Clinical and Immunological Effects of Granulocyte-Macrophage Colony-stimulating Factor Coadministered with Interleukin 2: A Phase IB Study

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ABSTRACT

Interleukin 2 (IL-2) and granulocytes-macrophage colony-stimulating factor (GM-CSF) are activators of the lymphocyte and granulocyte/macrophage series, respectively. We conducted a phase IB trial to identify the maximally tolerated dose and to assess immunological effects of the combination. Thirty-four patients with incurable cancers received 2.5, 5, or 10 µg/kg GM-CSF s.c. either before or concurrently with 1.5 or 3.0 million units/m²/day IL-2.

The most common laboratory and clinical side effects included an elevation of the total WBC or eosinophil count due to GM-CSF, and constitutional symptoms due to IL-2. Grade 3 or 4 toxicities included hypotension, thrombocytopenia, elevations in aspartate aminotransferase or bilirubin, renal toxicity, gastrointestinal hemorrhage, arrhythmia, and constitutional symptoms. Two patients receiving 5.0 µg/kg GM-CSF plus concurrent 3.0 million units IL-2 experienced dose-limiting grade 3 or 4 neurological toxicity, which reversed almost completely.

An increase in the serum-soluble IL-2 α chain receptor was observed with administration of GM-CSF, IL-2, or the combination. IL-2 therapy enhanced lymphokine-activated killer activity, antibody-dependent cellular cytotoxicity, and lymphocyte activation, with increased CD16 and CD56 expression. GM-CSF increased expression of human leukocyte antigen DR on peripheral blood monocytes and decreased surface expression of CD16 on circulating monocytes and polymorphonuclear cells. Lymphokine-activated killer activity and CD16 expression on monocytes and lymphocytes and CD56 expression on lymphocytes were significantly lower in patients receiving GM-CSF simultaneously with IL-2 than in patients receiving the sequential treatment.

Antitumor activity was observed in the lungs of four of eight renal cell carcinoma patients with pulmonary metastases treated with concurrent GM-CSF and IL-2. Although no or minimal shrinkage was observed in the patients' large primary tumors, these results warrant further study. The recommended initial Phase II dose and schedule is 1.25 µg/kg/day GM-CSF, given concurrently with 1.5 million Roche units/m²/day (4.5 x 10⁶ international units/m²/day) IL-2, with subsequent escalation of GM-CSF to 2.5 µg/kg/day after careful observation for toxicities.

INTRODUCTION

The failure of cytotoxic chemotherapy to induce long-lasting remissions in the majority of solid tumors has prompted investigation of new treatment modalities, including antitumor immunotherapies. The ability of various biological response modifiers, such as IL-2 and IFNs, to enhance cellular immunity suggests a potential therapeutic role for these agents in certain neoplastic diseases, such as melanoma and renal cell carcinoma (1-5). IL-2, a M, 15,000 protein produced by helper T cells following activation by antigens or mitogens, can activate effector T-cell populations and NK cells to lyse tumor cells in vitro and in vivo and can augment ADCC in vitro when used in combination with MoAbs (6-10). As a single agent, it can induce antitumor responses in 10-15% of patients with renal cell carcinoma or metastatic melanoma (1-5, 11). Toxicities, however, are significant, thus promoting continued efforts at identifying methods to enhance antitumor effects by increasing the specificity of the immune-mediated tumor destruction while modifying treatment programs to reduce toxicity (2, 11).

One possible method for enhancing antitumor effects includes combining IL-2 with other immunotherapies. Although IL-2 has been combined with IFNs and certain MoAbs, such as the anti-CD3 MoAb, with limited success (12-16), IL-2 has not been studied extensively in combination with CSFs. CSFs are a family of regulatory glycoproteins that were first identified because of their ability to stimulate precursor hematopoietic...
cells to form colonies of progenitor cells. However, it is now clear that they are not simply proliferative stimuli but can regulate the functional activity of mature hematopoietic cells (17). GM-CSF, for example, is necessary for cell survival in vitro of both progenitor and mature granulocytes and macrophages and also can affect the activation, cell mobility, phagocytic activity, and ADCC of these cells (17). The effects of GM-CSF and M-CSF on neutrophils and monocytes may translate into antitumor effects in vitro or in preclinical models (18–25). Clinically, treatment with GM-CSF or M-CSF can enhance monocyte activation and induce monocytosis (26–28).

Combining IL-2, a lymphocyte activator, with GM-CSF, an activator of the monocyte/macrophage and granulocyte lineage, may be a reasonable approach to the treatment strategy of activation of multiple effector cell populations. Therefore, we conducted a Phase IB trial of GM-CSF and IL-2 to determine the maximally tolerated dose, to identify toxicities, and to assess the immunological effects of the combination.

MATERIALS AND METHODS

Reagents

IL-2 and GM-CSF

Human recombinant IL-2 and GM-CSF for clinical treatment and in vitro studies were provided by Hoffmann-LaRoche Inc. (Nutley, NJ) and Immunix Corp. (Seattle, WA), respectively, through the Cancer Therapy Evaluation Program of the National Cancer Institute. IL-2 and GM-CSF are highly purified products produced in Escherichia coli and yeast expression vectors, respectively, using recombinant DNA techniques. IL-2 units are expressed in BRMP or Hoffmann-LaRoche units, and this compares to the international standard (in which 1 BRMP unit = ~3 international units; Ref. 29). Thus, the 1.5 × 10^6-unit/m²/day regimen used here with Hoffmann-LaRoche IL-2 would correspond to 4.5 × 10^6 international units/m²/day commercially available Aldesleukin (IL-2; Chiron, Inc., Emeryville, CA).

Clinical Protocol

Patients

Thirty-four patients were enrolled in this Phase I trial (National Cancer Institute BRMP protocol B92–0001). All patients had refractory cancers, for which other proven effective treatments were not available, and all signed approved informed consent forms. Patients had an Eastern Cooperative Oncology Group performance status of 0–1 (Karnofsky, 80–100) and a life expectancy of at least 12 weeks. Eligibility criteria included normal hematological parameters (hemoglobin, ≥10 g/dl; granulocyte count, >2000/mm³; and platelet count, ≥100,000/mm³), adequate liver function (total serum bilirubin, <2.0 mg/dl; and/or creatinine clearance, >50 ml/min). Criteria for exclusion included major surgery within the last 3 weeks or treatment with cytotoxic chemotherapy, radiation therapy, or other immunosuppressive therapy within 4 weeks of entry into this clinical trial. Patients with clinically significant cardiac abnormalities or significant pleural effusions were ineligible.

Patients with significant central nervous system disease, central nervous system metastases, or serious recent infections, or those who required continued therapy with corticosteroids, aspirin, or nonsteroidal antiinflammatory agents were ineligible. Patients with histories of chronic obstructive pulmonary disease were required to have adequate ventilatory function (forced expiratory volume, ≥60% of predicted). All patients underwent placement of in-dwelling venous catheters.

Treatment Program

Patients were scheduled to receive 2.5, 5, or 10 µg/kg GM-CSF and either 1.5 or 3.0 × 10^6 units/m²/day IL-2 on a sequential or concurrent schedule (Table 1). Patients were entered in groups of six to ensure adequate numbers for statistical evaluation of the immunological studies. Patients without dose-limiting toxicity or progressive disease in the first course were eligible to receive a second course of treatment following a 2-week rest period.

Patients were registered sequentially to receive one of two different treatment schedules (Table 1 and Fig. 1): GM-CSF for 12 days followed by IL-2 (sequential treatment) or concurrently with IL-2 starting on day 8.

Sequential GM-CSF Followed by IL-2. To assess the effects of GM-CSF and IL-2 individually, the first 12 patients received GM-CSF followed by IL-2 (Fig. 1). GM-CSF was given s.c. on days 1–12 or until hematological toxicity occurred, as defined below. Following a 3-day rest period, three consecutive weekly 4-day continuous infusions of IL-2 commenced (days 15–18, 22–25, and 29–32).

GM-CSF Administered Concurrently with IL-2. To determine the clinical and biological effects of a combination of IL-2 and GM-CSF, patients 13–34 received IL-2 as a 4-day continuous infusion for 3 weeks (days 1–4, 8–11, and 15–18) with GM-CSF starting the second week of IL-2 (day 8). Again, GM-CSF was administered for 12 days (days 8–19) or until treatment-limiting toxicity occurred (Fig. 1).

Toxicity Grading and Dose Modifications

The Common Toxicity Criteria were used for grading of toxicities. The University of Wisconsin Comprehensive Cancer Center clinical toxicity grading scale was used for weight gain, systolic blood pressure, temperature, and decline in performance status (8, 12). Mild (grade 1) toxicity corresponded to a ≤20-mm Hg decrease in systolic blood pressure, a 5–10% weight gain, temperature ≤38°C, and a decline in performance status of two grades. Moderate toxicity corresponded to grade 2 toxicity of a 21–39-mm Hg decrease in systolic blood pressure, 11–14% weight gain, temperature of 38.1–39.9°C, and a decline of

<table>
<thead>
<tr>
<th>Sequence</th>
<th>No. of patients</th>
<th>Dose of GM-CSF (µg/kg/day)</th>
<th>Dose of IL-2 (million units/m²/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequential, IL-2</td>
<td>6</td>
<td>5.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Concurrent, IL-2</td>
<td>6</td>
<td>10.0</td>
<td>3.0</td>
</tr>
<tr>
<td>GM-CSF + IL-2</td>
<td>4</td>
<td>2.5</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>5.0</td>
<td>1.5</td>
</tr>
</tbody>
</table>
three grades in performance status. Severe toxicity corresponded to grade 3 with a ≥40-mm Hg decrease in systolic blood pressure, ≥15% weight gain, temperature ≥40°C, and a decrease of four grades in performance status. For grade 3 toxicity, treatment was withheld until the toxic reaction(s) improved to grade 1 or symptoms returned to baseline. Treatment was then resumed at a 50% dose.

Initially, GM-CSF was administered for 12 days or until the absolute neutrophil count reached 50,000/mm³. Because of a grade 4 central nervous system toxicity occurring in patient 17, associated with an eosinophil count of 73,000/mm³, the protocol was amended so that dose modifications were to occur for an eosinophil count of >20,000/mm³ and/or a total WBC count of >50,000/mm³. Patients 18–20 were considered to have dose-limiting toxicity if this occurred. Starting with patient 21, the protocol was amended so that doses of IL-2 and/or GM-CSF would be reduced or held for these parameters; however, this was not considered dose-limiting toxicity.

Response Criteria
A partial response was defined as a 50% or greater shrinkage in the sum of the areas of all known disease, lasting at least 1 month. A complete response was defined as a complete disappearance of all known disease for at least 1 month.

Immunological Monitoring

Sample Times
Two baseline blood samples were obtained before treatment from all patients. Blood samples to analyze the in vivo effects of GM-CSF followed by IL-2 were obtained on days 2, 6, 9, 13, 20, and 34 of treatment. To assess the effects of GM-CSF combined with IL-2, blood samples were obtained on days 6, 9, 13, 16, and 20 (Fig. 1).

PBM and PMN Cell Isolation
PBM cells from patients and healthy donors were separated from whole blood by centrifugation on a Ficoll-hypaque gradient and washed twice with PBS. PMN cells (granulocytes) were obtained from the dense RBC layer of the gradient by adding 4 ml of the patient’s plasma (saved from the gradient) per 10 ml whole blood and 1.6 ml dextran (4.5% in 0.85% NaCl with 0.2% Tris, adjusted to pH 7.4, and sterile filtered) per 10 ml whole blood at room temperature. After allowing the preparation to settle for 40–60 min, the upper dextran layer containing PMN cells was obtained, and the contaminating RBCs were lysed with a lysis buffer. Neutrophils were not separated from this population. After centrifuging, the PMN pellet was washed with cold PBS.

ADCC
All ADCC assays were performed in RPMI-HS (RPMI 1640 supplemented with 10% human serum, 25 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate) and in RPMI-HS supplemented with IL-2 or GM-CSF. Effector cells in a total volume of 50 μl were plated in quadruplicate into 96-well, U-bottom microliter plates at E:T ratios of 50:1, 16.7:1, and 5.6:1 for PBM cells and 60:1, 20:1, and 6.7:1 or 180:1, 60:1, and 20:1 for PMN cells. Fifty microliters of RPMI-HS, RPMI-HS supplemented with IL-2 at a final concentration of 100 units/ml (to stimulate PBM cells), or RPMI-HS supplemented with GM-CSF at a final concentration of 1 μg/ml (to stimulate PMN cells) were added to effector cells and incubated for 20–30 min at 37°C. Just prior to the addition of target cells (LA-N-5 neuroblastoma cell line), 50 μl chimeric antibody 14.18 were added to quadruplicate wells at a final concentration of 0.25 μg/ml. Effector cells in media and in IL-2 or GM-CSF alone were also plated to determine the effectors’ ability to mediate lysis of target cells in the absence of antibody. Target
cells were labeled with 250 μCi 51Cr in 0.2 ml RPMI-HS. Target cells were mixed every 30 min during labeling to keep the cells in suspension. After washing twice, 5 × 10^3 target cells were added to wells containing effector cells and centrifuged at 200 × g for 5 min. The plates were incubated at 37°C for 4 h, and the supernatants were harvested using the Skatron Harvesting System (Skatron, McLean, VA). Percent cytotoxicity values were calculated for each E:T ratio as: %cytotoxicity = 100 × [(experimental release − spontaneous release)/(maximum release − spontaneous release)].

The maximum 51Cr release was measured by lysing target cells with 2% centriplate solution (Sigma Chemical Co., St. Louis, MO), and spontaneous release was measured with targets incubated in RPMI-HS alone. The results are expressed as lytic units in which 1 lytic unit is the number of effector cells necessary to achieve 20% lysis of 5 × 10^3 targets with maximum lysis of 100% by centriplate treatment.

**LAK Cell Functional Assays**

To test PBM cells for LAK cell function, PBM cells were assayed for their ability to kill the NK-cell-resistant Daudi target. PBM cells in 50 μl RPMI-HS were plated in quadruplicate in 96-well, U-bottom microtiter plates at E:T ratios of 50:1, 16.7:1, and 5.6:1. RPMI-HS or RPMI-HS supplemented with IL-2 at a final concentration of 100 units/ml was added to quadruplicate wells. Target and effector cells were handled as described above.

**Surface Marker Analysis**

All fluorescence labeling was performed at 4°C in the dark for 30 min. Patient PBM and PMN cell populations were characterized by incubating PBM and PMN cells in 100 μl HBSS and 1% fetal bovine serum with FITC-conjugated or phyco-erythrin-conjugated antibodies (Becton Dickinson, Mountain View, CA) at the recommended concentrations. The cells were washed of unbound antibody by adding 2 ml cold PBS and 1% fetal bovine serum. One hundred microliters of propidium iodide were added just prior to analysis on FACS to exclude dead cell populations from the analysis. The percentages of lymphocytes, monocytes, and granulocytes making up the PBM cell population were characterized by CD45 and CD14. The Fc receptor (CD16) and NK marker (CD56) were examined on the population were characterized by incubating PBM and PMN cells in 100 μl HBSS and 1% fetal bovine serum. One hundred microliters of propidium iodide were added just prior to analysis on FACS to exclude dead cell populations from the analysis. The percentages of lymphocytes, monocytes, and granulocytes making up the PBM cell population were characterized by CD45 and CD14. The Fc receptor (CD16) and NK marker (CD56) were examined on the population gated for lymphocytes. The lymphocytes and monocyte populations were examined for expression of MHC class II (HLA-DR) molecules and Fc receptors. In addition, the monocyte population was examined for the Fc receptor (32.2). PMN cells were examined for Fc receptors I and III using antibodies (32.2 and Leu 11a) and for the expression of MHC class II molecules (HLA-DR).

**Soluble IL-2R-α ELISA**

A sandwich assay using two monoclonal anti-Tac antibodies, American Type Culture Collection clones HB8784 and GL439 from R. Robb (DuPont, Boston, MA), was developed. This assay was standardized according to the commercially available kit from T-Cell Diagnostics (Cambridge, MA). Briefly, 96-well, C-bottom polystyrene plates (MaxiSorp; Nunc, Roskilde, Denmark) were coated with 150 μl/well antibody HB8784 at a concentration of 2 μg/ml in carbonate buffer (pH 9.6) overnight at 4°C, then washed and blocked with 5% nonfat dry milk in PBS for 1 h at room temperature. After washing with PBS/0.05% Tween 20, 100 μl/well standards, controls, and test samples were incubated overnight at 4°C in a wet chamber. Following washing, biotinylated secondary antibody GL439 was added, and the plate was incubated for 3 h at room temperature and washed as above; alkaline phosphatase-conjugated ExtrAvidin (Sigma) was added (1:5000 dilution in Tris/Tween), and plates were incubated for 1 h at room temperature. After washing with PBS/Tween, 1 mg/ml substrate (p-nitrophenyl phosphate; Sigma) in diethanolamine buffer (pH 9.8) was added. After a 30-min incubation at room temperature in the dark, the enzymatic reaction was stopped, and color intensity was measured at 405 versus 490 nm using an ELISA reader (EAR 400 AT; SLT Lab Instruments, Salzburg, Austria), and the concentration of soluble IL-2R was determined using SOFT-2000 software (SLT). Positive samples assayed previously were included in each assay to provide internal controls, allowing for comparison of soluble IL-2 results between different assays.

**Statistical Analysis**

Assessments of immunological effects within treatment groups were made using paired t tests. A comparison of effects between treatment groups were made using a two-sample t test.

**RESULTS**

Thirty-four patients were enrolled on the protocol. Their characteristics are shown in Table 2. Twenty-seven patients were evaluable for biological response assessment. Nine patients went off study prior to completion of one cycle of therapy,
Clinical Cancer Research 323

Table 3 Number of courses with grade 3 toxicities in patients receiving sequential GM-CSF followed by IL-2

<table>
<thead>
<tr>
<th>GM-CSF dose (μg/kg)</th>
<th>IL-2 dose (million units/m²/day)</th>
<th>No. of courses administered</th>
<th>GM-CSF</th>
<th>IL-2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>3</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>10</td>
<td>3</td>
<td>12</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Pain/reaction at injection site 0 0 0
Bone pain/myalgia 0 0 0
Hepatic
Aspartate aminotransferase 0 0 0
Bilirubin 0 0 0
Cardiac
Hypotension 0 0 0
Arrhythmias 0 0 0
Hematological
WBC >60,000 0 0 0
20,000-50,000 0 0 0
>50,000 0 0 0
Thrombocytopenia 0 0 0
Neurological 0 0 0
Other
Renal 0 0 0
Performance status 0 0 0
Fever >40°C 3 1 4
Hypocalcemia
Total 0 2 2
Corrected 0 0 0
Diarrhea 0 1 1
Gastrointestinal hemorrhage 0 0 0

Table 4 Number of courses with grade 3 toxicities in patients receiving concurrent GM-CSF plus IL-2

<table>
<thead>
<tr>
<th>GM-CSF dose (μg/kg)</th>
<th>IL-2 dose (million units/m²/day)</th>
<th>No. of courses administered</th>
<th>GM-CSF</th>
<th>IL-2</th>
<th>Total</th>
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<tr>
<td>2.5</td>
<td>2.5</td>
<td>13</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>5.0</td>
<td>5.0</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

Hepatic
Aspartate aminotransferase 0 1 1
Bilirubin 1 1 0
Cardiac
Hypotension 0 1 1
Arrhythmias 0 0 0
Hematological
WBC >60,000 0 1 1
WBC >40,000 0 0 0
Eosinophils
20,000-50,000 5 2 7
>50,000 0 0 0
Thrombocytopenia 1 2 3
Neurological 0 1 1
Other
Renal 0 1 1
Performance status 0 1 1
Fever >40°C 3 0 4
Hypocalcemia
Total 0 2 2
Corrected 0 0 0
Diarrhea 0 1 1

*Grade 4 toxicities.

six due to toxicity and three due to progressive disease. All patients were considered evaluable for toxicity and response.

Clinical Results

Toxicities

Toxicities secondary to treatment are shown in Tables 3 and 4. The majority of toxicities were elevations in total WBC or eosinophil count secondary to GM-CSF. Other nonhematological toxicities included fevers, hypotension, diarrhea, increases in hepatic enzymes, and arrhythmias.

No dose-limiting (grade 4) toxicities of sequential GM-CSF and IL-2 (Table 3) were observed. Dose-limiting toxicities of concurrent GM-CSF and IL-2 were primarily neurological and cardiovascular (Table 4).

Grade 3 neurological toxicity was observed in patient 17, who received concurrent GM-CSF (5 μg/kg) and IL-2 (1.5 million units/m²/day). The patient was a 48-year-old male who began experiencing forgetfulness, confusion, and lethargy on day 18 of his treatment. At that time, his WBC count was noted to be 75,800/mm³ with an absolute eosinophil count of 57,000/mm³ and a platelet count of 67,000/mm³. The GM-CSF was held, and IL-2 was stopped. Nonetheless, the patient’s neurological symptoms continued to worsen, and on day 19, he was experiencing dizziness, lethargy, confusion, headaches, hallucinations, short-term memory loss, right-hand motor difficulties, and intermittent mild, expressive aphasia. The maximum total WBC count was 99,400/mm³, and the maximum total eosinophil count was 73,000/mm³ on day 19, 31 h after the last dose of GM-CSF. As it was thought that these symptoms were probably due to the high WBC count with the eosinophilia, the patient was given 2 g hydroxyurea p.o. on day 19, with a resultant decrease in the WBC count to 46,000/mm³ and 23,000/mm³ over the next 2 days. Head MRI revealed multiple small lesions in the white matter in the front lobe bilaterally, with smaller lesions in the medial aspect of the right parietal lobe and right parietal temporal junction.

The patient’s neurological findings resolved slowly. Approximately 1 month later, he was seen in the clinic, where he was found to have some residual deficits in his attention span and ability to perform math calculations, clumsiness of his right hand, and some visual complaints. A computed tomographic scan of his chest showed an improvement in his pulmonary nodules. Two months following IL-2 and GM-CSF, neurological symptoms and complaints had completely resolved, with the exception of a visual-field defect, which was thought to be related to an occipital lesion, and subtle fine motor deficiencies in his right hand. Head MRI revealed no change in the ischemic lesions. The patient refused all but palliative treatment subsequent to GM-CSF and IL-2, and he developed a spinal cord compression secondary to bone metastases 11 months later.

Another patient (patient 33) on the concurrent arm of 5 μg/kg GM-CSF and 1.5 million units/m² IL-2 developed grade 4 neurological toxicity. This 53-year-old male who had renal cell carcinoma with nodal and adrenal metastases received six doses of GM-CSF during his second week of IL-2 therapy (two doses at 5 μg/kg, two at 2.5 μg/kg, and two at 1.25 μg/kg, with dose reductions due to grade 3 diarrhea and eosinophilia). On day 18 of his therapy, the patient became confused and lethargic, with weakness in all extremities, especially the right arm, and he developed total vision loss. Treatment with IL-2 and GM-CSF was stopped. This patient’s maximum total WBC and
Table 5  Antitumor effects of GM-CSF and IL-2 by groups and dose level

<table>
<thead>
<tr>
<th>GM-CSF dose (µg/kg)</th>
<th>Sequential, GM-CSF → IL-2</th>
<th>Concurrent, GM-CSF + IL-2</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>IL-2 dose (million units/m²/day)</td>
<td>5 10 2.5 2.5 5.0</td>
<td>3 3 1.5 3.0 1.5</td>
<td>6 6 6 4 12 34</td>
</tr>
<tr>
<td>No. of patients per dose level</td>
<td>2 5 3 1 4 15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of patients with metastatic renal cell carcinoma and pulmonary metastases only</td>
<td>0 1 1 0 3 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of responses</td>
<td>0 0 0 0 0 0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A

B

Fig. 2  Fold increase from baseline in soluble IL-2Rs in patients receiving sequential (A) or concurrent (B) GM-CSF and IL-2. The abscissa shows the day samples were obtained in relation to IL-2 and GM-CSF administration.

eosinophil counts were 43,800/mm³ and 24,000/mm³, respectively, on day 18. Head MRI showed multiple new foci of abnormalities in the cerebral cortex consistent with thrombosis or multiple small vessel emboli; a lumbar puncture and transesophageal echocardiogram were negative. The patient’s neurological signs began improving on day 19, and vision was totally resolved within 24 h. The majority of his symptoms resolved gradually over the next 3 months, and he was left with a residual mild weakness in the right arm. Neither of the two patients experiencing neurological toxicity had any predisposing risk factors or any evidence on head MRI of predisposing atherosclerotic disease.

Grade 4 cardiac toxicity was observed in patient 15 while receiving concurrent GM-CSF (5 µg/kg) and IL-2 (1.5 million units/m²/day). The patient was a 65-year-old male with renal cell carcinoma who had a prior history of atrial flutter and was taking digoxin; however, he had passed an exercise thallium test prior to going on study. On day 10, the patient began experiencing hypotension and tachycardia. He was found to have an elevated creatinine level (2.9 mg/dl) and hyperkalemia (6.0). In addition, an electrocardiogram showed 2:1 atrial flutter. The patient was transferred to the cardiac intensive care unit for monitoring, where he received i.v. fluids and Kayexalate. The patient spontaneously converted back to normal sinus rhythm, and his serum potassium returned to normal; his cardiac enzymes remained normal. He remained in the intensive care unit overnight and did not experience any more problems; he was not retreated.

Antitumor Effects

None of the 34 patients had a >50% shrinkage of the sum of all disease. Fifteen patients had stable disease at the end of two courses of therapy, and 19 patients developed progressive disease over the same time interval.

Although no patient had a partial response, as defined by a >50% shrinkage of the sum of all disease, the number of patients with metastatic renal cell carcinoma who had reductions in their pulmonary disease is of note (Table 5). Overall, 15 patients had metastatic renal cell carcinoma with pulmonary metastases; of these, 11 patients had their primary renal tumors intact. Of the 15 patients with metastatic renal cell carcinoma, 5 (33%) had >50% reduction in the size of their pulmonary metastases, although none of them qualified as a partial responder because of a lack of measurable response in the kidneys or other sites of metastases. Also noteworthy was that 4 of 8 patients with renal cell carcinoma and pulmonary metastases on the GM-CSF and concurrent IL-2 regimen had >50% reduction in the size of their pulmonary metastases.
Soluble IL-2R

Increases in the soluble IL-2R α chain of 3.5- and 6.3-fold were observed following 6 and 12 days, respectively, of treatment with GM-CSF in the 12 patients in the sequential arm (P < 0.0001; Fig. 2). These data demonstrate that GM-CSF alone induces a significant rise in this receptor level. The soluble IL-2R continued to rise with subsequent continuous infusion of IL-2 such that at day 34, there was a 28.6-fold increase from baseline (P < 0.0001).

Soluble IL-2R increased 16.2-fold in patients in the sequential group when measured 24 h after completion of the first 96 h continuous infusion of IL-2. This change was significantly greater than the 6.6-fold increase following 1 week of IL-2 alone in patients in the concurrent arm. These results indicate that GM-CSF given prior to one 96 h continuous infusion of IL-2 results in a higher overall release of soluble IL-2R than does treatment with IL-2 alone. Note, however, that the patients receiving the sequential treatment were treated with IL-2 at 3 × 10⁶ units/m², whereas only 4 of the patients in the concurrent group received this dose. The overall change following 3 weeks of IL-2 for the two groups was not significantly different, with a 28.6-fold increase on day 34 for the sequential arm and a 28.5-fold increase at day 20 for the concurrent arm (P = 0.11).

LAK and ADCC Activity

Twelve days of GM-CSF alone did not induce LAK activity in patients in the sequential arm. Following 12 days of GM-CSF, treatment for 96 h with IL-2 alone induced LAK activity when measured in the presence of IL-2 during the 4-h ⁵¹Cr release assay. LAK activity was also detected in patients in the concurrent arm following a 96-h continuous infusion of IL-2 prior to initiation of GM-CSF (day 6). This activity remained elevated through administration of GM-CSF. Although LAK activity seemed to be declining from the peak level noted at day 6, these differences were not statistically significant (P = 0.14).

The LA-N-5 neuroblastoma target was used to measure ADCC activity with the chimeric 14.18 human and mouse antibody, which recognizes the ganglioside GD2 on the neuroblastoma target. Treatment for 12 days with GM-CSF in the sequential group demonstrated that there was a slight down-modulation of ADCC activity from baseline to days 6 and 13. Initiation of IL-2 induced a significant increase in ADCC noted at days 20 and 34. In the concurrent group, ADCC increased after an initial week of continuous infusion of IL-2 and remained elevated following 2 weeks of IL-2 combined with GM-CSF.

When comparing LAK activity in the two groups following 3 weeks of IL-2, LAK activity in patients receiving GM-CSF simultaneously with IL-2 was significantly lower than that seen in patients receiving the sequential treatment (Table 6). This was noted against the Daudi target with PBM cells tested in medium (P = 0.03) and PBM cells tested on both Daudi and LA-N-5 targets in IL-2 (P = .03 and 0.012, respectively) (Table 6). There was no significant difference in the augmentation of ADCC activity induced with the two treatment regimens.

Surface Marker Analysis

Lymphocyte, monocyte, and PMN cell (granulocyte) populations were assessed for phenotypic changes and expression of activation antigens (Fig. 3).

Effects of GM-CSF Alone. As seen in Fig. 4, GM-CSF alone did not affect the expression of CD16, CD56, or HLA-DR on lymphocytes. However, CD16 was significantly down-modulated in both monocytes and PMN cells. In addition, GM-CSF induced an increase in the monocyte population expressing the HLA class II (DR) antigen.

Effects of IL-2 with and without GM-CSF. Lymphocytes. As noted in previous studies, the percentage of lymphocytes expressing CD56 and CD16 increased following IL-2 administration in patients in both the concurrent and sequential treatment groups. In addition, the percentage of lymphocytes expressing HLA-DR was also increased in both groups during the time of IL-2 administration.

Monocytes. IL-2 alone induced a significant increase in CD16 expression on monocytes in patients in the sequential group. No increase in the percentage of monocytes positive for CD16 was observed when IL-2 was given concurrently with GM-CSF. Although treatment with GM-CSF alone induced increased expression of HLA-DR on monocytes, this returned to baseline during the IL-2 infusion in patients receiving sequential treatment. There was no increase in monocyte DR expression in the concurrent patients following 1 week of IL-2. Increased DR expression was noted following the second week of IL-2; however, at the end of the third week of IL-2, the level was not significantly above the pretreatment level.

PMN Cells. IL-2 alone induced a significant decrease in CD16 expression on PMN cells in patients in both the sequential and concurrent groups. This level continued to fall during concurrent IL-2 and GM-CSF treatment. No significant changes were noted in cells expressing the Fc 1 receptor.

The expression of HLA-DR on PMN cells was examined.

### Table 6 Significant differences in immunological parameters between patients in the sequential and concurrent groups following cycle 1

<table>
<thead>
<tr>
<th>Surface markers</th>
<th>Change in % of cells positive from baseline to completion of IL-2</th>
<th>Sequential, day 34</th>
<th>Concurrent, day 20</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocyte CD16</td>
<td>34</td>
<td>22</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Lymphocyte CD56</td>
<td>50</td>
<td>28</td>
<td>0.0009</td>
<td></td>
</tr>
<tr>
<td>Monocyte CD16</td>
<td>28</td>
<td>1</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Change in lytic units from baseline to completion of IL-2</th>
<th>Sequential, day 34</th>
<th>Concurrent, day 20</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAK activity</td>
<td>14</td>
<td>2</td>
<td>0.03</td>
</tr>
<tr>
<td>Daudi, medium</td>
<td>233</td>
<td>62</td>
<td>0.03</td>
</tr>
<tr>
<td>LA-N-5, medium</td>
<td>78</td>
<td>5</td>
<td>0.08</td>
</tr>
<tr>
<td>LA-N-5, IE-2</td>
<td>295</td>
<td>28</td>
<td>0.012</td>
</tr>
<tr>
<td>LA-N-5, antibody</td>
<td>480</td>
<td>306</td>
<td>0.5</td>
</tr>
<tr>
<td>LA-N-5, + IL-2</td>
<td>735</td>
<td>552</td>
<td>0.56</td>
</tr>
</tbody>
</table>
only in patients treated in the concurrent arm. Resting PMN cells did not express HLA-DR. Following 1 week of IL-2 alone, there was a significant increase, with 10% of the PMN cells expressing HLA-DR. With the combination of IL-2 and GM-CSF, this increased to 34% at day 13 and was at 23% on day 20.

**Comparative Analyses between Groups**

Comparisons of changes between immunological parameters at the end of treatment in the concurrent group compared with the sequential group are shown in Table 6. The increase in CD16 and CD56 expression on lymphocytes and CD16 expression on monocytes were significantly greater in the patients receiving the sequential regimen. In addition, LAK cytotoxicity against the Daudi and LA-N-5 targets was significantly greater in this group. This increased expression of CD16 and CD56 and LAK cytotoxicity in the sequential group also may be due to the higher dose of IL-2 received by these patients, making any direct comparison difficult. However, there was no significant difference in the ADCC activity when comparing the sequential and concurrent arms. Because only a fraction of cells mediating LAK activity can mediate ADCC (30), this result may suggest differential activation of the LAK and ADCC effectors.

Significant differences in immunological parameters between the 18 patients in the concurrent treatment arm who showed no antitumor effects from treatment compared with the 4 patients who had at least 50% reductions in their pulmonary nodules (responding patients) are shown in Fig. 4 (P < 0.05). Although both responding and nonresponding patients had significant increases in lymphocyte
CD16 and CD56 expression, the responding patients did not have increases as great at day 20. The eosinophil counts in the responding patients were obtained in relation to IL-2 and GM-CSF administration.

**DISCUSSION**

Preclinical studies indicate that the CSFs, which activate monocytes, macrophages, and granulocytes, have immunological effects that may result in antitumor activity. M-CSF enhances ADCC in cynomolgus monkey, human, and murine monocytoid cells with a >50% reduction in GM-CSF and IL-2 (1, P < 0.05). The abscissa shows the day samples were obtained in relation to IL-2 and GM-CSF administration.

GM-CSF and M-CSF as monocyte activators when the gene is introduced directly into cells are ongoing (32-34). Clinical studies with M-CSF indicate that this molecule has biological and immunological activity. A Phase I study of M-CSF demonstrated that M-CSF increased the percentage of peripheral blood monocytes expressing the CD16 low-affinity Fc-γ receptor for aggregated immunoglobulin and the CD14 receptor (28). Systemic M-CSF induces peripheral blood monocytes and thrombocytopenia (28, 35-37).

The data regarding the effectiveness of GM-CSF as a monocyte activator in humans are somewhat limited. A Phase I study demonstrated that pretreatment with GM-CSF led to a statistically significant enhancement in direct monocyte cytotoxicity against HT29 cells, but without increasing serum TNF-α or IL-1β levels and without any consistent in vitro induction of TNF-α or IL-1β (26). One Phase I study of GM-CSF demonstrated activation of monocyte-mediated tumorlytic properties in only one of seven patients and failed to demonstrate stimulation of IL-1 or TNF by peripheral monocytes (27). However, patients received variable amounts of GM-CSF, and GM-CSF was administered as a continuous infusion. In another Phase I study using the 17-1A MoAb, ADCC and the number of Fc receptor-bearing monocytes increased during therapy with GM-CSF (38).

GM-CSF currently has practical advantages over M-CSF in that it is commercially available, and its toxicities and schedules of administration for hematopoietic stimulation are well defined. Therefore, the objective of this protocol was to determine the biological and immunological effects of this molecule on circulating blood lymphocytes and peripheral neutrophils and monocytes alone and in combination with IL-2. Simultaneous activation of multiple immune system effector cells has the theoretical advantage of enhanced direct antitumor cytotoxicity as well as activation of effectors capable of mediating ADCC for eventual use with tumor-specific MoAbs.

In our study, we observed an increase in soluble IL-2Rs with administration of GM-CSF, IL-2, or the combination. Expected effects of IL-2 on LAK activity, ADCC, and lymphocyte activation were also observed and did not seem to be qualitatively different following pretreatment with GM-CSF when compared with results obtained in our laboratory from other clinical IL-2 trials (7-9, 12, 15). Interestingly, these stimulatory effects on circulating lymphocytes were decreased with concurrent GM-CSF treatment, with a significantly lower level of LAK activity in patients receiving simultaneous treatment compared with sequential GM-CSF. GM-CSF increased expression of HLA-DR on monocytes; this was not maintained with concurrent or subsequent IL-2 therapy.

Intriguingly, surface expression of CD16 on monocytes and PMN cells decreased during GM-CSF treatment, whereas the morphology of the circulating PMN cells showed signs of activation (i.e., toxic granulations) clearly while on therapy. Although GM-CSF may cause down-regulation of CD16 in neutrophils (39), GM-CSF is also known to cause more rapid production and release of PMN precursors from the bone marrow, and Fc receptors are expressed late in PMN cell maturation (40). Thus, we postulate that the increase in circulating PMN cell and monocyte numbers with the decreased expression of Fc receptors on these cells represents two concurrent changes from the baseline physiological status: first, the appearance in the blood of immature PMN cells and monocytes that have not yet
expressed their Fc receptors; and second, the more rapid egress from the circulation of these activated PMN cells and monocytes due to their stimulation by GM-CSF and due to endothelial activation by the associated cytokines released in response to IL-2 and GM-CSF. This should result in a greater number of activated cells that are maturing and expressing CD16 within the tissues. This could not be tested directly in this study and emphasizes difficulties in evaluating changes in cell behavior in tissue compartments based solely on measurement of serum or peripheral blood cells from patients undergoing biological therapy.

Interestingly, several studies indicate that GM-CSF may activate lymphocytes either directly or indirectly in vivo. An increase in the absolute lymphocyte count, serum IL-2R, serum CD8 levels, and CD25+ and CD4+ lymphocytes was observed in patients with malignant lymphoma receiving GM-CSF (41). GM-CSF potentiated the long-term growth of nonactivated T cells in the presence of IL-2 and short-term proliferation of lectin-stimulated T cells (42) and augmented low-dose IL-2 induction from murine splenocytes (43). In our study, we did not observe any effects of GM-CSF on the induction of LAK or ADCC activity or on lymphocyte activation as assessed by increases in CD56+, CD16+, or HLA-DR-positive lymphocytes in the peripheral blood. An increase in soluble IL-2Rs was observed, indicating that GM-CSF may have an effect on lymphocyte activation, although monocytes also may have been the source of the soluble IL-2R.

Four of 5 patients who had 50% or greater reduction in their pulmonary metastases were receiving concurrent treatment with GM-CSF and IL-2. The percentage of lymphocytes positive for CD16 and CD56 in these patients was not as great as the increase noted in the 18 nonresponding patients. Soluble IL-2Rs and ADCC and LAK activity were not decreased in this subgroup of patients, indicating that immunological activation still occurred. Although the numbers of patients are small, it is tempting to speculate that CD16+ and CD56+ lymphocytes were less prevalent in the circulation because of greater egress into tissues, possibly including sites of pulmonary disease.

Clinically, the treatment was well tolerated, with side effects typical of those commonly seen with IL-2 or GM-CSF. The etiology of the two grade 3 or 4 neurological toxicities is unclear. Although we have seen similar toxicity in more than 150 patients treated with IL-2 at our institution, 6 other patients receiving IL-2 at other institutions, none of whom were also receiving GM-CSF, have been described as having similar toxicities; patients had confusion, delirium, coma, ataxia, hemiparesis, and cortical blindness, with multiple cerebral lesions on MRI (44). In this study, the neurotoxicity was not uniformly the result of the elevated WBC count and eosinophilia, because these were markedly elevated in only one of the two patients with neurotoxicity. Similarly, they were not clearly related to the dose of GM-CSF, because the second patient had received two doses of 5 μg/kg, two doses of 2.5 μg/kg, and two doses of 1.25 μg/kg GM-CSF.

Although the etiology of this neurotoxicity is not clear, thromboembolic cerebrovascular accidents have been described in patients with hypereosinophilia (45, 46). Most of the described patients had endocardial disease associated with idiopathic hypereosinophilic syndrome. Slungaard et al. (47) showed that eosinophilic cationic proteins, in particular major basic protein, inhibit anticoagulant activities of the glycosylated form of thrombomodulin potently. Endothelial and endocardial cells maintain an anticoagulant surface, in part through the expression of thrombomodulin on their surfaces (48–50); therefore, inhibition of thrombomodulin by hypereosinophilia may lead to thromboembolic cerebrovascular accidents. It remains unclear whether this mechanism or other possible mechanisms are responsible for the observed neurological toxicity in this study. Because the neurological toxicity was not related clearly to the dose of GM-CSF or the total WBC or eosinophil count, we recommend the Phase II starting dose of 1.25 μg/kg/day GM-CSF with 1.5 million Roche units/m2/day IL-2, with subsequent escalation of GM-CSF to 2.5 μg/kg/day after careful observation for toxicities.

We observed an increase in soluble IL-2Rs with administration of GM-CSF, IL-2, or the combination. IL-2 enhanced LAK activity, ADCC, and lymphocyte activation with and without pretreatment with GM-CSF; LAK activity was lower in patients receiving concurrent GM-CSF and IL-2 treatment. GM-CSF increased the expression of HLA-DR on monocytes; this was not maintained with concurrent or subsequent IL-2 therapy. GM-CSF decreased the surface expression of CD16 on monocytes and PMN cells. The antitumor responses observed in four of eight renal cell carcinoma patients with pulmonary metastases treated with concurrent GM-CSF and IL-2 are provocative and warrant further study. Although the relationship between the neurological toxicity and the dose and schedule of the combination, if any, is unclear, the recommended initial Phase II dose and schedule is 1.25 μg/kg/day GM-CSF, given concurrently with 1.5 million Roche units/m2/day IL-2, with subsequent escalation of GM-CSF to 2.5 μg/kg/day after careful observation for toxicities.

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REFERENCES


