Daily Subcutaneous Injection of Low-Dose Interleukin 2 Expands Natural Killer Cells in Vivo without Significant Toxicity


ABSTRACT

We aimed to determine the toxicity and immunological effects of daily s.c. administered low-dose interleukin (IL)-2. Adult cancer patients received a single daily s.c. injection of IL-2 as outpatients for 90 consecutive days. Cohorts of four to nine patients were treated at escalating IL-2 dose levels until the maximum tolerated dose (MTD) was defined. Peripheral blood mononuclear cell phenotyping, IL-2 serum levels, and the presence of anti-IL-2 antibodies were investigated. Thirty-eight patients were treated at seven IL-2 dose levels ranging from 0.4 to 1.75 million International Units (mIU)/m^2 daily. The MTD was 1.25 mIU/m^2 with constitutional side effects, vomiting, and hyperglycemia dose limiting. Severe toxicity did not occur at or below the MTD, although mild local skin reaction and mild constitutional side effects were common. Objective tumor regressions were not observed during this Phase 1 trial. Low-dose IL-2 resulted in natural killer (NK) cell (CD3^-CD56^+) expansion at all dose levels. This effect was dose dependent (P < 0.01), ranging from a 154 to 530% increase over baseline. Peak NK levels were achieved at 6–8 weeks and sustained through 12 weeks of therapy. As predicted by in vitro studies of IL-2 receptor structure-activity relationships, the subset of NK cells that constitutively express high-affinity IL-2 receptors (CD3^-CD56^bright+) showed more profound dose-dependent expansion, with increases ranging from 368 to 2763% (P = 0.015). NK expansion occurred at peak IL-2 levels <10 pg/mL (2.3 IU/ml). Three patients developed nonneutralizing anti-IL-2 antibodies. Thus, we concluded that selective expansion of NK cells may be achieved in vivo with daily s.c. injections of low-dose IL-2 with minimal toxicity.

INTRODUCTION

IL-2 is the first biological agent that acts through host immunomodulation to be approved by the U. S. Food and Drug Administration for the treatment of cancer. The initial clinical development of IL-2 involved high-dose i.v. therapy (~10–100 × 10^6 units/day) with substantial toxicity (1), and this has limited the widespread acceptance of IL-2 in clinical practice. The antitumor effect of IL-2 is mediated in part by activated NK cells (2). Recent descriptions of the IL-2 receptor complex, and the functional consequences of receptor-ligand interactions, have raised the possibility that more effective and less toxic methods of treatment with IL-2 are possible.

The IL-2 receptor complex has three known components (3). The α chain binds IL-2 with low affinity and does not by itself transmit an intracellular signal. The β and γ chains associate noncovalently to form an intermediate affinity IL-2 receptor. A high-affinity IL-2 receptor is formed as a heterotrimer of α, β, and γ receptor subunits. The intermediate- and high-affinity receptors transduce signal following ligand binding. NK cells, which comprise approximately 15% of PBMCs, are the only lymphocytes that constitutively express functional IL-2 receptors (3). Ten percent of NK cells constitutively express a high-affinity IL-2 receptor in addition to an intermediate-affinity IL-2 receptor.

The functional consequences of IL-2 binding on NK cells is dependent on the specific receptor complexes present (4). Activation of the high-affinity heterotrimeric receptor with μM concentrations of IL-2 provides a proliferative stimulus, without augmenting cytotoxicity. In contrast, nM concentrations of IL-2 that bind the intermediate-affinity β-γ IL-2 receptor complex result in augmented effector cell cytotoxicity, with little effect on proliferation. These functional results of IL-2 binding to its receptors are time dependent as well, with prolonged stimulation producing more pronounced effects (3).

A continuous i.v. infusion of low-dose IL-2 (0.5–6.0 × 10^5 units/m^2/day) that selectively binds high-affinity receptors results in expansion of NK cells with minimal systemic toxicity, but with the inconvenience, infection risk, and expense associated with permanent central venous catheters and ambulatory pump apparatus (5–7). The present study was undertaken in an attempt to reproduce the immunomodulatory effects of protracted i.v. infusions of IL-2, with outpatient daily low-dose s.c. IL-2 administration.

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The abbreviations used are: IL, interleukin; NK, natural killer; PBMC, peripheral blood mononuclear cell; MTD, maximum tolerated dose; LAK, lymphokine-activated killer; ADCC, antibody-dependent cellular cytotoxicity.
PATIENTS AND METHODS

Patient Eligibility

Subjects were at least 18 years old, with histologically confirmed malignancy. They were ambulatory at least 50% of the time, and had a life expectancy of at least 3 months. Previous cancer treatment was permitted at least 3 weeks prior to enrollment (6 weeks for nitrosoureas or mitomycin). Adequate hepatic (bilirubin <3.0 mg/dl), renal (creatinine <2.0 mg/dl), and hematological (neutrophils > 1,000/μl, hemoglobin ≥ 10 g/dl, platelets > 50,000/μl) function were required. Patients were excluded if they had uncontrolled infection, acute cardiac disease, were pregnant or lactating, or had central nervous system malignancy. This study was approved by the Roswell Park Cancer Institute Institutional Review Board, and written informed consent was obtained for all patients.

Treatment Schema

Subjects received a single daily s.c. injection of recombinant human IL-2 [Proleukin (aldesleukin), supplied by Chiron Corporation, Emeryville, CA; specific activity, 1.8 × 10^6 IU/mg protein] for 90 consecutive days as outpatients. Dose escalations were not permitted during this 90-day course. Self-administration was taught to all patients, with a standard injection site rotation schedule including deltoids, abdomen, thighs, and buttocks. Daily patient diaries were used to monitor toxicity and site of injection. A physical examination and interval history was performed at 2-week intervals. In addition, every 2 weeks a complete blood count with WBC differential; serum chemistries including creatinine, lactate dehydrogenase, alkaline phosphatase, hepatic transaminases, and bilirubin; and IL-2 pharmacokinetics and PBMC phenotyping were obtained. At baseline and at week 10, anti-IL-2 antibody titers were measured.

Cohorts of at least three patients were enrolled at escalating IL-2 dose levels. Toxicity was graded according to the NIH Common Toxicity Criteria (8). Constitutional toxicity was graded as follows: fever (°C)—grade 1, 37.1-38; grade 2, 38.1-40; grade 3, >40 for < 24 h; and grade 4, >40 for at least 24 h; chills—grade 1, mild; grade 2, moderate; grade 3, severe; and grade 4, intractable; and fatigue—grade 1, mild; grade 2, moderate; and grade 3, severe. For grade 3 or 4 toxicity, therapy was held until resolution, then restarted at a 33% IL-2 dose reduction. After grade 4 toxicity, patients were permitted to reenroll at a higher dose level 3 weeks after completion of their initial 90-day course. Toxicity data for the second dose level in these patients were not included in the definition of the MTD.

Laboratory Analyses

IL-2 Serum Levels. Every 2 weeks, serum was collected following s.c. injection of IL-2 at t = 0, 1, 4, and 6 h. Samples were stored at −70°C for batch analysis of the IL-2 concentration. IL-2 levels were determined using an ELISA (Advanced Magnetics, Cambridge, MA) per the manufacturer’s recommendations. A standard curve was generated with each ELISA and was linear between 10 and 100 pg IL-2.

Phenotypic Analysis. At baseline and every 2 weeks thereafter, 20 ml blood were collected in heparinized tubes immediately before IL-2 administration. RBCs were lysed, and fresh cells were prepared for phenotypic analysis as previously described (9). Monoclonal antibodies used included FITC-conjugated CD3 (Becton Dickinson, San Jose, CA) and phycoerythrin-conjugated CD56 (Coulter Immunology, Hialeah, FL). Samples were subsequently collected on a FACScan and analyzed using the Winlist software program (Verity Software House, Inc.). NK cells were identified as CD3−CD56+ using a lymphocyte gate. Cells showing high-density expression of CD56 (CD56high) were the NK subset with expression of high-affinity IL-2 receptors (10).

Cytotoxicity Assays. Frozen PBMCs that were obtained from patients before and during therapy with IL-2 were thawed in a 37°C water bath, washed twice, and resuspended in medium containing RPMI 1640 supplemented with 10% human AB serum (C-six Diagnostics, Mequon, WI), antibiotics, and anti-PPLO agent (GIBCO-BRL, Gaithersburg, MD) and plated for the cytotoxicity assay (10% human AB serum).

Target cell lines were COLO 205, a NK-resistant human colon adenocarcinoma cell line, and P815, a NK-resistant murine mastocytoma cell line (American Type Culture Collection, Rockville, MD). Both lines were grown in RPMI 1640 media supplemented with 10% heat-inactivated FCS (Sigma, St. Louis, MO), antibiotics, and anti-PPLO agent.

LAK and ADCC ⁵¹chromium release cytotoxicity assays were performed as previously described (11, 12). In both assays, 4 × 10⁴ patient PBMCs were placed into 96-well V-bottomed plates (Costar, Cambridge, MA) in 200 μl 10% HAB and 1 nm (230 IU/ml) IL-2 (specific activity, 1.53 × 10⁷ U/mg; Hoffmann LaRoche, Nutley, NJ) and incubated for 18 h at 37°C. For LAK assays, 4 × 10⁵ ⁵¹Cr-labeled COLO 205 cell targets were added to each well. Likewise, 4 × 10⁵ ⁵¹Cr-labeled P815 cell targets were added to each well for ADCC assays. For ADCC assays, P815 cells were preincubated with a 1:100 dilution of polyclonal rabbit antimouse lymphocyte serum (Accurate Chemical and Scientific Corp., Westbury, NY). An E:T ratio of 10 was used in all assays.

Anti-IL-2 Antibody Detection. The development of anti-IL-2 antibodies was measured at baseline and at week 10 using an ELISA. In brief, microtiter plates coated with Proleukin IL-2 were washed and loaded with either control samples of human IgG or IgM anti-IL-2, or serial dilutions of patient plasma. The reference samples were loaded at concentrations previously determined to yield a target absorbance of approximately 0.3. After incubation for 2 h at 21°C, plates were
washed, and horseradish peroxidase-conjugated goat antihuman IgG or IgM was added, with repeat incubation for 2 h at 21°C. After washing, ABTS (2,2'-azino-bis-[3-ethylbenz-thiazoline sulfonic acid]) substrate was added, plates were incubated for an additional 25 min, and absorbancies read at 405 nm.

A linear regression fit, log_{10} (absorbance) versus log_{10} (dilution), of each sample dilution series was performed for dilutions with absorbancies between 0.090 and 0.900. Using the linear regression equation, the dilution required to reach the target absorbance was calculated for each sample. The following conversion calculation was performed to standardize results: 1 titer unit = (dilution to reach target × 0.3) – 3.0. Binding studies (data not shown) indicate that 1 titer unit/μl can bind 3 ng Proleukin. Therefore, anti-IL-2 titers <1000 are not likely to have a clinical effect (13).

In samples with detectable anti-IL-2 antibodies, neutralizing activity was assessed in an HT-2 bioassay. A Proleukin IL-2 spike (50 IU/ml) was prepared in assay medium (RPMI 1640 containing 10% heat-inactivated FCS, 50 μM β-mercaptoethanol, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin). Prediluted patient serum and control samples were then heat treated at 56°C. Fifty μl patient and control samples were serially diluted in 50 μl assay medium in a flat-bottomed 96-well microtitre plate. Twenty μl spike IL-2 were added to each well, the plates were shaken for 1 min on a microplate shaker, wrapped in plastic, and incubated for 15 to 24 h at 2–8°C. Fifty μl of serially diluted samples were then transferred to a separate assay plate containing a dilution series of IL-2 standard and the spike IL-2. The HT-2 subclone ASE was transferred to a separate assay plate containing a dilution series of IL-2 and the spike IL-2. The HT-2 IL-2 dose levels, ranging from 0.4 to 1.75 mIU/m2/day. Five to nine subjects were treated at each dose level. Six patients were treated at two dose levels. Only the first course is included in the toxicity analysis.

**RESULTS**

Thirty-eight patients were treated in this study. The median age was 60 (range, 27–75) years. The cancer diagnoses were colorectal carcinoma (18 patients), soft tissue sarcomas (9 patients), carcinoma of the lung (3 patients), melanoma (4 patients), acute myeloid leukemia in remission (2 patients), and non-Hodgkin’s lymphoma and squamous cell carcinoma of unknown primary (1 patient each). Patient cohorts were enrolled at seven IL-2 dose levels, ranging from 0.4 to 1.75 mIU/m2/day. Five to nine subjects were treated at each dose level. Six patients were treated at two dose levels. Only the first course is included in the toxicity analysis.

**Toxicity.** Although constitutional side effects such as fever, chills, and fatigue were common at all dose levels, toxicity was generally mild. Grade 1–2 constitutional side effects occurred in 35 of 38 patients (92%) during their initial 90-day cycle of treatment, but no grade 3 or 4 constitutional side effects were observed. All 13 patients treated with at least 1.3 mIU/m2 IL-2 suffered constitutional side effects. Six patients were treated at a second dose level, and three of these second courses were discontinued for grade 3 constitutional toxicity (one at 1.25 mIU/m2 and two at 1.75 mIU/m2). Three other patients tolerated their second cycle with only minimal toxicity (one at 1.0 mIU/m2 and two at 1.75 mIU/m2).

Skin toxicity was present in 79% of the subjects and was not dose related. This local reaction was characterized by erythema, induration, and occasionally mild pain at IL-2 injection sites. Mild headache was reported in 6 of 38 patients, and mild sinus congestion was noted in 8 patients. One patient experienced recurrent palpitations that did not require specific intervention. Grade 3 vomiting occurred in one patient treated with 1.5 mIU/m2/day. Another patient with acute myeloid leukemia in remission developed grade 2 thrombocytopenia at 1.75 mIU/m2. A bone marrow examination showed a hypocellular marrow with decreased megakaryocytes and no evidence of recurrent leukemia. Grade 3 hyperglycemia developed in one patient treated with 1.5 mIU IL-2/m2 daily. This was controlled with insulin, and this patient was able to complete the study. There were no grade 4 or 5 toxicities observed during this study.

Reasons for study discontinuation or dose modification are outlined in Table 1. The most common reason for treatment discontinuation was constitutional symptoms, such as fever and fatigue. It is important to note that six subjects discontinued therapy for grade 2 constitutional symptoms that did not meet study criteria for dose modification, but were intolerable to patients. At IL-2 dose levels <1 mIU/m2/day, none of the 21 treated patients required dose modification or discontinuation for toxicity. At 1 mIU/m2/day, one of four patients did not tolerate the starting dose because of grade 2 fever. At the 1.25-mIU dose level, all patients treated for the first time tolerated the starting dose. Of three patients who tolerated a lower dose and were retreated at 1.25 mIU/m2, two had minimal toxicity and one had grade 3 constitutional side effects. The group of patients treated following the 1.25-mIU/m2 dose cohort
received a starting IL-2 dosage of 1.75 mIU/m² daily. However, three of the four patients at this level could not tolerate the dose because of constitutional symptoms. In addition, two patients who tolerated a lower dose of IL-2 experienced grade 3 constitutional toxicities when retreated at 1.75 mIU/m² IL-2 daily. A final dose level of 1.5 mIU/m² was added; however, this dosage also proved to be too toxic, with constitutional symptoms (two patients), vomiting (one patient), and hyperglycemia (one patient) accounting for dose intolerance in four of five patients. Therefore, the MTD was 1.25 mIU/m² daily. For patients discontinuing treatment because of constitutional toxicities, the mean duration (±SD) of treatment at the starting dose was 34 ± 27 days. For the three patients treated with 1.75 mIU/m², the mean duration of treatment was 18 days. In patients with measurable disease, no tumor responses were observed.

**Immunological Measures.** A representative flow cytometric analysis of a patient at baseline and after treatment with daily low-dose IL-2 is shown in Fig. 1. Table 2A gives the mean absolute number of NK cells at each IL-2 dose level with SEs calculated at baseline and at peak after treatment with IL-2. All patients who received treatment for at least 35 days were included. Thirty-three courses met this criteria, including two courses that were a patient’s second dose level. Daily s.c. administration of low-dose IL-2 resulted in NK cell expansion at all dose levels. The magnitude of the IL-2 response was dose dependent, with patients treated with at least 1 mIU/m² daily experiencing at least 3-fold increases in the NK cell number. This IL-2 dose response was statistically significant: there was a significant difference between the peak NK values among the six dose levels (P < 0.01). As shown in Fig. 2, patients with higher pretreatment NK cell levels tended to have higher peak levels, and thus a correction was made in the statistical analyses for baseline values (see “Patients and Methods”).

As predicted by in vitro studies of IL-2 receptor structure-activity relationships (4), treatment of patients with low-dose IL-2 had a more pronounced effect on the CD56<sup>bright</sup> NK cell subpopulation which constitutively expresses high-affinity IL-2 receptors, as compared to those NK cells expressing only intermediate affinity receptor, which require higher concentrations of IL-2 (Table 2B). At all but the lowest two dose levels, 1000-fold increases in CD56<sup>bright</sup> cells were observed. When the peak numbers of CD56<sup>bright</sup> cells for each of the six dose levels were compared using a one-way ANOVA, a significant dose-response effect was again observed (P = 0.015). The dose-response effects observed for both total NK cells and NK<sup>bright</sup> cells remained significant (P < 0.01 and P = 0.015, respectively) when the two patients that had received IL-2 at two dose levels were excluded from analysis. It should also be noted that the unique CD16 expression on CD56<sup>bright</sup> NK cells (i.e., CD16⁻ or CD16<sup>dimm</sup>) remains unchanged as this population expands (data not shown). Thus, the increase in CD56<sup>bright</sup> NK cell number is not secondary to increased surface density expression of CD56 on CD56<sup>dimm</sup> cells.

The time course of NK cell expansion is shown in Fig. 3. The mean NK cell number was calculated at 2-week intervals. Patients at all dose levels were included since the time course of NK expansion was not dose dependent. Increases in NK cell number were evident as early as 2 weeks, and reached a peak at 6–8 weeks that was maintained through week 12.

To determine whether expansion of NK cells was associated with an increase in functional activity, in vitro cytotoxicity assays were performed. Effector cytotoxicity at baseline and after daily IL-2 was compared using cryopreserved samples from five patients treated with a daily IL-2 dose of 1.25 mIU/m². Mononuclear cells in the patients tested showed (mean ± SE) 18.1 ± 2.4% cytotoxicity against COLO 205 cells (a NK-resistant, LAK-sensitive cell line) at baseline and 41.0 ± 3.4% cytotoxicity after daily IL-2 treatment (P < 0.0005, paired t test). In addition, patient effectors obtained after treatment with low-dose IL-2 showed increased killing of P815 targets (a NK-resistant, ADCC-sensitive cell line) that were preincubated with polyclonal antisera when compared with baseline samples (28.8 ± 2.5% versus 18.8 ± 3.2%, P < 0.005).

The effects of low-dose IL-2 on WBC counts are shown in Fig. 4. IL-2 produced a modest increase in total WBC counts (<30% at any dose level) but no effects on neutrophil or monocyte counts. Eosinophil counts increased during therapy, doubling even at the lowest IL-2 dose levels, and this was most pronounced at the higher doses tested, with 731 ± 292 and 689 ± 181% increases when patients were treated with 0.75–1.0 mIU/m² daily or 1.25–1.5 mIU/m² daily, respectively. T-cell counts were modestly elevated (Fig. 5), achieving a maximum increase at any one dose level of only 60% above baseline, and this effect was not dose dependent.

IL-2 serum levels were measured using an ELISA in 37 treatment courses. As shown in Fig. 6, peak IL-2 levels as measured with the first IL-2 dose showed great interpatient variability. At daily doses >1 mIU/m², 11 (73%) of 15 patients had peak concentrations in the 10–100 pm range, whereas 11 (50%) of 22 patients receiving lower doses of IL-2 had levels in this range. This peak was observed most commonly at 4–6 h. All assayed samples had detectable IL-2 levels.

The development of anti-IL-2 antibodies was measured in all patients at week 10 on study. One patient had a 79 titer IgG level at baseline, but no detectable antibodies at week 10. Three other patients who had negative titers at baseline developed antibodies against IL-2 during treatment (Table 3). Titers ranged from 78–177. Three patients had IgG antibodies and one patient had IgM antibodies. In a bioassay testing for neutralization of activity, all samples were negative. Three of four patients with anti-IL-2 antibodies showed at least a 2-fold NK cell expansion.

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### Table 1: Dose-limiting toxicities with daily s.c. IL-2

<table>
<thead>
<tr>
<th>IU/m²/day</th>
<th>Toxicity</th>
<th>No. affected&lt;sup&gt;a&lt;/sup&gt;/no. treated</th>
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<tr>
<td>&lt;1,000,000</td>
<td>None</td>
<td>0/21</td>
</tr>
<tr>
<td>1,000,000</td>
<td>Constitutional</td>
<td>1/4</td>
</tr>
<tr>
<td>1,250,000</td>
<td>Constitutional</td>
<td>0/4</td>
</tr>
<tr>
<td>1,500,000</td>
<td>Constitutional</td>
<td>2/5</td>
</tr>
<tr>
<td>1,750,000</td>
<td>Constitutional</td>
<td>1/5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Hyperglycemia</td>
<td>1/5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Constitutional</td>
<td>3/4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number affected refers to number of patients requiring dose modification or treatment discontinuation for toxicity.

<sup>b</sup> Controlled with insulin.
DISCUSSION

This study demonstrates that selective expansion of NK cells in vivo may be achieved with a single daily s.c. injection of low-dose IL-2 in patients with advanced malignancy. The dose-limiting toxicities of this therapy are largely constitutional, and side effects are mild at immunomodulatory doses. The effect of IL-2 on NK cell expansion is dose dependent as well as time dependent, with circulating NK cells increasing gradually over the first 6 weeks of treatment. Eosinophilia is also observed with treatment, but significant effects on absolute numbers of lymphocytes, monocytes, and granulocytes were not present.

We found that the MTD of IL-2 when administered as a single daily s.c. injection is 1.25 mIU/m²/day. It is interesting to note that although Common Toxicity Criteria grade 3–4 constitutional toxicity was not observed during initial treatment courses, these side effects proved to be dose limiting. In Phase I clinical trials, toxicities less than grade 3 do not generally preclude further dose escalation. As we observed in this study, however, with protracted treatment schedules, unremitting grade 2 toxicity, especially when more than one toxicity is present, may be unacceptable and therefore dose limiting.

Following s.c. administration, IL-2 peak plasma concentrations are generally reached at 2–4 h (14–16), with a half-life of approximately 4–5 h (15). Peak levels of IL-2 ≥ 10 pm were most consistently found at the highest dose levels. Nevertheless, NK expansion was present at all dose levels, confirming the importance of a prolonged duration of exposure rather than peak concentrations. This is consistent with the study of Thompson et
Table 2  NK cell expansion with low-dose IL-2

<table>
<thead>
<tr>
<th>IL-2 dose (IU/m²/day)</th>
<th>Baseline NK (×10⁰⁰0/µl ± SE)</th>
<th>Peak NK (×10⁰⁰0/µl ± SE)</th>
<th>Average of % change ± SE</th>
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</thead>
<tbody>
<tr>
<td>400,000 (7)*</td>
<td>0.21 ± 0.05</td>
<td>0.43 ± 0.09</td>
<td>154 ± 53</td>
</tr>
<tr>
<td>500,000 (8)</td>
<td>0.29 ± 0.09</td>
<td>0.60 ± 0.14</td>
<td>162 ± 36</td>
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<tr>
<td>750,000 (4)</td>
<td>0.28 ± 0.02</td>
<td>0.70 ± 0.03</td>
<td>153 ± 21</td>
</tr>
<tr>
<td>1,000,000 (4)</td>
<td>0.28 ± 0.09</td>
<td>0.92 ± 0.37</td>
<td>246 ± 61</td>
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<tr>
<td>1,250,000 (5)</td>
<td>0.21 ± 0.05</td>
<td>0.68 ± 0.24</td>
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<tr>
<td>1,500,000 (5)</td>
<td>0.18 ± 0.06</td>
<td>1.02 ± 0.17</td>
<td>530 ± 78</td>
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</table>

<table>
<thead>
<tr>
<th>IL-2 dose (IU/m²/day)</th>
<th>Baseline CD56bright (×10⁰⁰0/µl ± SE)</th>
<th>Peak CD56bright (×10⁰⁰0/µl ± SE)</th>
<th>Average of % change ± SE</th>
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<tr>
<td>400,000 (7)</td>
<td>0.04 ± 0.01</td>
<td>0.12 ± 0.03</td>
<td>368 ± 116</td>
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<td>750,000 (4)</td>
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<td>0.11 ± 0.03</td>
<td>1033 ± 406</td>
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<tr>
<td>1,000,000 (4)</td>
<td>0.02 ± 0.004</td>
<td>0.38 ± 0.22</td>
<td>2992 ± 2403</td>
</tr>
<tr>
<td>1,250,000 (5)</td>
<td>0.01 ± 0.005</td>
<td>0.23 ± 0.12</td>
<td>2400 ± 1545†</td>
</tr>
<tr>
<td>1,500,000 (5)</td>
<td>0.02 ± 0.01</td>
<td>0.49 ± 0.09</td>
<td>2763 ± 1018†</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, number of patients included in analysis. All patients completing at least 35 days of therapy are included.

† At each of these dose levels, one patient had nonmeasurable baseline CD56bright. The calculated percent increase in these cases would be infinite and was not included in the average of the percent change. A dose-dependent increase in peak NK levels (P < 0.01) and peak CD56bright levels (P = 0.015) was observed.

Fig. 2  Relationship between baseline and peak absolute NK cell count for patients treated with daily low-dose s.c. IL-2.

Fig. 3  Time course of NK cell expansion with daily low-dose s.c. IL-2. Numbers of patients at each time point are noted next to the mean values. Bars, SE.

frequently enough to calculate area under the concentration × time curve, which may have allowed identification of pharmacodynamic relationships. However, our data do demonstrate that IL-2 concentrations capable of engaging high-affinity receptors were achieved commonly at the doses tested. Gustavson et al. (15) observed that the bioavailability of s.c. administered IL-2 may decrease with increasing dose, suggesting that either multiple sites of injection or multiple daily doses may be required as doses are increased.

It is notable that the time course and magnitude of NK expansion in this study appears to be different than that previously described with a protracted venous infusion of low-dose IL-2 (11). With i.v. administration, absolute NK cell counts exceeded 2500/µl after 10 weeks of therapy with well-tolerated doses. This effect exceeds the maximum expansion of approx-
IgG antibodies. With five patients reported that 6 of 10 patients treated with 3-30 mIU/day developed IgG antibodies, with five patients having neutralizing activity. NK expansion in these patients ranged from 43 to 610% increases over baseline. In another study of a protracted course of s.c. IL-2, Whitehead et al. (16) reported that 6 of 10 patients treated with 3-30 mIU/day developed IgG antibodies, with five patients (50%) having neutralizing antibodies. It is possible that the higher doses used in that study resulted in a greater proportion of patients developing antibodies than we observed. With the doses and schedule described in the current study, clinically relevant anti-IL-2 antibody development is a rare event within the first 10 weeks of therapy. The significance of our finding of one patient who had anti-IL-2 antibodies before therapy that were undetectable at week 10 is uncertain. This patient had an increase in NK cells of approximately 1000/μl seen in the current study. In addition, our data suggest that a plateau may be reached after 6-10 weeks of s.c. administration. It is plausible that since s.c. administration results in intermittent saturation of IL-2 receptors, a proliferation "equilibrium" is ultimately reached. This contrasts with i.v. schedules that permit continuous receptor activation, and perhaps more prolonged and pronounced NK expansion. These conclusions are limited by patient numbers, with only a few subjects treated at any single dose level in these Phase I studies.

Only 3 (8%) of 37 patients tested developed anti-IL-2 antibodies, and all showed low titers. No patient plasma had IL-2 neutralizing activity. NK expansion in these patients ranged from 43 to 610% increases over baseline. In another study of a protracted course of s.c. IL-2, Whitehead et al. (16) reported that 6 of 10 patients treated with 3-30 mIU/day developed IgG antibodies, with five patients (50%) having neutralizing antibodies. It is possible that the higher doses used in that study resulted in a greater proportion of patients developing antibodies than we observed. With the doses and schedule described in the current study, clinically relevant anti-IL-2 antibody development is a rare event within the first 10 weeks of therapy. The significance of our finding of one patient who had anti-IL-2 antibodies before therapy that were undetectable at week 10 is uncertain. This patient had an increase in NK cells of 1200%.

Initial studies of IL-2 conducted at the National Cancer Institute (1, 18) showed antitumor responses in a variety of malignancies, including renal cell carcinoma (35%), melanoma (21%), and even tumor histologies traditionally thought to not be responsive to immunological approaches such as colorectal cancer (17%). These trials were based on the assumption of a dose-response effect with IL-2, and daily doses of IL-2 more than 100-fold higher than used in our study were used, with or without the infusion of mononuclear cells stimulated ex vivo with IL-2 (LAK cells). The toxicity observed with such high-dose IL-2 was substantial and probably has limited the integration of IL-2 into oncological practice. The antitumor activity in these trials was attributed to an IL-2-stimulated mononuclear population generated either in vivo or ex vivo, capable of non-MHC-restricted cytotoxicity. More recent phenotypic analyses have suggested that the effectors responsible for antitumor activity with high-dose IL-2, either with or without LAK cells, are largely activated NK cells (2).

The expression of CD16, the FcyII receptor, has been described on NK cells derived from patients treated with low-dose IL-2 (11, 19). Furthermore, ADCC has been reported with NK cells derived from patients treated with i.v. low-dose IL-2 (11). Therefore, an additional approach to improving the antitumor efficacy of low-dose IL-2 may be to combine this therapy with monoclonal antibodies that recognize epitopes present on tumor cells. Caron et al. (20) recently demonstrated ADCC against leukemic cells in vitro with humanized monoclonal antibody M195 (anti-CD33) plus low concentrations of IL-2 (~100 pm). In a Phase I study of IL-2 plus monoclonal antibody L6, Ziegler et al. (21) reported an antitumor response in a patient with colorectal cancer. Bispecific monoclonals reactive with both tumor antigens and CD56 would present another approach for bringing effector cells to the tumor site. The potential for IL-2-based therapy in solid tumors generally viewed as resistant to immunological approaches is also suggested by the report of a response in colon cancer to the combination of IL-2 plus IL-1 (22).

Using target cell lines, we demonstrated that numerical expansion of NK cells was associated with increased functional activity. In these experiments, LAK activity was generated with overnight incubation of lymphocytes with nm IL-2.
therapy with low-dose IL-2 resulted in increased LAK activity and ADCC in vitro, suggesting that periodic exposure to higher doses of IL-2 or antitumor antibodies could augment the cytotoxic potential of NK cells expanded in vivo with s.c. low-dose IL-2.

The data presented do not address the mechanism of NK expansion with low-dose IL-2. Proliferation of NK cells is augmented in vitro by pm IL-2 through stimulation of high-affinity receptors (4). However, a limited in vitro analysis of human PBMCs from cancer patients obtained after treatment with a protracted venous infusion of IL-2 did not show increased cycling by flow cytometry (11). A similar result was obtained when lymphocytes from acute myeloid leukemia patients in complete remission treated with i.v. low-dose IL-2 were analyzed (23). In four leukemia patients who had NK expansion with IL-2, there was no increase in the proportion of NK cells in the G2-M phase. NK cells cultured for 12 days in vitro with IL-2 showed decreased evidence of apoptosis when compared to cells cultured without IL-2. In addition, NK cells expanded in vivo in these patients showed high expression of bcl-2. Taken together, these preliminary data support the hypothesis that in vivo NK expansion with low-dose IL-2 may result in part from a lengthened life span of mature cells through prevention of programmed cell death. Alternatively, IL-2 could provide a proliferative stimulus for a noncirculating NK progenitor analogous to other hematopoietic growth factors, such as granulocyte-macrophage colony-stimulating factor, that increase proliferation in a mitotic compartment and differentiation of a postmitotic marrow precursor population.

The results of our study show that NK cells can be expanded in vivo with a convenient treatment schedule and mild toxicity. It is encouraging that modest antitumor activity has been observed with s.c. regimens of single-agent low-dose IL-2 in metastatic renal cell cancer (24–28). However, NK cell cytotoxicity requires activation of intermediate affinity receptors, and concentrations of IL-2 higher than those obtained with

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**Table 3 Development of anti-IL-2 antibodies with low-dose IL-2**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Dose level (IU/m²)</th>
<th>Week</th>
<th>Antibody titer</th>
<th>Baseline NK (×1000/μL)</th>
<th>Peak NK (×1000/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>400,000</td>
<td>0</td>
<td>IgG ND*</td>
<td>0.40</td>
<td>0.57</td>
</tr>
<tr>
<td>2</td>
<td>1,250,000</td>
<td>10</td>
<td>IgG ND</td>
<td>0.18</td>
<td>0.42</td>
</tr>
<tr>
<td>3</td>
<td>1,500,000</td>
<td>0</td>
<td>IgG 79</td>
<td>0.06</td>
<td>0.77</td>
</tr>
<tr>
<td>4</td>
<td>1,500,000</td>
<td>10</td>
<td>IgG ND</td>
<td>0.12</td>
<td>0.85</td>
</tr>
</tbody>
</table>

* ND, not detectable; titer calculation as described in text.

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Fig. 6 IL-2 peak values. Plasma IL-2 concentrations were obtained at 1–6 h after s.c. injection. Horizontal line at 10 M represents the lower limit of assay specificity.
low-dose IL-2 will likely be required to engage a substantial proportion of these receptors and, hence, maximize cytotoxicity. Given the current data and previous reports (4, 20) demonstrating that incubation of NK cells with nm IL-2 results in augmented tumor cell lysis in vitro, we are currently conducting a study of periodic brief pulses of higher dose IL-2 in patients whose effectors have been expanded with long-term low-dose IL-2 in an effort to develop a well-tolerated and effective outpatient antitumor regimen.

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REFERENCES


Daily subcutaneous injection of low-dose interleukin 2 expands natural killer cells in vivo without significant toxicity.


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