In Vivo Production of Interleukin-5, Granulocyte-Macrophage Colony-Stimulating Factor, Macrophage Colony-Stimulating Factor, and Interleukin-6 During Intravenous Administration of High-Dose Interleukin-2 in Cancer Patients

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Recombinant human interleukin-2 (IL-2), administered to cancer patients by continuous intravenous (IV) infusion (3 x 10^6 U/m^2/d), was found to induce the in vivo production of colony-stimulating factors (CSF). Plasma obtained from patients during IL-2 treatment stimulated in vitro colony formation of normal human bone marrow cells, depleted of mononuclear phagocytes and T lymphocytes. This colony-stimulating activity (CSA) was identified as IL-5, granulocyte-macrophage CSF (GM-CSF), and macrophage CSF (M-CSF), by the ability of specific antibodies against these factors to neutralize their effects. The presence of IL-2-induced GM-CSF and M-CSF was also demonstrated by specific radiocommunassays. During IL-2 treatment, plasma also contained detectable levels of IL-6, which was measured in a bioassay.

In vivo administration of recombinant human interleukin-2 (IL-2), applied as immunotherapy in patients with disseminated cancer, is accompanied by various hematologic side effects. Anemia, thrombocytopenia, eosinophilia, and lymphopenia, followed by a rebound lymphocytosis after discontinuation of IL-2 infusion, have been described in association with IL-2 treatment.1,2

The pathogenesis of these hematologic changes is not fully understood. In vitro studies have shown that IL-2 has no direct effect on the proliferation of hematopoietic progenitor cells (HPC),3 but IL-2 may induce peripheral blood mononuclear cells to produce a variety of cytokines, such as interferon gamma (IFN-γ),4 tumor necrosis factor-α (TNF-α),5 TNF-β,4 IL-1,6 IL-3,7 IL-5,6,8 granulocyte-macrophage colony-stimulating factor (GM-CSF).7 These factors can either suppress (IFN-γ, TNF-β), or stimulate (GM-CSF, IL-3, IL-5), in vitro colony growth of HPC. In mice, Talmedge et al11 demonstrated that IL-2 increased the frequency of bone marrow colony-forming unit-granulocyte-monocyte (CFU-GM). This phenomenon was probably secondary to the induction of CSF production, since IL-2 upregulated cytoplasmic mRNA for GM-CSF and IL-3. In humans, Etinghausen et al12 reported a decrease in the numbers of circulating erythroid (burst-forming unit–erythroid [BFU-E]) and myeloid (CFU-GM) progenitor cells during IL-2 treatment in conjunction with detectable serum levels of IFN-γ, suggesting a cytokine-mediated inhibition of hematopoiesis. However, the number of circulating HPC increased after discontinuation of IL-2 treatment.12 Recently we have studied this increase in more detail, showing that in comparison with pretreatment levels, the pool of circulating HPC expanded approximately 20-fold, reaching a maximum 5 days after completing IL-2 treatment (day 10 from the start of IL-2 administration).12

In the present study, we investigated the in vivo production and gene expression of the five myeloid CSF (M-CSF, G-CSF, GM-CSF, IL-3, and IL-5) during IL-2 treatment. We demonstrate that continuous infusion of high-dose IL-2 induced the in vivo production of circulating M-CSF, GM-CSF, and IL-5. In accordance, we show that IL-2 treatment induced the expression of mRNA for these factors in peripheral blood mononuclear cells. In addition, transcripts for IL-3, but not for G-CSF, were found during IL-2 administration.

MATERIALS AND METHODS

IL-2/lymphokine-activated killer cell treatment. Patients with metastatic renal cell carcinoma were treated with IL-2 and lymphokine-activated killer (LAK) cells according to a multicenter trial. From day 0 to 5, patients were given a priming course of recombinant human IL-2 (3 x 10^6 U/mg, EuroCetus, Amsterdam, The Netherlands), administered as a continuous infusion of 3 x 10^6 U/m^2/d (Fig 1). From day 7 to 10, peripheral blood leukocytes were harvested by four consecutive leukaphereses. Collected cells were incubated in vitro with IL-2 (1,000 U/mL) for 72 to 96 hours and reinjected from day 11 to 14. On day 11, a second course of IL-2 was started (108 hours), administered in an identical manner as the first course. After 3 weeks of rest, a second cycle as described above was given. Patients with responsive or stable disease received up to

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yielding suspensions with less than 1% T lymphocytes as defined as colonies consisting of hemoglobinized cells), and CFU-granulocyte, erythrocyte- and T lymphocyte-depleted bone marrow cells. After 18 days of culture, the numbers of CFU-G colonies (defined as aggregates of more than 20 monocytic cells), BFU-E colonies (defined as bursts of colonies during the last 16 hours of incubation). The limit of detection for IL-3 in plasma samples was 5 ng/mL. IL-6 was measured as hybridoma growth factor (HGF) using the 7TD1 murine hybridoma. One unit of HGF, giving half-maximal cell proliferation, corresponds to 1 pg IL-6. IFN-γ was assayed using an enzyme-linked immunosorbent assay (ELISA), the limit of sensitivity being 0.5 ng/mL. TNF-α was measured in a cytotoxicity assay on L929 cells. The limit of detection in plasma was 0.1 ng/mL.

Detection of CSF mRNA by a cDNA-PCR method. Cellular RNA from peripheral blood mononuclear cells was prepared using the guanidinium thiocyanate method. Samples containing 2 μg of total RNA were used to synthesize cDNA using moloney murine leukemia virus (M-MLV) reverse transcriptase and oligo(dT), as recommended by the supplier (BRL, Gaithersburg, MD) in a volume of 50 μL. The PCR was performed as described. Briefly, reaction mixtures of 0.1 mL contained 1 μL of the cDNA product, 200 μmol/L of each deoxynucleoside triphosphate (dNTP), 50 pmol of the two primers (see below), 2 mmol/L MgCl₂, 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.4, and 2 U of Taq polymerase (AmpliTaq, Perkin Elmer Cetus, Norwalk, CT). Samples were covered with 0.1 mL mineral oil, and denatured at 95°C for 5 minutes. Amplification was performed using a programmable Dri-Block (PHC-1, Technne, Cambridge, UK) for 33 cycles. Each cycle consisted of incubation for 1.5 minutes at 95°C, 2.5 minutes at 55°C, and 1 minute at 72°C. Ten microliters of the PCR products were electrophoresed on a 2.5% agarose gel, and visualized by ethidiumbromide fluorescence (0.5 μg/mL).

PCR primers for CSF genes were synthesized based on the
published sequences of the cDNA and genomic DNA. The various primer sets were chosen such that one could discriminate between amplified cDNA and contaminating DNA present in the RNA isolate based on the length of the amplicon (Table 1).

RESULTS

Effect of IL-2/LAK cell treatment on white blood cell counts. Six patients, who received a first cycle of IL-2 (days 0 to 5 and days 11 to 15) and LAK cell treatment (infused on days 11 to 14), were monitored for changes in white blood cell (WBC) and differential counts (Fig 1). Lymphopenia developed during IL-2 infusion, followed by a lymphocytosis after discontinuation of IL-2 therapy. Absolute numbers of neutrophils and eosinophils were elevated, in particular during the second part of the cycle. The monocyte counts did not change markedly (data not shown). Cell counts returned to normal 1 week after completing the IL-2/LAK cell cycle (day 23).

In vivo induction of CSA by IL-2. To investigate whether IL-2 induced the production of detectable levels of CSA in the circulation, plasma samples obtained before, during, and after IL-2 administration were added to cultures of normal human bone marrow cells that were depleted of mononuclear phagocytes and T lymphocytes. Before the start of IL-2 treatment (day 0), plasma contained a low background level of CSA, consisting of some BFU-E and CFU-M colony growth (Fig 2). During IL-2 infusions (days 2 and 4), plasma clearly stimulated in vitro colony growth, with a predominance of CFU-G and CFU-M. After discontinuation of IL-2 administration on day 5, the CSA levels diminished (day 7), reaching background levels at day 10. The possibility was considered that residual accessory cells in the cultures produced CSA after stimulation with plasma containing IL-2 or other CSF-inducing cytokines. However, in all experiments, control cultures stimulated with rhIL-2 (3, 10, 30, 100, 300 ng/mL) and rhIL-1 (10 ng/mL) yielded no colony growth (data not shown). Therefore, it was excluded that these cytokines were responsible for the stimulatory effect on colony formation.

Characterization of IL-2-induced CSA by RIA and bioassay. In four patients, plasma samples were assayed using specific RIAs for M-CSF, GM-CSF, and G-CSF, and an IL-3-sensitive cell line. IL-2 induced an increase of M-CSF production in all four patients (Table 2), although a constitutive level of M-CSF was present before the start of IL-2 treatment. IL-2-induced GM-CSF was found in one patient (6.9 ng/mL). Circulating levels of G-CSF and IL-3 could not be detected in any of the patients.

Characterization of IL-2-induced CSA using neutralizing antibodies. To further characterize the nature of the IL-2-induced CSA, neutralization experiments were performed using specific neutralizing antibodies against M-CSF, G-CSF, GM-CSF, IL-3, and IL-5. Plasma obtained during IL-2 administration (day 4) was preincubated with various combinations of antibodies, and tested for CSA. IL-2-induced CSA was totally neutralized by the combination of all five antibodies (Table 3). Preincubation with an anti-M-CSF antibody alone resulted in a strong decline of CFU-M colony growth, demonstrating the presence of bioactive M-CSF. Furthermore, we investigated the residual CSA when all but one CSF were neutralized. In this way, we showed that not only M-CSF, but also GM-CSF and IL-5, contributed to the observed IL-2-induced CSA. In those cultures in which all CSF but IL-5 were neutralized, all CFU-G colonies were eosinophilic, as determined by luxol fast blue staining. Addition of antibodies against M-CSF, G-CSF, IL-3, and IL-5 to control cultures stimulated with GM-CSF, and addition of the antibody against GM-CSF to cultures stimulated with IL-3, did not affect colony growth (data not shown), showing that the inhibition of colony formation was not due to nonspecific Fc receptor binding or toxicity of the antibodies.

Induction of CSA gene expression. Expression of CSA mRNA was investigated in peripheral blood mononuclear cells. Due to the low numbers of circulating mononuclear cells during IL-2 infusion, Northern blot analysis could not be performed. Therefore, a cDNA-PCR method was used. Before IL-2 treatment, CSA gene expression was not detected (Fig 3A); however, during IL-2 treatment, transcripts for M-CSF, GM-CSF, IL-3, and IL-5 were found (Fig 3B). G-CSF mRNA could not be detected in peripheral blood mononuclear cells during IL-2 treatment. As positive controls, we used cDNA obtained from phorbol myristate acetate (PMA)-stimulated mononuclear blood cells and from PHA-stimulated T lymphocytes, and these showed specific bands for all factors tested. A cell marker study in one patient during IL-2 treatment showed that peripheral blood mononuclear cells consisted of 52% T lymphocytes (CD3+), 23% natural killer (NK) cells (CD56+), CD3+, 9% B lymphocytes (CD20+), and 17% mononuclear phagocytes (Leu-M3+).

Effect of IL-2 treatment on the induction of IL-6. In four patients, plasma samples were assayed for the presence of IL-6. As demonstrated in Table 4, in vivo administration of IL-2 induced IL-6 bioactivity in the plasma of all these patients. This activity was identified as IL-6 by neutralization with a specific anti-IL-6 MoAb (data not shown).

Effect of IL-2 treatment on the induction of colony-inhibitory activity. Since IL-2 may induce the production of TNF and/or IFN-γ, cytokines that suppress in vitro he-

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Table 1. Characteristics of PCR Primers and Amplicons

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Position</th>
<th>Primer</th>
<th>Amplicon Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exon Nucleotides*</td>
<td>cDNA Genomic DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M-CSF</td>
<td>A 6 1,391-1,410</td>
<td>232 1,131</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B 8 1,603-1,622</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-CSF</td>
<td>A 4 1,132-1,151</td>
<td>714 878</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B 5 1,990-2,009</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM-CSF</td>
<td>A 2 943-962</td>
<td>423 1,918</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B 4 2,841-2,880</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-3</td>
<td>A 4 2,558-2,577</td>
<td>371 473</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B 5 3,011-3,030</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-5</td>
<td>A 3 1,883-1,902</td>
<td>379 485</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B 4 2,348-2,367</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>A 5 1,625-1,644</td>
<td>629 741</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B 6 2,346-2,365</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

*Position of primers (20-mer) based on numbering of nucleotides in the published genomic sequence (cDNA sequence in case of M-CSF); A, 5’ coding primer; B, 3’ antisense primer.
matopoiesis, we investigated the presence of colony-inhibitory activity in plasma in relation to IL-2 administration in three patients. Plasma samples were added to cultures that were maximally stimulated by a combination of plateau concentrations of GM-CSF, IL-3, M-CSF, and G-CSF. As shown in Table 5, colony-inhibitory activity was not found. Furthermore, no detectable levels of IFN-γ or TNF-α were found before, during, or after IL-2 treatment in the six patients studied (data not shown).

**DISCUSSION**

Immunotherapy with IL-2 is accompanied by a variety of hematologic changes. The patients we studied developed lymphopenia/lymphocytosis, eosinophilia, neutrophilia, and thrombocytopenia in relation to IL-2 treatment. To explain these changes, various mechanisms have been proposed. IL-2 may cause a capillary leak syndrome with extravasation of cellular elements, which may contribute to the observed lymphopenia and thrombocytopenia. Furthermore, studies in mice have shown that IL-2-induced lymphopenia resulted from an altered distribution pattern, with homing of peripheral blood lymphocytes to nonrecirculating compartments. In humans, decreased numbers of circulating HPC during IL-2 treatment and in vivo induction of IFN-γ and/or TNF, cytokines that suppress the in vitro proliferation of HPC, have been reported, suggesting an inhibitory effect of IL-2 on hematopoiesis.

In the present study, we demonstrate the in vivo induction of circulating IL-5, GM-CSF, M-CSF, and IL-6 during continuous infusion of high-dose IL-2 in cancer patients. These factors may be responsible for some of the hematologic effects observed during IL-2 treatment. IL-5 has a specific stimulatory effect on the production of eosinophils, whereas GM-CSF supports the production of eosinophils, as well as neutrophils. The marked contribution of IL-5 to the CSA of plasma for CFU-G colony growth, suggests a major role for IL-5 in the

**Table 2. In Vivo Induction of M-CSF Production During IL-2 Administration**

<table>
<thead>
<tr>
<th>Plasma Concentration of M-CSF (ng/mL)</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0*</td>
<td>6.95</td>
<td>6.46</td>
<td>8.57</td>
<td>9.08</td>
</tr>
<tr>
<td>Day 4</td>
<td>22.65</td>
<td>18.38</td>
<td>18.95</td>
<td>14.88</td>
</tr>
<tr>
<td>Day 7</td>
<td>8.31</td>
<td>9.13</td>
<td>11.72</td>
<td>10.74</td>
</tr>
<tr>
<td>Day 10</td>
<td>6.19</td>
<td>9.07</td>
<td>11.40</td>
<td>9.95</td>
</tr>
</tbody>
</table>

*Days after start of IL-2 treatment.

**Table 3. Effect of Preincubation With Neutralizing Antibodies on the CSA Present in Plasma During IL-2 Treatment (day 4)**

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>BFU-E</th>
<th>CFU-G</th>
<th>CFU-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-M</td>
<td>96 ± 12</td>
<td>92 ± 10</td>
<td>3 ± 5</td>
</tr>
<tr>
<td>α-M + α-GM + α-IL-3 + α-IL-5</td>
<td>37 ± 8</td>
<td>2 ± 1</td>
<td>0</td>
</tr>
<tr>
<td>α-M + α-G + α-IL-3 + α-IL-5</td>
<td>85 ± 7</td>
<td>50 ± 23</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>α-M + α-G + α-GM + α-IL-5</td>
<td>31 ± 5</td>
<td>2 ± 1</td>
<td>0</td>
</tr>
<tr>
<td>α-M + α-G + α-GM + α-IL-3</td>
<td>34 ± 4</td>
<td>56 ± 14</td>
<td>0</td>
</tr>
<tr>
<td>α-M + α-G + α-GM + α-IL-3 + α-IL-5</td>
<td>29 ± 3</td>
<td>1 ± 1</td>
<td>0</td>
</tr>
<tr>
<td>Medium control</td>
<td>31 ± 4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Data are expressed as a percentage (mean ± SD, n = 4) of the number of colonies obtained in the presence of plasma without addition of antibodies. Plasma from four different patients was tested in one experiment.

Abbreviations: α-M, anti-M-CSF; α-G, anti-G-CSF; α-GM, anti-GM-CSF; α-IL-3, anti-IL-3; α-IL-5, anti-IL-5.
IL-2–INDUCED CSF PRODUCTION IN VIVO

Fig 3. Expression of CSF mRNA (using the cDNA-PCR method) in peripheral blood mononuclear cells (A) before and (B) on day 4 after the start of IL-2 treatment. The strong upper band for IL-3 on day 4 represents contaminating genomic DNA (see Table 1); however, at 371-bp a weak signal for IL-3 cDNA is present (see arrow). Results shown are typical of three distinct experiments with cells from different patients. Photographic print from a diapositive.

Pathogenesis of eosinophilia observed in these patients. The in vivo significance of the increased plasma levels of M-CSF and IL-6 is less clear than for GM-CSF and IL-5. Although M-CSF is a growth factor for mononuclear phagocytes, we observed no significant changes in blood monocyte counts during IL-2 administration. A similar lack of increase in monocyte counts has been observed in patients treated with M-CSF in vivo. IL-6 may be involved in the regulation of hematopoiesis by acting synergistically with IL-3 on the proliferation of early HPC.

Recently, we demonstrated in the same patients that IL-2 has a biphasic effect on the numbers of circulating HPC, causing a decrease during IL-2 administration, followed by an increase after completing IL-2 treatment. Although the presence of detectable CSF in plasma during the days preceding the expansion of the pool of circulating HPC may imply a correlation between these two phenomena, the absence of detectable inhibitory bioactivity (or 1FN-γ or TNF-α levels) in plasma during the entire course of IL-2 treatment, shows that the pathogenesis of changes in the numbers of circulating HPC is more complex than a balance between circulating inhibitory and stimulatory activity. It seems likely that the decrease in the numbers of circulating HPC during IL-2 treatment is, analogous to the lymphopenia, due to an altered distribution pattern of HPC in response to IL-2 or IL-2–induced cytokines.

IL-2–induced production of CSF is in accordance with a study in mice, which demonstrated an increase in bone marrow CFU-GM during IL-2 administration. However, athymic nude mice do not produce myelostimulatory activity in response to IL-2, illustrating the important role of T lymphocytes in this effect. T lymphocytes can produce GM-CSF, IL-3, and IL-5. Unlike GM-CSF and IL-5, IL-3 bioactivity could not be detected in the circulation, but low expression of IL-3 mRNA was found in peripheral blood mononuclear cells during IL-2 administration. Since T lymphocytes do not produce M-CSF, other cell types are involved in the production of M-CSF. Monocytes can be induced to produce M-CSF and G-CSF. However, we did not detect G-CSF in the circulation, nor did we detect a transcript for G-CSF in mononuclear cells during IL-2 treatment. In accordance, independent regulation of M-CSF and G-CSF gene expression in human monocytes has been demonstrated, showing that IL-3 or GM-CSF induces M-CSF, without the concurrent induction of G-CSF in monocytes. NK cells may be another potential source for M-CSF. Recent studies have shown that under appropriate conditions of stimulation (a combination of IL-2 and CD16 ligands), NK cells express mRNA for M-CSF in the absence of G-CSF transcripts.

In summary, we have shown that continuous infusion of high-dose IL-2, administered to patients with disseminated cancer in the setting of IL-2/LAK cell immunotherapy, induces the in vivo production of various CSF, including IL-5, GM-CSF, M-CSF, and IL-6. Although M-CSF, as well as GM-CSF, exhibits tumoricidal effects in vitro, the significance of the production of these secondary cytokines in IL-2–induced immunomodulation is not clear. Our data demonstrate that in vivo administration of IL-2 may result in the production of factors that stimulate hematopoiesis. The induction of IL-5 may explain the mechanism of eosinophilia, whereas production of GM-CSF may be responsible for the neutrophilia observed during the course of IL-2 treatment.
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In vivo production of interleukin-5, granulocyte-macrophage colony-stimulating factor, macrophages colony-stimulating factor, and interleukin-6 during intravenous administration of high-dose interleukin-2 in cancer patients [see comments]

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