In Vivo Stimulation of Megakaryocytopoiesis by Recombinant Murine Granulocyte-Macrophage Colony-Stimulating Factor

By Alessandro M. Vannucchi, Alberto Grossi, Daniela Rafanelli, and Pierluigi Rossi Ferrini

Murine recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) was injected in mice, and the effects on bone marrow, splenic megakaryocytes, megakaryocyte precursors (megakaryocyte colony-forming units [CFU-Meg]) were evaluated. In mice injected three times a day for 6 days with 12,000 to 120,000 U rGM-CSF, no significant modification of both platelet levels and mean increase in blood neutrophils. However, the rate of platelet production, as assessed by the measurement of 

\[ ^{13} \text{selenomethionine incorporation into blood platelets,} \]

was significantly increased with the highest doses of rGM-CSF. On the contrary, administration of up to 384,000 U rGM-CSF significantly increased with the highest doses of rGM-CSF. The biologic activity of rGM-CSF has also been proved in vivo studies. When administered to normal mice, rGM-CSF was shown to be a potent stimulator of myelopoiesis, and also was able to increase the phagocytic activity of peritoneal macrophages. Furthermore, a dose-related increase in the number of splenic megakaryocytes occurred in mice receiving 60,000 to 120,000 U rGM-CSF, while a slight increase in the number of bone marrow megakaryocytes was observed in mice injected with 120,000 U rGM-CSF. The proportion of bone marrow megakaryocytes with a size less than 18 \( \mu m \) and greater than 35 \( \mu m \) resulted significantly higher in mice receiving rGM-CSF in comparison with controls; an increase in the percentage of splenic megakaryocytes greater than 35 \( \mu m \) was also observed. A statistically significant increase in the total spleen content of CFU-Meg was observed after administration of 90,000 and 120,000 U rGM-CSF three times a day for 6 days, while no effect on bone marrow CFU-Meg was recorded, irrespective of the dose delivered. Finally, 24 hours after a single intravenous injection of rGM-CSF, there was a significant increase in the proportion of CFU-Meg in S-phase, with the splenic progenitors being more sensitive than bone marrow-derived CFU-Meg. These data indicate that rGM-CSF has in vivo megakaryocyte stimulatory activity, and are consistent with previous in vitro observations. However, an effective stimulation of megakaryocytopoiesis in vivo, bringing about an increase in the levels of blood platelets, may require interaction of rGM-CSF with other cytokines. © 1990 by The American Society of Hematology.

MATERIALS AND METHODS

Mice. CBA T6/T6 male mice, 6 to 8 weeks old, were maintained under standard farm conditions, with water and chow ad libitum.

rGM-CSF. The recombinant murine GM-CSF preparation used in this study was the conditioned medium of COS cells transfected with a plasmid expressing murine GM-CSF. The units of rGM-CSF were determined by quantitation of murine CFU-GM growth in vitro after the addition of serial dilutions of the rGM-CSF stock solution to culture dishes. Seventy-five thousand nonadherent bone marrow cells were plated in 35-mm Petri dishes in 1-mL vol of McCoy's 5A medium (GIBCO, Grand Island, NY), 15% fetal calf serum (FCS; GIBCO), and 0.3% Bactoagar (DIFCO, Detroit, MI) (final concentrations). Cultures were incubated for 7 days at 37°C in a fully humidified atmosphere of 5% CO2 in air. GM colonies were enumerated at 40× magnification using an inverted microscope. Data obtained from two experiments, using four plates for each dilution of rGM-CSF, were pooled and a dose-response curve was constructed. Units of rGM-CSF were calculated from the linear portion of the curve, and the dilution that sustained the growth of 50% of the maximal colony number was considered to contain 50 U.

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Submitted December 28, 1989; accepted June 6, 1990.

Supported by Associazione Italiana per la ricerca sul Cancro (AIRC) and AIL (Florence).

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Blood, Vol 76, No 8 (October 15), 1990; pp 1473-1480

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With the Limulus lysate test for endotoxin, whose lower sensitivity limit is 0.5 ng/mL, we detected no significant endotoxin contamination of both the rGM-CSF solution and the sterile, isotonic saline supplemented with 1% bovine serum albumin (BSA) that was used for diluting the rGM-CSF stock solution (see below).

Moreover, in view of recent reports showing that both interleukin-1b (IL-1b) and IL-6 can affect megakaryocytes and platelet production after in vivo administration to normal mice, we looked for IL-1 and IL-6 activity in the preparation of rGM-CSF (COS conditioned medium) used in this study. No detectable IL-1 activity (as assayed by the thymocyte coproliferation assay32) or IL-6 activity (using the B9 cell line proliferation assay34) was observed in two different experiments, in which samples containing from 10,000 to 500,000 U rGM-CSF were used.

\( \text{rGM-CSF administration protocols.} \) rGM-CSF was diluted in sterile isotonic saline containing 1% BSA and filter-sterilized; dilutions from a stock solution were made in such a way to obtain the desired amount of rGM-CSF for each injection in a 0.1-mL vol.

Three to six mice per group were used in each experiment. Three different administration schedules were used, with either multiple or single injections. In the first of these, mice were injected intraperitoneally (i.p.) three times a day (at 8:00 AM, 2:00 PM, and 8:00 PM) for 6 days, and examined 12 hours after the last dose. In the single-injection protocol, which was adopted for experiments aimed at evaluating the cell cycle status of megakaryocytic progenitors (see below), mice received a single dose of rGM-CSF intravenously (IV), and were killed 24 hours later. In the third protocol, which was used for the analysis of thrombopoietin-like activity of rGM-CSF (see below), mice were injected i.p. two times a day (at 9:00 AM and 5:00 PM) for 2 days.

Control mice received 0.3 mL of sterile saline containing 1% BSA, using the same route and injection schedule as mice injected with rGM-CSF.

**Blood cell counts.** Blood was collected from the orbital plexus of unanesthetized mice using siliconized 20-μL micropipettes. White blood cell (WBC) counts were performed using hemocytometers, while platelet counts and platelet volume were obtained with an electronic Coulter counter (System 9000, Baken Instruments Corp, Allentown, PA). Differential cell counts were performed on blood films stained with May Gruenwald-Giemsa.

**Estimation of total spleen and femur cellularity.** For the estimation of total splenic cellularity, the entire spleen from a mouse was teased into small fragments in a minimum essential medium (α-MEM, GIBCO) supplemented with 2% FCS. One hundred thousand splenic cells/mL were seeded in a dish of Cycloheximide (CCh-MEM) containing 5% (vol/vol) heat-inactivated human plasma (SCM). This was prepared by incubating 10⁷ splenic cells/mL in α-MEM containing 5% (vol/vol) heat-inactivated human plasma and 2.5 μg/mL pokeweed mitogen (GIBCO). After 5 to 7 days of incubation, the content of each flask was centrifuged at 2,000g for 15 minutes at 4°C, the supernatant filtered through a 0.22-μm filter, and stored in aliquots at −30°C.

For the analysis of the size distribution of megakaryocytes, bone marrow cells were collected from one intact femur shaft by flushing with CATCH medium, and diluted to a final concentration of 0.75 to 1 × 10⁸/mL. Aliquots of 100 μL were immediately cytospun, and the centrifuge chamber washed once with CATCH medium. The slides were stained for acetylcholinesterase (AcChE) for 6 hours to allow detection of small AcChE-positive cells.37 Megakaryocyte size was measured using an eyepiece micrometer (Leitz Wetzlar, Wetzlar, FRG) at 1,000 x under phase contrast microscopy. Megakaryocyte diameter was calculated by determining the geometric mean of two perpendicular diameters of each AcChE-positive cell. Only cells that were free of intercellular contacts were considered, and at least 100 megakaryocytes for each mouse were sized and data pooled for that experimental group of mice.

Similar criteria were adopted for sizing splenic megakaryocytes, with the notable exception that splenic sections were stained with hematoxylin-eosin instead of AcChE; therefore, only obviously identifiable cells were considered.

**Effects of rGM-CSF on 75selenomethionine incorporation into blood platelets.** The effects of rGM-CSF on the rate of platelet production were quantified by means of the 75selenomethionine (75SeM; Amersham International, England; specific activity 9 mCi/mg) incorporation assay. rGM-CSF was administered i.p. as specified above for either 6 or 2 days, followed by the IV injection of 2 μCi of 75SeM 4 hours after the last dose. Mice were then killed 16 hours after isotope injection and exanguinated via cava in syringes containing 10 μL of 38% sodium citrate; platelet-rich plasma was prepared by differential centrifugation from citrated whole blood that had been diluted 1:5 with Tris-NaCl-EDTA buffer (Tris 150 mM/L, NaCl 77 mM/L, Na₂EDTA 1.4 mM/L, pH 7.4). To reduce contamination by radioactive blood proteins, the isolated platelets were washed through a cushion of isosmolar 10% Stractan (Sigma Chemical Co, St Louis, MO)40 before counting radioactivity in an LKB-Wallac ClinGammma 1272 counter (LKB, Bromma, Sweden). The percent incorporation of total injected radioactivity into circulating platelets was calculated as described.41

**Analysis of CFU-Meg growth ex vivo.** Cultures allowing the growth of megakaryocytic colonies were set up essentially as described by Williams et al.42 One femur and the spleen were obtained under sterile conditions, and a cellular suspension was prepared in α-MEM supplemented with 2% FCS. One hundred thousand bone marrow cells, or 5 × 10⁴ splenic cells, were cultured in McCoy’s 5A medium (GIBCO) supplemented with 2 mM L-glutamine, 8 mg/L L-serine, 1 mM/L sodium pyruvate, 16 mg/L L-asparagine, 15% FCS (final concentration), and penicillin-streptomycin (100 U/mL and 100 μg/mL, respectively); cultures were made semisolid by the addition of 0.3% Bacto-agar (final concentration), and incubated at 37°C in 5% CO₂, fully humidified atmosphere, for 7 days. Whole agar slide preparations were made by dehydrating the agar on a warm plate under a stream of warm air, after carefully layering a dish of 1 M Whatman filter paper over the surface of the agar. Megakaryocytic colonies were identified by AcChE staining using standard procedures. Aggregates of three or more cells were considered as a megakaryocytic colony. At least three dishes were prepared for each experimental point.

The growth-stimulating activity for CFU-Meg was provided by the addition to the cultures of 7% (vol/vol) conditioned medium obtained by pokeweed mitogen-stimulated splenic cells (PWM-SCM). This was prepared by incubating 10⁷ splenic cells/mL in α-MEM containing 5% (vol/vol) heat-inactivated human plasma and 2.5 μg/mL pokeweed mitogen (GIBCO). After 5 to 7 days of incubation, the content of each flask was centrifuged at 2,000g for 15 minutes at 4°C, the supernatant filtered through a 0.22-μm filter, and stored in aliquots at −30°C.
Cycle status of megakaryocyte progenitors (CFU-Meg). To determine the cell cycle status of megakaryocytic progenitors, hydroxyurea (Sigma), an S-phase specific drug, was used. An aliquot of nonadherent bone marrow or splenic cells was incubated at 37°C for 60 minutes in α-MEM supplemented with 5% FCS and containing 10 mmol/L hydroxyurea. The cells were then washed twice in α-MEM, 5% FCS and assayed for megakaryocytic colony growth. Control cells were incubated and processed as above but without the addition of hydroxyurea. The reduction in colony forming units (CFU) containing 10 mmol/L hydroxyurea was expressed as mean ± 1 SD of the values derived from each individual mouse. Mice were always processed singly, and cells from different mice, even belonging to the same group, were not pooled in any instance. A group of control mice was always run concurrently with the cells from each experimental group.

Statistical analysis. Data relative to each group of mice were expressed as mean ± 1 SD of the values derived from each individual mouse. Mice were always processed singly, and cells from different mice, even belonging to the same group, were not pooled in any instance. A group of control mice was always run concurrently with each group of mice receiving GM-CSF. The probability of significant differences between experimental groups and controls was determined with the use of the Student's t test. The chosen level of significance was P < .05.

RESULTS

Effects on WBC and platelet counts, and platelet volume. WBC counts were performed in mice receiving GM-CSF daily for 6 days. The level of WBCs did not modify significantly in comparison with controls, while the differential counts of neutrophils increased significantly in mice treated with 60,000 to 120,000 U from 1,050 ± 290/μL in controls to 1,950 ± 490/μL, 2,140 ± 580/μL, and 2,450 ± 470/μL in mice receiving 60,000, 90,000, and 120,000 U GM-CSF, respectively. No immature myeloid or nucleated red blood cells were observed in blood films. No modification of the hematocrit values was brought about by GM-CSF treatment. Both platelet count and mean platelet volume were similar in controls and mice injected with GM-CSF (Table 1).

Table 1. Platelet Counts and Platelet Volume in Mice Receiving GM-CSF

<table>
<thead>
<tr>
<th>Group</th>
<th>Platelet count (x10^11/μL)</th>
<th>Platelet Volume (fl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Two-day schedule</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline (12)</td>
<td>934 ± 229</td>
<td>4.9 ± 0.3</td>
</tr>
<tr>
<td>rGM-CSF, U per injection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>48,000 (6)</td>
<td>952 ± 157</td>
<td>4.8 ± 0.7</td>
</tr>
<tr>
<td>96,000 (6)</td>
<td>916 ± 73</td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>192,000 (6)</td>
<td>825 ± 160</td>
<td>4.9 ± 0.4</td>
</tr>
<tr>
<td>384,000 (6)</td>
<td>880 ± 210</td>
<td>4.9 ± 0.4</td>
</tr>
<tr>
<td>B. Six-day schedule</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline (12)</td>
<td>898 ± 177</td>
<td>4.8 ± 0.4</td>
</tr>
<tr>
<td>rGM-CSF, U per injection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12,000 (8)</td>
<td>937 ± 212</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>30,000 (12)</td>
<td>899 ± 240</td>
<td>5.0 ± 0.3</td>
</tr>
<tr>
<td>60,000 (12)</td>
<td>810 ± 133</td>
<td>4.7 ± 0.4</td>
</tr>
<tr>
<td>90,000 (12)</td>
<td>797 ± 310</td>
<td>4.5 ± 0.7</td>
</tr>
<tr>
<td>120,000 (12)</td>
<td>855 ± 190</td>
<td>4.6 ± 0.6</td>
</tr>
</tbody>
</table>

Mice were injected with the indicated amount of rGM-CSF using a 2-day or 6-day schedule (see text for details). Data shown are mean ± 1SD, and are derived from three (6-day protocol) or two (2-day protocol) experiments; the total number of mice examined is shown in parentheses.

Platelet levels and mean platelet volume were also determined in mice injected for 2 days with GM-CSF for the evaluation of thrombopoietin-like activity (see below). No modification of both platelet count and mean platelet volume was recorded, even using as much as 384,000 U twice a day per mouse for 2 days (Table 1).

Effects of GM-CSF on bone marrow and splenic cellularity, and on spleen weight. Injection of mice with 60,000 to 120,000 U three times a day for 6 days resulted in a dramatic increase in spleen weight up to 2.5-fold of the controls (from 103 ± 25 mg in controls to 265 ± 53 mg in mice receiving 120,000 U rGM-CSF, P < .01); this was accompanied by a parallel increase in total spleen cellularity up to 1.7-fold of the controls (from 252 ± 51 x 10^8 in controls to 429 ± 95 x 10^8 in mice receiving 120,000 U rGM-CSF, P < .05). On the contrary, the effects on bone marrow cellularity were less evident, and only a modest, although significant, increase in total cells per femur was observed in mice receiving 120,000 U rGM-CSF (from 22 ± 2 x 10^6 in controls to 30 ± 2.8 x 10^6, P < .05).

Effects of GM-CSF on the frequency and size of bone marrow and spleen megakaryocytes. The frequency of bone marrow and splenic megakaryocytes was determined in mice receiving GM-CSF for 6 days. As reported in Fig 1, a slight, although significant, increase in bone marrow mega-
karyocytes was observed in mice receiving 120,000 U rGM-CSF, while lower doses were ineffective. On the contrary, the increase in splenic megakaryocyte frequency was remarkable (up to 2.5-fold of the controls'), and generated a statistically significant dose-response curve ($r = .97; P < .01$) in mice injected with 60,000 to 120,000 U rGM-CSF.

The size distribution of bone marrow megakaryocytes from control mice and animals injected with rGM-CSF is shown in Fig 2. These data were pooled from mice injected with 60,000 to 120,000 U of rGM-CSF; these groups were significantly different from controls but not one to each other, while mice injected with 12,000 and 30,000 U did not behave differently from controls (data not shown). Although the mean size of smeared AchE-positive cells was similar in controls and mice injected with rGM-CSF (30.7 ± 10.2 μm and 26.8 ± 12.6 μm, respectively), the distribution of megakaryocytes in size classes was different between the two groups of mice. The frequency of small AchE-positive cells (diameter less than 18 μm) increased from 10.1% (±2.2) in controls to 20.1% (±3.0) in mice injected with rGM-CSF ($P < .01$), while the frequency of megakaryocytes with a diameter greater than 35 μm increased from 17.6% (±2.9) to 24.7% (±3.3) ($P < .05$). On the other hand, the proportion of cells in the 25- to 35-μm size class decreased from 52.0% (±4.0) in controls to 40.0% (±3.7) in mice injected with rGM-CSF ($P < .01$).

The size distribution of splenic megakaryocytes was derived from the measurement of morphologically recognizable megakaryocytes, due to the lack of AchE reaction in fixed splenic sections; therefore, this analysis underestimates small megakaryocytic cells that might have been lost by the use of morphologic criteria alone. As shown in Fig 3 (which is based on data pooled from mice injected with 60,000 to 120,000 U rGM-CSF), the proportion of splenic megakaryocytes with a diameter greater than 35 μm was doubled in mice receiving rGM-CSF in comparison with controls (from 20.0% [±5.2] to 40.5% [±9.4], $P < .01$), together with a concomitant reduction in the percentage of megakaryocytes belonging to the 25- to 35-μm size class (from 64.5% [±4.6] in controls to 50.5% [±4.0], $P < .05$).

Effects of rGM-CSF on platelet production. The modifications in the rate of platelet production induced in mice receiving rGM-CSF for either 6 or 2 days (see Materials and Methods for details) were quantified by the 3HMP incorporation assay.

As shown in Table 2, both the percent isotope incorpora-

![Fig 2. Effects of rGM-CSF on the size distribution of AchE-positive cells in the bone marrow of mice injected with 60,000 to 120,000 U three times a day for 8 days (see text for details): (*) and (**) denote values significantly different from controls ($P < .05$ and $P < .01$, respectively). (■), Controls; (■), rGM-CSF.](image)

![Fig 3. Effects of rGM-CSF on the size distribution of morphologically identifiable splenic megakaryocytes in mice injected with 60,000 to 120,000 U rGM-CSF three times a day for 8 days (see text for details): (*) and (**) denote values significantly different from controls ($P < .05$ and $P < .01$, respectively). (■), Controls; (■), rGM-CSF.](image)

<table>
<thead>
<tr>
<th>Group</th>
<th>% of Injected, $\times 10^{-2}$</th>
<th>cpm per platelet, $\times 10^{4}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Two-day schedule</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline (12)</td>
<td>3.49 ± 0.38</td>
<td>5.62 ± 0.90</td>
</tr>
<tr>
<td>rGM-CSF, U per injection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>48,000 (8)</td>
<td>4.01 ± 0.70</td>
<td>5.52 ± 1.67</td>
</tr>
<tr>
<td>96,000 (8)</td>
<td>3.41 ± 0.03</td>
<td>5.74 ± 0.71</td>
</tr>
<tr>
<td>192,000 (8)</td>
<td>3.95 ± 0.90</td>
<td>5.23 ± 0.88</td>
</tr>
<tr>
<td>384,000 (8)</td>
<td>3.20 ± 0.20</td>
<td>5.26 ± 0.20</td>
</tr>
<tr>
<td>B. Six-day schedule</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline (12)</td>
<td>3.07 ± 0.55</td>
<td>4.93 ± 0.79</td>
</tr>
<tr>
<td>rGM-CSF, U per injection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12,000 (6)</td>
<td>3.12 ± 0.34</td>
<td>4.83 ± 0.65</td>
</tr>
<tr>
<td>30,000 (8)</td>
<td>3.21 ± 0.21</td>
<td>5.02 ± 0.33</td>
</tr>
<tr>
<td>60,000 (8)</td>
<td>2.86 ± 0.85</td>
<td>4.45 ± 1.50</td>
</tr>
<tr>
<td>90,000 (8)</td>
<td>3.07 ± 0.38*</td>
<td>6.03 ± 0.62</td>
</tr>
<tr>
<td>120,000 (8)</td>
<td>4.58 ± 0.50†</td>
<td>6.64 ± 0.46†</td>
</tr>
</tbody>
</table>

Mice were injected with the indicated amount of rGM-CSF using a 2-day or 6-day schedule (see text for details); 4 hours after the last injection, 2 μCi of 3HMP were injected IV, and the incorporation measured 18 hours later. Data are expressed as mean ± SD; the total number of mice examined in two independent experiments is shown in parentheses. (*) and (**) indicate values significantly different from controls ($P < .05$, and $P < .01$, respectively).
tion and the amount of platelet-bound radioactivity (cpm/platelet) were significantly higher than the controls in mice receiving 90,000 or 120,000 U rGM-CSF three times a day for 6 days, although the increase was relatively small (the percent incorporation was 12% and 16% of the controls in mice receiving 90,000 and 120,000 U rGM-CSF, respectively). On the other hand, none of these parameters was modified in mice receiving high doses of rGM-CSF (up to 384,000 twice a day) for 2 days.

Effects of rGM-CSF on the number of bone marrow and splenic CFU-Meg. Table 3 reports the modifications induced by in vivo rGM-CSF administration on the number of megakaryocyte progenitors (CFU-Meg) in both the spleen and femur. There was no significant modification in the total number of bone marrow CFU-Meg, irrespective of the dose administered. Conversely, the number of splenic megakaryocyte progenitors was significantly increased in mice injected with 90,000 and 120,000 U rGM-CSF (121% and 143% of the controls, respectively). However, due to the increase in total splenic cellularity, the frequency of CFU-Meg did not vary at all (7.0/10^6 nucleated cells in controls; 6.7 and 7.0/10^6 in mice receiving 90,000 and 120,000 U, respectively).

Effects of rGM-CSF on the cycling status of bone marrow and splenic megakaryocyte progenitors. After a single IV injection of rGM-CSF there was a significant increase in the percentage of both femoral and splenic megakaryocyte progenitors in S-phase, as shown by the reduction in the number of megakaryocyte colonies after preincubation of cells with hydroxyurea. The percentage of CFU-Meg in S-phase decreased from control values of 12% (+4) to 18% (+4) in the bone marrow, and from 39% (+6) to 75% (+6) in the spleen of mice receiving 120,000 U rGM-CSF (Table 4). Thus, the magnitude of the increase was greater for the spleen (2.0-fold) than for bone marrow (1.5-fold); moreover, a significant increase in the proportion of splenic progenitors in S-phase was obtained using lower doses than those required for bone marrow-derived CFU-Meg (Table 4).

Simultaneous determination of CFU-GM growth showed that the percentage of GM progenitors in S-phase increased of a 2.3-fold factor in both the spleen and bone marrow of mice receiving 120,000 U rGM-CSF (data not reported in detail).

### DISCUSSION

A number of experimental data strongly suggest that hematopoietic growth factors overlap in their target cell specificity, which is generally broader than implied by their nomenclature. Among these, GM-CSF was initially characterized as a relatively specific in vitro stimulator of myeloid colonies composed of both granulocytes and macrophages, but more recently it has been shown to stimulate multipotent progenitors and committed progenitors of eosinophilic, erythroid, and megakaryocytic lineage.\(^5^\)\(^-^\)\(^14^\)

Although Williams et al\(^1^\) initially suggested that highly purified murine GM-CSF did not stimulate megakaryocytic colony growth in vitro, subsequent experiments by Quehenberry et al\(^1^4^\) Robinson et al,\(^1^3^\) and Metcalf et al,\(^6^\) who used either natural purified or recombinant murine GM-CSF, have established a role for this hematopoietin in the regulation of murine CFU-Meg growth. This effect is a direct one, as removal of accessory cells by plastic adherence and removal of T and B lymphocytes did not affect the growth of megakaryocytic colonies stimulated by murine rGM-CSF. Recent data for human megakaryocyte progenitors are in keeping with the observations made in the murine system. In fact, Mazur et al\(^1^5^\) reported a modest, but reproducible, stimulation of megakaryocyte progenitors derived from the peripheral blood of normal subjects, and Bruno et al\(^1^4^\) and Lu et al,\(^1^6^\) who used enriched populations of bone marrow megakaryocyte progenitors, observed a dose-related stimulation of megakaryocytic colonies by human rGM-CSF.

The availability of virtually unlimited amounts of both murine and human GM-CSF, made possible by the cloning and expression of the gene, offered for the first time the possibility to study the effects of rGM-CSF on hematopoiesis in vivo using murine\(^1^6^\)\(^-^\)\(^17^\) and nonhuman primate models,\(^1^8^\)\(^-^\)\(^2^0^\) and more recent phase I/II studies have supported the usefulness of human rGM-CSF administration in the clinical
setting. However, in all of these in vivo studies the effects of rGM-CSF on hematopoietic progenitors other than granulo-monocytic, and in particular on platelet precursors, were elusive. Metcalf et al. observed a dose-related increase in the number of splenic megakaryocytes in mice injected with 150 to 600 ng rGM-CSF daily for 6 days, while Monroy et al. have reported a dose-dependent increase in platelet levels in normal Rhesus monkeys treated with 12,500 to 50,000 U/kg/d rGM-CSF. Moreover, rGM-CSF was able to accelerate platelet recovery in monkeys heavily treated with radiations followed by bone marrow transplantation. Finally, Vadhan-Raj et al. reported a 2- to 10-fold increase in platelet counts in 3 of 8 patients with myelodysplastic syndromes treated with rGM-CSF, although this has not been confirmed in other studies.

The fact that GM-CSF plays a role in megakaryocytopoiesis is further stressed in a recent study by Hoffman et al., who showed that an acquired cyclic amegakaryocytic thrombocytopenic purpura in a young woman was due to an immunoglobulin selectively blocking the stimulatory action of GM-CSF on megakaryocyte progenitors.

In the experiments reported herein, we have injected normal mice with murine rGM-CSF to study the effects on megakaryocytes and megakaryocyte progenitor cells. Our data suggest that rGM-CSF has in vivo stimulatory effects on bone marrow and splenic megakaryocytic cells that are consistent with previous in vitro observations.

First, rGM-CSF enhanced the entrance of megakaryocyte progenitors in the S-phase of cell cycle, and expanded the pool of splenic megakaryocyte progenitors. Second, the total number of morphologically recognizable megakaryocytes increased in the spleen and, to a lesser extent, in the bone marrow. In addition, the size distribution of bone marrow megakaryocytes in mice injected with rGM-CSF was different in comparison with controls, in that there was a greater proportion of both small AchE-positive cells and megakaryocytes with a diameter greater than 35 μm. The percentage of megakaryocytes greater than 35 μm was also increased in the spleen. Third, the platelet production rate was slightly, although significantly, enhanced using the highest doses of rGM-CSF in the long-term administration protocol, while rGM-CSF had no immediate effect on platelet release from mature megakaryocytes. It is of interest that these modifications were not accompanied by any detectable increase in blood platelet counts. Therefore, effective megakaryocytopoiesis and thrombopoiesis may require the concomitant action of other factors in addition to GM-CSF. The most likely candidate may be thrombopoietin, a still ill-defined humoral regulator of thrombopoiesis, which has been found in animals and humans with thrombocytopenia, but not yet purified; or IL-6, which has been shown recently to be a potent thrombopoietic factor in vivo. In this respect, it may be also worth mentioning that an rGM-CSF-induced reticulocytosis has been observed only in anemic primates showing high levels of erythropoietin; on the other hand, platelet count was increased after rGM-CSF in two primates in which prolonged pancytopenia and bone marrow hypoplasia were suspected to stimulate the release of a thrombopoietic substance. Donahue et al. have reported that primates sequentially treated with rIL-3 for 7 days followed by rGM-CSF for another 7 days showed marked changes in platelet counts, while the same dose of rGM-CSF was virtually ineffective when used alone. On the other hand, when rGM-CSF administration preceded rIL-3, no significant effect was recorded, thus suggesting that the target cell of rGM-CSF is a more mature precursor than the one responding to IL-3. Similar observations have been reported by others.

The modifications observed in the size distribution of bone marrow megakaryocytes in mice injected with rGM-CSF are suggestive for a reduction in the transit time among different maturative stages. However, we suspect that these changes are the consequence of the stimulation of clonable megakaryocytic progenitors equivalent to the in vitro-defined CFU-Meg more than to a direct action on mature, postmitotic megakaryocytes. A definitive answer to this point awaits the demonstration of membrane receptors for rGM-CSF on target megakaryocytic cells. However, the observation that the rate of platelet production was enhanced in mice receiving rGM-CSF for 6 days, but unaffected in the short-term protocol routinely used for the in vivo assay of thrombopoiesis-stimulating substances, further suggests that rGM-CSF is not active at the level of mature, platelet-releasing megakaryocytes.

Some differences were noted in the effects of rGM-CSF on megakaryocyte progenitor cells derived from the bone marrow and the spleen. In fact, while the number of femoral megakaryocyte progenitor cells derived from the bone marrow and the spleen, in fact, while the number of femoral megakaryocyte progenitors was not significantly modified, a marked increase was observed in the spleen, together with a greater proportion of cells entering the S-phase of the cell cycle. These observations are in keeping with previous data suggesting that splenic megakaryocyte progenitors are kinetically different from the normal bone marrow counterpart, and may also show different proliferative and maturative responses to some hematopoietic growth factors, such as human recombinant erythropoietin or preparations of partially purified human urinary and plasma thrombopoietin.

Finally, it should be mentioned that our experimental design does not allow us to assess whether the described effects of rGM-CSF on megakaryocytopoiesis in vivo are the consequence of a direct action on megakaryocytes and megakaryocyte progenitors, or are, at least in part, mediated by indirect effects due to the stimulation of accessory, growth factor(s)-secreting cells by rGM-CSF. However, we have demonstrated that the administration in vivo of rGM-CSF to animals with normal bone marrow induces a number of proliferative and maturative changes in the megakaryocytic lineage, although other factor(s) appear necessary to obtain a full stimulation of megakaryocytopoiesis that ultimately leads to an increase in the levels of blood platelets.

ACKNOWLEDGMENT
This study has been made possible by the generous gift of murine rGM-CSF by Dr S.C. Clark (Genetics Institute, Boston, MA).


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In vivo stimulation of megakaryocytopenesis by recombinant murine granulocyte-macrophage colony-stimulating factor

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