A Single Dose of Granulocyte-Macrophage Colony-Stimulating Factor Induces Systemic Interleukin-8 Release and Neutrophil Activation in Healthy Volunteers


Granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) are frequently used in the clinical management of neutropenia. These cytokines not only enhance the proliferation of myeloid precursor cells but also influence the function of mature leukocytes. In a previous study, we found that the in vivo effects of G-CSF on neutrophils differed from those in vitro. In the present study, we investigated the effects of a single dose of recombinant GM-CSF (7.5 μg/kg, subcutaneously) on neutrophils, eosinophils, and monocytes in healthy volunteers. We analyzed leukocyte kinetics, phenotypical changes, neutrophil degranulation, and systemic cytokine production. After GM-CSF injection, phenotypical changes included upregulation of CD11b on all three cell types and a decreased expression of L-selectin and FcγRII on neutrophils. Neutrophil degranulation was evident from the increased plasma concentrations of lactoferrin and elastase. GM-CSF induced the release of interleukin-8 (IL-8), but not of IL-8 or tumor necrosis factor α. In comparison to the results from our previous study with G-CSF in healthy volunteers, GM-CSF induced a stronger activation of mature neutrophils but had a much less pronounced effect on the production and maturation of neutrophil precursors. These data may help to guide the choice between the two cytokines in different clinical situations.

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We studied the in vivo effects of a single dose of GM-CSF to gain more insight in the effects of this cytokine on the cells from the myeloid compartment in vivo. Previous in vivo work has shown that GM-CSF induces the upregulation of CD11b on neutrophils. Neutrophils from patients who were treated with GM-CSF also show an enhanced ex vivo production of superoxide anion. GM-CSF induces temporary neutrophil sequestration in the lungs, followed by specific granule release. To our knowledge, this is the first study that describes the effects of a single subcutaneous dose of GM-CSF on phagocyte kinetics, immunophenotype, degranulation, and systemic cytokine production in healthy volunteers. We analyzed our results in comparison to the results that we obtained in our earlier study on the effects of a single dose of G-CSF in healthy volunteers.

MATERIALS AND METHODS

Study design. Seven male volunteers (mean age, 33 years; range, 30 to 39 years) received a single subcutaneous dose of recombinant human GM-CSF (rhGM-CSF; Leucomax, 7.5 μg/kg; Schering-Plough/Sandoz, Basel, Switzerland). Medical history, physical examination, and routine laboratory investigations found normal results in all volunteers. No febrile illness had occurred 1 month before the treatment.

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study. Informed consent was obtained according to the rules of our hospital. All volunteers were observed clinically during the first 8 hours of the study.

Venous blood samples were drawn 3 days, 30 minutes, and immediately before GM-CSF was administered, as well as 5, 10, 15, and 30 minutes and 1, 2, 4, 8, 12, and 24 hours after GM-CSF was administered. Additional blood was drawn once daily during the first 8 days after injection; blood drawn 14 days after injection served as a control. Separate EDTA anticoagulated samples were drawn for leukocyte count and differentiation studies, for collection of plasma, and for immunophenotypical analysis. For leukocyte alkaline phosphatase (LAP) scores and serum GM-CSF levels, unpreserved blood was collected. The blood cell count and differentiation was determined using a routine laboratory procedure (H1 system; Technicon Instruments, Tarrytown, NY).

**Serum GM-CSF levels.** GM-CSF in serum was measured by enzyme-linked immunosorbent assay (ELISA). Plates were coated with monoclonal rat-anthuman GM-CSF (50 μL of 2 μg/mL in 0.1 mol/L NaHCO₃, pH 9.6; clone BV2D-23B8; PharMingen, San Diego, CA), followed by incubation with phosphate-buffered saline (PBS) containing 2% (vol/vol) milk. After the plates had been washed once, 50 μL of standard samples or sera was added to the wells and incubated at 37°C. Sera and standards were diluted in PBS containing in addition Tween 20 (0.02%, vol/vol), milk (2%, vol/vol), and normal human serum (2%, vol/vol). After 2 hours, the plates were washed and biotinylated rat-anthuman GM-CSF (1 μg/mL of antibody clone BV2D-21C11; PharMingen) was added. The antibody was diluted in PBS/Tween 20/milk plus 1% (vol/vol) normal mouse serum. After 45 minutes, the plates were washed and the antibody bound was detected with polymerized streptavidine-horseradish peroxidase (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service [CLB], Amsterdam, The Netherlands). As a standard, we used rhGM-CSF (Leucomax). The measuring range of this ELISA was 0.8 to 50 pg of GM-CSF per well. The recovery of GM-CSF added to serum was 100%. The interassay coefficient of variation was less than 8%.

**Tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and IL-8 concentrations.** The plasma concentrations of TNFα, IL-6, and IL-8 were measured with commercially available kits (CLB). The lower limits of detection are 3 pg/mL for TNFα and 5 pg/mL for IL-6 and IL-8.

**Immunophenotyping.** We used total white blood cell (WBC) suspensions for phenotypical analysis. Isolation and immunostaining of the WBC suspensions were performed strictly in the cold. This procedure minimizes the artifacts that are associated with granulocytes for phenotypical analysis. Isolation and immunostaining of the WBC suspensions were performed with a panel of mouse monoclonal antibodies (MoAbs) diluted in appropriate concentrations in PBS-BSA supplemented with 0.01% NaN₃ (wt/vol). The following MoAbs were used: OKM-1 (CD11b), CLB-FcRγI (FcγRIII, CD16), IV.3 (FcγRII, CD32), HP1/2 (VLA-4, CD49d), 84H10 (ICAM-1, CD54), Leu-8 (L-selectin, CD62L), CLB-435 (CD63), 32.2 (FcγRI, CD64), and B13.9 (CD66b, formerly CD67).

**LAP scores.** Unpreserved blood smears were dried in air, fixed with ice-cold 10% formaline methanol (vol/vol) for 1 minute, and then incubated with Fast-Blue solution for 15 minutes. Blood smears were stained according to Jenner-Giemsa. LAP was seen as blue deposits in neutrophils. The number of blue granules per cell was determined and rated on a scale of 0 to 4 (normal range, 0.25 to 0.5). Apart from characteristic forward scatter and side scatter patterns, monococytes were recognized on the basis of elevated CD9 expression and eosinophils on the basis of elevated CD14 expression.

**Flow cytometric analysis.** Samples were analyzed with a FACSscan (Becton Dickinson) flow cytometer. Linear mean cellular fluorescence intensities (MFI) were calculated from the logarithmic mean channel numbers (MCN) that were provided by the LYSISII software for data analysis, using MFI = 10^{MCN}. Apart from characteristic forward scatter and side scatter patterns, monococytes were recognized on the basis of high CD14 expression and eosinophils on the basis of elevated CD14 expression.

**Release of lactoferrin and elastase.** Levels of elastase-α1-antitrypsin (elastase:α1-AT) complexes and lactoferrin were detected in EDTA plasma with radioimmunoassays (RIAs), as previously described. Twenty volunteers experienced no adverse effects. Four volunteers reported mild flu-like symptoms of variable onset and duration on the first day. The reaction lasted for 20 minutes. Peripheral blood GM-CSF levels and leukocyte counts. The background serum GM-CSF concentration was less than 16 pg/mL. Between 15 and 30 minutes after the subcutaneous injection, the GM-CSF concentration increased to 1.6 ng/mL. The peak serum concentration of 5.4 ng/mL (n = 4).
was attained after 4 hours. After 24 hours, the serum GM-CSF concentration had returned to normal values in the five volunteers in which this was measured (data not shown). Neutrophils, eosinophils, and monocytes disappeared almost completely from the circulation within 15 minutes (Fig 1). Platelet, erythrocyte, and lymphocyte numbers did not change (data not shown). After 60 minutes, neutrophils were the first cells of the myeloid lineage that reappeared. Neutrophil numbers reached peak levels after 8 hours and returned to normal within 48 hours. Eosinophil and monocyte numbers peaked 24 hours after GM-CSF injection.

Neutrophil degranulation. Mature neutrophils have three types of intracellular granules. In the early maturation phase, azurophil granules are formed. Specific granules and secretory vesicles appear in the later phases of maturation. Upon activation, neutrophils degranulate. A rank order of exocytosis exists in vitro\(^2\) and in vivo.\(^2\) Secretory vesicles are mobilized before the specific granules, which in turn are mobilized before azurophil granules. A single subcutaneous injection of GM-CSF resulted in neutrophil degranulation (Fig 2). Plasma markers for released granule contents that were used in this study were lactoferrin (Fig 2A) for the specific granules and elastase (coupled to its natural inhibitor antitrypsin; Fig 2B) for the azurophil granules. Plasma concentrations of both proteins reached peak levels after 4 hours and normalized within 24 hours after GM-CSF injection.

Proteins that are associated with the membranes of distinct types of granules may also serve as markers of degranulation. Secretory vesicle membranes form the intracellular pool of LAP,\(^2\) FcγRIII,\(^15,28\) and the complement receptor type 1 (CR1, CD35).\(^29\) The decrease in the LAP score indicated translocation of alkaline phosphatase to the plasma membrane because the vesicle membrane fuses with the plasma membrane (Fig 2C). The upregulation of FcγRIII in the first 30 minutes after injection of GM-CSF (see below) was also a result of secretory vesicle mobilization.\(^15\) The expression of CD35 on the plasma membrane was also increased, with peak values reached at 60 minutes (data not shown). CD11b (C3bi-receptor, \(\alpha_{d}\beta_{2}\)-integrin) and CD66b (formerly CD67) antigens are present in the membranes of both secretory vesicles and specific granules.\(^30,31\) The upregulation of these proteins on circulating neutrophils further substantiated fusion of these granule compartments with the plasma membrane after GM-CSF injection (Fig 2D and E). Circulating neutrophils did not express CD63 on their membrane (Fig 2F). This antigen is a membrane protein associated exclusively with azurophil granules in resting neutrophils and becomes upregulated on the plasma membrane after azurophil granule release.\(^25\)

Neutrophil Fcγ receptor expression. Mature neutrophils express Fcγ receptors type IIA and IIIb. FcγRI is usually not present on circulating neutrophils, but its expression can be induced in vivo.\(^16,32\) A single dose of GM-CSF did not induce the expression of FcγRI on circulating neutrophils. The expression of FcγRII did not change significantly. Figure 3 depicts the changes in the expression of FcγRII after GM-CSF injection. Increased membrane expression of FcγRIII coincided with secretory vesicle release 30 minutes after GM-CSF injection. Hereafter, the expression of FcγRIII gradually decreased and the plasma concentration of the soluble form of the receptor began to increase. Nadir values of FcγRIII expression were reached after 24 hours.

Neutrophil L-selectin expression. GM-CSF decreased the expression of L-selectin on circulating neutrophils within 30 minutes (Fig 4).

Activation of eosinophils and monocytes. GM-CSF increased the expression of CD11b on circulating eosinophils and monocytes. Both cell types have secretory vesicles that, as in neutrophils, form an intracellular pool of \(\beta_2\) integrins in these cells.\(^33\) Expression of CD11b on monocytes increased from a preinjection value of 59 \(\pm\) 3 (MFI \(\pm\) SEM) to a maximum of 142 \(\pm\) 17 at 8 hours. The change in expression remained statistically significant during the 2- to 48-hour interval after injection. The expression of L-selectin did not change significantly (data not shown).

In blood samples obtained during the first 8 hours after...
Fig 2. Neutrophil degranulation in response to GM-CSF injection. Elevated plasma concentrations of lactoferrin (A; in nanograms per milliliter; n = 4) and elastase:AAT (B; in nanograms per milliliter; n = 4). Relative change in cellular alkaline phosphatase (C; percentage; n = 5). Upregulation of CD11b (D; MFI; n = 7), CD66b (E; MFI; n = 7), and CD63 (F; MFI; n = 7). Mean neutrophil counts are depicted as a dashed line. Values are expressed as the mean ± SEM. Asterisks indicate statistical significance (P < .05).
GM-CSF was too low to permit separate analysis (Fig 1). At 12 and 24 hours after injection, the mean eosinophil CD11b expression was elevated (preinjection MFI ± SEM, 160 ± 7.5; at 24 hours, 255 ± 55; P < .05; data not shown). The expression of L-selectin was significantly lower 24 and 48 hours after GM-CSF injection compared with the preinjection values (preinjection MFI ± SEM, 169 ± 7; at 24 hours, 91 ± 7; P < .01; at 48 hours, 91 ± 7; P < .01; data not shown).

The expression of the β1 integrin VLA-4 (CD49d) on monocytes and eosinophils did not change significantly.

Cytokine release. We studied the systemic release of TNFα, IL-6, and IL-8 in response to GM-CSF. Figure 5 shows the transient increase in the plasma IL-8 concentrations during the first 24 hours after GM-CSF injection. Plasma concentrations of TNFα and IL-6 did not change.

**DISCUSSION**

We have studied the effects of a single subcutaneous injection of 7.5 μg/kg of recombinant, Escherichia coli-derived, nonglycosylated GM-CSF (Leucotax) in healthy volunteers. The dose was chosen to match the recommended dose of GM-CSF in the clinical management of neutropenia (5 to 10 μg/kg/d). GM-CSF induced changes both in leukocyte numbers and phenotype and induced neutrophil degranulation. We have previously reported on the effects of a single dose of either cytokine (Table 1). In this discussion, we focus on differences and similarities we found between the effects of a single cytokine (Table 1). The study with G-CSF was completed before the present study was initiated, and different volunteers were used. Of course, this sets limitations to the comparison of the sets of data that were obtained with the two growth factors. However, because both in the G-CSF study and in the present study the interindividual
differences in the effects were rather small, we feel that the comparison is justified.

**Neutrophil activation.** When the early effects of GM-CSF and G-CSF injection on peripheral blood neutrophils are compared qualitatively, the effects are roughly similar. Both cytokines induced a transient neutropenia that was followed by an increase in the number of circulating neutrophils over the baseline value (Fig 1). Neutrophils in both studies had an increased expression of CD11b and CD66b and a decrease in the intracellular concentration of LAP. Furthermore, there was an increase in the plasma concentrations of lactoferrin and elastase, which indicated exocytosis of both specific granules and azurophil granules from neutrophils (Fig 2). As with G-CSF, in vivo shedding of FcγRIII was evident from an increase in the plasma concentration of the soluble form of the receptor after GM-CSF injection (Fig 3).

However, a quantitative comparison between the effects of both cytokines shows a stronger degree of neutrophil activation after GM-CSF injection. The neutropenia that followed GM-CSF injection lasted for approximately 2 hours, whereas the G-CSF–induced neutropenia resolved within 1 hour. GM-CSF, but not G-CSF, induced a partial decrease in the expression of L-selectin on the neutrophil membrane (shedding of L-selectin; Fig 4). Furthermore, neutrophils released more lactoferrin (peak plasma concentrations of 637 ± 86 ng/mL and 390 ± 56 ng/mL after GM-CSF and G-CSF injection, respectively) and more elastase (peak plasma concentrations of 224 ± 33 ng/mL and 115 ± 10 ng/mL, respectively) after an in vivo injection with GM-CSF. The maximal increase in the expression of CD11b on neutrophils was 470% after GM-CSF and only 150% after G-CSF injection. Similar results were found for CD66b (250% and 150%, respectively). Thus, GM-CSF induced a more pronounced activation of neutrophils than did G-CSF in vivo. A similar conclusion was reached by Yong and Linch, who used about 10-fold lower concentrations of GM-CSF than in the present study.

It is of interest that we saw no upregulation of the azurophil granule marker CD63 on circulating neutrophils after GM-CSF or G-CSF injection. Thus, the cells that reappeared in the circulation after the initial neutropenia were not the highly activated cells that had released their azurophil granules. The neutrophils that released their azurophil granules may have subsequently been induced to migrate from the circulation to the tissues, or they were already residing in the tissues at the time of GM-CSF injection. A different effect of GM-CSF on neutrophils that are in the circulation or attached to the extracellular matrix is supported by in vitro data. The effects of GM-CSF or G-CSF on adherent cells are markedly different from the effects of these cytokines on neutrophils in suspension.

**Enhanced production of neutrophils.** Lord et al have suggested that G-CSF enhances neutrophil production, matu-
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ration, and release from the bone marrow in vivo. From another study, these investigators concluded that GM-CSF does not greatly increase the production and maturation of neutrophils but that GM-CSF prolongs the circulation half-life of neutrophils. Continuous infusions of the cytokines were administered for several days in both studies; thus, these results cannot be extrapolated directly to the effects of a single subcutaneous dose of GM-CSF or G-CSF. However, our results suggest that even a single dose of G-CSF enhanced the production and maturation of neutrophils, whereas a single dose of GM-CSF did not enhance neutrophil production.

We found the following arguments for enhanced production and maturation of neutrophils in our G-CSF study. The number of circulating neutrophils increased from 2.4 × 10^9/L before the brief neutropenic period to a maximum of 21.7 × 10^9/L at 12 hours after injection. Eight hours after G-CSF, the circulating neutrophils had a low membrane expression of FcyRIII and a high intracellular LAP content and expressed FcyRI. Based on results from in vitro experiments, we concluded that the appearance of FcyRII-positive neutrophils reflected the effect of G-CSF on bone marrow precursor cells. The maturation time of these precursors had thus been shortened to 8 hours. FcyRIII colocalizes with LAP in secretory vesicles. We therefore proposed that the predominant localization of FcyRIII in these young neutrophils was intracellular. A second peak in the plasma concentrations of soluble FcyRIII and lactoferrin appeared after 6 hours. This was taken to reflect the tissue phase of the excess number of neutrophils that had been produced.

The strongest argument against extensive extracellular production of neutrophils after a single dose of GM-CSF is the absence of such a second peak in the plasma concentration of sFcyRIII several days after GM-CSF injection. Neutrophils release their FcyRIII when they undergo apoptosis in vitro. This finding supports the hypothesis that elevated plasma concentrations of sFcyRIII can reflect an increase in the number of neutrophils undergoing apoptosis in the tissues. In a more general concept, plasma sFcyRIII levels reflect the total number of neutrophils in the body. This sign of enhanced neutrophil production was absent in the volunteers who had received GM-CSF. Other data support this argument against overproduction of neutrophils after a single dose of GM-CSF. The maximum number of circulating neutrophils was only 10.3 × 10^9/L after GM-CSF injection (compared with 21.7 × 10^9/L after G-CSF). Furthermore, the neutrophils that were released into the circulation more than 12 hours after GM-CSF had a low membrane expression of FcyRIII but lacked the high intracellular LAP content characteristic of the very young neutrophils released after G-CSF administration.

It is also important to note that neutrophils with an activated phenotype (ie, an enhanced expression of CD11b and CD66b) had disappeared from the circulation 4 hours after G-CSF, whereas the expression of CD11b and CD66b on circulating neutrophils was still significantly elevated 12 hours after the injection of GM-CSF. This observation can be used as an argument in favor of a prolonged intravascular half-life of neutrophils after GM-CSF injection, as was suggested by Lord et al. The alternative explanation for this prolonged upregulation of CD11b and CD66b is that intravascular activation of neutrophils ceased only when the plasma concentration of GM-CSF had decreased to normal values between 12 and 24 hours after the injection.

Elevated production of IL-8. In the G-CSF study, we did not detect enhanced production of cytokines. In the present study, we observed that plasma levels of IL-8 began to increase 30 minutes after GM-CSF injection, reached peak concentrations of 50 pg/mL after 2 hours, and returned to normal values within 24 hours (Fig 5). The plasma half-life of IL-8 is low in animal models. In nonhuman primates, it is approximately 8 minutes. IL-8 binds to the chemokine receptor on erythrocytes and the endothelial cells that line the postcapillary venules and to glycosaminoglycan moieties present on endothelial cells and in the extracellular matrix. Thus, GM-CSF must have induced a substantial and continuous production of IL-8 to sustain the elevated plasma concentration of this cytokine for more than 8 hours.

The primary source of IL-8 production in our study is unknown. Monocytes/macrophages and endothelial cells are potent producers of IL-8, but granulocytes, lymphocytes, and a range of tissue cells can also produce IL-8. We did not detect increases in the plasma concentrations of IL-6 or TNFα, but this does not preclude the production of IL-8 by monocytes in our study. GM-CSF induces the production and release of IL-8, but not of IL-1, IL-6, or TNFα, from monocytes and neutrophils in vitro. Monocytes and neutrophils were activated in our study, and thus both cell types may have contributed to the systemic production of IL-8 after GM-CSF injection.

Possible effects of IL-8 production. The concentration of IL-8 that we measured in the plasma of our volunteers is too low to activate neutrophils directly under laboratory conditions. Nanomolar concentrations of IL-8 induce upregulation of CD11b and CD35, L-selectin shedding, actin polymerization, and priming of the respiratory burst in neutrophils in vitro.

However, intravascular IL-8 may impair neutrophil migration. Stimulated endothelial cells promote neutrophil transmigration in an IL-8-dependent manner. A haptotactic IL-8 gradient may play an important role in this process (haptotaxis: a hypothetical model that explains directed cellular movement by assuming that molecules of a chemottractant attached to a surface form a spatial gradient that directs movement along that surface). However, in animal models, intravascular IL-8 impairs neutrophil migration to sites of inflammation. In transgenic mice that overexpress IL-8, tissue migration of neutrophils seems severely impaired in a dose-dependent manner. Binding of IL-8 in high concentrations at the luminal side of the endothelial cells may have saturated the IL-8-binding capacity of the endothelial cells and may thus have prevented concentration-directed neutrophil movement. On the basis of the results from these studies, it is conceivable that GM-CSF–induced IL-8 production may have contributed to a prolonged intravascular neutrophil circulation time (see above) and may even help to explain impaired neutrophil migration during GM-CSF therapy. However, the production of IL-8 in pa-
patients treated with GM-CSF should be confirmed, and speculations about neutrophil migration and the possibility of synergistic effects of IL-8 and GM-CSF in neutrophil activation await further investigation.

Conclusions. We found three major differences between the in vivo effects of a single dose of GM-CSF and a single dose of G-CSF in healthy volunteers. First, GM-CSF induced a higher level of neutrophil activation. Second, in contrast to our results with G-CSF, we did not find evidence for a greatly enhanced production and maturation of neutrophils after GM-CSF injection. Third, GM-CSF induced the systemic release of IL-8.

Both G-CSF and GM-CSF can successfully shorten the neutropenic period after chemotherapy or after bone marrow transplantation. A new area of research is the use of these cytokines in non-neutropenic patients with severe infections. Although it is obvious that the effects of a single dose of a cytokine in healthy volunteers may differ from the effects of repeated doses of these cytokines in patients with different disease states, we feel that, in view of the increasing number of possible clinical applications, a deeper understanding of the differences and similarities between the effects that these cytokines can produce in vivo may help to determine which cytokine is best used under which circumstances.

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