Enhanced Survival but Reduced Engraftment in Murine Recipients of Recombinant Granulocyte/Macrophage Colony-Stimulating Factor Following Transplantation of T-Cell-Depleted Histoincompatible Bone Marrow

By Bruce R. Blazar, Michael B. Widmer, Christine C.B. Soderling, Steven Gillis, and Daniel A. Vallera

In vivo administration of murine recombinant granulocyte/macrophage colony stimulating factor (rGM-CSF) was evaluated for effects on survival and engraftment in an allogeneic murine bone marrow transplantation (BMT) model involving T-cell depletion of donor marrow. The model provides a high incidence of graft failure/rejection. Recipients of continuous subcutaneous infusions of rGM-CSF had a significant survival advantage when compared with untreated controls. However, a significantly lower incidence of donor cell engraftment was noted. Hematological parameters were not substantially affected. When rGM-CSF was administered intraperitoneally (IP), twice daily injections closely approximated the effects of continuous infusion on survival. Single IP injections were without significant effects on survival or engraftment. These results demonstrate that prolonged frequent in vivo exposure to rGM-CSF can significantly improve survival but significantly decreases donor cell repopulation in recipients of T-cell-depleted histoincompatible bone marrow grafts.

R ECOMBINANT colony-stimulating factors (CSFs) have recently been investigated as potential modalities for decreasing the period of leukopenia following chemotherapy and/or radiotherapy. Recent results of animal studies\(^1\) and preliminary human clinical trials\(^7\) using this approach in syngeneic or autologous bone marrow rescue experiments have been encouraging. However, the use of recombinant CSFs in allogeneic bone marrow transplantation (BMT) may yield different results due to allogeneic interactions of CSF-stimulated donor and radioresistant host cells.

We have previously described a murine BMT model in which histoincompatible donor marrow is pan T cell depleted and recipients are conditioned for BMT with 7.5 Gy of total body irradiation (TBI). In this model, a significant number of recipients experience graft failure/rejection and autologous recovery.\(^1\) Donor cell engraftment, survival, and hematological recovery can be independently assessed. In a previous report, we showed that the peritransplant use of rGM-CSF (either by ex vivo donor marrow incubation or as a single in vivo injection following marrow infusion) promoted engraftment\(^4\) without affecting hematological recovery or survival.

In the present study, continuous subcutaneous infusion of rGM-CSF over 14 days (beginning on the day of marrow infusion) significantly improved survival. Interestingly, long-term engraftment was inhibited as a result of rGM-CSF administration. These data suggest that the consequences of using growth factors differ, depending on the timing and mode of administration. This study provides the first data on the effect of rGM-CSF administration in the context of allogeneic BMT.

MATERIALS AND METHODS

Mice. BALB/c (H-2\(^d\)) and C57BL/6 (H-2\(^b\)) mice were bred and maintained at the University of Minnesota mouse colony. Animals were housed in conventional cages with filter lids and fed a fat supplemented diet and antibiotic supplemented water for two days before pretransplant immunosuppression and for 1 month posttransplant. Transplant recipients were at least 8 weeks old; donors were at least 6 weeks old. In each experiment, ten to 33 recipients were transplanted.

Recipient pretransplant conditioning. Our conditioning protocols have been previously described.\(^1\) Recipients were irradiated to a total dose of 7.5 Gy using a 220 KeV General Electric Maxipen-20 x-ray source at a dose rate of 0.45 Gy/min.

BMT procedure. Our procedure has been previously described in detail.\(^1\) BALB/c marrow (20 x 10\(^6\)/mL) was depleted of T cells with a pan T cell monoclonal antibody anti-Thy 1.2 plus complement (C). Similar treatment of thymocytes with this antibody results in the lysis of >95% of BALB/c thymocytes as assessed in a microcytotoxicity assay.\(^1\)

Complement-dependent microcytotoxicity assay for engraftment. Sixty to 69 days post-BMT, all surviving mice were sero-typed to determine engraftment. Peripheral blood mononuclear cells were collected by retroorbital venipuncture. Cells were reacted with non-crossreactive H-2 specific monoclonal antibodies and pre-screened baby rabbit C\(^+\) in a microcytotoxicity assay as previously described.\(^1\) Engraftment beyond day 60 is regarded as stable.\(^1\)

GM-CSF. rGM-CSF was purified by HPLC\(^1\) from the supernatant of yeast that was transformed with a plasmid capable of directing the expression of a murine cDNA for GM-CSF.\(^1\) The final 21 kd glycoprotein product was free of detectable endotoxin (<10 pg/10 µg GM-CSF). The specific activity of rGM-CSF was approximately 10\(^5\) U/mg of protein in colony-forming assays (1...
EFFECT OF rGM-CSF INFUSION IN MICE POST-BMT

The ability of rGM-CSF to augment the in vitro proliferation of both BALB/c and C57BL/6 bone marrow in our model has been reported. In vivo administration of GM-CSF. For continuous subcutaneous delivery of rGM-CSF, a 14-day miniosmotic pump (ALZA Corporation, Palo Alto, CA) was implanted (under general anesthesia) in control and experimental mice by a previously described surgical procedure. Two hundred microliter volumes containing 14 μg GM-CSF and albumin as a stabilizing protein were injected into the pump. Control pumps were injected with albumin. For comparison, intermittent delivery of GM-CSF was tested. Intrapenitoneal (IP) injections were administered for 14 days post-BMT either once or twice daily. Doses ranged between 0.0065 g and 6.5 μg per injection.

Hematological evaluation of recipients post-BMT. One hundred fifty to 250 μL of peripheral blood was obtained by retroorbital venipuncture on days 7, 14, and 28. Leukocyte number and morphology were determined by examination of Wright-Giemsa stained slides. Hematocrit values were determined by capillary tube red cell to plasma volume ratios after centrifugation.

Statistical analyses. Groupwise comparisons of continuous data were made by Student's t test. Survival data were analyzed by life-table methods using the Mantel-Peto-Cox summary of chi square.

RESULTS

The effect of 14-day continuous subcutaneous infusion of rGM-CSF on survival post-BMT. Recipients of T-cell-depleted histoincompatible donor marrow were infused with 1 μg/d rGM-CSF by miniosmotic pump to determine the effect of continuous administration of this growth factor on survival. The cumulative actuarial survival data for three experiments are depicted in Fig 1. Fifty-six recipients of rGM-CSF had a significantly greater (P < .01) 100-day actuarial survival rate (53%) than the 64 control recipients of pumps without rGM-CSF (36%). A decrement in survival among controls as compared with rGM-CSF-treated recipients was noted in each of the three experiments. Actuarial 100-day survival rates among controls were 39%, 13%, and 41% in experiments 1, 2, and 3, respectively. In contrast, 100-day survival rates among rGM-CSF-treated mice were 66%, 40%, and 53%, respectively. There was no clinical evidence of graft-versus-host disease (GVHD) (weight loss, ruing, or diarrhea) in rGM-CSF-treated or sham-treated recipients of T-cell-depleted donor marrow post-BMT, regardless of the dose, schedule, or route of administration.

The effect of 14-day continuous subcutaneous infusion of rGM-CSF on engraftment of donor marrow. To determine the effect of continuous administration of rGM-CSF on donor cell engraftment, cells from the animals of the above three experiments were serotyped (Table 1). Mean percentages of donor cells in the controls were higher (range, 68% to 85%) than in the rGM-CSF-treated mice (range, 44% to 58%). Correspondingly fewer host cells were present in controls (range, 11% to 28%) than in the experimental groups (range, 38% to 50%).

Data from experiments 1 through 3 were pooled for statistical analysis. Mean percent donor cells in controls (75 ± 5) were significantly higher (P = .01) than in rGM-CSF-treated recipients (51 ± 7). Mean percent of host cells for controls (22 ± 5) were significantly lower (P = .02) than in treated mice (43 ± 6).

The effect of 14-day continuous subcutaneous infusion of rGM-CSF on hematological recovery post-BMT. Leukocyte recovery data for experiments 1 through 3 are shown in Table 1. No significant differences in total leukocyte recovery or the morphology of circulating leukocytes were noted early post-BMT (day 7). On day 14 post-BMT in experiment 1, total circulating leukocytes were relatively (but not significantly) higher in the recipients of 14-day continuous infusion of rGM-CSF. In experiments 2 and 3, day 14 post-BMT mean leukocyte counts (12.9 ± 6.0 and 13.9 ± 3.3 x 10^3/μL) were higher in controls as compared with rGM-CSF-treated recipients (7.4 ± 2.6 and 8.8 ± 2.6 x 10^3/μL). These leukocyte counts on day 14 post-BMT are substantially higher than historical non-treated controls and concurrent controls receiving single daily IP injections (see below).

At no time during any experiment were differences noted for other hematological parameters, including hematocrits and percentages of reticulocytes, bands, eosinophils, or monocytes (data not shown).

The effect of 28 twice daily IP injections of rGM-CSF on survival post-BMT. In addition to continuous delivery of rGM-CSF, intermittent delivery was evaluated. Recipients of T-cell-depleted histoincompatible donor bone marrow were given IP injections twice daily of rGM-CSF for 14 days. The actuarial survival data are shown in Fig 2. Controls had lower actuarial 100-day survival rates (26%) than recipients of either of two different doses of rGM-CSF (41% and 58%). A statistically significant (P < .05) survival advantage was noted for recipients of 3.25 μg/dose of rGM-CSF. Recipients of 1 μg/dose twice daily had a superior but not significantly different survival rate (P = .13).

The effect of 28 twice daily IP injections of rGM-CSF on engraftment of donor marrow. To determine the effect of twice daily IP injections on engraftment, the animals from Fig 2 were serotyped (Table 2). Engraftment was not affected by rGM-CSF treatment (n = 7 to 8 per group) as

![Fig 1](image-url) Survival of irradiated C57BL/6 mice receiving continuous subcutaneous infusion of rGM-CSF (1 μg/d) immediately following transplant of T-cell-depleted BALB/c bone marrow cells. Animals were monitored for survival, engraftment, and hematological recovery. Survival data were plotted in an actuarial manner. The P value is shown.
compared with controls. Meaningful statistical analysis was not possible due to poor survival among controls.

The effect of 28 twice daily IP injections of rGM-CSF on hematological recovery post-BMT. Leukocyte recovery data for mice depicted in Fig 2 are summarized in Table 2. On day 7 post-BMT, no difference in total circulating leukocyte numbers or morphology of peripheral blood leukocytes was noted. On day 14, total leukocyte numbers and morphology were similar in all groups, although a mild depression in total leukocyte numbers was observed in the group receiving the highest dose of rGM-CSF. On day 28, recipients of 1 μg/dose rGM-CSF had a depression in circulating leukocytes and a lower percentage of neutrophils than the other groups. Overall, leukocyte alterations were not consistent with a rGM-CSF effect.

Leukocyte counts of control mice receiving albumin were not as high using the twice daily IP protocol as those in control mice implanted with pumps. However, the leukocyte counts observed using the twice daily IP protocol were elevated as compared with values observed in control mice using the once daily IP protocol shown in Table 3. Hematocrit values and reticulocyte numbers were not affected by rGM-CSF administration (data not shown).

The effect of 14 once daily IP injections of rGM-CSF on survival post-BMT. The effect of single daily IP injections was tested over a broad range of doses in log,0 increments from 0.0065 to 6.5 μg/dose. Actuarial survival data for each of the three experiments are shown in Fig 3. No significant differences were noted in survival rates for any of the recipient groups receiving rGM-CSF as compared with controls.

The effect of 14 once daily IP injections of rGM-CSF on engraftment post-BMT. Detailed engraftment data for each experiment in Fig 3 are shown in Table 3. No significant effects on the engraftment of recipients receiving rGM-CSF as compared with controls was noted. At the dose of 0.65 μg/injection, there was a trend toward improved engraftment that proved not to be reproducible on further experimentation.
The significantly higher survival rate observed in recipients of rGM-CSF as compared with controls could be ascribed to the known effects of in vivo GM-CSF on neutrophil and monocyte/macrophage function. This effect, in turn, may reduce the risk of mortality associated with infection during the early post-BMT period. Direct testing of neutrophil and monocyte function post-BMT in individual control and experimental mice (to correlate with survival) has not been feasible due to limitations in the number of cells obtainable without killing mice and pooling blood samples.

The presence of GVHD can also affect survival, especially in regards to the transfer of histoincompatible donor marrow. We cannot exclude the possibility that survival was prolonged by GM-CSF-mediated protection from GVHD. However, our T-cell depletion procedure is highly effective in reducing the incidence of GVHD. T cells undergoing a GVHD reaction would not be expected to have significant numbers of circulating host-type mononuclear cells post-BMT, and dying animals did not display the classical symptoms of GVHD.

In spite of its beneficial effect on survival, continuous rGM-CSF infusion led to a decreased incidence of engraftment. Thus, enhanced survival was obviously not attributed to a rGM-CSF-induced increase in donor progenitor cell recovery, as has been reported for syngeneic BMT recipients receiving a suboptimal marrow cell dose. We have previously shown in this model that both the number of marrow cells infused following T-cell depletion (10 to 18 x 10⁶ T-cell-depleted marrow cells) and the dose of TBI (LD75) favor autologous recovery and survival rather than lethal aplasia. The mechanism by which rGM-CSF reduces donor cell engraftment has not yet been determined. Since the dose of TBI used in these studies is not entirely myelosuppressive, rGM-CSF administration might facilitate autologous recovery by stimulating the repopulation of host progenitor cells. Thus, at higher irradiation doses, the deleterious effects of rGM-CSF on engraftment might be improved. rGM-CSF might also be mediating its effect by abrogating an antidonor marrow graft response, which can be observed in recipients of histoincompatible donor grafts.

Alternatively, rGM-CSF may be stimulating immunocompetent cells of the host. Graft rejection is a well-described complication of murine allogeneic recipients of T-cell-depleted marrow. Continuous subcutaneous infusion of rGM-CSF over a relatively prolonged period of time (14 days) may stimulate several types of immunocompetent cells known to be involved in graft rejection. Radiosensitive accessory cells could undergo direct stimulation by rGM-CSF binding to macrophage receptors. Stimulation of radiosensitive host T cells can occur indirectly by means of monocyte and macrophage-induced release of monokines such as interleukin-1, directed against the newly repopulating marrow graft. These monokines may also stimulate growth factor production by T cells, endothelial cells, and fibroblasts. The access of host progenitor cells to growth factors during a critical period of differentiation/proliferation could result in an increase in host progenitor cell repopulation and subsequent elevation in numbers of host anti-donor immunocompetent cells.

### Table 2. The Effect of 28 Twice Daily IP Injections of rGM-CSF on Donor Marrow Engraftment and Hematological Recovery

<table>
<thead>
<tr>
<th>rGM-CSF (μg/dose)</th>
<th>No. BMT</th>
<th>No. H-2 Typd</th>
<th>% Donor</th>
<th>% Host</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>12</td>
<td>3</td>
<td>37 ± 23</td>
<td>1.0 ± 0.2</td>
<td>75 ± 2</td>
<td>5.6 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>12</td>
<td>8</td>
<td>36 ± 15</td>
<td>0.8 ± 0.1</td>
<td>71 ± 2</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>3.25</td>
<td>12</td>
<td>7</td>
<td>52 ± 17</td>
<td>0.6 ± 0.2</td>
<td>67 ± 2</td>
<td>4.7 ± 1.0</td>
</tr>
</tbody>
</table>

Recipients of histoincompatible T-cell-depleted bone marrow were given 28 day twice daily IP injections of GM-CSF. Peripheral blood was collected and serotyped 60 to 69 days post-BMT. Blood was collected on days 7, 14, and 28 post-BMT for analysis.

*Data are presented as percent mean ± 1 SEM.

### Table 3. The Effect of 14 Once Daily IP Injections of rGM-CSF on Donor Marrow Engraftment and Hematological Recovery

<table>
<thead>
<tr>
<th>Experiment</th>
<th>rGM-CSF (μg/dose)</th>
<th>No. Transplanted</th>
<th>No. H-2 Typd</th>
<th>% Donor</th>
<th>% Host</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0</td>
<td>15</td>
<td>14</td>
<td>49 ± 10</td>
<td>0.3 ± 0.0</td>
<td>1.6 ± 0.4</td>
<td>2.3 ± 0.6</td>
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<tr>
<td>0.0065</td>
<td>15</td>
<td>11</td>
<td>13</td>
<td>64 ± 14</td>
<td>0.3 ± 0.0</td>
<td>1.7 ± 0.8</td>
<td>2.0 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>0.065</td>
<td>15</td>
<td>8</td>
<td>60 ± 13</td>
<td>0.4 ± 0.0</td>
<td>1.9 ± 0.4</td>
<td>1.4 ± 0.3</td>
<td>48 ± 4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>14</td>
<td>7</td>
<td>66 ± 18</td>
<td>0.2 ± 0.0</td>
<td>2.3 ± 0.4</td>
<td>32 ± 3</td>
<td></td>
</tr>
<tr>
<td>0.65</td>
<td>12</td>
<td>8</td>
<td>61 ± 16</td>
<td>0.7 ± 0.1</td>
<td>2.5 ± 0.6</td>
<td>3.2 ± 1.1</td>
<td>50 ± 3</td>
<td></td>
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<tr>
<td>6.5</td>
<td>12</td>
<td>6</td>
<td>68 ± 14</td>
<td>0.3 ± 0.0</td>
<td>2.8 ± 0.9</td>
<td>2.4 ± 0.6</td>
<td>32 ± 4</td>
<td></td>
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<tr>
<td>7</td>
<td>0</td>
<td>18</td>
<td>16</td>
<td>67 ± 10</td>
<td>0.6 ± 0.1</td>
<td>1.8 ± 0.4</td>
<td>52 ± 3</td>
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<tr>
<td>0.65</td>
<td>18</td>
<td>16</td>
<td>67 ± 10</td>
<td>0.7 ± 0.1</td>
<td>1.5 ± 0.3</td>
<td>3.9 ± 0.9</td>
<td>59 ± 4</td>
<td></td>
</tr>
<tr>
<td>Post (8-7)</td>
<td>0.65</td>
<td>32</td>
<td>26</td>
<td>56 ± 8</td>
<td>0.7 ± 0.1</td>
<td>1.9 ± 0.2</td>
<td>3.7 ± 0.7</td>
<td></td>
</tr>
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</table>

Recipients of histoincompatible T-cell-depleted bone marrow were given 14 once daily IP injections of GM-CSF. Peripheral blood was collected and serotyped 60 to 69 days post-BMT. Blood was collected on days 7, 14, and 28 post-BMT for analysis.

*Data are presented as percent mean ± 1 SEM.

**P < .05.

**P < .001.

**P < .01.
The hematological effects observed in the recipients of rGM-CSF were variable and partially dependent on mode of delivery. Mice implanted with miniosmotic pumps containing rGM-CSF or control protein had a striking leukocytosis 14 days post-BMT. The hematological findings support the theory that either the implantation of the bulky pumps or the trauma induced by frequent IP injections in irradiated mice are significant predisposing factors for infection-related complications in the control and rGM-CSF–treated mice. This made the assessment of hematopoietic effects difficult.

In contrast to more prolonged exposure to rGM-CSF by twice daily or continuous infusion, mice that received single daily IP injections (over a broad dose range) did not experience significant changes in engraftment or survival as compared with controls. However, since survival rates in controls receiving single daily IP injections were relatively high, detection of improvement was less likely. Given the probable reduced infection risk provided by single IP injections, a significant acceleration of leukocyte recovery on day 7 post-BMT was reproducibly observed in recipients of a single daily IP dose of rGM-CSF (0.65 μg/dose).

In summary, we have demonstrated that continuous subcutaneous infusion or twice daily IP injections of exogenous rGM-CSF for prolonged periods of time post-BMT (14 days) significantly improves post-BMT survival among recipients of allogeneic T-cell-depleted donor marrow. We also noted that continuous exposure to exogenously administered rGM-CSF can lead to a decreased incidence of engraftment, presumably due to stimulation of radioresistant host cells. Additional experiments varying the conditions of BMT, time of rGM-CSF administration, and the testing of other CSFs with more restricted reactivity (eg, G-CSF) will be required to understand the mechanisms involved in rGM-CSF–induced survival benefits and engraftment effects. Because ex vivo incubation of donor marrow results in an increased incidence of engraftment but does not affect survival, we conclude that timing and method of rGM-CSF delivery is important. Since the effects of rGM-CSF were different depending on whether it was delivered by infusion or IP, the mode of delivery is also important. The results of this study have implications on the use of rGM-CSF in patients receiving T-cell–depleted histoincompatible donor grafts.

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press)
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