, 2006), most of which contribute to defense against exogenous oxidative stress. However, the detailed intracellular survival mechanism needs further investigation.

Interestingly, live *C. neoformans* and *C. gattii* were also found to be expelled by macrophages as individual cells or extruded as biofilm-like microcolonies (Alvarez and Casadevall, 2006; Alvarez *et al.*, 2008; Ma *et al.*, 2006). This novel expulsive process has never been observed with any other pathogens parasitizing inside macrophages. Remarkably, expulsion does not kill either the expelled yeast or the host macrophage. The process is extremely rapid and yet can occur many hours after phagocytosis of the pathogen. The frequency of expulsion is both strain and host cell dependent (Alvarez and Casadevall, 2006; Ma *et al.*, 2006). Although the underlying molecular mechanism is unknown, the process seems to be cytoskeleton dependent, and is independent of phagosome maturation (Alvarez and Casadevall, 2006; Ma *et al.*, 2006). Compared with earlier studies showing intracellular proliferation followed by a lytic burst, this novel expulsive mechanism allows the pathogen to reemerge from the host cell in a more subtle way. Therefore, it may represent an important mechanism by which pathogens are able to escape from phagocytic cells without triggering host cell death and thus inflammation.

Although the expulsion event is independent of the initial route of the phagocytic uptake (Ma *et al.*, 2006), the outcome of the expulsion was affected by the mode of opsonization (Alvarez *et al.*, 2008). Extrusion of antibody-opsonized *C. gattii* and *C. neoformans* resulted in the release of a clump of yeast cells that remained attached to one another and continue to replicate extracellularly as a biofilm. In contrast, complement-opsonzed *C. neoformans* cells were released from macrophages dispersed as individual cells, which then continued to divide in the extracellular milieu as single cells. Therefore, the biofilm-like microcolony exit strategy of *C. neoformans* and *C. gattii* following antibody opsonization reduced fungal cell dispersion, suggesting that antibody agglutination effects persist even inside the phagosome to attach nascent daughter cells together and may thus contribute to antibody-mediated protection (Alvarez *et al.*, 2008).

Finally, *C. neoformans* has also been shown to spread from one macrophage to another directly without being exposed to the extracellular environment (Alvarez and Casadevall, 2007; Ma *et al.*, 2007). This so-called ''lateral transfer'' event needs further investigation, although it appears to be actin dependent (Alvarez and Casadevall, 2007) and

superficially resembles cryptococcal expulsion (Ma *et al.*, 2007). Despite the low rate of lateral transfer, it is possible that this process may have significant clinical implications, since it allows the pathogen to spread whilst remaining concealed from the immune system, and to move from weak to healthy phagocytes to ensure intracellular persistence even if the host cells starts to die (Ma *et al.*, 2007). Furthermore, it may represent a novel mechanism for *C. neoformans* to cross the blood–brain barrier (discussed in more detail in Section V).

B. Immunocompetent host

The host defense against *C. neoformans* is critically regulated by cell-mediated immunity (Lim and Murphy, 1980), especially T lymphocytes, which play a central role in eradicating this infection (Hill and Harmsen, 1991; Huffnagle *et al.*, 1991; Mody *et al.*, 1990). The mechanisms by which the lymphocytes facilitate elimination of cryptococci have not yet been elucidated. It is generally thought that lymphocyte clearance of *C. neoformans* acts indirectly through production of cytokines to enhance clearance of the organism by natural effector cells, particularly macrophages (Brodie *et al.*, 1994; Chen *et al.*, 1994; Kawakami *et al.*, 1995, 2000; Lindell *et al.*, 2005; Weinberg *et al.*, 1987; Zhang *et al.*, 1997).

Exposure to various pathogens can stimulate at least two patterns of cytokine production mainly by CD4+ T cells: Th1 and Th2. For *C. neoformans*, the balance between Th1 and Th2 cytokines markedly influences the outcome of infection. The predominant synthesis of Th1 cytokines over Th2 protects mice from infection was observed, whereas infection is exacerbated under Th2 dominant conditions (Hoag *et al.*, 1995; Huffnagle, 1996; Koguchi and Kawakami, 2002; Snelgrove *et al.*, 2006; Uicker *et al.*, 2005). Mice depleted of Th1 type cytokines are highly susceptible to cryptococcal infection (Huffnagle, 1996; Kawakami *et al.*, 1996), while the infection is less severe in mice lacking Th2 cytokines than in control mice (Blackstock and Murphy, 2004; Decken *et al.*, 1998).

During cryptococcal infection, the Th1/Th2 balance is maintained mainly by phagocytic effector cells (e.g., dendritic cells and neutrophils as discussed earlier) (Mednick *et al.*, 2003; Wozniak *et al.*, 2006) and some primary lymphocytes (e.g., natural killer T (NKT) cells and γδ antigen receptor-bearing T cells) (Kawakami, 2004; Nanno *et al.*, 2007; Zhang *et al.*, 1997). A remarkable feature of NKT cells is the abundant production of IFNγ and IL-4 upon stimulation via their antigen receptors. After cryptococcal infection, NKT cells were found to be recruited to the lung, and trigger a Th1-mediated, but not Th2-mediated, immune response (Kawakami *et al.*, 2001). In contrast, γδ T cells play a down-modulatory role in the development of Th1 responses and host resistance against *C. neoformans* (Uezu *et al.*, 2004). Therefore, γδ T cells may act to keep

the balance of Th1–Th2 responses in a proper manner by suppressing the exaggerated Th1 response caused by NKT cells (Kawakami, 2004). The contrasting roles of NKT and γδ T cells, and the fact the neutrophil depletion can enhance both Th1 and Th2 cytokines (Mednick *et al.*, 2003), suggest that these innate immune response are not only important for induction of proper host defense but also to balance the level of defense.

1. Antifungal effect of activated macrophages

When a T-cell mediated immune response is present, the majority of the intracellular cryptococci are eradicated (Fig. 5.4). Properly activated macrophages have a variety of microbicidal mechanisms that are potentially active against *C. neoformans*, including both oxidative and nonoxidative mechanisms and granuloma formation. The oxidative microbicidal mechanism involves the generation of reactive oxygen- and nitrogen-derived intermediates (ROI and RNI). ROI, such as

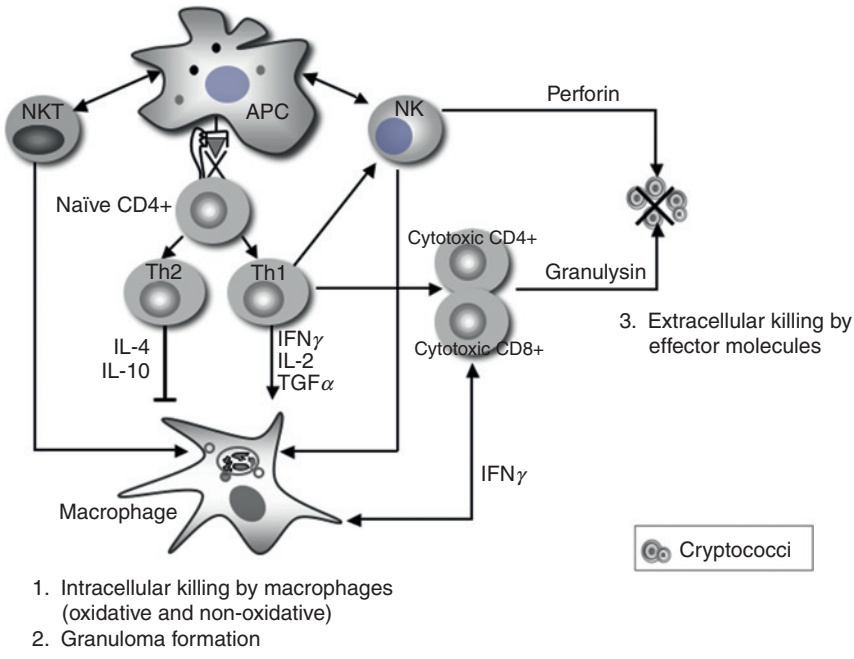

FIGURE 5.4 Killing of *C. neoformans* by the immune response in immunocompetent individuals. Killing can occur either intracellularly, when macrophages are activated by Th1 type cytokines, or extracellularly by effector molecules secreted by cytotoxic T lymphocytes (CD4+ and CD8+) and NK cells. Many other cells from the immune system also contribute to elimination of the cryptococci directly or indirectly by triggering and balancing Th1 type cytokine release.

superoxide anions, hydroxyl radicals, and hydrogen peroxide are generated as a result of the incomplete reduction of oxygen during respiratory metabolism (Turrens and Boveris, 1980). Nessa *et al.* showed that *C. neoformans* induced a markedly higher increase of oxidative metabolism in macrophages than did inert silica particles in an *in vivo* rabbit model of infection (Nessa *et al.*, 1997a). Such an increase was also observed with rat alveolar macrophages and *Candida* and *Aspergillus* species in *in vitro* studies (Nessa *et al.*, 1997b), indicating that production of ROI is a general mechanism of intracellular killing employed by macrophages. Cryptococcal strains lacking proteins (e.g., Aox1 and Skn7) that protect against reactive oxygen species inside macrophages, show reduced intracellular survival of *C. neoformans* and thus reduced virulence in animal models of infection (Akhter *et al.*, 2003; Coenjaerts *et al.*, 2006). RNI, produced by several mammalian cells, are also powerful antimicrobial molecules against intracellular *C. neoformans*. Nitric oxide, one of the key RNI molecules, is produced by macrophages through the action of inducible nitric oxide synthase on L-arginine (Tripathi *et al.*, 2007) and acts to suppress cryptococcal growth (Tohyama *et al.*, 1996). In addition, NK cells promote anticryptococcal activity of macrophages through enhancing nitric oxide activity (Kawakami *et al.*, 2000). Resistance to oxygen- and nitrogen-derived oxidants has been found to be a major factor in determining the outcome of infection with *C. neoformans* (Xie *et al.*, 1997), implying the importance of ROI and RNI in intracellular killing by macrophages.

In the presence of intact T cell function, macrophages also often form a histiocytic ring around *C. neoformans* cells and may fuse to form giant multinucleated cells in order to engulf heavily encapsulated yeast. This is called granuloma formation and has been demonstrated to be the most effective host response to localize the infection and prevent dissemination (Casadevall and Perfect, 1998; Hill, 1992). Furthermore, resolution of infection, when it occurs, almost always follows granuloma formation. For instance, intratracheal infection of rats with *C. neoformans* was found to elicit a strong granulomatous response and resulted in minimal or no dissemination (Goldman *et al.*, 1994, 1996; Kobayashi *et al.*, 2001). Granulomatous inflammation is more likely to be reported in non-HIV-associated cryptococcosis (Lee *et al.*, 1996; Mohanty *et al.*, 2003; Shibuya *et al.*, 2005), and there is evidence that a strong granulomatous response is dependent on intact T cell function (Clemons *et al.*, 1996; Hill, 1992), indicating a mechanism by which abnormalities of cell-mediated immunity can translate into poor inflammatory responses.

The fungistatic activity of macrophages can be enhanced by the presence of antibody. For example, antibody against capsular GXM seems to promote nitric oxide production in macrophages (Rivera *et al.*, 2002). Antibody-treated mice have been shown to have a more intense granulomatous

response than control mice, further supporting the concept that macrophage killing is enhanced in the presence of antibody (Casadevall and Perfect, 1998).

Nevertheless, a small number of cryptococci are able to survive and remain latent inside macrophages in immunocompetent individuals, despite the presence of Th1 cytokines and antibody. This latency is probably due to a combination of frequent lateral transfer events, the presence of cryptococcal anti-ROI/RNI factors and virulence factors (such as capsule, melanin, *AOX1*, *SOD1*, *CCP1*, *ISC1*, and *SKN7* (Akhter *et al.*, 2003; Alvarez and Casadevall, 2007; Coenjaerts *et al.*, 2006; Cox *et al.*, 2003; Giles *et al.*, 2005; Liu *et al.*, 2006; Ma *et al.*, 2007; Missall *et al.*, 2004; Zaragoza *et al.*, 2008)), rapid changes in virulence mediated by phenotypic switching and Th2-polarised responses later in infection to avoid tissue damages caused by the early Th1 response (Kawakami, 2004; Mednick *et al.*, 2003). This latent population is then able to trigger cryptococcosis later on in life when the host immune system becomes compromised (Garcia-Hermoso *et al.*, 1999).

2. Direct antifungal effects of T lymphocytes

Much evidence suggests that NK cells and T lymphocytes function as both regulators (by secreting cytokines, e.g., CD4+ T helper cell) and effectors (cytotoxic cells) during the immune response against *C. neoformans*. Hence, direct inhibition of cryptococcal cells by these host cells may be another important means of host defense against *C. neoformans*. Early studies by Levitz *et al.* demonstrated the competence of freshly isolated human CD4+, CD8+ lymphocytes, and CD16/56+ NK cells (but not B cells) to directly bind and inhibit the growth of *C. neoformans* in the absence of MHC restriction (Levitz and Dupont, 1993; Levitz *et al.*, 1994). These findings are in agreement with several previous studies (Hidore *et al.*, 1991; Murphy *et al.*, 1991, 1993; Nabavi and Murphy, 1985). Recent studies have improved our understanding of the underlying detailed mechanisms. These studies found that direct anticryptococcal activities of CD4+ and CD8+ cytotoxic cells are dependent on expression of granulysin after activation by CD4+ T helper cell (or IL-2/IL-15, which can substitute T cell helper) (Ma *et al.*, 2002; Zheng *et al.*, 2007), whereas NK cells used perforin instead (Ma *et al.*, 2004) (Fig. 5.4). Granulysin, a novel host defense protein, is able to increase membrane permeability of bacteria and fungi, and thus cause osmotic lysis (Ernst *et al.*, 2000). Granulysin expression in CD4+ cytotoxic T cells is controlled by PI3K and STAT5 signaling pathways through promoting IL-2Rβ induction (Zheng *et al.*, 2008). CD4+ cytotoxic T cells from HIV patients fail to induce granulysin expression due to defective PI3K and STAT5 pathways, resulting in inefficient killing (and growth inhibition) of *C. neoformans* (Zheng *et al.*, 2007). Similarly, CD8+ T cells express granulysin in the presence of IL-15 and CD4+ T cells, and the upregulation of granulysin correlated with the acquisition of

anticryptococcal activity (Ma *et al.*, 2002). Perforin, stored in secretory vesicles (granules) of T lymphocytes and NK cells, is another pore-forming effector molecule that acts by inserting into the target cell's plasma membrane, triggering lysis (Voskoboinik *et al.*, 2006). Perforin-mediated anticryptococcal killing is essential for NK cells, although both granulysin and perforin are constitutively expressed by this cell type (Ma *et al.*, 2004), and the killing is accompanied by activation of PI3K-ERK1/2 signaling pathway (Wiseman *et al.*, 2007).

C. Conclusion

C. neoformans is a facultative intracellular pathogen, capable of living both outside and inside cells. The current model of cryptococcal infection is based on five steps: internalization, dormancy, reactivation, proliferation, and dissemination (Fig. 5.3). In the initiation stage, *C. neoformans* interacts with and is internalized by lung phagocytes (mainly macrophages). Normally, in an immunocompetent individual, a T-cell mediated immune response (driven especially by CD4+ cells) develops. This leads to activation of macrophages via cytokine release and granuloma formation, resulting in either destruction of the intracellular fungus or containment in a latent state, which is probably maintained by lateral transfer of the yeast between host cells. Direct antifungal activity of lymphocytes also improves the host defense. Subsequently, when the individual becomes immunocompromised, *C. neoformans* can start proliferating inside the macrophage, followed by macrophage lysis and release of *C. neoformans*. The released organism can then enter other phagocytes, causing dissemination and increased proliferation. Long-term intracellular growth leads to enlargement of the capsule, which probably sequesters available complement proteins. The unopsonised organisms are poorly recognized by phagocytes and thus establish extracellular dominance. During prolonged infections, the yeast population can undergo microevolution, which results in both phenotypic and genotypic changes in order to be better adapted to local organs or environments (Lortholary *et al.*, 1999). The identification of genes and factors that contribute to either extra or intracellular proliferation of this pathogen may lead to the development of novel prevention and treatment strategies for cryptococcosis.

V. CURRENT UNDERSTANDING ON HOW *CRYPTOCOCCUS* CROSSES THE BLOOD–BRAIN BARRIER

Cryptococcal meningoencephalitis develops as a result of hematogenous dissemination of inhaled *Cryptococcus* from the lung to the brain. In order to penetrate into the brain, the yeast must cross the endothelium of the

blood–brain barrier, which is composed of brain microvascular endothelial cells connected by tight junctions between the cells (Rubin and Staddon, 1999).

Although the mechanisms of entry into the CNS for the majority of meningoencephalitis-causing microorganisms are not clear, three potential models have been described. Pathogens may cross the blood–brain barrier paracellularly (e.g., *Trypanosoma* species) (Grab *et al.*, 2004), transcellularly (e.g., *Streptococcus pneumoniae*) (Ring *et al.*, 1998), and by means of infected immune cells (Trojan horse mechanism, e.g., HIV) (Dallasta *et al.*, 1999; Erlander, 1995). In the case of *Cryptococcus*, several lines of evidence support the hypothesis that the yeast crosses the blood–brain barrier transcellularly. In 1995, an *in vitro* study by Ibrahim *et al.* demonstrated that *C. neoformans* (especially acapsular strains) was able to adhere to and then be internalized by endothelial cells, subsequently causing damage to endothelial cells. Furthermore, they found that internalization required the presence of a heat-labile serum factor, which could be one of the components of the classical complement pathway (Ibrahim *et al.*, 1995). Chretien and colleagues then reported for the first time that *in vivo*, *Cryptococcus* was phagocytosed by endothelial cells of leptomeningeal capillaries (Chretien *et al.*, 2002). Subsequently, Chen *et al.* and Chang *et al.* further proposed that *C. neoformans* was capable of altering the cytoskeletal morphology of human brain microvascular endothelial cell (HBMEC) through the ROCK-LIMK-cofilin pathway, and crossing the HBMEC layer transcellularly without affecting the monolayer integrity. Importantly, the virulence factor Skn7 has been demonstrated to coregulate the adaptive strategy of *Cryptococcus*, allowing intraphagocytic survival by conferring resistance to phagolysosomal killing in endothelial cells (Coenjaerts *et al.*, 2006). However, compared to *C. albicans*, the efficiency of adhesion and invasion is low (Chang *et al.*, 2004; Chen *et al.*, 2003; Jong *et al.*, 2001).

Several studies also demonstrated indirectly that phagocytes might act as a means of allowing *Cryptococcus* to invade the brain (Trojan horse mechanism). For example, microscopy of the leptomeninges of a mouse with severe meningoencephalitis showed cryptococci internalized both within mononuclear cells circulating within meningeal capillaries and within host cells touching the outer membrane of the capillaries. However, it was not determined whether the latter cells were microglial cells that had phagocytosed free cryptococci in the meningeal spaces or were macrophages derived from *C. neoformans*-infected monocytes (Chretien *et al.*, 2002). Furthermore, although direct transfer of *C. neoformans* from infected phagocytes to endothelial cells has not been demonstrated, such events have been observed between two macrophages (Alvarez and Casadevall, 2007; Ma *et al.*, 2007). When traveling throughout the host circulatory and lymphatic system, macrophage cells interact intimately with one another and with other cell types through transient contacts. It is possible that

internalized *C. neoformans* may use such transient contact in order to cross the blood–brain barrier by direct cell-to-cell spread from adherent infected macrophages to microvascular endothelial cells. In fact, spreading from macrophages to other cell types during dissemination has been demonstrated for other pathogens *in vitro*. For instance, *L. monocytogenes* can infect neurons by cell-to-cell spread from adherent macrophages, a more efficient process than direct invasion of neurons (Dramsi *et al.*, 1998). Intriguingly, cell-to-cell spread of bacteria from adherent infected phagocytes to endothelial cells of the CNS has also been reported (Drevets and Leenen, 2000) and it will clearly be of the great interest to investigate whether a similar process may occur during cryptococcosis.

Since cryptococcosis is very common in HIV-infected patients, it is not implausible to suspect that the presence of HIV may enhance cryptococcal entry into the CNS. Numerous studies have demonstrated that HIV is able to cause damage to the endothelial cell layer and thus facilitates other microorganisms to enter and infect the CNS (Dallasta *et al.*, 1999; Ricardo-Dukelow *et al.*, 2007; Toborek *et al.*, 2005). The interaction between HIV and *C. neoformans* has not been well studied, but a recent study reported an interesting interplay between the yeast and the HIV-1 protein gp41. Jong *et al.* demonstrated that the binding of *C. neoformans* to HBMEC could be enhanced by HIV-1 gp41 *in vitro* and also in a murine model. Therefore, they speculated that HIV-1 gp41 may play a role as a trans-predilection factor for *C. neoformans* invasion, thus resulting in a deteriorating meningoencephalitis in HIV-infected patients (Jong *et al.*, 2007).

In summary, there are three possible ways by which *Cryptococcus* can cross the blood–brain barrier and enter the CNS (summarized in Fig. 5.5). They are: (1) direct transcellular crossing, during which free cryptococci are internalized by endothelial cells and exit through the abluminal surface of the cells; (2) Trojan Horse mechanism, which proposes that cryptococci are engulfed by phagocytic cells at an early stage of infection and then trafficked by these host cells into the CNS; and (3) direct transfer from infected phagocytes into endothelial cells followed by exit at the abluminal surface of the cells. Moreover, the presence of HIV-1 may facilitate *Cryptococcus* to cross the blood–brain barrier by destroying the integrity of blood–brain barrier and/or by acting as a trans-predilection factor.

VI. ANIMAL MODELS

Analysis of molecular mechanisms by which a pathogen interacts with the human host is most commonly performed using a mammalian model of infection. However, several virulence-related genes previously shown to be involved in mammalian infection with *C. neoformans* have also been shown to play a role in the interaction of the pathogen with invertebrates,

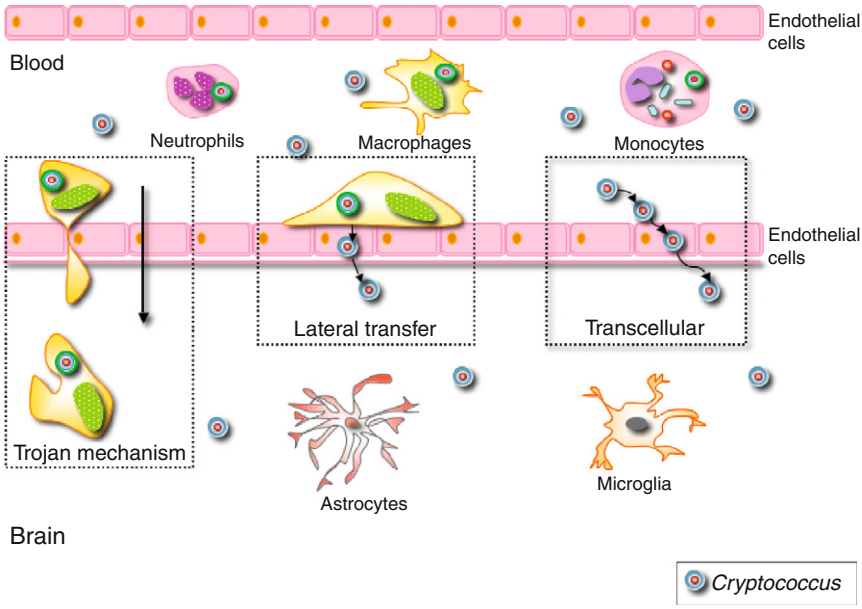

FIGURE 5.5 Possible routes for cryptococci to cross the blood–brain barrier: (1) Trojan horse mechanism; (2) Lateral transfer; and (3) Transcellular crossing.

including *C. elegans* (Mylonakis *et al.*, 2002; Tang *et al.*, 2005), *D. discoideum* (Steenbergen *et al.*, 2003), *D. melanogaster* (Apidianakis *et al.*, 2004), and *Galleria mellonella* (Mylonakis *et al.*, 2005). These organisms show promise for the study of *C. neoformans* pathogenesis, since they allow for high-throughput screening to identify novel loci related to mammalian pathogenesis (Tang *et al.*, 2005). Moreover, the interaction of these model organisms with *C. neoformans* suggest that the virulence factors important in human disease pathogenesis probably evolved through interactions with simpler organisms (discussed in Section II).

The routine mammalian systems used to study cryptococcal infection include mouse, rat, rabbit, and guinea pig (Barchiesi *et al.*, 2005; Clancy *et al.*, 2006; Cox *et al.*, 2000; da Silva *et al.*, 2006; Fries *et al.*, 2005; Perfect *et al.*, 1980; Torres-Rodriguez *et al.*, 2003; Zhou *et al.*, 2007). These models have been reviewed in detail recently (Carroll *et al.*, 2007), so they are not discussed here. It is important to point out, however, that although each model is robust and provides valuable insights into understanding of the host-pathogen interaction, there are many disagreements both between and within models. For instance, genes which are important for the virulence composite in one model are not in another (Cox *et al.*, 2000). Rats and mice are considered resistant and susceptible hosts respectively, partly due to difference in alveolar macrophage function in the two

species (Shao *et al.*, 2005). Within the murine model, there are several commonly used mouse laboratory strains including BALB/c, C57BL/6J, A/J, CBA/J, and DBA/J, and there is substantial variation in susceptibility to *C. neoformans* among these mouse strains (Huffnagle *et al.*, 1998). For instance, in an intranasal infection model with the same cryptococcal strains (WM276 and NIH444), WM276 was found to be more virulent in A/J mice (Fraser *et al.*, 2005), whereas the opposite was observed with BALB/c mice (Chaturvedi *et al.*, 2005). Early studies showed that the different resistance/susceptibility patterns of cryptococcal infection in various strains of mice were caused by differences in host factors (including genetic background, gender, and age) and were linked to differential phenotypes of the inflammatory responses (Hoag *et al.*, 1995; Huffnagle *et al.*, 1998; Lortholary *et al.*, 2002). A recent study exploring the immunological basis for differences in the susceptibility of BALB/c and CBA/J strains to *C. neoformans* infection demonstrated that the outcome of infection was also dependent on the route of infection: BALB/c mice are more resistant when infected intranasally, but both strains were equally susceptible when infected intravenously and, moreover, BALB/c mice were slightly more susceptible at higher intravenous infection doses. In addition, during intranasal infection, the resistance of BALB/c mice towards infection was associated with a stronger Th1 response (e.g., increased IL-12 but decreased IL-10), higher accumulation of CD4+ and CD8+ T cells in the lung and elevated antibody production during the early stage of infection. This may lead to the difference in macrophage activation profile, since significant differences in intracellular replication were observed between *C. neoformans* ingested by BALB/c versus CBA/J alveolar macrophages (Zaragoza *et al.*, 2007). Similarly, Chen *et al.* showed that BALB/c mice cleared infected *C. neoformans* more effectively compared to C57BL/6 mice, as a result of a protective Th1 response and greater numbers of CD4+ cells in the lungs following infection. Furthermore, they demonstrated that first-generation hybrid mice, unlike C57BL/6 mice, maintained the ability to clear cryptococci from the lungs, although their clearance was slower than that of BALB/c mice. Detailed analysis revealed F1 resistance was due to the inheritance of the propensity for a Th1 versus Th2 bias of the immune responses, but susceptibility versus resistance to *C. neoformans* infection was inherited in a complex fashion (Chen *et al.*, 2008a).

VII. PERSPECTIVES

The AIDS pandemic has resulted in a new wave of research on *C. neoformans* over the last 30 years, and our understanding of the biology of this pathogen has improved dramatically. As a result of technical improvements, such as

the development of three transformation techniques (electroporation (Chang *et al.*, 1996; Edman and Kwon-Chung, 1990), biolistic transformation (Alspaugh *et al.*, 1997; Odom *et al.*, 1997; Toffaletti *et al.*, 1993), and *Agrobacterium-mediated* transformation (McClelland *et al.*, 2005)) and the recent completion of several whole-genome sequences, many genes responsible for virulence in *C. neoformans* during infection have been identified and the mutants verified in robust animal models. Its clinical significance and well-defined virulence factors, along with advanced genome-wide analysis tools, have made *C. neoformans* an organism of choice for the study of fungal pathogenesis in general.

However, although the clinical management of cryptococcosis is possible, the morbidity and mortality remain high, and a critical challenge will be to develop novel treatments based upon advances in genomics, proteomics, and metabolomics. This requires a better understanding of host-pathogen interplay, and of the communication between the innate and adaptive immune response at the molecular level. The task for the future is to translate our growing biological understanding of this organism into real improvements in both therapeutic antifungals and preventative vaccines.

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