Neutrophil Migration Is Defective During Recombinant Human Granulocyte-Macrophage Colony-Stimulating Factor Infusion After Autologous Bone Marrow Transplantation in Humans

By William P. Peters, Ann Stuart, Mary Lou Affronti, Chul Soo Kim, and R. Edward Coleman

We have previously reported that continuous intravenous (IV) administration of recombinant granulocyte-macrophage colony-stimulating factor (rHuGM-CSF) to humans following high-dose alkylating agent chemotherapy and autologous bone marrow support (ABMS) results in myeloid bone marrow maturation, accelerated granulocyte recovery, and reduced treatment-related toxicity. However, we found that leukocyte counts declined rapidly after discontinuation of rHuGM-CSF therapy, which suggests possible growth factor effects on leukocyte margination and migration. For these reasons we studied granulocyte margination by using 111In-labeled autologous granulocytes and found similar granulocyte margination before and during rHuGM-CSF infusion (23.3% ± 9.8%). Phagocytosis of Cryptococcus neoformans and granulocyte hydrogen peroxide production was similar before and during rHuGM-CSF infusion and similar to patients treated with the same high-dose chemotherapy and ABMS but not receiving growth factor. However, migration of granulocytes to a sterile inflammatory site was markedly reduced during continuous rHuGM-CSF infusion (1.2 ± 0.9 WBCs/cm², 24 hr) as compared with baseline (39.6 ± 17.7 WBCs/cm²/24 hr; P < .0008). These findings may be of relevance when extravascular granulocytes are required for host defense.

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METHODS

Patients studied. Patients with histologically confirmed breast cancer or melanoma were studied before and during the intravenous (IV) administration of rHuGM-CSF during the conduct of a phase II evaluation of rHuGM-CSF. Five additional patients with metastatic malignancy (three with breast cancer, one each with lung cancer and ovarian cancer) receiving high-dose chemotherapy and bone marrow support but not rHuGM-CSF were studied as controls.

All patients received an indwelling, triple-lumen, right atrial catheter for venous access before treatment. The transplant preparative regimen in all patients receiving rHuGM-CSF was as follows: cyclophosphamide was administered as a one-hour IV infusion on days -6, -5, and -4 at a dose of 1.875 mg/m²/day; cisplatin (165 mg/m²) was administered with hydration by continuous infusion over 72 hours from days -6 to -3; carmustine (600 mg/m²) was administered on day -3 at a rate of 5 mg/m²/min. The chemotherapy program for the control patients was exactly the same as described earlier in the three control breast cancer patients and substituted etoposide (1,200 mg/m²) or thiotepa (300 mg/m²) for carmustine in the other two patients.

Three days following the completion of chemotherapy (day 1) cryopreserved autologous bone marrow was thawed and rapidly infused without further processing.

rHuGM-CSF. Complementary DNA encoding human GM-CSF was cloned and expressed by Genetics Institute, Inc (Cambridge, MA) and rHuGM-CSF was formulated as a lyophilized powder by Sandoz Pharmaceuticals (Basel, Switzerland). It was reconstituted in sterile water and added to 500 mL of 5% dextrose with 0.45% saline before administration. The specific activity was 8.0 x 10⁹ U/mg protein.

rHuGM-CSF was administered for 14 to 21 days by continuous IV infusion through a dedicated lumen of the right atrial catheter beginning three hours after bone marrow infusion.

Granulocyte margination. Margination of granulocytes was studied in four patients by a modification of the procedure described...
by Weiblen et al.12 Granulocytes were isolated from the peripheral blood of patients receiving high-dose combination alkylating agents and autologous bone marrow support before treatment and on the last day of rHuGM-CSF infusion. Granulocytes were isolated by Ficoll-Hypaque gradient centrifugation and red cells removed by sedimentation with 3 mL 6% hydroxyethyl starch to each 10 mL of granulocytes. The cells were washed twice with 1% human serum albumin. Granulocytes were labeled by mixing 50 µCi 111In oxine prepared as described by Thakur et al.13 and kept at room temperature for 20 minutes with gentle mixing. The labeled granulocytes were then withdrawn from the vial and injected into the recipient. Serial blood samples were obtained at 15, 30, 60, 120, 135, 150, 240, and 360 minutes. Epinephrine (0.3 mg subcutaneously [s.c.] was administered at 120 minutes to cause temporary demargination. The percentage of maximal cell-associated counts per minute appearing after epinephrine administration was used to calculate the percent margination.

Localized leukocyte mobilization. The ability of granulocytes to migrate to a peripheral sterile inflammatory site was measured by using a standardized skin chamber assay14,15 in 15 patients before chemotherapy and autologous bone marrow transplantation (baseline). Ten of these patients were again studied during continuous infusion of rHuGM-CSF at a time when the peripheral leukocyte count was comparable to baseline values; three patients treated with high-dose chemotherapy and autologous bone marrow support but who did not receive rHuGM-CSF were also studied at the time of hematopoietic recovery as controls. A 1-cm square abrasion was made on the volar surface of the forearm by using a rotary emery wheel (Dremel Moto Tool, Racine, WI). A sterile plastic Senn chamber16 was applied and fixed with glue and tape. The chambers were filled with 2.0 mL of autologous cell-free serum through the entry-exit port. Serum was generally obtained from one large bleed before the first control study, although serum obtained while the patient was receiving rHuGM-CSF showed no difference to pretreatment serum when studied in duplicate chambers. Leukocyte mobilization into the serum was determined by periodically draining the chamber through the port after opening the air vent. The chamber was then refilled with fresh autologous serum. Leukocyte mobilization was determined at intervals during the 24 hours and the cumulative value expressed as WBCs x 10⁶/cm² of abrasion surface. Differential cell counts performed on 100 cells stained with Wright-Giemsa reagent indicated that >98% of migrating cells were granulocytes, which is consistent with previous observations.14,15

Phagocytosis of Cryptococcus neoformans. Granulocytes were prepared from 30 mL peripheral blood by sedimentation with 4% dextran, removal of mononuclear cells by sedimentation through Ficoll-Hypaque gradients, and lysis of contaminating RBCs by 0.8% NH₄Cl. A quantity of 4 x 10⁶ granulocytes was added to each of six microtiters wells for each assay and incubated in 5% CO₂ for 30 minutes. C neoformans were grown overnight in Dubelcco’s modified Eagle’s medium (DMEM) with 250 µCi/mL H₂¹⁵³SO₄ with a specific activity of 43 Ci/mg S. Yeasts were washed with phosphate-buffered saline (PBS) three times by centrifugation and were resuspended at 8 x 10⁶ cells/mL in DMEM. ²⁴S-C neoformans (0.1 mL) was added to each assay well and incubated at 37°C for 90 minutes. Phagocytosis was performed with and without autologous serum. Extracellular yeasts were removed from the granulocytes by rinsing twice with PBS and then lysed by adding 1.0 mL 0.5% (wt/vol) sodium deoxycholate. Released cryptococci were resuspended, and 0.1 mL was mixed with 4 mL Biofluor (New England Nuclear, Boston) and counted in a liquid scintillation spectrometer (Beckman LS-800, Palo Alto, CA). The percentage of phagocytosis was calculated from the means of triplicate counts per minute recovered from the granulocytes divided by the counts per minute added to granulocytes and multiplied by 100. Results are expressed as mean percentages of input counts ingested for replicate samples ± SD. Granulocytes migrating into autologous serum-filled chambers as described earlier were also studied for their phagocytic capacity. Insufficient numbers of granulocytes mobilized to the skin chambers to allow an assay while patients were receiving rHuGM-CSF. Four patients who did not receive rHu-GM-CSF were studied at the time of granulocyte recovery following high-dose chemotherapy and autologous bone marrow infusion.

Granulocyte hydrogen peroxide production. Granulocytes from 13 patients were prepared from peripheral blood or obtained from localized leukocyte mobilization (LLM) chambers as described in the legend to Table 1. Aliquots of 1 x 10⁸ were added to each of three replicate microtiters wells. Basal and maximally stimulated oxidative metabolism was determined by using a modification of the method of Pick and Miezels by the addition of PBS or phorbol myristate acetate (PMA) (2.5 µg) and horseradish peroxidase (Sigma Chemical Co., St Louis) and the reactions incubated at 37°C for 60 minutes. Samples were read in a Titerhek Multiskan MC (Flow Laboratories, McLean, VA) spectrophotometer at 620 nm and hydrogen peroxide production calculated by comparison to a standard curve by using linear regression and expressed as nmol H₂O₂ produced/h/10⁷ granulocytes.

Statistical methods. Values are expressed as means ± SD. The statistical significance of differences was calculated by using the nonparametric Mann-Whitney test and the normal approximation Z score.

RESULTS

In these studies we sought to evaluate the effects of continuous infusion of rHuGM-CSF in humans on the function of mature granulocytes. Specifically, we analyzed granulocyte margination, migration, phagocytosis, and oxidative metabolism. To analyze the effect of rHuGM-CSF on leukocyte margination, we labeled purified autologous granulocytes with 111In and administered these labeled granulocytes to four patients with metastatic breast cancer or melanoma who were receiving high-dose alkylating agent chemotherapy and autologous bone marrow support. The patients were studied before the initiation of chemotherapy and again after 14 to 21 days of continuous IV rHuGM-CSF infusion at a time when peripheral blood counts were similar. The granulocyte half-life was calculated from the disappearance of cell-associated 111In from the circulation. Epinephrine (0.3 mg s.c.) was administered to produce temporary leukocyte demargination (Fig 1) and the marginating granulocyte fraction calculated. The margination of granulocytes was similar before (21.5% ± 13.4%) and during infusion (23.3% ± 9.6%) of rHuGM-CSF.

We next analyzed the ability of the granulocytes maturing during rHuGM-CSF infusion to phagocitize ³⁵S-labeled C neoformans. The serum-dependent ingestion of C neoformans was similar during the administration of rHuGM-CSF as compared with patients before chemotherapy and rHuGM-CSF administration (P < .17, Mann-Whitney test) and similar in patients not receiving rHuGM-CSF but treated with the same high-dose chemotherapy and bone marrow support (Table 1).

Granulocytes exert a major portion of their killing function through the generation of reactive oxygen reduction products. This coordinated sequence of biochemical reactions, known as the "oxidative burst," results in the one-
which electron reduction of oxygen to superoxide anions (O$_2^-$), which are subsequently converted to hydrogen peroxide (H$_2$O$_2$) by either spontaneous or enzyme-mediated dismutation. rHuGM-CSF has been shown in vitro to enhance the maximally stimulated production of superoxide anions.$^{7,8}$ We measured the basal and PMA-stimulated production of hydrogen peroxide in the basal state or in response to PMA is not significantly different in granulocytes obtained from patients during rHuGM-CSF treatment as compared with pretreatment granulocytes or patients not receiving rHuGM-CSF. Granulocytes migrating to LLM chambers displayed hydrogen peroxide production similar to granulocytes from the peripheral blood.

Finally, we evaluated the ability of granulocytes to migrate to a sterile inflammatory site by using a standardized skin chamber assay.$^{14,15}$ A 1-cm abrasion was made on the volar forearm surface and covered with a sterile plastic chamber. Migration of granulocytes into the patients' autologous serum was quantitated over a 24-hour period. In this assay, 95% to 98% of the cells appearing in the chambers were granulocytes. Patients were studied by using duplicate chambers at similar peripheral leukocyte counts before (mean: 5.0 ± 1.3) and during (mean: 4.0 ± 2.2) the continuous IV infusion of rHuGM-CSF. The results demonstrate that leukocyte migration to a sterile inflammatory site is markedly reduced during continuous rHuGM-CSF infusion (1.2 ± 0.9 WBCs/cm$^2$/24 h) as compared with baseline values (39.6 ± 17.7 WBCs/cm$^2$/24 h; $P < .0008$, Mann-

![Graph A](image1.png)

![Graph B](image2.png)

**Fig 1.** Determination of granulocyte margination before (A) and during (B) continuous infusion of rHuGM-CSF in one patient.

**Table 1. Phagocytosis of C neoformans by Granulocytes From Patients Receiving rHuGM-CSF**

<table>
<thead>
<tr>
<th>UPN</th>
<th>Study Day</th>
<th>Peripheral Blood</th>
<th>LLM Chamber</th>
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<tr>
<td></td>
<td></td>
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<td>With Serum</td>
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<tr>
<td>167</td>
<td>Pre</td>
<td>17.1 ± 7.7</td>
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<tr>
<td>171</td>
<td>Pre</td>
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<tr>
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<td>Pre</td>
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<tr>
<td>174</td>
<td>Pre</td>
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<td>27.6 ± 9.2</td>
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<tr>
<td>184</td>
<td>Pre</td>
<td>22.9 ± 5.6</td>
<td>28.3 ± 8.4</td>
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<tr>
<td>193</td>
<td>Pre</td>
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<tr>
<td>194</td>
<td>Pre</td>
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<tr>
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<tr>
<td>156</td>
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<tr>
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Abbreviations: Pre, granulocytes obtained before the initiation of chemotherapy and rHuGM-CSF; + rHuGM-CSF, granulocytes obtained after hematopoietic recovery but during the administration of continuous-infusion rHuGM-CSF; No GM-CSF, granulocytes obtained from patients after hematopoietic recovery from high-dose chemotherapy and autologous bone marrow infusion but who did not receive rHuGM-CSF at any time; LLM chamber, granulocytes migrating into the LLM chamber; ‑, test not performed; QNS, quantity not sufficient.
The studies presented here demonstrate a significant defect in granulocyte migration to a sterile inflammatory site during rHuGM-CSF infusion. The partial recovery of granulocyte migration after discontinuation of rHuGM-CSF therapy is consistent with this interpretation. The design of the migration studies in which each patient served as his own control and in which the studies were performed in duplicate provide a high degree of reliability to the observations. These data would suggest that continuous IV infusion may not be the optimal manner of administration. Evaluation of alternative modes of rHuGM-CSF infusion on granulocyte migration and function is being undertaken.

Other explanations of the defective migration are possible. It is possible that the reduced migration is an effect of high-dose chemotherapy or other supportive care and not directly an effect of rHuGM-CSF. Observations with patients not receiving rHuGM-CSF (Fig 2A) suggest that this possibility is not a complete explanation since patients with recovering counts after high-dose chemotherapy and bone marrow support without rHuGM-CSF mobilize granulocytes nearly normally. While further studies to define mechanisms are essential, particularly in normal individuals.

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Fig 2. (A) LLM before (-S. 28 chambers) and during rHuGM-CSF administration (—, 17 chambers) in patients treated with high-dose alkylating agents and autologous bone marrow support. LLM after leukocyte recovery in patients treated with the same chemotherapy but not receiving rHuGM-CSF is shown as open circles (four chambers); n, number of patients studied. Values are expressed as means ± SD. Studies were timed to occur at the end of rHuGM-CSF infusion or at an equivalent leukocyte count to the baseline study. (B) LLM before, during, and after rHuGM-CSF infusion for duplicate skin chambers on patient 167 (unique patient number [UPN]). The inset shows peripheral WBCs v day from marrow infusion. Symbols (■, □, △) and arrows in the inset define the times at which LLM in the major panel was performed. Each study was timed to be performed at the time of an equivalent peripheral leukocyte count.

where the effects of chemotherapy are not seen, the data presented show that granulocytes appearing with rHuGM-CSF infusion during recovery from high-dose chemotherapy and autologous bone marrow support (ABMS) do not migrate normally. This effect may be of relevance when extravascular granulocytes are of importance in defense against bacterial or fungal invasion during rHuGM-CSF infusion.

Continuous infusion of rHuGM-CSF did not appear to affect the marginating pool. These data are consistent with the in vitro observations that rHuGM-CSF does not affect the adherence of granulocytes to vascular endothelium\(^{18}\) in contrast to bacterial products, complement cascade components,\(^{21}\) and recombinant human tumor necrosis factor.\(^{32}\) Whether shorter infusions of rHuGM-CSF have a similar effect is unknown at present. Further evaluation of the acute effect of rHuGM-CSF administration is warranted.

The normal phagocytosis and hydrogen peroxide production would be expected from studies of rHuGM-CSF in vitro. The presence of an increased number of functionally normal cells in the peripheral blood provides an explanation for the reduced frequency of bacteremias in patients receiving rHuGM-CSF infusion.\(^{8}\) The presence of primed granulocytes in the peripheral circulation might reasonably be expected to enhance host resistance to bacteremia. However, the defect in migration might predict increased difficulty with soft-tissue infections. Soft-tissue infections are uncommon in this patient population, so a reduced ability to defend against such infections during rHuGM-CSF infusion might not be easily determined. There may be other systemic effects of rHuGM-CSF involved as well as in host defense. It may be that tissue macrophages also proliferate in response to the systematic administration of rHuGM-CSF and thereby provide enhanced host resistance to soft-tissue infection. Studies to evaluate these possibilities are in progress. The findings in this report emphasize, however, that increased circulating granulocyte counts do not necessarily imply normal function.

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