In Vivo Effects of Recombinant Human Granulocyte Colony-Stimulating Factor on Neutrophil Oxidative Functions in Normal Human Volunteers

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The effect of daily in vivo granulocyte colony-stimulating factor (G-CSF) treatment on neutrophil function was studied over a 14-day period using a luminescence system for differential measurement of oxidase and myeloperoxidase (MPO) dioxygenation activities in whole blood. Opsonin receptor-mediated phagocyte functions were also measured with this system. G-CSF produced a dose-dependent neutrophil leukocytosis and a proportional increase in oxidase activity per volume of blood. The oxidase activity per neutrophil remained relatively constant throughout the test period. However, both chemical- and opsonin-stimulated MPO oxygenation activities per neutrophil were greatly increased by treatment with maxima correlating temporally to initial G-CSF exposure during the early mitotic phase of neutrophil development. The possibility that peroxynitrite contributes to this maximum luminal-dependent activity was tested, but neither superoxide dismutase, a competitive inhibitor of peroxynitrite production, nor N-methyl-L-arginine, an inhibitor of nitric oxide synthase, exerted a significant inhibitory effect.

Granulocyte colony-stimulating factor (G-CSF) promotes the proliferation and differentiation of the progenitors of polymorphonuclear neutrophil leukocytes [1]. G-CSF also modulates the functional activity of mature neutrophils. Treatment with G-CSF is reported to significantly enhance phagocytosis and killing of bacteria and fungi in vitro [2, 3], to induce neutrophil IgG FcγRI (CD64) expression [4, 5], and to increase neutrophil mediated antibody-dependent cellular cytotoxicity activity [6].

In several neutropenic and nonneutropenic animal models of infection, G-CSF treatment increased the number and function of circulating blood neutrophils. In a mouse model of intramuscular Pseudomonas aeruginosa infection, G-CSF alone and in combination with cefazidine significantly increased survival [7]. G-CSF alone or in combination with antibiotics also significantly improved survival in a rat neonatal model of group B streptococcal infection [8, 9]. Likewise, G-CSF improved the survival of splenectomized mice with gram-positive Streptococcus pneumoniae pulmonary infection [10] and ethanol-treated rats with gram-negative Klebsiella pneumoniae pulmonary infection [11].

To investigate the possible effect of G-CSF on neutrophil function, we treated healthy human volunteers with recombinant human G-CSF daily for up to 2 weeks [12, 13]. Here we present the results of a detailed chronologic study of the effects of this treatment on neutrophil oxidase- and myeloperoxidase (MPO)-dependent dioxygenation activities in response to various chemical and opsonin stimuli, using a whole-blood luminescence methodology. This system also measures phagocyte opsonin receptor expression—dependent activities and provides a gauge of the in vivo state of immune activation [14, 15].

Methods

Study design and volunteers. Nineteen young (20–30 years) and 17 elderly (70–80 years) volunteers of both sexes participated. All the volunteers were nonsmokers, were taking no prescription medications, and had had no acute illness during the 6 weeks before the study. They had normal physical examinations and laboratory results for complete blood cell count (CBC), urinalysis, and blood chemistries. Some investigations of these subjects have been reported [12, 13].

Study design. The volunteers were randomized to one of three dosage groups: no drug (7 young, 5 elderly), 30 μg of recombinant human (rh) G-CSF/day (5 young, 6 elderly), and 300 μg of rhG-CSF/day (7 young, 6 elderly). After a 2-day baseline period (days 0 and 1), subjects received either no drug or G-CSF (Amgen, Thousand Oaks, CA) for 14 days. Three additional young subjects received 7 days of G-CSF at 300 μg/day to test the effect of in vitro inhibitors of neutrophil oxygenation activity. All injections of G-CSF were given subcutaneously between 7:00 and 9:00 A.M. daily. Throughout the test period, all subjects were monitored by daily vital signs and serial CBCs and differential leukocyte counts. Blood luminescence analysis was done on days 0, 1, 2, 3, 5, 6, 7, 9, 12, and 14 on blood samples drawn before G-CSF administration.

Differential measurement of phagocyte oxidase and myeloperoxidase activities. The metabolically linked oxygenation activities

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of circulating phagocytes, predominantly neutrophils, were measured in whole blood samples with the CORE/MORE automated luminescence system (EOE, Little Rock, AR) as described [12, 15–17]. This system uses low-dose phorbol 12-myristate 13-acetate (PMA)—coated tubes formulated to optimally stimulate neutrophil-specific (secondary) degranulation and oxidative activation and high-dose PMA-coated tubes formulated to stimulate extensive neutrophil degranulation, including azurophilic granules containing MPO. Oxidase activity was measured using low-dose PMA (10 pmol/tube) with dimethylacridinium (DBA**+, also called lucigenin; 120 nmol/tube) as the luminigenic substrate. Oxidase-driven MPO activity was measured using high-dose PMA (5 nmol/tube), and luminol (90 nmol/tube) was used as the luminigenic substrate [18].

Activity in K₃EDTA-anticoagulated blood samples was measured in triplicate within 4 h of collection. One microliter of blood was used per measurement, and the final test volume was 0.7 mL. Results are expressed as the total luminescence activity per neutrophil for the 20-min measurement period (counts/20 min/ neutrophil). Results can also be expressed as luminescence activity per phagocyte (counts/20 min/phagocyte), where the phagocytes are the total neutrophils, monocytes, and eosinophils in the blood. Luminescence activity in monocytes and eosinophils is similar to that in neutrophils, but typically these phagocytes are present in relatively low numbers, so the results are essentially the same using either expression [18].

Measurement of opsonin receptor expression—dependent activities. The expression of opsonin receptors, such as complement receptors CR3 (CD11b/CD18) and CR1 (CD35), on neutrophils and other phagocytes increases in proportion to the level of exposure to inflammatory mediators, such as C5a, platelet activating factor (PAF), leukotrienes, various cytokines, interleukins, and certain microbial products, such as formylmethionyl peptides. The circulating opsonin receptor (COR) expression of phagocytes in freshly drawn whole blood is measured as the initial luminescence response to a nonlimiting concentration of an opsonin stimulus (e.g., complement-opsonized zymosan), using a nonlimiting concentration of luminigenic substrate. The maximum opsonin receptor (MOR) expression of these blood phagocytes is measured by including an optimum quantity of opsonin receptor—activating agent (e.g., C5a or PAF) with the same nonlimiting quantity of opsonin stimulus and luminigenic substrate [14, 15]. For this study, the opsonin stimulus for both COR and MOR measurements was human complement—opsonized zymosan (hC-OpZ); ~2 x 10⁶ yeast cell wall particles were used per test, MOR activity was measured using the same quantity of hC-OpZ in combination with one of several different activating reagents, including C5a (20 pmol), PAF (10 pmol), LTB₄ (leukotriene B₄, 10 pmol), and FMLP (100 pmol) as previously described [14–17]. As for the other measurements, 1 μL of whole blood was used per test; however, the final test volume was 0.8 mL.

COR and MOR activities are opsonin receptor—dependent functions measured as the luminescence product of phagocyte metabolism. As such, the COR or MOR activity per phagocyte is dependent not only on opsonin receptor expression but also on the metabolic capacity of the phagocyte, and metabolic capacity changes in response to in vivo immune activation and marrow stimulation. For any given blood specimen, expressing the COR activity relative to the MOR activity (i.e., as the COR/MOR ratio) effectively normalizes for differences in phagocyte metabolic response capacity and focuses emphasis on the physiologic readiness of blood phagocytes to respond to opsonin stimuli. The lower the ratio, the greater the response capacity, that is, the greater the difference between circulating and maximum opsonin-stimulated activity. The ratio approaches unity as in vivo immune activation approaches maximum.

A four-letter code (XXYZ) is used to describe and differentiate the various luminescence measurements. The first two letters of the code (XX) describe the priming agent or stimulus used to coat the reaction tube: BK, PB, PA, FA, FB, and PF for uncoated and low-dose PMA—, high-dose PMA—, high-dose FMLP—, low-dose FMLP—, and PAF-coated tubes, respectively. The third letter (Y) describes the type of opsonin stimulus employed: N for none, and C for hC-OpZ. The forth letter (Z) describes the chemilumigenic substrate employed: L for luminol, and D for DBA**+ (lucigenin). Therefore, PBND represents the activity of blood in response to low-dose PMA (PB) with no opsonin stimulus (N) and with lucigenin (D) as the chemilumigenic substrate. BKCL represents activity in response to uncoated blank tubes (BK) with hC-OpZ (C) as the opsonin stimulus and luminol (L) as the chemilumigenic substrate.

Inhibition studies. The luminol luminescence reaction of neutrophils is mainly, but not exclusively, dependent on oxidase-driven MPO activity [18]. Luminol is also reported to react with other oxygenating agents, such as the peroxynitrite anion (OONO⁻), to yield luminescence [19]. The contribution of MPO to the luminol luminescence response was tested using azide, a potent inhibitor of both MPO microbicidal and luminescence activities [18, 20].

Peroxynitrite is produced by the reaction of nitric oxide (NO) with superoxide anion (O₂⁻) [21, 22]. As such, the inhibitory effects of N-methyl-D-arginine (NMLA), an inhibitor of NO synthase, and superoxide dismutase (SOD), an enzymatic scavenger of O₂⁻, were tested in an attempt to measure the possible contribution of OONO⁻ to the phagocyte luminol luminescence response.

Statistical analysis. SPSS version 6.1 software (SPSS, Chicago) was used for statistical analyses, including calculations of means and SDs, normal distributions, 95% confidence intervals, Student’s t test, Pearson’s correlation coefficients (r), coefficient of determination (R²), and probabilities (P).

Results

G-CSF neutrophilia and oxidase activity. The effects of G-CSF treatment on blood neutrophil count and function were measured ~24 h after injection of G-CSF. Treatment produced a dose- and time-dependent increase in the blood neutrophil count, which reached a plateau of ~25,000/μL. Correspondingly, G-CSF treatment produced a dose- and time-dependent increase in blood oxidase activity stimulated by low-dose PMA and measured with lucigenin as the luminigenic substrate (i.e., PBND activity). Figure 1 shows the chronologic effect of G-CSF treatment on both PBND activity (top) and neutrophil count (bottom). The pretreatment and treatment periods are described by days 0 and 1 and days 2-14, respectively.

PBND oxidase activity increased in proportion to the neutrophil count. Pearson’s correlation coefficient (r) relating neutro-
Figure 1. Effect of daily G-CSF injections on oxidase activity and absolute neutrophil count of blood over 2-week treatment interval. Top: effect of 300 μL of G-CSF, 30 μL of G-CSF, or no treatment on low-dose phorbol myristate acetate--stimulated oxidase activity of blood measured as luminescence product of dimethylbiacridinium (lucigenin) dioxygenation (PBND activity expressed as counts/20 min/μL of blood). Bottom: effect of treatment on neutrophil count/μL of blood. Error bars = 95% confidence interval for mean.

phil count to oxidase activity for all subjects on all days was 0.864. When PBND oxidase activity was analyzed relative to the total phagocyte count, the value of r for all subjects on all days was only slightly higher at 0.869. The data of figure 2 are derived from the composite information of figure 1 and are expressed as specific oxidase activity per neutrophil for each day tested. The PBND activities in the 30 μg of G-CSF group were significantly higher than those in controls (P < .01) on days 3 through 7, and the activities in the 300 μg of G-CSF group were significantly higher on days 2 through 6. However, the PBND activity per neutrophil remained relatively constant throughout the testing period. The maximum activity induced by G-CSF treatment never exceeded 1.5 times that of the pre-treatment and untreated control groups.

Figure 2. Effect of G-CSF treatment on specific oxidase activity per neutrophil. Data, expressed as counts/20 min/neutrophil, were derived by dividing PBND (low-dose phorbol myristate acetate, no opsonin, lucigenin substrate) activity/μL of blood by neutrophil count/μL of blood. Error bars = 95% confidence interval.

Myeloperoxidase activities. High concentrations of PMA (5 nmol/test) cause receptor-independent specific and azurophic degranulation with release of MPO and can be measured as the luminescence product of luminol dioxygenation (PANL activity). As shown in figure 3, G-CSF treatment produced large increases in PANL activity, expressed as counts/20 min/neutrophil, which were dose- and time-dependent. Although a significant (P < .001) increase in PANL activity was observed

Figure 3. Effect of G-CSF treatment on high-dose phorbol myristate acetate--stimulated neutrophil oxidase--driven myeloperoxidase activity measured as luminescence product of luminol dioxygenation (PANL activity expressed as counts/20 min/neutrophil). Error bars = 95% confidence interval.
by day 3, these early increases were small relative to the >3-fold increase observed on days 6 and 7 for the group treated with 30 µg of G-CSF and the 5-fold increase observed on day 5 for the group treated with 300 µg of G-CSF.

N-formylmethionyl peptides directly activate neutrophils by a receptor mechanism that is opsonin-independent. Figure 4 depicts the effects of high-dose-FMLP (1 µmol/test) stimulation of blood measured as the luminescence product of luminol dixygenation (FANL activity). Similar to what was observed with high-dose PMA, a relatively small but significant increase in FANL activity was seen by day 3. However, a >3-fold increase was observed on days 6 and 7 for the 30 µg of G-CSF group, and a 10-fold increase was observed on day 5 for the 300 µg of G-CSF group.

Opsonin receptor–dependent dixygenation activities. Under properly controlled test conditions, opsonin receptor expression exerts rate-limiting control over opsonin activation of neutrophil metabolism and the generation of oxygenating agents. When the opsonin stimulus is in excess, the initial rate of phagocyte metabolic activation is proportional to opsonin receptor expression and can be measured as the luminescence product of luminol dixygenation. The upper part of figure 5 depicts the frequency distributions of hC-OpZ–stimulated COR-dependent activities of blood neutrophils for the pretreatment period (days 0 and 1) and for the post–G-CSF (300 µg) treatment periods on days 2 and 3 and days 5 and 6 with luminol used as the luminogenic substrate (BKCL activity). Activity was measured for the initial 10-min period following exposure to hC-OpZ to minimize the potentially limiting effect of neutrophil metabolic capacity. Relative to days 0 and 1, COR activity was significantly increased by days 2 and 3 and remained increased throughout the period of G-CSF treatment. To a lesser degree, the same results were obtained for the group treated with 30 µg of G-CSF (data not shown).

The bottom part of figure 5 presents the frequency distributions for PAF-primed hC-OpZ–stimulated MOR activities of blood neutrophils measured as the luminescence product of luminol dixygenation (PFCL activity) on pretreatment days 0 and 1 and on post–G-CSF (300 µg) treatment days 2 and 3 and days 5 and 6. Note that the combined pretreatment PFCL activities of all subjects were much higher than the combined pretreatment BKCL activities. Also note that the increases in PFCL activity after G-CSF treatment paralleled the increases in BKCL activity, such that the ratio of the activities remained relatively constant.

This ratio of COR–to MOR-dependent activities (BKCL/PFCL ratio) provides a functional gauge of in vivo phagocyte priming. The value of the ratio increases toward unity in proportion to the degree of in vivo immune activation or inflammation. Figure 6 presents the results of no treatment and treat-
Figure 6. Effect of G-CSF treatment on ratio of circulating to maximum opsonin receptor–dependent activities (BKCL/PFCL ratio). Error bars = 95% confidence interval.

Figure 7. Effect of G-CSF treatment on platelet activating factor–primed, complement-opsonized zymosan–stimulated blood measured as luminescence product of luminol dioxxygenation (PFCL activity) expressed as counts/20 min/neutrophil. Error bars = 95% confidence interval.

dependent specific activities, but DBA+++-dependent specific activity remained relatively constant throughout the testing interval. When luminol is the luminogenic substrate, the luminescence activity of neutrophils from healthy control subjects is largely, but not completely, due to MPO [18]. Azide is a potent inhibitor of MPO microbicidal and luminescence activities [18, 20]. To further investigate the role of MPO with regard to the increased luminol luminescence capacity associated with G-CSF treatment, the concentration-dependent inhibitory effect of azide on PANL activity was studied in 3 additional subjects receiving 300 µg of G-CSF. The subjects were studied on pretreatment day 1 and days 2 (or 3), 5, and 8. Consistent with the importance of MPO in catalyzing luminol dioxygenation, azide produced a potent inhibition of PANL activity in all specimens tested. At a final concentration of 71 µM (50 nmol/tube), azide produced 87%, 84%, 75%, and 77% inhibition of PANL activity on days 1, 2–3, 5, and 8, respectively.

The top part of table 1 presents the azide inhibition results in the form of an equation, that is, luminescence = k(azide/neutrophil)^m, where luminescence is the PANL activity expressed as the counts/20 min/neutrophil, k is the proportionality constant, azide/neutrophil is the quantity of azide expressed relative to the quantity of neutrophils present in the blood specimen tested, and the exponential m is the negative slope or order of inhibition. The azide/neutrophil expression was used to normalize for the large differences in neutrophil counts observed in the different specimens tested. Azide inhibition was essentially the same for pretreatment day 1, when the neutrophil count was 3800/µL, as for days 2 and 3 of G-CSF treatment, when the count had risen to 17,000/µL. As indicated by a decrease in the negative slope of inhibition (m), azide was less effective as an inhibitor on day 5, the day of maximum

PFCG activity is measured as the luminescence product of luminol dioxygenation and, as such, is ultimately linked to oxidase-driven MPO activity. Figure 7 depicts the PFCG activity per neutrophil throughout G-CSF treatment. As with the opsonin receptor–independent PANL and FANL activities, the opsonin receptor–dependent PFCG activity was higher and occurred sooner for the 300 µg of G-CSF group relative to the 30 µg of G-CSF group, but the magnitudes of the increases were smaller than those of increases observed for the opsonin receptor–independent activities. Similar results were also obtained for C5aR, LTB4R, and FMLP-primed MOR activities (data not shown). These differences may be related to the fact that PANL and FANL activities per neutrophil are relatively low compared with PFCG and other MOR activities (i.e., even the maximum G-CSF–induced PANL and FANL activities were lower than the PFCG activity).

Effect of azide, SOD, and NO synthase inhibitor. G-CSF treatment causes large, temporally defined increases in luminol-
G-CSF treatment-induced PANL activity, and on day 8. This decrease in the effectiveness of azide inhibition suggests that the increased PANL activity observed following several days of G-CSF treatment might be caused by some MPO-independent dioxgenation activity that is not inhibited by azide.

Evidence for neutrophil NO synthase activity has been reported [23, 24], and it is possible that G-CSF treatment could induce neutrophil NO synthase. Although relatively unreactive with nonradicals, NO readily reacts with radicals such as \( \text{O}_2^- \), the product of NADPH oxidase, to yield nonradical peroxynitrite (\( \text{OONO}^- \)) [21, 22]. Peroxynitrite is a reactive microbicidal oxygenating agent and is reported to react with luminol, yielding luminescence [19]. Both NO and \( \text{O}_2^- \) are required for \( \text{OONO}^- \) synthesis, and SOD consumes \( \text{O}_2^- \). Therefore, SOD should exert a competitive inhibitory effect on neutrophil production of \( \text{OONO}^- \) [24], and this effect should be measurable as a decrease in PANL activity. As shown by data in the lower part of table 1, the inhibitory effect of SOD on PANL activity was minimal. In addition, attempts to directly inhibit NO synthase with NMLA were also unsuccessful. Neither NMLA nor its isomeric control, N-methyl-L-arginine, produced inhibition of PANL luminescence at final concentrations ranging from 10 to 800 \( \mu M \) (data not shown).

**Discussion**

G-CSF is an established agent for the treatment of neutropenic patients, and considerable evidence now suggests that it is largely responsible for maintenance of normal blood neutrophil counts and for neutrophilia in response to infections [25]. In addition to its hematopoietic effect, G-CSF also appears to modulate the function and microbicidal capacity of mature blood neutrophils [2, 3, 6, 7, 26–28].

In the present study, oxidase activity, stimulated by PMA and measured as lucigenin luminescence (PBND activity), was proportional to the number of neutrophils present in the volume of blood tested. The specific oxidase activity per neutrophil was only mildly increased in response to G-CSF treatment and remained relatively constant throughout the treatment period. In contrast, G-CSF treatment caused large, temporally defined increases in chemical- and opsonin-stimulated phagocyte oxygenation activities requiring MPO and being measured as luminol luminescence. These observations, in combination with the infection model data, suggest that G-CSF exerts a hypertrophic as well as a hyperplastic influence on neutrophils.

Neutrophil development can be divided into mitotic and postmitotic phases [29]. The mitotic phase of development can be further divided into a promyelocytic synthetic phase and a myelocytic synthetic phase. The promyelocyte phase is characterized by the synthesis of lysosomal enzymes including MPO and the other components that make up the azurphilic (primary) granules. The myelocyte stage that follows is characterized by the production and accumulation of peroxidase-negative specific (secondary) granules [30, 31]. The postmitotic development phase is characterized by an increase in heterochromatin and nuclear segmentation, involution of endoplasmic reticulum and mitochondria, and accumulation of glycogen.

Neutrophil specific granules provide ready-to-use membrane containing the components required for opsonin recognition and NADPH oxidase activation. In an unprimed neutrophil, a large percentage of complement receptor CR3 (CD11b) is localized to the specific granules [32–34]. Likewise, in unprimed neutrophils, 80%–85% of cytochrome b and the major part of NADPH oxidase are associated with the specific granules [35–37]. The transition from promyelocyte to myelocyte is characterized by cessation of azurophilic granule production and initiation of specific granule production, which continues throughout the myelocyte phase of development [30, 31]. As a result, mitoses in the myelocyte stage cause a de facto dilution in the number of azurophilic granules per cell.

### Table 1. Sodium azide and superoxide dismutase (SOD) inhibition of PANL luminescence.

<table>
<thead>
<tr>
<th>Day of G-CSF treatment</th>
<th>( k )</th>
<th>( m )</th>
<th>( R^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na azide*</td>
<td>3.8787</td>
<td>-0.3296</td>
<td>0.9573</td>
</tr>
<tr>
<td>Day 1, pretreatment</td>
<td>3.8714</td>
<td>-0.3031</td>
<td>0.9254</td>
</tr>
<tr>
<td>Days 2–3, G-CSF</td>
<td>4.1125</td>
<td>-0.2138</td>
<td>0.8122</td>
</tr>
<tr>
<td>Day 5, G-CSF</td>
<td>3.7419</td>
<td>-0.2196</td>
<td>0.5994</td>
</tr>
<tr>
<td>All days</td>
<td>3.9090</td>
<td>-0.3126</td>
<td>0.6929</td>
</tr>
<tr>
<td>SOD†</td>
<td>3.4854</td>
<td>-0.0028</td>
<td>0.0003</td>
</tr>
<tr>
<td>Day 1, pretreatment</td>
<td>3.6768</td>
<td>-0.0414</td>
<td>0.0271</td>
</tr>
<tr>
<td>Days 2–3, G-CSF</td>
<td>4.0217</td>
<td>-0.0029</td>
<td>0.0001</td>
</tr>
<tr>
<td>Day 5, G-CSF</td>
<td>3.4165</td>
<td>0.0524</td>
<td>0.0158</td>
</tr>
<tr>
<td>All days</td>
<td>3.7554</td>
<td>-0.1186</td>
<td>0.0943</td>
</tr>
</tbody>
</table>

* Counts/20 min/neutrophil = \( k \) (fmol of azide/neutrophil)*.
† Counts/20 min/neutrophil = \( k \) (mU of SOD/neutrophil)*.

**NOTE.** PANL = high-dose phorbol myristate acetate, no opsonin stimulus, and luminol substrate.
Daily treatment with 30 or 300 μg of G-CSF shortens the postmitotic pool transit time from 6.4 days to 4.5 and 3 days, respectively [13]. Such treatment also produces a similar, temporarily linked increase in MPO-dependent activity in response to chemical and opsonin stimuli, but oxidase activity is only minimally increased. Treatment with 30 μg of G-CSF induced a maximum 3-fold increase in high-dose PMA (PANL) and high-dose FMLP (FANL) stimulated activities on days 6 and 7 (i.e., after 5–6 days of treatment). For PAF-primed hC-OpZ-stimulated (PFCL) activity, a small but significant increase in maximum was also observed at day 7 (i.e., after 6 days of treatment). Treatment with 300 μg of G-CSF produced a 5-fold maximum increase in PANL activity, a 10-fold maximum increase in FANL activity, and a 1.5-fold maximum increase in PFCL activity. All of the increases were maximum on day 5 (i.e., after 4 days of G-CSF treatment). On the basis of temporal correlation, maximum specific activity per circulating neutrophil required exposure to G-CSF when its precursor cell was in the mitotic pool.

In healthy, unstimulated controls, transition through the myelocytic pool probably includes two mitotic divisions and takes ~4 days [29]. Although there is at present no direct evidence, G-CSF treatment may shorten the transit time through the mitotic pool and decrease the number of divisions in the myelocyte pool. Decreased mitotic activity in the myelocytic pool would decrease mitotic dilution of the azurophilic granules. The resulting mature neutrophils would have a higher azurophilic granule content and a higher MPO content. If G-CSF shortens transit time through the myelocytic pool in proportion to its effect on the postmitotic pool transit time, then these maximum activity neutrophils might have been initially exposed to G-CSF during the promyelocytic phase of their development. The promyelocyte phase is characterized by synthesis of MPO and other azurophilic granular components. Exposure of promyelocytes to a relatively high concentration of G-CSF might directly influence the quality or quantity (or both) of azurophilic granule synthesis.

The effect of G-CSF on the morphology of Wright- or Giemsastained blood neutrophils is similar to that observed in infection. At a high dose, G-CSF produces a left shift toward larger neutrophils with less nuclear segmentation, more obvious azurophilic granulation, and increased MPO content [38]. Such changes might explain the increased opsonin-stimulated PFCL activity and the increased microbicidal activity that followed G-CSF treatment. However, the increases in PANL and FANL activities seem too large to be solely explained by increased MPO.

Previous azide inhibition studies indicate that MPO is largely but not completely responsible for the luminol luminescence activity of neutrophils from healthy donors [18, 15]. The potent inhibitory effect of azide is also illustrated in the present study. The nature of azide inhibition of PANL activity was essentially the same for pretreatment day 1 specimens as for the early posttreatment days 2 and 3 specimens, despite a 4-fold increase in neutrophil count. By day 5 of G-CSF treatment, azide was less effective as an inhibitor as indicated by a decrease in the percentage and in the slope of inhibition, suggesting that activity was less dependent on MPO.

Alternatively, G-CSF treatment might induce or activate an additional enzymatic system capable of dioxygenating luminol to yield luminescence. In common with bacterial infections and treatment with interferon-γ, G-CSF induces neutrophil expression of high-affinity receptors for IgG (FcγR1, CD64) and increases neutrophil-mediated antibody-dependent cellular cytotoxicity activity [6]. In an analogous manner, G-CSF treatment might also induce neutrophil synthesis of NO synthase. NO is the major endothelium-derived relaxing factor responsible for regulation of vascular tone [39]. PMA, FMLP, and serum-opsonized zymosan are reported to stimulate neutrophil synthesis and release of NO [23, 24]. NO is a paramagnetic molecule and is highly reactive with other paramagnetic molecules, such as O₂⁻ [15]. Reaction of NO with O₂⁻ yields OONO⁻ [21], which can serve as a direct dioxygenating agent and may be the active agent responsible for NO-associated microbicidal action. The available evidence suggests OONO⁻ can directly dioxygenate luminol, yielding luminescence [19]. If so, G-CSF–induced NO synthase activity might in part explain the large increases in PANL and FANL activity that follow several days of G-CSF treatment.

OONO⁻ production requires the reaction of NO with O₂⁻; therefore, introduction of SOD, an enzyme that disproportionates O₂⁻ to O₂ and H₂O₂, would remove O₂⁻ and competitively inhibit neutrophil production of OONO⁻ [23, 24]. However, as illustrated by the data in table 1, the inhibitory effect of SOD on PANL activity was relatively small. Furthermore, direct attempts to inhibit NO synthase with NMLA were unsuccessful at concentrations ranging from 10 to 800 μM. Although they are interesting possibilities, we did not find evidence in support of G-CSF induction of NO synthase or that neutrophil generation of OONO⁻ is responsible for the increased luminescence resulting from luminol oxygenation.

Neutrophils are the primary effector phagocytes of host defense against infection. The neutrophil integrin CD11b/CD18 (i.e., Mac-1 or CR-3) plays a crucial role in the neutrophil-endothelial interaction required for neutrophils to migrate from the vascular space, recognize opsonized microbes, phagocytize these microbes, and activate the redox metabolism required to produce the oxidants and oxygenating agents that participate in microbe killing. Unprimed circulating blood neutrophils of healthy controls express only a small portion of their total CD11b on the surface membrane. CD11b is stored in the specific granules of the unprimed neutrophil [32–34]. A broad spectrum of agents, including the complement anaphylatoxin C5a, lipid-derived agents, such as PAF and leukotrienes (LTB₄), and cytokines, such as interleukin 8, are capable of causing full expression of neutrophil CD11b/CD18 at concentrations that do not directly activate respiratory burst metabolism [15].
G-CSF is reported to increase the expression of CD11b in vitro [40, 41]. Treatment of healthy subjects with G-CSF also causes an initial in vivo increase in the CD11b expression of circulating neutrophils, but expression returns to pretreatment levels with prolonged G-CSF administration [42–44]. Consistent with the increased antigenic CD11b expression, a mild transient increase in the COR/MOR ratio was caused by G-CSF. The COR-dependent activity per neutrophil (BKCL activity), should not be used alone as a direct gauge of CD11b expression, because COR activity is dependent on both opsonin receptor expression and neutrophil metabolic capacity, and as shown in the results section, neutrophil metabolic capacity is increased by G-CSF treatment. The data of figure 5 demonstrate that G-CSF treatment increases both COR and MOR activities. Since both COR and MOR activities are measured from the same blood specimen, expressing the COR activity relative to the MOR activity (i.e., as the COR/MOR ratio) normalizes the results with respect to any change in neutrophil metabolic capacity. The ratio provides an index of opsonin receptor response capacity and minimizes the distortion introduced by differences in neutrophil metabolic and oxygenation capacity, such as is observed during the temporal course of G-CSF treatment [14, 15].

A low antigenic CD11b expression and a relatively low functional COR/MOR ratio are consistent with a low level of in vivo activation. Measurement of low integrin expression in unstimulated circulating neutrophils does not necessarily imply impaired capacity or function. Any assessment of neutrophil phenotypic or functional opsonin receptor capacity should be based on measurement of optimally primed phagocytes in order to guarantee MOR expression per neutrophil.

References

28. Baldwin GC, Fuller ND, Roberts RL, Ho DD, Golde DW. Granulocyte- and granulocyte-macrophage colony-stimulating factors enhance neu-


