Expansion and Manipulation of Natural Killer Cells in Patients with Metastatic Cancer by Low-Dose Continuous Infusion and Intermittent Bolus Administration of Interleukin 2

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ABSTRACT

Interleukin 2 (IL-2) administered at low doses for prolonged periods can markedly expand the number of CD56+ natural killer (NK) cells in patients with metastatic cancer. The cytotoxic capacity of NK cells obtained from patients receiving IL-2 in vivo can be dramatically augmented by additional exposure to IL-2 in vitro. These observations formed the basis of a clinical trial in which patients with metastatic cancer were treated with low-dose continuous daily infusions of IL-2 to increase the number of their NK cells in conjunction with intermittent boluses of additional IL-2 to stimulate this expanded pool of cytotoxic cells. Twenty-three patients were registered to receive IL-2 at 4.5 × 10^5 units/m^2/day for 8 weeks by continuous i.v. infusion. After 4 weeks of "priming" with low-dose continuous infusion IL-2, cohorts of three to five patients received 5 weekly 2-h boluses of IL-2 at doses ranging from 2.5 × 10^5 units/m^2 to 1.0 × 10^6 units/m^2. Low-dose continuous infusion IL-2 was usually well tolerated; 2-h bolus infusions of IL-2 were often associated with high fevers and constitutional symptoms that resolved after several hours. Low-dose continuous infusion IL-2 resulted in the progressive expansion of circulating CD56+CD3- NK cells. In contrast, each bolus infusion of IL-2 resulted in an immediate dramatic decrease in both the number of NK cells and activated T lymphocytes with recovery noted within 24 h. Bolus doses of IL-2 as low as 2.5 × 10^5 units/m^2 were capable of producing these effects. Cytolytic activity against NK-sensitive and -resistant targets correlated with the presence of circulating activated NK cells. Our results demonstrate that NK cells expanded by low-dose continuous infusions of IL-2 can be further activated in vivo by exposure to very low doses of IL-2 as a 2-h i.v. bolus. This capacity to manipulate human NK cells in vivo through varying the dose and schedule of IL-2 administration may help in defining the therapeutic potential of these cytotoxic effectors in the treatment of both neoplastic and infectious diseases.

INTRODUCTION

The identification, characterization, and biosynthesis of immunoregulatory proteins capable of stimulating cytolytic activity of PBMCs has fueled considerable interest in applying these cytokines to the treatment of patients with malignant disease. The most widely studied of these products has been interleukin-2 (IL-2) (1-5). The relationship between the immunomodulatory effects of IL-2 therapy, its antitumor effects, and its deleterious side effects remains incompletely understood. Presumably, IL-2 acts by stimulating certain cellular elements, such as cytotoxic T lymphocytes or NK cells to kill tumor cells, either by direct cell-cell contact or via the elaboration of secondary cytokines, such as tumor necrosis factor, γ-IFN, or IL-1 (6, 7). The relative contributions of NK cells and T lymphocytes to either the beneficial or toxic effects of IL-2 therapy have yet to be completely determined. In an attempt to partially address this question, we conducted a clinical trial in which we administered very low doses of IL-2 to patients with metastatic cancer for prolonged periods (8). The trial was designed to determine the clinical and immunological consequences of administering IL-2 at doses sufficiently low as to result in the preferential stimulation of NK cells without affecting T cells. This approach was based on the observation that purified populations of NK cells and T lymphocytes obtained from the peripheral blood of normal volunteers respond differently in vitro when exposed to low concentrations of IL-2 (9). Specifically, DNA synthesis and cellular proliferation increase dramatically in NK cells when they are incubated at concentrations of IL-2 several logs lower than those required to stimulate T cell proliferation.

In our initial clinical studies, we found that very low doses of IL-2 (1.5-4.5 × 10^5 units/m^2/day) could be delivered safely to patients by continuous infusion for an extended period (up to 90 days). Moreover, these doses of IL-2, which were <5% of the daily dose delivered in conventional IL-2 treatment protocols, resulted in a 10-20-fold expansion of the number of NK cells found in the peripheral blood of patients with advanced metastatic cancer. In contrast, there was essentially no change in the number of T lymphocytes in these patients. Although the cytolytic activity of these expanded NK cells increased when tested in vitro against NK-sensitive targets (e.g., K562), activity against NK-resistant targets (e.g., Daudi, COLO 205) increased only a small degree. However, when cells from patients who...
were receiving IL-2 in vivo were exposed to additional IL-2 in vitro, killing increased dramatically, much more so than it did in cells obtained from these same patients prior to initiating IL-2 therapy (10, 11).

Based on these observations, we designed a new clinical study using weekly boluses of escalating doses of IL-2 to activate a primed NK cell population already expanded in vivo with low-dose continuous infusion IL-2. We find that this approach is clinically tolerable and provides a basis for selectively manipulating NK cells in vivo for immunotherapeutic purposes.

PATIENTS AND METHODS

Patient Characteristics. Patients were eligible for entry into this study if they had evidence of metastatic cancer demonstrable either by physical examination or radiographical imaging study. Eligibility criteria also included age >18 years, Eastern Cooperative Oncology Group performance status 0–2, normal or near-normal parameters of renal, hepatic, and pulmonary function, and life expectancy exceeding 3 months. Twenty-three patients were registered. There were 18 men and 5 women. Their median age was 55 (range, 23–69) years. The majority of patients had either renal cell carcinoma (n = 10) or melanoma (n = 6). Other diagnoses included lung cancer (n = 2), colon cancer (n = 2), sarcoma, nonseminomatous germ cell tumor, and chronic myelogenous leukemia (n = 1).

IL-2 Treatment. The schema outlining the planned treatment protocol is depicted in Fig. 1. Patients received recombinant IL-2 (Hoffmann LaRoche, Nutley, NJ) by continuous i.v. infusion through an indwelling central catheter for 8 consecutive weeks at a dose of 4.5 × 10⁵ units/m²/day. Drug was delivered by a portable computerized ambulatory pump (Pharmacia/Deleec Model 5100 HF; Pharmacia/Delecc, St. Paul, MN). The supply of IL-2 was renewed every 7 days by the outpatient pharmacist. After 4 weeks of treatment had elapsed, patients were given weekly i.v. boluses of additional IL-2 through the end of the eighth week. Boluses were administered over a 2-h period in the outpatient clinic. The bolus dose was initially only 2.5 × 10⁵ units/m² (level 1) but was escalated in cohorts of three to five patients. Escalation continued until dose-limiting toxicity was encountered. Because we intended to maintain an outpatient program, we considered dose-limiting toxicity to include any complication directly related to IL-2 administration which required hospitalization. Patients were not routinely treated with any prophylactic antipyretic or anti-inflammatory agents while receiving continuous infusion IL-2. However, they were given acetaminophen and antihistamines prior to the administration of IL-2 boluses. Tumor responses were evaluated after the 8-week treatment program. A partial response was defined by a 50% reduction in the size of presumed tumor masses as measured in both the horizontal and vertical dimensions. A complete response was defined by total regression at all tumor sites.

Immunophenotypic Studies. PBMCs for immunological studies were obtained weekly. During weeks that patients received IL-2 boluses, samples were obtained before the bolus, immediately after completion (0 h), 3 h after completion, and 24 h after completion. Blood was collected in preservative-free heparin. PBMCs were obtained following Ficoll-Hypaque density gradient sedimentation. PBMCs were analyzed by direct immunofluorescence for reactivity with a series of monoclonal antibodies using standard techniques. Cells were analyzed for reactivity with a panel of monoclonal antibodies, including T3 (CD3), T4 (CD4), T8 (CD8), NKH1 (CD56), Tac (CD25), Mo1 (CD11b), and Ia (DR) (Coulter Immunology). Single- and dual-color immunofluorescence reactivity were determined by automated flow cytometry analyzing 10⁵ cells in each sample (ELITE; Coulter Electronics).

Cytotoxicity Assays. Cryopreserved PBMCs were thawed and evaluated for their ability to lyse NK-sensitive (K562) and NK-resistant (COLO 205) tumor cell targets. After thawing, effector cells were incubated for 18 h in media alone (RPMI 1640 with 10% heat-inactivated human AB serum, 2% glutamine, 1% penicillin-streptomycin, and 1% sodium pyruvate) or media enriched with IL-2 (500 units ml, 3 nm; Amgen, Inc., Thousand Oaks, CA). Four-h chromium release assays against K562 and COLO 205 were performed at E:T ratios of 40:1, 20:1, and 10:1 as previously described (11). Assays were performed on cells prior to beginning IL-2 therapy, while receiving continuous infusion IL-2 prior to IL-2 bolus, immediately after IL-2 bolus, and 24 h after the IL-2 bolus. Samples from patients were run simultaneously for comparative purposes.

RESULTS

Toxicity of IL-2 Treatment. Continuous i.v. administration of low dose (4.5 × 10⁵ units/m²/day) IL-2 was well tolerated by the majority of patients in the study. In 3 of 23 patients, the dose of continuous infusion IL-2 was reduced to 3.0 × 10⁵ units/m²/day because of fever and constitutional symptoms significant enough to make outpatient administration difficult to tolerate. Eighteen of 23 patients went on to receive bolus infusions of IL-2. Of the five patients who were withdrawn early, four (two melanoma and two renal cell carcinoma) had rapidly progressive metastatic disease over a 3-week period and one chose to discontinue for personal reasons.

Boluses of IL-2 were administered to 18 patients after they had completed 4 weeks of continuous infusion therapy and were repeated weekly until the end of the eighth week of continuous IL-2 treatment. Side effects were far more commonly observed in association with bolus infusion than during continuous administration (Table 1). All patients experienced fevers (>38.5°C) and rigors within 1 h after the 2-h bolus infusion was

Fig. 1 Low-dose IL-2 treatment schedule. Schematic representation of the 8-week treatment program with low-dose continuous and bolus infusions of IL-2. Bolus dose was 0.25–1.0 × 10⁶ units/m²/day.

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Table I  Toxicities of low-dose IL-2 by continuous and bolus infusion

<table>
<thead>
<tr>
<th>Toxicity</th>
<th>Continuous Infusion ( (n=23) )</th>
<th>Bolus Infusion ( (n=18) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever &gt;38°C</td>
<td>12 (52%)</td>
<td>18 (100%)</td>
</tr>
<tr>
<td>Fatigue</td>
<td>15 (65%)</td>
<td>18 (100%)</td>
</tr>
<tr>
<td>Anorexia</td>
<td>7 (30%)</td>
<td>16 (89%)</td>
</tr>
<tr>
<td>Rigors</td>
<td>2 (9%)</td>
<td>14 (78%)</td>
</tr>
<tr>
<td>Nausea/vomiting</td>
<td>2 (9%)</td>
<td>8 (44%)</td>
</tr>
<tr>
<td>Dyspnea</td>
<td>3 (13%)</td>
<td>4 (22%)</td>
</tr>
<tr>
<td>Rash</td>
<td>3 (13%)</td>
<td>3 (17%)</td>
</tr>
<tr>
<td>Hypotension</td>
<td>0 (0%)</td>
<td>2 (11%)</td>
</tr>
<tr>
<td>Arrhythmias</td>
<td>1 (4%)</td>
<td>1 (6%)</td>
</tr>
<tr>
<td>Abnormal LFTs</td>
<td>3 (13%)</td>
<td>1 (6%)</td>
</tr>
<tr>
<td>Azotemia</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

\( ^a \) 4.5 \times 10^6 \text{ units/m}^2/\text{day}.

\( ^b \) 2.5–10.0 \times 10^5 \text{ units/m}^2/\text{dose}.

\( ^c \) Requiring vasopressors, dose, 1.0 \times 10^6 \text{ units/m}^2.

completed. Nausea, vomiting, and diarrhea were occasionally observed, more commonly at higher bolus dose levels. Hypotension requiring the administration of vasopressor support and necessitating 24-h hospital admission occurred in two patients receiving a bolus dose of 1.0 \times 10^6 \text{ units/m}^2. This toxicity defined the maximum tolerated outpatient bolus dose in our schema of IL-2 administration. Bolus doses were not escalated above 1.0 \times 10^6 \text{ units/m}^2. Virtually all side effects subsided within 12 h of bolus administration. Side effects occurred with equal intensity through all five planned bolus treatments. During the weekly intervals between boluses, patients tolerated the continuous daily administration of IL-2 as easily as they had during the first 4 weeks of the treatment program.

Twelve of 18 patients received all five planned IL-2 bolus infusions. Two patients were withdrawn because of disease progression, one developed a hypersensitivity reaction manifested primarily by severe urticaria, and three developed sepsis related to indwelling catheters. Overall, bacterial infections were documented in 6 of the 23 patients. One patient developed streptococcal cellulitis at the site of a prior melanoma excision. Five patients had documented bacteremia (two polymicrobial) suspected to be secondary to their catheters. Bacterial isolates included both Gram-positive (\(n=4\)) and Gram-negative (\(n=3\)) species.

Of the 18 patients who received IL-2 boluses as part of this protocol, there were no partial or complete responses. Nine patients had stable disease, and nine showed evidence of progression within 1 month of completing treatment. Two patients with stable disease were retreated for another 8 weeks, but did not achieve a complete or partial response.

**Hematological Effects of IL-2 Treatment.** Low-dose continuous infusion IL-2 resulted in increased WBC counts in the majority of patients. The change in WBC was attributable to increments in the number of circulating lymphocytes, monocytes, and eosinophils. Essentially no change was observed in granulocyte number. Platelet counts either remained unchanged or decreased slightly (<25%) during therapy. In no cases did patients require platelet transfusion.

In contrast, when IL-2 was administered by 2-h bolus infusion to patients who had been receiving it by continuous infusion for 4 weeks, a dramatic and immediate reduction in WBC was observed. The average number of lymphocytes detected in the circulation of patients after administration of the IL-2 bolus was reduced by 75% compared with the number immediately prior to the bolus (Fig. 2). A greater than 50% reduction in the numbers of monocytes and eosinophils was also observed immediately after the completion of the 2-h bolus whereas the change in the number of neutrophils was far less

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**Fig. 2** Effect of continuous infusion/bolus IL-2 on circulating lymphocytes. The mean absolute lymphocyte count over the 8-week course of the study is displayed. SE was <10% of the mean for each data point.

**Fig. 3** Effect of IL-2 bolus on circulating leukocytes. The mean absolute neutrophil, lymphocyte, eosinophil, and monocyte counts during the 24 h following a 2-h IL-2 bolus in patients primed with low-dose continuous infusion IL-2 is displayed.
Effects of IL-2 on NK Cells

The dose of the IL-2 bolus did not appear to exert a major influence on the extent of lymphocytopenia. As bolus doses increased from 0.25 to 1.0 $\times$ $10^6$ units/m$^2$, the relative degree of lymphocytopenia observed at 0 and 3 h after bolus infusion did not significantly change. However, at the higher IL-2 bolus doses, lymphocyte recovery did not appear to be fully complete by 24 h (Fig. 4). Similar patterns were observed for monocytes and eosinophils.

Effect of Treatment on Lymphoid Subsets. We have previously reported that low doses of IL-2 can selectively expand the number of circulating NK cells in patients with malignant disease (10). This phenomenon was again observed in the current trial. After only 5–6 weeks of continuous i.v. treatment with IL-2 at 4.5 $\times$ $10^6$ units/m$^2$/day, the percentage of CD56$^+$ NK cells in peripheral blood of our patients increased from an average of 12.2 $\pm$ 2.4% to 51.5 $\pm$ 5.6%. Overall, the total number of circulating NK cells increased approximately 10-fold. These NK cells were predominantly CD56$^+$CD3$^-$CD2$^+$CD16$^+$. The majority also expressed CD8 on their surface. Although there was a relative decrease in the fraction of circulating T cells in the peripheral blood of these patients, no significant change in the absolute number of T cells was noted, owing to the increase in the absolute lymphocyte count.

The effect of a 2-h bolus of IL-2 on the lymphoid composition of peripheral blood was quite different from that observed during continuous i.v. administration. Immediately after the IL-2 bolus was completed, there was a dramatic reduction in the number of all lymphoid subsets, but most notably NK cells. Whereas the majority of cells from patients receiving continuous infusion IL-2 at 4.5 $\times$ $10^6$ units/m$^2$/day had been CD56$^+$ NK cells, most of the cells remaining in the circulation immediately after the bolus infusion were T lymphocytes, specifically CD4$^+$ cells (Fig. 5). NK cells recovered significantly after 3 h and returned to prebolus levels within 24 h. This effect was observed with each successive IL-2 bolus and at all bolus doses. As little as 2.5 $\times$ $10^5$ units/m$^2$ of IL-2 reduced the average number of NK cells from 1693 $\pm$ 204 $\times$ $10^6$ NK cells/liter to 68 $\pm$ 13 $\times$ $10^6$ NK cells/liter (96% decrease).

Although the absolute number of circulating T cells also was decreased after bolus IL-2 administration, the immediate decrease was far less profound, dropping from 1333 $\pm$ 124 $\times$ $10^6$ T cells/liter to 598 $\pm$ 46 $\times$ $10^6$ T cells/liter (55% decrease). There did appear to be a preferential loss of circulating T cells that expressed activation antigens such as CD54 (ICAM-1). Within our cohorts of patients, the number of CD54$^+$ T cells decreased from a mean of 245 $\pm$ 31 cells/liter to 38 $\pm$ 10 cells/liter (85% decrease) after bolus IL-2 infusion. There was also a marked drop in the number of T cells expressing the IL-2 receptor $\beta$ chain (163 $\pm$ 17 cells/liter to 13 $\pm$ 3 cells/liter, 92% decrease). In contrast, T cells expressing CD25 (the IL-2 receptor $\alpha$ chain) were far less affected by the IL-2 boluses, decreasing from an average of 272 $\pm$ 39 cells/liter to 135 $\pm$ 28 cells/liter (50% decrease).

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**Fig. 4** IL-2 bolus dose and lymphocyte recovery. IL-2 was administered as a 2-h bolus at doses between 0.25 and 1.0 $\times$ $10^6$ units/m$^2$. All doses produced transient lymphopenia. Higher doses were associated with slower lymphocyte recovery.

**Fig. 5** Effect of IL-2 bolus infusion on lymphoid subsets. The effect of a 2-h IL-2 bolus on the fraction of PBMCs that are CD3$^+$ T cells and the percentage that are CD56$^+$ NK cells is depicted in a representative patient. Prior to the bolus, the predominant cells were CD56$^+$ NK cells. Immediately after the bolus, CD4$^+$ T cells predominated. Within 3 h NK cells were again recoverable from the peripheral blood.
DISCUSSION

NK cells have been suggested to play an important role in host defenses against both malignant tumor growth and viral infection. In experimental models, NK cell number and activity have been found to correlate with resistance to the metastatic spread of tumor. In humans, diminished NK cell function has been associated with cancer progression of both solid and hematological malignancies. After allogeneic marrow transplantation, IL-2-activated NK cells can mediate MHC-nonrestricted killing of tumor cells obtained from patients prior to bone marrow transplantation. Such activity has been correlated with subsequent freedom from relapse post-after bone marrow transplantation. It is possible that exploitation of the cytotoxic activity of NK cells may be an effective way to approach the control of disease in patients with metastatic cancer.

Our current study demonstrates that NK cells which have been expanded in patients receiving low-dose continuous infusion IL-2 maintain their ability to respond to IL-2 boluses in vivo. Moreover, these primed NK cells are sensitive to bolus IL-2 at extremely low doses, doses which are easily tolerable in outpatient settings. The schedule of IL-2 administration that we have tested is one which allows large numbers of NK cells to be generated and subsequently activated in vivo. More than 1 × 10^10 NK cells can be manipulated in vivo in this fashion. Although it is not clear what happens to these expanded NK cells during bolus administration, a reasonable hypothesis might be that the bolus infusion of IL-2 results in the up-regulation of adhesion molecules followed by the margination and extravasation of activated NK cells from the circulation. Such a process might theoretically allow an expanded population of activated NK cells to exercise their cytolytic activity directly at sites of tumor metastases.

The responses of circulating NK cells to the 24-h continuous infusion and the 2-h bolus infusion of IL-2 are clearly different. Low doses of IL-2 given over 24 h resulted in NK cell expansion, whereas similarly low doses of IL-2 when administered over 2 h resulted in the temporary disappearance of these cells from the circulation, presumably as a consequence of NK cell activation. These distinct functional effects can be attributed to differences in the expression of IL-2 receptors on the NK cell surface. Low-dose continuous infusion IL-2 presumably exerts its effect by stimulating a small subset of NK cells that constitutively express the high-affinity form (αβγ) of the IL-2 receptor. Selective engagement of this high-affinity receptor appears to primarily signal NK cell proliferation. Although the dose of IL-2 administered by the 2-h bolus was essentially equivalent to the continuous infusion dose, bolus administration presumably produced serum IL-2 levels high enough to activate the intermediate affinity (βγ) IL-2 receptor on the surface of NK cells. Engagement of the intermediate affinity IL-2 receptor on NK cells can trigger NK cell activation and initiate the cytolytic cascade. By combining continuous infusion with intermittent bolus administration of IL-2, NK cells can thus be sequentially expanded and then activated in vivo.

Although symptoms arising during the continuous administration of IL-2 were minimal, significant toxicities were associated with the bolus infusion phase of this study. However, it is not clear whether NK cells were responsible for the observed side effects. In addition to affecting NK cells, bolus IL-2 infusion also produced marked decreases in the number of monocytes, eosinophils, neutrophils, and activated T cells in patients in the study. It is likely that these cells, which also express the intermediate affinity IL-2 receptor (βγ) on their surface, can be activated by transiently elevated levels of IL-2 present after a 2-h bolus.

The elaboration of secondary cytokines by NK cells during bolus administration, a reasonable hypothesis might be that the bolus infusion of IL-2 results in the up-regulation of adhesion molecules followed by the margination and extravasation of activated NK cells from the circulation. Such a process might theoretically allow an expanded population of activated NK cells to exercise their cytolytic activity directly at sites of tumor metastases.
cancer patients, they may prove useful in adjuvant settings when tumor burden is low. Indeed, recent experience at our institution suggests that low-dose IL-2 may decrease relapse rates and improve disease-free survival in patients undergoing T-cell-depleted allogeneic bone marrow transplantation for hematological malignancy (26). We have also recently initiated a trial in which we are administering the same continuous/bolus infusion schedule of low-dose IL-2 described herein to patients with acute myelogenous leukemia in first complete remission following intensification therapy with high-dose cytosine arabinoside (27). In addition to a potential role in limiting neoplastic disease, NK cell manipulation may also be valuable in new strategies to combat infectious agents, such as viral and mycobacterial organisms (17, 28).

As an alternative to using IL-2 to manipulate an expanded population of NK cells, other cytokines capable of stimulating NK cells expanded in vitro by continuous infusion IL-2 may also prove useful. For example, IL-12 can dramatically augment the in vitro cytolytic activity of NK cells obtained from patients receiving IL-2 (29, 30). Phase 1 clinical studies of IL-12 are now underway in humans. By evaluating the efficacy of these and similar clinical models, we may be able to design effective strategies employing NK cells in the control of malignant and infectious disease.

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