Effect of Recombinant Human Granulocyte-Macrophage Colony-Stimulating Factor Administration on the Lymphocyte Subsets of Patients With Refractory Aplastic Anemia

By Mohamed Faisal, William Cumberland, Richard Champlin, and John L. Fahey

Human recombinant granulocyte-macrophage colony-stimulating factor (rhGM-CSF) was administered to 14 patients with refractory aplastic anemia (AA). The effect of rhGM-CSF therapy on the lymphocyte phenotype; on the proliferative responses to the mitogen phytohemagglutinin, Candida albicans, and tetanus toxoid antigens; and on the natural killer (NK) activity of the circulating lymphocytes was studied. Samples were collected before (baseline) and twice during the rhGM-CSF administration. The absolute number of circulating lymphocytes remained relatively constant during the first period, but experienced a significant increase (P < .001) during the second period. The increase was most prominent in B cells (P < .001), but the T cells (P < .016) also increased. Detailed investigation of lymphocyte subsets showed an increase of the markers CD38 (Leu17), HLA-DR, and the transferrin receptor throughout the treatment course giving evidence of lymphoid cell activation. The NK cell activity was suppressed (P < .008) throughout the treatment. However, proliferative responses to phytohemagglutinin, Candida antigen, and tetanus toxoid were unaffected. Although the mechanism is not yet defined, GM-CSF does induce activation and increase in absolute lymphoid cell number, especially B cells, together with a decrease in NK cytotoxicity. The implication of these immune cell changes in relation to host resistance to microorganisms remains to be established.

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COLONY-STIMULATING factors (CSFs) are a group of glycoprotein (GP) hormones that primarily regulate hematopoiesis by promoting proliferation and differentiation of progenitor cells. Among these, granulocyte-macrophage CSF (GM-CSF), produced by activated T lymphocytes, is a stimulator of pluripotent as well as granulocytic/monocytic precursor cells. The gene for GM-CSF was successfully cloned and recombinant human GM-CSF (rhGM-CSF) is being produced in amounts sufficient to allow its clinical use as a therapeutic agent in hematologic disorders. rhGM-CSF has been administered to patients with acquired immunodeficiency syndrome, following chemotherapy, with aplastic anemia and myelodysplasia syndrome, and most recently to patients with refractory aplastic anemia. In each of these studies, administration of the rhGM-CSF resulted in an increase in the number of circulating neutrophils, eosinophils, and monocytes.

Because of the crucial role lymphocytes play in the immune defense mechanism, and since lifelong treatment trials with rhGM-CSF are planned, it is important to know the effect of continuous rhGM-CSF administration on lymphocytes. To this end, we investigated the changes in lymphocytes' phenotypes and functions during treatment, with rhGM-CSF, of a group with refractory aplastic anemia.

MATERIALS AND METHODS

Patients and treatment protocol. Fourteen patients, 16 to 70 years old with acquired refractory aplastic anemia, received rhGM-CSF (activity of 5.4 × 10^6 U/mg GPs Sandoz Co, Hanover, NJ) by constant intravenous infusion in normal saline as described by Champlin et al. Treatment was administered for 2 weeks (first induction phase, IP1) at an initial dose level. The dose was then increased to the next higher dose level if grade >2 toxicity did not occur for an additional 2 weeks (second induction phase, IP2). Patients with complete or partial responses continued on maintenance treatment at the same dose level, while patients who showed no improvement after 1 month did not continue rhGM-CSF therapy. In several patients, dose was reduced because of toxicity. The dose levels used ranged from 4 to 64 μg/kg/d. For lymphocyte analysis, samples were taken at three time points: directly before the drug administration (baseline), at the end of the first induction phase (IP1), and at the end of the second induction phase (IP2). One to three samples taken from four patients during the maintenance period were also analyzed.

The study was approved by the UCLA Human Subject Protection Committee and informed consent was obtained from all patients or their legal guardians.

Flow cytometry. To monitor the changes in the major lymphocyte subpopulations following rhGM-CSF administration, the fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies (MoAbs) against CD3 (Leu-4), CD4 (Leu-3), CD8 (Leu-2), and CD20 (Leu-16) (Becton-Dickinson Immunocytometry Systems, Mountain View, CA) were used. For a subgroup of eight patients, a dual-color analysis of their lymphocyte subsets was performed using FITC and phycoerythrin (PE)-labeled MoAbs. The antibodies (from Becton-Dickinson unless otherwise indicated) used were those (FITC/PE) against: TCR-1 (T-cell receptor)/CD3; CD4/Leu-8; CD4/CD8 (Coulter Immunology, Hialeah, FL); CD8/HLA-DR; CD8/CD38 (Leu-17, OKT10); Leu7 (HNK-1)/CD8; CD8/Leu-15; transferrin receptor/CD20; Leu-7/Leu-19 (NKH-1); CD16 (Leu-11)/Leu9; Leu7/CD16; and as an isotype control IgG1-IgM/ IgG2. Staining was done by incubating 3 × 10^6 leukocytes with the appropriate dilution of the MoAbs for 30 minutes at 4°C in the dark. Samples were then washed twice in phosphate-buffered saline-2% fetal calf serum buffer, containing 0.1% sodium azide. Red blood
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cells were lysed in an ammonium chloride-based lysing solution and the lymphocyte markers were analyzed using Coulter EPICS-C flow cytometer.

Functional Assays

Peripheral blood was diluted 1/1 (vol/vol) with sterile saline, and mononuclear cells (PBMNCs) were separated by centrifugation over Histopaque 1077 (Sigma, St Louis, MO).

Natural killer (NK) cell activity analysis was performed using standard techniques. Briefly, carbonyl-iron phagocytosis was used to deplete the PBMNCs population from monocytes. The NK activity was assayed using 31Cr-labeled K562 cells incubated for 3 hours with lymphocyte effector cells at effector to target cell ratios of 25:1 and 10:1. The percent of specific release was calculated and the data were transformed into lytic units with the aid of a nonlinear equation program. A lytic unit (LU20) is denoted as the number of effector cells required to achieve 20% cytotoxicity of 1 × 10⁶ target cells.

The proliferative responses to mitogens and antigens were determined according to the method described by Spina et al. Briefly, 2.5 × 10⁴ PBMNCs/well were stimulated with the mitogen phytohemagglutinin (PHA) (Wellcome, Burroughs, UK), at a dilution of 1:50. The cells were then incubated for 48 hours, after which 1 μCi of ³H-thymidine/well was added. The cells were harvested 24 hours later, and counts per minute (cpm) were obtained with a scintillation counter. The proliferative response to antigens was performed by stimulating 5 × 10⁴ PBMNCs/well with Candida albicans antigen (Hollister Star, Los Angeles, CA) at a final dilution of 1:5,000, and tetanus toxoid (State Laboratory Institute, Jamaica Plain, MA) at a...
final dilution of 1:4,000. The cells were then incubated for 6 days before pulsing with thymidine.

**Statistical analysis.** To see the different effects during both induction phases, ratios of the values at the end and beginning of each phase for each patient were calculated. Box-Whisker plots of these ratios with the medians, 25th, and 75th percentiles were done for total lymphocytes, myeloid cells, and selected lymphocyte subsets. Tests for changes during the period (ie, a ratio different from one) were performed by first taking logarithms of the ratios and then using the Wilcoxon signed-rank test. This transformation (which is equivalent to taking differences of the logarithms of the original measurements) helps to achieve symmetry of the distribution of the measurements, which is a necessary condition for applying this test. P values are for two-sided alternatives.

**RESULTS**

A clear difference emerged in the changes of leukocytes during the first 2-week induction phase (IP1) as compared with those during the second induction phase (weeks 3 and 4) (IP2). In Fig 1, we have plotted the ratios for the end of IP1 over baseline, the end of IP2 over IP1, and overall (ie, end of IP2 over baseline) of the white blood cell (WBC) count, myeloid cells, and lymphocytes. The WBC count showed a sharp increase during IP1 \( (P < .0001) \); it continued rising during IP2 \( (P < .001) \), resulting in a large overall increase from baseline \( (P < .0001) \). Closer inspection showed that the effect on lymphocytes and myeloid cells was quite different during these two periods. The myeloid cells increased sharply during IP1 \( (P < .0001) \) followed by a smaller increase during IP2 \( (P < .001) \). The increase in granulocytes and monocytes occurred in each patient. On the other hand, lymphocytes showed little changes during IP1 (median 1), but increased significantly during the second phase \( (P < .001) \). There was no correlation between changes in granulocytes or monocytes and increment in lymphocytes.

To investigate whether GM-CSF has a selective effect, we first looked at the major lymphocyte subsets: T, B, and NK (expressed as CD16+ cells). T and B cells showed the same general pattern (Fig 2) of changes as seen in total lymphocytes, ie, nonsignificant changes during IP1 and a significant increase during IP2 \( (P < .016, .001 \text{ respectively}) \), so that an increase over the baseline was evident. These changes were observed to be greater in B \( (P < .008) \) than in T \( (P < .025) \); the ratio at the end of period 2 to baseline ranged, in the case of T cells, from 0.65 to 7.7 (median 1.43); and in the case of B cells from 0.7 to 8.5 (median 1.88). The level of NK cells remained constant, ie, they did not increase with the rise in B or T cells and did not appear to be influenced by rhGM-CSF. The B cell increase was confirmed by a small follow-up study of four patients monitored at frequent intervals during the maintenance period of rhGM-CSF administration. In these patients, B cells remained elevated relative to the baseline, and this elevation was higher than those of the T or NK cells (Fig 3).

Monitoring the absolute numbers of CD4 (T helper/inducer) and CD8 (T suppressor/cytotoxic) cells showed the
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Fig 3. GM-CSF administration induced a relatively higher elevation of B than T or NK cells. The graph represents the follow-up of the patient J.R. throughout induction and maintenance phases.

same pattern as in total T cells: a nonsignificant decrease during IP1 followed by a significant increase during the second induction (P < .025 for CD8 and .021 for CD4 T cells). The increase over the baseline was also significant (P < .021).

The effect of rhGM-CSF on T-cell subsets as well as on activation and maturity markers was evaluated by dual-color immunofluorescence analyses. The data of eight patients showed that TCR and CD3 changed together as did 4B4 and CD4, Leu-8 and CD4, Leu-15 and CD4, and Leu-7 and CD8. The activation markers CD38 (Leu-17), TFR, and HLA-DR, were increased by GM-CSF. The percentage of Leu 17⁺ in the non-CD8 lymphocytes demonstrated a significant increase during IP1 (P < .016) with no obvious trend during IP2. HLA-DR also showed a significant increase in percent (P < .008) and a possible increase in absolute number (P < .03 single sided) during the first phase, followed by a significant decrease in percent during IP2 (Table 1). Further analysis of the DR⁺ cells in relation to the CD8⁺ subpopulation indicated that the effect on the CD8⁺DR⁺ subset was different from the CD8⁻ DR⁻ cells. During IP1, both the percent of CD8⁻ DR⁻ (P < .008) and the absolute number increased (P < .016) mainly in DR⁺ dim, ie, low antigen density. During IP2 there was a moderate decrease in percent but the count showed no clear trend. CD8⁺ DR⁻ showed the same pattern as total CD8. Similarly, the cells expressing the transferrin receptors showed a significant increase at IP1 (P < .008) which occurred due to TFR⁺ non-B cells.

However, NK cytotoxicity consistently showed a decrease (P < .008) in all patients during the first induction phase (Fig 4), and the activity remained low during IP2. The

<p>| Table 1. Ratios Indicating Changes During the First and Second Induction Phases (IP1 and IP2) of the Activation Markers CD38, HLA-DR, and Transferrin Receptor (TFR) |
|-------------|-----|-----|-----|</p>
<table>
<thead>
<tr>
<th>Marker</th>
<th>IP1</th>
<th>IP2</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total CD38</td>
<td>%</td>
<td>1.5 (0.5-3.5)*</td>
<td>0.9 (0.8-1.2)</td>
</tr>
<tr>
<td>Abs</td>
<td></td>
<td>1.1 (0.5-2.4)</td>
<td>1.4 (1.2-2.5)</td>
</tr>
<tr>
<td>CD38⁺ nonCD8</td>
<td>%</td>
<td>1.9 (0.9-4.1)*</td>
<td>0.9 (0.7-1.1)</td>
</tr>
<tr>
<td>Abs</td>
<td></td>
<td>1.5 (0.6-4.8)</td>
<td>1.3 (1.2-2.2)</td>
</tr>
<tr>
<td>CD38⁺ CD8⁺</td>
<td>%</td>
<td>1.1 (0.7-2.6)</td>
<td>1.1 (0.8-1.1)</td>
</tr>
<tr>
<td>Abs</td>
<td></td>
<td>0.9 (0.5-3.4)</td>
<td>1.5 (0.9-2.9)*</td>
</tr>
<tr>
<td>Total HLA-DR</td>
<td>%</td>
<td>1.7 (0.7-4.8)*</td>
<td>0.8 (0.6-1.0)</td>
</tr>
<tr>
<td>Abs</td>
<td></td>
<td>1.3 (0.7-3.2)</td>
<td>1.1 (0.9-2.6)</td>
</tr>
<tr>
<td>HLA-DR⁺ nonCD8</td>
<td>%</td>
<td>2.1 (1.3-5.0)*</td>
<td>0.7 (0.6-1.0)</td>
</tr>
<tr>
<td>Abs</td>
<td></td>
<td>1.9 (0.8-4.6)*</td>
<td>1.1 (0.7-2.1)</td>
</tr>
<tr>
<td>HLA-DR⁺ CD8⁺</td>
<td>%</td>
<td>1.4 (0.8-19.0)</td>
<td>1.2 (0.8-2.0)</td>
</tr>
<tr>
<td>Abs</td>
<td></td>
<td>1.0 (0.3-2.6)</td>
<td>2.1 (0.8-2.2)</td>
</tr>
<tr>
<td>TFR</td>
<td>%</td>
<td>2.4 (0.6-30.0)*</td>
<td>0.8 (0.3-1.5)</td>
</tr>
<tr>
<td>Abs</td>
<td></td>
<td>2.8 (0.2-111.0)*</td>
<td>1.2 (0.4-1.6)</td>
</tr>
<tr>
<td>TFR⁺ nonB</td>
<td>%</td>
<td>2.0 (0.6-70.0)*</td>
<td>0.8 (0.3-1.5)</td>
</tr>
<tr>
<td>Abs</td>
<td></td>
<td>2.3 (0.6-111.0)*</td>
<td>1.2 (0.9-1.5)</td>
</tr>
<tr>
<td>TFR⁺ B</td>
<td>%</td>
<td>1.0 (0.0-17.0)</td>
<td>0.8 (0.0-6.0)</td>
</tr>
<tr>
<td>Abs</td>
<td></td>
<td>0.8 (0.0-6.0)</td>
<td>0.8 (0.0-2.6)</td>
</tr>
</tbody>
</table>

Data are presented as medians (min-max) of the ratios of eight aplastic anemic patients.
Abbreviation: Abs, absolute number.
*P < .05.
proliferative responses to \textit{C albicans} antigen or tetanus toxoid showed no changes. No statistically significant change was observed for response to PHA, even though some individuals showed substantial increases.

**DISCUSSION**

GM-CSF stimulates proliferation of granulocytes, myeloid progenitors, and enhances the function of mature monocytes and eosinophils.\textsuperscript{13}-\textsuperscript{16} Although lymphocytes do not express GM-CSF receptors, many cytokines that influence the immune system are produced by monocytes. These studies examined whether rhGM-CSF administration would affect the phenotypic and functional activity of circulating lymphocytes. We observed a continued increase in leukocytes during the 4-week course of GM-CSF, while lymphocyte number increased only during the last 2 weeks. The lymphocyte increase was greater in B lymphocytes than in T (helper and suppressor) cells. The increase occurred in both mature (4B\textsuperscript{4+}) and immature (4B\textsuperscript{4-}) lymphocytes. A direct effect of GM-CSF on lymphocytes seems unlikely because these cells do not express GM-CSF receptors.\textsuperscript{17} The effect could be indirect via GM-CSF stimulation of granulocytes and macrophages to produce cytokines. Monokines, including interleukin-1, interleukin-6, and tumor necrosis factor, are produced by GM-CSF–activated monocytes,\textsuperscript{18} which can influence lymphocyte growth, differentiation, and activation.\textsuperscript{19,20} NK cell activity was suppressed throughout the treatment course. The mechanism of this effect is uncertain.

Increased expression of the activation markers CD38, HLA-DR, and transferrin receptor occurred during GM-CSF treatment. Activation of lymphocytes as indicated by these markers occurred earlier than their increase in absolute number. This early lymphocyte activation may result from the cytokines produced by monocytes and granulocytes stimulated by GM-CSF.

**REFERENCES**

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