Granulocyte-macrophage colony-stimulating factor and interferon-γ prevent dexamethasone-induced immunosuppression of antifungal monocyte activity against Aspergillus fumigatus hyphae

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Treatment with corticosteroids is an important risk factor for development of invasive aspergillosis. We evaluated the effect of dexamethasone (DEX) on superoxide anion (O₂⁻) release and damage caused by elutriated human monocytes (EHM) on unopsonized hyphae of Aspergillus fumigatus. In addition, we studied the effects of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon-γ (IFN-γ) on these functions of DEX-treated EHM. Treatment of EHM with concentrations of DEX ranging from 5 to 500 nM (1.4–140 ng ml⁻¹) for 48 h suppressed O₂⁻ release in response to phorbol myristate acetate in a dose-dependent fashion. Similarly, DEX significantly suppressed hyphal damage caused by EHM as measured by colorimetric MTT assay. Both GM-CSF (5 ng ml⁻¹) and IFN-γ (1.2 ng ml⁻¹) added at day 0 to the EHM together with DEX (500 nM) significantly enhanced O₂⁻ release and percentage hyphal damage, preventing the DEX-induced suppression of EHM function. Thus, GM-CSF and IFN-γ prevented the deleterious effects of DEX on antifungal activity of EHM against Aspergillus suggesting a potential therapeutic role in patients at risk for or suffering from invasive aspergillosis.

Introduction

Treatment with corticosteroids is an important risk factor for development of invasive aspergillosis leading to excessive morbidity and mortality among immunocompromised patients [1–4]. Aspergillus fumigatus is the most frequently isolated species from these cases. Peripheral blood polymorphonuclear and mononuclear phagocytes play a critical role in antifungal host defences against Aspergillus spp. by secreting microbicidal oxidative metabolites and causing damage to hyphae. In cooperation with pulmonary alveolar macrophages, which inhibit germination of inhaled conidia of Aspergillus spp., they prevent the establishment of invasive disease [5–7].

Pharmacological concentrations of corticosteroids have been previously found to cause immunological defects in vivo and in vitro [8]. In particular, corticosteroids can suppress certain monocyte/macrophage functions, including release of oxidative and non-oxidative metabolites (9–11), as well as antibacterial and antifungal activities [12–14]. Of note, DEX has been recently shown to decrease the amount of GM-CSF secreted by lipopolysaccharide-treated monocytes [15]. In addition, mononuclear cells from normal donors treated with corticosteroids in vitro have shown suppressed antifungal activity against hyphae of Aspergillus spp. [16]. Moreover, animal studies of experimental aspergillosis have demonstrated increased mortality following treatment with corticosteroids [17,18].

Defining the effects of corticosteroids on antifungal activity of elutriated human monocytes (EHM) could further elucidate the increased susceptibility of corticosteroid-treated patients to aspergillosis and may lead to design of preventive strategies by use of immunomodulators. For example, IFN-γ has been shown to
upregulate specific surface receptors and functions of corticosteroid-treated monocytes [10,19]. Whether GM-CSF and IFN-γ can prevent the corticosteroid-associated defects of monocytes against hyphae of *Aspergillus* spp. is not known but may have important therapeutic implications for patients receiving immuno-suppressive doses of these frequently used compounds. Therefore, the goals of this study were to evaluate the effect of dexamethasone (DEX) on oxidative burst and on antifungal activity of EHM and the effects of IFN-γ and GM-CSF in preventing potential dexamethasone-mediated suppression of these functions.

**Materials and methods**

**Elutriated monocytes**

Monocytes were separated from venous blood of healthy adult volunteers by use of a two-step procedure consisting of automated leukapheresis and counterflow centrifugal elutriation in a Fenwal CS3000 continuous flow separator (Fenwal Laboratories, Deerfield, IL) at the Transfusion Medicine Department, Clinical Center, NIH, Bethesda, MD [20]. The EHM were counted on a haemocytometer and resuspended in HBSS without Ca²⁺ and Mg²⁺. The viability of resulting cell preparations was higher than 95% by Trypan blue exclusion and > 90% of cells were monocytes as found by modified Wright–Giemsa stain and/or nonspecific esterase stain.

**Treatment of EHM with DEX and cytokines**

Endotoxin-free dexamethasone was purchased from Sigma Chemical (St Louis, MO). Recombinant human GM-CSF produced in yeast was kindly provided by Immunex (Seattle, WA), and had a specific activity of > 5 × 10⁷ U mg⁻¹ of protein. Recombinant human IFN-γ was kindly provided by Genentech (South San Francisco, CA), and had a specific activity of 4 × 10⁷ U mg⁻¹. Both preparations contained < 0.1 ng mg⁻¹ of protein endotoxin as tested by the limulus amoebocyte lysate assay.

The EHM were cultured in tissue-culture flasks (Costar, Cambridge, MA) containing RPMI-1640 supplemented with 10% FCS, 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin in a cell density of approximately 10⁶ cells per ml of medium at 37°C and 7% CO₂ for up to 4 days. DEX and cytokines were present during the total period of culture, and medium was not changed. Prior to assessing the antifungal activity, monocytes were gently scraped off the flask, washed, resuspended in HBSS free of Ca²⁺ and Mg²⁺ and counted on a haemocytometer.

**Organism**

Strain #4215 of *A. fumigatus* isolated from a cancer patient with invasive aspergillosis was used in these studies. The strain was preserved on frozen potato dextrose agar slants at -70°C. Conidia were harvested by scraping the surface of the slants and suspending them in phosphate buffered saline as previously described in detail [21]. One day before experiments, 1 ml aliquots of a suspension of 10⁵ conidia per ml in yeast nitrogen base supplemented with 2% glucose were plated in each of the wells of 24-well plates (Costar), and incubated approximately 16 h at 30°C to permit germination.

**Superoxide anion release by EHM**

Release of superoxide anion (O₂⁻) by EHM in response to phorbol myristate acetate (PMA; Sigma Chemical) was assessed by the superoxide dismutase-inhibitable reduction of cytochrome C [22]. One million EHM were mixed with 50 µM cytochrome C (Sigma Chemical) and 500 ng ml⁻¹ PMA as stimulus in 1 ml HBSS; this mixture was incubated on a shaker at 37°C for 15 min. The change in absorption at 550 nm was measured on a Gilford 260 spectrophotometer (Ciba-Corning Diagnostics, Oberlin, OH).

**Damage of hyphae caused by EHM**

The hyphal damage caused by EHM was assessed by the colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as previously described in detail [23,24]. The amount of MTT reduction has been found to be proportional to the number of live hyphae existing in wells [24]. Briefly, the supernatants of wells seeded with fungi were aspirated and EHM, that had been incubated with either culture medium only or medium containing DEX and/or GM-CSF and/or IFN-γ, were added to the wells in final effector-to-target cell (E/T) ratios of 1/1, 5/1, 10/1 and 20/1. After 2 h incubation at 37°C, supernatants were aspirated, EHM were lysed by adding 300 µl of 0.5% sodium deoxycholate and hyphae were washed three times with sterile water. Subsequently, 1 ml of RPMI-1640 without phenol red containing 0.5 mg ml⁻¹ MTT was added to each well, and the plates were further incubated at 37°C for 3 h. The wells were then aspirated dry, 200 µl of isopropanol was used to extract the dye in each well, volumes of 150 µl were transferred into the wells of a 96-well plate, and the colour was measured on a Titerette Multiscan microplate spectrophotometer (Flow Laboratories, McLean, VA) at the dual wavelength 570/690 nm. A well containing isopropanol alone was used as a blank. Control wells containing hyphae and buffer only but not EHM were
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included in each experiment. Antifungal activity (hyphal damage) was calculated using the formula:

\[
\% \text{ MTT reduction} = \frac{\text{OD of control wells} - \text{OD of test wells}}{\text{OD of control wells}} \times 100
\]

where controls wells were the wells containing hyphae only. Each condition was tested in duplicate or quadruplicate and the results were averaged.

**Statistics**

To evaluate differences in the amount of \( O_2^- \) that was released or in the degree of MTT reduction that was achieved at particular concentrations from baseline, Student’s \( t \)-test was used. To compare multiple concentrations from baseline, unbalanced analysis of variance (ANOVA) was used. The \( P \) values reported are two-sided and derived from \( t \)-tests unless otherwise stated.

**Results**

**Effects of DEX on EHM superoxide release and antifungal activity**

The \( O_2^- \) release and antihyphal activity of EHM were first evaluated after *in vitro* treatment with DEX. Following 2 days of incubation, DEX significantly inhibited \( O_2^- \) release in a concentration-dependent manner (Fig. 1). Therapeutically achievable concentrations as low as 5 nM showed statistically significant inhibition (by ANOVA, \( P < 0.05 \)). Lower concentrations of 0.05 or 0.5 nM of DEX also demonstrated a consistent trend toward inhibition.

The percentage suppression of \( O_2^- \) release caused by incubation of EHM with 500 nM of DEX for 1 day (11.8 ± 3.7% below baseline) versus 2 days (46.9 ± 6.2% below baseline) were compared. Two days but not 1 day of incubation of EHM with DEX resulted in significant suppressive effect (different from baseline, \( P < 0.05 \)).

In addition, DEX significantly decreased antifungal activity of EHM at E/T ratios 10/1 (Fig. 2A) and 20/1 (Fig. 2B). The results presented were obtained by incubating EHM with 500 nM of DEX for 2 days. The differences between DEX-treated EHM and untreated controls were significant at both E/T ratios (\( P < 0.05 \)).

Similar suppressive effects of 500 nM of DEX on antifungal activity of EHM were observed at E/T ratios 1/1 and 5/1 after 2 days of incubation (data not shown). In addition, time-course experiments with 1, 2 and 4 days of culture showed that the effect of DEX peaked and became statistically significant only after 2 days of incubation of EHM with the corticosteroid. For example, after 2 days of incubation, DEX-treated EHM induced an MTT reduction of 15.5 ± 17.9% compared with 41.2 ± 8.8% for controls (\( P < 0.05 \)).

**Effects of IFN-γ and GM-CSF on superoxide release and antifungal activity of DEX-treated EHM**

The \( O_2^- \) release and antihyphal activity of EHM were then evaluated after *in vitro* simultaneous treatment with DEX and either GM-CSF or IFN-γ. To this aim, monocytes were incubated with DEX and either GM-CSF or IFN-γ for 2 days and the abilities of these cytokines to prevent DEX-induced suppression of \( O_2^- \) release and antifungal activity were evaluated. Figure 3 illustrates the effects of GM-CSF and IFN-γ on \( O_2^- \) released by DEX-treated EHM in response to PMA. Both GM-CSF (0.5 ng ml\(^{-1}\)) and IFN-γ (1.2 and 2.4 ng ml\(^{-1}\)) significantly counteracted DEX-induced inhibition of \( O_2^- \) release by EHM (\( P < 0.05 \)). Similarly, GM-CSF (5 ng ml\(^{-1}\)) enhanced antifungal activity of DEX (500 nM)-treated EHM against *A. fumigatus* hyphae as compared to DEX-treated controls at both E/T ratios 10/1 and 20/1 (Fig. 4). IFN-γ (1.2 ng ml\(^{-1}\)) enhanced the antifungal activity of EHM significantly only at E/T ratio 10/1.
Fig. 2 Hyphal damage of A. fumigatus caused by elutriated human monocytes incubated with either medium alone or with medium containing 500 nM dexamethasone (DEX) for 2 days. Monocytes that were either preincubated with DEX or with medium alone were added into wells containing 10^5 hyphae per well without serum and were incubated at 37 °C for 2 h. The various symbols connected with thin lines represent different monocyte donors (different experiments). The thick line connects means of donors and vertical bars indicate standard errors of means. The symbols of means are slightly moved to the right of actual positions for clarity in depicting the points of actual experiments. The effector-to-target cell ratios used in these experiments was 10/1 (A) and 20/1 (B). At both E/T ratios, * indicates differences of DEX-treated monocytes from controls significant with P < 0.05.

Discussion

In this study, we found that DEX suppressed the oxidative burst of EHM as well as the antifungal activity against hyphae of A. fumigatus in a concentration-dependent manner. Furthermore, we found that GM-CSF and IFN-γ counteracted the immunodeficiency in both functions of DEX-suppressed EHM.

As aspergillosis is an important complication of corticosteroid therapy, it was important to evaluate the underlying phagocytic activities against this organism during DEX treatment and the potential for modulation of these effects by recombinant cytokines. Our results demonstrating suppressive effects of DEX on oxidative burst and antifungal activity of elutriated monocytes are consistent with the results previously obtained with mixed mononuclear cells in vitro [16] and further offer an explanation of the increased frequency of invasive aspergillosis in corticosteroid-treated patients. Of note, considerable variation of individual donors' EHM functions and responses to DEX was observed. The relevance of this variability in DEX-induced suppression may be related to variable susceptibility of corticosteroid-treated individuals to aspergillosis.

The suppressive effect of DEX on O_2^- released by EHM was observed with concentrations as low as 5 nM (1-4 ng ml^-1). These concentrations can be easily achieved in clinically used dosages [25,26] and underscore the high degree of susceptibility of monocytes to corticosteroids.

GM-CSF and IFN-γ have enhancing effects on various functions of normal monocytes. For example, GM-CSF augments autocandidal and antiprotozoal activities of monocytes or monocyte-derived macrophages [27-29].

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The mechanisms by which GM-CSF and IFN-γ prevent the suppressive effect of DEX warrant further investigation. The findings of this study imply that activated monocytes become less susceptible to the action of DEX. It is possible that GM-CSF and IFN-γ upregulate the number and/or the affinity of the same receptors (e.g. Fcγ) on the surface of monocytes that DEX downregulates. As a direct NADPH oxidase stimulus (PMA) has been used, it is not possible to locate the modulatory effects from the surface of the phagocyte up to the oxidase. Further work is needed to elucidate the signal transduction pathway(s) of these cytokines in corticosteroid-treated phagocytes.

These findings enhance our understanding of the roles of GM-CSF and IFN-γ on host defences against *A. fumigatus* and also carry therapeutic implications. As peripheral blood monocytes constitute active effector cells against hyphal forms of important opportunistic fungi, such as *Aspergillus*, their cytokine-induced upregulation may lead to more efficient hyphal killing and prevention of establishment of infection at an early stage when a limited number of hyphae have invaded the tissues. Thus, the findings that corticosteroid-induced suppression of monocyte function is prevented by GM-CSF and IFN-γ may have clinical significance for prevention of invasive aspergillosis during corticosteroid treatment. In addition, the recent progress of separation by elutriation of large numbers of relatively pure monocytes from normal donors coupled with their property of longer life may make their transfusions to corticosteroid-treated patients with established aspergillosis in conjunction with cytokine treatment a potential therapeutic adjunct to antifungal chemotherapy. Further experimental animal and clinical studies are warranted to investigate this potential modality of immune reconstitution.

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**References**


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