RAPID COMMUNICATION

Effective Stimulation of Donors for Granulocyte Transfusions With Recombinant Methionyl Granulocyte Colony-Stimulating Factor

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Effective granulocyte transfusion (GT) therapy has been hampered by the low yield of neutrophil granulocytes (PMN) obtainable from normal donors even by use of corticosteroid pretreatment, hydroxyethyl starch (HES), and modern leukapheresis (LA) techniques. To increase the PMN yield we performed LA in 22 healthy volunteer donors after a single subcutaneous administration of 300 μg of granulocyte colony-stimulating factor (G-CSF) 12 to 16 hours before LA. Five to 7 L of blood was processed within 3 to 3 hours using the standard CS-3000Plus (Baxter, Deerfield, IL) LA protocol including HES. The mean number of PMN harvested was 44.32 ± 15.5 X 10^9, corresponding to 6.88 ± 2.1 X 10^9/L of blood processed. In the final product PMN functions (in vitro: chemotaxis, phagocytosis, chemiluminescence, superoxide anion production; in vivo: chemiluminescence, half-life) were at least normal. In all donors G-CSF induced a consistent increase of white blood cell (mean 16.46 ± 3.8 X 10^9/L) and PMN counts (15.94 ± 3.6 X 10^9/L). No G-CSF-related side effects were observed and LA was well tolerated. G-CSF prestimulation allows to harvest three to five times higher numbers of functionally normal PMN by LA compared with corticosteroid pretreatment. This may help to overcome one of the major limitations of an effective PMN support.

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GRANULOCYTE transfusions (GT) have been used successfully to support patients with persistent agranulocytosis or neutrophil granulocyte (PMN) function defects. Benefit has been shown in serious bacterial and fungal infections unresponsive to appropriate antimicrobial therapy. In these studies a dose-response relationship has been demonstrated, with higher total cell dose leading to improved clearance of infection and survival. However, GT obtained by leukapheresis (LA) from normal donors contain 0.5 to 3 X 10^6 PMN only, despite the use of erythrocyte sedimenting agents and corticosteroid pretreatment. This amount corresponds to only 10% to 20% of the daily PMN production of a healthy adult and even less of a patient with infection. Therefore, a simple method allowing to harvest larger numbers of fully functional PMN would be highly desirable.

For this purpose we evaluated the feasibility and effectiveness of granulocyte colony-stimulating factor (G-CSF) (recombinant methionyl G-CSF, filgrastim; Amgen/Roche, Basel, Switzerland) stimulation in normal donors and tested the function of the collected PMN.

METHODS, DONORS, AND RECIPIENTS

PMN Collection

LA was performed by means of the Standard Procedure 2 on the CS-3000 Plus Blood Cell Separator (Baxter, Deerfield, IL). For each collection, 5 to 7 L of ACD-A anticoagulated blood were processed by continuous-flow centrifugation at a flow-rate of 50 mL/min. Red blood cell (RBC) sedimentation was facilitated by hydroxyethyl starch (Plasmasteril 6%, Fresenius, Germany; 1:13 vol/vol). The final product was tested for complete blood cell counts and PMN function and was then subjected to irradiation with 15 to 40 Gy.

Cell Counts

Complete blood cell counts were performed on the H*l flow cytometer (Technicon Instruments, Tarrytown, NY). In addition, 200-cell differentials were performed microscopically on stained blood films. Cell counts are given as means ± 1 standard deviation. Leukocyte (Lc) and PMN recoveries are calculated as follows:

\[
\text{Cells in the Final Product} = \frac{\text{mean of pre- and post-LA cell counts}}{\text{blood volume processed (L)}} \times 100
\]

PMN Function Tests

The function of the collected PMN was tested by several standard methods: Chemotaxis under agarose, phagocytosis of opsonized particles, chemiluminescence, and superoxide anion (O_2^-) production.

Chemotaxis. Migration under agarose was tested as previously described. Agarose plates were prepared (Agarose 1%, Hanks' balanced salt solution [HBSS] and HEPES buffer) and punched to obtain 3 lines of 0.4-mm holes. Migration of the PMN (1.5 × 10^6 cells in HBSS) from the holes in the middle line toward the upper holes filled with HBSS (not stimulated) and toward the lower holes containing zymosan-activated serum (stimulated) was measured after 90 minutes of incubation at 37°C in 5% CO_2. The results are given in micron migrated per 90 minutes.

Phagocytosis. PMN, 10^5, were added to a mixture of 100 μL of fresh plasma 15% and 100 μL HBSS containing 2.5 × 10^3 yeast particles. The phagocytized particles were counted microscopically after 30 minutes incubation at 37°C. The results are given in phagocytosed particles/100 PMN.

Chemiluminescence. Luminol-dependent chemiluminescence was measured as described elsewhere using an LKB Wallac 1251 luminometer (Wallac, Turku, Finland). Stimulation of 10^6 PMN was effected with opsonized zymosan 1 mg/mL. The results are given in mV/10^6 PMN/min.

O_2^- production. Continuous and quantitative measurement of the O_2^- production was performed on a diode array spectrophotometer (Hewlett Packard, Fort Collins, CO) by measuring the change in the absorbance of cytochrome C at 550 nm as a function of time. PMN, 10^6, in cytochrome C-buffer were exposed to stimulation by phorbol...
nyristate acetate 100 ng/mL with formyl-methionyl-leucyl-phenylalanine 1 μmol/L. The results are shown in μmol/10^6 PMN/min.

The number of O2 producing cells was evaluated by flow-cytometry using dihydrorhodamine (DHR) 123 as the indicator. Briefly, 5 × 10^8 PMN in 200 μL medium were activated by addition of 20 μL PMA (2 mg/mL stock solution). After 15 minutes at 37°C, DHR 123 was added to a final concentration of 150 ng/mL. After a further 15-minute incubation at 37°C cells were measured in a flow-cytometer (FACScan; Becton-Dickinson, Basel, Switzerland) and compared with PMA unstimulated cells.

Intravascular survival. Cell counts and differentials were performed before as well as 1, 3, 6, 9, 12 to 14, and 24 hours post GT.

Donors

Twenty-two healthy adult donors were recruited from the regular volunteer donor pool of the Swiss Red Cross. They had tested negative for antibodies against human immunodeficiency virus (HIV) I/II, hepatitis C virus, cytomeglovirus, and for hepatitis B surface-antigen. After informed consent was obtained, G-CSF (filgrastim, Amgen/Roche) was injected subcutaneously (sc) at 300 pg fixed dose (3 to 5 pg/kg body weight [BW]) 12 to 16 hours before LA. A blood cell count was performed before injection and immediately before LA.

Recipients

GT were administered after irradiation with 15 to 40 Gy using a linear accelerator as source to three patients with profound aplasia (two children after allogeneic bone marrow transplantation, one adult after myeloablative therapy for acute myelogenous leukemia) and severe unresponsive bacterial or fungal infections.

Statistics

Significance was calculated using the unpaired t-test.

RESULTS

Leukocyte and Granulocyte Yields

The total blood volume processed was 7 L (15 donors), 6 L (2), or 5 L (5) in a processing time of 1.9 to 3.0 hours. The average total yield of Lc was 54.78 ± 16.9 × 10^9 (range 31 to 119.49) and of PMN 44.32 ± 15.2 × 10^9 (range 21.39 to 102.68) (Fig 1A).

The average yield of Lc per liter of processed blood was 8.52 ± 2.4 × 10^9 (range 5.17 to 17.05) and the yield of PMN 6.88 ± 2.1 × 10^9 (range 3.57 to 14.67) (Fig 1B). The Lc and PMN yields per hour of LA were 20.49 ± 6.2 × 10^9 (range 10.33 to 39.8) and 16.55 ± 5.4 × 10^9 (range 7.13 to 34.23), respectively.

The Lc and PMN recovery (n = 7) were 47.76% ± 5.1% (range 39.32 to 56.52) and 45.23% ± 3.5% (range 41.96 to 51.25), respectively. The final product also contained 4.07 ± 0.9 × 10^11 thrombocytes and had a hematocrit of 12.6% ± 7.8%.

To assess the true benefit for G-CSF stimulation the last 104 consecutive PMN collections performed at our institution using the same standard LA technique (CS-3000, procedure 2; hydroxyethyl starch; blood volume processed 5 to 7 L) but with dexamethasone-stimulated donors (6 and 3 mg administered 12 and 3 hours before LA, respectively) were analyzed retrospectively. Twenty-three of the 104 LA (22%) could not be evaluated because of incomplete apheresis protocols (9), missing white blood cell differentials (13), or clot formation...
(1) of the final product. In the remaining 81 LA the mean volume of donor blood processed was 6.78 L (compared with 5.96 L in the study donors). The average total PMN yield (17.4 ± 6.8 × 10⁹) and the mean PMN yield per liter of processed blood (2.7 ± 1.0 × 10⁹) were significantly lower after prestimulation with dexamethasone than after G-CSF (P < .0001).

PMN Function Tests

Results of function tests performed with nine final PMN products obtained after G-CSF prestimulation of the donors are shown in Table 1 both before (top) and after (bottom) the 15 to 40 Gy irradiation (Table 1).

PMN function tests for the analyzed PMN yield results comparable with those of the same tests performed with PMN from 10 normal unstimulated adults. The measurements of the respiratory burst (chemiluminescence, O₂ formation) even showed values exceeding the normal range.

In Vivo Data

Collected PMN were stored at room temperature and transfused within 2 hours. PMN survival data of seven GT administered to two aplastic patients with persistent severe infection are shown in Fig 2. The intravascular half-life was 9 to 12 hours in both of them. (The remaining 15 GT were not transfused [n = 2] or incompletely documented [n = 3] or were given to a patient with chronic granulomatous disease [CGD] before myeloablative therapy [n = 10] whose pretransfusion PMN values varied considerably from day to day [from 0.25 to 3.62 × 10⁹/L], most probably reflecting his severe uncontrolled infection. Because of this inconstant endogenous PMN production and/or turnover, evaluation of posttransfusion PMN survival was not meaningful for these GTs.) After 3 of the 4 GT depicted in the top panel of Fig 2, administered to a patient suffering from CGD with defective endogenous O₂ production, the in vivo function of the transfused PMN could be tested by flow cytometry using DHR 123 as respiratory burst indicator. Transfused PMN, 90% to 96%, were shown to be functionally intact with respect to O₂ production over their circulation time (results not shown).

Effects on the Donors

Twenty-two donors were stimulated with a single dose of 300 µG G-CSF (3 to 5 µg/kgBW) administered sc. There were no G-CSF-related side effects. The number of Lc, PMN, and monocytes before and 12 to 16 hours after G-CSF are shown in Fig 3.

The Lc count increased from 7.38 × 10⁹/L (range: 4.17 to 11.09) to 23.17 × 10⁹/L (range: 16.69 to 32.27). The average increase was 16.48 ± 3.8 × 10⁹/L (range 11.02 to 23.66; P < .0001) or by a factor of 3.37 ± 0.6 (range 2.24 to 5.10). The main effect was an increase of the PMN count, which increased from 4.24 × 10⁹/L (range 2.13 to 7.97) before G-CSF administration to 20.18 × 10⁹/L (range 14.54 to 28.63) 12 to 16 hours thereafter. The average increase was 15.94 ± 3.6 × 10⁹/L (range 10.70 to 23.18; P < .0001) corresponding to an increment of 5.05 ± 1.5 (range 2.99 to 10.38). Morphologically, the PMN were normal except for a "shift to the left" without immature forms and a trend to toxic granulation. We also observed a consistent increase of blood monocytes from 0.49 × 10⁹/L (range 0.24 to 0.77) to 1.25 × 10⁹/L (range 0.53 to 2.12), corresponding to an average increase of 0.63 ± 0.41 × 10⁹/L (range 0.12 to 1.60; P < .0001) or an increment of 2.40 ± 1.0 (range 1.25 to 5.16). The other cell lines (eosinophils, basophils, lymphocytes, hematocrit, and reticulocytes) were not significantly changed (1 < P < .6;

Table 1. In Vitro Function Tests of Collected Neutrophils (PMN)

<table>
<thead>
<tr>
<th>No.</th>
<th>Migration</th>
<th>Spontaneous (μM/90 min)</th>
<th>Stimulated (μM/90 min)</th>
<th>Phagocytosis (Particles/100 PMN)</th>
<th>Chemiluminescence (mV/10⁹ PMN/min)</th>
<th>O₂ Production (μmol/10⁹ PMN/min)</th>
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<td></td>
<td></td>
<td>308 ± 87</td>
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<td>555 ± 210</td>
<td>10.5 ± 1.8</td>
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<td>Before irradiation</td>
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<td></td>
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<td>1</td>
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<td>Mean</td>
<td></td>
<td>680 ± 62</td>
<td>1,126 ± 193</td>
<td>716 ± 106</td>
<td>1,986 ± 468</td>
<td>17.28 ± 2.2</td>
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<tr>
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<td>1,135</td>
<td>775 ± 43</td>
<td>1,856 ± 438</td>
<td>18.29 ± 0.9</td>
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</table>

Mean ± SD; n = 9.

Abbreviations: Contr, normal values of 10 unstimulated adult controls (mean ± SD); ND, not done.
PMN $\times 10^9/\text{L}$

Pat 1

0 6 12 18 24 30 hours

0 0.3 0.6 0.9 1.2

PMN $\times 10^9/\text{L}$

Pat 2

0 6 12 18 24 30 hours

0 10 20 30

**Fig 2.** In vivo neutrophil (PMN) survival in patient 1 (age 11 years, top) and patient 2 (age 6 months, bottom), each line depicting a separate PMN transfusion.

data not shown). The LA procedure was generally well tolerated by the donors. Some donors experienced slight citrate toxicity during apheresis necessitating oral calcium supplementation. After LA no donor showed a thrombocyte count below $130 \times 10^9/\text{L}$.

**DISCUSSION**

Pharmacokinetic studies of sc G-CSF administration have shown maximal serum levels after 3 to 6 hours lasting for 10 to 16 hours.\(^{16-18}\) The few animal studies on the course of PMN levels after a single sc dose of G-CSF or after two injections separated by 12 hours have shown a 3-fold and 10-fold increase of circulating PMN, respectively.\(^{19,20}\) A single G-CSF administration to two human subjects lead to a sixfold increase of PMN counts within 8 hours.\(^{21}\) On the basis of these observations, we decided to test whether a single dose of G-CSF 3 to 5 $\mu\text{g/kgBW}$ administered sc would bring about an increase in the circulating PMN levels, thereby optimizing the numbers of PMN harvested by LA from normal donors.

Our results show that a single sc injection of 300 $\mu\text{g}$ G-CSF resulted in a substantial increase of the circulating PMN level. When the donors were subjected to LA starting 12 to 16 hours after G-CSF administration, a mean yield of $44.9 \pm 15.1 \times 10^9$ PMN ($6.9 \times 10^9/\text{L}$ of processed blood) was obtained. This is about 10 times higher than the yields reported for LA without any donor stimulation or 3 to 5 times higher than those usually reported for donors receiving dexamethasone and LA performed with hydroxyethyl starch.\(^{3,5-10}\) With longer apheresis even higher yields seem to be possible (data not shown). Moreover the optimal dose and timing of G-CSF administration awaits further investigation. Assuming a daily PMN production rate of $1.5 \times 10^9/\text{kgBW},^{11,22}$ the harvest of nearly 1 day's adult PMN production seems within reach by use of G-CSF stimulation and modern LA techniques. Because the relatively small number of PMN transfused to severely infected patients in earlier studies is one of the major criticisms of GT therapy this improvement may be of clinical relevance. Moreover, the shorter processing time may ameliorate the acceptance of LA by volunteer donors and reduce the financial burden of GT.

The PMN stimulated by short courses of G-CSF are reported to be functionally normal\(^{23,24}\) and their in vivo survival remains unaffected.\(^{25}\) Moreover, G-CSF is very well tolerated. Doses up to 100 $\mu\text{g/kgBW}$ have been administered to primates for periods of 2 weeks without toxicity.\(^{26}\) Our studies confirm these earlier observations and point to the efficacy and safety of G-CSF stimulation for PMN collection from normal donors. The functions tested in vitro were not affected by up to 40 Gy gamma irradiation. The transfused PMN showed a calculated intravascular half-life of about 10 hours, something longer than previously reported.\(^{22}\) An initial accumulation of G-CSF–stimulated PMN in the pulmonary capillary system followed by subsequent redistribution may be postulated as a possible explanation for this observation, as has previously been shown for GM-CSF–stimulated PMN.\(^{27}\) As was expected from earlier in vitro and ex vivo studies on G-CSF–prestimulated PMN, all in vitro functions tested after LA were normal (chemotaxis, phagocytosis) or even enhanced (respiratory burst: chemiluminescence, $O_2$ production).\(^{28,29}\) Because G-CSF does not directly stimulate the release of superoxide, but primes cells for specific responses triggered via receptor-mediated mechanisms,\(^{29}\) targeted deposition of oxygen radicals onto opsonized microbes is expected instead of premature activation of the respiratory metabolism with detrimental collateral damage. The preserved $O_2$ formation of the transfused PMN observed in our patient with CGD also indicates a normal function in vivo.

A single sc G-CSF administration to normal donors was regularly followed by a consistent increase of the PMN counts 12 to 16 hours later. We are not aware of other similar studies in humans. Significant effects on other blood cell lines have not been documented after short-term application of G-CSF in humans, whereas monocyte levels doubled during prolonged treatment periods with higher doses.\(^{28}\) We observed...
a regular increase of monocytes to more than twice the baseline values after a single G-CSF injection. Monocytes may play an important role in the clinical efficacy of GT because they have a much longer tissue half-life than PMN, and retain the ability to differentiate into macrophages.30

We conclude that the stimulation of donors for leukapheresis by 300 µg G-CSF in a single sc dose is feasible and safe. It results in a substantial increase of circulating PMN, allowing higher PMN yields to be obtained by standard LA procedures. Whether a higher number of PMN per GT will result in improved support of severely infected patients with bone marrow failure remains to be shown.

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Effective stimulation of donors for granulocyte transfusions with recombinant methionyl granulocyte colony-stimulating factor [see comments]

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