Accumulation of Adoptively Transferred Adherent, Lymphokine-activated Killer Cells in Murine Metastases

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Summary

While close contact between lymphokine-activated killer (LAK)/adherent, lymphokine-activated killer (A-LAK) cells and tumor cells is believed to be a prerequisite for initiating the events leading to tumor cell lysis, clear evidence for the ability of these effector cells to infiltrate tumors or tumor metastases in vivo still has to be obtained. In the present study, we report that a significant fraction of adoptively transferred A-LAK cells, labeled with fluorochromes for identification, accumulates in lung and liver metastases of the B16 melanoma, the MCA 102 sarcoma and the Lewis lung carcinoma lines. Thus, 5- to 10-fold higher numbers of A-LAK cells were found in the malignant lesions compared to the surrounding normal tissue. The infiltration seemed very heterogeneous after intravenous injection of moderate numbers of A-LAK cells (15×10^6). However, after adoptive transfer of 45 million A-LAK cells, an A-LAK cell/tumor cell ratio higher than 1:1 in most metastases was observed. Surprisingly, approximately 5% of the lung metastases seemed totally resistant to infiltration even though neighboring metastases were highly infiltrated.

While substantial infiltration of lung metastases was seen after i.v. injection, significant infiltration of liver metastases was seen only after intraportal injection of the A-LAK cells indicating impaired traffic of intravenous injected A-LAK cells through the lung capillaries.

These results present direct evidence that A-LAK cells, upon a proper route of administration, have the potential to migrate to and heavily infiltrate metastases from murine tumors of different origin.

Results obtained from immunotherapy with IL-2 in combination with lymphokine activated killer (LAK)[1] cells have indicated dramatic reduction in the number of established metastatic lesions in several animal systems (1–3). LAK cell therapy has also been successful in some advanced cancer (most notably so in melanoma and renal cell carcinoma), with complete or partial responses in 20–30% of the patients. Recent studies have shown that NK cells comprise the major cell type that responds to IL-2 to generate LAK cell activity (4). Upon culture with IL-2, NK cells develop high levels of cytotoxic activity against a wide range of tumor target cells. Many of these activated cells adhere to plastic, allowing for the isolation and rapid expansion of highly enriched populations of cells with NK-phenotype (5). These cells, termed adherent LAK (A-LAK) cells, show a higher potential of in vitro cytotoxicity (5) and are more effective than unpurified LAK cells in therapy of lung and liver metastases in a rat model (6). Despite these promising results, a better understanding of the mechanism(s) behind the in vivo anti-cancer effect of LAK and A-LAK cells is still wanted with the aim of further increasing the frequency and durability of the anti-tumor responses.

The extent of accumulation of effector cells within, or in the near vicinity of, malignant lesions might be decisive for the efficacy of adoptive immunotherapy of cancer. While much is known about adhesion molecules responsible for the in vivo distribution and selective uptake in certain organs of other effector cell types, mainly T cells, only scarce information regarding LAK and A-LAK cells is published. However, characterization of the in vivo migration LAK/A-LAK cells is a prerequisite for further investigation of the molecular mechanisms determining LAK/A-LAK cell adhesion and migration to sites of infection or malignant disease.

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[1] Abbreviations used in this paper: A-LAK, adherent, lymphokine-activated killer cells; AsGM1, asialo-GM1; LAK, lymphokine-activated killer cells; rhodamine, tetramethylrhodamine isothiocyanate; st-LAK, standard lymphokine-activated killer cells.

Even though several studies have addressed this question by analyzing the distribution of injected effector cells (7–14), convincing evidence whether LAK/A-LAK cells actually migrate to, and infiltrate malignant lesions in numbers sufficient to eradicate tumors or tumor metastases is still missing. One major limitation to interpretation has been the utilization of radioisotopes for identification of the injected cells. In addition to problems concerning reutilization of radiolabel released by dead cells (15, 16), they may not be suitable for precise detection of small proportions of the injected cells accumulating in malignant lesions located in organs such as lung, liver, and spleen, where considerable accumulation of effector cells is reported to occur even in the absence of tumors (7, 8). Furthermore, radiolabels do not easily permit discrimination between infiltration of malignant and normal tissue within a specimen.

We therefore studied the in vivo distribution of A-LAK cells labeled with the fluorescent dye rhodamine, in tumor bearing animals. This method, previously used in lymphocyte traffic studies of Butcher et al. (17, 18), allows for precise identification of injected, fluorescent cells in frozen sections of various organs and makes it possible to determine their exact relation to the malignant lesions.

Materials and Methods

Animals and Cell Lines. Male C57BL/6 mice, 10–14 wk-of-age, were used. The sublines F1 and F12H(1.4) of the B16 melanoma (C57BL/6 origin) and the P815 mastocytoma (DBA origin) were maintained in vitro in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 0.8 g/l streptomycin and 1.6×10^5 U/L penicillin, hereafter referred to as complete medium. Adherent cells were detached by exposure to 0.02% EDTA for 5–8 min and washed three times in PBS. The cells were resuspended in RPMI 1640 to a final concentration of 10×10^5 per ml. Cell viability was always >95% as judged by trypan blue dye exclusion test. Pulmonary metastases of B16-F1 melanoma cells were established by tail vein injection of 2×10^5 cells in a volume of 0.2 ml. Liver metastases were induced by left ventricular injection of 0.5×10^6 B16-F12H(1.4) melanoma cells into 10–14 wk old male C57BL/6. The B16-F12H(1.4) subline was established in our laboratory from liver metastases arising from B16-F1 and induces a considerably higher number of liver metastases than the B16 F-1 line after left ventricular injection.

Preparation of LAK and A-LAK Cells. Standard LAK (st-LAK) cells were prepared as described by Rosenstein et al. (19). Briefly, spleens were harvested and single-cell suspension was prepared in RPMI 1640. RBCs were lysed by incubation with ammonium chloride-potassium buffer and the cells were thereafter washed two times. Cells were transferred into T150 plastic flasks and cultured for 3–5 d (37°C, 5% CO$_2$) in 50 ml of complete medium supplemented with nonessential amino acids and 5×10^{-5} M 2-ME and human recombinant IL-2 (1000 U/ml - kindly provided by the Cetus Corporation, Emeryville, CA and EuroCetus, Amsterdam, the Netherlands). The preparation of A-LAK cells has been described elsewhere (6). Briefly, spleen cells suspensions were prepared and incubated with IL-2 as described above. After 2–3 d of incubation, nonadherent spleen cells were removed and the flasks gently washed with prewarmed (37°C) complete medium to remove cells not firmly attached to the plastic. 50 ml fresh complete medium, supplemented as described above, were added and the cells

were cultured for an additional 2–3 d. After at total of 5 d of culturing, cells were harvested after a short treatment with 0.02% EDTA and washed twice in RPMI 1640 before use. Routinely, the A-LAK cells were phenotypically >95% large, granular lymphocytes, >98% asialo-GM$_1$+, 60–95% NK1.1 but only 4–9% Lyt 2+ and <2% L3T4+, in agreement with previous findings (4, 6).

Labeling of A-LAK Cells with Rhodamine and PKH26. $3–6 \times 10^8$ A-LAK cells were incubated with 15 μg rhodamine (TRITC; Sigma Chemical Co., St. Louis, MO) in 50 ml RPMI 1640 (0.3 μg/ml) for 30 min at 37°C (this low dose of rhodamine does not alter the proliferative properties or the cytotoxic capacity of the A-LAK cells). After labeling, cells were washed twice in RPMI 1640 and adjusted to 75×10^6 cells/ml. In selected experiments, A-LAK cells were stained with PKH26 (Zynaxis Cell Science, Inc., Malvern, PA). Briefly, 5×10^7 pelleted A-LAK cells were resuspended in 1 ml of diluent for PKH26. Immediately thereafter, 1 ml of a 20 μM solution of PKH26 in diluent was added and allowed to react with the A-LAK cells for 2 min at room temperature. The cells were washed twice in RPMI 1640 containing 10% fetal bovine serum. Fifteen million A-LAK cells resuspended in 200 μl RPMI 1640 were injected into the lateral tail vein. Lungs were removed 16 h after injection of A-LAK cells and frozen sections were prepared. To inhibit leakage of PKH26, one drop of cyanocryolit (Duro Super Glue, Loclite corp., Cleveland, OH) was used as mounting media for coverslips. After fluorescence photomicrographs were taken, the coverslips were removed by soaking the slides in acetone for 24–48 h. The sections were counterstained with Mayer's haematoxylin and microphotographs were made under a light microscope.

Adoptive Transfer of A-LAK Cells - Intravenous Administration. On day 3, 6, 9, or 12 of tumor growth, each mouse received 15 or 45×10^6 rhodamine labeled A-LAK cells i.v. and 20,000 U IL-2 i.p. at 0 and 8 h. One or 16 h after injection, lungs were removed and processed as described below.

Adoptive Transfer of A-LAK Cells - Intraportal Administration. On day 9 of tumor growth, each mouse received 15×10^6 rhodamine labeled A-LAK cells intraportally. Mice were anesthetized with Nembutal and the anterior abdominal wall was opened. A branch of the portal vein system was identified and 200 μl RPMI 1640 containing 15×10^6 A-LAK cells were injected using a 30 gauge needle. Bleeding was stopped by light compression. The abdominal wall was closed with surgical clips. Mice were given i.p. injections of 20.000 u of IL-2 every 8 h. One or 16 h after injection, livers were removed and processed as described below.

Fluorescent Microscopy. One or 16 h after injection of the A-LAK cells, lungs were fixed in 4% formalin (installed via the trachea) for 18 h and subsequently placed in 30% sucrose for additional 18 h. Livers were fixed in 4% formalin for 24 h and thereafter placed in 30% sucrose for additional 18 h. After fixation, the tissues were snap-frozen in n-Hexane at −70°C and 8 μm cryo-sections were made. Rhodamine or PKH26 labeled A-LAK cells were identified by use of a Zeiss Fluorescence Microscope with a HBO 200 w/4 mercury vapor luminator and with filter combinations for rhodamine (BP 546, FT 580 and LP 590). Metastatic lesions were easily identified by light microscopy due to the high content of melanin produced by the melanoma cells.

Electron Microscopy. Lung lobes were fixed for microscopy 2.5% glutaraldehyde in 0.05 M Na cacodylate buffer, pH 7.2. The fixative was installed into the bronchial tree until the lobes assumed a shape corresponding to maximum inhalation. Specimens were kept in aldehyde for 2 d and were treated with OsO$_4$, stained en bloc, dehydrated and infiltrated with Agar 100 resin according to routine procedures. Ultrathin sections were cut with a Reichert

Ultracut E microtome and were examined in a Zeiss CEM 902 electron microscope.

Enumeration of Tumor Infiltrating A-LAK Cells. One and 16 h after i.v. injection of 15 × 10⁶ rhodamine or PKH26 labeled A-LAK cells, multiple sections (8 μm) of lungs and livers were made from animals bearing day 3, 6, 9, or 12-d-old B16-F1 lung or 9-d-old B16-F12H(1.4) liver metastases (3 animals from each group). The number of infiltrating A-LAK cells in individual metastatic lesions as well as the number of A-LAK cells per sq mm of the surrounding normal tissue were counted in animals bearing 6–12-d-old lung or 9-d-old B16-F12H(1.4) liver metastases. The area of the metastases was measured and the average number of A-LAK cells/sq mm metastatic tissue was calculated. The very small number of tumor cells within day 3 lung metastases prevented precise quantization of the area of the lesion (distinct nodules were seldom formed) and therefore the term "infiltration" is misleading. Consequently, tumors of a size less than 40 × 40 μm were placed in the center of a square of this size and all of the A-LAK cells within this area were counted. Therefore, the number of A-LAK cells per sq.mm of tumor and tumor associated tissue is presented as: the number of A-LAK cells in the 40 × 40 μm area/0.0016 mm.

Estimation of the A-LAK Cell:Tumor Cell Ratio in Day 9 Lung and Liver Metastases. The A-LAK cell:tumor cell ratio in individual day 9 lung and liver metastases 16 h after inoculation (i.v. and intraportally, respectively) of 15 × 10⁶ rhodamine A-LAK cells was estimated as follows: (area of metastases × 0.008/average volume of the tumor cell)/number of infiltrating A-LAK cells, where 0.008 is the section thickness in mm. The average volume of one tumor cell was determined from in vitro cultured B16 melanoma cells and was found to be 2.03 × 10⁻⁶ mm³ (average diameter = 15.7 μm). More than 100 lung or liver metastases from 6 different animals were analyzed.

Flow Cytometric Analysis. The following antibodies were used: rabbit anti-asialo GM1 serum (Wako Chemical Company, Dallas, TX), mAB NK1.1 (PK136, American Type Culture Collection), fluorescein-conjugated rat mAB anti–Lyt-2 and rhodamine-conjugated rat mAB L3T4 (Becton-Dickinson and Co., Sunnydale, CA), fluorescein-conjugated swine anti-rabbit IgG and fluorescein-conjugated goat anti–mouse IgG (F205 and F313, Dakopatts, Denmark). Cells were harvested and resuspended at 5 × 10⁵ per tube in staining buffer (PBS, 2% AB serum). After centrifugation, antiserum or mAB were added to the pellet and incubated 30 min at 4°C. The cells were washed twice and incubated with FITC-labeled anti-IgG of the primary antibodies for an additional 30 min at 4°C. After washing, cells were fixed in 1% paraformaldehyde. Flow cytometric analysis were performed using a Profile flow cytometer with logarithmic amplification (Coulter Electronics Limited, Luton, UK).

Results

The Rhodamine Label. Immediately after incubation with rhodamine, the A-LAK cells are strongly fluorescent, but their intensity declines rapidly over time, limiting the use of this technique to about 20–24 h after labeling (17, 18). It cannot be excluded that binding of rhodamine to intracellular proteins interferes with various cell functions, but upon labeling with low doses of rhodamine, as used here (<0.5 μg/ml), no changes in adherence, proliferation or cytolytic capacity of the labeled A-LAK cells were detected (data not shown).

Accumulation of Intravenous Injected A-LAK Cells in Lung Metastases. One hour after i.v., injection of rhodamine-labeled

A-LAK cells into C57 BL/6 mice bearing experimental lung metastases from the syngeneic B16 melanoma, most of the injected A-LAK cells were evenly distributed in the lung tissues (Fig. 1, *a* and *b*). At this time, there was no significant difference between the number of A-LAK cells per sq mm of normal and metastic tissue, with the exception of very small (day 3) tumors (Fig. 2). In contrast, a significant increase (p < 0.001) in the numbers of A-LAK cells infiltrating the lung metastases was observed at 16 h after injection, where the number of A-LAK cells in 6-, 9-, and 12-d-old metastatic tumor tissue rose to more than 500/sq.mm, clearly demonstrating a time dependent redistribution of A-LAK cells towards the malignant tissue (Fig. 1, *c* and *d*, and Fig. 2). Simultaneously, the A-LAK cells gradually disappeared from the surrounding normal lung tissue, where less than 100 A-LAK cells per sq mm could be found at 16 h (Fig. 2). This distribution pattern was not restricted to the B16 melanoma model of metastasis, since we observed similar levels of accumulation of A-LAK cells in lung metastases induced by either MCA 102 sarcoma or Lewis Lung carcinoma cells (data not shown).

Infiltration of Lung Metastases by Various Cell Types. To compare infiltration of lung metastases by different subpopulations of LAK cells and to investigate whether the accumulation of rhodamine-labeled cells in the metastases might be due to non-specific entrapment of cells (i.e., whether other cell types than LAK cells accumulate in the lung metastases) rhodamine-labeled fresh splenocytes, st-LAK cells grown for 3 or 5 d (st-LAK-3 and -5 respectively), A-LAK cells grown for 5 d, as well as P815 and B16-F1 tumor cells were injected i.v. into animals bearing 12-d-old lung metastases. No uptake of fresh spleen cells, P815 or B16-F1 cells in the metastases was seen 0–16 h after injection (Table 1). Splenocytes were rapidly cleared from the lung tissue and redistributed mainly to the white pulp areas of the spleen. In contrast, st-LAK-3 cells were able to infiltrate the lung metastases. This infiltration was, however, less efficient than the infiltration by A-LAK and st-LAK-5 cells (Table 1).

Comparison between Rhodamine and PKH26. To ensure that the infiltration of the lung metastases by the A-LAK cells was not attributable to some metabolic effect of the rhodamine label on the A-LAK cells, in selected experiments we employed the fluorescent dye PKH26, which binds mainly to the cell membrane (20, 21), thus displaying distinct physical and binding characteristics as compared to rhodamine, which binds mainly to intracellular proteins (17). A similar pattern of infiltration of lung metastases by A-LAK cells was observed using this dye (data not shown), indicating that these fluorescent dyes did not influence the distribution pattern of the A-LAK cells. Since the half-life of the PKH26 on labeled cells is much longer than the half-life of rhodamine this new dye might be very useful in long term studies where labeled cells are to be followed by more than 16–24 h.

Fate of Rhodamine and PKH26 Released from Dead Cells. Since accumulation of rhodamine or PKH26 in the metastases due to uptake, e.g., by tumor infiltrating macrophages, of dye released from dead or dying A-LAK cells could not be ruled out, pre-killed A-LAK cells, labeled with either rhodamine or PKH26, were injected i.v. into animals bearing
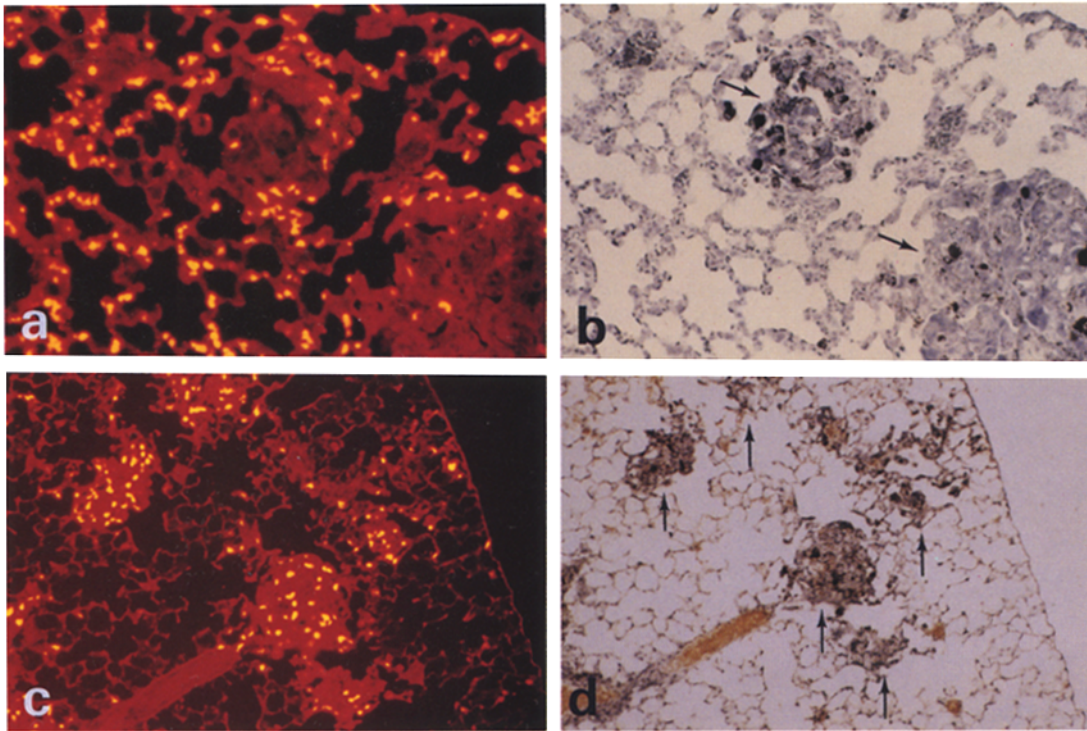
Figure 1. (*a*) Fluorescence micrograph of lung tissue one hour after i.v. injection of 15 × 10⁶ rhodamine labeled A-LAK cells. The A-LAK cells are evenly distributed in the lung tissue and do not show any accumulation in the malignant tissue (×140). (*b*) Haematoxylin staining of the section shown in (*a*). Note metastatic nodules of melanin-producing B16 melanoma cells (*arrows*). (*c*) Fluorescence micrograph of lung tissue 16 h after i.v. injection of 15 × 10⁶ rhodamine labeled A-LAK cells, showing accumulation of A-LAK cells in metastatic nodules. Only a few A-LAK cells are now present in the normal lung tissue (×60). (*d*) Haematoxylin staining of the section shown in (*c*). Arrows indicate metastatic nodules.

9-d-old lung metastases. These pre-killed A-LAK cells were cleared from the lungs within a few hours and no uptake in the metastases of dye or dye-containing cell-debris was seen 0–16 h after injection. These experiments clearly show that neither rhodamine, nor PKH26 accumulates non-specifically in the metastases.

Analysis of Infiltrated Lung Metastases by Electron Microscopy. To further validate the accumulation of A-LAK cells in the metastases, lung sections were examined by electron microscopy. Probable killer cells, i.e., cells with typical LGL morphology, were found in most metastases of mice, which had received A-LAK cells and were treated with IL-2 (Fig. 3). No cells with LGL morphology were identified in metastases from animals that were given IL-2 only. The infiltrating A-LAK cells were located both in capillaries and in various stages of exit from the microvessels into the interstitium of the metastases.

Accumulation of A-LAK Cells in Liver Metastases. Injection of A-LAK cells by the intravenous route into mice bearing lung metastases can be regarded as regional delivery of the A-LAK cells to the lung metastases, since all i.v. injected cells initially reach the lungs. In contrast, to reach liver metastases, A-LAK cells must first migrate through the lung vasculature and, for those cells reaching the liver via the portal vein, the capillary bed of the intestinal tract must also be traversed. We have previously reported that intra-arterial (left ventricular) inoculation of tumor cells resulted in increased distri-

bution to the liver when compared to i.v. injection (22). Furthermore, since A-LAK cells are large, rigid and less deformable than other lymphoid cells (23), it appears that the capillaries of the lungs and the intestinal tract might be limiting factors for the subsequent circulation of i.v. injected cells towards the liver. Indeed, in animals bearing day 9 liver metastases, very few A-LAK cells were seen in normal and metastatic tissue after i.v. injection (Fig. 4 *a*). The mean number of A-LAK cells in the normal liver tissue remained constantly low (<40 cells/sq mm) one to 16 h after inoculation (Fig. 5). The number of i.v. injected A-LAK cells infiltrating the liver metastases rose slightly from <50 cells/sq mm at 1 h to approximately 100 cells/sq mm by 16 h after inoculation (Fig. 5).

To test whether a more direct delivery of A-LAK cells to the liver could enhance the uptake of A-LAK cells into the liver and improve infiltration of liver metastases, rhodamine-labeled A-LAK cells were inoculated directly into the portal system, thus by-passing the lungs and the intestinal tract. By this route, more than five times as many cells were seen in the normal liver tissue 1 h after injection compared to the situation after i.v. injection (∼200 and ∼40 per sq mm, respectively - Fig. 4 *b* and Fig. 5). By 16 h, the number of A-LAK cells in the normal liver tissue decreased to ∼100/sq mm, whereas the number of A-LAK cells per sq mm tumor tissue rose to >500 per sq mm (Fig. 4 *c*, Fig. 5), i.e., more than 5 times as many as seen after i.v. injection.

Ratio of A-LAK Cells and Target Cells in Individual Metastases.
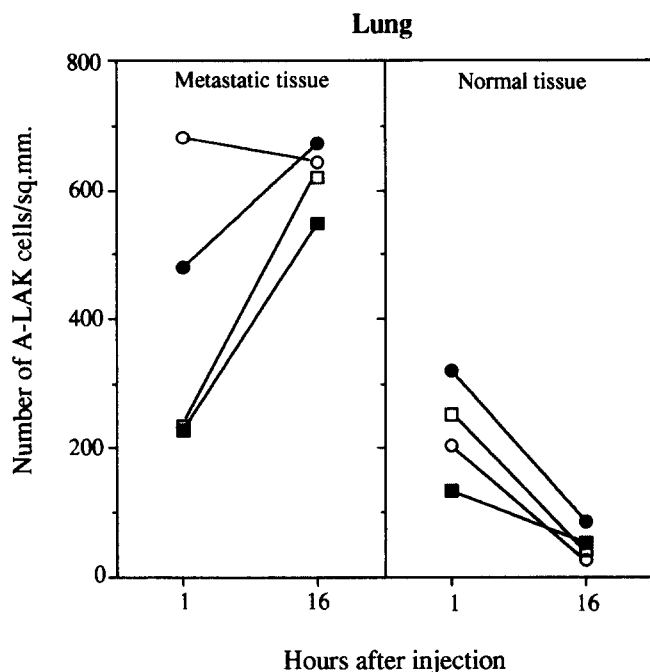
Lung

Figure 2. Number of A-LAK cells per sq mm normal or metastatic lung tissue 1 and 16 h after i.v. injection of 15 × 10⁶ rhodamine labeled A-LAK cells. Animals bearing 3-d-old metastases (*open circles*), 6-d-old metastases (*black circles*). 9-d-old metastases (*open squares*) or 12-d-old metastases (*black squares*). Statistical analysis (by Students double tailed *t* test): Number of A-LAK cells in tumor tissue one hour after injection vs. 16 h after injection; day-3: NS, day 6: p <0.03, day-9 and day-12: p <0.001. Number of A-LAK cells in normal tissue one hour after injection vs. 16 h after injection; p <0.001 in all groups.

As shown in Table 2, the ratio between A-LAK cells and tumor cells (called; E/T ratio) varied considerably among individual metastases in both lungs and liver when a moderate number (15 × 10⁶) of A-LAK cells was injected. The E/T ratio in ∼35% of the lung metastases was between 1:1 and 1:5. Less than one A-LAK cell per five tumor cells was seen in the remaining lung metastases, and some 3–5% of the lung metastases were totally without infiltration, even though they were often located near well-infiltrated lesions. Serial sections of such nodules revealed that lack of infiltration was not restricted to certain areas of the metastases, but rather included the entire lesion. The noninfiltrated nodules were of the same size (or even larger) than the infiltrated lesions and no necrosis or infiltration by inflammatory cells were seen, i.e. it is unlikely that these nodules represent regressed metastases.

In the liver, the E/T ratio was as high as 1:1 to 1:5 in 6% of the metastases following intraportal injection, i.e., the infiltration of liver metastases was less efficient than the infiltration of lung metastases. Metastases totally free of infiltration, as seen in the lung, were not observed in the liver following intraportal injection.

The E/T ratio within most lung metastases could be substantially improved simply by injecting more effector cells. Thus, forty-five million A-LAK cells injected i.v. raised the number of infiltrating effector cells in lung metastases to more

Table 1. *Infiltration of Lung Metastases by Different Cell Types*

Cell type*	No. of infiltrating cells per sq.mm tumor tissue (SD)‡	Phenotype of the adoptively transferred cells (%)		
		NK1.1	LYT-2	AsGM1
splenocytes	<10	4	12	7
st-LAK-3	404 (187)§	29	31	44
st-LAK-5	637 (370)	50	52	91
A-LAK-5	611 (281)	89	7	92
B16	0	0	0	0
P815	0	0	0	0

* Splenocytes, fresh spleen cells; st-LAK-3 and st-LAK-5, spleen cells cultured in IL-2 for 3 and 5 days respectively; A-LAK-5 cells, adherent LAK cells cultured in IL-2 for 5 days (see Materials and Methods); B16, B16 F1 melanoma; P815, a mastocytoma cell line.
‡ The number of cells infiltrating day 12 lung metastases was counted 16 hours after inoculation of 20 × 10⁶ rhodamine labeled cells. Each animal received i.p. injections of 50,000 U IL-2 at 0, 4, and 8 hours af cell inoculation. 40–50 metastases were examined in each group.
§ Significantly different from A-LAK-5 and st-LAK-5 by the student's *t* test (p < 0.005).

than 2,000/sq mm or an average E/T ratio ∼1:1. In many lesions, the number of infiltrating cells was even higher, the A-LAK cells comprising an almost confluent layer (Fig. 6, *a* and *b*). In this situation, precise enumeration of infiltrating A-LAK cells became impossible. Even when these high numbers of A-LAK cells were administrated, some 5% of the metastases were still totally resistant to infiltration by A-LAK cells (Fig. 6, *c* and *d*).

Discussion

Many investigations have been directed towards elucidating the mechanisms of lymphocyte-mediated cytotoxicity and motility in vitro (24, 25). However, in vivo distribution of adoptively transferred LAK or A-LAK cells has only been dealt with in a few papers. Some of these have demonstrated accumulation of a small number of injected effector cells within the area of malignant lesions (1, 10–13) while others have suggested that the effector cells do not at all migrate towards sites of tumor growth (14, 26).

Using the fluorescent dyes rhodamine and PKH26, which allow for detailed analysis on single-cell level, we here unambiguously demonstrate that A-LAK cells accumulate within malignant tissue upon adoptive transfer. Infiltration of the metastatic lesions was confirmed by electron microscopy, clearly demonstrating A-LAK cells in close contact with melanoma cells. Intra-lesional accumulation of A-LAK cells was not restricted to metastases of B16 melanoma origin but was also seen in lung metastases of Lewis Lung carcinoma or MCA 102 sarcoma origin. The ability of spleen cells to accumulate within metastases seemed in some way to depend on the time
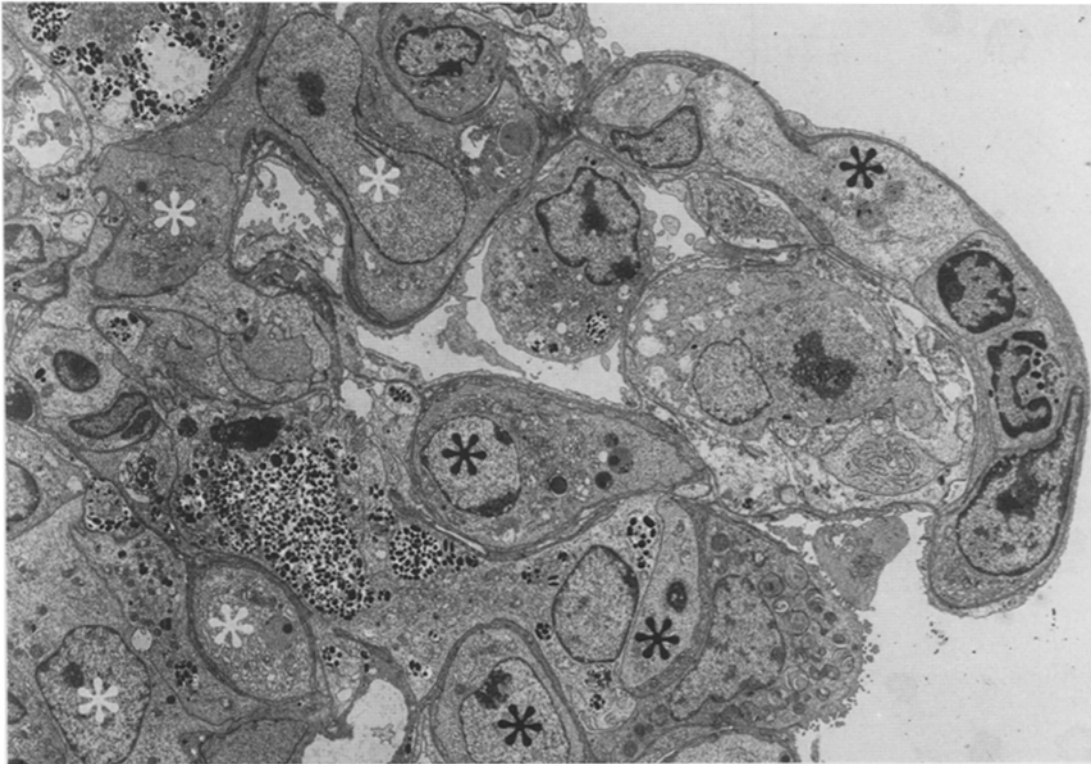
Figure 3. Lowpower electron micrograph of subpleural melanoma metastasis (×1800). A-LAK cells were injected 16 h before sacrifice. The identification of A-LAK cells (*asterisks*) depended on the presence of specific granules in this or adjacent sections as seen at higher magnification. Melanoma cells are recognized by their content of melanin.
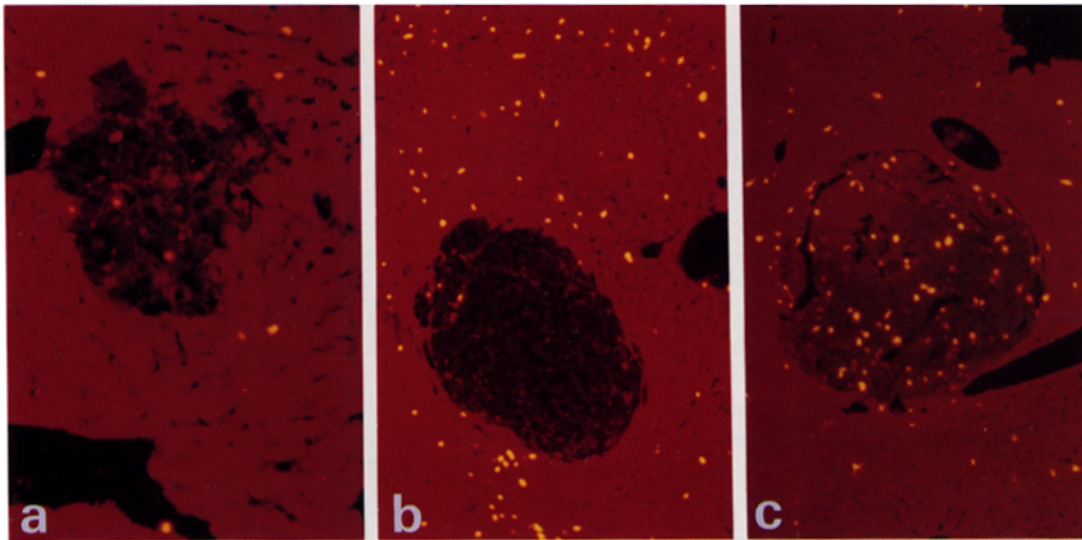
Figure 4. (*a*) Fluorescence micrograph of liver tissue 16 h after i.v. injection of 15 × 10⁶ rhodamine labeled A-LAK cells, showing a few A-LAK cells infiltrating a metastatic nodule. Few A-LAK cells are present in the normal liver tissue (×140). (*b*) Fluorescence micrograph of liver tissue 1 h after intraportal injection of 15 × 10⁶ rhodamine labeled A-LAK cells. The A-LAK cells are evenly distributed in the liver tissue, but do not show any accumulation in the malignant tissue (×60). (*c*) Fluorescence micrograph of liver tissue 16 hours after intraportal injection of 15 × 10⁶ rhodamine labeled A-LAK cells, showing accumulation of A-LAK cells in a metastatic nodule. Numerous A-LAK cells are still present in the surrounding normal liver tissue (×60).
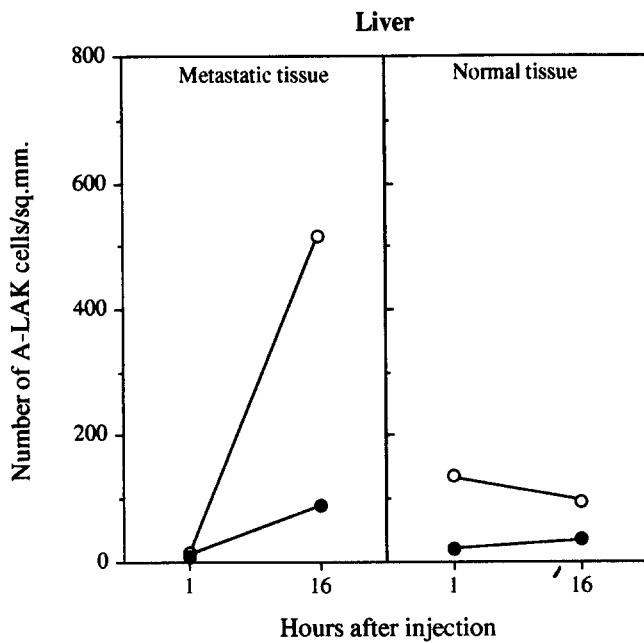
Liver

Figure 5. Number of A-LAK cells per square mm normal or day-9 metastatic liver tissue one and 16 h after i.v. (*black circles*) or intraportal (*open circles*) injection of 15 × 10⁶ rhodamine labeled A-LAK cells. Statistical analysis (by Student's double tailed *t*-test): Number of A-LAK cells in tumor tissue one hour vs. 16 h after i.v. injection $p < 0.01$; after intraportal injection: $p < 0.001$. Number of A-LAK cells in normal tissue 1 h vs. 16 h after i.v. injection: $p < 0.01$; after intraportal injection: $p < 0.001$.

of incubation with IL-2. Fresh, unactivated spleen cells did not infiltrate the metastases at all, whereas both A-LAK cells and st-LAK cells, grown in IL-2 for 5 d, infiltrated lung metastases better than LAK cells cultured in IL-2 for only 3 d. After 5 d in culture, NK (A-LAK cells) and T cells (st-LAK-5 cells) infiltrate the metastases equally well suggesting that expression of NK1.1 and Lyt2 is not crucial for infiltration. On the other hand, expression of the asialo-GM1 gan-

Table 2. *Variation in A-LAK cell:tumor cell ratio among individual lung and liver metastases*

| A-LAK cell:tumor cell ratio*	% of metastases‡	
	Lungs	Liver
1:1–1:5	37.8	6.4
1:5–1:10	44.9	28.6
1:10–1:15	7.1	22.2
1:15–1:100	6.1	42.8
No infiltration	4.1	0

* The tumor cell to A-LAK cell ratio in individual day 9 lung and liver metastases 16 hours after inoculation of 15 × 10⁶ rhodamine A-LAK cells was estimated as described in Materials and Methods.
‡ Lung metastases were induced by i.v. injection of 2 × 10⁵ B16-F1 melanoma cells. Liver metastases were induced by intraportal injection of 2 × 10⁵ B16-F12H(1.4) melanoma cells.

glioside seems to correlate with the infiltrative potential of the LAK cells indicating a role of this structure in the migration process (27, 28). However, whereas no clear evidence exists concerning the therapeutical effect of these two cell subpopulations in the murine system, Schwartz et al. (3) has shown, that A-LAK cell therapy in a rat system is superior to st-LAK cells.

While substantial infiltration of lung metastases was seen after i.v. injection, significant infiltration of liver metastases was seen only following intraportal injection of the A-LAK cells. Tumor infiltrating lymphocytes as well as inflammatory cells are thought to circulate in the blood until they reach their target tissues. If this was the case for A-LAK cells too, the degree of infiltration of liver metastases would be expected to be similar regardless of the route of administration. Since the accumulation of A-LAK cells in the liver metastases was much higher after local (i.e., intraportal) administration than after i.v. injection, it seems more likely that at least part of the tumor infiltrating A-LAK cells redistribute within the organ, from normal liver tissue into metastases. The low number of A-LAK cells reaching the liver after i.v. injection could be due to the pronounced rigidity of A-LAK cells (23), i.e., the A-LAK cells might simply become stuck in the lung capillaries. Sequestration of A-LAK cells in the lung capillaries might also be mediated by cell surface receptors as suggested by Maghazachi et al. (26, 29), who found that preincubation of A-LAK cells with carbohydrates (e.g., mannose) resulted in redistribution of A-LAK cells from the lungs to the liver. Thus, augmented uptake of A-LAK cells into the liver and higher efficacy in treatment of liver metastases might be obtained either by modulation of homing receptors or by injecting the A-LAK cells directly into the portal vein (or into the hepatic artery for tumors nourished mainly by arterial blood). This is supported by Lafreniere and Rosenberg (30), who found intraportal administration of LAK cells significantly more effective in therapy of liver sarcoma (MCA 105) metastases than i.v. administration of the same cells.

The mechanisms responsible for the in vivo tumoricidal effect of LAK and A-LAK cells remain rather obscure, but several potential possibilities exist: (*a*) A-LAK cells may interact directly with and lyse tumor cells. (*b*) A-LAK cells in metastatic lesions may release cytokines such as tumor necrosis factor that could mediate tumor cell damage and/or microvascular coagulopathy and hemorrhagic necrosis. (*c*) A-LAK cells may damage or activate tumor microvasculature and lead to extravasation of other effector cells or cytolytic molecules with resultant hemorrhagic necrosis. It is not possible yet to determine which lytic mechanism plays the predominant role in vivo, but if LAK and A-LAK cell-mediated killing of tumor cells in vivo requires direct contact between the killer cells and the tumor targets, as is the case in vitro, then the ratio between killer cells and tumor targets within the malignant lesions becomes a critical factor for the efficacy of the antitumor effect, especially if the LAK or A-LAK cells are not capable of unlimited recycling from tumor cell to tumor cell (31). After injection of a moderate number of A-LAK cells (15 × 10⁶), the ratio between A-LAK cells and tumor cells varied considerably among individual metastases in both lungs
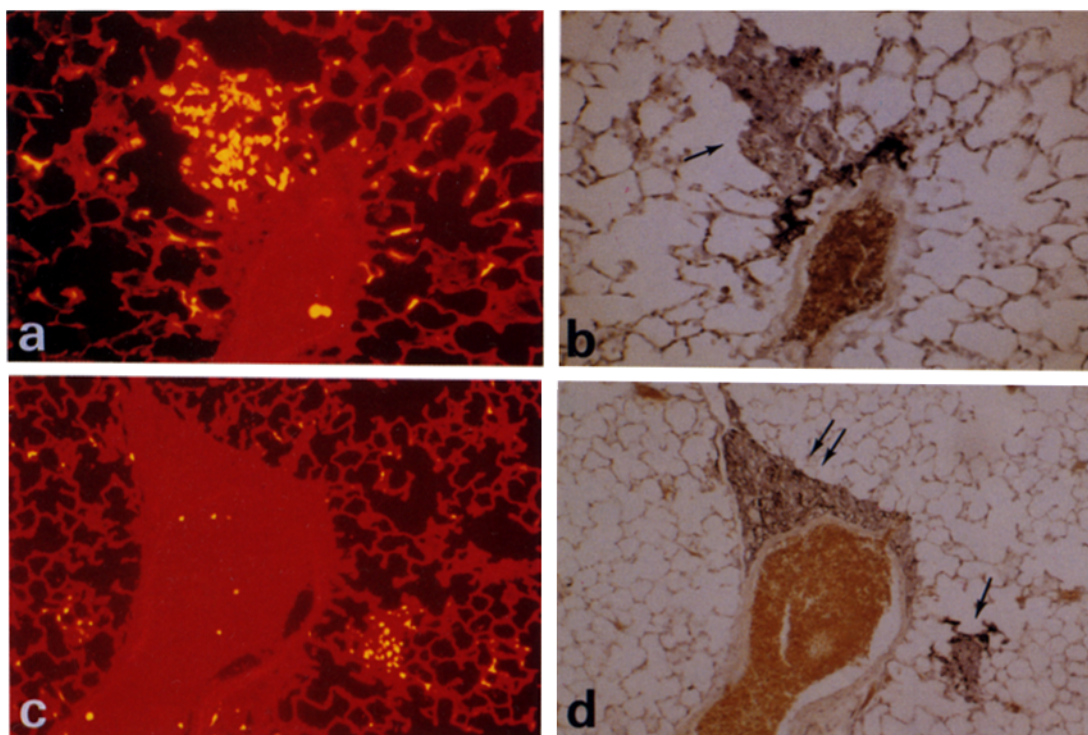
Figure 6. Fluorescent micrographs (Fig. 6, *a* and *c*) of lung tissue 16 h after i.v. injection of 45 × 10⁶ rhodamine-labeled A-LAK cells. The majority of the lung metastases are highly infiltrated (Fig. 6, *a* and *c*, *single-arrows*), but few of the metastases remain free of infiltration (Fig. 6 *c*, *double-arrow*). Fig. 6 *b* and *d* show haematoxylin staining of the sections shown in Fig. 6 *a* and *c* (×60).

and liver (Table 1) being as high as 1:1–5 in only 35–40% of the lung metastases. Thus, it is of interest that adoptive transfer of 15 × 10⁶ A-LAK cells together with IL-2, reduced the number of B16 melanoma lung metastases by ∼40% (data not shown), indicating that at least a 1:5 ratio of A-LAK cells and tumor cells was needed to induce regression of a tumor nodule. After injection of higher numbers of A-LAK cells (∼45 × 10⁶), the vast majority of lung metastases became highly infiltrated (>2000 cells/sq mm or an average E/T ratio ∼1:1) suggesting a dose dependent infiltration of the metastases. Interestingly, a few (4–5%) of the lesions, often those located around major vessels, remained totally devoid of infiltrating A-LAK cells even though they were often surrounded by highly infiltrated lesions. The existence of such noninfiltrated lesions, which are perhaps due to heterogeneity in vascular access (32, 33), might in part explain why LAK or A-LAK cell therapy, although capable of an 80–95% numerical reduction of metastases, seldom result in complete eradication of tumor burden (1–3).

In conclusion, the present communication demonstrates the novel finding that adoptively transferred LAK cells accumulate within lung and liver metastases upon a proper route of injection. Our observation provides an important basis and support both recent and future studies dealing with the molecular effector-mechanisms behind LAK and A-LAK cell-mediated cytotoxicity, because further in vivo analysis of those molecules (e.g., pore-forming cytolysin) and enzymes (e.g., proteases), which have been shown to be involved in cell-mediated lysis in vitro, would have no direct relevance if the effector cells could not bring themselves in close contact with the target cells. Due to the selective accumulation of A-LAK cells in malignant tissue compared to normal tissue, these studies further indicate that A-LAK cells, in addition to their direct or indirect cytolytic effector mechanisms, should also constitute a vehicle by which anti-tumor drugs, attached to A-LAK cells, can be selectively delivered to established micrometastatic deposits.

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