Filgrastim Induces Lymphocytosis and Increases Ex Vivo Interleukin-2 Production in HIV-Infected Patients

Thomas Hartung, David L. Pitrak, MaryAnn Foote, Edward M. Shatzen, Stephen C. Verral, and Albrecht Wendel

Filgrastim induces lymphocytosis, including all T cell subsets, and increased ex vivo interleukin-2 (IL-2) release as well as lymphocyte proliferation. Since Filgrastim is increasingly used in patients with human immunodeficiency virus (HIV) infection, the effect of Filgrastim on ex vivo cytokine production was determined. Whole blood from 8 healthy volunteers, 5 high-risk volunteers, and 31 HIV-infected outpatients was assayed for cytokine production in response to endotoxin (LPS) or staphylococcal enterotoxin B (SEB) in the presence of absence of 100 ng/mL Filgrastim. LPS-inducible blood cytokine release of HIV-infected patients was different from that of normal or high-risk volunteers. The suppressive effect of Filgrastim on LPS-inducible blood tumor necrosis factor-α and interferon-γ formation in normal volunteers was not found in HIV-infected patients. Patients with advanced HIV infection showed reduced IL-2 and IL-4 release in the presence of SEB. In the presence of Filgrastim, IL-2 production was partially restored.

Filgrastim (granulocyte colony-stimulating factor, r-metHuG-CSF) is a hematopoietic growth factor primarily of neutrophilic granulocytes. It is used clinically to treat neutropenia due to myelosuppressive chemotherapy or radiation therapy, severe chronic neutropenia, human immunodeficiency virus (HIV) infection, and other diseases [1]. HIV-infected patients may develop neutropenia because of the myelosuppressive effects of HIV or because of some of the drugs used to treat these patients [2]. Both HIV-infected patients and HIV-infected leukocytes have shown a reduced ability to produce G-CSF [3–5]. Neutropenia appears to be a risk factor and contributes to the increased incidence of infections in neutropenic HIV-infected patients [6–8], and Filgrastim treatment has been shown to reverse neutropenia in HIV-infected patients and to decrease infection [9–14]. A number of functional defects of neutrophils have been reported for HIV-infected patients [15–17] that could be reversed by Filgrastim [18]. In addition, retrospective studies have demonstrated that Filgrastim has a beneficial effect on survival of patients with AIDS, which does not appear to be entirely due to prevention of infections [9, 19].

HIV-infected patients are known to develop major defects of the immune signaling system [20], in particular of the cytokine-mediator system. Some proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interferon (IFN)-γ, are found to be increased [5, 21–23] and have been implicated in the promotion of viral replication [24]. More recently, lymphokines, such as interleukin (IL)-2, have been thought to play an important role in the progression of HIV infection to AIDS. IL-2 formation by CD4 T cells appears to be mandatory in maintaining CD8 T cell control of viral replication [25]. A switch from Th1-type lymphokines (primarily IL-2) to Th2-type lymphokines (IL-4, IL-10) was suggested to represent the change from asymptomatic HIV infection to AIDS [26].

Studies have shown that G-CSF has a widespread effect as an immunomodulator controlling the functions and activity of mature neutrophilic granulocytes [27, 28] and has profound antiinflammatory effects on monocytes [29]. In healthy volunteers treated with Filgrastim, the ex vivo cytokine release pattern in stimulated whole blood was switched toward an antiinflammatory pattern. In another volunteer trial, we have studied the effect of Filgrastim treatment on lymphocyte functions (unpublished data). We found an increase not only in lymphocyte counts but also, in particular, in all T cell populations starting after 5 days of daily Filgrastim injection, with a relative maximum at day 8. In addition, 24 h after initiation of Filgrastim treatment, lymphocytic IL-2 release capacity was augmented, and ex vivo proliferation increased immediately after initiation of treatment.

With regard to the frequent use of Filgrastim in HIV-infected patients and the crucial role of IL-2 formation as well as T cell counts in these patients, pilot experiments with HIV-infected patients were carried out. We assessed the effect of Filgrastim added in vitro to HIV-infected patient blood stimulated to release cytokines. Blood from patients at advanced stages of HIV disease (as determined by low CD4 cell counts) was stimulated
Table 1. Demographic and hematologic distribution of study population.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>High-risk control</th>
<th>CD4 cell count/µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>38 ± 13</td>
<td>38 ± 3</td>
<td>&gt;500</td>
</tr>
<tr>
<td>AMC</td>
<td>.5 ± .1</td>
<td>.7 ± 2</td>
<td>200–500</td>
</tr>
<tr>
<td>ANC</td>
<td>4.3 ± 1.4</td>
<td>4.2 ± 1.8</td>
<td>100–199</td>
</tr>
<tr>
<td>APC</td>
<td>5.0 ± 1.3</td>
<td>5.2 ± 1.8</td>
<td>&lt;100</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black</td>
<td>2</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>White</td>
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<td>3</td>
<td></td>
</tr>
<tr>
<td>Risk factor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVDA</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Homosexual</td>
<td>0</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Heterosexual</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Transfusion</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Note. AMC = absolute monocyte count; ANC = absolute neutrophil count; APC = absolute phagocyte count; IVDA = intravenous drug abuse.

Materials and Methods

Patient population. The study population consisted of healthy volunteers (n = 8), people at high risk of HIV infection (n = 5), and HIV-positive patients as diagnosed by EIA (n = 29) (table 1). Outpatients of the Infectious Diseases Clinic were enrolled, and patients with HIV infection were stratified according to disease severity, as indicated by CD4 cell count: >500/µL (n = 8), 200–500/µL (n = 9), 100–200/µL (n = 6), and <100/µL (n = 8). All subjects with a current infection or history of Mycobacterium avium infection, or who were receiving Filgrastim, steroid, or non-steroidal antiinflammatory drug therapy, were excluded. Patients with HIV were allowed to continue standard care for their infection.

The high-risk control group consisted of patients who had risk factors for HIV infection but were seronegative for anti-HIV antibodies by EIA. These subjects were enrolled at the time of posttest counseling. We have found that this group shows evidence of immune activation, even in the absence of any symptoms or signs of any active infection or other inflammatory process.

Study design and laboratory measurements. The protocol was an open-label study. Citrate-treated blood samples were taken on a single occasion during routine visits to the outpatient clinic of the hospital. A portion of the blood was exposed in vitro to endotoxin (lipopolysaccharide, LPS) in parallel in the presence and in the absence of Filgrastim (100 ng/mL). These samples were incubated for 24 h before collecting supernatants for cytokine determination. In parallel, another portion of the blood was incubated for 48 or 72 h in the presence of SEB before assessing IL-2 and IL-4 levels.

Whole blood cytokine release. To assess whole blood cytokine response in a clinical setting, we used the following simplified experimental setup: Sterile glass 10-mL vials for blood collection (Vacutainer; Becton-Dickinson, Heidelberg, Germany) were filled with 4 mL of RPMI 1640 (Biochrom, Berlin) supplemented with penicillin-streptomycin and 2.5 IU of heparin (Liquemin; Hoffmann-La Roche, Grenzach-Wyhlen, Germany). In some vials, 50 µg of LPS from Salmonella abortus equi (Sigma, Deisenhofen, Germany) or 5 µg of SEB (Sigma) was added. One milliliter of freshly drawn citrated blood was injected into the vial with a 1-mL syringe. Control and LPS vials were incubated at 37°C for 24 h. SEB and more control vials were incubated similarly for 48 or 72 h. To end the incubations, the vials were shaken and centrifuged (300 g, 5 min). Cell-free supernatants were collected, and aliquots were frozen for cytokine measurements by EIA. Commercial EIA kits were used throughout to determine cytokine levels: IL-2, TNF-α, IL-4 (®gure 1C). Therefor 24 h before collecting supernatants for cytokine determination. was no significant difference in LPS-inducible TNF-α and IL-10 release from the different groups. The capacity of blood from healthy volunteers to produce IFN-γ in response to LPS exceeded that of high-risk controls (P < .01) and HIV-infected patients (P < .001). The stage of disease, as characterized by

by staphylococcal enterotoxin B (SEB) to release lymphokines, and the IL-2 formation was compared with that in blood from normal volunteers.

Results

Blood cytokine response to LPS. Incubation of diluted human whole blood in the presence of 10 µg/mL LPS resulted in the release of significant amounts of monokines such as TNF and IL-10 (figure 1A, B). A subsequent stimulation of lymphocytes led to the formation of IFN-γ (figure 1C). There was no significant difference in LPS-inducible TNF-α and IL-10 release from the different groups. The capacity of blood from healthy volunteers to produce IFN-γ in response to LPS exceeded that of high-risk controls (P < .01) and HIV-infected patients (P < .001). The stage of disease, as characterized by
However, in blood from HIV-infected patients, Filgrastim did not affect in vitro TNF release. LPS-inducible IL-10 release was not changed in any blood donor group in the presence of Filgrastim during the incubation. IFN-γ release in response to LPS was suppressed in the presence of Filgrastim in normal volunteer blood (mean 43% reduction; \( P = .008 \)), but no significant effect was seen in blood from high-risk volunteers or HIV-infected patients (figure 1C).

**Blood lymphokine response to SEB.** The restricted sensitivity of available EIAs does not allow determination of IL-2 serum levels as a measure of endogenous production of this prime T lymphocyte growth factor. We therefore used a whole blood assay to determine lymphokine release capacity. We used the superantigen SEB to induce significant amounts of the lymphokines IL-2 and IL-4 after 48 h of incubation. Assayed amounts of IL-4 increased by extending the incubation time to 72 h.

IL-2 release by whole blood is prone to considerable interindividual variances. However, these variances appear to reflect individual characteristics, since these differences remained unchanged in a study group of 20 healthy volunteers followed for several months (data not shown). Even among healthy blood donors, some failed to produce any significant amount of IL-2. In our study population, 3 of 7 normal volunteers and 3 of 5 high-risk volunteers failed to release significant amounts of IL-2 after 48 h of incubation in the presence of SEB. In HIV-infected patient blood, the number of nonproducers tended to increase with the stage of disease: 2 of 8 blood donors with CD4 cell counts \( \geq 500 \), 5 of 9 with counts between 200 and 500, 3 of 6 with counts between 100 and 200, and all 8 with \( < 100 \) CD4 cells/\( \mu L \) failed to produce significant amounts of IL-2 in response to SEB. In the presence of Filgrastim, however, 3 subjects with counts of 200–500, who did not respond to SEB alone, released significant amounts of IL-2.

The SEB-stimulated blood samples from HIV-infected patients who had CD4 cell counts \( < 500/\mu L \) produced significantly less IL-2 than did samples from healthy volunteers or HIV-infected patients with CD4 cell counts \( \geq 500/\mu L \) from the same study population (table 2) (\( P = .02 \)). This impaired IL-2 production was partially restored in vitro by Filgrastim. Significantly higher levels of IL-2 were detected when whole blood of HIV-infected patients with CD4 cell counts \( < 500/\mu L \) was incubated with SEB in the presence of Filgrastim (100 ng/mL), compared with IL-2 levels seen in blood incubated without the addition of Filgrastim (\( P = .037 \)) (figure 2). Filgrastim did not affect SEB-stimulated IL-2 production in blood from healthy or high-risk volunteers or HIV-infected patients with CD4 cell counts \( > 500/\mu L \) (table 2).

Significant amounts of IL-2 were produced by blood samples from patients with CD4 cell counts \( < 100/\mu L \) only after 72 h of incubation (figure 3A). Even in patients with advanced HIV infection, as evidenced by low CD4 cell counts, a trend toward increased IL-2 production in the presence of Filgrastim was observed (\( P = .078 \)).
Monokine (TNF-α) and lymphokine (IL-2 and IL-4) release in response to SEB.

Discussion

In conclusion, the in vitro effects of Filgrastim on blood cytokine response differ between healthy volunteers, in whom it exerts antiinflammatory activities, and HIV-infected patients, in whom it increases the formation of the lymphocyte growth factor IL-2.

G-CSF is a hematopoietic growth factor that also has immunomodulatory effects on mature neutrophils, such as increased oxidative burst and phagocytosis. These effects have led to the clinical use of Filgrastim in HIV-infected patients with neutropenia due to myelosuppressive drugs, such as zidovudine, or to HIV infection itself [9, 13]. The general augmentation of neutrophilic host defense by Filgrastim might have a bearing for opportunistic infections in patients with AIDS. Furthermore, a functional defect of neutrophils from HIV-infected patients has been reported [15, 16], which was restored with Filgrastim treatment [18]. These findings have raised hopes for clinical efficacy of Filgrastim beyond mere reversal of neutropenia, which were substantiated by retrospective case-control studies suggesting prolonged survival times [8, 19].

We have previously described that, when blood of healthy volunteers treated with Filgrastim was stimulated with a variety of immune activators, there was a change in the balance from proinflammatory to antiinflammatory cytokines [29]. Recently, a study in which volunteers were challenged with LPS 24 h after rhUG-CSF injection proved that this is also true in vivo [30]. HIV-infected patients are known to express increased amounts of proinflammatory cytokines, such as TNF-α [31] and IFN-γ [31, 32]. These proinflammatory mediators have been shown to represent endogenous stimuli promoting HIV replication [33–35]. Thus, Filgrastim treatment could also reduce the formation of proinflammatory factors in HIV-infected patients. However, we found that the suppression of TNF-α and IFN-γ formation did not occur in HIV-infected patients’ blood. However, it has to be noted that in our previous healthy volunteer studies [29], TNF-α and IFN-γ suppression by Filgrastim was much more pronounced when treating donors compared with mere addition of Filgrastim to blood in vitro. A study of Filgrastim treatment of HIV-infected patients is currently being undertaken to determine these antiinflammatory activities.

Table 2. Staphylococcal enterotoxin B–inducible interleukin (IL)-2 release in blood taken from patients with HIV and healthy volunteers in presence and absence of Filgrastim.

<table>
<thead>
<tr>
<th>Patient group</th>
<th>No Filgrastim added</th>
<th>Filgrastim (100 ng/mL) added</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal subjects</td>
<td>385 ± 101</td>
<td>369 ± 180</td>
<td>NS</td>
</tr>
<tr>
<td>HIV-positive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4 cells &gt;500/μL</td>
<td>553 ± 244</td>
<td>355 ± 77</td>
<td>NS</td>
</tr>
<tr>
<td>CD4 cells &lt;500/μL</td>
<td>158 ± 43</td>
<td>356 ± 133</td>
<td>.037</td>
</tr>
<tr>
<td>CD4 cells &lt;200/μL*</td>
<td>63 ± 22</td>
<td>199 ± 93</td>
<td>.078</td>
</tr>
</tbody>
</table>

NOTE. Data are mean ± SE. Blood was incubated in presence of 1 μg/mL staphylococcal enterotoxin B for 48 h. IL-2 was determined by ELISA. Only those whose samples released detectable amounts of IL-2 are included. NS = not significant (paired two-tailed Wilcoxon test).

* Exposed for 72 h rather than 48 h.

SEB-inducible IL-4 was determined as an indicator of Th2 lymphokines. In normal volunteers, 9 ± 2 pg of IL-4/mL of blood was released after 48 h in response to SEB. IL-4 amounts further increased to 41 ± 18 pg/mL of blood when the incubation time was extended to 72 h (figure 3B). IL-4 response of blood from HIV-infected patients tended to decrease in parallel with the decrease in CD4 cell counts. Blood from HIV-infected patients with <100 CD4 cells/μL released only 1.4 ± 0.8 and 2.1 ± 1.0 pg/mL of blood at 48 and 72 h of incubation, respectively (figure 3B). Addition of Filgrastim in vitro did not significantly alter SEB-inducible IL-4 release; that is, all 22 HIV-infected patients released 14 ± 5 pg of IL-4/mL of blood on SEB stimulation, while 16 ± 7 pg of IL-4/mL of blood was found on SEB stimulation in the presence of Filgrastim. Thus, increased formation of the Th1 lymphokine IL-2 in the presence of Filgrastim is not accompanied by a concomitant decrease in release of the Th2 lymphokine IL-4.

Discussion

Patients at advanced stages of HIV infection showed reduced lymphokine (IL-2 and IL-4) release in response to SEB. Monokine (TNF-α, IL-10) and IFN-γ release in response to LPS was not changed. In the presence of Filgrastim, a reduction in LPS-inducible TNF-α and IFN-γ release capacity was noted in healthy volunteers but was not found in blood from HIV-infected patients. In contrast, Filgrastim increased SE-inducible IL-2 formation in HIV-infected patient blood, while it had no effect in blood from healthy volunteers or HIV-infected patients with CD4 cell counts >500/μL. In conclusion, the in vitro effects of Filgrastim on blood cytokine response differ between healthy volunteers, in whom it exerts antiinflammatory activities, and HIV-infected patients, in whom it increases the formation of the lymphocyte growth factor IL-2.
The increased lymphocyte and CD4 cell counts is therefore of potential therapeuti
cbenefit in the treatment of HIV disease.

The mechanism by which Filgrastim improves IL-2 formation in HIV-infected patient blood remains obscure. Since this effect was not observed when Filgrastim was added to blood of healthy donors, a specific responsiveness of HIV-infected patient leukocytes must be assumed. Lymphocytes, at least from normal donors, in contrast to neutrophils and monocytes, do not bind G-CSF. Thus, an indirect effect by factors of either G-CSF-responsive leukocyte is most likely. However, monokine release was not significantly different in the presence of Filgrastim. The fact that Filgrastim restored IL-2 release to control donor levels argues in favor of G-CSF counteracting a suppressor present in HIV-infected patient blood. Future mechanistic studies will address the IL-2 release in response to immunostimuli other than SEB, IL-2 mRNA expression, and the effect of Filgrastim on isolated leukocyte populations.

Reduced IL-2 formation in HIV-infected patients is well known [23, 31, 37] and appears to be the cause of decreased lymphocyte proliferation and generation of cytotoxic T lymphocytes [38]. Whether this phenomenon, however, represents a crucial step in the pathogenesis of HIV disease is currently under discussion [39]. High levels of IL-2 formation were correlated with long-term nonprogression in HIV infection [40], and lymphocyte responsiveness is an independent predictor for survival in patients with HIV and AIDS [41, 42]. The fact that Filgrastim treatment of volunteers allowed ex vivo IL-2 release raised the question whether this drug might also alter IL-2 formation of HIV-infected patients. Patients with HIV infection have decreased CD4 cell counts, and treatment designed to increase IL-2 formation and CD4 cell counts may be particularly relevant to this patient population. Attenuated production of IL-2 in advanced HIV infection appears to play a major role in the loss of T helper cells. One part of this study, therefore, examined the effect of Filgrastim in vitro on IL-2 production in blood from patients with HIV infection. IL-2 is a lympho-
kine, the major growth factor for lymphocytes. In addition to the proliferation of CD4 and CD8 lymphocytes, IL-2 also enhances cytolytic activity of suppressor cells, enhances NK cell activity, and increases IFN-γ production. IL-2 infusions and subcutaneous injections cause a marked increase in the absolute CD4 lymphocyte count in patients with HIV infection. There is also potential benefit of increased numbers and cytolytic activity of CD8 lymphocytes, as these are the major effector cells in the host response against HIV infection.

An impairment in IL-2 production in patients with CD4 cell counts <500/μL was reversed by the presence of Filgrastim which is not associated with the flu-like symptoms observed with subcutaneous injections of IL-2. Endogenous IL-2 formation is believed to play a key role in the pathogenesis of HIV infection. The CD8 cells maintained by CD4 cell-derived IL-2 are known to suppress, in turn, HIV replication in CD4 cells [25, 43]. In line with these observations, recent studies
have documented a benefit from exogenous low-dose recombinant human IL-2 administration in HIV-infected patients [44]. The loss of sufficient IL-2 production capability was suggested to be the turning point for the progression from an asymptomatic to a symptomatic phase of HIV disease [45–47]. Reduced IL-2 formation by HIV-infected patient blood was suggested to reflect general loss of CD4 cells [48]. The fact that Filgrastim did restore IL-2 formation, however, argues in favor of additional blockades of lymphocytic IL-2 formation, which can be overcome in the presence of G-CSF. Increased endogenous IL-2 formation should contribute to an increase in CD4 cells, an observation of previous findings in HIV-infected patients treated with Filgrastim [49–51].

Additional studies designed to examine IL-2 production and lymphocytosis in Filgrastim-treated patients with HIV infection are warranted. Augmentation of endogenous IL-2 production in HIV-infected patients might be an alternative therapeutic approach to administration of recombinant human IL-2 with fewer side effects. In addition, Filgrastim, because of its well-known antinfectious effects, may improve the quality of life of HIV-infected patients and the course of their disease.

References


