Enhancement of Oxidative Response and Damage Caused by Human Neutrophils to *Aspergillus fumigatus* Hyphae by Granulocyte Colony-Stimulating Factor and Gamma Interferon

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Invasive aspergillosis is a serious fungal infection caused by the proliferation and invasion of *Aspergillus* hyphae in tissue. Neutrophils (PMNs) are the most important line of defense against *Aspergillus* hyphae. To investigate the role of granulocyte colony-stimulating factor (G-CSF) and gamma interferon (IFN-γ) against *Aspergillus fumigatus*, we studied the effects of the two cytokines on the oxidative burst and the capacity of normal human PMNs to damage hyphae of the organism. G-CSF enhanced PMN oxidative burst measured as superoxide anion (O$_2^-$) production in response to N-formylmethionyl leucyl phenylalanine, serum opsonized hyphae, and nonopsonized hyphae by 75, 37, and 24%, respectively, compared with control PMNs ($P < 0.015$). IFN-γ also induced increases of 52, 71, and 96%, respectively, in response to the same stimuli ($P < 0.006$). In addition, the capacity of PMNs to damage hyphae as measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric metabolic assay was significantly enhanced by G-CSF and IFN-γ ($P < 0.01$ and $< 0.05$, respectively). The enhancement was achieved irrespective of serum opsonization of the hyphae, suggesting upregulatory actions of the two cytokines on signal pathways specific for opsonized and nonopsonized hyphae. The combination of the two cytokines exhibited an additive effect at the higher concentrations compared with the effects of the cytokines alone ($P < 0.05$). Pretreatment of PMNs with protein synthesis inhibitors showed that IFN-γ activates PMN function through transcriptional regulation, whereas the effect of G-CSF does not require new proteins. These in vitro effects suggest modulatory roles for G-CSF and IFN-γ in the host defense against *Aspergillus* hyphae irrespective of serum opsonization and a potential utility of the cytokines as adjuncts for the prevention and possible treatment of invasive aspergillosis.

Invasive aspergillosis has emerged as an important cause of mortality in persistently neutropenic patients with cancer or aplastic anemia and in patients with phagocytic defects such as chronic granulomatous disease (12, 47, 48). An increased susceptibility to *Aspergillus* infections has also been described in patients with AIDS (14). *Aspergillus fumigatus* is the most common isolate from these patients. Although the alveolar macrophages have been shown to be the first-line effector cells that inhibit germination of the inhaled *Aspergillus conidia*, neutrophils (PMNs) are the cells that damage the escaping hyphae by secretion of microbial oxidase and possibly nonoxidative metabolites and thus prevent the establishment of invasive disease (15, 43). The study of PMN-hypha interactions has been largely facilitated by the recent adaptation of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric method (25, 32, 38) for the measurement of hyphal damage. Previous studies have shown that hyphae of other fungi such as *Candida albicans* are killed by PMNs following an oxidative metabolic burst (16, 26). Furthermore, in the case of *C. albicans*, PMNs respond to both opsonized and nonopsonized pseudohyphae with activation of the oxidative metabolic burst and subsequent release of microbial metabolites such as hydrogen peroxide ($\text{H}_2\text{O}_2$), hypochlorous acid, and chloramines. Hyphae of *Aspergillus* spp. are also susceptible to these microbicidal substances (24) and may elicit similar responses from the attacking PMNs.

Granulocyte colony-stimulating factor (G-CSF) and gamma interferon (IFN-γ) have been shown to upregulate certain antimicrobial PMN functions such as oxidative metabolic burst in response to soluble stimuli (9, 23), phagocytosis, and bactericidal activity of PMNs (19, 41, 44). In addition, our previous work has suggested that each of these cytokines enhances the oxidative metabolic burst of PMNs in response to particulate stimuli, such as *C. albicans* blastoconidia and pseudohyphae (40).

Little is known, however, about whether the effects of cytokines such as G-CSF and IFN-γ on the oxidative burst and fungicidal activities of PMNs against hyphae of *Aspergillus* spp. are similar to their effects on other fungi. Diversity of results among different fungi and cytokines has been previously reported by a number of investigators (17, 20, 29, 41, 42) and may be critical in pathogenesis and host defenses against specific fungal infections. Thus, in this study, we undertook to assess the effects of the two cytokines on the oxidative burst of normal human PMNs in response to hyphae of *A. fumigatus* and compare them with...
the soluble stimulus N-formylmethionyl leucyl phenylalanine (FMLP). In addition, we examined the effects of the cytokines alone or in combination on the fungicidal activity of PMNs (the National Institutes of Health, Bethesda, Md. Human AB normal serum was purchased from GIBCO Laboratories, Grand Island, N.Y.

Separation of PMNs. PMNs were prepared from heparinized (5 to 10 ml) venous blood by dextran sedimentation followed by centrifugation over Ficoll and hypotonic lysis of erythrocytes as previously described (4, 39). They were then suspended in HBSS without Ca²⁺ and Mg²⁺. The resulting cell preparations consisted of more than 95% viable PMNs as judged by trypsin blue exclusion and counting on a hemacytometer.

Priming of PMNs. Before the assays, PMNs (2 × 10⁶/ml) were incubated with various concentrations of G-CSF or IFN-γ in HBSS without Ca²⁺ and Mg²⁺ at 37°C for various periods. After this pretreatment, PMNs were used for assays without any further washing or other treatment. For some selected experiments, PMNs were pretreated with the protease synthesis inhibitors cycloheximide and actinomycin D for 15 min prior to and during the 90-min priming of the cells with cytokines. The concentrations of the inhibitors used were 1 and 5 μg/ml, respectively.

Superoxide production by PMNs. Production of superoxide anion by PMNs in response to the synthetic tripeptide FMLP and to hyphae of A. fumigatus was assessed by the superoxide dismutase-inhibitable reduction of cytochrome c spectrophotometrically (39). PMNs (10⁶) were mixed with 50 μM cytochrome c. As a stimulus, either 0.5 μM FMLP or hyphae in an effector-to-target cell (E/T) ratio of 1/1 were added to the PMNs in 1 ml of HBSS, and the mixture was incubated on a shaker at 37°C for 15 min. In some experiments, the hyphae were preopsonized by incubation in 50% AB human serum at 37°C for 20 to 30 min. The serum was derived from normal donors, and its complement activity was not defined. Control tubes containing all of the constituents listed above plus superoxide dismutase (40 μg/ml) or hyphae alone were also included. After incubation, the reaction tubes were centrifuged and the supernatants were removed. The amount of O₂⁻ produced was measured in the supernatants as the difference in A₅₅₀ for the reaction mixture and the control. For this purpose, a Gilford 260 spectrophotometer (Ciba-Corning Diagnostics Corp., Oberlin, Ohio) was used. Superoxide produced by 10⁶ PMNs was then calculated by using the millimolar extinction coefficient for reduced cytochrome c. For each individual donor, the amount of superoxide produced by control PMNs was subtracted from the amount of superoxide produced by cytokine-pretreated PMNs, and the absolute difference was calculated.

PMN-induced damage of hyphae. The damage to hyphae caused by PMNs was assessed by the MTT colorimetric assay previously described in detail for assessing viability of tumor cells (32) and fungi (25, 38). This assay takes advantage of the fact that metabolically active (growing) hyphae convert the yellow tetrazolium salt MTT to a blue formazan derivative with maximum absorbance at 560 to 570 nm, whereas damaged hyphae fail to do so (25, 38). Briefly, the supernatant of the wells was aspirated with a 19-gauge needle connected to a vacuum aspirator, leaving the hyphae attached at the bottoms of the wells. Particular care was taken not to let the hyphae dry for more than 1 to 2 min. The hyphae adhered firmly to the bottoms of the wells and were not detached from the plates by repetitive washings. PMNs that had previously been treated with buffer or various concentrations of G-CSF or IFN-γ at a final E/T ratio of 5/1, 10/1, or 20/1 and in a final volume of 1 ml of HBSS containing 20 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) buffer were added to the wells. In those experiments, in which assessment of the effect of cytokines on the killing of opsonized hyphae was the goal, 10% AB human serum (100 μl) was also added to each well. After the appropriate time of incubation at 37°C, supernatants were completely aspirated from the wells and PMNs were lysed by adding 300 μl of 0.5% sodium deoxycholate. The hyphae were then washed with sterile distilled water three times, 1 ml of RPMI 1640 with l-glutamine and without phenol red but containing 0.5% mg of MTT per ml was added to each well, and the plates were further incubated at 37°C for three additional hours. The plates were then aspirated dry, 200 μl of isopropanol was added to each well, and the plates were swirled gently until all of the blue precipitate had dissolved.
Volumes (150 μl) of isopropanol were transferred to the wells of a 96-well Immulon plate (Dynatech Laboratories, Chantilly, Va.), and the color was measured on a Titertek Multiscan microplate spectrophotometer (Flow Laboratories, McLean, Va.) at the wavelengths 570 and 690 nm. A well containing only isopropanol was used as a blank. Control wells containing hyphae and buffer only but not PMNs were included in each experiment. Fungicidal activity (damage) was calculated by using the following formula: % damage of hyphae = [(OD of control wells - OD of test wells)/OD of control wells] × 100, where OD is optical density and control wells are the wells containing hyphae and buffer only. Each condition was tested in duplicate or triplicate, and the results were averaged.

**Statistics.** Differences in superoxide production and hyphal damage between baseline and individual concentrations were assessed by unbalanced two-way analysis of variance, with PMN donor and concentration as factors. Least-squares regression analysis was employed to test for linear trends as functions of log(10 + concentration) or log(1 + concentration). In every regression model, the baseline for each donor was included as a parameter to be estimated. The distributions of residuals were consistent with normality. All $P$ values reported are two tailed and are derived from the analysis of variance unless otherwise stated.

**RESULTS**

**Effects of G-CSF on the oxidative metabolic burst.** We first investigated the oxidative metabolic burst of PMNs in response to *A. fumigatus* and whether this response is modulated by the two cytokines. Therefore, we measured the release of O$_2^-$ in response to either serum-preopsonized or nonopsonized hyphae, and we compared these responses with that to the soluble stimulus FMLP. Baseline superoxide productions were 3.43 ± 0.60 (13 experiments), 2.48 ± 0.41 (6 experiments), and 2.38 ± 0.70 (6 experiments) mmol/10$^6$ PMNs in response to FMLP, opsonized hyphae, and nonopsonized hyphae, respectively. In this study, we confirmed previous findings (23) that G-CSF enhances the O$_2^-$ production in response to FMLP (Fig. 1A). After 10 min of pretreatment with G-CSF, PMNs exhibited an enhanced oxidative burst in response to FMLP. However, the degree of enhancement was maximized after 30 min of pretreatment and remained high after as long as 120 min. Thus, in subsequent experiments, we used 30 to 90 min as the priming time.

In addition, G-CSF enhanced the oxidative metabolic burst in response to opsonized and nonopsonized hyphae (by regression analysis, $P < 0.015$). The stimulation of PMNs by opsonized and nonopsonized hyphae was slightly slower than that by FMLP, with 15 min being the minimum incubation period showing some degree of stimulation. The peak of enhancement against hyphae appeared to be achieved by concentrations of 1,000 to 4,000 U of G-CSF per ml (Fig. 1C and E). In contrast, enhancement with FMLP caused a different pattern of response to the cytokine, with higher concentrations of G-CSF (i.e., 10,000 U/ml) inducing an even higher degree of oxidative burst. For example, while concentrations of G-CSF as low as 100 U/ml slightly increased the oxidative burst in response to FMLP in three of four donors, only higher concentrations (1,000 to 10,000 U/ml) yielded statistically significant increases above the controls. Indeed, pretreatment with 10,000 U/ml had the highest overall effect on FMLP-stimulated O$_2^-$ production (an increase of 75%) compared with pretreatment of control PMNs with buffer ($P < 0.001$). The enhancement in response to both opsonized and nonopsonized hyphae was smaller (peak increases of 37% at 4,000 U/ml and 24% at 1,000 U/ml, respectively).

**Effects of IFN-γ on the oxidative metabolic burst.** As shown in Fig. 1B, IFN-γ significantly enhanced the production of O$_2^-$ in response to FMLP within a wide range of concentrations (100 to 5,000 U/ml) (by regression analysis, $P < 0.006$). In this series of experiments, the baseline superoxide productions, derived from 6 to 13 experiments, were 3.17 ± 0.50, 1.66 ± 0.45, and 1.63 ± 0.31 nmol/10$^6$ PMNs in response to FMLP, opsonized hyphae, and nonopsonized hyphae, respectively. The differences between the baseline values given above and those from experiments with G-CSF are not statistically significant. The concentration of IFN-γ that induced the highest degree of overall enhancement of FMLP-stimulated O$_2^-$ production appeared to be 5,000 U/ml. The optimal time of pretreatment of PMNs was 90 min, and no further enhancement was noted with longer pretreatment times. The greatest enhancement of FMLP-stimulated O$_2^-$ production observed (90 min of pretreatment with 5,000 U/ml) was 52% above the O$_2^-$ production of control cells ($P = 0.007$).
IFN-γ also enhanced the oxidative metabolic burst in response to opsonized A. fumigatus hyphae in a pattern similar to that of G-CSF (Fig. 1D). While the slope of enhancement of O$_2^-$ production in response to FMLP continued to rise with concentrations higher than 1,000 U/ml, the curve of O$_2^-$ production in response to either serum-preopsonized or nonopsonized hyphae reached a peak in the range of concentrations from 100 to 1,000 U/ml. In contrast to G-CSF, however, the increase of O$_2^-$ production in response to hyphae was larger than that in response to FMLP. For example, a 71% peak increase over that with control PMNs was achieved in response to opsonized hyphae after pretreatment of the cells with 1,000 U of IFN-γ per ml. The peak increase of O$_2^-$ production in response to nonopsonized hyphae achieved at 100 U/ml was 96% above the control.

Effects of G-CSF on the fungicidal activities of PMNs against opsonized hyphae. Since hyphal damage correlates with release of oxidative metabolites by PMNs (15), we also evaluated the effects of the two cytokines on the fungicidal activities of PMNs against A. fumigatus hyphae. Figure 2A illustrates the effects of various concentrations of G-CSF on the damage to opsonized hyphae caused by PMNs at three different E/T ratios. At all three E/T ratios, hyphal damage was increased after treatment with relatively low concentrations of G-CSF (500 to 1,000 U/ml), with no further enhancement at higher concentrations. The E/T ratio that showed the most significant differences, however, was 5/1, with concentrations of 1,000 and 10,000 U/ml giving results statistically different from those with the control (P = 0.001 and 0.002, respectively). To test for any possible direct fungicidal effects of the two cytokines on the survival or metabolic activities of the hyphae, hyphae were incubated with the cytokines at the highest concentrations used in this study. Neither of the two cytokines had an adverse direct effect on the hyphae of A. fumigatus.

In additional time course experiments with PMNs and opsonized hyphae, no enhancing effect of G-CSF on PMN antihyphal activity was found at the early stages of the reaction (30 and 60 min of incubation). There was a consistent difference between control and G-CSF-primed PMNs at a later stage of incubation (120 min) in all the PMN donors. However, because of the small number of experiments, this difference did not reach significance (Fig. 2B). The difference at 120 min was due instead to a longer-lasting capacity of the G-CSF-treated PMNs to inhibit the further growth of hyphae.

Effects of IFN-γ on the fungicidal activities of PMNs against opsonized hyphae. Figure 3A shows the results of five experiments examining the effects of various concentrations of IFN-γ on the damage caused by PMNs on opsonized hyphae. At an E/T ratio of 5/1, hyphal damage was significantly increased by concentrations of IFN-γ ranging from 100 to 1,000 U/ml. Thus, at 100 U/ml, the increase of fungicidal activity was 2.76 times higher than that with the control, whereas at 1,000 U/ml, the fungicidal activity was 3.28 times higher than that with the control (P = 0.025 and 0.027, respectively).

Time course experiments on the PMN-opsonized hyphal reaction were also performed to further explore the way IFN-γ enhances PMN antihyphal activity. IFN-γ-primed PMNs significantly differed from the controls by damaging hyphae sooner after the initiation of the incubation (30 and 60 min), whereas there was no significant difference between primed and unprimed PMNs at 120 min of incubation (Fig. 3B). This pattern of enhancement was different from that of G-CSF (Fig. 2B), the latter cytokine inducing a more delayed and prolonged increase in antihyphal activity during the PMN-hypha reaction and keeping PMNs active longer.

Effects of G-CSF and IFN-γ on the fungicidal activities of PMNs against nonopsonized hyphae. Since nonopsonized hyphae of A. fumigatus are able to stimulate primed PMNs for enhanced O$_2^-$ production, the effects of the two cytokines on the fungicidal activities of PMNs against nonopsonized hyphae were also studied. Figure 4A shows the effect of G-CSF on PMN activity against nonopsonized hyphae. At an E/T ratio of 5/1 and after 120 min of incubation at 37°C, the cytokine significantly enhanced the fungicidal activities of the PMNs against nonopsonized hyphae (P = 0.019). Similarly, IFN-γ also enhanced the antihyphal activities of PMNs against nonopsonized A. fumigatus hyphae (Fig. 4B; P < 0.001).
FIG. 3. (A) Percent hyphal damage of A. fumigatus caused by PMNs pretreated with either buffer or various concentrations of IFN-γ. PMNs were pretreated with the indicated concentrations of IFN-γ for 90 min and were then incubated with 105 hyphae in the presence of 10% human AB serum for 60 min at 37°C. An E/T ratio of 5/1 was used. (B) Time course study of the effect of IFN-γ on the percent hyphal damage caused by PMNs against A. fumigatus. PMNs were pretreated either with buffer (●) or with 1,000 U of IFN-γ per ml (○) for 90 min at 37°C. IFN-γ had no direct effect on the survival or growth of A. fumigatus hyphae. Results were derived from five experiments. Vertical bars indicate standard errors of the means. *, difference from the control, P < 0.05.

Effects of the combination of G-CSF and IFN-γ on the fungicidal activities of PMNs. Judged from the data given above, each cytokine appeared to have the capacity to enhance PMN activity against A. fumigatus hyphae. To test the hypothesis that the two cytokines combined might be synergistic, we studied the effects of the combination of the two cytokines on the antihyphal activities of PMNs and compared this effect with those obtained by each cytokine separately and with the antihyphal activities of the control PMNs. The data presented in Fig. 5, which were generated by using nonopsonized hyphae and an E/T ratio of 5/1, confirm the previous findings that both G-CSF and IFN-γ enhance the antihyphal activities of PMNs. In addition, they show that the combination of G-CSF (4,000 U/ml) and IFN-γ (1,000 U/ml) has an additive effect in priming PMNs against nonopsonized hyphae compared with the effect of the two cytokines alone at similar concentrations. For example, while IFN-γ (1,000 U/ml) alone caused 35.0% ± 6.5% hyphal damage and G-CSF (4,000 U/ml) alone caused 39.8% ± 6.5% damage, the combination of G-CSF (4,000 U/ml) and IFN-γ (1,000 U/ml) caused 46.3% ± 4.8% (difference between IFN-γ alone and IFN-γ plus G-CSF, P < 0.05).

Effects of protein synthesis inhibitors on the immunomodulatory abilities of the two cytokines. Since both G-CSF and IFN-γ have the capacity to enhance PMN activity against hyphae of A. fumigatus (although patterns of optimal times of priming, concentrations of the cytokines, and times of PMN-hyphae incubation to obtain maximal antihyalp activity are different), it seemed important to study the steps in which the two cytokines act in the PMNs. For this purpose, the protein synthesis inhibitors actinomycin D, which inhibits DNA-dependent mRNA transcription, and cycloheximide, which inhibits protein synthesis at a posttranscriptional level, were employed. PMNs were first treated with either cycloheximide or actinomycin D and then with the two cytokines or buffer, following which the antihyalp activities of these cells were assessed.

After the cells had been treated with the two inhibitors and then primed with the cytokines, diverse effects caused by the two inhibitors were observed. As shown in Fig. 6 (top panel), G-CSF (4,000 U/ml), IFN-γ (100 U/ml), and the combination of both cytokines (4,000 and 100 U/ml, respectively) enhanced the fungicidal activity of PMNs. Indeed, in
FIG. 5. Effects of G-CSF and IFN-γ alone or in combination on the antihyphal activities of PMNs against nonopsonized A. fumigatus hyphae. The data are presented as means ± standard errors of the means of 12 or 13 separate duplicate experiments. PMNs were pretreated with buffer, G-CSF (4,000 U/ml), IFN-γ (100 or 1,000 U/ml), or a combination of the two cytokines (4,000 U of G-CSF and 100 or 1,000 U of IFN-γ per ml) for 90 min. The antihyphal activities of PMNs were assessed after a 2-h incubation of the cells with 10⁵ nonopsonized hyphae at 37°C. The E/T ratio used was 5/1.

In these experiments, the results of the experiments shown in Fig. 5 were confirmed; i.e., the combination of G-CSF and IFN-γ is more active than either of the two cytokines alone. When cycloheximide was used to pretreat the cells, both unprimed and IFN-γ-primed but not G-CSF-primed PMNs showed a dramatic inhibition of their antihyphal activities (middle panel). However, when actinomycin D was used to pretreat the cells before they were primed with cytokines, only IFN-γ-primed PMNs were inhibited, whereas unprimed and G-CSF-primed PMNs were not affected (lower panel). Cells pretreated with the combination of G-CSF and IFN-γ showed intermediate results compared with cells treated with the two cytokines separately.

These data suggest that the enhancing effect of G-CSF on the antihyphal activities of PMNs does not require the synthesis of new proteins (probably occurring through up-regulation of already existing receptors or messengers), whereas the enhancement due to IFN-γ occurs through regulation of the transcription of new mRNA (as suggested by actinomycin D-induced inhibition). The finding that the antihyphal activity of unprimed PMNs is inhibited by cycloheximide but not by actinomycin D suggests that new proteins participating in the oxidative metabolic pathway of the cell for the killing of the organism may have to be constructed at a posttranscriptional level.

DISCUSSION

In this study, we have shown that (i) both G-CSF and IFN-γ enhance the oxidative metabolic bursts of PMNs in response to Aspergillus hyphae; (ii) these effects are independent of preopsonization of the hyphae with serum; (iii) the enhancement of the oxidative metabolic burst is associated with increased hyphal damage caused by PMNs, also regardless of the presence of serum for opsonization; (iv) the combination of the two cytokines has an additive effect at higher concentrations; (v) there are differences in time and concentrations required for the two cytokines to exert their effects on PMNs; and (vi) the biologic step of activation of PMNs is quite distinct for G-CSF and IFN-γ.

The results of our study of the effects of G-CSF and IFN-γ on PMN activity against Aspergillus spp. are consistent with the findings of previous studies that have examined the effects of these two cytokines on other aspects of PMN function in vitro and in vivo. Those studies have shown that the two cytokines have enhancing properties against a variety of bacteria both in vivo and in vitro (1, 8, 10, 11, 21–23, 27, 28, 41, 49). Their modulatory effects on the fungicidal activity of phagocytes, however, have been found to be more variable, being cytokine dependent and organism specific. For example, G-CSF has been found to enhance bactericidal activity against Staphylococcus aureus but not fungicidal activity against C. albicans blastoconidia (1, 41). More recently, G-CSF has been found to enhance the PMN oxidative burst in response to C. albicans pseudohyphae (40).

In addition, IFN-γ has been found to enhance a number of different microbicidal functions of PMNs such as expression of high-affinity FcyRI receptors (35), phagocytosis of latex beads (34, 46), oxidative metabolic burst (2, 34, 46), and bactericidal activity against S. aureus (19). However, its effects on the fungicidal activities of phagocytes are variable. IFN-γ enhances fungicidal PMN activity against Blastomyces dermatitidis, whereas the findings with C. albicans blastoconidia are discordant (18, 29, 30, 34). Similarly, IFN-γ does not affect the fungicidal activities of human macrophages against Histoplasma capsulatum conidia (20) or of corticosteroid-treated macrophages against Aspergillus conidia (42). In contrast, IFN-γ has recently been found to enhance PMN oxidative burst in response to C. albicans pseudohyphae (40) and PMN fungicidal activity against C. albicans pseudohyphae (17).

Our findings demonstrate that the two cytokines play an important role in PMN host defenses against Aspergillus spp., modulating the phagocytes' activity in response to...
were able to challenge with studying. Despite the interexperimental variation of the baseline PMN activity, we were able to demonstrate significant increases of fungicidal activity within the same set of experiments and PMN donors due to cytokine pretreatment of PMNs. Our results with IFN-γ are paralleled by a recently reported ex vivo study of patients with chronic granulomatous disease (known to have defective PMN function) receiving IFN-γ who showed an increase in antiphagocytic activity (37). In this study, we correlated enhancement of fungicidal activity with an increase of oxidative burst in response to the same organism. Moreover, we demonstrated that G-CSF possesses a similar property that enhances fungicidal activity and that the two cytokines are synergistic. Potential effects of the two cytokines on nonoxidative fungicidal mechanisms, however, have not been investigated here and are worth studying. Nonoxidative mechanisms do contribute to the fungicidal activities of PMNs. Up to this time, however, to our knowledge, there have been no studies investigating the effects of cytokines on specific components of the nonoxidative microbial armamentarium of the PMN.

Our data suggest that PMN activity against *Aspergillus* hyphae (both oxidative metabolic burst and hyphal damage) can be enhanced by lower concentrations of G-CSF (500 to 1,000 U/ml) than are required for other PMN activities such as oxidative metabolic burst in response to FMLP and bactericidal activity (the peak occurring around 4,000 U/ml) (41) and PMN activity against *C. albicans* blastoconidia (significant enhancement not obtained by concentrations up to 10,000 U/ml). The basis for these organism-specific differences remains to be elucidated.

Opsonized *A. fumigatus* hyphae stimulate the phagocytic cells by a mechanism different from that of the soluble stimulus FMLP. Thus, while FMLP stimulates the cell membrane by interaction with specific receptors, opsonized hyphae stimulate the cell by attachment and interaction of opsonins with Fc and C' receptors. Our findings suggest that serum-opsonized *A. fumigatus* hyphae stimulate the oxidative burst of normal PMNs but with a somewhat lower production of O₂⁻ than occurs after stimulation with FMLP. The finding of enhanced PMN stimulation against opsonized hyphae by the two cytokines is consistent with previous reports that G-CSF and IFN-γ modulate FcyRII receptors on the PMN membrane and that IFN-γ additional increases the expression of FcyRI receptors (6, 7). The increased expression of the receptors may lead to a more efficient interaction of the cell with opsonized organisms and consequently to enhanced oxidative metabolic burst in response to these organisms.

Of note, the two cytokines were able to enhance the oxidative burst and hyphal damage even in response to nonopsonized particulate stimuli. To our knowledge, this is a novel finding: that is, cytokines can enhance PMN stimulation in response to organisms through non-opsonin-specific receptors and intracellular pathways. Such receptors have been found in the case of *C. albicans* pseudohyphae (manose receptors) (13). Similar receptors may be involved in the case of *Aspergillus* spp., and G-CSF and IFN-γ may upregulate their numbers and/or affinities in a manner analogous to the upregulation of FMLP receptors. However, to our knowledge, PMN receptors for cell wall surface components of *Aspergillus* hyphae have not yet been described. Another possibility is that the two cytokines modulate the early responses that are elicited after initial stimulation of the cellular membrane receptors, although it has recently been reported that only minor enhancement by IFN-γ occurs at the early steps of the oxidative burst in response to *C. albicans* hyphae (17). Nevertheless, this independence from serum is biologically intriguing and may be clinically useful in the activation of PMNs against *Aspergillus* hyphae at sites with limited supply of opsonins.

The finding of differential effects of protein synthesis inhibitors on the augmentation of antiphagocytic activity of PMNs by the two cytokines helps in understanding the intracellular pathways involved in priming the PMN by G-CSF and IFN-γ. Very little is known about the signal transduction of the two cytokines. G-CSF appeared not to require synthesis of new proteins (probably acting through upregulation of already existing receptors and protein kinase C), IFN-γ appeared to prime the microbialic mechanisms of PMNs through regulation of the transcription of new mRNA. In agreement with our results, the effect of IFN-γ on the fungicidal activity of murine macrophages has recently been found to be inhibited by cycloheximide (5).

The maximal enhancement in response to G-CSF is achieved by concentrations ranging from 500 to 4,000 U/ml. These concentrations are well within the range of levels in serum achieved in patients receiving the recombinant cytokine intravenously or subcutaneously and correlate with PMN response (3, 31, 45). In contrast, although the concentrations of IFN-γ that were shown in this study to have an effect (100 to 1,000 U/ml) are achievable clinically, there does not appear to be a correlation between levels of IFN-γ in serum and degree of in vivo phagocytic activation (33).

The findings of this study may have clinical implications and suggest that PMNs can be primed by G-CSF and IFN-γ to possess enhanced activity against *Aspergillus* spp., a property that may prevent invasive aspergillosis in immunocompromised patients. Whether the results presented in this report correlate with in vivo activation is not known at present. Nevertheless, since PMNs are the main effector cells against hyphal forms of important opportunistic fungi such as *Aspergillus* spp., these in vitro effects may be associated with increased in vivo stimulation of PMNs, increased subsequent killing of the hyphae, and clearing from the infected tissues. In support of our data, administration of G-CSF was recently reported to prolong survival in normal and immunocompromised mice infected with *A. fumigatus* (36). Thus, they may be particularly important in amplifying host defenses against invasive aspergillosis in immunocompromised hosts. These cytokines alone or combined with antifungal agents may also have an effect on the treatment of established invasive disease. In the future, G-CSF and IFN-γ may offer an adjunct to antifungal chemotherapy in prevention and treatment of fungal infections in immunocompromised patients, and appropriate studies appear worthy of investigation.

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