Interferon-γ and Granulocyte-Macrophage Colony-Stimulating Factor Augment the Activity of Polymorphonuclear Leukocytes against Medically Important Zygomycetes

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Zygomycetes cause serious invasive infections, predominantly in immunocompromised and diabetic patients with poor prognoses and limited therapeutic options. We compared the antifungal function of human polymorphonuclear leukocytes (PMNLs) against hyphae of Rhizopus oryzae and R. microsporus, the most frequently isolated zygomycetes, with that against the less frequently isolated Absidia corymbifera. We then evaluated the effects of interferon (IFN)-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF), alone or combined, on PMNL antifungal function against these zygomycetes. Both PMNL oxidative burst in response to hyphae and PMNL-induced hyphal damage were significantly lower in response to Rhizopus species than in response to A. corymbifera. Incubation of PMNLs with IFN-γ and GM-CSF alone or combined for 22 h increased the PMNL-induced hyphal damage of all 3 species. The treatment of PMNLs with the combination of IFN-γ and GM-CSF significantly increased the release of tumor necrosis factor–α in response to R. microsporus and A. corymbifera hyphae. IFN-γ significantly reduced interleukin-8 release in response to all zygomycetes. Although Rhizopus species demonstrate a decreased susceptibility to the antifungal activity of human PMNLs, 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 zygomycosis.
Cytokines, Neutrophils, and Zygomycetes

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Materials and Methods

PMNL isolation. PMNLs were isolated from heparinized whole blood of healthy young adult volunteers [19]. Blood was immediately allowed to settle with 3% dextran T500 (Pharmacia Biotech AB) in a 2:1 volume/volume proportion. PMNLs were separated by centrifugation over a ficoll (Lymphocyte Separation Medium, Gibco BRL Life Technologies) cushion. Contaminating erythrocytes were hypotonically lysed, and the PMNL suspension was washed in Hanks’ balanced salt solution without Ca²⁺ and Mg²⁺ (HBSS⁺; Gibco). The cells were resuspended in HBSS⁺ and counted on a hemocytometer.

Cytokine treatment. PMNLs were incubated at a concentration of 5 × 10⁶ cells/mL in RPMI 1640 supplemented with 10% pooled human serum (in the case of 2-h incubation) or 10% autologous serum (in the case of 22-h incubation) in the presence or absence of 100 ng/mL IFN-γ (Boehringer-Ingelheim) and/or 100 ng/mL GM-CSF (Schering-Plough; donated by Tore Abrahamsen, University of Oslo, Oslo, Norway), at 37°C and 5% CO₂. At the end of the 22-h incubation, PMNLs were counted using a hemocytometer, and the viability was assessed with trypan blue staining, ensuring >95% viability.

Fungi. Three clinical isolates of zygomycetes were used in these studies. R. oryzae (RO 27) was isolated from a cut-down wound site (Fungus Testing Laboratory, University of Texas Health Sciences Center, San Antonio), R. microsporus var. rhizopodiformis (AZN 1185) was isolated from an invasive infection, and A. corymbifera (AZN 4095 and CBS 271.65) was a clinical isolate from an undetermined site of infection. The 2 latter isolates were donated by Paul Verweij (University Medical Center St. Radboud, Nijmegen, The Netherlands).

Fungi from frozen stocks were inoculated on potato dextrose agar (Merck Darmstadt) plates and grown for 4 days. Sporangiospores were then harvested by scraping the surfaces of the plates, suspended in HBSS⁺, and filtered through sterile gauze. After centrifugation at 400 g for 10 min, supernatant was aspirated, the pellet was resuspended in normal saline, and cells were counted on a hemocytometer. They were kept at 4°C for no longer than 3 weeks.

Hyphae were generated for each of the fungi by placing 200 µL of a suspension containing 7.5 × 10⁶ (for superoxide anion [O₂⁻] production assays) or 7.5 × 10⁵ (for hyphal damage and cytokine release assays) sporangiospores/mL in yeast nitrogen base (YNB; Difco Laboratories) medium in each well of a 96-flat-bottom well cell-culture plate (Corning). The plate was then incubated at 25°C (Rhizopus species) or 32°C (A. corymbifera), for 14 h. These hyphae were used as stimuli in all of the experiments.

O₂⁻ quantification. The oxidative burst evidenced by the production of O₂⁻ was measured as reduction of cytochrome c from horse heart (Sigma Chemical Co.) [28]. Hyphae were prepared as described above. Once the hyphal network was established, in the case of O₂⁻ assays with serum-opsonized hyphae, the plate was centrifuged at 400 g at 25°C for 30 min. YNB was then replaced by 50% human serum in HBSS⁺, and the plate was rotated at 37°C for 30 min, for opsonization. Because zygomycete hyphae were loosely attached to the bottom of the wells, plates were always centrifuged before aspiration of supernatant during washing. The plate was washed 3 times, and PMNLs were added at an effector cell:target (E:T) ratio of 1:1 to a final volume of 200 µL of HBSS⁻ containing 60 µmol/L cytochrome c with serum-opsonized or nonopsonized hyphae. Basal O₂⁻ production was assessed in the absence of stimuli. After incubation at 37°C on a rotator for 1 h, 100 µL was transferred to another plate, and absorbance was read in a spectrophotometer at 550 nm, with a reference of 690 nm. The extinction coefficient of cytochrome c at 550 nm was taken as 29.5 × 10⁴ L/mmol·cm.
**Hyphal damage assay.** PMNL-induced hyphal damage was assessed by use of a modification of the XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide sodium salt; Sigma) plus coenzyme Q₉ (2,3-dimethoxy-5-methyl-1,4-benzoquinone; Sigma) assay [29]. Hyphae were generated as described above. Once the hyphal network was established, the plates were centrifuged at 400 g at 25°C for 30 min. YNB in each plate was then replaced by 200 μL of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, and PMNLs were added at the corresponding E:T ratios. After incubation at 37°C with 5% CO₂ for 2 or 22 h, the plates were centrifuged at 400 g at 10°C for 30 min. One hundred ninety microliters of RPMI 1640 was then replaced with H₂O, and the plates were centrifuged again, to lyse the cells without aspiration of hyphae. After a second wash performed in this manner, H₂O in each plate was replaced by 150 μL of PBS (Biochrom KG) containing 25 mg/mL XTT and 40 μg/mL coenzyme Q₉. Absorption of the wells was performed very gently with a multichannel pipette. Because of the possibility of aspiration of hyphae, wells were set in octuplicate. After incubation with XTT, the wells of the plate were observed under the microscope, to ensure that hyphae were not accidentally aspirated from the wells. After incubation with XTT at 37°C with 5% CO₂ for 1 h, 100 μL from each plate was transferred to a new plate, and the change in color (absorbance) was assessed spectrophotometrically at 450 nm by use of a 690-nm reference. Antihyphal activity was calculated according to the following formula: percentage of hyphal damage = (1 – X/C) × 100, where X is the absorbance of experimental wells and C is the absorbance of control wells with hyphae only.

**Cytokine release from PMNLs.** Release of IL-8 and TNF-α from IFN-γ- and GM-CSF–treated PMNLs was measured by incubating PMNLs for 4 h in microtiter plate wells containing 200 μL of HBSS with 1.5 × 10⁶ PMNLs/well. PMNLs were added to wells containing hyphae of the 3 zygomycetes (generated from sporangiospores, as described above) at an E:T ratio of 10:1. For baseline release of IL-8 and TNF-α, freshly isolated PMNLs were also incubated for 2 or 22 h without IFN-γ and GM-CSF. Untreated and treated PMNLs were added at the same E:T ratio to microtiter plate wells containing fungal hyphae and were suspended in HBSS at 37°C for 4 h. At the end of the incubation period, supernatants were stored at −20°C until testing for IL-8 and TNF-α concentrations. TNF-α and IL-8 production was assessed using Quantikine ELISAs (R&D Systems). The sensitivities of the TNF-α and IL-8 assays were <4 and <10 pg/mL, respectively.

**Statistical analysis.** Each experiment was performed with the cells of 1 donor and by use of quadruplicate or octuplicate wells for each condition. The mean value of these replicate wells was taken as the value for this particular donor/experiment. The means of the replicate wells of each experiment were then used in the data analysis to calculate the mean ± SE of all the experiments at the same conditions. The statistics program Instat (version 3.0; Graphpad) was used for analysis. Differences in PMNL response to the zygomycetes were evaluated by repeated-measures analysis of variance (ANOVA) with Bonferroni posttest. Differences in antifungal effects between the cytokine-treated PMNLs and the untreated controls were performed using repeated-measures ANOVA with Dunnett’s posttest for multiple comparisons. A 2-sided P < .05 indicated statistical significance.

**RESULTS**

**O₂⁻ production.** We evaluated the oxidative burst of PMNLs immediately after their isolation, by use of serum-opsonized or nonopsonized hyphae as stimuli (figure 1). Both serum-opsonized and nonopsonized hyphae of R. oryzae, R. microsporus, and A. corymbifera stimulated PMNLs to produce increased amounts of O₂⁻, compared with production by unstimulated PMNLs (P < .05). Nonopsonized hyphae of A. corymbifera stimulated PMNLs to release significantly more O₂⁻ than did nonopsonized hyphae of either Rhizopus species (P < .05). This difference tended to be significant (P < .1) in the case of serum-opsonized hyphae.

Opsonized hyphae of R. oryzae and R. microsporus stimulated PMNLs to produce higher amounts of O₂⁻ than did nonopsonized hyphae of these fungi (P < .001). A trend of higher O₂⁻ production in response to serum-opsonized A. corymbifera hyphae than in response to nonopsonized hyphae also was evident.

**Figure 1.** Superoxide anion (O₂⁻) production by freshly isolated human polymorphonuclear leukocytes (PMNLs) challenged with no stimulus (vertically striped bars) or with nonopsonized (white bars) or serum-opsonized (diagonally striped bars) hyphae of Rhizopus oryzae (RO), R. microsporus (RM), and Absidia corymbifera (AC). PMNLs were added at an effector cell:target ratio of 1:1. Columns represent the means ± SEs of values derived from 7 experiments. Asterisks (*) indicate significant differences between nonopsonized Rhizopus species and A. corymbifera, evaluated by repeated-measures analysis of variance (ANOVA) with Bonferroni posttest. Daggers (†) indicate significant differences between all 3 species and nonstimulated PMNLs, evaluated by repeated-measures ANOVA with Dunnett’s posttest for multiple comparisons (P < .05).
Effects of cytokines on hyphal damage. PMNLs induced hyphal damage of each of the 3 species in a E:T ratio–dependent manner, showing a significantly linear trend (P < .005) (figure 2). PMNLs damaged hyphae of *A. corymbifera* to a significantly higher degree than they damaged hyphae of *Rhizopus* species, at E:T ratios of 5:1 and 10:1 (P < .001). A similar trend was observed at an E:T ratio of 20:1 (P = .087).

Effects of cytokines on PMNL oxidative burst. After incubation for 2 h, IFN-γ and GM-CSF alone or combined significantly increased O2− production by PMNLs in response to serum-opsonized hyphae of *A. corymbifera* (P < .01) (figure 3). In addition, GM-CSF and its combination with IFN-γ augmented PMNL oxidative burst in response to nonopsonized *A. corymbifera* hyphae (P < .01). However, only GM-CSF significantly increased the PMNL oxidative burst in response to nonopsonized hyphae of *R. oryzae* at 2 h (P < .01), and no cytokine affected the oxidative burst in response to serum-opsonized hyphae of *R. oryzae* at 2 h. In the case of *R. microsporus*, both GM-CSF and its combination with IFN-γ augmented the oxidative burst in response to nonopsonized hyphae (P < .01), whereas only GM-CSF showed an enhancing effect in response to serum-opsonized hyphae (P < .05). These effects were not evident after incubation for 22 h, since treated PMNLs released amounts of O2− similar to those released by untreated PMNLs.

Effects of cytokines on hyphal damage. PMNLs that had been treated with IFN-γ, GM-CSF, or their combination for 22 h exhibited a significantly increased capacity to induce hyphal damage of all 3 zygomycetes (figure 4). Whereas untreated PMNLs damaged 43.2% ± 8.4% of hyphae of *A. corymbifera*, PMNLs treated with IFN-γ, GM-CSF, or their combination damaged 78.4% ± 2.0%, 78.4% ± 2.0%, and 76.1% ± 3.8% of hyphae, respectively (P < .01). Further, whereas untreated PMNLs damaged 13.2% ± 10.1% of hyphae of *R. oryzae*, those treated with IFN-γ, GM-CSF, or their combination damaged 40.1% ± 6.9%, 40.6% ± 9.8%, and 47.4% ± 6.4% of hyphae, respectively (P < .01). Similar augmentation of hyphal damage was shown by cytokine-pretreated PMNLs against *R. microsporus* hyphae (P < .05).

The augmenting effects of IFN-γ and GM-CSF were time dependent, requiring prolonged incubation of PMNLs with the cytokines. Thus, PMNLs that had been pretreated with IFN-γ, GM-CSF, or their combination for only 2 h did not show a significant increase in induction of hyphal damage for any of the 3 species, with the exception of IFN-γ-pretreated PMNLs and subsequent challenge with *R. microsporus* hyphae (P = .027; data not shown).
After incubation of PMNLs with the combination of IFN-γ and GM-CSF for 2 h, TNF-α release from PMNLs in response to \( R. \) microsporus and \( A. \) corymbifera hyphae was significantly increased, compared with that from untreated PMNLs (figure 5, upper panel). Whereas TNF-α release from untreated PMNLs in response to \( R. \) microsporus hyphae was 477 ± 89 pg/mL, TNF-α release from PMNLs treated with the combination of IFN-γ and GM-CSF was augmented to 1222 ± 236 pg/mL (\( P < .05 \)). Similarly, when untreated PMNLs were challenged with \( A. \) corymbifera hyphae, TNF-α release was 392 ± 118 pg/mL, whereas, in the case of PMNLs treated with the combination of IFN-γ and GM-CSF, it increased to 849 ± 286 pg/mL (\( P < .05 \)). A significant enhancing effect of the combination of IFN-γ and GM-CSF also was observed in response to \( R. \) microsporus after 22 h (TNF-α release from untreated PMNLs was 354 ± 78 pg/mL, vs. 905 ± 193 pg/mL from PMNLs treated with both cytokines; \( P < .05 \)), but there was a trend of enhancement in response to \( A. \) corymbifera hyphae (figure 5, lower panel). Comparable amounts of TNF-α were measured in untreated PMNLs and in PMNLs treated with each cytokine alone after 2 or 22 h in response to \( R. \) microsporus and \( A. \) corymbifera. When PMNLs treated for 2 or 22 h with IFN-γ, GM-CSF, or their combination were challenged with \( R. \) oryzae hyphae, there was no effect on TNF-α release.

PMNLs treated with IFN-γ for 2 h had significantly decreased IL-8 release in response to \( R. \) oryzae, \( R. \) microsporus, and \( A. \) corymbifera hyphae, compared with that of untreated PMNLs (\( P < .01 \)) (figure 6, upper panel). The suppression of IL-8 release in response to all 3 zygomycetes became even more pronounced after incubation of PMNLs with IFN-γ for 22 h (\( P < .01 \)) (figure 6, lower panel). Treatment of PMNLs with GM-CSF or its combination with IFN-γ for 2 or 22 h did not produce a significant effect on IL-8 release in response to any of these 3 zygomycetes; the only exception was GM-CSF treatment of PMNLs for 2 h and stimulation by \( A. \) corymbifera.

**DISCUSSION**

In this study, we have found that human PMNLs have a reduced capacity to mount an oxidative burst in response to both Rhizopus species and to induce hyphal damage of these zygomycetes, in comparison with its response to \( A. \) corymbifera. However, IFN-γ and GM-CSF augmented PMNL-induced hyphal damage of all 3 zygomycetes, in a time-dependent manner. Furthermore, treatment of PMNLs with the combination of cytokines enhanced the release of TNF-α in response to \( R. \) microsporus and \( A. \) corymbifera but not in response to \( R. \) oryzae hyphae. By comparison, IFN-γ inhibited IL-8 release in response to hyphae of the 3 zygomycetes. To our knowledge, this is the first time that intergenus differences in host response to zygomycetes have been reported and that the effects of IFN-γ and GM-CSF on PMNL response to these fungi have been described.
**Figure 6.** Release of interleukin-8 (IL-8) by human polymorphonuclear leukocytes (PMNLs) treated without cytokines (white bars) or with interferon-γ (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), or both (black bars), as assessed by ELISA. Supernatants were assayed after incubation of PMNLs with or without 100 ng/mL IFN-γ and/or 100 ng/mL GM-CSF at 37°C for 2 h (upper panel) or 22 h (lower panel) and subsequent incubation of PMNLs with Rhizopus oryzae (RO), R. microsporus (RM), and Absidia corymbifera (AC) for 4 h. Columns represent means ± SEs of data derived from 5 experiments. Differences between cytokine-pre-treated and untreated PMNLs were evaluated by repeated-measures analysis of variance with Dunnett’s posttest for multiple comparisons. *P < .05; †P < .01.

Despite the long-standing clinical interest in zygomycetes as causative agents of serious and frequently fatal infections in immunocompromised and diabetic patients, little has been accomplished in recent years to improve the understanding of host defenses against zygomycetes and the role of cytokines. Indeed, the antifungal activity of PMNLs and macrophages, as well as the mechanisms involved, were investigated in pioneering studies 2 decades ago [9–13]. With the exception of 1 study demonstrating the ex vivo effects of granulocyte colony-stimulating factor in up-regulating PMNL-induced hyphal damage of *R. arrhizus* (synonymous with *R. oryzae*) [30], no studies have examined the effects of any cytokines on multiple species of zygomycetes. The finding that both *Rhizopus* species stimulated PMNL oxidative burst less often than did *Absidia corymbifera* and suffered less hyphal damage from PMNLs may be related to the relatively high frequency of *Rhizopus* species and the comparatively lower frequency of *A. corymbifera* as causative agents of zygomycosis [1–5]. These data suggest that reduced susceptibility to innate host defense by members of the genus *Rhizopus* may contribute to their greater prevalence as pathogens in immunocompromised patients. Differences in cell-wall constituents and ligands on the surface of the hyphae of the different genera of zygomycetes may account for the differences in the stimulation of PMNLs and the susceptibility to their antifungal activity. An alternative explanation that may account for such differences is that organism-related factors (e.g., diffusible components of the fungal surface or production of toxins by *Rhizopus* hyphae) compromise any effects of PMNLs challenged with that fungus. That soluble factors from *R. oryzae* may enhance the PMNL respiratory burst was suggested in the study by Liles et al. [30]. Similar to hyphae of other filamentous fungi, such as *A. fumigatus*, *Fusarium* species, and *Scedosporium* species [12, 31, 32], hyphae of zygomycetes induced an increase in $O_2^-$ production, compared with nonstimulated PMNLs, and the antityphal activity induced by PMNLs was E:T ratio dependent.

In this study, we describe—for the first time, to our knowledge—a modification of the XTT assay previously used for the study of hyphal damage of other filamentous fungi [29, 31–33], to study PMNL-zygomycete interactions. Since hyphae of zygomycetes adhere to the plastic surface of wells very loosely, we centrifuged the plates before each aspiration and took care to leave a small amount of $H_2O$ in the wells during the washes. With this modification, XTT, an easily performed nonradioisotopic assay, could be used for the study of hyphal damage of zygomycetes instead of a radioactive assay, which previous investigators were required to use [13]. IFN-γ and GM-CSF are among the cytokines most frequently used for prevention and treatment of invasive fungal infections [18]. This and the fact that they, in theory, have many favorable features for immune up-regulation against zygomycosis prompted us to investigate their activities against these organisms. Indeed, GM-CSF has been found to augment phagocytosis and oxidative burst and to increase the number and membrane expression of several classes of surface receptors on PMNLs, such as FMLP, CD11b, and C3bi [23, 34, 35]. Additionally, GM-CSF increases PMNL fungicidal activity against several pathogenic species of fungi [20, 21, 36–38] and protects mice in 2 models of deep candidiasis [39, 40]. Similarly, IFN-γ induces a Th1 response, which favors resistance to invasive fungal infections [18, 21, 41]. This cytokine primes $O_2^-$ production of PMNLs in response to FMLP, in a time-dependent manner, by a mechanism that involves synthesis of proteins [42, 43]. Additionally, IFN-γ enhances PMNL fungicidal and fungicidal activities against certain fungal pathogens [21, 37, 38, 44–46]. Administration of this cytokine to experimental animals with fungal infections has yielded encouraging results [47–50]. In patients with chronic granulomatous disease, IFN-γ reduced the risk of infections by a mechanism independent from $O_2^-$ production [51], and administration of IFN-γ to healthy volunteers improved their phagocytic host defense, indicating that this cytokine may be useful in the treatment of patients with other immune disorders [52].
We found a time dissociation between augmentation of \( \mathrm{O}_2^- \) production and hyphal damage by treatment of PMNLs with IFN-\( \gamma \) and GM-CSF. Whereas the optimal time for increase of \( \mathrm{O}_2^- \) production was 2 h, the optimal time for increase of hyphal damage was 22 h. This time dependency in response to stimuli, which has previously been noted, to a lesser extent, with other filamentous fungi [53, 54], may be due to (1) increased amounts of myeloperoxidase or other antifungal proteins, as a result of augmented signal transduction and synthesis of proteins induced by the cytokines; (2) posttranslational processing of these proteins; and/or (3) an increased rate of degranulation of PMNLs releasing antifungal proteins. These data also demonstrate a potentially critical role for nonoxidative mediators (e.g., antimicrobial peptides) in mediating the enhanced PMNL host response to *Rhizopus* and *Absidia* species. Indeed, although IFN-\( \gamma \) had no effect on \( \mathrm{O}_2^- \) production in response to *Rhizopus* species, it substantially enhanced hyphal damage of all 3 zygomycetes.

The combination of IFN-\( \gamma \) and GM-CSF showed no better effects than IFN-\( \gamma \) or GM-CSF alone in enhancing hyphal damage or \( \mathrm{O}_2^- \) production. However, the combination induced differentiation of PMNLs into major histocompatibility complex class II–expressing antigen-presenting cells [55–58], enabling them to participate in the subsequent T cell response. Although these results are derived from in vitro models, we suggest that the combination may have therapeutic potential that warrants further evaluation in animal models of zygomycosis.

Our study also demonstrated species-specific increases of TNF-\( \alpha \) release as a result of treatment with the combination of IFN-\( \gamma \) and GM-CSF, in contrast to the decreased IL-8 release from IFN-\( \gamma \)-treated PMNLs. These data reflect different immunoregulatory mechanisms in host defenses against each of the 3 zygomycetes. Perhaps the down-regulation of IL-8 expression by IFN-\( \gamma \)-treated PMNLs is induced by a negative feedback pathway to modulate the inflammatory response against zygomycetes.

The data provided in this study contribute to a better understanding of the pathogenesis of zygomycosis. Because of the difficulty in treatment and the high mortality of patients with infections caused by zygomycetes, IFN-\( \gamma \) and GM-CSF may prove to be useful therapeutic adjuncts. Further investigations in immunocompetent and immunosuppressed animal models (e.g., corticosteroid-treated or NADPH-oxidase–knockout mice) and, ultimately, clinical trials are warranted to assess the utility of IFN-\( \gamma \) and GM-CSF as adjuncts to conventional antifungal chemotherapy.

References


