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Update on mucormycosis pathogenesis

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Abstract

Purpose of review—Mucormycosis is an increasingly common fungal infection with unacceptably high mortality. The recent sequencing genome projects of Mucorales and the development of gene manipulation have enabled significant advances in understanding the pathogenesis of mucormycosis. Therefore, we review the pathogenesis of mucormycosis and highlight potential development of novel diagnostic and therapeutic modalities against this lethal disease.

Recent findings—Much of the work has been focused on the role of iron uptake in the virulence of Mucorales. Additionally, host receptors and fungal ligands involved in the process of tissue invasion as well as sporangiospore size and sex loci and their contribution to virulence of Mucorales are discussed. Finally, the role of innate and adaptive immunity in protection against Mucorales and new evidence about drug-induced apoptosis in these fungi are discussed.

Summary—Recent discoveries introduce several potentially novel diagnostic and therapeutic modalities, which are likely to improve management and outcome for mucormycosis. Future preclinical and clinical research is warranted to develop these diagnostic and therapeutic strategies.

Keywords

innate/adaptive immunity; invasion; iron uptake; mucormycosis

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Epidemiology/Risk Factors

Mucormycosis is an uncommon, life-threatening infection caused by fungi belonging to the subphylum Mucormycotina, order Mucorales [1,2]. Among organisms responsible for causing mucormycosis, *Rhizopus* species are the most common cause of infection, followed by *Mucor* and *Lichtheimia* species [1,2]. Although the infection afflicts immunocompromised patients with hematologic malignancies, organ transplantation, and cancer chemotherapy, patients with uncontrolled diabetes or ketoacidosis and other forms of acidosis are uniquely susceptible to infection [3]. Also, another patient category that is uniquely predisposed to mucormycosis includes patients who are treated with deferoxamine for treating iron toxicity mainly because of renal failure [4,5]. Finally, trauma patients can also contract mucormycosis because of contamination of wounds with Mucorales. For example, the recent reports of outbreaks of mucormycosis in victime of natural disasters such as during the Joplin tornado [6] and the outbreak of mucormycosis among soldiers following combat-related injuries [7] highlight this mechanism of acquisition of severe infection by Mucorales.

The infection is characterized by rapid tissue destruction, advancement across tissue planes (Fig. 1a), pleiotropic clinical manifestations and propensity for dissemination [8–12]. Despite aggressive antifungal therapy and in selected cases extensive, disfiguring surgical debridement, the overall mortality of mucormycosis remains approximately 40% or more. In patients with hematologic malignancy or hematopoietic stem cell transplantation, mortality rates exceed 65% and 90%, respectively [8–12].

Recent data have demonstrated a notable increase in the number of reported cases of mucormycosis [10]. For example, there has been an alarming rise in the incidence of mucormycosis at major transplant centers and the number of cases over a 15 year period has more than doubled [11,12]. In fact, in high-risk patients the prevalence of mucormycosis is up to 8% in autopsied patients with leukemia [13]. A recently published population based study demonstrated a 70% increase in mucormycosis cases between 1997 and 2006 [14]. Further, data from a tertiary care center demonstrated an increase of 400% or more in mucormycosis incidence, mainly among diabetic ketoacidosis (DKA) patients, between 1991 and 2007 [15,16]. These studies are part of an explosion in the number of published studies on this devastating opportunistic fungal infection over the past decade (Fig. 1b). Owing to the rising prevalence of diabetes, cancer, and organ transplantation in the ageing US population, the number of patients at risk for this deadly infection is expected to continue to rise. Clearly, new strategies to prevent and treat mucormycosis are urgently needed. Such strategies could originate from better understanding of the pathogenesis of mucormycosis, which could enable novel therapeutic and/or diagnostic modalities. Therefore, this review will focus on the present understanding of mucormycosis pathogenesis and the possibility of translating this knowledge into novel strategies to diagnose, prevent and/or treat mucormycosis.

Pathogenesis

The recent completion of *Rhizopus delemar* 99-880 (aka *R. oryzae*) genome sequence revealed the existence of putative virulence factors that are likely to be critical for invasion and survival of the fungus in the host during infection [17]. Additionally, the epidemiology, risk factors and clinical hallmarks of the disease point to the critical role of quantitative and/or qualitative phagocytic defects, as well as the high iron and glucose concentrations, in mediating angioinvasion during mucormycosis. We will discuss and elucidate the role of these factors below with emphasis on the major recent advancements in this field. These virulence traits are summarized in Table 1 [5,17,18–23,24■,25,26,27■,28,29■].

The genome of R. oryzae

The genome of *R. oryzae* is highly repetitive with abundant transposable elements, comprising approximately 20% of the genome [17]. The entire genome underwent an ancestral whole-genome duplication (WGD) at an early point in its evolution and retained two copies of three sophisticated systems involved in energy generation and utilization associated with respiratory electron transport chains, the V-ATPase and the ubiquitin–proteasome systems. The retention of redundant protein complexes involved in energy generation could explain the versatility, including the rapid growth, of *Rhizopus* compared with other fungi. The ancient WGD, together with recent gene duplications, has led to the expansion of gene families related to pathogen virulence such as the presence of secreted aspartic protease (28 genes) and subtilase protein (23 genes) families, which are involved in substrate degradation from the host and likely to contribute to the angioinvasive nature of the disease. Also, unlike dikaryotic fungi, the cell wall of R. oryzae and other Mucorales contains a high percentage of chitin and chitosan, which are synthesized by chitin synthases (23 genes) and chitin deacetylases (34 genes), respectively. Furthermore, the duplication of the ergosterol biosynthetic pathwayencoding genes could contribute to the variable and modest susceptibility of *Rhizopus* to azole antifungal drugs. Specifically, although the ergosterol fungal biosynthesis pathway is conserved in Mucorales, about half the genes, including the major azole target, lanosterol 14a-demethylase (*ERG11*, RO3G_11790, RO3G_16595), are present in multiple copies, which might explain the variability of susceptibility of Mucorales to different azoles because acquisition of azole resistance in a clinical strain of *Candida albicans* reflected amplification of *ERG11* in a gene copy-dependent manner [30,31].

Moreover, the number of GTPase superclasses and their regulators involved in cellular processes including protein synthesis, membrane trafficking, cytoskeletal dynamics, signaling and cell division far exceed the number of genes in the other genomes (e.g. those reported for *Saccharomyces cerevisiae*, *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*). Such an increase in gene copy numbers might provide the organism an enhanced capacity for coordinating growth and metabolism under highly varied environmental conditions.

Sex loci

Data obtained from *Mucor circinelloides* and *R. oryzae* demonstrate that the sex locus that orchestrates sexual reproduction in Mucorales consists of a high mobility group (HMG) transcription factor flanked by genes encoding a triose phosphate transporter homolog and an RNA helicase [32-34]. The HMG domain proteins are designated *SexP* for the (+) and *SexM for* the (−) mating types (MAT). The sequences of the genes encoding *SexP* and *SexM* are divergent but allelic in the (+) and (−) MAT, in contrast to the idiomorphic nature of MAT in many ascomycetes and basidiomycetes encoding entirely divergent proteins [35]. A study investigating the role of the sex locus in *M. circinelloides f. lusitanicus* virulence found that spore size and shape differ between (−) and (+) MAT isolates, with (−) strains producing larger, irregularly shaped spores whereas (+) isolates are smaller in spore size [26]. The larger (−) spores were consistently more virulent in the wax moth host compared with the smaller (+) spores and were shown to germinate inside and lyse macrophages, whereas the smaller ones did not [26]. However, *sexM* deletion mutants, which were sterile, still produced larger spores that retain their virulence potential, suggesting that either the sex locus is not involved in virulence/spore size or the *sexP* allele plays an inhibitory role.

Role of iron in the virulence of mucormycosis

Risk factors for mucormycosis clearly implicate the presence of increased iron availability as a risk factor for mucormycosis. For example, patients with DKA, other forms of acidosis or those treated with the iron deferoxamine are uniquely susceptible to mucormycosis [1-3,36]. In all these patient populations, a markedly enhanced availability of iron in tissues or serum promoted aggressive invasive growth of acquired Mucorales spores [1]. For example, the excessive glycosylation of proteins such as transferrin and ferritin, owing to poorly controlled diabetes, results in decreased affinity of these proteins to bind iron, making that important element available for Mucorales [1]. Similarly, lower blood pH due to ketoacidosis and other forms of acidosis compromises the affinity of transferrin to bind iron [37]. Finally, deferoxamine is a bacterial siderophore and is actually utilized by Mucorales as a xenosiderophore for acquiring iron from the host [22]. In contrast, the modern iron chelators, such as deferiprone and deferasirox, which lack xenosiderophore activity for *Rhizopus*, induce an ironstarvation effect to the fungus and are protective in a *Drosophila* fly model [38], animal models [5,18,19] of mucormycosis and in anecdotal reports of human infection when used as an adjunctive therapy [20]. The key role of iron utilization in pathogenesis of mucormycosis is further illustrated by the fact that conditional inactivation of *R. oryzae* high-affinity iron permease gene, *FTR1*, renders the fungus nonpathogenic in mice and anti-Ftr1 protein antibodies protect against mucormycosis [21]. Genes involved in other iron uptake systems such as siderophore synthesis (rhizoferrin), and iron uptake from heme, have been identified by the genome sequencing project of *R. Oryzae*, but their role in mucormycosis pathogenesis remains unstudied. However, a recently completed phase II, double-blind, randomized, placebo-controlled trial of adjunctive deferasirox therapy failed to demonstrate a benefit of the combination regimen in patients with mucormycosis [39]. Twenty patients with proven or probable mucormycosis were randomized to treatment with liposomal amphotericin B plus deferasirox (20 mg/kg/day for 14 days) or liposomal amphotericin B plus placebo. Although reported adverse effects were similar between the

two study groups, significantly higher mortality rates were found in patients randomized to receive deferasirox at 30 (45 vs. 11%) and 90 days (82 vs. 22%, *P*=0.01). However, patients in the deferasirox arm were more likely than placebo patients to have active malignancy, neutropenia or corticosteroid therapy, and less likely to have received additional antifungals, making the results of this pilot trial less conclusive [40]. Another possibility is that adjunctive benefits of deferasirox are more pronounced in patients with underlying DKA vs. profound neutropenia [41]. Thus, conclusions regarding the use of deferasirox cannot be drawn from this small study; only a large, phase III trial, potentially enrolling only diabetic or corticosteroid-treated patients, and excluding cancer/neutropenia patients, could further elucidate the safety and efficacy of initial, adjunctive deferasirox for the treatment of mucormycosis.

Host–pathogen interactions: adhesin and invasion

As discussed above, a hallmark of mucormycosis is the extensive angioinvasion with resultant vessel thrombosis and tissue necrosis. This angioinvasion is often characterized by limited inflammatory immune response [42]. Therefore, interaction of fungal cells with the endothelium lining blood vessels represents a critical step in the progression of the disease. Mucorales can adhere to, invade and then damage endothelial cells in vitro [43]. Surprisingly, viable cells are not essential for causing host cell damage, which suggests that a toxinlike substance in Mucorales is important for early adhension events. The polyketide toxin, rhizoxin, is known to be secreted by the bacterium *Burkholderia*, which lives symbiotically with *Rhizopus* [44]. However, subsequent studies showed that this toxin has little to do with virulence of Mucorales [45,46], thereby the extensive tissue necrosis and the ability to cause host cell death independent of viability must be attributed to other Mucorales toxin-like substance(s).

A study investigating the interactions of Mucorales with human endothelial cells identified glucose-regulated protein 78 (GRP78) as a novel host receptor that selectively and specifically interacts with Mucorales during invasion and subsequent damage of host tissues [4]. Of interest, elevated glucose and iron levels upregulate GRP78 expression and promote endothelial cell invasion and damage by *R. oryzae* in a receptor-dependent manner. Importantly, anti-Grp78 serum protected DKA mice; these mice have increased expression of GRP78 and are more susceptible to mucormycosis than normal mice [25]. These results provide insights into why patients with DKA are uniquely susceptible to mucormycosis infections and point to new directions for therapeutic interventions. More recently, CotH proteins, which are present in Mucorales and absent in other pathogens, act as the fungal ligands that bind to GRP78 during invasion of endothelial cells. *R. oryzae* mutants that have reduced expression of CotH proteins are defective in invasion of endothelial cells, or Chinese hamster ovary cells overexpressing GRP78 [23], and have attenuated virulence in the DKA mouse model of mucormycosis [24■]. Interestingly, anti-CotH antibodies blocked invasion and subsequent damage to host cells in vitro and protected DKA mice from mucormycosis [24■]. The unique presence of CotH in Mucorales further explains the specific susceptibility of DKA patients to mucormycosis. Furthermore, CotH– GRP78 interaction is a promising therapeutic target for mucormycosis.

Immunopathogenesis

In immunocompetent individuals, mononuclear and polymorphonuclear phagocytes efficiently eliminate fungal spores and hyphae by oxidative and nonoxidative killing mechanisms [47–49]. Quantitative (i.e. neutropenia) or qualitative (i.e. associated with glucocorticoids, hyperglycemia and/or acidosis) defects in phagocytic cell activity permit unrestricted growth of the hyphal form and invasive infection. In particular, both hyperglycemia and acidosis are known to impair chemotaxis and the killing activity of phagocytic cells against Mucorales by impairing oxidative and nonoxidative mechanisms [50]. Likewise, corticosteroids impair migration, ingestion and phagolysosome fusion in human macrophages [47]. However, receipt of corticosteroids per se is not a predominant risk factor in the absence of underlying severe immunosuppression, and the molecular mechanisms that account for attenuated phagocytic function in patients with hyperglycemia, ketoacidosis or steroids are not characterized. For example, in contrast to *Aspergillus* spp., Mucorales rarely infects chronic granulomatous disease patients, and direct genetic evidence for a role of NADPH-dependent reactive oxygen species (ROS) production in ex-vivo killing of Mucorales by phagocytes is missing. Thus, comparative genetic studies on the mechanisms of phagocytosis, phagosome maturation and killing of *Aspergillus* vs. Mucorales by phagocytes are clearly required.

Of interest, when compared with *Aspergillus*, Mucorales hyphae display inherent resistance to killing by both *Drosophila* [51] and human phagocytes [52], which might partially account for the increased virulence of these fungi and ability to infect a broad range of hosts. Whether this resistance to killing by phagocytes is related to the release of virulent factors or is a result of differential cell wall composition is unknown.

Also, little is known about innate sensing of Mucorales spp. by immune and/or nonimmune (e.g. endothelial) cells and the role of adaptive immunity in patients with pulmonary mucormycosis. Of interest, similar to *Aspergillus*, β-glucan exposure during germinating growth of *Rhizopus* triggers dectin-1 signaling in human dendritic cells and results in robust induction of the interleukin-23/T_H17 responses [53]. Until recently, the role of adaptive immunity in mucormycosis was underappreciated because acquired T-cell deficiency is not associated with increased susceptibility to infection by the Mucorales. However, investigators recently indicated the presence of Mucorales-specific T cells in patients and healthy individuals, which enhanced effector function of professional phagocytes and could be harnessed for adoptive immunotherapy strategies and diagnosis [28,29■]. Furthermore, studies on natural killer (NK) cells demonstrated that despite the immunosuppressive effect of *R. oryzae* hyphae on NK cells, these innate immunity cells can directly damage the fungal hyphae, at least in part, by perforin [27■].

Mechanisms of Azole Activity Against Mucorales

Azoles have variable activity against Mucorales, with some drugs being ineffective against this group of fungi (e.g. fluconazole and voriconazole) and others reported to have activity (e.g. itraconazole and posaconazole), albeit to varying degree, against Mucorales. Azole activity is mainly characterized by a ‘static’ effect rather than cidality in vitro. However, emerging awareness that noxious environmental cues such as hyperthermia [54■], inhibition

of homeostatic mitochondrial function [55] and inhibition of stress response pathways such as calcineurin pathway [56] render static azole drugs to be more potent and rapidly cidal in vitro through a mechanism that involves induction of Mucorales apoptosis (Fig. 2). Apoptosis induction under the above conditions was correlated with ROS accumulation and was a caspase-dependent phenomenon. These recent data can be an area of further investigation in order to understand how Mucorales are killed by drugs *in vivo* and might inform novel modalities of mucormycosis treatment.

Implications of Pathogenesis to Diagnosis and Treatment

Despite the paucity in mucormycosis pathogenesis research compared with other fungal infections, recent major discoveries highlight the possibility of translating this knowledge into possible novel therapies and diagnostic tools urgently needed to improve the care for this lethal infection. For potential novel therapies, passive immunization targeting virulence genes of Mucorales such as iron acquisition through high affinity iron permease [21] or proteins involved in mediating host cell invasion [24■,25] has proven to be effective against experimental mucormycosis. Additionally, despite the failure of using deferasirox in the small Phase II study, animal data and anecdotal cases (mainly in diabetics with ketoacidosis) warrant the continued investigation into the development of iron chelation therapy into treating mucormycosis either by the use of another iron chelator and/or targeting a defined patient population (i.e. patients with elevated available serum iron such as DKA or other forms of acidosis) with this treatment. Equally important, research into the immunopathogenesis of mucormycosis likely will pave the road for new treatment modalities that include adoptive immunotherapy using Mucorales-specific T cells and/or NK cells [27■,29■]. Additionally, pathogenesis research has led to the possibility of using Mucorales-specific T cells as a potential diagnostic test for mucormycosis. These specific cells could be detected only in patients with mucormycosis both at diagnosis and throughout the entire course of the disease, but neither before nor for long after resolution of the infection [28]. Finally, the discovery of the CotH proteins, which are widely present in *Mucorales*, but not other organisms, raises the hope that these proteins can be exploited in the future for early detection and tracking of mucormycosis.

Conclusion

Mucormycosis is an emerging infection in immunocompromised patients, and the mortality with standard therapy remains unacceptably high. Although research into mucormycosis pathogenesis is considered to be in its infancy, major advances have been achieved recently on how Mucorales causes disease and survives in the host. Most of the work has focused on fungal iron uptake mechanisms, molecular mechanisms of adhesion/invasion, spore size and sex loci and role of innate and adaptive immunity in host defense against Mucorales. Although many putative virulence traits remain unstudied (Table 1) and represent appealing avenues for research, promising new treatments and potential diagnostic markers have been recently identified. The major hurdle in developing these promising technologies, or any new technologies, into actual therapies and/or diagnostics is the lack of interest from pharmaceutical companies in a disease that represents a small market revenue opportunity.

Consequently, novel promising treatment/diagnostic discoveries for uncommon diseases that represent a threat to the public must be supported by federally funded research mechanisms.

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Key Points

- Mucormycosis is an increasingly encountered infection with poor outcome.
- Fungal iron metabolism, host-pathogen interaction and natural killer and T cells have been identified as targets for potential novel diagnostic and/or treatment modalities.
- Continued preclinical and clinical research is warranted to develop these promising targets into patient care.

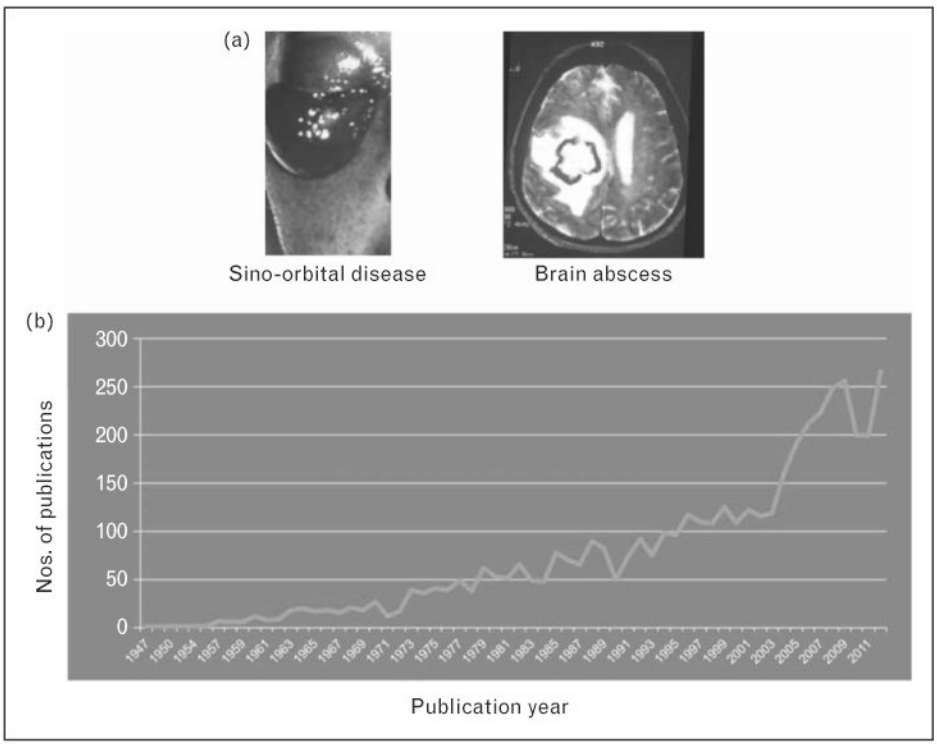

Figure 1.

(a) Mucormycosis can rapidly progress across tissue planes and does not respect anatomic boundaries. (b) Annual number of published articles on mucormycosis since 1975 (SCOPUS, accessed July, 2013).

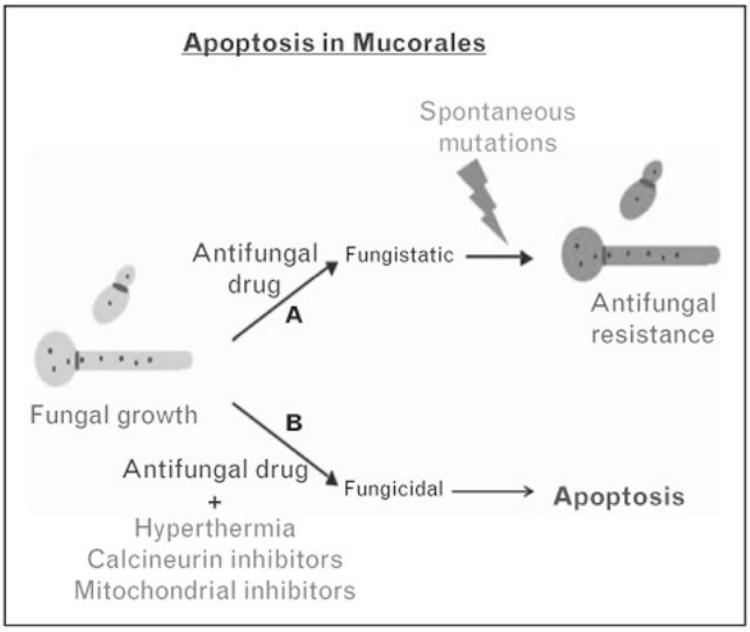

Figure 2.

A model depicting the effect of extreme noxious environmental cues on the conversion of azole effect from static to cidal *in vitro*.

Table 1

Virulence traits of Mucorales and their potential use for development of diagnostics and therapeutics for mucormycosis

Virulence traits	Function	Role in virulence/immunopathogenesis	Potential for diagnostics	Potential for therapeutics	References
Iron uptake					[18–20]
Reductase/permease	Iron uptake in iron depleted environments	Proven			[21]
Permease (FTR1)		Proven	ND		[21]
Reductases		putative	ND	ND	
Cu-oxidases		putative	ND	ND	
Siderophore	Siderophore-mediated iron uptake	Proven			[5,22]
Rhizoferrin		putative	ND	ND	
Deferoxamine		Proven	ND	ND	[5,22]
Heme oxygenase	Iron-uptake from heme	Putative	ND	ND	
CotH	Host cell invasion	Proven			[23,24■]
GRP78	Host cell receptor	Proven	ND		[25]
Proteinases	Protein lysis	Putative	ND	ND	[17]
Chitin/chitosan	Cell and structure assembly	Putative	ND	ND	[17]
Ergosterol biosynthesis	Cell membrane fluidity and azole resistance	Putative	ND	ND	[17]
Cell size		Proven	ND	ND	[26]
Sex loci	Mating	Putative	ND	ND	[26]
NK cells	Host defense	Proven	ND		[27■]
Mucorales specific T cells	Host defense	Proven			[28,29■]

ND, not determined; NK, natural killer.

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