

REGRESSION OF ESTABLISHED PULMONARY METASTASES
AND SUBCUTANEOUS TUMOR MEDIATED BY THE
SYSTEMIC ADMINISTRATION OF HIGH-DOSE
RECOMBINANT INTERLEUKIN 2

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We have previously demonstrated (1–4) that the incubation of human peripheral blood lymphocytes or murine splenocytes in the lymphokine, interleukin 2 (IL-2)¹, leads to the generation of cells capable of lysing fresh syngeneic or autologous tumors in short-term chromium-release assays. These lytic cells have been termed lymphokine-activated killer (LAK) cells, and they exhibit broad lytic specificity for a variety of fresh tumors, but they do not lyse fresh normal cells. Recombinant IL-2 produced in *E. coli* and purified to apparent homogeneity is highly effective in generating LAK cells from both human and murine lymphocytes (5). We have recently demonstrated (6, 7) that the intravenous injection of LAK cells in conjunction with recombinant IL-2 can mediate the regression of established pulmonary metastases from a variety of sarcomas, as well as from the B16 melanoma.

Because recombinant IL-2 is capable of efficiently generating LAK cells *in vitro*, and because the systemic administration of LAK cells has potent antitumor activities, we have studied the possible use of high-dose recombinant IL-2 administered directly to tumor-bearing mice, aiming to generate LAK cells *in vivo* and mediate tumor regression. In these studies, we have shown that the systemic administration of high doses of recombinant IL-2 leads to the generation of LAK cells in the spleens of recipient mice and mediates the regression of established pulmonary metastases and subcutaneous implants from a variety of tumors. Tumor regression appears to be mediated by endogenously activated lymphocytes, presumably LAK cells, present at the tumor site and in the organs of mice receiving high-dose recombinant IL-2.

Materials and Methods

Animals. Female C57BL/6 mice were obtained from the Small Animal Section, Veterinary Resources Branch, National Cancer Institute, Bethesda, MD, or from The Jackson Laboratory, Bar Harbor, ME. All mice were 12–16 wk old when used in experiments.

Tumors. The MCA-105 and MCA-106 tumors were induced in our laboratory by

¹ *Abbreviations used in this paper:* HBSS, Hank's balanced salt solution; IL-2, interleukin 2; LAK, lymphokine-activated killer cells; NK, natural killer cells.

intramuscular injection of 0.1 ml of 0.1% 3-methylcholanthrene in sesame oil. These tumors were serially transplanted into syngeneic C57BL/6 mice by intramuscular injection of a tumor cell suspension prepared by mechanical means. At the first transplant generation, multiple vials of tumor were cryopreserved. For all experiments reported here, the tumor was used within the first seven transplant generations. When a tumor reached the seventh transplant generation, a new vial from the first generation was thawed and transplanted. These tumors grew progressively, and were invariably fatal in normal syngeneic mice.

The B16 melanoma is a long-transplanted tumor syngeneic to C57BL/6 mice. Multiple vials of tumor from a single tissue culture passage were cryopreserved, and a fresh vial was thawed for each experiment.

Preparation of Single-cell Suspensions from Solid Tumor. Intramuscular tumors were excised when they reached 1–1.5 cm in diameter, and were dissected free of necrotic tumor and connective tissue. The remaining tumor was minced into 2 mm fragments and digested, with constant stirring, in 40 ml of Hank's Balanced Salt Solution (HBSS) containing 4 mg deoxyribonuclease, 40 mg collagenase, and 100 U hyaluronidase (Sigma Chemical Co., St. Louis, MO) for 3 h at room temperature, as previously described (7). The supernatant cell suspension was then harvested, washed three times in HBSS, and adjusted to the desired concentration.

The B16 melanoma was thawed and placed in culture for ~18 h before harvesting the cell monolayer using 0.25% trypsin in Dulbecco's phosphate-buffered saline without calcium and magnesium. The cell suspension was washed three times, and suspended in HBSS at the desired cell concentration.

Tumor Therapy Protocol for Pulmonary Metastases. 2×10^5 – 3×10^5 tumor cells, in single-cell suspension in HBSS, were injected, in 1 ml volumes, into the tail veins of mice. After 3 d, micrometastases were present in the lungs and were invading into the pulmonary alveoli. By 10 d after tumor cell injection, grossly visible metastases were present on the surface of the lung. At 3 or 10 d after tumor cell injection, therapy with intraperitoneally injected IL-2 was begun. At 15–18 d after tumor cell injection, mice were ear-tagged, randomized, and killed. The lungs were removed by opening the thoracic cavity, injecting a 15% solution of India ink into the trachea, and "bleaching" with Fekette's solution, using a technique described previously (8). The white tumor nodules in the black lung were counted without knowledge of the treatment received. After all data were recorded the mice and their tissues were identified. All treatment groups contained at least five mice.

Tumor Therapy Protocol for Subcutaneous Tumor. 2×10^4 MCA-105 tumor cells, in single-cell suspension in HBSS, were injected, in 0.05 ml volumes, in the belly skin of mice. The tumor was injected as superficially as possible, raising a "bleb" in the skin. Animals were examined three times weekly, and perpendicular diameters were measured with a vernier caliper.

IL-2. The gene for IL-2 was isolated from a high-producer Jurkat cell line, inserted into *E. coli*, and expressed at high levels, as previously described (5). The IL-2 was purified to apparent homogeneity, as demonstrated by the presence of a single band of ~15,000 mol wt. The recombinant IL-2 for these studies was kindly supplied by the Cetus Corporation, Emeryville, CA. IL-2 activity was measured using a standard bioassay, as previously described (9). The endotoxin level in purified preparations was <0.1 ng/ 10^6 U IL-2, as measured by a standard Limulus assay.

Assessment of LAK Activity in Splenocytes. Spleens were excised and dissociated into single-cell suspensions in HBSS by pressing with the blunt end of a 10-ml plastic syringe plunger. Erythrocytes were lysed in ammonium chloride–potassium lysing buffer (Media Production Section, NIH) and were washed three times in HBSS. The resultant cell suspension was filtered through a double layer of no. 100 nylon mesh (Nitex), and resuspended in medium for assay of cytotoxicity against fresh MCA sarcoma cells in a standard 4-h chromium-release assay.

Statistical Analysis. Statistical analyses were performed using a Wilcoxon rank sum test (10). All *P* values reported are two-sided values.

TABLE I
Induction of LAK Activity in Spleens of Mice Treated with Recombinant IL-2

IL-2*	Lytic activity against MCA-102	
	100:1	E:T [‡] 20:1
<i>U</i>		% [§]
0	-1 ± 2	-3 ± 3
75,000	14 ± 1	-2 ± 1
100,000	17 ± 2	1 ± 2
150,000	19 ± 1	9 ± 1
200,000	21 ± 4	11 ± 1
400,000	32 ± 2	16 ± 4

* IL-2 was administered for 5 d in 0.5 ml i.p., approximately every 8 h.

[‡] Effector/target ratio in 4-h ⁵¹Cr-release assay.

[§] Percent lysis ± SEM.

Results

Systemic Administration of Recombinant IL-2 Generated LAK Cells in the Spleen. To test the ability of recombinant IL-2 to induce LAK cell activity *in vivo*, mice were injected with varying doses of IL-2 approximately every 8 h for 5 d. The IL-2 was given by intraperitoneal injection in 0.5 ml HBSS, and was injected at approximately 8:00 am, 4:00 pm, and 11:00 pm of each day. On the morning of the sixth day, mice were killed, and LAK activity in spleens was assessed using a fresh MCA sarcoma single-cell suspension as target. As shown in Table I, increasing doses of recombinant IL-2 resulted in a successive increase in the lytic activity in the spleen against fresh sarcoma cells.

Systemic Administration of Recombinant IL-2 Reduced the Number of Established 3-d Pulmonary Metastases. 3 d after the intravenous injection of MCA-105 tumor cells in the tail vein, mice were treated with varying doses of recombinant IL-2 in 0.5 ml i.p., approximately every 8 h for 5 d. Mice were killed at 15 d, and the number of pulmonary metastases in the lung was determined. A characteristic experiment is shown in Fig. 1. Recombinant IL-2 at a dose of 50,000 or 100,000 U was capable of significantly reducing the number of pulmonary metastases. Mice receiving either HBSS alone, or 5,000 or 20,000 U of IL-2 had a mean number of metastases of >250, 212, and 214, respectively (no significant differences between these groups). Mice receiving 50,000 or 100,000 U of IL-2 had a mean of 19 and 61 metastases, respectively ($P = 0.002$ and $P = 0.003$, respectively, compared to HBSS group).

The ability of high-dose recombinant IL-2 to reduce the number of established 3-d pulmonary metastases was highly reproducible. The results of 36 consecutive experiments, using doses between 3,000 and 170,000 U of recombinant IL-2 administered three times daily for 5 d is shown in Fig. 2. The percent reduction in pulmonary metastases of mice treated with IL-2 at three dosage ranges is compared to mice treated with HBSS alone. Mice receiving between 3,000 and 8,000 U of IL-2 intraperitoneally three times daily had $5.7 \pm 5.0\%$ (mean ± SE) reductions in pulmonary metastases, mice receiving 20,000–50,000 U of IL-2

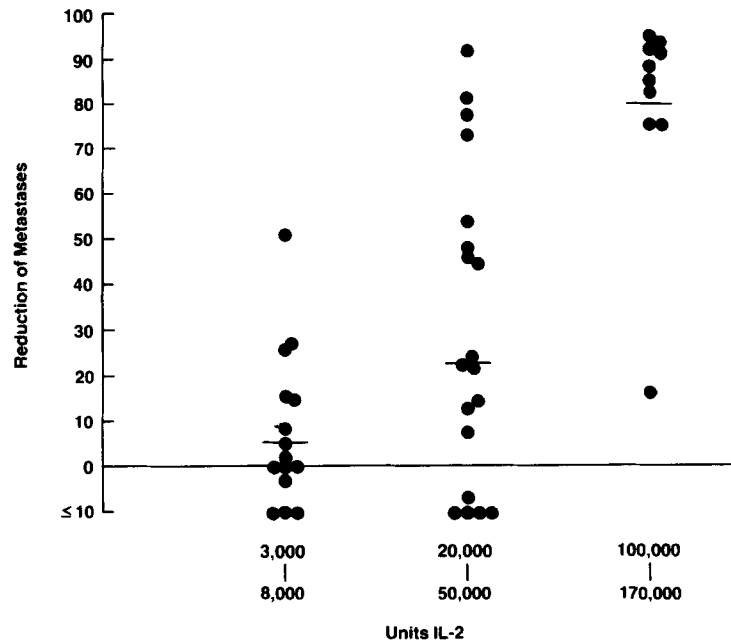


FIGURE 2. Results of 36 consecutive experiments evaluating the ability of varying doses of recombinant IL-2 to reduce day 3 pulmonary metastases. Each dot represents a separate experimental measurement in which mice receiving IL-2 were compared to mice treated with HBSS alone. Therapy with varying doses of IL-2 was initiated 3 d after the intravenous injection of MCA-105 sarcoma cells. IL-2 was administered intraperitoneally approximately every 8 h for 5 d. Doses of 100,000–170,000 U IL-2 significantly reduced pulmonary metastases in 9 of 10 experiments.

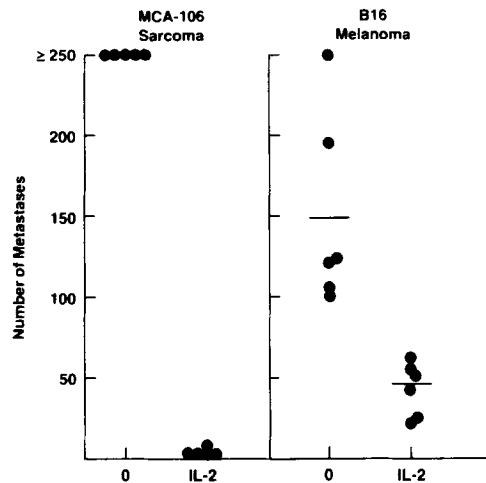


FIGURE 3. Reduction of pulmonary metastases of the MCA-106 sarcoma (*left*) and the B16 melanoma (*right*) by administration of recombinant IL-2. 3 d after intravenous injection of either MCA-106 sarcoma or B16 melanoma cells, therapy was initiated with 100,000 U recombinant IL-2 given intraperitoneally approximately every 8 h for 5 d. On day 15, mice were killed and pulmonary nodules were counted. Each dot represents the measurement of a single mouse. A significant reduction in the number of pulmonary metastases was seen in the treatment of both tumors when IL-2 was administered ($P = 0.006$ for the MCA-106 sarcoma, and $P = 0.005$ for the B16 melanoma).

TABLE II
Reduction of Established (10-d) Pulmonary Metastases in Mice by Treatment with Recombinant IL-2

IL-2*	Number of metastases					Mean	P [‡]
U							
Exp. 1							
0	>250,	>250,	>250,	>250,	>250	>250	—
1,000	>250,	>250,	150,	138,	103	178	0.10
4,000	>250,	>250,	>250,	113,	78	188	0.18
20,000	48,	37,	24,	5,	5	24	0.007
100,000	33,	19,	13,	11,	3	16	0.008
Exp. 2							
0	>250,	>250,	>250,	197,	189	227	—
1,000	>250,	>250,	>250,	164,	126	208	0.72
6,000	>250,	>250,	207,	147,	2	171	0.50
20,000	>250,	22,	5,	2,	0	56	0.07
100,000	32,	6,	4,	1,	0	9	0.01

* IL-2 was administered in 0.5 ml i.p., three times daily for 5 d, starting 10 d after tumor injection.

‡ Wilcoxon rank sum test of group receiving IL-2 compared to group receiving no IL-2 (two-sided).

times a day for 5 d. In these experiments, mice were usually killed on the 18th day after tumor cell injection, and the number of pulmonary metastases was determined. Two representative dose-titration experiments are shown in Table II. In exp. 1, a statistically significant reduction in pulmonary metastases was seen at 20,000 U (mean = 24) and 100,000 units (mean = 16) of IL-2 administered three times daily for 5 d, compared to mice treated with HBSS (mean >250; $P = 0.007$ and $P = 0.008$, respectively). In exp. 2, a reduction in the number of pulmonary nodules was again seen at 20,000 (mean = 56) and 100,000 U (mean = 9) of IL-2 administered three times daily, compared to mice treated with HBSS (mean = 227; $P = 0.07$ and $P = 0.01$, respectively).

Reduction of 10-d pulmonary metastases by IL-2 therapy was highly reproducible. The results of 15 consecutive experiments using recombinant IL-2 to treat 10-d metastases are shown in Fig. 4. 1–6,000 U IL-2 reduced the number of pulmonary nodules by $34.6 \pm 13.3\%$. 20,000–50,000 U IL-2 reduced 10-d pulmonary metastases by $89.4 \pm 3.9\%$, and 100,000–200,000 U IL-2 reduced pulmonary metastases by $79.6 \pm 4.7\%$.

A comparison of results using IL-2 to treat day 3 (Figs. 1 and 2) and day 10 (Table II and Fig. 4) pulmonary metastases suggested that lower doses of IL-2 were more effective against day 10 tumors than against day 3 tumors. Two experiments designed to test this observation directly are presented in Table III. MCA-105 tumor was injected intravenously on day 0, and beginning either 3 or 10 d later, therapy with IL-2 was initiated for 5 d. All mice were killed on day 15, and the number of pulmonary tumors was determined. In exp. 1 (Table III), control mice (HBSS alone) had a mean of 56 metastases compared to a mean of 47 metastases in mice that started IL-2 treatment at 6,000 U three times daily on day 3 ($P = 0.23$), and 8 metastases in mice that started IL-2 treatment on day 10 ($P = 0.02$). Similarly, in exp. 2, control mice had a mean of 45 metastases compared to 54 metastases in mice starting treatment with 20,000 U IL-2 three

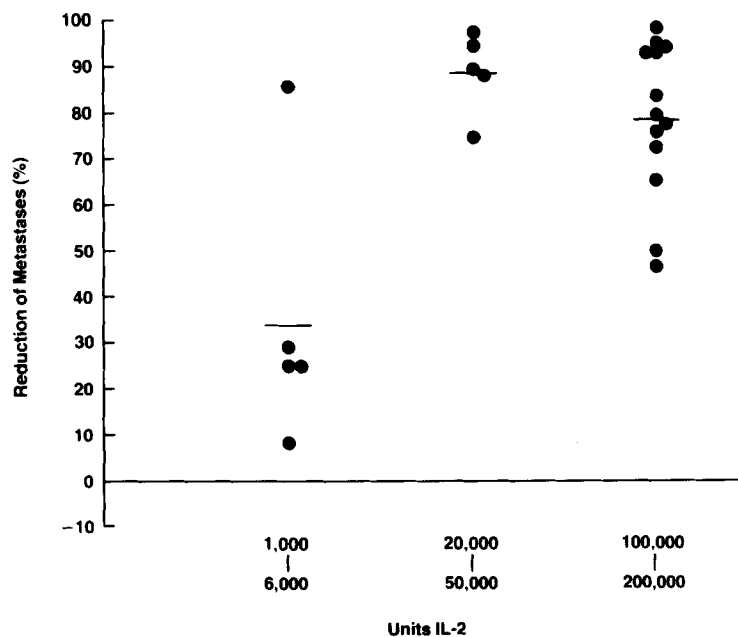


FIGURE 4. Results of 15 consecutive experiments evaluating the ability of treatment with recombinant IL-2 to reduce the number of 10-day pulmonary metastases. Each dot represents a separate experimental measurement in which mice receiving IL-2 were compared to mice treated with HBSS alone. 10 d after intravenous injection of MCA-105 sarcoma cells, therapy was initiated with varying doses of recombinant IL-2 given intraperitoneally approximately every 8 h for 5 d. On days 15–18, mice were killed and pulmonary metastases were counted. Doses >20,000 U administered on this schedule were consistently able to reduce the number of pulmonary metastases compared to that of control mice.

TABLE III
Treatment of Established (Day 3 and 10) Pulmonary Metastases with Recombinant IL-2

IL-2*	Mean number of metastases, with treatment begun on:	
	Day 3	Day 10
<i>U</i>		
Exp. 1		
0	56	—
6,000	47 (<i>P</i> = 0.23) [‡]	8 (<i>P</i> = 0.02)
Exp. 2		
0	45	—
20,000	54 (<i>P</i> = 0.79)	6 (<i>P</i> = 0.006)

* IL-2 was administered in 0.5 ml i.p. three times daily for 5 d, starting either 3 or 10 d after tumor injection.

[‡] Wilcoxon rank sum test comparing group receiving IL-2 to group receiving no IL-2 (two-sided).

TABLE IV
Treatment of Established (Day 3 and 10) Pulmonary Metastases with Recombinant IL-2

IL-2*	Mean number of metastases, with treatment begun on:			
	Day 3		Day 10	
	Normal mice	Irradiated [‡] mice	Normal mice	Irradiated mice
U				
0	>250	>250	172	>250
100,000	61	>250	7	>250
	(P = 0.003) [§]		(P = 0.02)	

* IL-2 was administered in 0.5 ml i.p. three times daily for 5 d, starting either 3 or 10 d after tumor injection (separate experiments).

[‡] 500 rad total body irradiation administered 4 h before tumor cell injection.

[§] Wilcoxon rank sum test comparing group receiving IL-2 to group receiving no IL-2 (two-sided).

times daily on day 3 ($P = 0.79$) and 6 metastases in mice starting treatment on day 10 ($P = 0.006$). These experiments demonstrated that day 10 metastases from the MCA-105 sarcoma were more susceptible to IL-2 therapy than were day 3 metastases.

Antitumor Effect of Recombinant IL-2 Was Eliminated in Preirradiated Mice. To evaluate the role of host factors in the antitumor response mediated by recombinant IL-2, mice received 500 rad total body irradiation from a ¹³⁷Cs source, 4 h before injection of tumor into the tail vein. 3 or 10 d later, therapy was begun with 100,000 U of IL-2, injected intraperitoneally three times daily for 5 d (Table IV). When treatment was initiated on day 3, IL-2 treatment reduced the number of pulmonary metastases in normal, nonirradiated mice from a mean of >250 metastases in controls to 61 metastases in IL-2-treated mice ($P = 0.003$). In preirradiated mice, however, treatment with IL-2 resulted in no significant reduction in the number of pulmonary metastases (>250 metastases in both groups). Similarly, in mice beginning treatment on day 10, a significant effect of IL-2 was seen in normal, nonirradiated mice (172 metastases in controls, compared to 7 in IL-2-treated mice; $P = 0.02$), although no reduction in pulmonary metastases was seen in preirradiated mice (>250 metastases in both groups). This inability of recombinant IL-2 to mediate a reduction in the number of metastases in irradiated mice was shown in three additional experiments, and in two experiments using thymectomized mice that received 1,000 rad total body irradiation and were reconstituted with T cell-depleted bone marrow.

Histologic Assessment of the Regression of Pulmonary Metastases Mediated by Recombinant IL-2. A histologic assessment of the lungs during the regression of pulmonary metastases mediated by IL-2 was conducted by sequentially killing mice every other day, beginning at the initiation of IL-2 treatment on day 3 or day 10 after intravenous tumor injection. All studies were performed in duplicate. Control groups consisted of tumor-bearing mice receiving a comparable volume of HBSS, and of otherwise healthy mice treated with IL-2 alone. All tissues were fixed in 10% buffered formalin and processed routinely in paraffin. Sections were stained with hematoxylin and eosin. Histologic interpretations were made by a pathologist (C. Reichert).

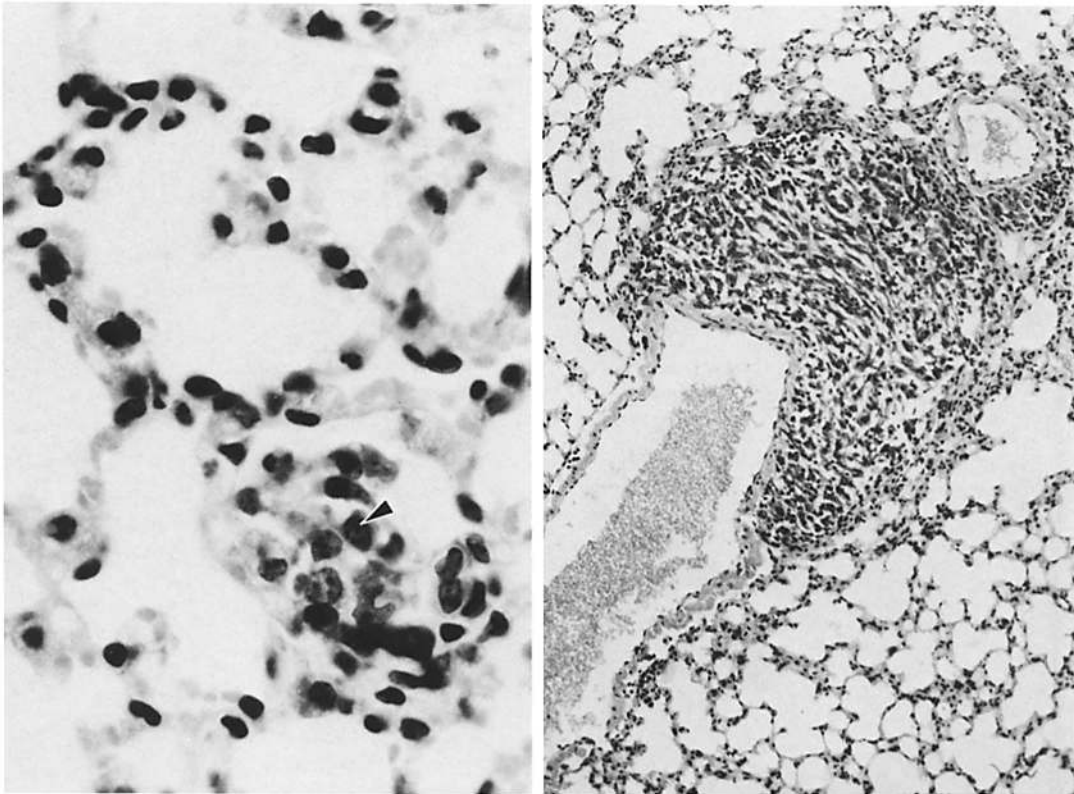


FIGURE 5. Histologic appearance of pulmonary metastases from the MCA-105 sarcoma (*left*). Typical appearance of a 3-d-old, untreated pulmonary metastasis. Growth of hematogenously disseminated tumor cells (arrow) has resulted in expansion of an alveolar septum. (Hematoxylin and eosin, original magnification $\times 860$). 10-d-old, untreated pulmonary metastasis located within a dilated perivenular lymphatic channel (*right*). (Hematoxylin and eosin, original magnification $\times 130$).

3 d after tail vein injection of tumor cells, numerous micrometastases, ranging up to 10 tumor cell diameters, were detected in necropsy specimens of lung. Most of the tumor cell aggregates were located within alveolar septae; many had ruptured into the alveolar air spaces. The characteristic appearance of one such metastasis is shown in Fig. 5, *left*. In those experiments where IL-2 immunotherapy was delayed until 10 d after tumor injection, the tumor nodules were grossly visible, and, microscopically, ranged up to 50 tumor cell diameters. At this time most of the tumor nodules were located within perivenular and peribronchial lymphatics, and subpleural spaces (Fig. 5, *right*). By 4 d after the thrice daily intraperitoneal injections of 100,000 U IL-2, many activated lymphocytes were noted within the lungs, primarily within lymphatic channels of the interlobular pulmonary septae (Fig. 6, *left*). Cytologically, these lymphocytes had larger nuclei, more irregular nuclear contours, and more open chromatin patterns (Fig. 6, *right*) than typical small, mature lymphocytes. Mitoses were frequent among IL-2 stimulated lymphocytes. After 6 d of IL-2 administration, numerous activated lymphocytes were seen circulating within alveolar capillaries (Fig. 7), as well as

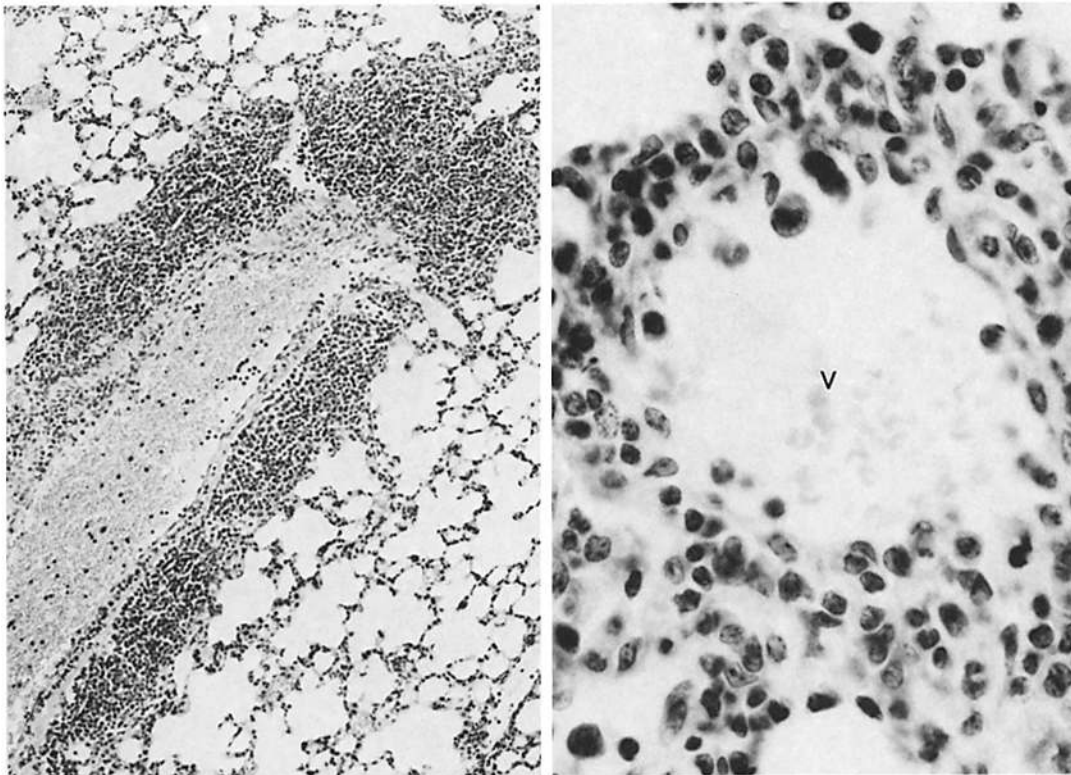


FIGURE 6. Mice repeatedly stimulated by intraperitoneal injections of IL-2 show a marked accumulation of activated lymphocytes, primarily in a perivascular lymphatic distribution (*left*). (Hematoxylin and eosin, original magnification $\times 130$). High-power view of activated lymphocytes in a dilated perivascular lymphatic within the lung of an IL-2-treated mouse (*right*). Some of the lymphocytes are seen within the lumen of a pulmonary vein (V). (Hematoxylin and eosin, original magnification $\times 860$).

within multiple parenchymal organs. Overall numbers of lymphocytes appeared to be increased over non-tumor bearing controls that received IL-2.

With respect to the histologic appearance of tumor in IL-2-treated vs. control mice, the primary differences were in the number, size, and extent of lymphocytic infiltration of the tumor metastases (Fig. 8). Micrometastases contained lymphocytes within the centers of the tumor masses as well as at the perimeters, with far greater numbers of lymphocytes present in the IL-2-treated mice. Increasing numbers of activated lymphocytes were apparent in the tumor nodules as therapy with IL-2 proceeded and lesions regressed (Fig. 8, *right*). Those few tumor nodules that failed to regress in IL-2-treated mice showed a predilection for subpleural localization, but were otherwise histologically indistinguishable from untreated controls.

Treatment of Subcutaneous Sarcoma by Recombinant IL-2. Two treatment models were studied to evaluate the ability of recombinant IL-2 treatment to affect the growth of subcutaneous tumors. First, 2×10^4 MCA-105 sarcoma cells were injected subcutaneously, and 3 d later IL-2 therapy was given for ~ 5 d. No

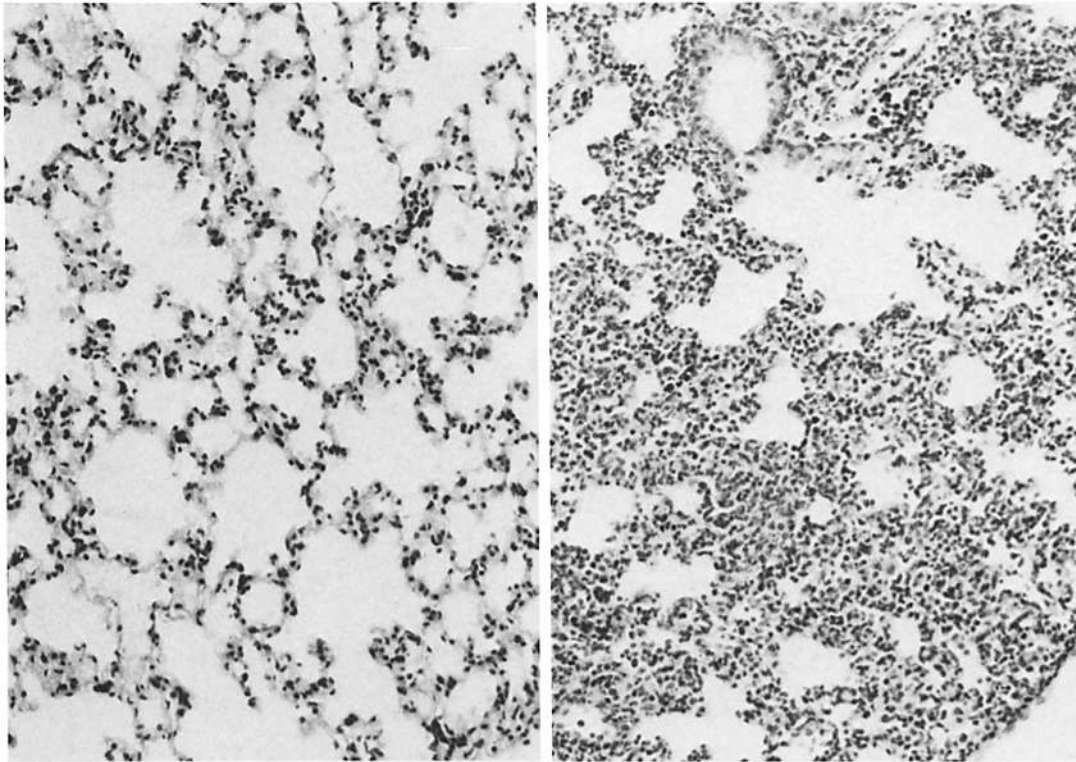


FIGURE 7. Appearance of lung parenchyma 6 d after the initiation of IL-2 immunotherapy (*right*). Note the diffuse lymphoid infiltrate. A normal mouse lung is provided for comparison (*left*). (Hematoxylin and eosin, original magnification $\times 220$).

palpable tumor was evident at the onset of therapy. A characteristic experiment is shown in Fig. 9. The latent period of tumor appearance, defined as the time to development of palpable tumor in more than half of the mice, was prolonged by 16 d ($P = 0.02$) by treatment with 170,000 U IL-2 i.p. three times a day. The results of four consecutive experiments are shown in Table V. The latent period was prolonged from ~ 10 d to 19–35 d in the four experiments. In each experiment, all animals treated with HBSS developed tumors, while 17–40% of mice treated with recombinant IL-2 never developed tumors, over a 2-mo observation period.

Recombinant IL-2 could also be used to successfully treat palpable tumors. By 10 d after tumor cell injection, tumors were palpable, with a diameter of 2–4 mm. Initiation of IL-2 therapy at this time caused a dramatic reduction in the growth rate of tumors, as well as the disappearance of tumors in some animals. A representative experiment is shown in Fig. 10. In this experiment, mice received 200,000 U i.p. thrice daily for 13 d, although occasional doses were skipped if mice appeared lethargic. Tumor grew progressively in all six mice treated with HBSS alone. In mice receiving IL-2, tumor growth was substantially inhibited in all six mice, and tumor regressed completely in three mice.

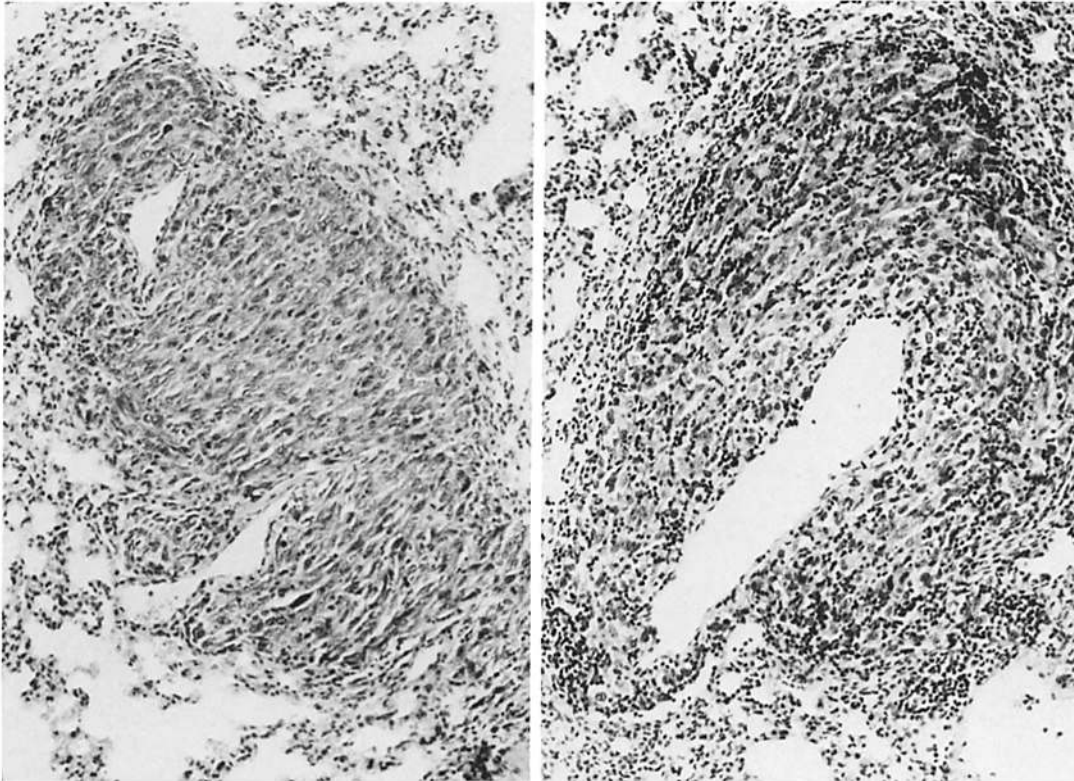


FIGURE 8. Appearance of an 18-d-old pulmonary metastasis from an untreated mouse (*left*). (Hematoxylin and eosin, original magnification $\times 170$). Appearance of an 18-d-old pulmonary metastasis from a mouse treated with IL-2 (*right*). Note the increased numbers of lymphocytes associated with the spindle-shaped sarcoma cells. (Hematoxylin and eosin, original magnification $\times 170$).

Discussion

IL-2 is a lymphokine, with a mol wt of $\sim 15,000$, that is produced by T lymphoid cells (11). In vitro, IL-2 has a broad range of immunoregulatory activities that appear to stem from the ability of IL-2 to expand selected subpopulations of T cells that bear IL-2 receptors (12). The systemic administration of IL-2 in vivo also has a broad range of immunologic effects, including the induction of specific T helper cells, cytotoxic cells, and autoantibody production in nude mice (13–15), and the enhancement of the restoration of immune function in irradiated rats (16) or in mice treated with cyclophosphamide (17). System injection of IL-2 is capable of specifically enhancing alloimmune responses in either normal or primed (18, 19) mice, and has been shown to enhance the antitumor activity of adoptively transferred, expanded, IL-2-dependent, specifically immune lymphocytes (20, 21).

In 1980, we first noted an unusual effect of IL-2 on resting lymphoid populations in vitro. Incubation of either murine splenocytes or human peripheral blood lymphocytes from normal or tumor-bearing hosts in lymphokine preparations containing IL-2 resulted in the generation of lymphoid cells capable of

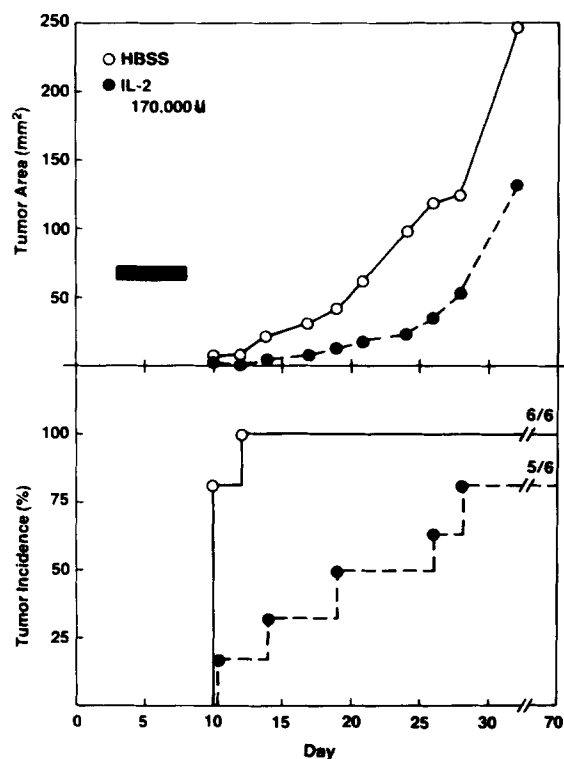


FIGURE 9. Treatment with recombinant IL-2 inhibited growth of day 3 subcutaneous tumor. 2×10^4 MCA-105 sarcoma cells were injected subcutaneously, and 3 d later therapy was initiated with 170,000 U recombinant IL-2 given intraperitoneally approximately every 8 h for 5 d. Mean tumor size (the product of perpendicular diameters) is presented in the upper panel, and time of tumor appearance is presented in the lower panel. The black bar indicates the interval of treatment with IL-2. A significant delay in tumor appearance was seen in animals treated with recombinant IL-2 ($P = 0.02$).

TABLE V
Treatment of Intradermal Tumor with Recombinant IL-2

Exp.	IL-2*	Duration of treatment	Latent period [‡]		Final tumor incidence (number of mice with tumor/total) [§]	
			HBSS	Recombinant IL-2	HBSS	Recombinant IL-2
	<i>U</i>	<i>d</i>	<i>d</i>			
1	100,000	3-8	11	19	5/5	3/5
2	100,000	3-8	10	26	6/6	5/6
3	100,000	3-8	10	21	6/6	5/6
4	200,000	3-6	10	35	6/6	3/4
	400,000	3-6	—	35	—	3/5

* IL-2 was administered in 0.5 ml i.p. three times daily.

[‡] Latent period was defined as the time to development of palpable tumor in more than half of mice.

[§] Mice observed for >2 mo.

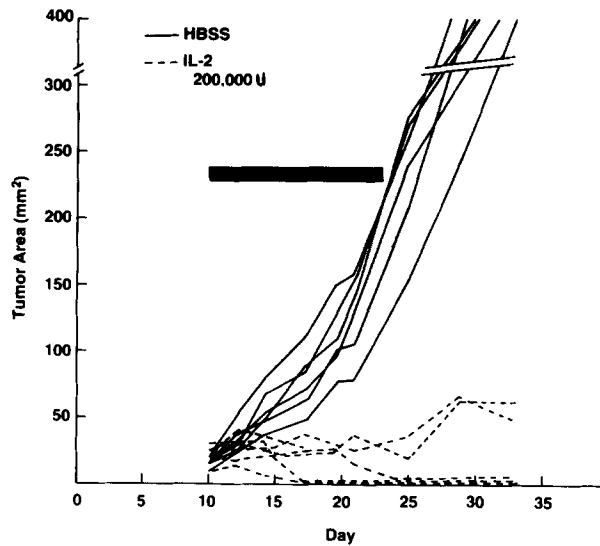


FIGURE 10. Treatment of established subcutaneous tumor with recombinant IL-2. 10 d after the injection of 2×10^4 MCA-105 sarcoma cells, palpable nodules were present in the subcutaneous area. At this time therapy was initiated with 200,000 U of recombinant IL-2 administered intraperitoneally approximately every 8 h for 13 d. The black bar indicates the interval of treatment with IL-2. Toxicity due to IL-2 administration necessitated skipping occasional doses. Each line represents the growth of tumor in an individual mouse. Whereas tumor grew progressively in all control mice injected with HBSS alone, all mice receiving IL-2 exhibited decreased tumor growth, and tumor disappeared completely in three mice.

lysing fresh autologous or syngeneic tumor cells in short term chromium-release assays (1-4). We have subsequently characterized these lytic cells, and have termed them LAK cells (4, 22). In both mouse and human, LAK cells are lytic for a broad range of autologous, syngeneic, and allogeneic fresh tumor cells, but they do not lyse fresh normal cells, such as peripheral blood lymphocytes, or lung or liver cells. This broad lytic specificity has been seen both in direct lytic assays, as well as by using cold-target inhibition techniques. LAK cells appear to represent a lytic system distinct from that of natural killer (NK) cells and cytotoxic T lymphoid cells, although this is an area of some controversy. Precursors of LAK cells in humans are negative for characteristic markers of NK cells, such as OKM1 and Leu-7, and are negative for T cell markers such as Leu-1, OKT3, OKT4, and OKT8. The mature LAK effector cell generated following incubation in IL-2, however, bears many of the characteristic markers of classic cytolytic T lymphoid cells. In humans, LAK effector cells are OKM1⁻, OKT3⁺, 4⁻, 8⁺, and are Leu-1⁺ (22, 23). In the mouse, LAK effector cells are Thy-1⁺, Lyt-1⁻, 2⁺ (4). IL-2 alone appears to be sufficient for the induction of LAK cells, because highly purified IL-2 preparations obtained from recombinant *E. coli* are capable of generating both human and murine LAK cells (5). The relationship of LAK cells to other forms of anomalous cytotoxicity induced in vitro by a variety of other techniques, such as incubation with lectins (24), pooled allogeneic stimulation (25-27), or incubation in heterologous serum (28), is unclear, although it is possible that IL-2 stimulation represents a final common pathway for generating these lytic activities in lymphocytes in vitro.

Because of the high degree of lytic specificity of LAK cells for fresh tumor, and due to the relative ease of generation of LAK cells, we explored the ability of LAK cells to mediate antitumor effects when injected *in vivo*. We have previously demonstrated (6) that the injection of LAK cells was capable of mediating the regression of established pulmonary metastases from B16 melanoma that were induced either by the intravenous injection of B16 melanoma cells, or from spontaneous metastases of B16 melanoma growing in the hind limb. More recently (7), we have demonstrated that the systemic administration of LAK cells to mice bearing a variety of sarcomas resulted in the regression of established day 3 and day 10 pulmonary metastases, when LAK cells were injected in combination with the systemic administration of recombinant IL-2.

The recent availability of recombinant-derived IL-2 resulting from the insertion into *E. coli* of the gene coding for human IL-2 has provided large amounts of highly purified IL-2 for *in vivo* studies (5). Preliminary experiments, in which mice received continuous infusions of recombinant IL-2 either intraperitoneally or subcutaneously, using an osmotic pump, indicated that LAK cells could be generated in the spleen of mice *in vivo* (29). The *in vivo* antitumor effect of LAK cells in combination with recombinant IL-2, and the suggestion that systemically administered recombinant IL-2 generated LAK cells *in vivo* have led us to explore the ability of high-dose IL-2 administration alone to mediate the regression of established syngeneic tumors.

Large doses of IL-2 were required in these studies. We previously (29, 30) showed that the serum half-life of natural and recombinant IL-2 in mice was ~2 min after intravenous injection (29, 30). The primary mechanism for IL-2 clearance appeared to be metabolism in the renal tubules, since clamping of both renal pedicles extended the half-life of IL-2 ~40-fold, while ureteral ligation did not significantly change the half life of IL-2. Further, we were not able to isolate IL-2 from urine of mice given high doses of this material (30).

In these studies, we have demonstrated that the intraperitoneal administration of high doses of recombinant IL-2 was capable of generating LAK cells in the spleens of mice. Intraperitoneal administration of 100,000 U recombinant IL-2 approximately every 8 h resulted in the generation of significant levels of lytic activity against a variety of MCA-induced sarcomas in chromium-release assays. Using this dosage regimen, sequential measurement of serum levels of IL-2 in these mice revealed IL-2 levels of 3,000–4,000 U/ml in serum for the first several hours after intraperitoneal injection, followed by a drop in titer to ~5–10 U/ml in serum by the time the next dose was given (data not shown). These findings are in accord with our previous observations (29, 30) concerning the absorption of IL-2 administered intraperitoneally.

Administration of high doses of recombinant IL-2 consistently mediated the regression of established tumor in both lungs and subcutaneous areas. In the treatment of mice 3 d after tumor injection, at a time when pulmonary metastases were firmly established in the lung, ~100,000 U IL-2 given intraperitoneally thrice daily was required to mediate the inhibition of pulmonary metastases from the MCA-105 tumor. At this IL-2 dose, significant inhibition of the syngeneic MCA-106 sarcoma as well as of the syngeneic B16 melanoma was also seen. Administration of 3,000–8,000 U IL-2 had little effect on 3-d pulmonary

metastases, and in only occasional experiments did 20,000–50,000 U IL-2 injected intraperitoneally three times a day mediate antitumor effects. At doses of 100,000 U IL-2 intraperitoneally three times daily, >70% reduction of pulmonary metastases was seen in 9 of 10 experiments.

Somewhat surprisingly, pulmonary metastases established 10 d before the onset of therapy appeared to be more sensitive to treatment with IL-2. By 10 d after intravenous injection, the tumor nodules were grossly visible in the lung, and were comprised of many hundreds of tumor cells. 20,000 U recombinant IL-2 intraperitoneally three times daily was capable of substantially inhibiting pulmonary metastases in this 10-d tumor model; dosages from 20,000 to 50,000 U IL-2 showed >70% reduction of pulmonary metastases in five of five experiments. In two experiments, a direct comparison of the treatment of day 3 and day 10 metastases with relatively low doses of recombinant IL-2 confirmed this increased sensitivity of larger metastases to the effects of recombinant IL-2. Since mice that were treated 10 d after tumor injection had grossly visible metastases on the lung surface that could be counted, these experiments indicated that IL-2 not only inhibited tumor growth, but was capable of actually mediating the regression of established metastases. This phenomenon was also apparent in the treatment of subcutaneous sarcomas. The treatment of mice 3 d after the subcutaneous injection of MCA-105 sarcoma in the belly skin resulted in a substantial increase in the latency period of tumors, and cured 15–40% of mice. When treatment was instituted 10 d after injection of subcutaneous tumor, at a time when tumor was easily palpable, tumor growth was substantially inhibited in all mice, and in ~50% of mice, tumor regression was noted, with long term cure of these mice. Mice cured by these means were then capable of resisting a subsequent tumor challenge (data not shown).

The mechanism by which IL-2 mediates the regression of established tumor is not clear. Although IL-2 is capable of mediating the generation of LAK cells *in vivo*, we have no direct evidence that these LAK cells are responsible for the antitumor effects seen. A possible mechanism of the antitumor activity of IL-2 involves the *in vivo* generation of LAK cells, either in lymphocytes infiltrating the tumor, or at other sites, with subsequent migration to the tumor. The increased number of lymphocytes in larger, compared to smaller, pulmonary metastases may explain the increased sensitivity of larger tumors to IL-2 administration. It is quite clear, however, that the antitumor effects of IL-2 are eliminated if mice received a prior dose of 500 rad total body irradiation. The inability of IL-2 to mediate antitumor effects in these irradiated mice implies that the IL-2 is not directly mediating tumor regression, but, rather, is acting through a radiosensitive host component.

Further evidence supporting the role of LAK cells in the rejection of established tumor comes from a histologic assessment of regressing pulmonary metastases. Within 2 d after the injection of IL-2 into mice bearing 10-d pulmonary metastases, lymphocytes that look activated are seen infiltrating the periphery of the tumor. With progressive treatment, activated lymphocytes are seen infiltrating throughout resolving tumor. By 6–8 d after the onset of therapy, many tumor nodules had undergone complete regression. Activated lymphocytes have been noted not only in the lung, but in the liver, spleen, and kidney as well.

While we have as yet no evidence that these activated lymphocytes have lymphokine-activated killing activity, an attractive hypothesis is that the systemic administration of IL-2 is leading to the generation of LAK cells *in vivo* from precursor lymphocytes within the growing tumor, which then mediate tumor destruction. Larger tumors may have an increased relative number of infiltrating lymphoid cells, and this may account for the increased sensitivity of day 10 tumors to IL-2 therapy, compared to the sensitivity of day 3 tumors.

Other possible mechanisms for the antitumor effect of IL-2 should be mentioned. It has been shown (20, 21) that IL-2 is capable of enhancing the antitumor activity of specifically immune cells. If small numbers of specifically immune lymphoid cells are present in growing tumor nodules, then IL-2 may be capable of expanding the numbers of these cells *in vivo*. *In vitro* stimulation of lymphoid cells with IL-2 leads to the secretion of interferon, and it is likely that the systemic administration of IL-2 leads to the secretion of a variety of other lymphokines from T lymphoid cells *in vivo* (31–33). In our initial clinical trials of recombinant IL-2 administration to humans, circulating levels of interferon have been noted. It is thus possible that IL-2 mediates antitumor effects, not by the induction of LAK cells, but rather by its ability to mediate the secretion of other lymphokines by T lymphoid cells.

The antitumor effects of IL-2 we have noted *in vivo* and the ready availability of recombinant IL-2 makes treatment with this lymphokine an attractive approach to the treatment of human cancer. We have recently completed phase I clinical studies of the administration of natural and recombinant IL-2 by both continuous infusion and bolus injection into humans, and we are currently initiating trials to explore the antitumor efficacy of maximally tolerated doses of recombinant IL-2 in humans with advanced cancer. The use of recombinant IL-2 directly, as well as in conjunction with LAK cells, is an attractive new approach to the treatment of human cancer that warrants active investigation.

Summary

Incubation of resting lymphoid cells with recombinant interleukin 2 (IL-2) *in vitro* leads to the generation of lymphokine activated killer (LAK) cells capable of lysing fresh tumor cell suspensions in short-term chromium-release assays. Our previous studies (7) have demonstrated that the injection of LAK cells plus low doses of recombinant IL-2 were capable of inhibiting the growth of pulmonary metastases. We have now explored the ability of high doses of recombinant IL-2, administered systemically, to generate LAK cells *in vivo*, and to mediate antitumor effects directly. Administration of increasing doses of recombinant IL-2 intraperitoneally resulted in the generation of LAK cells in the spleens of recipient mice. Doses of 100,000 U recombinant IL-2 administered intraperitoneally approximately every 8 h for 5 d were capable of dramatically inhibiting established 3-d pulmonary metastases from the MCA-105 and MCA-106 syngeneic sarcomas and the syngeneic B16 melanoma in C57BL/6 mice. Grossly visible metastases present at 10 d after tumor injection also underwent regression following IL-2 therapy. Surprisingly, established 10 d pulmonary metastases were more susceptible to the effects of IL-2 than were the smaller 3 d pulmonary metastases. All antitumor effects of the systemic administration of recombinant

IL-2 were eliminated if mice received prior treatment with 500 rad total body irradiation. The administration of high doses of recombinant IL-2 was also capable of inhibiting the growth of 3-d established subcutaneous tumors from the MCA-105 sarcoma, and of mediating the inhibition of growth and regression of established palpable subcutaneous MCA-105 sarcomas. Lymphocytes, which appeared morphologically to be activated, were present at the site of regressing tumor, and it appears that the mechanism of the antitumor effect of recombinant IL-2 administered systemically is via the generation of LAK cells *in vivo*, although this hypothesis remains to be proven. The ready availability of high doses of recombinant human IL-2, and the demonstration of antitumor effects seen in animal models have led us to the initiation of the clinical trials of recombinant IL-2 in humans.

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