ABSTRACT

Interleukin-2 (IL-2) at high doses or at low doses in concert with lymphokine-activated killer (LAK) cells can produce regression of established pulmonary and hepatic metastases from a variety of tumors in mice. IL-2 appears to mediate its antitumor effect through the generation of LAK cells in vivo from endogenous lymphocytes and by the stimulation of host and transferred LAK cell proliferation in tissues. In this paper we have investigated different strategies for IL-2 administration to determine which regimen produced maximal in vivo proliferation and optimal immunotherapeutic efficacy of LAK cells. Tissue expansion of lymphoid cells was assessed using an assay of in vivo labeling of dividing cells by the thymidine analogue, 5-[125I]iododeoxyuridine. The therapeutic effect of the different IL-2 administration protocols was determined by evaluating their efficacy in the treatment of established, 3-day pulmonary metastases from sarcomas in mice. The selection of IL-2 injection regimens for evaluation was based upon pharmacokinetic studies of IL-2 in mice. A single i.v. or i.p. dose yielded high peak IL-2 levels that could be measured for only a few hours after injection, while IL-2 given i.p. thrice daily produced titers that were detectable throughout the study periods (≥5 units/ml of serum after 100,000 units of IL-2 i.p. thrice daily).

Using the proliferation and therapy models, we tested the same cumulative daily dosage of IL-2 administered by i.v. or i.p. once daily, or i.p. thrice daily regimens. The i.p. thrice daily protocol stimulated greatest lymphoid cell proliferation in the lungs, for example, than did the other regimens. Similarly, 300,000 units of IL-2 divided i.p. thrice daily were more successful in reducing metastases (n = 16) than was the entire dose given i.v. once daily (n = 190; P < 0.05) or i.p. once daily (n = 71; P < 0.05). When compared to the i.v. or i.p. once daily protocols, the i.p. thrice daily regimen for IL-2 also produced greater proliferation of exogenous LAK cells, as well as a more effective therapeutic outcome when IL-2 was combined with transferred LAK cells. Thus, sustained, lower levels of IL-2 were more effective than brief, high peak titers for stimulation of proliferation and antitumor activity.

We then evaluated the effect of duration of IL-2 treatment as well as the number of LAK cell injections in the two models. When IL-2 was given i.p. thrice daily together with LAK cells, proliferation in the lungs and therapeutic effect were greatest after 6 days of IL-2 as compared with 1 or 3 days. Similarly, two infusions of LAK cells yielded higher levels of 5-[125I]iododeoxyuridine uptake in tissues and a greater reduction of lung metastases than did one LAK cell injection.

The establishment of optimal regimens for LAK cell and IL-2 immunotherapy in the murine system has played an important role in the design of similar treatments in ongoing human trials.

INTRODUCTION

We have previously reported that the incubation of murine splenocytes or human lymphocytes in medium containing IL-2 leads to the production of LAK cells. These cells may be generated from lymphocytes obtained from normal or cancer-bearing hosts, are capable of lysing a wide variety of fresh, NK cell-resistant tumor cell targets, but not normal cells in short-term [51Cr] release assays (1-8). Recent investigations have elucidated the phenotypic characteristics of the LAK precursor (Thy 1+, Ia-; asialo-GM1+) and effector (Thy 1+, Ia+, Lyt 2+, Ia-, γ-Fc+) cell populations (9). Initial in vivo studies with these cells have shown that the adoptive transfer of LAK cells (generated with natural, nonrecombinant IL-2) to C57BL/6 mice can significantly reduce spontaneous and artificial pulmonary metastases from a B16 melanoma (10). With the availability of recombinant human IL-2 (11, 12), further study of LAK cells in the therapy of growing pulmonary and hepatic metastases from a broad range of sarcomas, carcinomas, and melanomas has demonstrated the requirement for the concurrent administration of IL-2 to mice to observe significant antitumor effects (Footnote 3; Refs. 13-17). A similar enhancement by IL-2 of the in vivo antitumor activity of specifically immune, cultured T-cells has been shown by our laboratory and others in the FBL-3 lymphoma system (18-21).

Studies to define the in vivo role of IL-2 have documented that the lymphokine can promote the expansion of transferred LAK cells (22) and immune T-cells (21, 23). The administration of IL-2 alone to mice also stimulates the proliferation of endogenous lymphoid cells which, upon recovery from tissues, possess lytic activity for fresh tumors in in vitro assays (24). Furthermore, endogenous lymphocytes and transferred LAK cells expanding in response to IL-2 have specific tissue sites of growth including lungs, liver, spleen, mesenteric lymph nodes, and kidneys (22, 24).

The present study was conducted to optimize in vivo LAK cell proliferation and antitumor activity after adoptive transfer by evaluating the effects of different routes and schedules of IL-2 administration as well as the number of LAK cell infusions. To quantitate in vivo lymphoid cell expansion, we used a previously described technique for the labeling of proliferating cells with [125I]iodo[125I]uridine, a γ-emitting nucleoside incorporated into the DNA of dividing cells (22, 24). Then, after establishing the schedules of IL-2 administration and LAK cell infusion which produced the highest levels of lymphoid expansion, we tested the various regimens for their antitumor effect in mice bearing 3-day pulmonary metastases from a transplantable sarcoma. Our findings demonstrated that low, sustained concentrations of IL-2 rather than high, brief peak levels of the lymphokine evinced in the highest levels of expansion of endogenous lymphoid and transferred LAK cells. Multiple injections of LAK cells were more effective in therapy than single injections. Furthermore, these proliferative responses directly paralleled the therapeutic effects of LAK cells and IL-2 in reducing pulmonary metastases.

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2. To whom requests for reprints should be addressed, at Surgery Branch, National Cancer Institute, Building 10, Room 2842, Bethesda, MD 20892.

3. The abbreviations used are: IL-2, interleukin-2; LAK, lymphokine activated killer; NK, natural killer; [125I]iodo[125I]uridine; MCA, 3-methylcholanthrene; HBSS, Hanks' balanced salt solution; CM, complete medium; FdUrd, 5-fluoro-2'-deoxyuridine; PI, proliferation index.

IN VIVO GROWTH AND ANTITUMOR EFFECT OF LAK CELLS

MATERIALS AND METHODS

Mice. Eight to 16-wk-old, female C57BL/6 mice (Animal Production Colonies, NIH, Bethesda, MD) were used in experiments. Splenectomies for LAK cell generation were obtained from female C57BL/6 retired breeders.

Recombinant IL-2. The human recombinant IL-2 used in these experiments was kindly supplied by the Cetus Corporation (Emeryville, CA). The biological, immunological, and biochemical properties of this material have been previously described (12, 25). The lyophilized IL-2 was reconstituted in sterile distilled water before use.

Collection of Serum Samples and Assay of IL-2 Activity. Two mice per time point were bled from the inferior tail artery into microfuge tubes (Eppendorf, Westbury, CT). After clot retraction, the samples were centrifuged at 15,000 rpm for 1 min. The sera were harvested and stored briefly at 4°C until assayed for IL-2 levels. IL-2 activity in test serum was then determined by assaying [3H]thymidine incorporation by an IL-2-dependent cell line as previously described (26).

Tumor. The MCA-101 and MCA-105 sarcomas, syngeneic to the C57BL/6 mouse, were derived in our laboratory by the i.m. injection of 0.1 ml of 1% MCA in sesame oil (27). These tumors have been maintained in our laboratory as previously described (13-16) and were used during the fourth to tenth transplant generations in the current experiments.

In order to produce pulmonary metastases, viable tumor was excised from the flank of a C57BL/6 mouse in which the sarcomas were serially passaged. A single cell suspension was then made by finely mincing the tumor and gently stirring for 3 h at room temperature in a mixture of hyaluronidase, DNase, and collagenase (Sigma, St. Louis, MO) as previously described (13). After washing the cell suspension in HBSS (Biofluids, Rockville, MD), the tumor was doubly filtered through 100-μm nylon mesh (NiteX; Lawshe Industrial Co., Bethesda, MD) and viable cells were counted by trypan blue exclusion before injection into mice. Three to 5 x 10⁶ tumor cells were then injected i.v. in 1 ml of HBSS to produce pulmonary metastases.

Preparation of LAK Cells. Splenectomies were removed aseptically and gently crushed in CM with the flat end of a sterile syringe. CM consisted of RPMI-1640 (Biofluids), 10% heat-inactivated fetal calf serum (Biofluids), 0.03% glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml) (all from NIH Media Unit), 0.1 mM nonessential amino acids, 1 μM sodium pyruvate, gentamicin (50 μg/ml) (all from M. A. Bioproducts, Walkersville, MD), 5 x 10⁻⁵ M mercaptoethanol (Aldrich, Milwaukee, WI), and Fungizone (0.5 μg/ml) (Flow Laboratories, McLean, VA). The cells were passed through nylon mesh and then placed in buffered ammonium chloride solution (NIH Media Unit) to osmotically lyse erythrocytes. After washing the cell suspension in CM, LAK cells were prepared by placing 5 x 10⁸ viable splenocytes (as assessed by trypan blue exclusion) together with 175 ml of CM and 175,000 units of IL-2 in 175-ml tissue culture flasks (Falcon, Oxnard, CA). The flasks were incubated supine at 37°C in humidified 5%CO₂ for 72 h. The LAK cells were harvested by passage over Ficoll gradients (Lympholyte-M; Cedarlane, Hornby, Ontario, Canada) and washed in HBSS. The cell suspension was then filtered through Nitex and adjusted to the desired number in HBSS after counting viable cells in trypan blue. LAK cells which were injected i.v. into mice also demonstrated the ability to lyse fresh, NK-resistant, tumor targets in short-term ³¹Cr release assays as previously reported (2).

In Vivo Assay of Lymphoid Proliferation in Tissues. On Day 0, mice were randomized to receive either whole-body irradiation (500 rads from a ¹³⁷Cs source) or no irradiation, followed 3 to 5 h later by the i.v. administration of either 10⁵ viable LAK cells or no cells. The selection of 10⁵ cells for adoptive transfer was based on previous work which demonstrated that 10⁵ LAK cells per infection provide the optimal therapeutic effect in the metastasis model and are the maximal number of cells tolerated per infusion (14). Immediately afterwards, similar cumulative daily doses of IL-2 in 0.5 ml of HBSS (or HBSS alone) were injected into mice i.v. or i.p. once daily, or i.p. thrice daily for 1 to 6 days. Doses of IL-2 varied between experiments and are reported in the table footnotes and figure legends. On Day 13 or 14, mice were ear-tagged and randomized. After animal sacrifice, lungs were insufflated with 15% India ink suspension, removed, and bleached in Fekete's solution as previously described (31). Metastases, visualized as distinct, white nodules on the black background of normal lung parenchyma, were then counted in a blinded fashion. Metastases numbering greater than approximately 250 per pair of lungs were too numerous to count accurately and were thus arbitrarily assigned the value of 250. After all data were recorded, the codes were broken. Four to ten mice were included in each treatment group in all experiments.

Statistical Analysis. The significance of differences in cpm of tissues between treatment groups was assessed with Student's t test (32). Analyses of differences in numbers of metastases between groups were determined by the Wilcoxon rank sum test (33). All results are presented as 2-sided P values.

RESULTS

In Vivo Lymphoid Proliferation and Successful Immunotherapy Produced by LAK Cells and IL-2. Prior work from our laboratory has shown that the administration of high dose IL-2 alone or low doses of IL-2 in combination with LAK cells to tumor-bearing mice mediates the reduction of established pulmonary metastases (13-15, 34), and that IL-2 stimulates the in vivo proliferation of endogenous lymphocytes and transferred LAK cells in tissues of normal mice (22, 24). In Experiment 1 of Table 1 (14, 34), administration of two doses of 10⁵ LAK cells on Days 3 and 6 plus 20,000 units of IL-2 i.p. thrice daily from Days 3 to 8 to normal mice bearing 3-day pulmonary micrometastases significantly reduced the mean number of metastases (n = 19) on Day 14 in contrast to HBSS (n = 250; P < 0.005) or IL-2 alone (n = 214; P = 0.001). IL-2 alone at higher doses of 50,000 and 100,000 units i.p. thrice daily was also capable of dramatically reducing the number of metastases (n = 19 and 61, respectively). The combination of LAK cells plus IL-2 at 100,000 units resulted in 11 metastases compared to 61 metastases in mice treated with IL-2 alone (P > 0.05).

Irradiation of mice with 500 rads prior to tumor cell injection and subsequent therapy abrogated any significant antitumor effect produced by 50,000 or 100,000 units of IL-2 alone (209 and 250 metastases, respectively; P > 0.05 versus HBSS). However, if LAK cells were transferred to irradiated mice in combination with multiple injections of IL-2 at doses of 20,000 or 100,000 units, a significant reduction in metastases was still apparent when compared to HBSS controls (44 and 67 metas-
or preirradiated mice received one injection of i0@LAK cells or no cells on Day 0 followed by 100,000 units of IL-2 (or IL-2 alone (P1 = 8.3) when compared with background activity peaked within the first 2 mm and rapidly declined, so that no IL-2 was measurable after 2 h. A similar IL-2 dose given i.p. resulted in lower peak levels but in a significant prolongation of detectable activity over time. In other experiments, a direct comparison of the duration of measurable IL-2 activity in serum after i.v. and i.p. administration of 10,000 to 20,000 units of IL-2 showed a mean duration of 1.3 ± 0.3 (SE) h versus 3.0 ± 0.3 h, respectively (25).

Since the i.p. route yielded more prolonged levels of IL-2 in the serum, we examined the time course of IL-2 activity after multiple i.p. injections of high dose IL-2. Mice received 100,000 units of IL-2 in 0.5 ml of HBSS i.p. thrice daily (e.g., 8 a.m., 4 p.m., 10 p.m.) for 2 days. Serum samples were collected from two mice per time point at 0.5, 1, 2, 4, and 6 h following the 8 a.m. and 4 p.m. injections of IL-2 on 2 successive days. Fig. 1 depicts the serum titers of IL-2 during the 2 days of IL-2 injections. Thirty min after each i.p. dose, IL-2 activity was >2000 units/ml. The IL-2 titer declined rapidly but was still detectable (≥6 units/ml) 6 to 10 h later, at the time of the next injection. Moreover, a gradual rise in the absolute trough level of IL-2 was apparent over time, with 7 units/ml after the initial dose increasing to 34 units/ml after the fifth injection.

Proliferative and Immunotherapeutic Responses to Similar Cumulative Doses of IL-2 Administered i.p. or i.v. Once Daily, or i.v. Thrice Daily. In order to determine whether high peak levels or low sustained concentrations of IL-2 were more effective at yielding the highest levels of lymphoid proliferation, normal mice received the same total daily doses of 150,000 units of IL-2 as one injection by either the i.v. or i.p. route or as three divided doses i.p. per day from Days 0 to 5. Twenty-four h after FdUrd and [125I]IdUrd injection, the lungs, liver, spleen, and mesenteric lymph nodes were assayed on Day 5. As presented in Fig. 2 in two separate experiments, markedly increased [125I]IdUrd uptake was seen in the lungs, liver, and mesenteric lymph nodes when IL-2 was administered i.p. thrice daily as compared with the other treatment schedules. In the lungs from animals in Experiment 1, for example, the PI was 26.0, 8.5, and 7.1 after the same cumulative doses of IL-2 were administered i.p. thrice daily, i.v. once daily, and i.p. once daily, respectively. In the spleen, there appeared to be no major difference between high peak levels of IL-2 after i.v. doses once daily or low sustained levels with the i.p. thrice daily schedule.

In both experiments there was little difference in proliferation between the i.p. and i.v. routes when IL-2 was given as a single dose, although radiolabel uptake in the liver and mesenteric
tases, respectively, versus 250; P < 0.005).

Because of the pivotal role of IL-2 in antitumor treatment by itself and in concert with LAK cells, we have previously investigated the mechanisms by which IL-2 exerted its immunotherapeutic effects in vivo. By using an assay of in vivo cell proliferation utilizing [125I]IdUrd (see "Materials and Methods"), we found that IL-2 stimulated the in vivo proliferation of endogenous lymphoid cells as well as adoptively transferred LAK cells. In Experiment 2 of Table 1 (22), non-tumor-bearing, normal or preirradiated mice received one injection of 106 LAK cells or no cells on Day 0 followed by 100,000 units of IL-2 (or HBSS) i.p. thrice daily from Days 0 to 3. On Day 2, FdUrd and [125I]IdUrd were administered, and lungs were assayed for uptake of radiolabel on Day 3. As shown, the lungs of normal, nonirradiated mice receiving LAK cells plus IL-2 displayed significantly greater levels of [125I]IdUrd incorporation (9817 cpm) then did lungs of animals treated with HBSS alone (280 cpm; P < 0.01) or IL-2 alone (2334 cpm; P < 0.02). Furthermore, a dramatic increase in radiolabel uptake was produced by IL-2 alone (PI = 8.3) when compared with background incorporation. Following preirradiation of the animals, the level of proliferation after treatment with 100,000 units of IL-2 alone was largely eliminated (452 cpm), while high levels of [125I]IdUrd incorporation were restored iF LAK cells were transferred to mice also receiving IL-2 (9573 cpm).

A comparison of these two experiments, which are characteristic of many others performed with similar results, demonstrates that the cellular proliferation in the lungs, previously shown to be endogenous lymphoid cells as well as transferred LAK cells, paralleled the immunotherapeutic effects in the metastases model with IL-2 alone as well as with LAK cells plus IL-2.

**Table 1** In vivo effects of LAK cells and IL-2: immunotherapy of established pulmonary sarcoma micrometastases and stimulation of in vivo proliferation of lymphoid cells

<table>
<thead>
<tr>
<th>Experimenta,b</th>
<th>Pretreatment of mice</th>
<th>HBSS</th>
<th>IL-2 alone</th>
<th>LAK' + IL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>20,000 units</td>
<td>50,000 units</td>
</tr>
<tr>
<td>1. Immunotherapy of pulmonary sarcoma metastases (mean no. of metastases)</td>
<td>None</td>
<td>280 ± 98d (A)</td>
<td>NDd</td>
<td>2334 ± 27 (8.3)d (B)</td>
</tr>
<tr>
<td>2. Assay of in vivo proliferation of lymphoid cells in lungs</td>
<td>None</td>
<td>280 ± 98d (A)</td>
<td>NDd</td>
<td>2334 ± 27 (8.3)d (B)</td>
</tr>
</tbody>
</table>

a Experiment 1 adapted from Refs. 14 and 34. Experiment 2 adapted from Ref. 22.
b Statistical analysis of differences between groups—in Experiment 1: A versus B, not significant; A versus C, P < 0.005; A versus D, P < 0.005; A versus E, P < 0.005; A versus F, P < 0.005; B versus C, P < 0.005; B versus D, P < 0.005; B versus E, P < 0.005; B versus F, P < 0.005; C versus D, P < 0.005; C versus E, P < 0.005; C versus F, P < 0.005; D versus E, P < 0.005; D versus F, P < 0.005; E versus F, not significant; G versus H, not significant; G versus J, not significant; G versus K, not significant; G versus L, P < 0.005; G versus M, P < 0.005; H versus J, not significant; H versus K, not significant; H versus L, P < 0.005; J versus K, not significant; K versus M, P < 0.005; L versus M, not significant; in Experiment 2: A versus B, P < 0.005; A versus C, P < 0.01; B versus C, P < 0.02; D versus E, P < 0.005; D versus F, P < 0.005; E versus F, P < 0.001.

c LAK cells (106) in 1 ml of HBSS were injected i.v. on Days 3 and 6 in Experiment 1 and on Day 1 in Experiment 2.
d Mean ± SE (cpm).

e ND, not done.
f Numbers in parentheses, PI.
lymph nodes tended to be somewhat higher after i.p. injection.

Since the [3H]thymidine assay showed that different levels of lymphoid cell proliferation were produced by the three IL-2 administration regimens, we tested the three IL-2 treatment protocols in the pulmonary metastasis model. Mice bearing 3-day lung tumors were given injections of similar cumulative daily doses of IL-2 (150,000 units in Experiment 1 and 300,000 units in Experiment 2) by the i.v. or i.p. once daily, or i.p. thrice daily regimens from Days 3 to 8 after tumor cell injection. In Experiments 1 and 2 as presented in Table 2, the i.p. thrice daily schedule for IL-2 significantly reduced the number of pulmonary metastases when compared with HBSS-treated animals (n = 10 versus 137, P < 0.005; and n = 16 versus 180, P < 0.05), respectively). In neither experiment did the reduction of metastases by the administration of IL-2 i.v. or i.p. once daily achieve statistical significance compared to control mice. In Experiment 2, the i.p. thrice daily regimen showed significantly greater immunotherapeutic effect over IL-2 given i.v. once daily (n = 16 versus 190, P < 0.05) and i.p. daily (n = 16 versus 71, P < 0.05).

**In Vivo Proliferation of LAK Cells in Irradiated Mice Receiving IL-2 by i.v. and i.p. Routes Once Daily, or i.p. Thrice Daily.** Since the i.p. thrice daily schedule for IL-2 stimulated the greatest level of proliferation by endogenous lymphocytes and produced the greatest immunotherapeutic effect, we studied the ability of the three IL-2 schedules to induce the in vivo proliferation of transferred LAK cells. Non-tumor-bearing mice were irradiated with 500 rads just prior to adoptive transfer to prevent significant proliferation by endogenous lymphoid cells (see Experiment 2, Table 1), thereby allowing assessment of the expansion of only the transferred LAK cells. LAK cells (10^6) or no cells were injected i.v. on Day 0, and HBSS or 60,000 units of IL-2 were administered from Days 0 to 5 as a single daily i.v. or i.p., or split into three doses of 20,000 units i.p. per day. A representative experiment, as shown in Fig. 3, demonstrates that the levels of proliferation achieved in the lungs, liver, spleen, and mesenteric lymph nodes were significantly greater than these same tissues in animals receiving HBSS or IL-2 given once daily by either the i.v. or the i.p. route. The results are reported as the mean cpm of three mice per treatment group. The PI is presented at the top of each column.

![Image](image-url)

**Fig. 1.** Serum IL-2 levels in mice receiving high dose IL-2 i.p. thrice daily. Mice were given 100,000 units of IL-2 i.p. thrice daily (e.g., 8 a.m., 4 p.m., and 10 p.m.) for 2 consecutive days. The combined sera of two mice per time point were collected at 0, 0.5, 1, 2, 4, and 6 h after the 8 a.m. and 4 p.m. doses of IL-2. IL-2 activity (units/ml) was then determined by a standard bioassay as described in “Materials and Methods.” The vertical arrows indicate the times of IL-2 injections.

**Fig. 2.** In vivo proliferation of endogenous lymphoid cells in tissues of mice treated with IL-2 injected by different dose schedules and routes of administration. In Experiments 1 and 2, normal non-tumor-bearing mice received HBSS i.p. thrice daily (C) or 150,000 units of IL-2 i.v. once daily (D) or i.p. once daily (E), or divided into three i.p. doses per day (B) from Days 0 to 5. On Day 4, 25 μg of FdUr and 1 μCi of [3H]thymidine were given i.p. Twenty-four h later, the lungs, liver, spleen, and mesenteric lymph nodes were harvested and counted in a gamma analyzer. The results are reported as the mean cpm of three mice per treatment group. The PI is presented at the top of each column.

![Image](image-url)

**Table 2.** Immunotherapy of 3-day pulmonary metastases from MCA-105 sarcoma using IL-2 given by different dose schedules and routes of administration

<table>
<thead>
<tr>
<th>Experiment</th>
<th>HBSS (A)</th>
<th>i.v. once daily (B)</th>
<th>i.p. once daily (C)</th>
<th>i.p. thrice daily (D)</th>
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<tr>
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<td>2,000</td>
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</table>

* Cumulative daily IL-2 dose was 150,000 units and 300,000 units per mouse in Experiments 1 and 2, respectively.

* Statistical analysis of differences between groups—in Experiment 1: A versus B, not significant; A versus C, not significant; A versus D, P < 0.005; B versus D, P < 0.05; C versus D, not significant; in Experiment 2: A versus B, not significant; A versus C, not significant; A versus D, P < 0.005; B versus D, P < 0.05; C versus D, P < 0.05.
IN VIVO GROWTH AND ANTITUMOR EFFECT OF LAK CELLS

![Fig. 3. In vivo proliferation of LAK cells in tissues of irradiated mice treated with IL-2 injected by different dose schedules and routes of administration. Whole-body irradiated (500 rads) mice received 10^5 LAK cells i.v. (or no cells) on Day 0, followed by 60,000 units of IL-2 administered i.v. once daily (iv qd), or i.p. once daily (ip qd), or divided into three i.p. injections per day (ip tid) from Days 0 to 5. Control mice were given HBSS i.p. thrice daily. On Day 4, 25 μg of FdUrd and 1 μCi of [125I]IDUrd were injected i.p. Twenty-four h later, the lungs, liver, spleen, and mesenteric lymph nodes were harvested and counted in a gamma analyzer. The results are presented as the mean cpm from three mice per treatment group. The PI is presented at the top of each column.](image)

![Table 3 Immunotherapy of 3-day pulmonary metastases from sarcomas using LAK cells and IL-2 given by different dose schedules and routes of administration.](table)

**Table 3.** Immunotherapy of 3-day pulmonary metastases from sarcomas using LAK cells and IL-2 given by different dose schedules and routes of administration.

MCA sarcoma cells were injected i.v. on Day 0, and IL-2 (or HBSS) was administered from Days 3 to 8. LAK cells (10^5) were injected i.v. on Days 3 and 6. On Day 14 or 15, lungs were harvested, and metastases were counted in a blinded fashion. The number of mice per group ranged from 4 to 6 in Experiment 1 and from 6 to 8 in Experiment 2.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Tumor</th>
<th>Dose schedule and route of IL-2 or HBSS administration</th>
<th>IL-2^a (mean no. of metastases)</th>
<th>LAK + IL-2 (mean no. of metastases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 MCA-101</td>
<td>i.v. once daily</td>
<td>250 (B)' 166 (C)</td>
<td>137 (F) 12 (G)</td>
<td></td>
</tr>
<tr>
<td>i.v. once daily</td>
<td>232 (D) 88 (E)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.p. once daily</td>
<td>250 (A)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.p. thrice daily</td>
<td>137 (F) 12 (G)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 MCA-105</td>
<td>i.v. once daily</td>
<td>162 (B) 60 (C)</td>
<td>137 (A) 75 (F) 3 (G)</td>
<td></td>
</tr>
<tr>
<td>i.v. once daily</td>
<td>148 (D) 52 (E)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.p. once daily</td>
<td>137 (A) 75 (F) 3 (G)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.p. thrice daily</td>
<td>137 (A) 75 (F) 3 (G)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a In Experiment 1, 4 x 10^5 MCA-101 cells and in Experiment 2, 3 x 10^5 MCA-105 cells injected i.v. on Day 0.

^b Cumulative daily dose of IL-2 was 30,000 units per mouse in both experiments.

^c Statistical analysis of differences between groups—in Experiment 1: A versus B, not significant; A versus C, not significant; A versus D, not significant; A versus E, P < 0.05; A versus F, P < 0.01; A versus G, P < 0.01; C versus G, P < 0.05; E versus G, not significant; in Experiment 2: A versus B, not significant; A versus C, not significant; A versus D, not significant; A versus E, not significant; A versus F, not significant; A versus G, P < 0.005; A versus H, P < 0.005; C versus G, P < 0.005; E versus G, P < 0.005.

Immunotherapy Using i.v. LAK Cells Plus IL-2 Administered i.v. or i.p. Once Daily, or i.p. Thrice Daily. We next studied the capacity of LAK cells to treat established pulmonary metastases in combination with IL-2 injected i.v. or i.p. once or i.p. thrice daily. Mice received 3 to 4 x 10^5 MCA-101 or MCA-105 sarcoma cells on Day 0 and 10^5 LAK cells (or no cells) on Days 3 and 6. From Days 3 to 8, IL-2 (or HBSS) was given as 30,000 units i.v. or i.p. once daily, or 10,000 units i.p. thrice daily. Lungs were removed on Day 14 or 15, and metastases were counted in a blinded fashion. In both experiments shown in Table 3, the comparison of the number of metastases in the HBSS group with the LAK cell plus IL-2 i.p. thrice daily group showed a significant immunotherapeutic effect, while the groups receiving IL-2 alone i.v. or i.p. once daily, or LAK cells plus IL-2 i.v. once daily did not achieve statistically significant differences compared to HBSS treatment. In Experiment 2, the combination of LAK cells plus IL-2 administered i.p. thrice daily reduced metastases (n = 3) significantly more than did LAK cells plus IL-2 i.v. once daily (n = 60, P < 0.005) or i.p. once daily (n = 52, P < 0.005). In both experiments the once daily dose of IL-2 injected i.p. appeared to have a greater impact on metastases than did the i.v. route.

In Vivo Proliferation of LAK Cells Stimulated by IL-2 Administered for Varying Durations. Since the i.p. thrice daily injection regimen for IL-2 achieved the optimal levels of lymphoid proliferation and the best therapeutic effect, we then examined whether the duration of IL-2 administration could affect the levels of proliferation. Mice used in these experiments were preirradiated with 500 rads in order to assess [125I]IDUrd uptake by the transferred LAK cells alone. LAK cells (10^5) or no cells were injected i.v. on Day 0, and 7500 units of IL-2 (or HBSS) were injected i.p. thrice daily for 1, 3, or 6 consecutive days beginning on Day 0. Animals were sacrificed, and lungs were removed to quantitate [125I]IDUrd uptake on Days 2, 4, 6, and 8. Each treatment group contained three mice per time point. Fig. 4 presents the data in mean cpm for the lungs of mice treated by the four treatment regimens. The administration of IL-2 for 6 days to mice which also received LAK cells induced the highest level of incorporation of the radiolabel (14,023 cpm on Day 4) when compared to animals receiving IL-2 for 3 days, 8,083 cpm on Day 2) or 1 day, 1,644 cpm on Day 4) plus LAK cells. Minimal levels of [125I]IDUrd uptake above background were noted in mice treated with LAK cells plus HBSS or IL-2 alone. LAK cells plus 6 days of IL-2 also yielded the most sustained levels of proliferation (4,806 cpm or PI = 7.9 on Day 8).

Expansion of LAK cells was clearly dependent upon the

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IN VIVO GROWTH AND ANTITUMOR EFFECT OF LAK CELLS

Table 4 Immunotherapy of 3-day pulmonary metastases from MCA-105 sarcomas using LAK cells and IL-2 administered for different durations

MCA-105 sarcoma cells (3 × 10^5) were injected i.v. on Day 0 and, 7500 units of IL-2 (or HBSS) were injected i.p. thrice daily beginning on Day 3. LAK cells (10^6) were administered i.v. on Day 3. On Day 13, lungs were harvested, and metastases were counted in a blinded fashion. The number of mice ranged from 5 to 9 per group.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>IL-2 (mean no. of metastases)</th>
<th>LAK + IL-2 (mean no. of metastases)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 (A) x 1 day</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>214</td>
<td>159</td>
</tr>
<tr>
<td>2</td>
<td>140</td>
<td>88</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>x 3 days</th>
<th>164</th>
<th>119</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x 6 days</td>
<td>70</td>
<td>6</td>
</tr>
</tbody>
</table>

*Statistical analysis of differences between groups—in Experiment 1: A versus B, not significant; A versus C, not significant; A versus D, not significant; A versus E, not significant; A versus F, P < 0.05; A versus G, P < 0.01; A versus H, P < 0.005; B versus H, P < 0.05; C versus H, P < 0.01; D versus H, P < 0.005; E versus H, P < 0.005; F versus H, P < 0.05; G versus H, not significant; in Experiment 2: A versus B, not significant; A versus C, not significant; A versus D, not significant; A versus E, not significant; A versus F, not significant; A versus G, P < 0.01; A versus H, P < 0.01; B versus H, P < 0.005; C versus H, P < 0.005; D versus H, not significant; E versus H, P < 0.05; F versus H, P < 0.005; G versus H, not significant.

continued administration of IL-2 as best demonstrated by the group receiving LAK cells plus IL-2 for 3 days. By Day 6 (or 3 days after the completion of IL-2 injections), [125I]IdUrd incorporation by LAK cells had reached background levels.

Immunotherapy Using LAK Cells and IL-2 Administered for 1, 3, or 6 Days. We next evaluated the therapeutic efficacy of LAK cells when IL-2 was given for 1, 3, or 6 consecutive days in the pulmonary metastasis model. Normal, nonirradiated mice received 3 × 10^5 MCA-105 sarcoma cells on Day 0 and one injection of 10^6 LAK cells i.v. (or no cells) on Day 3. IL-2 (7500 units) (or HBSS) was given i.p. thrice daily on Day 3, Days 3 to 5, or Days 3 to 8. Lungs were harvested on Day 13, and metastases were counted blindly. Table 4 shows the results of two experiments. The longer the duration of IL-2 therapy, whether administered in conjunction with LAK cells or, less dramatically, by itself, the greater the reduction in metastases. For example, in Experiment 1, LAK cells alone (HBSS) or followed by IL-2 for 1, 3, or 6 days resulted in a mean number of metastases of 159, 116, 32, and 11, respectively, compared to 214 metastases in HBSS alone-treated mice. A statistically significant reduction in lung tumors was seen in mice treated with LAK cells and 6 days of IL-2 compared to animals treated with LAK cells plus 1 day of IL-2, LAK cells plus HBSS, HBSS alone, and IL-2 alone for 1 or 3 days in both experiments.

In Vivo Proliferation and Immunotherapeutic Effects of One versus Two Doses of LAK Cells. We next studied the differences between one versus two doses of LAK cells plus IL-2 using the [125I]IdUrd assay to determine in vivo lymphoid proliferation and the metastasis model to examine therapeutic effects. In the proliferation assay, normal, nonirradiated mice received 10^6 LAK cells (or no cells) on Day 0 or Days 0 and 3, and 6000 units of IL-2 (or HBSS alone) i.p. thrice daily from Days 0 to 6. The lungs, livers, and spleens from three mice per treatment group were assayed for [125I]IdUrd incorporation on Days 3, 5, 7, and 9. Fig. 5 presents the mean cpm (top) and P1 (bottom) for the three tissues studied. In mice receiving LAK cells plus IL-2, two injections of cells produced higher [125I]IdUrd uptake than one injection in tissues on Day 5 and in the lungs and spleen on Day 7. In the liver on Day 7 and in all tissues on Day 9 (or 3 days following completion of IL-2 injections), there were no major differences between one or two doses of LAK cells plus IL-2, IL-2 alone, or the control group. As previously shown in Fig. 4, lymphoid proliferation was again strongly dependent upon continued administration of IL-2, since radio-label uptake declined in the IL-2 alone group and, particularly, in the LAK cell plus IL-2 group after discontinuation of IL-2 on Day 6.

Since two doses of LAK cells produced the higher degree of proliferation than did one injection, we compared the same treatments in the immunotherapy model. Mice bearing established pulmonary metastases received 10^6 LAK cells on Day 3 alone or on Days 3 and 6, and 34,000 units or 25,000 units (Table 5, Experiments 1 and 2, respectively) of IL-2 i.p. thrice daily (or HBSS) from Days 3 to 8. Pulmonary metastases were counted in a blinded fashion on Day 15 in Experiment 1 and on Day 13 in Experiment 2. The mean number of metastases for the treatment groups in two separate experiments is shown in Table 5 (14). In Experiments 1 and 2, two injections of LAK cells plus IL-2 markedly reduced metastases (n = 5 and 4, respectively) and achieved statistical significance when com-
Table 5 Immunotherapy of 3-day pulmonary metastases from MCA-105 sarcoma using IL-2 and one versus two injections of LAK cells

MCA-105 sarcoma cells (3 x 10^6) were injected i.v. on Day 0, and IL-2 (or HBSS) was injected i.p. thrice daily from Days 3 to 8. Lungs were removed on Days 15 and 13 in Experiments 1 and 2, respectively, and metastases were counted in a blinded fashion. The number of mice per group ranged from 6 to 9 in Experiment 1 and was 5 in Experiment 2.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>HBSS (A)</th>
<th>IL-2 (B)</th>
<th>IL-2 (1 injection) (C)</th>
<th>IL-2 (2 injections) (D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>211</td>
<td>103</td>
<td>49</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>214</td>
<td>186</td>
<td>43</td>
<td>4</td>
</tr>
</tbody>
</table>

* Adapted from Ref. 14.
* IL-2 dose was 34,000 units in Experiment 1 and 25,000 units in Experiment 2.
* LAK cells (10^6) were injected i.v. on Day 3 in Group C and on Days 3 and 6 in Group D.
* Statistical analysis of differences between groups—in Experiment 1: A versus B, not significant; A versus C, P < 0.01; A versus D, P < 0.005; B versus C, not significant; B versus D, P < 0.005; C versus D, P < 0.005; in Experiment 2: A versus B, not significant; A versus C, P < 0.01; A versus D, P < 0.01; B versus C, P < 0.05; B versus D, P < 0.01; C versus D, P < 0.01.

Mean no. of metastases

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>211</td>
<td>103</td>
<td>49</td>
<td>5</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>214</td>
<td>186</td>
<td>43</td>
<td>4</td>
</tr>
</tbody>
</table>

In the current work, we investigated the relationship between proliferation and antitumor activity of LAK cells, varying the schedule and route of IL-2 administration and the number of injections of LAK cells so as to maximize in vivo LAK cell growth and improve therapeutic results in the pulmonary metastasis model. From this work, we concluded that the IL-2-mediated antitumor activity of LAK cells was highly correlated with their proliferative activity in vivo. These experiments were of central importance in the selection of dose schedules currently used in our clinical trials with LAK cell immunotherapy (35).

Optimal proliferation and immunotherapy results in this study were highly dependent upon IL-2 pharmacokinetics. Several reports have investigated the serum half-life of IL-2 for material obtained from both supernatants of lectin-stimulated EL-4 thymoma cells (18, 26) and recombinant sources (25). Murine studies (25) of recombinant IL-2 administered i.v. have revealed a short half-life on the order of 1.6 min with no detectable activity after 2 h following a single dose of 10,000 units. The i.p. route for recombinant IL-2 resulted in lower peak concentrations but in a prolongation of detectable levels in the serum; serial measurements showed ≥10 units of IL-2 per ml for 3 to 4 h after 10,000 units given by the i.p. route. Because IL-2 is rapidly cleared from the serum, probably by renal tubular metabolism (26), the major factor determining the duration of measurable activity is related to release of IL-2 from the site of administration.

In the current study, we found that 100,000 units of IL-2 injected i.p. thrice daily yielded continually detectable levels of IL-2 in the serum, ranging from > 2000 units/ml 30 min after each dose to ≥6 units/ml just prior to each successive IL-2 dose. Thus, single daily injections of IL-2 by either the i.v. or i.p. route resulted in measurable IL-2 levels for a maximum of several hours, with higher peak levels after i.v. injection. If, however, IL-2 was administered i.p. in three doses evenly spaced throughout the day, IL-2 activity was maintained in the detectable range over the entire 24-h interval.

Having established the characteristic time courses for the different IL-2 routes and schedules, we then determined which IL-2 injection protocol provided optimal in vivo activity, as assessed by stimulation of lymphoid cell proliferation and antitumor effect. To quantitate proliferation, we utilized a method of in vivo labeling of the DNA of dividing cells by [125]IdUrd. Following its incorporation into cells, this γ-emitting thymidine analogue is not reutilized to any significant degree (22, 29, 30). Tissues were then easily counted, and radiolabel uptake was compared between groups. We also used our standard model (13) of pulmonary metastases from MCA sarcomas to determine the antitumor benefit of each therapy protocol tested.
We found that maximal levels of proliferation as well as optimal therapeutic results were highly correlated and were obtained when IL-2 was given i.p. thrice daily rather than i.p. or i.v. once daily. Comparable results were seen when IL-2 was administered together with exogenous LAK cells. In addition, the antitumor activity of LAK cells was enhanced if IL-2 was administered for 6 consecutive days rather than for shorter durations. Thus, the in vivo exposure of endogenous lymphocytes and transferred LAK cells to sustained, low concentrations of IL-2 was superior to brief, high peak levels of the lymphokine in promoting in vivo lymphoid cell expansion and tumor cytolysis. Similar findings have also been noted with LAK cell immunotherapy in a peritoneal sarcomatosis model where reduction of tumor load by LAK cells was more successful if the same total daily dose of IL-2 was given 4 times a day rather than twice or once a day.2 Furthermore, Cheever et al. (36), investigating specifically sensitized T-cells, have demonstrated that immune T-cells exhibited greater expansion in the spleen and ascites of congenic murine hosts when the same total s.c. dose of IL-2 was administered every 8 h rather than every 24 or 48 h. In addition, they found that the recovery of donor cells in spleen and ascites 8 days after transfer was greatest if IL-2 was injected i.p. rather than i.v.

The length of exposure of T-cells to IL-2 has important ramifications for T-cell growth both in vitro and in vivo (20, 23, 36, 37). In vitro, cellular expansion is dependent in part on IL-2 receptor induction and stimulation, which in turn is influenced by the concentration and duration of exposure to IL-2 (37). An association between T-cell expansion and function as mediated by IL-2 has been reported in vivo for long-term cultured T-cells (20, 23). Moreover, proliferation of LAK cells (22) as well as specifically immune T-cells (21, 23) is augmented in a dose-dependent manner by IL-2.

The benefit of low, sustained serum levels of IL-2, as generated by the i.p. thrice daily schedule and by prolonged administration of IL-2 over several days, may be related to IL-2 receptor kinetics (36). Initial dosing of mice with IL-2 in the current study presumably led to early saturation of IL-2 receptors on T-cells, including those on LAK cells (8). However, rapid appearance of newly created receptors on growing cells required that additional IL-2 be available to bind to these receptors to maintain maximal growth and function. Thus, single daily injections of IL-2 either by the i.v. or i.p. routes provided suboptimal conditions for T-cell expansion leaving IL-2 receptors unbound for significant periods of time.

The finding that two doses of LAK cells yielded higher levels of lymphoid proliferation than were noted following one injection is further evidence that maximization of in vivo LAK cell expansion is important for antitumor effect. This hypothesis is supported by the observation that IL-2 produces a dramatic increase in number of lytic units in mouse lungs following LAK cell transfer. This IL-2 effect is the result of expansion in numbers of cells with similar lytic activity and not due to augmentation of the lytic capacity per individual cell (22).

Finally, the importance of defining the optimal means by which to administer IL-2 has major importance for future application to the immunotherapy of human cancer (35). Early trials with IL-2 in humans have documented major toxicity due to fluid retention, resulting from a poorly understood capillary leak syndrome and renal insufficiency (38). Results from the murine tumor models suggest that the antitumor efficacy of LAK cells is directly related to IL-2 doses and schedules. The maximum daily dose of IL-2 achieved in humans has been 100,000 units/kg every 8 h for 3 to 4 days before limited by toxicity (35). In contrast, the minimum IL-2 requirement for producing a significant reduction in metastases using LAK cells in the mouse model has been 5,000 units i.p. thrice daily or approximately 250,000 units/kg/dose (13–17). In humans, larger amounts of IL-2 (up to 10⁶ units/kg) have been infused as a single dose with much less toxicity (38). The results described in the current paper, however, strongly suggest that IL-2 doses should be distributed throughout the day and should not be administered as single daily injections. Future investigations to enhance LAK cell proliferation and associated antitumor activity by altering IL-2 administration schedules in the murine model may have significance in the development of effective immunotherapies for human cancer.

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Immunotherapy of Murine Sarcomas Using Lymphokine Activated Killer Cells: Optimization of the Schedule and Route of Administration of Recombinant Interleukin-2

Stephen E. Ettinghausen and Steven A. Rosenberg


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