Granulocyte Colony-Stimulating Factor Administered In Vivo Augments Neutrophil-Mediated Activity against Opportunistic Fungal Pathogens

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Granulocyte colony-stimulating factor (G-CSF) not only increases neutrophil (polymorphonuclear leukocyte, PMNL) production but also modulates PMNL biologic function. To assess the ability of G-CSF administered in vivo to enhance PMNL activity against opportunistic fungal pathogens, the antifungal activity of PMNL obtained from normal human volunteers before and after G-CSF administration was compared. In vivo, G-CSF significantly enhanced PMNL-mediated killing of *Aspergillus fumigatus* and *Rhizopus arrhizus* by 4-fold and 15-fold, respectively (*P* < .05). In contrast, PMNL-mediated killing of *Candida albicans* was unaffected by G-CSF. The ability of aqueous fungal extracts to induce the PMNL respiratory burst was evaluated by luminol-enhanced chemiluminescence. G-CSF in vivo primed PMNL for sustained chemiluminescence in response to extracts of *Candida*, *Aspergillus*, and *Rhizopus* organisms. These data demonstrate that G-CSF in vivo augments antifungal activities of PMNL, thereby implicating a possible therapeutic role for G-CSF as a biologic response–modifying agent during opportunistic fungal infection.

Neutrophils (polymorphonuclear leukocytes, PMNL) represent the first and most important line of host defense against pathogenic opportunistic fungi in humans [1, 2]. Prolonged neutropenia and neutrophil dysfunction are among the strongest predictors for serious, life-threatening infections from these agents [1]. Despite aggressive antimicrobial therapy, severe morbidity and mortality are common clinical sequelae of these infections.

Recent studies have demonstrated that granulocyte colony-stimulating factor (G-CSF) not only increases the production of PMNL but also modulates the functional activity of developing and mature PMNL [3]. When PMNL from human immunodeficiency virus (HIV)–infected or bone marrow transplant patients are cocultivated with G-CSF in vitro, enhanced killing of *Candida albicans* has been observed [4, 5]. However, investigations examining the ability of G-CSF in vitro to promote PMNL-mediated killing of *C. albicans* have yielded variable results [6–9]. This increase in PMNL-mediated activity against *C. albicans* has been attributed, at least in part, to G-CSF priming of the inducible respiratory (or oxidative) burst [4–6, 10]. Similar effects have been observed in studies examining the effect of G-CSF in vitro on fungicidal activity of both normal and defective PMNL against *Aspergillus fumigatus*, a medically important opportunistic mold [11–13]. These results suggest that G-CSF may serve as a clinically useful immunomodulatory agent for the prevention and treatment of opportunistic fungal infections in selected patient populations [3–6, 8–13].

While the effects of G-CSF in vitro on PMNL-mediated antifungal activity have been well documented, the ability of G-CSF administered in vivo to modulate PMNL antifungal activity has received less attention in published reports. To assess whether G-CSF in vivo potentiates PMNL antifungal activity, we performed the present study, in which PMNL were obtained from normal human volunteers both before and after the administration of G-CSF. We then compared the fungicidal activity of these two types of PMNL against conidia from 3 species of medically important opportunistic fungi: *C. albicans*, *A. fumigatus*, and *Rhizopus arrhizus* (agent of zygomycosis [mucormycosis]). Furthermore, the ability of G-CSF in vivo to prime the inducible respiratory burst of PMNL in response to aqueous extracts from both the hyphal and conidial forms of these opportunistic fungi was evaluated.

**Materials and Methods**

**Materials.** Recombinant human G-CSF was provided as a gift by Amgen (Thousand Oaks, CA). *A. fumigatus* and *R. arrhizus* were stock strains from the National Committee for Clinical Laboratory Standards. *C. albicans* was a patient strain isolated from a
throats surveillance culture at the Fred Hutchinson Cancer Research Center. Stock fungal strains were maintained by standard microbiologic methods.

**Study design.** Three normal human volunteers recruited from the community (age, 24–35 years; weight, 65–74 kg) received 300 μg of G-CSF subcutaneously each morning for 5 consecutive days. All of the volunteers were nonsmokers, had normal physical examinations, were receiving no concomitant medications, and had not experienced an acute illness during the 6 weeks preceding the study. All volunteers underwent laboratory screening.

**PMNL purification.** Venous blood was collected from the normal human volunteers on day 0 before G-CSF administration and on day 6 (following administration of G-CSF for 5 consecutive days) and 24 h after the last dose of G-CSF, using 0.2% dipotassium EDTA as anticoagulant. PMNL were isolated by sequential sedimentation in Dextran T-500 (Pharmacia LKB Biotechnology, Piscataway, NJ) in 0.9% sodium chloride, centrifugation in Histopaque-1077 (Sigma, St Louis), and hypotonic lysis of erythrocytes

**Aqueous fungal extracts.** Fungal extracts were prepared by filter separation of culture filtrates. Conidia from *C. albicans*, *A. fumigatus*, and *R. arrhizus* were grown separately in 1-L volumes of RPMI 1640 (American Biorganics, Niagara Falls, NY) for 7 days at 37°C with constant aeration. The predominant growth forms of the mycelia under these conditions were blastospores for *C. albicans*, conidia for *A. fumigatus*, and sporangiogospores for *R. arrhizus*. However, hyphae were easily visible in all aliquots examined. The culture supernatant was separated from the mycelial mat by vacuum filtration through a sterile 0.2-μm pore size disposable filter (Zapcap-S; Schleicher & Schuell, Keene, NH), followed by a single wash with 500 mL of sterile water. This final filtrate product, designated as ‘extract,’ was frozen at −20°C in 2-mL aliquots until use. Protein concentrations of the fungal extracts were measured colorimetrically (BCA protein assay reagent; Pierce, Rockford, IL).

**Luminol-enhanced chemiluminescence.** Luminol-enhanced chemiluminescence was used as a sensitive measure of the respiratory burst of PMNL as previously described [15]. At the start of the assay, 10 μM luminol and the designated fungal extract (100 ng of protein/mL) was added to the reaction mixture containing 10^6 PMNL. Luminol-enhanced chemiluminescence was read for 10-s intervals at the designated times with a luminometer (Monolight 2001; Analytical Luminescence Laboratory, San Diego) set to integration mode. Chemiluminescence is reported as relative light units/10^6 PMNL/min.

**PMNL fungicidal activity.** Fungicidal activity was evaluated by serial dilution colony-forming unit (cfu) inhibition assay in a microtiter plate format as previously described [5]. Mycelia in resting phase were suspended in sterile water and stored at 4°C until use. Aliquots containing 10^6 conidia (determined by counting microscopically) of the designated fungus were incubated with 10^6 PMNL in RPMI plus 10% human serum for 2 h. Fungicidal activity is expressed as the percentage of cfu inhibition, where % fungal death = 100 − (cfu from experimental wells/cfu from control wells [no PMNL]) × 100.

**Statistical analysis.** Statistical differences were determined using Student’s two-tailed t test for independent means (not significant, *P* > .05).

**Results**

The absolute neutrophil count (ANC) in the venous blood of the 3 normal volunteers increased from a baseline value of 4025/μL (range, 2948–5346/μL) before administration of G-CSF to a mean value of 24,878/μL (range, 18,330–31,020/μL) on day 6 of the protocol after 5 consecutive daily doses of G-CSF. This increase in ANC induced by G-CSF in vivo was predominantly due to a large increase in morphologically mature PMNL. Bands were the least mature granulocytes identified in the peripheral blood smear and accounted for <5% of the ANC in each volunteer at the end of the protocol. The regimen of G-CSF was generally well-tolerated by the volunteers, with the only reported side effect being mild bone pain reported by 1 donor on day 5 of the study protocol.

The effect of G-CSF in vivo on PMNL-mediated fungicidal activity against opportunistic fungi was evaluated by cfu inhibition assay (figure 1). Fungicidal activity against *C. albicans* was not significantly affected by G-CSF, with 60%–70% killing observed with PMNL isolated from the normal volunteers both before and after administration of G-CSF in vivo. In contrast, G-CSF in vivo significantly enhanced PMNL-mediated killing of both *A. fumigatus* and *R. arrhizus* conidia (*P* < .05). Killing of *A. fumigatus* was increased ~4-fold, from 17% to 84%, in PMNL following the administration of G-CSF in vivo. The augmentation in PMNL-mediated killing of *R. arrhizus* was even more striking, where G-CSF in vivo increased killing of *R. arrhizus* ~15-fold (pre–G-CSF, 3%; post–G-CSF, 45%).
The ability of G-CSF to prime the PMNL respiratory burst in response to aqueous extracts from both the hyphal and conidial forms of opportunistic fungi was evaluated by luminol-enhanced chemiluminescence (figure 2). Negligible chemiluminescence was induced in control PMNL in response to extracts of *C. albicans*, *Aspergillus fumigatus*, and *Rhizopus arrhizus*. When control PMNL were preincubated for 60 min with G-CSF (1 µg/mL) in vitro, each of these extracts induced a rapid chemiluminescence response, which peaked at 10–15 min and declined to baseline values by 60 min. Chemiluminescence kinetics were markedly different following administration of G-CSF in vivo. In contrast to the relatively brief chemiluminescence primed by G-CSF in vitro, administration of G-CSF in vivo resulted in a population of PMNL capable of undergoing a sustained chemiluminescence, which was still evident 90 min after stimulation by each of the fungal extracts (figure 2A).

The maximal rate of PMNL chemiluminescence induced by specific fungal extracts was differentially affected by G-CSF in vitro and in vivo (figure 2B). Although peak PMNL chemiluminescence in response to each fungal extract was significantly
increased by G-CSF whether administered in vitro or in vivo (P < .05), the maximal rate of PMNL chemiluminescence induced by Candida extract was 4-fold greater in PMNL primed by G-CSF in vitro than in PMNL isolated following G-CSF in vivo. In contrast, extracts of both A. fumigatus and R. arrhizus induced greater peak rates of chemiluminescence in PMNL obtained from volunteers following G-CSF in vivo.

Discussion

This study demonstrates that G-CSF administered in vivo is capable of enhancing PMNL-mediated antifungal activity against some opportunistic fungi. Specifically, G-CSF in vivo potentiated fungicidal activity of normal PMNL against conidia of A. fumigatus and R. arrhizus but not C. albicans (figure 1). It should be noted, however, that these results do not exclude the possibility that G-CSF in vivo could enhance the candidial activity of defective PMNL, as in patients following bone marrow transplantation [5]. Moreover, hyphae of A. fumigatus and R. arrhizus were not used in our fungicidal assays.

The inducible respiratory burst is recognized to play an essential, but possibly not sufficient, role in the fungicidal activity of PMNL [1, 14]. In the present study, PMNL obtained following G-CSF administration in vivo demonstrated enhanced respiratory burst activity in response to extracts of hyphae and conidia of all 3 opportunistic fungi examined (figure 2). In contrast to the relatively rapid but brief respiratory burst primed by G-CSF pretreatment of PMNL in vitro, the respiratory burst responses induced by fungal extracts were generally slower in onset but sustained in PMNL following G-CSF in vivo (figure 2A). This potentiation of respiratory burst activity, resulting in greater production of reactive oxygen intermediates, may contribute to enhanced PMNL fungicidal activity. However, our results do not exclude the possibility that administration of G-CSF in vivo modulates other antifungal activities of PMNL aside from the respiratory burst [14].

This study extends our understanding of G-CSF modulation of PMNL activity against opportunistic fungi. Previous in vitro studies have established that G-CSF is capable of potentiating PMNL activity against Candida species and A. fumigatus [4–13]. Our results demonstrate that G-CSF in vivo favorably modulates PMNL host defense activity against 3 medically important opportunistic fungi. These findings provide further support for G-CSF as a potential therapeutic adjunct to conventional antifungal chemotherapy in the treatment of infections due to specific opportunistic fungal pathogens.

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References