Effect of Recombinant Granulocyte Colony-Stimulating Factor on Neutrophil Kinetics in Normal Young and Elderly Humans

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Recombinant granulocyte colony-stimulating factor (G-CSF) was administered to healthy young (n = 32) and elderly (n = 19) volunteers (0 µg/d, 30 µg/d, or 300 µg/d) to determine its effect on neutrophil production, blood kinetics, and tissue migration. Measurements included blood counts (daily), marrow neutrophil pool sizes and neutrophil tissue migration (baseline and day 5), blood kinetics (day 6), and marrow transit time while on drug (days 6 to 14). G-CSF markedly expanded the marrow neutrophil mitotic pool and shortened the transit time of the postmitotic pool (control, mean = 6.4 days; 300 µg/d, mean = 2.9 d). G-CSF increased neutrophil production without significantly altering blood neutrophil half-life or margination. Compared to control, neutrophil accumulation in skin chambers decreased by about 50% in the 300 µg/d group in both young and elderly subjects. G-CSF induced neutrophilia by stimulating proliferation of marrow neutrophil precursors and accelerating neutrophil entry into the blood. Decreased neutrophil inflammatory responses measured with the skin chamber technique may be because of the relative immaturity of the circulating cells or to alterations in neutrophil phenotype induced by G-CSF.

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MATERIALS AND METHODS

Subjects. Subjects were young (age 20 to 30) or elderly (age 70 to 80) normal volunteers. Some data on these subjects and their response to G-CSF has been reported previously. All subjects were nonsmokers who, at entry, had normal blood counts and no history of hematologic abnormality, recent infectious disease, use of medication, or significant medical illness. All subjects gave informed consent for this study, which was approved by the University of Washington Human Subjects Review Committee.

Study design - overview. Each subject had baseline measurements including a medical history, physical examination, complete blood count, BM aspiration for determination of marrow pool sizes, and skin chamber test for measurement of the in vivo neutrophil inflammatory response. In addition, buccal neutrophils were determined from an oral wash with saline. Nineteen young and 19 elderly subjects were separately randomized to receive 30 µg/d G-CSF (14 subjects), 30 µg/d G-CSF (14 subjects), or no G-CSF (10 subjects). G-CSF was administered subcutaneously at 8 AM on study days 1 through 14. Complete blood counts were determined daily just before G-CSF administration. On day 5 a repeat marrow aspiration for marrow neutrophil quantitation was performed as were repeat skin chamber and oral wash studies. Blood neutrophil kinetic studies using tritiated diisopropylfluorophosphate (H-DFP) labeled autologous neutrophils were performed on day 6. On day 7 subjects were given an injection of tritiated thymidine and samples drawn over the next 8 days for determination of marrow postmitotic pool transit time. Blood neutrophil kinetics studies were performed in an additional eight young subjects who had not received G-CSF. To examine further the effects of G-CSF on the in vivo neutrophil inflammatory response, skin chamber studies were performed on an additional 13 young subjects receiving 300 µg/d G-CSF. Marrow culture studies (CFU-GM assays) were performed on these same additional subjects. G-CSF (300 µg/mL) was supplied by Amgen Corp (Thousand Oaks, CA).

BM pool sizes. Marrow differentials were determined by counting 500 nucleated cells on Wright's stained smears using standard techniques. Results for each marrow neutrophil compartment were expressed as a percent of the total nucleated cell population. Results were also normalized to the total marrow erythroblast population and expressed as neutrophil/erythrocyte ratios.

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BM culture studies. Heparinized BM aspirates were obtained from the posterior iliac crest under local anesthesia. The cell suspension was diluted 1:1 with NCTC 109 tissue culture medium, and the mononuclear cell population was isolated by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) sedimentation (400 g) and washed three times with TC199 or Hanks' balanced salt solution (HBSS). In vitro colony formation was determined in a serum-containing system as previously described. Adherent cells were removed by two 1-hour incubations in 75 cm² plastic flasks (Corning, Corning NY) containing 20 × 10⁶ cells in 20 mL TC199 supplemented with 10% (vol/vol) fetal calf serum (FCS) at 37°C in 5% CO₂. The nonadherent cells (2 × 10⁷ cells/mL) were cultured in 0.3% agar containing 20% FCS with or without the addition of growth factors. Maximum growth was stimulated by a mixture of three recombinant hematopoietic growth factors: interleukin-3 (IL-3) (40 pmoVL), granulocyte-macrophage-CSF (GM-CSF) (50 pmoVL), and G-CSF (282 pmoVL) (all provided by Amgen) or by phytohemagglutinin-stimulated leukocyte conditioned medium. Colony growth was determined on day 14.

Neutrophil transit time. The transit time through the marrow postmitotic pool was measured following a study day 7 injection of 10 μCi/kg ³H-thymidine (50 to 90 Ci/mmol, New England Nuclear, Boston, MA) as previously described. Twenty milliliter blood samples for determination of neutrophil specific activity were obtained daily for 2 days, then q12 hours for a total of 8 days. Neutrophils were isolated from each blood sample by Ficoll-Hypaque sedimentation (Pharmacia), dextran sedimentation, and NH₄Cl lysis of contaminating red cells. The radioactivity of a measured number of cells was determined by liquid scintillation counting. Cell specific activity was plotted as a function of time after injection of the isotope, the resulting curve representing the sum of influx of labeled cells into the circulation from the marrow and the efflux of labeled cells into the tissues as indicated by the formula:

\[ g(t) = cH(t) + H'(t) \]

in which \( g(t) \) equals the rate of entry of labeled cells from the marrow at time \( t \); \( c \) is a constant determined by dividing \( H(t) \) by the transit time of the postmitotic pool; \( H(t) \) is the neutrophil specific activity at time \( t \); and \( H'(t) \) is the slope of the curve at time \( t \). The transit time of the postmitotic pool was obtained by subtracting from the maximum \( g(t) \) the average time required for 50% of the last myelocyte generation to enter the postmitotic pool. The latter figure was assumed to be 8 hours based on the data of Cronkite et al.

Blood neutrophil kinetic studies. The blood recovery and survival of autologous neutrophils was measured on day 5. Approximately 500 mL of blood was withdrawn into a plastic bag containing acid-citrate dextrose, formula A (ACD-A) or citrate-phosphate-dextrose-adenine (CPDA1) anticoagulant (Fenwal PL130, Baxter Corporation, Deerfield, IL). The neutrophils were labeled in vitro with 100 μCi of ³H-DHP as previously described. The isotope (³H-DHP, 6 to 10 Ci/mmol) was obtained from the manufacturer (New England Nuclear) as a radiochemical and was sterilized and diluted in propylene glycol to a concentration of 1 mCi/mL; 0.1 mL of this mixture was diluted in 10 mL saline immediately before use. After removing an aliquot of the labeled blood for determination of neutrophil specific activity (previously described), the labeled blood was reinfused into the subject over a 5 to 10 minute period. Twenty milliliter blood samples were obtained at 10 minutes and at 1, 2, 3, 4, 6, 8, 11, and 24 hours after infusion for determination of neutrophil specific activity. Blood neutrophil half-time was determined by the method of least squares through the most linear portion of a semilogarithmic plot of these data points. Neutrophil recovery was defined as the ratio of infused cells circulating at time zero, determined by the value of the y-intercept of the extrapolated survival curve. The fraction of infused cells not recovered in the circulation was considered to represent the marginal pool. Neutrophil turnover was calculated as outlined by Athrens et al.

In vivo measurement of inflammatory response. For measurement of neutrophil accumulation in skin chambers, a 2 cm² area of the volar forearm was abraded by scraping with a surgical scalpel blade and was covered with a glass chamber, which was fastened in place with adhesive compound and tape. The chamber was filled with a mixture of 10% autologous serum and saline to which had been added 100 μL streptokinase/streptodornase (Behringwerks AG; Marburg/Lahn, Germany) to prevent neutrophil clumping as previously described. The chamber fluid was replaced at 4 and 8 hours and removed at 24 hours. The number of white cells in each fluid sample was determined by an electronic particle counter (Coulter, Hialeah, FL). Skin chamber studies were performed on the first 38 subjects at baseline and at day 5. Studies to determine the acute effects of G-CSF in 13 additional subjects were done on day 1, beginning immediately after a dose of 300 μg G-CSF.

Neutrophil accumulation in the oral cavity was assessed by the method of Wright et al. The subject was asked to swish his/her mouth with 25 mL of saline for 30 seconds, return the specimen to a spumet cup, and to immediately repeat the procedure to obtain a duplicate sample. Specimens were centrifuged at 200g for 10 minutes within one hour of collection. The pellet was resuspended in 1 mL HBSS containing 2 μg/mL acridine orange and incubated 15 minutes in a 37°C shaking water bath. The cells were then thoroughly resuspended and the number of neutrophils determined by a hemocytometer count using fluorescence microscopy.

Statistical analysis. Results are expressed as mean ± 1 SEM unless otherwise specified. Student's t-test or paired t-test was used to determine the significance of differences between groups.

RESULTS

Hematologic values. The effect of G-CSF on routine hematologic values in these subjects has been reported previously. In summary, there was a dose and time dependent increase in blood neutrophil counts, the 8 AM (Pre-G-CSF dose) count averaging 20.5 × 10⁹/L and 28.6 × 10⁹/L after 5 and 15 days of 300 μg/kg/d G-CSF, respectively. Bands and segmented neutrophils both increased over this time period, but the relative immaturity of the neutrophils, represented by the fraction of the cells that were bands, increased only with higher dose of the drug. After G-CSF administration, neutrophils on the peripheral blood smear appeared larger than normal with very blue cytoplasm, as reported previously. No consistent or significant changes were seen in blood lymphocyte, monocyte, basophil, or eosinophil counts. Hematocrit changes were only seen at day 15, undoubtedly because of the fact that 500 to 750 mL of blood had been withdrawn between day 5 and day 15 for laboratory measurements, and there were no differences between subjects given different doses of the drug. Platelet counts were not affected by 15 days of G-CSF. There were no substantive differences between the values for the young and elderly subjects.

Marrow neutrophil pool sizes. The effect of 5 days of G-CSF therapy (300 μg/d) on marrow differential counts in these subjects has been previously reported and indicated an expansion of the mitotic compartment with a relative depletion of the segmented neutrophils. When these values are expressed in relation to the erythroid compartment (Fig 1), a relative increase in all morphologic types of neutrophils is apparent with significant increases in promyelocytes, myelocytes, and metamyelocytes. Because the absolute size of
the marrow erythroid compartment would not have been expected to change appreciably over the first 5 days of G-CSF therapy, this normalization of the myeloid values to a constant erythron size provides a more absolute measure of changes in the size of the neutrophil pools for this time point. The fact that a smaller effect is seen in the maturational compartments than in the proliferative compartment is attributable, at least in part, to an acceleration of mature cell movement through the marrow and into the blood, as indicated by the results for the marrow transit times (discussed later). There were no differences in marrow pool size measurements for young and elderly subjects (data not shown).

Marrow culture studies. Marrow myeloid progenitor cell assays were performed on 11 young normal subjects receiving 300 μg/d G-CSF. With maximal growth stimulation (IL-3, GM-CSF, G-CSF), CFU-GM values after 5 days of G-CSF (18 ± 5/10⁶ cells plated) were slightly reduced from baseline (26 ± 8/10⁶ cells plated), although the difference was not statistically significant. However, the mononuclear cell preparations used for these cultures contained all nucleated cells up to and including the metamyelocyte stage. Because CFU-GM are expressed as colonies per nucleated cells plated, the expansion of the myeloblast-metamyelocyte pools seen with G-CSF administration will serve to dilute the progenitor cells plated and may result in an underestimation of the CFU-GM pool size.

Marrow neutrophil transit time. The transit time of the marrow postmitotic pool, measured by the pattern of emergence of labeled neutrophils in the blood after intravenous injection of tritiated thymidine, is shown in Fig 2. In normal subjects not treated with G-CSF the mean transit time is 6.4 ± 0.3 days, a value similar to that obtained by Dancey et al. The value is shortened substantially on G-CSF treatment, reaching means of 4.3 ± 0.2 days and 2.9 ± 0.1 days after treatment with 30 μg/d G-CSF and 300 μg/d G-CSF, respectively. The differences between the values at all three drug levels are statistically significant (P < .01).

Neutrophil blood kinetics. Results of blood kinetic measurements are shown in Table 1. There was no significant effect of G-CSF treatment on the fraction of labeled neutrophils accounted for in the circulation (% recovery), an indicator of the distribution of blood neutrophils between the circulating and marginal pools. Mean blood neutrophil half-disappearance time was slightly prolonged in subjects receiving G-CSF, but the values were not statistically significantly different from those in the controls in this relatively small group of subjects. Calculation of the neutrophil turnover, the daily production of neutrophils by the marrow, suggests that the kinetic mechanism responsible for the neutrophilia is principally that of increased neutrophil production by the marrow. The effect of G-CSF to increase neutrophil production was the same in young and elderly subjects (data not shown).

Inflammatory response. The neutrophil inflammatory response was assessed by measuring neutrophil accumulation in a skin chamber and in the oral mucosa (Table 2). Skin chamber neutrophils after 5 days of G-CSF decreased in proportion to the dose, reaching less than half the baseline value in both young and elderly subjects receiving 300 μg/ day. There was no apparent acute effect of G-CSF on neutrophil localization to the skin chamber because results obtained in skin chambers placed at the time of the first 300 μg G-CSF dose (125.9 ± 35.0 × 10⁶, n = 11) were not different from baseline values. Buccal neutrophils were more plentiful in elderly subjects who were not edentulous, presumably

<table>
<thead>
<tr>
<th>Dose</th>
<th>n</th>
<th>PMN/μL</th>
<th>Recovery (%)</th>
<th>t₁/₂ (hr)</th>
<th>PMN Turnover (×10⁷/kg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12</td>
<td>3.5 ± 0.4</td>
<td>71 ± 9</td>
<td>10.4 ± 1.5</td>
<td>64 ± 11</td>
</tr>
<tr>
<td>30 μg</td>
<td>11</td>
<td>8.2 ± 0.6†</td>
<td>87 ± 10</td>
<td>15.9 ± 2.1</td>
<td>103 ± 23</td>
</tr>
<tr>
<td>300 μg</td>
<td>9</td>
<td>23.7 ± 1.9††</td>
<td>62 ± 10</td>
<td>13.5 ± 1.7</td>
<td>389 ± 47††</td>
</tr>
</tbody>
</table>

Abbreviation: PMN, polymorphonuclear leukocyte.
† Different from control (P < .01).
†† Different from 30 μg G-CSF (P < .01).
related to gingival inflammation. There was neither a significant increase or decrease in buccal neutrophils with G-CSF treatment. As shown in Table 2, there was a marked contrast between the elevated blood neutrophil counts, the simultaneous diminution of skin chamber neutrophil localization, and the constant or slight reduction in buccal neutrophil localization in the young and elderly subjects, respectively.

Adverse effects. Mild bone aching was reported by several subjects, both elderly and young, during the period of G-CSF administration. It was not necessary to discontinue treatment in any subject. No other side effects attributable to the drug were seen.

**DISCUSSION**

G-CSF is a potent stimulator of in vivo granulopoiesis and is an essential factor for maintenance of normal blood neutrophil counts. Serum levels of G-CSF, for example, are elevated in infection and animals lacking G-CSF are neutropenic. Previous studies have shown that G-CSF stimulates proliferation of marrow neutrophil precursors concomitant with increases in the blood neutrophil count. Neutrophil transit time through the marrow postmitotic pool has been reported to be shortened by G-CSF, but the magnitude of the effect has been controversial. Studies in two patients with metastatic breast cancer suggested that the transit time was shortened to one day, ~20% of the normal value, but more recent studies in mice have suggested a more modest 55% reduction, similar to the findings in this report. G-CSF is not thought to affect neutrophil blood kinetics, but direct measurements of neutrophil margination and survival have not been reported previously. Limited studies have suggested that G-CSF does not affect neutrophil localization to an inflammatory site. Few of these studies have been performed on normals, thus raising the possibility that some of the findings were influenced by other abnormalities in the subjects. Although it is known that G-CSF stimulated mobilization of marrow neutrophils is different for elderly than for young subjects, no other information is available on a possible influence of age on G-CSF effects.

As has been previously reported, in the present study there was a dose dependent increase in blood neutrophil count seen with G-CSF administration, an effect that was identical for young and elderly subjects. A relative increase in immature neutrophils was seen only at the highest doses of the drug. There was no consistent effect of G-CSF on blood levels of other white blood cells or platelets. The decrease in hematocrit seen after 15 days of G-CSF administration undoubtedly reflected blood loss caused by phlebotomy and not an effect of G-CSF.

Marrow neutrophil pool sizes were measurably increased after 5 days of 300 µg/d G-CSF. This increase was most apparent in the mitotic pool compartments where absolute pool sizes were increased approximately 150%. The apparent lesser effect on the postmitotic neutrophil pool sizes was most likely caused by the accelerated delivery of these immature neutrophils into the circulation, shown by the dose dependent shortening of the marrow transit time seen in Fig 2. This shortened maturation time seen with G-CSF is similar to that seen in other situations where neutrophils are mobilized from the marrow storage pool such as infection or inflammation or administration of substances such as corticosteroids or endotoxin.

Blood kinetic studies indicate that neutrophil margination is not appreciably affected by G-CSF in the doses studied. There is at most a modest prolongation of blood neutrophil survival, although the values are not significantly different from normal. The data suggest that the increased blood neutrophil count seen with G-CSF administration is principally attributable to increased delivery of neutrophils from the marrow to the blood, whether the result of early mobilization or increased granulopoiesis. Although not shown, there are no differences seen between young and elderly subjects in these blood kinetic measurements.

Previous studies have suggested that tissue neutrophil localization is impaired after the administration of GM-CSF but is unaltered by the administration of G-CSF. In the present study, it is apparent that the tissue inflammatory response, as measured by neutrophil localization to skin chambers, was significantly reduced if measured after 5 days
of high-dose G-CSF (300 µg/d) in both young and elderly subjects, despite marked blood neutrophilia. Similarly, buccal neutrophils were not increased in proportion to the dramatic increase in circulating neutrophil counts seen with G-CSF stimulation. The apparent decrease in buccal neutrophils was not statistically significant, although the failure to detect a real difference may be because of the variability of the measurements and the relatively small sample size. Skin chamber accumulation was not different from normal when studied immediately after the first 300 µg dose of G-CSF, suggesting that reduced localization is not caused by G-CSF per se; rather it appears more likely to reflect the fact that neutrophils released from the marrow after several days of G-CSF stimulation have different levels of surface adhesion proteins than do neutrophils released from the marrow without stimulation. Differences that may affect the neutrophil's ability to migrate from the blood into the tissues. The reduced neutrophil response seen in the skin chamber studies does not necessarily imply that these neutrophils would be unavailable to a patient with an infection. These cells made under the influence of G-CSF have been shown to be capable of upregulation of cell surface markers on activation by inflammatory mediators; an in vivo infectious challenge may well provide the same activation of the circulating neutrophils. That G-CSF stimulated neutrophils are capable of responding to an infection normally is suggested by the various clinical studies performed in both animals and humans suggesting that G-CSF treatment may have a positive effect on survival and resolution of infection. The failure of neutrophilia per se to affect the quantity of neutrophils accumulating at the inflammatory sites suggests that when neutrophils are adequate in number, the number of cells localized is primarily dependent upon the inflammatory stimulus, not simply the number of available neutrophils. The observation that tissue infiltration by neutrophils is minimal in transgenic mice with marked G-CSF induced neutrophilia is consistent with this formulation.

The effect of G-CSF was no different in young and elderly subjects in any of the results reported here. The only observed difference between the different age groups was that elderly subjects had higher numbers of oral neutrophils. This was true whether or not G-CSF had been administered and was easily attributable to differences in gingival health.

These studies have shown that G-CSF stimulates marrow neutrophil delivery into the blood by stimulating proliferation of both progenitor cells and the identifiable marrow mitotic pool and by accelerating the transit time through the postmitotic pool. Blood kinetics, as reflected by margination or survival of neutrophils, is unaffected by G-CSF therapy, and the neutrophilia observed in these subjects was entirely accounted for by increased marrow neutrophil production. The neutrophils circulating after several days of G-CSF stimulation are different from those produced in the absence of G-CSF. They are larger, their complement of surface molecules is different, and their ability to localize to an inflammatory site, at least in the absence of a major inflammatory stimulus, is reduced. In view of clinical studies suggesting a salutary effect of G-CSF administration during infection, it is plausible that under a more severe inflammatory stimulus, neutrophil delivery may be normalized or even enhanced.

REFERENCES


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