Phase I Trial of Combined Immunotherapy with Subcutaneous Granulocyte Macrophage Colony-stimulating Factor, Low-Dose Interleukin 2, and Interferon α in Progressive Metastatic Melanoma and Renal Cell Carcinoma

Gijsbert C. de Gast,¹ Heinz-Joseph Klümpen, Florry A. Vyth-Dreese, Marie-José Kersten, Natascha C. V. Verra, Johan Sein, Dian Batchelor, Willem J. Nooijen, and Jan H. Schornagel
Division of Medical Oncology, Antoni van Leeuwenhoek Hospital/ Netherlands Cancer Institute, 1066 CX Amsterdam, the Netherlands

ABSTRACT
The purpose of our study was to determine the maximally tolerated dose (MTD) and DLT of combined administration of granulocyte macrophage colony-stimulating factor (GM-CSF), low-dose interleukin 2 (IL-2) and IFN-α in patients with progressive metastatic melanoma or renal cell carcinoma (RCC). In addition, the activation and expansion of effector cells were measured. Cohorts of three patients were treated with increasing doses of IL-2 (1, 4, and 8 MIU/m²) and GM-CSF (2.5 and 5 µg/kg) with a constant dose of IFNα (5 million units) s.c. for 12 days every 3 weeks. An additional six patients were treated at the MTD. Immune activation was monitored during the first cycle. Response was evaluated after two cycles. The MTD was found to be 2.5 µg/kg GM-CSF, 4 MIU/m² IL-2, and 5 mega units of IFNα. DLT was grade 4 fever, chills with hypotension, grade 3 fatigue/malaise, and fluid retention. Dose reduction of IL-2 to 2 MIU/m² was necessary in three of nine patients who initially received the MTD. Treatment was initiated in the hospital but could be continued at home after 3–4 days. Significant increases in lymphocytes, (activated) T cells (CD4+ and CD8+), NK cells, monocyte DR expression, neutrophils, and eosinophils were found. CD8+ T-cell activation (sCD8) and NK cell expansion was mainly present in patients receiving 2 or 4 MIU/m² IL-2. Of eight patients with progressive metastatic RCC after nephrectomy, three achieved a complete remission, and 1 of 7 patients with metastatic melanoma achieved a partial remission. In our study, the MTD of combined immunotherapy with GM-CSF, IL-2, and IFNα was established; DLT was: (a) grade 4 fever with hypotension needing i.v. fluid support; and (b) grade 3 fluid retention and/or fatigue/malaise. The scheme resulted in considerable expansion and/or activation of various effector cells. The complete responses in RCC patients are promising but need to be confirmed in Phase II studies.

INTRODUCTION
Melanoma and RCC² are considered the most immunogenic human tumors. Whereas metastatic melanoma responds to chemotherapy in 15–20% of cases, without an effect on survival, chemotherapy in metastatic RCC has never shown a response rate of more than 5% (1, 2). Both IL-2 and IFNα and the combination have shown responses in metastatic melanoma and RCC (3, 4). High-dose IL-2 i.v. can result in long-term survivors in about 10% of cases but at the cost of considerable toxicity (3, 5–8). The addition of chemotherapy to immunotherapy has resulted in a higher number of responders in metastatic melanoma but not in a better long-term survival rate (9–12). To develop a more effective immunotherapy than IL-2 + IFNα and to avoid the toxicity of high-dose IL-2, we performed a Phase I trial of the combination of GM-CSF, IL-2, and IFNα given s.c. for 12 days. We started with a dose of 2.5 µg/kg GM-CSF, 1 MIU/m² IL-2, and 5 MU of IFNα regardless of body weight, as this was well tolerated without serious (grade 3 or 4, common toxicity criteria) toxicity in an outpatient setting in a Phase II study in metastatic melanoma.¹ In that trial, no clear CD8+ T- or NK-cell activation was found. The present trial was conceived to find out whether such cells, thought to be important for antitumor activity, could be activated by increases in IL-2 and/or GM-CSF. Therefore, the rationale of the combination was to induce nonspecifically the activation and expansion of all of the putative effector cells without serious toxicity and to maintain

¹ To whom requests for reprints should be addressed, at Division of Medical Oncology, Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, the Netherlands. Phone: 31-20-5122570; Fax: 31-20-5122572; E-mail: irene@nk.nl.

² The abbreviations used are: RCC, renal cell carcinoma; IL, interleukin; GM-CSF, granulocyte macrophage colony-stimulating factor; PD, progressive disease; SD, stable disease; PR, partial response; CR, complete response; IL-2R, IL-2 receptor; sIL-2R, soluble IL-2R; CVA, cerebrovascular accident; LN, lymph node; MTD, maximal tolerated dose; sCD8, soluble CD8; PE, phycoerythrin; LAK, lymphokine-activated killer.

such activation for a longer time than previously achieved. Experimental data have shown that GM-CSF and low-dose IL-2 induce T-LAK without inducing NK-LAK and its inherent toxicity of vascular-leak syndrome, as seen with high-dose IL-2 i.v. (13–16). However, little is known about the toxicity of this combination and the immunological effects in humans.

PATIENTS AND METHODS
Human recombinant IL-2 was provided by Chiron Benelux [Amsterdam, the Netherlands (Aldesleukin)] and is expressed as 106 international units (MIU)/m2/day. GM-CSF (Molgramostim) was produced by Escherichia coli and IFN-α 2b (Intron-A) in yeast expression vectors, using recombinant techniques (Schering-Plough, Maarssen, the Netherlands).

Clinical Protocol
Patients. Eighteen patients were enrolled in this Phase I trial (Netherlands Cancer Institute, protocol N981RM), which was approved by the local Ethics committee. All of the patients had progressive metastatic RCC (n = 11), chemotherapy (Dacarbazine)-resistant skin melanoma (n = 5), or uveal melanoma (n = 2). Three patients with metastatic RCC had their primary tumor in situ. Patients had a WHO performance status of 0–2 and a life expectancy of at least 12 weeks.

Eligibility criteria included: (a) adequate hematological parameters (hemoglobin, >6.0 mmol/liter; WBC, >4.0/nl; and platelets, >100/nl); (b) adequate liver function (total serum bilirubin, <1.5 μmol/liter; and serum glutamic oxalacetic transaminase, <3 times the upper limit of normal); (c) adequate renal function (serum creatinine, <180 μmol/liter); and (d) written informed consent. Exclusion criteria included: (a) major surgery; (b) cytotoxic chemotherapy or radiation therapy within 4 weeks of entry into this clinical trial; and (c) past or present autoimmune disease and HIV antibodies. Patients with clinically significant cardiac, pulmonary, or metabolic disease were ineligible, as well as patients with symptomatic central nervous system disease, serious recent infections, or requirement of systemic steroids or nonsteroidal anti-inflammatory agents.

Treatment Program. Patients were scheduled to receive simultaneously s.c. injections of GM-CSF (2.5 or 5 μg/kg), low-dose IL-2 (1, 4, or 8 MIU/m2) and IFNαs (5 MU regardless of body weight). Saline (500 ml) was given the first day at the start of the injections as a prophylactic measure against hypotension during fever. Acetaminophen (up to 4 g/day) was given prophylactically 1 h after the injections (1 g) and at the start of chills (1 g) and later on, therapeutically, to mitigate fever and chills. Patients were entered in groups of three. During the first 3–4 days, the s.c. injections were given in the hospital to monitor toxicity and to instruct the patients or family members to prepare and administer the s.c. injections, which were given in different places (GM-CSF, upper left leg; IFNαs, upper right leg; IL-2, abdomen). The cycle was continued at home for a total of 12 days. A second cycle of 12 days was given at home after 9 days of rest if no dose-limiting toxicity or PD disease was present. Response was evaluated after two cycles; patients with response or with SD were eligible for another two cycles.

Toxicity Grading and Dose Modifications. The common toxicity criteria (CTC version 2.0) were used for grading toxicity. In case of grade 4 fever (>40°C) with hypotension requiring i.v. fluids, the patient received parenteral fluid per continuous infusion, and treatment was withheld until the toxic reaction improved to grade 1 or symptoms returned to baseline. Subsequently, dose reduction was applied, which allowed patients to be discharged and safely continue treatment at home. Other grade 3 toxicities were managed accordingly with symptomatic treatment. Patients were discharged from hospital if ≤grade 2 toxicity was achieved with the exception of grade 3 fever and chills. If GM-CSF induced leucocytosis of >30/nl, GM-CSF was stopped for the remainder of the cycle.

Response Criteria. A CR was defined as a complete disappearance of all of the known measurable disease for at least 1 month; a PR, as a 50% or greater decrease in the sum of the products of the largest diameters of all evaluable disease, lasting 1 month; SD as <50% decrease or <25% increase in evaluable lesions without new lesions, lasting 1 month; and PD as >25% increase of known disease or appearance of new lesions.

Immunological Monitoring
Sample Times. Blood samples were obtained before treatment and on days 8, 12, 15, and 22 after the start of treatment. Cells for cytofluorography were collected in EDTA; plasma for cytokine measurements was centrifuged immediately.

Flow Cytometry. To detect cellular differentiation markers on peripheral blood cell samples taken before, during and after immunotherapy, WBCs were collected from heparinized peripheral blood by lysis of red cells with ammonium chloride. Triple staining was performed by the incubation of cell samples in a mixture of fluorescein (FITC)-, R-PE-, and PE-Cy5-conjugated mouse monoclonal antibodies, directed to cellular differentiation markers, for 20 min at room temperature followed by washing in PBS containing 0.2% BSA and 0.02% azide. FITC and PE conjugates were obtained from Becton & Dickinson Immunocytometry Systems (San Jose, CA) and cytochrome-conjugates from Coulter Immunotech (Mijdrecht, the Netherlands.) To prevent aspecific binding via Fc-receptors, incubation was carried out in the presence of 1% normal mouse serum (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands). Fluochrome-labeled isotype control antibodies were included in each assay to determine background staining. Lymphocyte (CD3+, CD19+, and CD16+56+) and monocyte (CD14+) populations were gated according to their differential forward and side scatter, which was confirmed by back-gating. Fluorescence was measured on a FACScan flow cytometer (Becton & Dickinson, Mountain View, CA), and data were analyzed using Cellquest software (Becton & Dickinson).

Cytokine Assays. Cytokine levels were determined in plasma derived from EDTA-treated blood that had been kept directly on ice after collection and promptly centrifuged and frozen at −20°C, according to the manufacturer’s instructions. The following cytokines were assayed by ELISA: sIL-2R (Eurogenetics, Tesserderlo, Belgium) and soluble CD8/sCD8 (T cell Diagnostics, Cambridge, MA). The measurable threshold levels were 100 units/ml for sIL-2R and 100 units/ml for sCD8.
Levels of circulating cells in peripheral blood and of cytokines at various time points were compared by Student's t test. P ≤ 0.05 were considered significant.

RESULTS

From April 1 until September 1, 1998, 18 patients were included—12 in the Phase I trial and 6 additional patients at the MTD established in Phase I. All of the patients had progressive metastatic RCC or melanoma (five skin melanoma and two uveal melanoma). The five patients with skin melanoma were chemotherapy (DTIC)-resistant. Three patients with RCC still had their primary tumor in situ. Details of the patients are given in Table 1. Performance score was 0 or 1, except in one melanoma patient (patient 2). All of the patients received at least one cycle of immunotherapy and were evaluable for toxicity and response. Mean number of cycles was 3.0 (range, 1–6).

Clinical Results

Toxicities. As shown in Table 2, only 1 of 3 patients at dose level 1 developed grade 3 fever. At dose level 2, 3 of 3 had grade 3 fever and chills, but no other grade 3 toxicity except fatigue and malaise with a decrease in performance status to WHO grade 3. At dose levels 3 and 4, all of the patients developed grade 4 fever with chills and hypotension the first day, which could be corrected by i.v. fluids within 12 h. In all of the patients the dose of GM was reduced to 2.5 mg/kg (level 3) and the dose of IL-2 to 4 MIU/m² (level 4). A further dose reduction of IL-2 was necessary in three patients (in one patient, to 2 MIU/m²; in two, to 1 MIU/m²) to maintain systolic blood pressure at ≥100 mm Hg without i.v. fluids (see Table 1). These results defined dose level 2 as the MTD. Six additional patients were entered at this dose level to clearly establish the MTD. In 1 of 6 patients, dose reduction of IL-2 to 2 MIU/m² was necessary because of grade 4 fever with hypotension. In two other patients, a similar dose reduction of IL-2 was necessary because of fluid retention that was not responsive to diuretics. Thus, in 3 of 9 patients starting at the MTD, dose reduction of IL-2 to 2 MIU/m² was necessary. No further toxicity requiring dose reduction was seen during subsequent cycles at home. Dose-limiting toxicity was mainly grade 4 fever with chills and hypotension on the first day and fluid retention or severe malaise/fatigue with anorexia after 7–10 days. One patient (patient 7) had angioedema at the start of the second cycle, which was relieved rapidly by clemastine. A rechallenge with IL-2 and IFNα did not produce similar complaints, identify-
Phase I Trial of s.c. GM-CSF, Low-Dose IL-2, and IFN-α

<table>
<thead>
<tr>
<th>Immune activation before and after combined immunotherapy</th>
<th>Before treatment</th>
<th>At day 12</th>
<th>P (t test)</th>
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<tr>
<td>Lymphocytes</td>
<td>1,344 ± 142a</td>
<td>2,852 ± 309a</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD3</td>
<td>957 ± 109</td>
<td>1,946 ± 239</td>
<td>0.002</td>
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<tr>
<td>CD3/DR</td>
<td>221 ± 39</td>
<td>885 ± 111</td>
<td>&lt;0.001</td>
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<tr>
<td>CD4/DR</td>
<td>93 ± 18</td>
<td>467 ± 56</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD8/DR</td>
<td>56 ± 12</td>
<td>263 ± 68</td>
<td>0.008</td>
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<tr>
<td>NK cells</td>
<td>214 ± 49</td>
<td>662 ± 102</td>
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<tr>
<td>Monocytes</td>
<td>665 ± 83</td>
<td>802 ± 106</td>
<td>n.s.</td>
</tr>
<tr>
<td>CD14/DRa</td>
<td>774 ± 61</td>
<td>1,230 ± 144</td>
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<td>Eosinophils</td>
<td>243 ± 109</td>
<td>2,881 ± 655</td>
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<td>Neutrophils</td>
<td>4,906 ± 368</td>
<td>19,606 ± 1,677</td>
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<td>sIL-2R</td>
<td>148/590</td>
<td>233/515</td>
<td>790/11,669</td>
</tr>
<tr>
<td>sCD8</td>
<td>12/26</td>
<td>515/68</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

a Absolute number of cells × 10⁶/liter.

Effect of IL-2 Dose on Immunological Parameters

Because the IFNα dose was kept constant and the GM-CSF dose had to be reduced immediately from 5 µg/kg to 2.5 µg/kg after the first day at dose level 3, the effect of the different IL-2 doses could be studied; 1 MIU/m² was used as the final dose in five patients, 2 MIU/m² in four patients, and 4 MIU/m² in nine patients (Tables 1 and 4).

The increase in the number of NK cells and sCD8 seemed proportional to the IL-2 dose. Patients receiving 4 MIU IL-2/m² reached significantly higher sCD8 and NK values than those receiving 1 MIU/m² (P = 0.02).

DISCUSSION

Toxicity. In this Phase I trial of combined immunotherapy the MTD was established at 2.5 µg/kg GM-CSF, 4 MIU/m² IL-2, and 5 MU IFNα given s.c. for 12 days. Dose-limiting toxicity was grade 4 fever with hypotension requiring i.v. fluids and grade 3 fluid retention with weight gain and severe fatigue and malaise. Most IL-2-induced toxicities seem to be dose-related, with the highest doses frequently requiring intensive-care-unit support (17–21). s.c. IL-2 gives lower peak levels and less toxicity, even with the same doses (21–25). Low-dose IL-2 s.c. can induce T cell activation without NK cell activation because activated T cells have a high affinity for IL-2R in contrast to NK cells, which have an IL-2R of intermediate affinity (26–28).
There has been only one report on the combination of GM-CSF s.c. and IL-2 i.v. (15), in which the MTD was 2.5 μg/kg GM-CSF and 4.5 MIU/m² IL-2. Dose-limiting toxicity in that study of concurrent GM-CSF and IL-2 were primarily neurological and cardiovascular, apart from fever. In our study, we also observed one patient with a fatal CVA, which is a serious concern. Because of the data, we stopped GM-CSF when leukocytes rose above 30/nl; this was seldom necessary, perhaps because we combined it with IFNα. It is not clear whether a high number of leukocytes, especially eosinophils, is responsible for CVAs. In an ongoing Phase II studies in RCC (30 patients) and melanoma (40 patients), no CVAs have been observed. Most of the toxicities associated with s.c. administration of GM-CSF, low-dose IL-2, and IFNα seem to be manageable. Treatment could be continued at home after the first days in hospital. The combination of GM-CSF and low-dose IL-2 seemed powerful in T cell activation and the induction of T-LAK without NK-LAK, which may explain the limited toxicity due to vascular leak syndrome (13, 20).

**Immunological Effects.** An important aspect of this study was in monitoring the response to document which effector cells were expanded and activated. A significant increase in circulating (activated) T cells (both CD4 and CD8), NK cells, neutrophils, and eosinophils were found after 12 days of treatment. Circulating monocyte numbers did not increase, but we did find an increase of monocyte DR expression, probably due to GM-CSF. We assume that DR expression is increased as well on dendritic cells, because GM-CSF promotes differentiation of DC (29). The increases in activated CD3 T cells (both CD4+ and CD8+) and NK cells are probably relevant for the antitumor effect, but differences between patients and the correlation with response should be studied in a larger series. The role of eosinophils, which are clearly produced and activated by GM-CSF and IL-2, is unclear, but they are often found as infiltrating cells in tumor sites. In the study with GM-CSF and IL-2, eosinophilia seemed to correlate with response (15). Eosinophils may be important in view of the production of IL-12 and histamine. They can produce similar amounts of IL-12 as macrophages (30), which can skew T cell differentiation to the TH-1 and TC-1 direction, thought to be important in view of the production of IL-12 and CSF and IL-2, is unclear, but they are often found as infiltrating and CD8+ DC (29). The increases in activated CD3 T cells (both CD4+ and CD8+) and NK cells were expanded and activated. A significant increase in circulating monocyte numbers did not increase, but we did find an increase of monocyte DR expression, probably due to GM-CSF. We assume that DR expression is increased as well on dendritic cells, because GM-CSF promotes differentiation of DC (29). The increases in activated CD3 T cells (both CD4+ and CD8+) and NK cells are probably relevant for the antitumor effect, but differences between patients and the correlation with response should be studied in a larger series. The role of eosinophils, which are clearly produced and activated by GM-CSF and IL-2, is unclear, but they are often found as infiltrating cells in tumor sites. In the study with GM-CSF and IL-2, eosinophilia seemed to correlate with response (15). Eosinophils may be important in view of the production of IL-12 and histamine. They can produce similar amounts of IL-12 as macrophages (30), which can skew T cell differentiation to the TH-1 and TC-1 direction, thought to be important for antitumor response (31). Histamine has been shown to counteract the depression of cytotoxicity of NK cells and T cells due to O2 radicals and H2O2 production by local macrophages (32), sIL-2R and sCD8 both increased significantly during combined immunotherapy and probably reflect not only activation of CD4-T and CD8-T in the circulation, but also in LNs and tumor. Similar increases in sIL-2R and sCD8 have been described in patients with malignant lymphoma treated with GM-CSF alone after intensive chemotherapy (33).

**Relationship of Immunological Effects and IL-2 Dose.** IFNα was kept constant; GM-CSF was doubled in dose level 3 (with 4 MIU/m² IL-2) but had to be reduced immediately from 5 μg/kg to 2.5 μg/kg because of grade 4 fever with hypotension. Therefore, the effect of the IL-2 dose could be studied in patients receiving 1 MIU/m² (n = 5; in three patients as starting dose, in two patients after reduction because of grade 4 fever with hypotension), 2 MIU/m² (n = 4; patients started with 8 or 4 MIU/m² but reduced to 2 MIU/m²), and 4 MIU/m² (n = 9; starting with that dose or reduced from 8 MIU/m²). Combined immunotherapy with 1 MIU/m² did induce T cell activation (CD3/DR and sIL-2R increases) but it remained restricted to CD4+T. Neither CD8+T cells nor NK cells were significantly expanded or activated. The higher dose of IL-2 (2 or 4 MIU/m²) resulted not only in CD4+T, but also in CD8+T and NK cell expansion and activation. A larger series and longer follow-up is necessary to determine whether there are significant differences between 2 and 4 MIU/m² IL-2. Prolonged administration of low-dose IL-2 can indeed induce an increase in NK cell number and cytotoxic activity (34).

Remarkably, three of eight patients with progressive metastatic RCC after nephrectomy achieved CR. Efficacy is now being tested in Phase II trials in progressive metastatic RCC and in therapy-naive metastatic melanoma patients (in the last category combined with chemotherapy). It seems feasible to give combined immunotherapy with these cytokines at home after hospitalization during the first days to establish the tolerated dose of IL-2.

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