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Antitumour efficacy of lymphokine-activated killer cells loaded with ricin against experimentally induced lung metastases*

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Summary. Adoptive transfer of tumour-specific T lymphocytes loaded with ricin into tumour-bearing mice exerts a transient therapeutic effect against locally induced tumours [Cerundolo et al. (1987) Br J Cancer 55: 413]. As transferred cells preferentially locate in the lung, we studied the therapeutic effect of ricin-loaded, lymphokine-activated killer (LAK) cells on lung metastases induced by M4 or B16-F1 (F1) tumour cell injection. In vitro studies demonstrated that ricin-treated LAK cells were about 100-fold more efficient than untreated LAK cells in inhibiting growth of the ricin-sensitive M4 tumour cell line. This effect was most likely due to the released ricin, as treated and untreated LAK cells inhibited the relatively toxin-resistant F1 cell line to the same extent. Ricin treatment did not alter the tissue distribution of intravenously (i.v.) injected LAK cells, which selectively localized in the lung early after inoculation, whether or not metastases were present. Adoptive transfer experiments showed that ricin-treated LAK cells were significantly more efficient than untreated LAK cells in inhibiting M4- but not F1-induced lung metastases. These results indicate that LAK cells are able to deliver a therapeutic concentration of antineoplastic compounds directly to the lung.

Key words: LAK cells – Ricin – Lung metastases

Introduction

The immunotherapeutic efficacy of interleukin-2(IL-2)-activated killer (LAK) cells alone or in combination with recombinant (r)IL-2 has been extensively studied in experimental tumour models as well as in patients with advanced cancers [17, 25, 27, 28]. Large numbers of LAK cells in combination with high amounts of rIL-2, which must be transferred to achieve maximal anti-tumour activity, often cause severe toxic effects, which limit the widespread use of this therapeutic approach in clinical trials. To enhance the efficacy of antineoplastic agents, alternative strategies were proposed, such as the use of liposomes [12, 13, 30] or red blood cells [9, 31], to deliver toxin or drugs selectively to the tumour site. Therapeutic agents encapsulated in liposomes or red blood cells, on the one hand, are protected from attack by inactivating factors and, on the other, are released slowly and locally, which might reduce their toxicity. However, carriers used so far lack tumour cell specificity, and are mostly trapped in the liver, thus allowing limited therapeutic applications. Numerous studies have addressed the possibility of conferring specificity to the carriers; among these, the strategy of liposome conjugation with antitumour monoclonal antibodies (immunoliposomes) [16, 18] is, however, vitiated by the moderate in vivo stability of immunoliposomes, as well as their inability to extravasate and reach solid tumours or metastases [23, 30].

We recently showed that T lymphocytes specific for Moloney murine sarcoma virus (M-MSV) can internalize ricin, a plant toxin, and release it without loss of its toxicity [6]. We also observed that transfer of a low number of M-MSV-specific, ricin-treated T lymphocytes into tumour-bearing hosts exerted a transient therapeutic effect on locally induced sarcomas [6], comparable to that achieved using large doses of untreated cells [5].

A major problem common to all adoptive immunotherapeutic approaches based on the use of lymphocytes activated in vitro is the migration pattern of the transferred cells, which, following intravenous (i.v.) injection, localize principally in the host's lungs, and then redistribute in the liver and spleen [8, 19]. Nevertheless, in our previous therapeutic model, even if only a few tumour-specific ricin-treated lymphocytes reached peripherally localized tumours, an evident therapeutic effect was achieved.

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In this study, we utilized the preferential distribution of adoptively transferred cells to the lungs to explore the therapeutic potential of ricin-treated LAK cells against pulmonary metastases that had been induced by i.v. injection of highly metastasizing tumour cell lines. We found that transfer of toxin-treated LAK cells was associated with a significant reduction in the number of M4-induced metastases, compared to control groups injected with untreated LAK cells or free ricin. This therapeutic effect was most likely due to the ricin delivered by the LAK cells, as metastatic growth of the relatively ricin-resistant B16-F1 (F1) cell line was unaffected.

Materials and methods

Mice. Inbred C57BL/6 (B6) and DBA/2 6- to 8-week old mice were purchased from the Charles River Laboratories (Calco Como, Italy). The weight of the B6 mice used in the adoptive immunotherapy experiments ranged between 15 g and 17 g/mouse.

Tumour cell lines. P815 (H-2^d), a chemically induced mastocytoma, was maintained by weekly passages of the ascites form in syngeneic DBA/2 recipient mice. The M4 tumour cell line (H-2^b), kindly provided by Dr. A. Mantovani, Mario Negri Institute, Milano, Italy, was originally derived from spontaneous metastases of benzopyrene-induced sarcoma mFS6 [22]. The B16-F1 (F1) tumour cell line (H-2^b), obtained from the Cancer Institute, Bologna, Italy, was originally established from lung metastases induced by the B16-A melanoma [11]. Both the M4 and F1 tumour cell lines were maintained in monolayer cultures in 250-ml tissue-culture flasks (Falcon 3024, Falcon, Los Angeles, Calif.) in complete medium. Complete medium consisted of Dulbecco's modified essential medium (D-MEM, Flow Laboratories, Irvine, Scotland, UK) supplemented with L-glutamine (2 mM final concentration) HEPES (10 mM final concentration), 2-mercaptoethanol (50 μM), antibiotics and 10% heat-inactivated fetal calf serum (FCS, Flow Laboratories).

For immunotherapy experiments, M4 and F1 cells were thawed from our cryopreserved stock, and grown in complete medium for approximately 1 week before injection into syngeneic mice.

Toxin. Ricin, extracted from the seeds of *Ricinus communis* [1] and purified to homogeneity, was kindly provided by Prof. F. Stirpe, University of Bologna, Italy. To evaluate its uptake and release from LAK cells, 50 μg ricin in 0.1 M sodium borate buffer (pH 8.5) was iodinated by Bolton Hunter reagent (Amersham International Ltd., UK) and then passed through a Sephadex G-25 column to separate free ^{125}I from the labelled protein. The specific activity of the iodinated ricin was 1.2×10^3 cpm/ng.

LAK cell generation and loading with ricin. LAK cells were generated by culturing B6 spleen cells at 3×10^6 cells/ml with 100 U/ml rIL-2 (Glaxo Institute for Molecular Biology S. A., Geneva, Switzerland) for 5 days at 37° C in a water-saturated atmosphere containing 5% CO_2 in air. Cell loading with ricin was performed as previously described with a minor modification [6]. Briefly, LAK cells at 10×10^6/ml were incubated 30 min at 37° C with 3 μg/ml ricin in serum-free medium. To remove the toxin bound to the cell surface at the end of incubation, LAK cells were washed twice with medium containing lactose (100 mM), and twice with medium supplemented with 3% FCS. The amount of incorporated ricin was determined in LAK cells incubated with ^{125}I-labelled ricin (15×10^6 cpm/ml) and washed as reported above. Aliquots of labelled cells, at a final concentration of 1×10^6/ml, were placed in 0.5 ml medium; the radioactive ricin released in 100 μl medium after different time intervals was determined in triplicate samples using a gamma counter (Packard Instruments Co., Illinois) and expressed as a percentage of the total amount of cell-bound toxin. The estimated amount of incorporated ricin was 0.5 ± 0.2 pg/100 cells. Approximately 30% of the total internalized toxin was released from cells within the first 30 min of incubation; thereafter, a steady but lower release was observed. In both in vitro and in vivo experiments, ricin-treated LAK cells were allowed to stand for 30 min in order to eliminate the toxin, which was rapidly released during this period.

Cytolytic assay. LAK cell cytolytic activity was measured by incubating serial dilutions of effectors with 2×10^3 ^{51}Cr-labelled ($Na_2{}^{51}CrO_4$, NEN, Dreiech, FRG) target cells in triplicate wells of round-bottomed microtiter plates (Sterilin, Teddington, Middlesex, UK) in a final volume of 0.2 ml, as described previously [7]. After 4 h of incubation the plates were centrifuged and 0.1 ml supernatant was removed for counting. The percentage of specific ^{51}Cr release was calculated according to the expression:

$$100 \times \frac{\text{(experimental release–spontaneous release)}}{\text{(maximum release–spontaneous release)}}$$

Tumour cell proliferation assay. M4 or F1 tumour cells (2.5×10^4/ml) were incubated with serial dilutions of ricin-treated or untreated LAK cells or ricin alone at various concentrations in a final volume of 0.2 ml in triplicate wells of flat-bottomed microtiter plates (Sterilin). After 48 h, the cultures were pulsed with 1 μCi [^3H] dThd/well (NEN), and collected 8 h later on filter paper using a cell harvester (Skatron AS, Norway). Radioactivity was determined in a beta scintillation counter (Beckman Instruments Inc., Irvine, Calif.).

In vivo distribution of LAK cells. LAK cells (10×10^6/ml) were labelled with 100 μl ^{51}Cr and at the same time loaded with 3 μg ricin as reported above. Ricin-treated or only ^{51}Cr-labelled LAK cells (10×10^6 cells in 0.2 ml medium) were injected i.v. into syngeneic normal mice. Cell viability prior to injection was routinely greater than 90%. At 1 h or 6 h following injection, mice were sacrificed and organs were excised and counted in a gamma scintillation counter. The radioactivity recovered from each organ was calculated as the percentage of total radioactivity injected.

Adoptive immunotherapy. For induction of lung metastases, M4 and F1 cells were harvested with medium containing 0.25% trypsin and 0.2% EDTA, washed three times, suspended in cold (4° C) phosphate-buffered saline (Oxoid, England) at the indicated concentrations, and then injected i.v. into B6 mice. Ricin-treated LAK cells were injected i.v. into tumour-bearing mice 4 days after tumour cell injection. Prior to their inoculation, the LAK cells were kept at 37° C for 30 min in order to eliminate that aliquot of ricin which is rapidly released, then washed, and resuspended in cold medium at a concentration of 7×10^6 in 0.2 ml. The animals were sacrificed 10 days after tumour cell injection, and their lungs were carefully removed, rinsed in water and fixed. F1 metastases formed black nodules on the lung surface and were easily counted after fixation; M4-infiltrated lungs were fixed in Bouin's solution, which outlined metastases as opalescent white areas. In both cases, the number of metastases was determined by counting peripheral pulmonary nodules under a dissecting microscope. Significant differences in the number of lung metastases were established by using the Mann-Whitney Wilcoxon test.

Other groups of animals were instead injected i.p. with 5-fluorodeoxyuridine (FdUrd), 25 μg/mouse, to reduce endogenous availibility of thymidine and then received 1 μCi $^{125}IdUrd$ (Amersham) 1 h later [3]. Lungs were removed 24 h later, and tissue-associated radioactivity was evaluated by beta scintillation counting.

Results

In vitro activity of ricin-treated LAK cells

The cytotoxic potential of LAK cells against P815 cells was evaluated at different times following ricin loading. As previously observed with tumour-specific T lymphocytes, ricin treatment did not affect LAK cell cytotoxic activity at

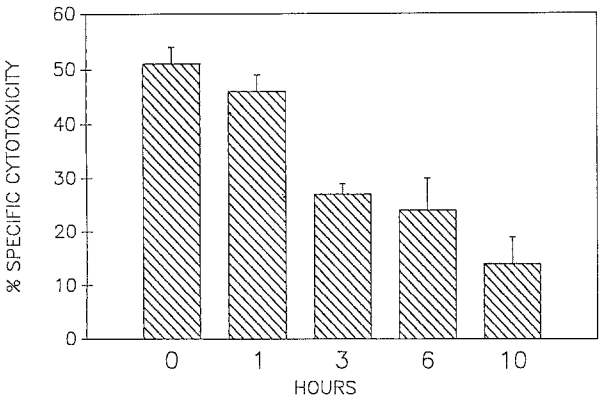

Fig. 1. Lytic activity of ricin-treated lymphokine-activated killer (LAK) cells evaluated against P815 target cells at different times after ricin treatment. The percentage cytotoxicity was determined in a 4-h ^{51}Cr-release assay at an effector/target ratio of 50:1. Untreated LAK cell cytotoxic activity ranged between 49% and 57% at the same effector/target ratio, at all times tested

the end of the incubation period; a gradual loss of activity was observed in the hours following, and by 10 h little cytotoxicity was present (Fig. 1).

To assay the activity of the ricin released by LAK cells, different numbers of ricin-treated LAK cells were added to cultures of 5×10^3 M4 or F1 cells; 48 h later [^{3}H]dThd uptake of tumour cells was compared to that in control cultures, where untreated LAK cells or various concentrations of free toxin had been added. As shown in Fig. 2A the addition of untreated LAK cells results in a similar growth inhibition of both cell lines (open symbols). The presence of ricin-treated LAK cells (closed symbols) led to a 100-fold reduction in the LAK cell number required to induce 50% growth inhibition of M4 cells (circles), while F1 cell growth inhibition was similar to that observed when untreated LAK cells were added (triangles).

This different susceptibility of the M4 and F1 cell lines to the ricin-treated LAK cells was exclusively due to the toxin, which was released in active form; in fact, 50% inhibition of M4 cell growth required a ricin dose 1000 times lower than that needed to achieve a similar effect on F1 cells (Fig. 2B). On the other hand, a short-term ^{51}Cr-release assay showed that both tumour cell lines were equally sensitive to LAK cell cytotoxic activity (about 60% specific lysis at an effector/target ratio of 50:1 with both target cells; data not shown).

In vivo distribution of ricin-treated LAK cells

Following i.v. injection into syngeneic recipients, in vitro activated lymphocytes are trapped in the lungs within 30 min, and later redistributed to the liver [19]. A similar pattern of localization was also observed after i.v. inoculation of tumour-specific ricin-treated T lymphocytes [6].

We assessed the tissue distribution of ricin-treated LAK cells after i.v. inoculation in normal recipients or in mice bearing lung metastases (4-day tumours) induced by injection of M4 tumour cells. To trace the homing of cells into various tissues, we labelled the LAK cells with ^{51}Cr, which provides a convenient measure of early in vivo cell distribution [20]. After 1 h or 6 h, recipient mice were sacrificed, and different organs were analysed by gamma counting for tissue-associated radioactivity. As shown in Fig. 3A, after 1 h about 35% of the injected radioactivity had accumulated in the lungs of normal recipient mice, while after 6 h maximal recovery was obtained in the liver (about 25% of the injected radioactivity); low amounts of radioactivity were detected in other organs including the spleen, kidney and gastrointestinal tract (not shown). Control mice injected with untreated LAK cells showed a similar pattern of localization (not shown). Furthermore, the presence of 4-day lung metastases did not appear to alter the above-reported kinetics of migration, nor the percentage of radioactivity recovered in the lungs at every time tested (Fig. 3B).

Adoptive immunotherapy with ricin-treated LAK cells

The therapeutic effect of ricin-treated LAK cells was evaluated in B6 mice bearing lung metastases induced by i.v. injection of M4 or F1 tumour cells. Preliminary testing, in which different doses of ricin-treated LAK cells or various

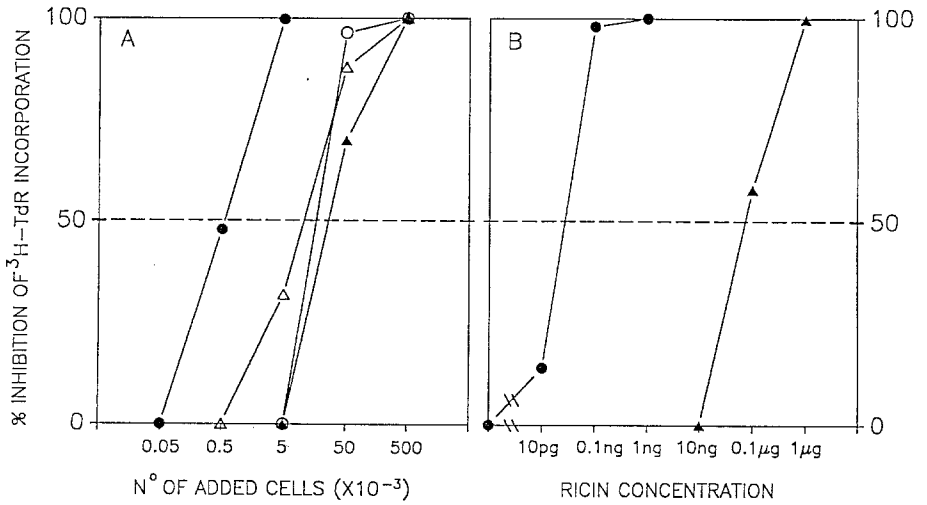

Fig. 2A, B. Inhibition of [^{3}H]dThd incorporation of M4 and F1 tumour cells. **A** Different numbers of untreated (○, △) or ricin-treated (●, ▲) LAK cells cocultured with M4 (○, ●) or F1 (△, ▲). **B** M4 (●) and F1 (▲) cells cultured in the presence of various concentrations of free ricin. The results are expressed as percentage inhibition with respect to the control cultures containing only tumour cells

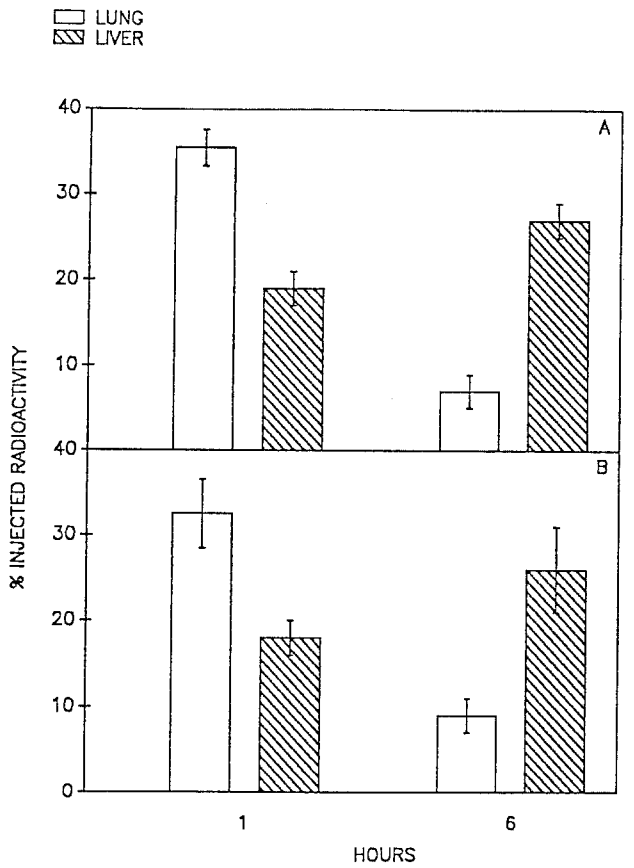

Fig. 3. Distribution of ricin-treated ^{51}Cr-labelled LAK cells into the lungs or liver, 1 h and 6 h after i. v. injection into syngeneic normal mice (**A**) or into 4-day tumour-bearing mice (**B**). Results are expressed as the percentage of injected radioactivity (cpm). Each point represents the mean (±SD) of eight mice

amount of ricin were injected (not shown), indicated that the LD_{50} was 10^7 treated LAK cells or 30 ng free ricin, respectively. Consequently, 7×10^6 ricin-treated LAK cells, which carried approximately 25 ng ricin, were injected i.v. into syngeneic mice that had been inoculated 4 days earlier with M4 or F1 cells; these mice were killed 6 days later, and the efficacy of the transferred LAK cells was evaluated by counting lung metastases. In some experiments, lung uptake of ^{125}IdUrd was also employed to evaluate tumour cell growth, as a direct relationship between isotope uptake values and extent of neoplastic growth was demonstrated in several tumour models [3].

Three representative experiments are shown in Table 1; 10 days after i.v. injection of 1.2×10^4 (experiment 1) or 2.5×10^4 (experiment 2) M4 cells, 59±18 and 149±25 lung metastases, respectively, were observed. The injection of ricin-treated LAK cells reduced the number of metastases by 32% and 30% in experiments 1 and 2 respectively (P <0.001 compared to controls). Injection of the same number of untreated LAK cells, or 25 ng free ricin did not bring about a significant decrease in the number of metastases; moreover, no reduction was seen when untreated LAK cells were transferred together with free ricin (25 ng) or sonicated ricin-treated LAK cells were injected.

These results were confirmed by ^{125}IdUrd uptake experiments; in fact, lung uptake levels in mice receiving ricin-treated LAK cells were lower than levels in control mice, which were injected with untreated LAK cells (12220±4394 cpm vs 27060±9147 cpm, P <0.01; data not shown).

On the other hand, when mice were injected with the relatively ricin-resistant F1 cell line (Table 1, experiment 3) 58±14 metastases were counted. However, no significant decrease in this number occurred after injection of 7×10^6 ricin-treated LAK cells.

Discussion

The low tumour specificity and the poor in vivo stability of carriers designed to deliver cytotoxic agents specifically to the tumour site constitute serious drawbacks in their application to cancer immunotherapy. In the light of our previous findings in mice bearing local M-MSV-induced sarcomas and injected with tumour-specific ricin-treated T lymphocytes [6], in this study we evaluated the therapeutic effect of ricin-treated LAK cells on experimental lung metastases. This model is based on the observation that most lymphoid cells following i.v. injection are immobilized in

Table 1. Reduction in the number of M4-induced lung metastases by adoptive transfer of ricin-treated lymphokine-activated killer cells (LAK)

Expt. no.	Tumour cells[a]	No. of metastases after indicated treatment (mean±SD)[b]					
		A None	B LAK cells[c]	C Ricin-treated LAK-cells[c]	D Free ricin[d]	E Sonicated LAK cells[e]	F LAK cells and free ricin[f]
1	M4	59±18	61±20	19±12	48±15	45±14	49±15
2	M4	149±25	115±32	45± 4	130±16	123±24	135±15
3	F1	58±14	41±13	40±21	52± 9	ND	ND

[a] The number of M4 cells injected i.v. was 1.2×10^4 in expt. 1 and 2.5×10^4 in expt. 2; the number of F1 cells was 2×10^5. All experiments contained eight mice per group

[b] Statistical significance of differences in expts. 1 and 2: A vs B, D, E and F, not significant; A vs C, P <0.02; B vs C, P <0.01

[c] 7×10^6 untreated or ricin-treated LAK cells were injected i.v. on day 4 after tumour cell injection. In all experiments, LAK cell cytotoxic activity, tested before infusion, ranged between 56% and 68% at an E/T of 50:1

[d] 25 ng/mouse free ricin was injected i.v. on day 4 after tumour cell injection

[e] Ricin-treated LAK cells were sonicated for 15 s at 100 W immediately before injection

[f] 7×10^6 untreated LAK cells and 25 ng ricin were separately injected

the lung capillaries; this phenomenon is particularly evident when cells cultured in vitro are used [4, 8, 15]. This altered in vivo traffic of i.v. transferred cells limits the number of effectors that will reach peripheral tumours, but could favour their accumulation in tumours that arise or metastasize in the lung.

In vitro studies demonstrated that ricin uptake and subsequent release by LAK cells was similar to that observed in normal lymphoid cells and tumour-specific T lymphocytes [6, 24]. LAK cell viability, evaluated by the dye exclusion test, remained high (90%) even 8–10 h after ricin loading, despite a gradual decline in cytotoxic activity a few hours after such treatment, most likely because of a ricin-induced inhibition of protein synthesis. Although lytic activity was impaired, the loading of LAK cells with ricin dramatically increased their antitumour activity, as treated LAK cells were about 100-fold more efficient than untreated cells in causing 50% inhibition of M4 cell line growth in vitro. On the other hand, both ricin-treated and untreated LAK cells inhibited growth of the relatively ricin-resistant F1 cell line to the same extent.

Several in vivo studies showed that significant differences in the tissue distribution pattern depended on the marker used to trace the i.v. injected cells [14, 21, 26, 29]; the highest variability was observed when cell homing was determined at a later time. As ^{51}Cr labelling proved to be a reliable tool to monitor cell distribution early after inoculation [20], we used this radioisotope to evaluate LAK cell localization 1 h and 6 h after administration. In line with previous data we found no differences in localization between ricin-treated and untreated LAK cells [6]. Interestingly, in our experimental model, the presence of M4-cell-induced lung metastases apparently did not modify the initial trapping in the lung and subsequent redistribution to the liver. However, the possibility cannot be excluded that the radioactivity increase observed at 6 h in the liver of normal and tumour-bearing mice, besides representing the localization of intact transferred cells could also be due to the accumulation of radiolabel released by disrupted cells. Similarly, Felgar and Hiserodt [10] observed that the migration pattern of adherent LAK cells in rats bearing experimentally induced lung metastases was not different from that in control animals even in the presence of large tumour burdens. These results are partialy in contrast with findings reported by Basse et al. [2], who demonstrated a time-dependent infiltration of lung metastatic lesions by LAK cells; the use of effectors in combination with repeated administration of high IL-2 doses, as well as differences in the tumour model adopted, could account for this discrepancy.

The therapeutic efficacy of ricin-treated LAK cells was evaluated against M4- and F1-induced lung metastases; these tumour cell lines exhibit the same susceptibility to LAK-cell-mediated lysis, but a highly different ricin sensitivity. The transfer of 7×10^6 ricin-treated LAK cells brought about a remarkable reduction in M4- but not F1-induced lung metastases, thus suggesting that the effect on the former depended mainly on the activity of the ricin that was delivered by LAK cells. It is noteworthy that an amount of free ricin (25 ng/mouse) equivalent to that carried by 7×10^6 LAK cells produced no significant decrease in the number of metastases, while higher doses (30 ng/mouse) killed 50% of the mice within 3 days after administration.

On the whole these results indicate that two main factors could contribute to the therapeutic effect of ricin-treated LAK cells. First, the preferential lung localization of transferred cells enables selective delivery of ricin directly to the tumour site, and this results in local ricin concentrations that could not otherwise be achieved. In addition, ricin could be slowly released by accumulated LAK cells, and toxin levels could be maintained in the proximity of tumour cells for a longer period.

In conclusion, our observations indicate that cytotoxic cells used as carriers of antineoplastic agents may be advantageous in achieving drug delivery to the tumour site, especially in organs, such as the lungs, which are a preferential target for adoptively transferred effectors. This approach entails a considerable reduction in the number of cells required to induce a therapeutic effect, as well as in the systemic toxicity of an antineoplastic drug. However, the narrow difference between therapeutic and toxic concentrations still constitutes a major disadvantage to ricin use in this approach, so that further improvments of this tumour-targeted therapy, using compounds with high efficiency of entrapment in cytotoxic cells but less toxic effects, are currently being investigated (manuscript in preparation).

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