In Vivo Induction of Lymphokine-activated Killer Cells by Interleukin-2 Splenic Artery Perfusion in Advanced Malignancy

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ABSTRACT

In an effort to stimulate in vivo LAK cell activity at relatively nontoxic doses, 20 patients with advanced metastatic malignancy (13 renal cell carcinoma, 6 melanoma, 1 lymphoma) were treated with recombinant human interleukin-2 (IL-2) by continuous 5-day splenic artery perfusion using the femoral approach. Two treatment cycles were administered 3 weeks apart; IL-2 doses ranged from 1.5-4 × 10⁴ Cetus units/kg/day. Peripheral blood lymphocyte cytotoxicity in a 4-h ⁵¹Cr release assay was measured using as tumor cell targets K562 for natural killer (NK) activity, Daudi for LAK, and Daudi plus in vitro IL-2 for inducible LAK (I-LAK). For the 20 patients, an increase in mean peak percent cytotoxicity from pretreatment levels was seen for NK (36% to 53%), LAK (8% to 37%) and I-LAK (20% to 53%) activity, all significant at P = 0.001. On day 43, 16 days after completing the second cycle of treatment, NK activity remained elevated at 47% and I-LAK at 40% (P = 0.008 and 0.01, respectively). Lymphocyte phenotype analysis by flow cytometry demonstrated increases from pretreatment levels in Leu 11* (13 to 23%), Leu 19* (10 to 21%), Leu 11* 19+ (7 to 17%), IL-2r* (4 to 17%), and HLA-DR* (12 to 25%) subsets, all significant at P ≤ 0.01. Dose effect was studied at 3 dose levels: 1.5, 3, and 4 × 10⁴ Cetus units/kg/day. At the higher doses mean peak NK (57%) and I-LAK (57%) activity were greater than at the low dose (42 and 31%, respectively), both significant at P < 0.05. A trend to positive dose effect was seen in LAK activity (P = 0.08). Splenic artery perfusion with IL-2 can result in significant in vivo peripheral LAK cell generation as well as enhancement of I-LAK and NK activity that persists at least 16 days after the cessation of treatment. Such sustained activity would not be expected with conventional high dose i.v. therapy.

INTRODUCTION

IL-2² has well-demonstrated anticancer activity in murine models of established lung and liver metastasis (1, 2). A number of studies have now reported responses to this agent in phase I and II clinical trials (3–6). Although the exact mechanism by which IL-2 causes tumor regression is as yet unclear, a number of functions of IL-2 have now been defined. These include the stimulation of proliferation of specific subsets of T-cells (helper, suppressor, cytotoxic), augmentation of cytoxic T-cell function, induction of other lymphokine secretion (γ-interferon, tumor necrosis factor, B-cell growth factors), increase in NK cell cytotoxicity, and the induction of LAK cell activity (7–13).

Preclinical work suggested that LAK cell cytotoxicity, defined as the ability to lyse fresh NK cell-resistant primary and metastatic tumor cells in vitro, was the critical step in obtaining tumor regression. Human LAK cells can be easily generated in vitro by incubating PBL with IL-2. In clinical studies with IL-2, the ability to generate LAK cells in vivo has been inconsistent but appears to be necessary for obtaining tumor response (14–17). However, the high systemic doses of IL-2 required have resulted in significant toxicity.

Given this background, our approach was to consider directed therapy with IL-2 administered by splenic artery perfusion. The spleen is a large lymphocyte reservoir which derives its entire arterial supply through the splenic artery. Kinetic studies have shown it to contain 25% of the total exchangeable T-lymphocyte pool and 10–15% of the exchangeable B-lymphocytes (18). Approximately 50% of circulating lymphocytes pass through the periarteriolar lymphatic sheaths and germinal centers (18, 19). In addition, true “null” lymphocytes, the group within which LAK precursor cells are thought to reside, are infrequent in the peripheral circulation but present in large numbers among splenocytes in the mouse (20). This suggests that appropriate precursor cells can be exposed to high intrasplenic IL-2 concentrations, while limiting systemic exposure to relatively low IL-2 concentrations with a very brief half-life. The generation of in vivo LAK activity might then be achieved at a lower level of systemic toxicity. In addition, a murine model of methylcholanthrene-induced fibrosarcoma treated with tumor-specific transplantation antigen, cyclophosphamide, and IL-2 given by different routes did demonstrate that intrasplenic continuous infusion IL-2 was superior in terms of tumor growth retardation and survival gain (21). In this report we examine the effects of IL-2 splenic artery perfusion on lymphocyte phenotype and function as measured in cytotoxicity assays in vitro against NK-sensitive and NK-resistant target cells.

MATERIALS AND METHODS

Clinical Methods

Twenty patients with metastatic malignancies were entered in a phase I trial of IL-2 splenic artery perfusion between November 1987 and October 1988. Histological diagnoses were renal cell carcinoma in 13 patients, malignant melanoma in 6 patients, and low grade non-Hodgkin’s lymphoma in 1 patient. All had extensive bulky visceral disease. Percutaneous catheterization was accomplished via the femoral artery with guidance into the distal splenic artery under direct visualization in an angiography suite. Patients were treated with IL-2 (kindly provided by Cetus Corporation, Emeryville, CA) continuously infused for 5 consecutive days at 3-week intervals for 2–4 cycles, depending on response. Four patients each were treated at doses of 1.5, 3, and 4 × 10⁴ Cetus units/kg/day. The next 8 patients all received 3 × 10⁴ Cetus units/kg/day, a dose found to be tolerable for administration outside of an intensive care setting. The specific activity of Cetus IL-2 is 18 × 10⁴ IU/mg (1 Cetus unit = 6 IU) (22).

Immunological Assays

Sample Collection. Timing of blood samples for each of two treatment cycles included pretreatment (days 1 and 22), during treatment (days 3, 5, 24, and 26), and posttreatment (days 8, 15, 29, 36, and 43). Automated complete blood cell counts with a manual differential WBC and platelet count were performed at each assay point.

Lymphocyte Separation. Thirty ml of peripheral blood was collected by venipuncture into a sterile container with 15–20 sterile glass beads. Defibrination was accomplished by gentle inversion for 10 min. After centrifugation at 400 × g for 10 min and serum removal and dilution with 50% serum volume of 0.15 M PBS, the cell suspension was added to Sepragel-Mn (Seprotech Corporation, Oklahoma City, OK) in 15-ml siliconized glass tubes and centrifuged at 1500 × g for 20 min (23). The mononuclear layer was removed and subjected to the Sepragel procedure a second time. A suspension of pure lymphocytes that was
free of monocytes, confirmed on flow cytometry, was then removed and washed twice in RPMI 1640 before resuspension in assay medium (RPMI 1640 supplemented with 20% fetal calf serum). Cells were kept at 4°C until assay performance on the same day.

**LAK Cytotoxicity**. Daudi cells were radiolabeled with 51Cr to serve as targets for LAK cell lysis. To 2.5 x 10⁶ lymphocytes in each 12 x 75-mm test tube were added 10⁶ 51Cr-labeled Daudi cells, yielding an effector to target ratio of 25:1. All assays were performed in triplicate. Following 4 h incubation in 500 l of assay medium at 37°C in 5% CO₂, 1 ml of cold (4°C) PBS was added, and the cells were centrifuged at 400 x g for 5 min. Cell pellets and decanted supernatants were analyzed in a γ-ray spectrometer. Percentage of lysis for each specimen was calculated as follows:

$$\frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}} \times 100$$

where spontaneous cpm represents 51Cr release in the absence of effector cells, and maximum cpm was the total cpm added (precipitate + supernatant).

**Inducible-LAK Cytotoxicity (I-LAK)**. This assay was used to evaluate LAK precursors. The procedure was identical to that described for LAK cytotoxicity except that 1000 units/ml of IL-2 was added to each test tube at the beginning of the 4-h incubation period.

**NK Cytotoxicity**. The procedure was identical to that described for LAK cytotoxicity except that 51Cr-labeled K562 erythroleukemia cells were used as targets for NK cells.

**Lymphocyte Phenotyping**. Lymphocytes were analyzed using a two-color direct immunofluorescence assay with FITC- and PE-conjugated monoclonal antibodies (Becton-Deckinson, Mountain View, CA) on a Coulter EPICS V flow cytometer. Twenty µl of conjugated antibodies was added to 10⁵ lymphocytes in 50 µl of phosphate-buffered minimal essential medium containing 1% sodium azide and 2% fetal calf serum for each pair of assays. After the cells were mixed, equilibrated for 45 min on ice, and washed twice with the same medium, they were fixed with 1% paraformaldehyde in PBS. The fixed cells were kept at 4°C before flow cytometry and analyzed within 5 days of labeling. The following pairs of markers were examined: Leu-3 (CD4)-FITC/Leu-8-PE, Leu-2a (CD8)-FITC/Leu-15 (CD11)-PE, Leu-19-FITC/Leu-11c (CD16)-PE, IL-2 receptor (CD25)-PE/HLA-DR-FITC.

**Statistical Analysis**

Statistical programs from the SPSS/PC software system (SPSS Inc., Chicago, IL) were used. The paired t test was used to test for significant differences between pretreatment control and all values obtained after starting treatment. Dose response for each variable was tested by linear regression analysis of the three dose levels.

**RESULTS**

**Clinical Results**

In the 20 patients entered in this phase I trial, 1 partial and 2 minor responses were seen. A patient with renal cell carcinoma had a partial response with complete disappearance of 3 hepatic metastases, while a previously irradiated mass in the head of the pancreas, unchanged over the 12 months prior to IL-2 treatment, remained. In one minor response, a 3.3-cm hepatic mass completely resolved, while the renal primary and lung metastases remained unchanged. The patient with lymphoma had a 40% decrease in the sum of the products of all measurable nodes. The detailed clinical effects, toxicity, and clinical laboratory evaluation of these patients will be reported separately.³

**Immunological Assays**

**Peripheral Blood Cell Counts**. Fig. 1 shows the changes in mean lymphocyte, monocyte, neutrophil, and eosinophil counts for all 20 patients during 2 cycles of treatment. Mean lymphocyte counts decreased significantly on day 5 during IL-2 perfusion (P = 0.005) and then dramatically increased on day 8 (P = 0.0001), 48 h after completing the first cycle of treatment, to >4 x 10⁹ cells/liter. Lymphocyte counts gradually returned to baseline and displayed similar fluctuation during the second cycle. Mean monocyte counts followed a similar pattern with statistically significant elevations on days 8 and 29. Granulocyte counts were significantly elevated at day 5 (P = 0.007) during treatment and day 8 (P = 0.01), with a return to baseline on day 22. In the second cycle the elevation was more sustained with a mean of 7.5 x 10⁹ granulocytes/liter observed as late as day 29. While mean eosinophil counts were only modestly increased on day 8 (P = 0.03), there was a dramatic increase in the second cycle to 3.5 x 10⁹/liter (P = 0.003 on day 26). Two patients achieved absolute values of 15 x 10⁹ cells/liter. There were no statistically significant differences in any of the cell counts by dose level.

**LAK Cytotoxicity**

The values for mean LAK, I-LAK, and NK cytotoxicity for all 20 patients are displayed in Table 1 and Fig. 2. LAK cytotoxicity reached peaks of 37% lysis on day 5 and 25% lysis on day 26 (P < 0.0001 and 0.008, respectively) with a mean pretreatment value of 7%. In each cycle this represented the fifth day of IL-2 perfusion, a time when peripheral blood lymphocyte counts were significantly reduced. On day 8 the LAK cytotoxicity remained significantly elevated (P = 0.001), but the mean value had decreased to 18%. Since lymphocyte counts increased approximately 10-fold during the same time period, the total LAK activity in peripheral blood would have been greatest on day 8. LAK activity returned to the pretreatment range by day 22.

There was no statistically significant difference in LAK cytotoxicity by dose level, although there was a trend (P = 0.08) toward higher activity at higher doses during the first treatment cycle.

**Inducible LAK Activity (I-LAK)**

During the first IL-2 treatment there was a modest increase in LAK cytotoxicity induced by adding exogenous IL-2 during the assay (Fig. 2 and Table 1). For example, LAK activity on day 5 was 37% and adding IL-2 to the assay increased this to an I-LAK of 52%. I-LAK values remained higher than baseline throughout the study period and starting on day 8 this induction

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³ R. J. Klasa and H. K. B. Sliver, manuscript in preparation.
was substantial. Even on day 43, 16 days after completing the second 5-day IL-2 perfusion, I-LAK was double the pretreatment value at 40% (P = 0.01). Peak values were seen on days 8 and 26 (P = 0.0001 for both) at the time of rebound lymphocytosis 48 h after IL-2 was discontinued. At the time of day 8 peak activity a positive dose-response effect was seen by linear regression analysis (P = 0.03).

**NK Cytotoxicity**

The general pattern of NK activity was similar to both LAK and I-LAK (Fig. 2 and Table 1). Mean values reached 53% lysis and were significantly greater than baseline during perfusion on days 5 and 24 (P = 0.001 and 0.006, respectively), as well as 48 h after perfusion on days 8 and 26 (P = 0.001 and 0.006, respectively). Of interest, 8 of the 20 patients sustained >25% elevation of activity above their baseline on day 22 and 3 patients remained above that level on day 43. Although not statistically significant, it is of interest that some patients did maintain high NK activity 16 days after completion of a cycle of treatment. A positive dose-response effect was seen at the times of peak activity on days 5 and 26 (P = 0.04 and 0.03) as well as on day 43 (P = 0.008).

**Lymphocyte Phenotype**

Leu-3 and Leu-8. Within the helper/inducer (Leu-3+) compartment, Leu-3+8+ cells are thought to represent inducers of T-suppressor cells and Leu-3+8- amplifiers of that induction (24, 25). No consistent pattern of change was seen in these subsets with treatment and no statistically significant relationship to dose emerged.

Leu-2a and Leu-15. Within the cytotoxic/suppressor (Leu-2a+) compartment, Leu-2a+15- cells are identified with suppressor function and Leu-2a+15+ with cytotoxicity (26). No statistically significant changes were seen with IL-2 treatment. Although the trend on dose analysis was toward lower percentages of Leu-2a+15+ suppressor cells in the high dose group, there was no statistical significance.

**Leu-11 and Leu-19.** These markers have been associated with NK and LAK cell cytotoxicity (27-31). As can be seen in Fig. 3 and Table 2, Leu-11+, Leu-19+, and Leu-11+19+ circulating cells all increased during or soon after IL-2 administration with peak values of 23, 29, and 17%, respectively. Statistically significant elevations were present on days 5, 8, 26, and 29 (P values all between 0.001 and 0.04). Expressed as absolute numbers of marker-positive lymphocytes, the pattern was similar but with higher positivity seen during the first cycle of treatment (Fig. 4). Of interest, these values remained high on day 43, 16 days after completing the second IL-2 perfusion (P values between 0.007 and 0.02). No dose response was apparent.

IL-2r and HLA-DR. Both IL-2r and HLA-DR expression was increased with IL-2 treatment (Fig. 5 and Table 2). IL-2r+ cells peaked at 17% and were significantly elevated on days 8, 26, 29, and 36 (P = 0.0001, 0.02, 0.003, and 0.04, respectively). HLA-DR+ cells reached 37% and were significantly increased on days 5, 8, and 26 (P = 0.02, 0.001, and 0.01, respectively). No dose response was demonstrated.

**Responders versus Nonresponders**

Laboratory values for the 3 responding patients were compared with results for the 17 nonresponders. Fig. 6 shows results for LAK and I-LAK activity. Although the number of responders was small, both LAK and I-LAK were significantly higher for responders at the end of the observation period on day 43 (P = 0.007 and 0.03, respectively). There was no difference in level of NK activity between the two groups. Responders also showed higher expression of HLA-DR on day 8 (P = 0.05). Mean granulocyte counts were higher in responders during the second cycle of treatment on days 22 and 43 (P = 0.02 and 0.04, respectively). Mean peak lymphocyte counts and all other lymphocyte marker studies were not significantly different in the two groups.

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**Table 1** NK, LAK, and I-LAK cytotoxicity for 20 patients through 2 cycles of IL-2 treatment

<table>
<thead>
<tr>
<th>Treatment Day</th>
<th>NK</th>
<th>LAK</th>
<th>I-LAK</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 3 5 8 15 22 24 26 29 36 43</td>
<td>36.5 ± 2.9</td>
<td>7.8 ± 1.5</td>
<td>20.1 ± 3.2</td>
</tr>
</tbody>
</table>

* Mean % lysis ± SE.
LAK INDUCTION WITH IL-2 SPLENIC ARTERY PERFUSION

Table 2 Changes in peripheral blood lymphocyte phenotype for 20 patients through 2 cycles of IL-2 treatment

<table>
<thead>
<tr>
<th>IL-2 treatment on day:</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>8</th>
<th>15</th>
<th>22</th>
<th>24</th>
<th>26</th>
<th>29</th>
<th>36</th>
<th>43</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu-11*</td>
<td>9.3 ± 1.4*</td>
<td>10.0 ± 3.6</td>
<td>13.4 ± 2.8</td>
<td>15.7 ± 1.8</td>
<td>16.2 ± 1.4</td>
<td>13.4 ± 2.1</td>
<td>11.7 ± 4.0</td>
<td>22.7 ± 3.0</td>
<td>15.0 ± 2.0</td>
<td>25.4 ± 3.0</td>
<td>23.1 ± 1.4</td>
</tr>
<tr>
<td>Leu-19*</td>
<td>7.1 ± 1.6</td>
<td>7.4 ± 3.1</td>
<td>15.4 ± 4.0</td>
<td>16.8 ± 3.2</td>
<td>9.9 ± 3.3</td>
<td>8.4 ± 1.8</td>
<td>11.0 ± 4.6</td>
<td>28.5 ± 8.1</td>
<td>16.4 ± 3.9</td>
<td>16.3 ± 3.5</td>
<td>20.8 ± 2.8</td>
</tr>
<tr>
<td>Leu-11<em>19</em></td>
<td>4.6 ± 1.2</td>
<td>4.9 ± 2.3</td>
<td>8.6 ± 2.7</td>
<td>9.2 ± 2.0</td>
<td>6.8 ± 2.5</td>
<td>6.3 ± 1.5</td>
<td>8.5 ± 4.2</td>
<td>15.8 ± 3.6</td>
<td>9.7 ± 2.1</td>
<td>13.4 ± 3.4</td>
<td>17.0 ± 2.5</td>
</tr>
<tr>
<td>IL-2r</td>
<td>4.6 ± 0.9</td>
<td>3.4 ± 0.9</td>
<td>7.4 ± 2.2</td>
<td>16.7 ± 2.6</td>
<td>7.7 ± 3.2</td>
<td>7.9 ± 1.5</td>
<td>11.8 ± 3.4</td>
<td>9.4 ± 2.1</td>
<td>17.5 ± 2.6</td>
<td>11.4 ± 1.9</td>
<td>13.6 ± 4.7</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>12.1 ± 2.0</td>
<td>14.1 ± 4.0</td>
<td>17.2 ± 3.6</td>
<td>24.2 ± 3.4</td>
<td>11.0 ± 2.8</td>
<td>13.1 ± 2.6</td>
<td>12.6 ± 2.4</td>
<td>36.9 ± 6.0</td>
<td>23.1 ± 3.3</td>
<td>13.5 ± 2.0</td>
<td>19.3 ± 7.2</td>
</tr>
</tbody>
</table>

* Mean % marker-positive cells ± SE.

DISCUSSION

The most striking finding was the development of circulating LAK effector cells even at the lowest daily dose of IL-2, 1.5 × 10^4 Cetus units/kg/day, within 48–96 h of initiating treatment. The peak mean LAK cytotoxicity reached 37% for the entire study group on day 5 of treatment. The starting daily IL-2 dose was 5% of that published in the early IL-2 + LAK cell studies (3) and, nevertheless, resulted in this marked increase in cytotoxicity which was associated with phenotypic alterations in the peripheral blood lymphocyte pool. Although direct comparisons are difficult because different methods are used to calculate and express the degree of cytotoxicity, it is worth comparing these findings with studies in which IL-2 is given i.v.

In a dose escalation study of weekly IL-2 by either s.c. injection, 2-h i.v. infusion, or 24-h infusion, Thompson et al. (15) found no increase in LAK precursor (comparable to I-LAK), NK, or LAK activity. The highest dose used was 3 × 10^7 Biological Response Modifiers Program (BRMP) units and there were no clinical responses among the 24 patients studied. A subsequent investigation (16) compared 2- and 24-h infusions 5 days/week for 4 weeks. On this more intensive regimen all patients developed LAK precursors, but only those at the highest dose level (3 × 10^6 units/m^2/day) developed circulating LAK effectors. Both toxicity and LAK activity were more marked with continuous infusion. Of the 23 patients studied one had a mixed clinical response. Hank et al. (17) administered IL-2 at doses ranging from 10^3–10^7 BRMP units/m^2/day by i.v. bolus or continuous infusion for 7 consecutive days in a phase I study of 25 patients. There was an increase in LAK precursor and NK activity with IL-2 treatment, but no LAK effector cells were generated. The same group (6) treated 11 patients with 4 consecutive weekly cycles of IL-2 by continuous infusion 4 days/week. With the longer treatment time, they were now able to show an increase in LAK activity. One patient sustained a measurable response. Creekmore et al. (32) examined intermittent 24-h infusions at doses up to 3 × 10^7 BRMP units/m^2/day for 4 weeks. Infusions were once weekly for 4 weeks and then twice weekly for an additional 4 weeks. NK enhancement and circulating LAK activity were only seen at the highest doses given twice weekly. Compared with the above studies, we were able to show that relatively low doses of IL-2 delivered to the spleen can result in marked peripheral LAK activation and enhancement of I-LAK and NK activity.

A unique feature of splenic perfusion is the persistence of LAK and I-LAK activity after withdrawal of IL-2 (Fig. 2). This has not been reported for i.v. administration. The kinetics of IL-2 decay may be altered in the spleen or a large pool of LAK and precursor cells may be generated which can then be released gradually into the systemic circulation. In either event it appears that splenic perfusion permits sustained activity with intermit-
tent treatment. Considering the remarkable persistence of primed I-LAK cells, it may be possible to achieve significant augmentation of LAK cytotoxicity by modest systemic IL-2 doses between splenic perfusions.

Analysis of lymphocyte phenotypes showed increases in Leu-11+, Leu-19+, and Leu-11+19+ cells with treatment, consistent with other reports (33). This phenotype was temporally related to the appearance of LAK and increase in I-LAK and NK activities. Expression of IL-2 receptors and HLA-DR both increased during treatment. In contrast, there was a striking lack of consistent changes within either the helper/inducer or cytotoxic/suppressor cell compartments. Leu-2a (CD8) expression, specifically, did not increase with IL-2 treatment.

Since the number of responses was small and one of these was at the lowest dose, it is not altogether surprising that no clinical dose-response effect could be demonstrated. However, even with this small patient population there was evidence of a dose response for NK and I-LAK activities as has been noted even with this small patient population there was evidence of a dose response for NK and I-LAK activities as has been noted by others. In our limited study the best laboratory correlates of clinical response were sustained LAK and I-LAK activities at doses between splenic perfusions.


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