**Human Phagocytic Cell Responses to Scedosporium apiospermum (Pseudallescheria boydii): Variable Susceptibility to Oxidative Injury**

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_Scedosporium apiospermum_ (Pseudallescheria boydii) is an emerging opportunistic filamentous fungus that causes serious infections in both immunocompetent and immunocompromised patients. To gain insight into the immunopathogenesis of infections due to _S. apiospermum_, the antifungal activities of human polymorphonuclear leukocytes (PMNs), mononuclear leukocytes (MNCs), and monocyte-derived macrophages (MDMs) against two clinical isolates of _S. apiospermum_ were evaluated. Isolate SA54A was amphotericin B resistant and was the cause of a fatal disseminated infection. Isolate SA1216 (cultured from a successfully treated localized subcutaneous infection) was susceptible to amphotericin B. MDMs exhibited similar phagocytic activities against conidia of both isolates. However, PMNs and MNCs responded differently to the hyphae of these two isolates. Serum opsonization of hyphae resulted in a higher level of superoxide anion (\(O_2^-\)) release by PMNs in response to SA54A (amphotericin B resistant) than that seen in response to SA1216 (amphotericin B susceptible; \(P < 0.001\)). Despite this increased \(O_2^-\) production, PMNs and MNCs induced less hyphal damage to SA54A than to SA1216 (\(P < 0.001\)). To investigate the potential mechanisms responsible for these differences, hyphal damage was evaluated in the presence of antifungal oxidative metabolites as well as in the presence of a series of inhibitors and scavengers of antifungal PMN function. Mannose, catalase, superoxide dismutase, dimethyl sulfoxide, and heparin had no effect on PMN-induced hyphal damage to either of the two isolates. However, azide, which inhibits PMN myeloperoxidase activity, significantly reduced hyphal damage to SA1216 (\(P < 0.001\)) but not to SA54A. Hyphae of SA1216 were slightly more susceptible to oxidative pathway products, particularly HOCl, than those of SA54A. Thus, _S. apiospermum_ is susceptible to antifungal phagocytic function to various degrees. The selective inhibitory pattern of azide with respect to hyphal damage and the parallel susceptibility to HOCl suggests an important difference in susceptibility to myeloperoxidase products that may be related to the various levels of pathogenicity and amphotericin B resistance of _S. apiospermum_.

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**Materials and Methods**

**Isolation of phagocytes.** PMNs were purified from whole blood of healthy young adult volunteers. The blood was heparinized and immediately allowed to settle with 3% dextran TS500 (Pharmacia Biotech AB, Uppsala, Sweden) in a 2:1 (vol/vol) proportion. PMNs were separated by centrifugation over a Ficoll (lymphocyte separation medium; Gibco BRL Life Technologies Ltd., Paisley, Scotland) cushion. Contaminating erythrocytes were hypotonically lysed with distilled water, and the PMN suspension was washed in Hanks' balanced salt solution.
counted on a hemocytometer. They were kept at 4°C for 18 h prior to and during the plates. Conidia were suspended in HBSS, and grown for 7 days. Conidia were harvested by gently scraping the surfaces of the PDA plates. The percentage of monocytes that consisted of RPMI 1640 supplemented with 25% human AB serum (Gibco) prior to and during the plates. Conidia were suspended in HBSS, and grown for 7 days. Conidia were harvested by gently scraping the surfaces of the PDA plates. The percentage of monocytes that consisted of RPMI 1640 supplemented with 25% human AB serum (Gibco) was dissolved in H2O and stored at −20°C. SOD (2,000 U/ml) was dissolved in H2O and stored at 4°C. DMSO (1 μM) was stored at the ambient temperature and diluted in HBSS immediately before use. DIP, SOD, SOD plus CAT, and DMSO each were added to wells containing hyphae immediately before the addition of PMNs in E:T ratios of 20:1. At the concentrations employed in these studies, none of the above-stated compounds (excluding H2O2 and HOCl) had an adverse effect on the viability of PMNs (assessed as the exclusion of trypan blue staining from the PMNs). Control wells contained inhibitors and hyphae without PMNs.

Phagocytosis assay. For early differentiation to MDMs, 1 million monocytes purified as mentioned above and resuspended in CM were placed on 18-mm-diameter sterile round glass coverslips (Fisher). The coverslips were incubated at 37°C with 5% CO2 for 2 to 3 days. After this incubation, the wells with the coverslips in them were washed with HBSS and 1 ml of a suspension containing 106 conidia/ml of CM was added. After incubation at 37°C with 5% CO2 for 1 h, the wells were again washed with warm HBSS three times. For measurement of the percentage of phagocytosis and the phagocytic index, the coverslips were removed, dried, and subjected to modified May-Grünwald-Giemsa staining. Cells having or not having phagocytosed conidia were counted microscopically as previously described (14). Conidia that remained attached after three washes were counted only if they had been ingested at a rate of more than 50%. The results were calculated according to the following formulas:

\[
\text{Percentage of phagocytosis} = \left( \frac{B + C + D + E + F}{A + B + C + D + E + F} \right) \times 100
\]

\[
\text{Phagocytic index} = \left( \frac{B + 2C + 3D + 4E + 5F}{B + C + D + E + F} \right)
\]

where the phagocytic index represents the average number of phagocytosed conidia per phagocytosing cell. A represents the number of cells that ingested no conidia, B represents the number of cells that ingested one conidium, C represents the number that ingested two conidia, D represents the number that ingested three conidia, E represents the number that ingested four conidia, and F represents the number that ingested more than four conidia.

Superoxide anion release assay. The oxidative burst evidenced by the production of O2− was measured as the SOD-inhibitable reduction of cytochrome c. A total of 200 μl of a suspension containing 1.5 × 106 conidia/ml in yeast nitrogen base medium (YNB; Difco Laboratories, Detroit, Mich.) was plated in each of 96 flat-bottom wells in cell culture clusters (Corning Inc., New York, N.Y.) and incubated at 32°C for 18 h. In the case of O2− assays with serum-opsonized hyphae, YNB was replaced by 50% pooled human serum in HBSS and incubated for 30 min at 37°C for opsonization. Pooled human serum was obtained from healthy young adult volunteers and kept at −35°C for no longer than 1 month. The plates were washed three times, and phagocytes were added at an E:T ratio of 1:1 to a final volume of 300 μl of HBSS containing 50 μM cytochrome c (Sigma) (from horse heart) with serum-opsonized or unopsonized hyphae. Basal O2− production was assessed in the absence of stimuli. After incubation at 37°C with 5% CO2 for 1 h, 200 μl was read in a spectrophotometer at a wavelength of 550 nm. The extinction coefficient of ferricytochrome c at 550 nm was taken as 29.5 × 103 M−1 cm−1.

Hyphal damage assay. Phagocyte-induced hyphal damage was assessed with XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide sodium salt; Sigma) plus coenzyme Q10 (2,3-dimethoxy-5-methyl-1,4-henzoquinone; Sigma). A total of 200 μl of a suspension containing 7.5 × 106 conidia/ml in YNB was plated in each of 96 flat-bottom wells in cell culture clusters (Corning) and incubated at 32°C for 18 h. YNB was then replaced by HBSS, and phagocytes (either PMNs or MNCs) were added at different E:T ratios. Following incubation at 37°C with 5% CO2 for 2 h, cells were lysed by washing with H2O and shaking for 5 min three times. A total of 150 μl of phosphate-buffered saline (Biochrom KG, Berlin, Germany) containing 0.25 mg of XTT/ml with 40 μg of coenzyme Q10/ml was added, and plates were again incubated at 37°C with 5% CO2 for 1 h. A total of 100 μl of aliquots from each well was transferred to a new plate and read at 450 nm with a reference wavelength of 690 nm. Antifungal activity was calculated according to the following formula: percentage of hyphal damage = (1 − [D]/[C]) × 100, where D represents the optical density of test wells and C represents the optical density of control wells with hyphae only. Each set of conditions was tested in duplicate, control wells were set up in quadruplicate, and the results were averaged.

In studies examining the antifungal activity of individual oxidative compounds (H2O2 and HOCl), conidia were germinated as described above. YNB was then replaced by HBSS, and HBSS or HBSS containing the above-stated compounds were added to the conidia. Following incubation at 37°C with 5% CO2 for 2 h, 150 μl of phosphate-buffered saline containing 0.25 mg of XTT/ml with 40 μg of coenzyme Q10/ml was added.
Similarly, the phagocytic indices were similar for the two strains.

RESULTS

Phagocytosis of conidia by MDMs. To evaluate the ability of MDMs to phagocytose conidia of S. apiospermum, MDMs were incubated with conidia at an E:T ratio of 1:1 for 1 h (Fig. 1). No differences were detected in the percentages of conidia phagocytosed by MDMs from either strain SA1216 and SA54A (Fig. 1, upper panel). Similarly, the phagocytic indices were similar for the two strains (1.3 ± 0.08 and 1.3 ± 0.05 for SA1216 and SA54A, respectively).

Oxidative burst in response to hyphae. The O$_2^-$ released by PMNs and MNCs in response to the presence of both serum-opsonized and nonopsonized hyphae of both isolates was measured (Fig. 2). Opsonization of hyphae with pooled human serum induced a significant increase in PMN O$_2^-$ production over that seen with nonopsonized hyphae (P < 0.001). Moreover, serum-opsonized hyphae of the SA54A isolate elicited significantly more O$_2^-$ from PMNs than those of SA1216 (P < 0.001).

When the oxidative burst of MNCs was evaluated (Fig. 2, lower panel), opsonization of hyphae with pooled human serum significantly increased superoxide release over that seen with nonopsonized hyphae of isolates SA1216 and SA54A (P < 0.011). There also was a tendency for the opsonized SA54A isolate to elicit more O$_2^-$ from MNCs than the SA1216 isolate (P = 0.07).

Damage of hyphae. Hyphal damage induced by PMNs (Fig. 3, upper panel) or by MNCs (Fig. 3, lower panel) was assessed at E:T ratios of 1:1, 5:1, 10:1, and 20:1. When challenged with either type of phagocytic cell, the isolates under investigation were damaged in a concentration-dependent manner, with a statistically significant linear trend (P < 0.001 for both strains and both types of phagocytes, as determined by repeated measurement of ANOVA and posttest for linear trend).

However, the SA54A isolate was more resistant to PMN- and MNC-mediated hyphal damage than the SA1216 isolate. For instance, while PMNs caused 53.7% ± 6.4% hyphal damage to SA1216 at an E:T ratio of 5:1 they caused only 26.7% ± 4.5% hyphal damage to SA54A hyphae (P < 0.001, as determined by repeated measurement using ANOVA with Dunnett’s correction for multiple comparisons used was used for comparisons between the two strains, as these data followed a normal distribution. Repeated measurement of analysis of variance (ANOVA) with Dunnett’s correction for multiple comparisons used was used for comparisons of each strain with the control. A two-sided P value of <0.05 indicated statistical significance.
Effects of superoxide pathway products. There was a direct concentration-response relationship between the oxidative metabolites (H$_2$O$_2$ and HOCl) and percent hyphal damage to both isolates of S. apiospermum. Although the SA54A isolate exhibited slightly more susceptibility to H$_2$O$_2$-mediated hyphal damage at 1.0 mM, SA1216 was more susceptible to HOCl-mediated hyphal damage at 0.1 mM (Table 1). At high concentrations, however, both strains were highly susceptible (hyphal damage > 94%) to both H$_2$O$_2$ and HOCl.

Effects of inhibitors and scavengers of PMN antifungal functions on hyphal damage. To further investigate the differences of isolates in host response, we assessed the damage to both isolates induced by PMNs at an E:T ratio of 20:1 in the presence of various inhibitors and scavengers of PMN functions, i.e., DPI, MAN, CAT, SOD, DMSO, AZI, and HEP. In these experiments, MAN, CAT, SOD (or CAT plus SOD), and HEP had no effect on PMN-induced hyphal damage to the two isolates (Table 2). In addition, DMSO (2 and 20 mM) had no significant effect on PMN-induced hyphal damage to the two isolates. None of the above-named compounds had any adverse effect on damage to hyphae of either strain. By comparison, at 5 μM DPI alone had a direct adverse effect on hyphae of both strains, causing 62.2% damage to the SA54A isolate and 41.8% to SA1216. When DPI was added to PMNs and hyphae, the level of hyphal damage was similar to that seen with DPI alone.

In contrast, AZI exerted a significant effect on PMN-induced damage to SA1216 hyphae. At 0.1 mM, a concentration previously reported to inhibit MPO activity (8, 9, 36), the presence of AZI resulted in a significant inhibition (from 79.8% ± 7.1% to 26.6% ± 3.5%) of the damage to isolate SA1216 hyphae ($P < 0.01$). The presence of AZI resulted in only an insignificant reduction (from 34.9% ± 8.0% to 26.6% ± 3.5%) of the damage to SA54A hyphae caused by PMNs ($P > 0.05$). In the presence of AZI, the levels of damage to both

<table>
<thead>
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<th>Inhibitor or scavenger</th>
<th>SA1216</th>
<th>SA54A</th>
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<tbody>
<tr>
<td>None</td>
<td>79.8 ± 7.1$^b$</td>
<td>34.9 ± 8.0</td>
</tr>
<tr>
<td>MAN</td>
<td>80.5 ± 7.2</td>
<td>35.5 ± 7.1</td>
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<td>CAT</td>
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<td>65.0 ± 6.0</td>
<td>26.1 ± 7.0</td>
</tr>
<tr>
<td>AZI</td>
<td>27.4 ± 8.0$^b$</td>
<td>27.9 ± 3.5</td>
</tr>
<tr>
<td>HEP</td>
<td>75.0 ± 6.0</td>
<td>42.2 ± 6.4</td>
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$^a$ PMNs were added to germinated conidia at E:T ratio 20:1. PMNs were preincubated for 15 min in the presence of 0.1 mM AZI or HBSS at 37°C before being added to germinated conidia. The inhibitors MAN (100 mM), CAT (6,000 U/ml), SOD (2,000 U/ml) and HEP (0.5 U/ml) were added immediately before incubating the PMNs with the hyphae at the same E:T ratio. The results are indicated as means ± SE derived from six experiments. None of the inhibitors and scavengers listed caused direct hyphal damage. However, DPI caused 62.2% and 41.8% direct hyphal damage to SA54A and SA1216, respectively. The level of hyphal damage caused when DPI was added to PMNs and hyphae was similar to that caused by DPI alone.

Significant ($P < 0.01$) difference in the damage to strain SA1216 induced by PMNs in the presence of AZI from that seen with control PMNs without inhibitors.
isolates induced by PMNs became equivalent and SA1216 then displayed resistance to hyphal injury similar to that demonstrated by SA54A.

**DISCUSSION**

In this study, we found that phagocytes respond actively to challenge by *S. apiospermum*. Specifically, (i) macrophages are capable of phagocytosing *S. apiospermum* conidia, (ii) both PMNs and MNCs release O$_2^-$ in response to the presence of serum-opsonized hyphae of this fungal pathogen, and (iii) these cells damage hyphae of *S. apiospermum* in a concentration-dependent manner. Additionally, we observed that the hyphae of two strains that caused infections with different clinical outcomes in patients had different levels of susceptibility to MPO products.

*S. apiospermum* is a filamentous fungus characterized by oval, 6- to 12-μm-long conidia. The in vitro susceptibility of this fungus to conventional and novel antifungal drugs has been studied extensively (4, 5, 10, 23, 40). Despite interlaboratory discrepancies, in general these studies suggest that *S. apiospermum* is frequently resistant to amphotericin B. Determination of antifungal drug MICs for the two clinical isolates used in this study showed that strain SA54A was 32-fold more resistant to amphotericin B than strain SA1216 (amphotericin B MICs = 4 and 0.125 μg/ml, respectively).

As macrophages are the first phagocytic cells to encounter conidia of *A. fumigatus*, their primary role in antifungal defense appears to be prevention of conidial germination (38). When macrophages fail to prevent germination, hyphae transfix these cells and grow through them. In that case, PMNs and circulating MNCs attempt to damage hyphae extracellularly by potent oxidative and nonoxidative fungicidal mechanisms (31, 38).

The fact that host defenses against *S. apiospermum* are poorly understood prompted us to evaluate the antifungal activities of phagocytes against conidia and hyphae of this organism. To gain insight into the immunopathogenesis of infection due to *S. apiospermum*, two clinical strains (isolated from human infections with very different outcomes and characterized by distinct colonial morphologies and antifungal drug susceptibility features) were used to compare the innate immune responses to each of them.

Phagocytosis of *S. apiospermum* conidia by MDMs does not differ from phagocytosis of *A. fumigatus* conidia by MDMs, as assessed in similar experiments using the same methodology in our laboratory (27). While macrophages phagocytosed conidia of the two *S. apiospermum* isolates in equivalent manners, hyphae of the two isolates studied showed distinct differences in their interactions with and susceptibilities to PMNs and MNCs. For example, when the oxidative burst in response to opsonized hyphae of both isolates was evaluated the SA54A isolate elicited a significantly higher amount of O$_2^-$ from PMNs than SA1216. While MNCs displayed a significant oxidative burst in response to opsonized hyphae of both isolates, the level of O$_2^-$ generated in response to SA54A tended to be higher than that seen with SA1216. Although SA54A elicited production of more O$_2^-$ than did SA1216, this did not translate into more hyphal damage, suggesting a relatively high level of resistance of this strain to oxidative injury.

When hyphal damage induced by both PMNs and MNCs was assessed, the level of damage inflicted on isolate SA54A was found to be significantly lower than that seen with SA1216. Under some experimental conditions this difference in susceptibility to hyphal damage approached 40%. As the patient infected with SA54A had a fatal outcome whereas the patient infected with SA1216 was cured, we hypothesized that the difference in the hyphal damage was related to distinct differences in the susceptibilities of these isolates to certain components of the antifungal host defense mechanism. To further investigate this, we used oxidative pathway antifungal products and inhibitors or scavengers of various steps of PMN antifungal functions to determine whether this difference resides in one or more of these pathways.

Hyphae of the SA1216 strain appeared to be slightly more susceptible to HOCl at ≥0.1 mM than those of SA54A. This finding in a cell-free system correlates with the finding of a higher level of susceptibility of SA1216 to MPO products (as shown by AZI inhibition) in our PMN-hyphal system. By (presumably) acting on the hyphal surface, both antifungal oxidative compounds of phagocytes damaged hyphae of the susceptible strain more efficiently, a finding similar to that of the higher degree of susceptibility to amphotericin B, an agent also acting on the surface of the fungi (ergosterol of the fungal membrane).

The differences in susceptibility to amphotericin B between these isolates of *S. apiospermum* parallel the differences in vulnerability to oxidative metabolites and to hyphal damage. As susceptibility to amphotericin B-induced membrane injury is dependent upon lipoperoxidation (3), it is possible that the greater susceptibility to oxidative injury by some isolates of *S. apiospermum* is related. Resistance to lipoperoxidative injury by amphotericin B may be enhanced by increased expression of CAT (32). However, this appears not to be the mechanism of resistance of SA54A to amphotericin B or to H$_2$O$_2$ since there was a concentration-dependent response to H$_2$O$_2$ and even a trend toward heightened H$_2$O$_2$ activity at 1 mM. Assessment of potential differences in membrane sterol composition may further elucidate these mechanisms.

MAN receptors have been shown to mediate attachment and subsequent phagocytosis of various fungi (13, 33, 34, 39) and can be inhibited by the use of MAN (18). It appears that MAN receptors are not involved in the attachment of PMNs to *S. apiospermum* hyphae.

Although expected to create a cellular environment similar to that of chronic granulomatous disease PMNs by inhibiting the NADPH oxidase activity (21), DPI exerted a direct adverse effect on survival of *Scedosporium* hyphae, thus precluding an assessment of its effects on PMN hyphal damage. This may have been due to an effect of DPI on NADPH-like enzymes of *Scedosporium* hyphae (21).

Further, in previous studies CAT was successfully used to disrupt H$_2$O$_2$ at concentrations similar to those used in the present study (9, 41). H$_2$O$_2$ has been found to play a role in inducing destruction of fungi, including damage to *A. fumigatus* conidia and hyphae (8, 9, 22, 44). Using H$_2$O$_2$ as the substrate, MPO generates hypohalides, such as HOCl and chloramines, which are highly toxic compounds (1, 15). It is unclear whether the antifungal role of H$_2$O$_2$ is due to its own
fungicidal activity or to the activity of its downstream metabolic products. Wagner et al. reported similar results when assessing the damage to C. albicans pseudohyphae caused by PMNs (37). They proposed that CAT might be excluded from the area of attack at the fungal surface, since it has been previously demonstrated that proteins with a molecular mass over 50 kDa are excluded from sites of attachment of macrophages (42) and CAT is a high-molecular-mass protein. SOD (alone or in combination with CAT) as well as DMSO did not induce significant differences in hyphal damage between the two strains. It is possible that SOD with or without CAT is unable to eliminate all superoxide and H₂O₂ in the presence of PMNs. As noted in Table 1, a concentration of H₂O₂ of <0.1 mM was able to inhibit hyphal injury on Scedosporium spp. It is possible that at low concentrations, the oxidative metabolites O₂⁻, H₂O₂, HOCl, and MPO function additively or synergistically to mediate hyphal damage in the presence of these inhibitors.

Furthermore, HEP has been shown to bind to α-defensins (members of the cationic proteins) and inhibit their function (2, 16). We did not find an inhibitory effect of HEP on PMN-induced hyphal damage to either the SA54A or SA1216 isolate. Supporting our results, Diamond et al. suggested that all cationic proteins may be involved in damaging Rhizopus oryzae hyphae, they do not appear to have an effect on PMN-induced damage to A. fumigatus hyphae (9). Since S. apiospermum is more closely related to A. fumigatus than to R. oryzae, it is probable that α-defensins are not a key component in cell damage of this organism.

Among all seven inhibitors and scavengers used, only AZI exerted an inhibitory effect on PMN-induced hyphal damage and this inhibition was significant only with SA1216 isolate. Since the aim of these experiments was to maximize differences in hyphal damage between the two isolates, we used a relatively high E:T ratio. Smaller differences would be expected at lower E:T ratios. The concentration of AZI used in this study has been shown previously to inhibit MPO enzyme without altering other PMN functions (8, 9, 36). This finding suggests that the isolate SA54A is resistant to MPO products. This resistance may occupy an important role in the differences in susceptibility to innate host defense and, thus, may be partially responsible for the differences in clinical outcome for the patients infected with these isolates.

MPO deficiency is a common phagocytic disorder in the general population and is asymptomatic in the vast majority of persons. These individuals are not at significant risk for invasive infection due to filamentous fungi. However, when exposed to an uncommon pathogen such as S. apiospermum, an MPO-deficient host may be at greater risk for refractory infection.

In conclusion, this study has demonstrated a selective activity of phagocytic host defense against S. apiospermum, enhancing our understanding of the immunopathogenesis of infection due to this emerging fungal pathogen. The study findings indicate the need for further studies on the effects of immunomodulatory agents to augment host response and thus improve therapy to combat the devastating infections caused by S. apiospermum.

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