SUPPLEMENT ARTICLE

Host Defenses Against Zygomycetes

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Mucormycosis is a devastating disease and can occur in patients with a variety of risk factors, the most important of which are immunosuppression, anatomic barrier breakdown, iron overload, and hyperglycemia/acidosis. Similarly to what occurs with *Aspergillus*, the host stimulates an innate immune response against the challenging sporangiospores and invading hyphae of Zygomycetes. This article discusses the host defense to different Zygomycetes, its augmentation, and its subsequent impact on the outcome of mucormycosis.

Infections caused by Zygomycetes have been reported with increasing frequency in recent years [1–4] and still have unacceptably high morbidity and mortality. A number of risk factors are known to be associated with invasive mucormycosis, including hematologic malignancies and transplantation, iron overload, diabetes and ketoacidosis, birth prematurity, and possibly prior exposure to certain *Aspergillus* active antifungal agents [5, 6]. Among clinically relevant Zygomycetes, the most frequent species are *Rhizopus oryzae* and *Rhizopus microsporus*. *Cunninghamella bertholetiae* is less commonly encountered but is associated with more severe infections. By comparison, *Absidia* (*Mycocladus* or *Lichtheimia*) *corymbifera* is a less virulent and infrequent pathogen. This article discusses the host defense to different Zygomycetes and its augmentation and subsequent impact on outcome of mucormycosis.

ENTRY OF ZYGOMYCETES

The most frequent portal of entry for Zygomycetes is the respiratory tract. Sporangiospores released by sporangiophores with 3–11-μm diameters are easily aerosolized and are readily dispersed throughout the environment. They are inhaled and are continuously cleared by mucociliary transport [7]. Thus, the first barriers are ciliated bronchial cells and their mucus that together with cough lead sporangiospores away from alveoli. The second line of defense is pulmonary alveolar macrophages that can phagocytose and destroy sporangiospores before they terminate to become hyphae.

Sporangiospores may also infect patients through the gastrointestinal tract or by direct inoculation through sites of skin breakdown (trauma, burn) or exit sites of central venous catheters [8]. Thus, intact skin and mucosal surfaces are another important barrier against mucormycosis. As sporangiospores enter and infect various parts of the host, they may challenge different innate immune cells (ie, microglial, Langherhans, or Kupffer cells) and lead to variable host responses. The function of these cells is both to damage the invading organisms and to regulate innate immune response through secretion of cytokines and chemokines.

In addition to immunosuppression and physical barrier breakdown, a number of other risk factors are involved in the establishment and dissemination of mucormycosis. Hyperglycemia and low pH are among the most important factors. For several reasons, the detailed analysis of which is beyond the scope of this review, these conditions help Zygomycetes to evade host defenses. Furthermore, iron overload is an important growth factor of these organisms. In this regard, deferoxamine therapy has been associated with mucormycosis cases

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due to freeing iron from transferrin. By keeping iron steadily away from the organism, the newer iron chelator deferasirox not only protects host from infection but may play a therapeutic role against mucormycosis as well. However, this role has not been proved in a recently completed, small randomized study and requires further study.

HOST IMMUNE RESPONSE

The main line of innate host response to filamentous fungi consists of circulating polymorphonuclear neutrophils (PMNs), mononuclear cells (MNCs), and macrophages, particularly pulmonary alveolar macrophages. A number of pathogen-associated molecular patterns on the surface of fungal spores or hyphae bind to pattern-recognition receptors of phagocytes and generate the molecular signal for the proinflammatory and antifungal activities of phagocytes. Toll-like receptors (TLRs) together with other receptors play a critical role in the recognition of the fungal patterns and the intracytoplasmic transduction of the signals [9].

Phagocytes are capable of damaging fungal spores and hyphae through oxygen-dependent and oxygen-independent mechanisms. The oxygen-dependent mechanisms consist of a series of reactions starting with the production of superoxide anion (O_2^-), which is dismutated into hydrogen peroxide. Myeloperoxidase then catalyzes the conversion of hydrogen peroxide and halides to generate hypohalides, such as hypochlorite and chloramines, which exert potent antifungal activities [10, 11]. Cationic peptides (defensins and cathelicidins) are part of the oxygen-independent pathway of phagocytic cells [12–14]. A variety of cytokines, chemokines, and growth factors play an important role in the host response against filamentous fungi [15]. Most of these in vitro studies have been performed with immune cells obtained from healthy volunteers, because their primary objective was to elucidate the basic properties of normal host response against fungal pathogens. However, because effector cell activity from immunocompromised patients may vary greatly as a function of different types and degrees of immunosuppression, careful consideration of these variables is important in treating these host populations.

Little had been known about host immune responses against Zygomycetes until recently. Historically, three decades ago Diamond and Clark [16] showed that *Rhizopus* hyphae are damaged by healthy human leukocytes. Fifteen years later, Liles et al [17] showed that *R. oryzae* is killed by healthy human neutrophils (PMNs) much less than *Aspergillus fumigatus*. More recently, Warris et al [18] showed that *R. oryzae* stimulates healthy human MNCs to release more interleukin (IL) 6 and tumor necrosis factor (TNF) α than all *Aspergillus* species including *A. fumigatus*, suggesting a more pronounced proinflammatory response to this fungus than to *Aspergillus*.

Because Zygomycetes share some common features with *A. fumigatus* in terms of using the same portals of entry into human body, having similar clinical features and histopathology, as well as causing therapy-refractory and life-threatening infections, especially in immunocompromised patients, reasonably analogous immune mechanisms may apply to the 2 fungal pathogens. However, key differences between *Aspergillus* and Zygomycetes are that the latter are less common opportunistic pathogens and can cause disease in patients with no immunosuppression more frequently than *Aspergillus* spp.

Whereas *A. fumigatus* is recognized by both TLR2 and TLR4 [19], hyphae of *R. oryzae* are recognized only by TLR2 [20]. The significance of Zygomycetes recognition for the development of infection has been demonstrated in a mucormycosis model of *Drosophila melanogaster* flies [21]. In contrast to most other fungi, which are nonpathogenic for *Drosophila* (eg, *A. fumigatus*), Zygomycetes rapidly infect and kill wild-type flies [21]. Phagocytic cells from wild-type *Drosophila* display decreased phagocytosis and cause less hyphal damage to Zygomycetes compared with *A. fumigatus* [21]. Furthermore, phagocytosis-defective Toll-deficient flies display an even higher susceptibility to Zygomycetes. These results taken together suggest that Toll-dependent and Toll-independent innate immune responses to Zygomycetes and subsequently substantial differences in handling zygomycosis from aspergillosis by host defense

Recognition of fungi is followed by up- or down-regulation of a great number of relevant genes. The genes that are regulated in response to *R. oryzae* are fewer than to *A. fumigatus*. Using microarray technology, among 6125 genes differentially expressed, 348 (5.7%) of the genes of MNCs from healthy volunteers were found to be significantly modulated by both *A. fumigatus* and *R. oryzae*. Biologic categories enriched in these 348 differentially expressed genes included proteins with cytokine activity and the inhibitor of κB/nuclear factor-κB pathway. *A. fumigatus* and *R. oryzae* also induced 4287 and 1142 genes, respectively, that were not shared between the 2 pathogens. *A. fumigatus* conidia induced a 4-fold greater number of differentially expressed genes in MNCs than *R. oryzae* [22]. Furthermore, in the *Drosophila* model, *R. oryzae* down-regulated genes related to immune response compared with *A. fumigatus* [21]. The genes that are differentially regulated by the 2 fungal pathogens may provide pathways by which innate immunity responds to these different fungal infections.

Whereas the above studies have been performed in whole-genome level of human or fly cells, other studies have measured protein release of individual cytokines important in the antifungal host response. Among secreted cytokines, *R. oryzae* has been shown to induce significantly more TNF-α and IL-6 release by healthy human MNCs than do *Aspergillus* spp. in vitro (Table 1). This could be attributed to the specific composition of the cell wall of *R. oryzae,* which contains more chitin than

Table 1. Summarized Comparison of Host-Pathogen Interactions Between *Aspergillus* and Various Zygomycetes

Function	*Aspergillus fumigatus*	*Rhizopus oryzae*	*Rhizopus microsporus*	*Absidia corymbifera*	*Cunninghamella bertholetiae*
PAMP recognition	TLR2 and TLR4	TLR2	ND	ND	ND
MNC genes regulated only by organism, No.	4287	1142	ND	ND	ND
IL-6 secretion	+	+++	ND	ND	ND
IL-8 secretion	ND	++	++	++	+
TNF-α secretion	+	+++	+	+	++
Phagocytosis	++	+	ND	ND	ND
O_2^- production	++	+	+	++	ND
Hyphal damage	+++	++	++	+++	+

Plus signs denote various degrees of activity.

Abbreviations: IL, interleukin; MNC, monocytes; ND, no data; O_2^-, superoxide anion; PAMP, pathogen-associated molecular pattern; TLR, Toll-like receptor; TNF, tumor necrosis factor.

in other fungi [23], and this may be a more potent stimulatory pathogen-associated molecular pattern to phagocytes [18].

Because both *Aspergillus* spp. and Zygomycetes enter through the respiratory tract most frequently, the identity and expression of locally released cytokines are important data. During inhalation of *A. fumigatus* by immunocompetent mice, IL-18, IL-12, and TNF-α were found to be released. These cytokines exert their modulatory effects on both intrapulmonary immunoregulatory pathways and on effector cells that inhibit growth of *A. fumigatus* [24]. Similar studies with Zygomycetes have not been performed.

In addition, healthy human phagocytes ingest *R. oryzae* sporangiospores less efficiently than *A. fumigatus* conidia [22]. Likewise, they damage hyphae of *R. oryzae* less efficiently than *A. fumigatus* [20]. Among different species of Zygomycetes, there are differences in hyphal damage. For example, *R. oryzae* and *R. microsporus* are equally susceptible to PMNs, whereas *A. corymbifera*, a less virulent species, is damaged much more by phagocytes [25]. Likewise, PMN oxidative burst in response to hyphae was significantly lower in response to *Rhizopus* spp. than in response to *A. corymbifera* [25]. Hydrocortisone has been found to exert a suppressive effect on PMN-induced hyphal damage of *R. oryzae* at concentrations ≥3 mmol/L [26]. Likewise, dexamethasone (100 μM) suppressed hyphal damage and phagocytosis of *R. oryzae* in a *D. melanogaster* embryonic phagocytic cell line [21].

NUMERICAL AUGMENTATION OF HOST DEFENSE

Both granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) increase the number of circulating PMNs by stimulating the proliferation and differentiation of myeloid progenitor cells, and they reduce the depth and duration of chemotherapy-induced neutropenia, diminishing the frequency of infections [27]. Their administration would be potentially beneficial in fighting mucormycosis. As a general principle, the use of recombinant G-CSF or GM-CSF for acceleration of recovery from neutropenia is biologically sound and supported by randomized trials. Whereas individual reports of adjuvant therapy for mucormycosis with G-CSF and GM-CSF have been published [28–30], the efficacy of recombinant cytokines in neutropenic or nonneutropenic patients with mucormycosis has not been evaluated through adequately powered randomized controlled trials. Their use in such cases should be individualized.

Granulocyte transfusions are an alternative approach for augmentation of innate phagocytic host defenses against invasive fungal infections [17, 31]. Treatment of donors with G-CSF with or without corticosteroids increases the yield of PMNs, allowing for as much as 10- to 100-fold increase in yield of transfused cells and sustained concentrations of circulating leukocytes [32–35]. At this time, efficacy data for PMN transfusion in fungal infections are limited, and potential benefits should be weighed against known complications, including respiratory distress, alloimmunization, and anaphylaxis. Nonetheless, PMN transfusions with cytokine augmentation may provide critical support to a neutropenic host with a life-threatening infection until recovery from neutropenia ensues [36]. This strategy might be worthwhile as secondary prophylaxis in selected neutropenic patients with history of or active mucormycosis, who undergo cytotoxic chemotherapy (D. Kontoyiannis, unpublished data).

FUNCTIONAL AUGMENTATION OF HOST DEFENSE

In Vitro Evidence

Immune recovery may be accelerated by treatment with G-CSF, GM-CSF, and interferon (IFN) γ. G-CSF and GM-CSF stimulate

production of PMNs and MNCs and enhance their antifungal activity [25, 37]. IFN-γ directly enhances the antifungal activity of host effector cells and induces development of T-helper 1 cell responses, which further augments innate defenses against fungi [15]. Ex vivo incubation of PMNs derived from transplant recipients with G-CSF enhances the oxidative respiratory burst against *Rhizopus* sporangiospores [37]. Likewise, GM-CSF and IFN-γ augment the antifungal activity of PMNs from healthy volunteers against *R. oryzae*, *R. microsporus*, and *A.* (*Mycocladus* or *Lichtheimia*) *corymbifera* [25]. Both PMN oxidative burst in response to hyphae and PMN-induced hyphal damage were significantly lower in response to *Rhizopus* spp. than in response to *A. corymbifera*. Incubation of PMNs with IFN-γ and GM-CSF alone or combined increased the PMN-induced hyphal damage of all 3 species. The treatment of PMNs with the combination of IFN-γ and GM-CSF significantly increased the release of TNF-α in response to *R. microsporus* and *A. corymbifera* hyphae. IFN-γ significantly reduced IL-8 release in response to all Zygomycetes. Although *Rhizopus* spp. demonstrate a decreased susceptibility to the antifungal activity of human PMNs, in comparison with *A. corymbifera*, IFN-γ and GM-CSF augment the hyphal damage of all 3 Zygomycetes, suggesting a role for IFN-γ and GM-CSF in the management of invasive mucormycosis.

To assess the ability of G-CSF administered in vivo to enhance PMN activity against opportunistic fungal pathogens, the antifungal activity levels of PMNs obtained from normal human volunteers before and after G-CSF administration were compared. G-CSF significantly enhanced PMN-mediated killing of *A. fumigatus* and *R. oryzae* by 4-fold and 15-fold, respectively, in contrast to *Candida albicans*. G-CSF primed PMNs in vivo for sustained respiratory burst in response to extracts of *Candida, Aspergillus*, and *Rhizopus* organisms. These data suggested that G-CSF may have a possible therapeutic role as a biologic response-modifying agent during opportunistic fungal infection [17]. It is not known whether a similar enhancement occurs with immunosuppressed phagocytes.

In Vivo Evidence

G-CSF, GM-CSF, or IFN-γ has been administered together with antifungal agents for the treatment of experimental mucormycosis. GM-CSF enhanced the efficacy of liposomal amphotericin B (LAMB) in a neutropenic murine model of disseminated infection by *R. oryzae*, significantly prolonging survival and reducing tissue burden. The use of IFN-γ alone was ineffective, and IFN-γ combined with LAMB did not improve the results obtained with LAMB alone [38].

In another neutropenic model of murine mucormycosis by *R. microsporus*, posaconazole (PSC) monotherapy and PSC combined with G-CSF were studied. PSC and combination therapy significantly reduced the fungal burden in the kidneys, whereas a moderate reduction of fungal burden was observed for the rest of the organs. Combining G-CSF with PSC did not substantially affect the antifungal efficacy of PSC [39].

Immunopharmacology

Certain antifungal agents exert immunomodulatory effects on host innate immunity against *Candida* and *Aspergillus* [40]. A potential up-regulation of the host response to Zygomycetes would be clinically important. The antifungal activity of PMNs from healthy volunteers in combination with LAMB, amphotericin B lipid complex, voriconazole (VRC), and PSC against *R. oryzae* and *R. microsporus* were studied and compared with the less pathogenic *A. corymbifera*. Whereas *A. corymbifera* was more susceptible to PMNs than the 2 *Rhizopus* species, *R. microsporus* appeared to be the most susceptible to combined effects of amphotericin B formulations with PMNs. LAMB exhibited synergistic activity with PMNs in inducing hyphal damage to *R. microsporus* but not to the other fungi. In contrast, amphotericin B lipid complex exhibited synergistic or additive activity with PMNs against all 3 fungi. Among triazoles, VRC but not PSC exhibited additive effect with PMNs against *R. microsporus* [41]. These in vitro results suggest that there are Zygomycetes-specific and antifungal agent–specific (even for agents belonging to the same class) differences in immunopharmacologic effects and might support the concomitant administration of antifungal agents and PMN transfusions to persistently neutropenic patients with invasive mucormycosis.

Zygomycetes

Among Zygomycetes, *C. bertholletiae* infection occurs less frequently as an etiologic agent of human disease but causes more aggressive, refractory, and fatal infections despite antifungal therapy. The differential innate host response against *Cunninghamella* and other Zygomycetes in the presence of antifungal agents, the activity of healthy human PMNs alone or in combination with caspofungin, PSC, and VRC against hyphae of *R. oryzae*, *R. microsporus*, and *C. bertholletiae* were studied. *C. bertholletiae* was more resistant to PMN-induced hyphal damage than either *Rhizopus* species. The hyphal damage caused by caspofungin at 0.1 mg/L or PSC and VRC at 0.5 mg/L with *C. bertholletiae* and *R. oryzae* and by caspofungin against *R. microsporus* ranged from 18% to 29%. The PMN-induced hyphal damage was not modulated by combination with antifungal agents. *C. bertholletiae* induced significantly decreased IL-8 but increased TNF-α release from PMNs, compared with both *Rhizopus* species. No IL-6 was released from PMNs exposed to the 3 Zygomycetes [42].

Lamaris et al [43] found that preexposure to caspofungin enhances PMN-mediated hyphal damage of a number of *Aspergillus* and non-*Aspergillus* filamentous fungi, including *R. oryzae*. In particular, previous exposure of *R. oryzae* to high

concentration of caspofungin (32 mg/L) before the addition of PMNs induced β-glucan unmasking and significantly increased PMN-induced hyphal damage. These findings support the immunopharmacologic mode of action of echinocandins.

CONCLUSIONS AND FUTURE DIRECTIONS

Phagocytes recognize Zygomycetes mainly by TLR2 and respond by releasing cytokines and causing fungal damage. Although cytokines enhance antifungal activity of phagocytes in vitro, such augmentation is yet to be proved convincingly in vivo. Echinocandins and amphotericin B formulations exert distinct immunomodulatory activity on phagocytes.

Studies on the role of endogenous cytokines in host defense to mucormycosis should be an important direction of future preclinical research because they may ultimately provide new strategies for adjunct therapy in immunocompromised patients. A better understanding of the normal host response to these pathogens should establish objective targets for immune augmentation in immunocompromised hosts. It is especially important to investigate to what extent, if any, there exists intergenus and interspecies variability in host-fungus interaction. Knowledge of the virulence degree of these species in immunocompetent as well as immunocompromised subjects and of their response to the host innate immune response and antifungal agents could be useful in the selection of appropriate treatment of the severe and refractory infections caused by Zygomycetes. Innovative in vivo and clinical trial designs will be needed to translate these advances from bench to bedside. Because host gene expression differs between *A. fumigatus* and *R. oryzae*, cytokine expression profiling through future easy-to-perform microarray technology may assist in earlier diagnosis and monitoring response to antifungal therapy of mucormycosis.

Notes

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