Adoptive Immunotherapy of Established Pulmonary Metastases with LAK Cells and Recombinant Interleukin-2

Abstract. *The activation of human peripheral blood leukocytes or murine splenocytes with interleukin-2 (IL-2) generated cells that were lytic in vitro for a variety of fresh tumor cells. The adoptive transfer of such lymphokine-activated killer (LAK) cells to mice with established pulmonary sarcoma metastases was highly effective in reducing the number (and size) of these tumor nodules when combined with repeated injections of recombinant IL-2. These findings provide a rationale for clinical trials of the infusion of human LAK cells generated with recombinant IL-2 as well as Phase I trials of the infusion of recombinant IL-2 systemically into humans.*

We reported previously that activation of human peripheral blood leukocytes or murine splenocytes with interleukin-2 generates cells that are lytic for a variety of fresh tumor cells (*1–4*). These lymphokine-activated killer (LAK) cells are lytic for fresh autologous, syngeneic, and allogeneic primary or metastatic tumor cells (regardless of natural killer cell susceptibility) but not for normal cells. In the mouse, the cell surface phenotype of the LAK effector cell is Thy-1$^+$, Lyt-1$^-$2$^+$ (*4*). Likewise in humans, LAK cells express the OKT-3 and OKT-8 phenotypic markers characteristic of allogeneic cytotoxic T lymphocytes (*3*). Because LAK cells have apparent tumor specificity in vitro and are easily generated, we have undertaken a series of studies to test the efficacy of LAK cells in vivo against pulmonary metastases from multiple sarcomas of relatively poor immunogenicity. Further, the availability of large amounts of recombinant interleukin-2 (RIL-2) with known biologic activities (*5*) now makes it feasible to evaluate the antitumor effectiveness of this lymphokine in vivo either alone or in concert with LAK cells. We now show that, although LAK cells or RIL-2 alone are relatively ineffective, the two administered in combination result in significant reduction in the number of established pulmonary sarcoma metastases in mice.

The syngeneic tumors used in these studies were induced in our laboratory by intramuscular injection of 3-methylcholanthrene and were always used in the first seven transplant generations. C57BL/6 mice, injected intravenously with MCA-105 sarcoma cells, were given LAK cells, fresh normal splenocytes (NL), or no lymphocytes on days 3 and 6 of tumor growth. Multiple foci of pulmonary sarcoma metastases were clearly established at the time of LAK cell infusion as assessed by histologic examination of lung tissue removed for biopsy 3 days after tumor injection. For this experiment we used LAK cells derived from 3-day cultures of normal syngeneic splenocytes incubated with RIL-2. From days 3 through 8, the mice received intraperitoneal injections of Hanks balanced salt solution (HBSS) or 25,000 units of RIL-2 (specific activity, 3×10^6 to 4×10^6 unit/mg; Cetus) in HBSS every 8 hours. We assayed the effect of these treatments on metastasis development on day 13. In the groups receiving either RIL-2 alone, or normal splenocytes plus HBSS or plus RIL-2, or LAK cells plus HBSS, no significant antimetastatic effect occurred compared to the group given no therapy (that is, HBSS alone) (Table 1). In contrast, the group receiving the combination of LAK cells and RIL-2 had significantly fewer lung metastases (mean = 13) when compared to the group receiving HBSS alone (mean = 141). The pulmonary metastases that remained in mice receiving LAK cells plus RIL-2 were generally smaller in size than those in lungs of the group receiving no treatment. In several experiments we noticed a trend, although not statistically significant, in which RIL-2 alone or LAK cells alone appeared to reduce the number of metastases (see Table 2, experiments 1, 3, and 9). Thus, although LAK cells or RIL-2 alone had little if any impact, the combination of these two treatments significantly reduced the number (and the size) of pulmonary metastases of MCA-105 sarcoma (Fig. 1). Further, this reduction was dependent on LAK cells because the combination of fresh normal splenocytes plus RIL-2 was ineffective.

We next studied the kinetics of generation of LAK cells in vitro that would be effective in vivo. Normal C57BL/6 splenocytes were activated in vitro with RIL-2 for 1, 2, 3, or 4 days. These LAK cells were then tested for their therapeutic effect in vivo against MCA-105 pulmonary metastases. Normal splenocytes had to be cultured for at least 2 days in RIL-2 to generate LAK cells capable of reducing the number of metastatic nodules when combined with RIL-2; LAK cells cultured with RIL-2 for 2, 3, and 4 found 2×10^5 to 5×10^5 cells to be optimal. A single cell suspension was prepared for injection by excising fresh sarcoma growing intramuscularly in syngeneic mice (second to seventh passage in vivo), mincing the tissue in HBSS, and stirring it in a triple-enzyme mixture [deoxyribonuclease (4 mg), collagenase (40 mg), and hyaluronidase (100 units; Sigma) in HBSS (35 ml)] for 3 hours at room temperature. The dispersed tumor cells were collected, passed through 100-gauge nylon mesh (Nitex), and washed three times in HBSS. The cells were incubated for 2 hours at room temperature and were washed once more before adjusting the mixture to the appropriate cell concentration for intravenous injection. LAK cells or normal splenocytes (NL) were suspended in HBSS (10^8 cells per milliliter) and injected into the tail veins of recipient mice 3 and 6 days after tumor injection. Normal splenocytes were prepared as described (*8*). LAK cells were generated by placing 5×10^8 normal C57BL/6 splenocytes (erythrocytes lysed osmotically) into 750-ml flasks (Falcon) containing 175 ml of complete medium [RPMI 1640 with nonessential amino acids (0.1 m*M*), gentamicin (50 μg/ml), sodium pyruvate (1 μ*M*), 2-mercaptoethanol (5×10^{-5}*M*), streptomycin (100 μg/ml), penicillin (100 unit/ml), glutamine (0.03 percent), and heat-inactivated fetal calf serum (10 percent)] and 250,000 units of RIL-2 (*5*). The flasks were incubated horizontally at 37°C in a 5 percent CO_2 atmosphere for 72 hours. The LAK cells were then harvested, passed over a Ficoll filter (Lympholyte-M; Cedarlane) to remove dead cells, washed three times, and resuspended in HBSS for intravenous injection. Portions of LAK cells were tested for cytotoxicity in vitro (*1, 4*). From days 3 through 8, mice were given intraperitoneal injections (0.5 ml every 8 hours) of either HBSS or 25,000 units of RIL-2 in HBSS; units of RIL-2 activity were determined as described (*5, 11*). The amount of RIL-2 was selected on the basis of dose-response experiments; 4000 units of RIL-2 was ineffective in augmenting LAK activity in vivo. On day 13 after tumor induction, the mice were ear-tagged, randomized, and killed for enumeration of metastatic pulmonary nodules as described (*13*). Complete enumeration (blind fashion) of metastases was possible because of the discrete white nodules formed on the blackened surface of the lungs (insufflated with a 15 percent solution of India ink) when bleached by Fekette's solution. Statistical difference between group treated with HBSS and with LAK + RIL-2 : $P < 0.02$ (two-sided P value from a standard normal table; Wilcoxon rank-sum test).

Treatment	Metastases on day 13 (mean number)
HBSS	141
RIL-2	115
NL + HBSS	215
NL + RIL-2	138
LAK + HBSS	75
LAK + RIL-2	13

Table 1. Effect of lymphokine-activated (LAK) cells and recombinant interleukin-2 (RIL-2) on established pulmonary sarcoma metastases in mice (n = 5 per group). C57BL/6 mice (12 to 18 weeks old) were injected intravenously with MCA-105 sarcoma cells suspended in Hanks balanced salt solution (HBSS; Gibco) (3×10^5 cells per milliliter). We had initially titrated the dose of tumor cells required to give countable numbers of pulmonary metastases after 12 to 15 days and

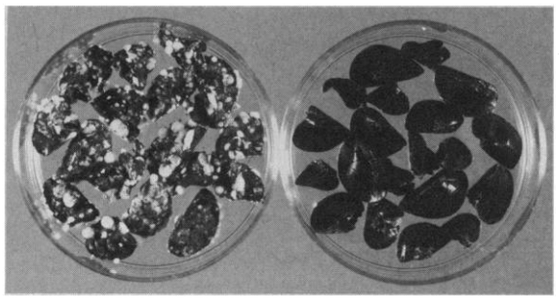

Fig. 1. Representative lungs of four mice (see experiment 8 in Table 2) from the group given HBSS (left) or LAK plus RIL-2 (right).

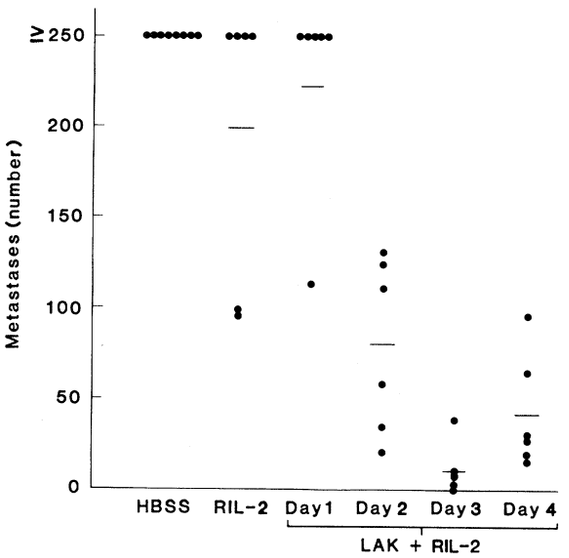

Fig. 2. LAK cells that reduce established pulmonary sarcoma metastases are generated by normal splenocytes cultured for 2 days in vitro with RIL-2. C57BL/6 mice (10 to 12 weeks old) were injected intravenously with 3×10^5 MCA-105 cells. They were then infused intravenously with 10^8 LAK cells on days 3 and 6 after tumor injection. The LAK cells were generated in vitro (see legend to Table 1 for details) from normal splenocytes cultured in RIL-2 for 1, 2, 3, or 4 days before infusion. Mice serving as controls received no LAK cells. RIL-2 (25,000 units) was administered intraperitoneally three times a day from days 3 through 8; controls received HBSS. The number of pulmonary metastases was then counted on day 15 after MCA-105 injection. Statistical differences between groups treated with HBSS and with LAK + RIL-2: day 1, N.S.; day 2, $P = 0.006$; day 3, $P = 0.003$; day 4, $P = 0.006$ (two-sided P values from a standard normal table; Wilcoxon rank-sum test).

Table 2. Immunotherapeutic effect of LAK cells plus RIL-2. Experimental conditions were as described in the legend to Table 1. MCA-105 sarcoma cells were used for all experiments except 9a (MCA-102) and 9c (MCA-106). The number of sarcoma cells injected intravenously into recipient mice was 3×10^5 (experiments 1 through 8), 5×10^5 (experiments 9a to 9c), or 2×10^5 (experiment 10). Sarcomas were induced by 0.1 ml of 1 percent 3-methylcholanthrene in sesame oil injected intramuscularly (*14*). Experiment 1 is also shown in Table 1; experiment 8 is shown in greater detail in Fig. 2.

Experiment*	Days after tumor injection	Metastases (mean number)				P§
		HBSS	LAK + HBSS†	RIL-2‡	LAK + RIL-2	
1	13	141	75	115	13	<0.02
2	15	250	250	250	62	<0.004
3	14	122	69	39	1	<0.14
4	14	233	191	221	62	<0.006
5	18	250	166	250	21	<0.003
6	15	194		220	52	<0.001
7	15	250		250	21	<0.004
8	15	250		199	11	<0.003
9a	15	26		31	5	<0.009
b	15	168		164	17	<0.02
c	15	85		46	12	<0.03
10	14	151			11	<0.004

*n = 5 to 9 in all experiments except the group given LAK + RIL-2 in experiment 3 (n = 3). †LAK cells (10^8) were injected intravenously on days 3 and 6 after tumor injection in experiments 1 through 5, 7, 8, and 10; on day 3 in experiment 6; and on days 3, 6, and 9 in experiments 9a to 9c. On day 6 in experiment 6, 5×10^7 cells were injected intravenously; on days 3 and 6 in experiments 9a to 9c, 10^8 cells were injected intraperitoneally. ‡RIL-2 was injected intraperitoneally on days 3 through 8 after tumor injection (experiments 1 through 8 and 10) or on days 3 through 9 (experiments 9a to 9c) as described (see legend to Table 1). Mice received (per injection) 10,000 units of RIL-2 in experiments 9a to 9c; 20,000 units in experiments 5, 7, and 10; 25,000 units in experiments 1, 2, 4, 6, and 8; and 125,000 units in experiment 3. §Significance of difference between groups treated with HBSS and LAK + RIL-2 (two-sided P values from a standard normal table; Wilcoxon rank-sum test). Lungs with metastases too numerous to count were assigned an arbitrary value of 250 because we were able to count reliably only numbers of metastases approaching 250.

days appeared to be relatively similar in effectiveness (mean = 80, 11, and 42, respectively) compared to HBSS (mean = 250) (Fig. 2).

The results of ten separate experiments (Table 2) showed that, in most instances, pulmonary metastases were significantly reduced but not eliminated by the combined treatment. In experiment 9 (Table 2) we tested the efficacy of LAK cells plus RIL-2 in the treatment of pulmonary metastases of sarcomas other than MCA-105. For this experiment we used two additional MCA sarcomas generated in our laboratory: MCA-102, which exhibited no immunogenicity in standard transplantation tests (*6*), and the more immunogenic MCA-106. Treatment with LAK cells plus RIL-2 significantly reduced the numbers of established pulmonary metastases of both MCA-102 and MCA-106 sarcomas as well as the MCA-105 sarcoma (Table 2). This treatment also reduced the number of hepatic metastases that developed along with pulmonary metastases in most of the mice, although we have not evaluated the efficacy of LAK cell transfer on tumors at other sites.

Our results have led us to the following conclusions. (i) Human RIL-2 produced in *Escherichia coli* generates murine LAK cells with antimetastatic capacity in vivo. (ii) The combination of LAK cells plus RIL-2 is necessary for the successful reduction of established pulmonary sarcoma metastases. We and others have previously shown that administration of nonrecombinant IL-2 in vivo can enhance the antitumor therapeutic efficacy of adoptively transferred, long-term cultured, specifically sensitized T lymphocytes (*7*). It is of interest that, in our earlier studies, LAK cells alone could substantially inhibit pulmonary metastases of the B16 melanoma (*8*), although little impact of LAK cells alone was seen on MCA sarcoma metastases in the absence of IL-2. LAK cells may require exogenous IL-2 for their prolonged survival and maintenance of activity in vivo to have therapeutic value against sarcoma metastases. Recently, administration of IL-2 in vivo has been shown to induce the growth of antigen-activated T lymphocytes in vivo (*9*). The cytotoxicity of LAK cells in vitro rapidly diminishes when IL-2 is no longer provided (*6*). (iii) This immunotherapeutic approach appears to be effective against multiple sarcomas, including one of no detectable immunogenicity. (iv) A reduction in the number of pulmonary metastatic nodules can be achieved without prior treatment of the hosts with chemotherapy or radiation, approaches known

to be effective in decreasing those suppressor mechanisms activated in vivo by tumor antigens that interfere with specific adoptive immunotherapy (*10*). We realize, however, that administering low doses of cyclophosphamide or radiation may have a synergistic effect when combined with LAK cells plus RIL-2.

The combination of normal splenocytes and RIL-2 or RIL-2 alone was ineffective in reducing established pulmonary sarcoma metastases. This finding suggests that LAK cells are not generated in vivo in sufficient amounts by intraperitoneal administration of RIL-2 at the doses used in these experiments. The serum half-life of IL-2 in vivo is short (3 to 4 minutes) in the murine host (*11*), and serum inhibition of IL-2 activity, as reported previously (*12*), may limit the usefulness of IL-2 administration in vivo.

Because LAK cells are nonspecifically generated by activation with IL-2 alone, they are more easily obtainable than lymphoid cells specifically sensitized in vivo or in vitro to unique tumor antigens. This is particularly important when considering human tumors that have poor immunogenicity and in which the availability and the preparation of suitable human tumor material for sensitization in vitro is a major problem. By using recombinant IL-2, human LAK cells with properties in vitro identical to those of murine LAK cells can readily be generated in vitro (*2, 3, 5*). Clinical trials of the infusion of human LAK cells generated with RIL-2 as well as Phase I trials of the infusion of RIL-2 systemically into humans have recently begun.

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Inhibition of Collagen Fibril Formation in Vitro and Subsequent Cross-Linking by Glucose

Abstract. *Glucose inhibits collagen fibril formation in vitro. A linear dose response was observed, with half-maximum inhibition of fibril formation occurring at 50 mM glucose. Nonfibrillar collagen cannot be cross-linked by lysyl oxidase, an enzyme that catalyzes the initial cross-linking reaction. The degree of decreased fibril formation correlated with the loss of ability of the collagen to serve as a substrate for lysyl oxidase. Collagen that is not cross-linked is unstable and more susceptible to collagenolytic attack. Interference with collagen cross-linking and more rapid degradation may explain the decreased amounts of interstitial collagen and the poor healing of wounds associated with diabetes mellitus.*

A compromised vascular supply due to thickened capillary basement membranes is the mechanism usually invoked to explain the slow and poor healing of wounds in diabetic patients. However, there is a lower content of interstitial collagen in wounds (*1*) and the skin (*2*) of diabetics than in normal subjects. The tensile strength of wounds and the collagen content of wound chambers is reduced in streptozotocin-induced diabetic rats as compared to control animals (*1*). The mechanism by which this occurs is unknown, but accelerated catabolism of interstitial collagen in streptozotocin-induced diabetic rats probably acts in this process (*3*).

The rate of degradation of interstitial collagen is related to the degree of collagen cross-linking. Cross-linked collagen is more resistant to collagenase degradation than the polymer that is not cross-linked (*4*). Fibril formation is the critical step in the cross-linking of collagen (*5*), and glucose and other sugars have been shown to inhibit collagen fibril formation in vitro (*6*). We now describe our experiments to test the hypothesis that glucose may decrease cross-linking through the inhibition of fibril formation and present evidence that decreased cross-linking and enhanced susceptibility to proteolytic attack may be the mechanism of the connective tissue defect in diabetes.

The effect of glucose on collagen cross-linking was investigated with the use of an in vitro model. Isotopically labeled native collagen was obtained from cultured embryonic chick calvaria incubated in vitro with [6-^{3}H]lysine. The initial reaction in the cross-linking of collagen by the enzyme lysyl oxidase is the formation of ε-aldehydes from the ε-amino groups in certain lysyl and hydroxylysyl residues (*7*). Formation of aldehydes at the 6 position of lysyl or hydroxylysyl residues of the [6-^{3}H]lysine-labeled collagen substrate results in release of ^{3}H from that position and formation of ^3H_2O (*7*). The amount of released ^3H_2O correlates directly with the formation of aldehydes, which are the actual precursors of the lysine-derived cross-linkages in collagen (*7*). Therefore, by measuring the amount of released ^3H_2O, we estimated the rate of aldehyde formation obtained under our experimental conditions.

Lysyl oxidase activity is dependent on the physical state of the collagen substrate. This activity is high with precipitated collagen fibrils and lower with soluble collagen (*5*). The effect of glucose on fibril formation and the subsequent substrate activity was measured by incubating the substrate with various concentrations of glucose at 37°C for 1 hour and then adding lysyl oxidase and incubating the mixture again. The amount of ^3H_2O released after the reaction with lysyl oxidase was suppressed in the presence of glucose (Fig. 1). A linear relation between glucose concentration and dose response was observed up to 100 m*M*. Glucose inhibited release of ^3H_2O at concentrations as low as 5 m*M*, and half-maximum inhibition occurred at 50 m*M*. At 200 m*M*, the amount of ^3H_2O released was less than 10 percent of the control reaction.

To determine whether glucose affected the cross-linking reaction by acting directly on the inhibiting lysyl oxidase, we added glucose to the isotopically labeled collagen substrate after the collagen had been precipitated and reconstituted as a fibril. The rate of ^3H_2O release was not affected by the presence of glucose, even at concentrations as high as 500 m*M* (Fig. 2). To determine whether the inhibitory effect of glucose was reversible, we added glucose to the substrate and