Modulation of Neutrophil-Mediated Activity Against the Pseudohyphal Form of *Candida albicans* by Granulocyte Colony-Stimulating Factor (G-CSF) Administered In Vivo


Renewed interest in neutrophil transfusions has emerged with the development and clinical use of granulocyte colony-stimulating factor (G-CSF). G-CSF not only increases neutrophil (polymorphonuclear leukocyte, PMNL) production but also modulates various physiological properties of PMNL. The effects of G-CSF on PMNL-mediated fungicidal activity were evaluated by administration of G-CSF (300 µg/day subcutaneously) to 5 healthy volunteers for 6 days. G-CSF significantly enhanced PMNL-mediated damage of *Candida albicans* pseudohyphae by 33% (*P* = .007) on day 2 and by 44% (*P* = .04) on day 6 at a 10:1 effector:target ratio. In contrast, the ability of PMNL to induce damage of hyphae from either *Paecilomyces liliae* or *Aspergillus fumigatus* did not significantly change during the study period. These data demonstrate that G-CSF administered in vivo modulates PMNL-mediated fungicidal activity against the pseudohyphal form of *C. albicans*, thereby suggesting potential utility of G-CSF as a biologic response–modifying therapy in some opportunistic fungal infections.

With the advent of dose-intensive chemotherapy and bone marrow transplantation during the last 2 decades, an increase in the incidence of life-threatening bacterial and fungal infections has been observed [1, 2]. *Candida albicans*, a true yeast, and *Aspergillus fumigatus*, a true mold, are responsible for the majority of invasive fungal infections in this group of immunocompromised patients [3]. Prolonged neutropenia and abnormal neutrophil function are the major risk factors for opportunistic mycotic infections [4], which cause high morbidity and mortality despite currently available antifungal therapy [2, 3].

Neutrophils (polymorphonuclear leukocytes, PMNLs) play a key role in damaging and eventually killing fungal hyphae and pseudohyphae, the predominant tissue forms of opportunistic fungi during invasive infections [5]. Recent studies have demonstrated that granulocyte colony-stimulating factor (G-CSF) not only increases PMNL production but also modulates various antimicrobial functions of developing and mature PMNLs, including respiratory (oxidative) burst activity, microbialicidal activity, degranulation, and expression of FcγR1 (CD 64) [6].

We reported previously that in vivo administration of G-CSF to healthy volunteers effectively primed PMNL for sustained production of reactive oxygen species, as measured by luminol-enhanced chemiluminescence, in response to aqueous extracts of *A. fumigatus*, *Rhizopus arrhizus*, and *C. albicans* [7]. Furthermore, G-CSF significantly enhanced PMNL-mediated killing of *A. fumigatus* and *R. arrhizus* conidia [7]. Administration of G-CSF to individuals infected with human immunodeficiency virus–type 1 (HIV-1) has also been reported to enhance PMNL-mediated fungicidal activity against *C. albicans* and *Cryptococcus neoformans* [8]. In vitro, G-CSF has been shown to prime normal and defective PMNLs for increased antifungal activity against *A. fumigatus* hyphae [9, 10]. However, in vitro experiments evaluating the ability of G-CSF to prime PMNLs for enhanced fungicidal activity against *C. albicans* blastoconidia and pseudohyphae have yielded conflicting results [7, 11, 12].

Lately, a renewed interest in granulocyte transfusion therapy has evolved, based largely on the ability to obtain high yields of PMNLs by leukapheresis after stimulation of donors with G-CSF [13, 14]. Although the effects of G-CSF on PMNL-mediated fungicidal activity have been studied in vitro, much less attention has been focused on the effect of G-CSF administered in vivo on PMNL-mediated antifungal activity. Because hyphae (or pseudohyphae) are the predominant stage of opportunistic fungi in host tissues during invasive infection, we administered G-CSF (300 µg/day subcutaneously for 6 days) to healthy volunteers, to evaluate its effect on PMNL-mediated antifungal activity against hyphal and pseudohyphal forms of...
three medically important opportunistic fungi: *A. fumigatus*, *Fusarium solani*, and *C. albicans*.

Materials and Methods

**Organisms.** The isolates of *A. fumigatus*, *F. solani*, and *C. albicans* used in this study are well-characterized clinical isolates obtained from patients with invasive fungal infections at the Fred Hutchinson Cancer Research Center (Seattle). Stock fungal isolates were maintained by standard microbiologic methods. Conidia (*A. fumigatus* and *F. solani*) and blastoconidia (*C. albicans*) were suspended in RPMI 1640 without phenol red, containing 10 mM HEPES, pH 7.4 (RPMI/HEPES; Life Technologies, Gibco BRL, Grand Island, NY). Conidia and blastoconidia were counted in a hemocytometer and adjusted to a final concentration of $1 \times 10^7$ conidia/mL and $1 \times 10^4$ blastoconidia/mL. Aliquots of 100 μL were dispensed into 96-well flat-bottomed plates (Costar, Cambridge, MA), and incubated for 16 h to form hyphae and pseudohyphae (*A. fumigatus* at 37°C; *F. solani* and *C. albicans*, 25°C). More than 95% of conidia and blastoconidia germinated (hyphal length, 160–200 μm; pseudohyphal length, 80–100 μm).

**Reagents.** Recombinant human G-CSF (1 $\times 10^5$ U/mg) was provided by Amgen (Thousand Oaks, CA). A 2,3-bis-(2-methoxy-4-nitro-5-sulphonylphenyl)-2H-tetrazolium-5-carboxanilide solution (XTT; Sigma, St. Louis) was prepared fresh daily in Dulbecco’s PBS (Life Technologies) at 0.5 mg/mL with heating at 55°C for 20 min, 2,3-dimethoxy-5-methyl-1,4-benzoquinone (coenzyme Q; Sigma) was added at 50 μg/mL, and the solution was passed through a 0.22-μm filter.

**Study design.** Five healthy human volunteers (age, 25–49 years; weight, 67–85 kg) received 300 μg of G-CSF subcutaneously each morning for 5 consecutive days. All volunteers were nonsmokers, had normal physical examination findings, were receiving no concomitant medications, and had not experienced an acute illness during the 6 weeks preceding the study. All volunteers underwent screening consisting of physical examination and complete blood count.

**PMNL purification.** Venous blood was collected from healthy human volunteers on day 0 before G-CSF administration, on day 2 (approximately 18 h after the second dose of G-CSF), and on day 6 (approximately 18 h after the sixth dose of G-CSF), with 0.2% K$_2$ EDTA used as anticoagulant. PMNLs were isolated by sequential centrifugation on Histopaque-1077 (Sigma), sedimentation in 3% Dextran (Sigma) in 0.9% sodium chloride, and hypotonic lysis of remaining erythrocytes. After separation, PMNLs were suspended in RPMI/HEPES. PMNL purity was >98%, and cell viability was >98% as determined by trypan blue exclusion.

**Hyphal damage assay.** PMNL-mediated hyphal damage was measured by modification of an XTT-based assay system described by Meshulam et al. [15]. PMNLs suspended in RPMI/HEPES plus 20% autologous serum were dispensed in 100-μL aliquots to achieve effector:target (E:T) ratios of 10:1 and 1:1. Each condition was performed in quadruplicate. PMNLs were gently sedimented to the bottom of the well by centrifugation at 400 g for 3 min, and then the plates were gently rocked at 37°C for 2 h. Blank wells, containing only RPMI/HEPES plus serum, and control wells with only hyphae or cells were also prepared and subjected to the same experimental conditions as the test wells. After incubation, 100 μL of fluid was aspirated and PMNLs lysed by addition of 100 μL of ice-cold distilled water, followed by centrifugation at 2000 g for 7 min at 1°C. One hundred microliters of fluid was then aspirated, and the procedure was repeated once. Microscopic examination confirmed that cells were completely lysed by this process.

We assessed fungal viability by adding 100 μL of XTT solution to each well and rocking the plates for 1 h at 37°C. Absorbance of each well was determined at 450 nm by use of a microplate spectrophotometer (Automated Microplate Reader, Model EL311, Bio-Tek Instruments, Inc., Winooski, VT). The percentage of fungal damage was defined by the equation (1 – [Abs of fungi incubated with cells – Abs of cells alone]/[Abs of fungi alone]×100.

**Statistical analysis.** Paired Student’s 2-tailed t test was used for statistical analysis. Statistical significance was defined as $P < .05$.

**Results**

The absolute neutrophil count (ANC) in the venous blood of the 5 healthy volunteers increased from a mean baseline value of 3402/μL (range, 2600–5248/μL) before administration of G-CSF to a mean value of 21,275/μL (range, 14,025–34,400/μL) after the second dose of G-CSF, and to a mean value of 24,450/μL (range, 17,085–36,720/μL) after the sixth consecutive daily dose of G-CSF (table 1). The increase in ANC observed after G-CSF administration was predominantly due to enhancement in the population of mature neutrophils. Bands were the least mature cells of the neutrophil lineage observed in the peripheral

### Table 1. Leukocyte counts in healthy donors administered G-CSF (300 μg/day subcutaneously) for 6 days.

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>Day 0</th>
<th>Day 2</th>
<th>Day 6</th>
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<tbody>
<tr>
<td></td>
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<td>ANC</td>
<td>WBC</td>
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<td>2.6</td>
<td>20.8</td>
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<td>5.2</td>
<td>39.1</td>
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<tr>
<td>5</td>
<td>5.2</td>
<td>3.4</td>
<td>20.6</td>
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<tr>
<td>Mean ± SE</td>
<td>5.5 ± 0.8</td>
<td>3.4 ± 0.5</td>
<td>25 ± 4.1</td>
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NOTE. Data are cells/μL; n = 5 healthy donors. WBC, total leukocyte count; ANC, absolute neutrophil count; SE, standard error.
blood smear. G-CSF treatment was well tolerated by the volunteers, with only 1 volunteer reporting mild low back pain on days 5 and 6 of the study period.

The effect of in vivo administration of G-CSF on PMNL-mediated fungicidal activity against opportunistic fungi was evaluated by hyphal/pseudohyphal damage assay using an XTT assay (figure 1). In vivo administration of G-CSF significantly enhanced PMNL-mediated fungicidal activity against C. albicans pseudohyphae. At baseline (day 0) prior to administration of G-CSF, 57% ± 10% (mean ± SE) and 47% ± 11% pseudohyphal damage was observed at E:T ratios of 10:1 and 1:1, respectively. After the second dose of G-CSF (day 2), mean hyphal damage increased to 76% ± 10% at a 10:1 E:T ratio (P = .007) and to 68% ± 13% at a 1:1 E:T ratio. Enhanced PMNL-mediated activity against C. albicans persisted throughout the duration of G-CSF administration. After the sixth dose of G-CSF (day 6), mean hyphal damage increased to 82% ± 9% at a 10:1 E:T ratio (P = .04) and to 67% ± 17% at a 1:1 E:T ratio.

In contrast, fungicidal activity against F. solani hyphae was not significantly affected by G-CSF. At baseline, PMNL-mediated hyphal damage was 31% ± 4% at a 10:1 E:T ratio and 18% ± 5% at a 1:1 E:T ratio. After the second dose of G-CSF, 37% ± 9% hyphal damage was observed at a 10:1 E:T ratio and 24% ± 8% damage at a 1:1 E:T ratio. After the sixth dose of G-CSF, PMNL-mediated hyphal damage remained unchanged, with 36% ± 9% damage observed at a 10:1 E:T ratio and 16% ± 5% at a 1:1 E:T ratio. Likewise, PMNL-mediated fungicidal activity against A. fumigatus hyphae was not significantly affected by in vivo administration of G-CSF. On day 0, the hyphal damage was 43% ± 5% and 34% ± 3% at 10:1 and 1:1 E:T ratios, respectively. After the second dose of G-CSF, the mean hyphal damage was 43% ± 1% at a 10:1 E:T ratio and 35% ± 5% at a 1:1 E:T ratio. After the sixth dose of G-CSF, the mean hyphal damage was 40% ± 5% at a 10:1 E:T ratio and 31% ± 6% at a 1:1 E:T ratio.

Discussion

We reported elsewhere that in vivo administration of G-CSF failed to augment PMNL-mediated killing of C. albicans blastoconidia subsequently measured in vivo. In contrast, the present study demonstrates that in vivo administration of G-CSF to healthy volunteers effectively enhances PMNL-mediated antifungal activity against C. albicans pseudohyphae, which predominate at sites of invasive tissue infection. After the second dose of G-CSF (day 2), the degree of PMNL-mediated fungal damage was significantly enhanced by 33% above the baseline (P = .007) at a 10:1 E:T ratio. After the sixth dose of G-CSF (day 6), PMNL-mediated pseudohyphal damage was further enhanced to 44% (P = .04) above the baseline antifungal activity. Although G-CSF had a beneficial effect on PMNL-mediated fungicidal activity against C. albicans at the 2 different E:T ratios studied, the difference in pseudohyphal damage was significant only at the 10:1 E:T ratio, suggesting that the number of PMNLs at the site of infection plays a pivotal role in the resolution of invasive candidiasis.

Although the administration of G-CSF in vivo failed to significantly enhance PMNL-mediated hyphal damage of A. fumigatus and F. solani, PMNLs retained fungicidal activity against these organisms for the duration of G-CSF treatment. PMNL-mediated antifungal activity against A. fumigatus hyphae was essentially unchanged after the second and the sixth doses of G-CSF. On the other hand, PMNL-mediated hyphal damage of F. solani was slightly increased at a 10:1 E:T ratio to 20% above baseline after the second dose of G-CSF and to 16% above baseline after the sixth dose of G-CSF. However, these results do not exclude the possibility that G-CSF in vivo

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Assessment of PMNL-mediated hyphal/pseudohyphal damage of opportunistic fungi after granulocyte colony-stimulating factor (G-CSF) treatment in vivo. Healthy donors received G-CSF (300 μg/day) subcutaneously for 6 days. Percentage hyphal damage (mean ± standard error) of isolated PMNLs against C. albicans (A), F. solani (B), and A. fumigatus (C), was evaluated at baseline (day 0), after second dose of G-CSF (day 2), and after sixth dose of G-CSF (day 6). Striped bars, 10:1 E:T ratio; open bars, 1:1 E:T ratio. *Significant difference in hyphal/pseudohyphal damage compared with day 0 (baseline; P < .05).
could increase the fungicidal activity of defective PMNLs, as reported in HIV-1-infected patients [8, 10].

With the increasing use of dose-intensive chemotherapy for solid tumors and myeloid malignancies, as well as the increasing number of marrow transplantations being done, a growing number of neutropenic patients develop serious and often fatal invasive fungal infections [1, 3]. The use of G-CSF in healthy donors not only allows the collection of adequate numbers of PMNLs for transfusion but also preserves and, in some cases, enhances PMNL-mediated activity against opportunistic fungal hyphae or pseudohyphae that predominate at sites of invasive tissue infection. However, only controlled clinical trials can establish the proper role of G-CSF as an adjunctive immunomodulatory agent for the treatment of patients with invasive infections by opportunistic fungal pathogens.

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References