Antitumor Effects of Interleukin 2 Liposomes and Anti-CD3-Stimulated T-Cells against Murine MCA-38 Hepatic Metastasis

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ABSTRACT

The stimulation of murine splenocytes with the monoclonal antibody anti-CD3 and interleukin 2 (IL-2) results in the propagation of large numbers of T-activated killer (T-AK) cells which demonstrate high therapeutic efficacy when infused with IL-2 into mice bearing pulmonary metastases. Interleukin 2 infusions are required to maintain the function of the adoptively transferred cells. Recent data demonstrate that the therapeutic efficacy can be enhanced by encapsulating IL-2 in liposomes. The present work tested the combination of T-AK cells with IL-2 liposomes in an immunotherapy model utilizing the MCA-38 murine colon adenocarcinoma.

Expansion of murine splenocytes was achieved with anti-CD3 monoclonal antibody plus IL-2 and was consistently greater than 50-fold during a 9-day culture period. Cytolytic activity of the murine T-AK cells was mediated primarily by Lyt-2+ cells. In vivo results demonstrate synergistic therapeutic efficacy of the combination of IL-2 liposomes and T-AK cells. Evaluation of the in vivo distribution of these T-AK cells utilizing congenic mice demonstrates that Lyt-2+ cells from these in vitro cultures infiltrate hepatic metastases in vivo. The activation of lymphocytes with anti-CD3 monoclonal antibody and IL-2 appears to be a reproducible and convenient method of producing cells capable of producing antitumor effects in models of adoptive immunotherapy.

INTRODUCTION

Immunotherapy with a combination of IL-2 and adoptive transfer of LAK cells has been shown to be effective against a limited number of human cancers (1, 2). However, the combination of IL-2 and LAK cells results in toxicity in 100% of patients, with less than 20% demonstrating a significant response (3). In addition, imaging studies have shown that adoptively transferred LAK cells do not home to tumor sites but appear to accumulate nonspecifically in the lungs, liver, and spleen (4). Several subpopulations of lymphocytes develop LAK activity, depending on the specific signals used to activate the cells. The addition of IL-2 alone generates cells with lytic activity that are predominantly NK cells (5–8). The addition of anti-CD3 MoAb in combination with IL-2 results in large numbers of rapidly proliferating T-AK cells (9–12). We have concentrated our efforts on these activated T-cells. The T-AK cells have been tested for efficacy against pulmonary metastases of MCA-106 sarcoma cells and have antitumor efficacy equivalent to that of LAK cells generated with IL-2 (13).

Major systemic toxicity has been associated with high-dose, prolonged IL-2 administration in humans. This toxicity is manifested as malaise, fever, and hepatic and renal dysfunction as well as a pulmonary capillary leak syndrome. Liposomes can modify toxicity by altering the absorption and distribution of the entrapped drug (14). We have recently reported that IL-2 encapsulated in liposomes is significantly more effective in tumor reduction than free IL-2 in the murine pulmonary metastases model with MCA-106 (15).

The present report describes the antitumor efficacy of the combined use of T-AK cells and IL-2 liposomes in a murine hepatic metastases model utilizing the colon adenocarcinoma MCA-38. The results demonstrate a synergistic effect between IL-2 and T-AK cells when the lymphokine is encapsulated in liposomes. Infusion of IL-2 encapsulated in liposomes alone induces an infiltration of the hepatic tumor by Lyt-2+ cells of host origin. However, after the adoptive transfer of T-AK cells and IL-2 liposomes the donor Lyt-2+ cells constitute the predominant infiltrating population in the hepatic metastases in this model. We further demonstrate that the lytic activity of these CD3-activated Lyt-2+ cells is not MHC restricted. Thus it appears that the cells activated in the murine T-AK cultures are activated T-cells with nonspecific lytic activity, rather than activated NK cells found in LAK cultures.

MATERIALS AND METHODS

T-AK Cells. C57BL/6 murine splenocytes were harvested and activated with anti-murine CD3 MoAb 145-2C11 (a generous gift of Dr. J. Bluestone) (16) and IL-2 (specific activity, 1.5 × 10^7 units/mg; a generous gift of Hoffmann-LaRoche Inc., Nutley, NJ) as previously described (13). Briefly, fresh splenocytes were incubated in culture flasks at a concentration of 1.0 × 10^6 cells/ml of TC1 consisting of RPMI 1640 supplemented with 25 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mm l-glutamine, 5% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 10 mm nonessential amino acids, 100 mm sodium pyruvate (GIBCO, Grand Island, NY), and 25 mM 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO). To generate T-AK cells 2 µg of 145-2C11 MoAb were added per ml of TC1. In addition, IL-2 was added to the TC1 at a concentration of 100 units/ml. Anti-CD3 MoAb was added only at the start of cultures. All cultures were supplemented with new IL-2-containing TC1 to maintain a cell density of 0.2–0.5 × 10^6 cells/ml every 2 to 3 days. Cultures were sampled on days 3, 5, 7, and 9; cell viability and increases in cell number were determined by trypan blue exclusion and light microscopy.
microscopy. Cells utilized in adoptive immunotherapy experiments were from days 3 and 5 of culture.

IL-2 Incorporation into Liposomes. IL-2 (specific activity, 1.5 x 10^7 units/mg; Hoffmann-La Roche) containing 25 mg human serum albumin/1 x 10^4 units IL-2 was diluted in HBSS and added to DMPC (Avanti Polar Lipids, Pelham, AL). Utilizing a freeze/thaw and bath sonication technique, we formulated IL-2 liposomes as previously described (15). The resulting IL-2 liposomes were multilamellar vesicles (<5 μm), with over 95% of IL-2 consistently associated with the liposome fraction. To standardize the quantity of lipid injected with each concentration of IL-2 tested, IL-2 liposomes were synthesized utilizing a constant ratio of lipid to IL-2 solution of 15 mg DMPC/0.2 ml IL-2 solution. All mice were inoculated with 15 mg of liposome/animal. The concentration of IL-2 varied from 1.0 x 10^7 to 5.0 x 10^8 units/0.2 ml in the dose-response experiments of IL-2 liposomes. All other experiments used 5.0 x 10^7 units IL-2/0.2 ml DMPC.

Tumor Preparation and Tumor Cell Lines. MCA-38 was provided by Dr. J. Mulé (National Cancer Institute, Bethesda, MD) and maintained by s.c. passage in C57BL/6 mice. MCA-38 is a weakly immunogenic murine colon adenocarcinoma that was induced by the s.c. injection of dimethylnitrosamine into C57BL/6 mice (17). The murine tumor cell lines MCA-38 (H-2d; colon adenocarcinoma), YAC-1 (H-2b; lymphoma), Renca (H-2b; renal adenocarcinoma), and MBL2 (H-2d; lymphoma) were subcultured in TC10 with 10% fetal calf serum. YAC-1, Renca, and MBL2 were provided by Dr. R. Wiltrout (National Cancer Institute, Frederick, MD). M5076 (H-2b; ovarian reticulum cell sarcoma) was maintained by i.p. passage in C57BL/6 mice. On the morning of a cytotoxicity assay, ascites containing a single cell tumor preparation of M5076 were harvested, washed, and chromated labeled.

Cytotoxic Assays. Tumor targets (2 x 10^6 cells in 0.5 ml media) were incubated with 150 μCi Na^25CrO_4 (1000 μCi/ml; New England Nuclear Research Products, Boston, MA) at 37°C for 60 min. The targets were then washed twice with TCM, resuspended in media, and counted. T-AK effector cells were washed twice and then aliquoted in triplicate in U-bottomed microtiter plates (CoStar) and serially diluted 2-fold to yield effector:target cell ratios from 25:1 to 3:1; 5000 targets were added per well. Spontaneous release wells contained media only; maximum release wells contained detergent. Plates were incubated for 4 h at 37°C in 5% CO_2. The percentage of specific cytotoxicity was determined as

\[
\text{Experimental mean cpm - spontaneous mean cpm} \times 100
\]

\[
\text{Maximal mean cpm - spontaneous mean cpm} = \% \text{ of cytotoxicity}
\]

Hepatic Metastases Model. This is a variation of a model described by LaFreniere and Rosenberg (18). Single cell suspensions of MCA-38 tumor were prepared with DNase, hyaluronidase, and collagenase digestion of solid tumor that had been passed s.c. as previously described (13). Eight- to 12-week-old C57BL/6 mice were anesthetized with 0.2-ml i.p. injection of 1.4 mg pentobarbital. A left subcostal incision was made, and the spleen was treated by injection of 3.0 x 10^7 cells in 0.5 ml of HBSS with a 30-gauge needle. The spleen was gently held for 90 s with sterile gauze to obtain hemostasis and then returned to the peritoneal cavity. The incision was closed with wound clips in one layer. Operative and perioperative mortality was consistently between 0 and 4% for all experiments performed. With this model, untreated mice die between days 13 and 17 after tumor inoculation. The number of hepatic metastases was evaluated in each therapeutic group on day 11 after tumor inoculation. From 1 to 2 ml of a 15% India ink solution were injected with a 27-gauge needle into the superior mesenteric vein of mice anesthetized with pentobarbital. The liver was then removed and placed in Fekete’s solution (300 ml 70% ethanol, 30 ml formalin, and 15 ml glacial acetic acid). Hepatic metastases were counted blind by 2 individuals. Student’s unpaired t test was used to evaluate the significance of differences between treatment groups.

Immunotherapy with IL-2 Liposomes and T-AK Cells. Each experimental group contained 10 C57BL/6 mice. All mice had 14 mg/ml indomethacin (Sigma) added to their drinking water beginning on the day of surgery and continuing for the duration of the experiment (19). Experimental protocols involved once-daily i.p. injections of empty liposomes, IL-2 liposomes, or free IL-2 at varying concentrations in 0.2 ml of HBSS on days 3–7 following tumor inoculation. Groups receiving adoptive immunotherapy received T-AK cells i.v. on days 3 and 5. Cells utilized were always from days 3 and 5 of culture.

Antibodies. Monoclonal antibodies were purified from ascites with saturated ammonium sulfate, dialyzed against PBS, and adjusted to a final concentration of 500 μg/ml in PBS. The antibody recognizing Lyt-2.2 is 2.43 (catalogue no. TIB 210, American Type Culture Collection, Rockville, MD) and that recognizing L3T4 is GK1.5 (catalogue no. TIB 207, American Type Culture Collection). Rat anti-Thy-1.2 FITC was obtained from Becton Dickinson (Mountain View, CA) and anti-MAC-1 was from Boehringer Mannheim (Indianapolis, IN). Monoclonal antibody NK 1.1 FITC was provided by L. Mason (National Cancer Institute, Frederick, MD). Second antibodies were goat anti-rat FITC from Pel-Freeze Biologicals (Rogers, AR), and rabbit anti-goat FITC was from Chappel (Cochraneville, PA).

Congenic Mouse Experiments. C57BL/6 mice (Thy-1.2) and B6-Pl-Thy-1a/Cy mice (Thy-1.1) were obtained from The Jackson Laboratory (Bar Harbor, ME). B6-Pl-Thy-1*/Cy mice were inoculated i.s. with a single cell suspension of MCA-38 as previously described. T-AK cells were generated from splenocytes of normal Thy-1.2* C57BL/6. The tumor-bearing B6-Pl-Thy-1/Cy mice were then treated with IL-2 liposomes alone, T-AK cells alone, or a combination of IL-2 liposomes and T-AK cells. B6-Pl-Thy-1*/Cy mice express Thy-1.1 on their lymphocytes, allowing their differentiation from the adoptively transferred Thy-1.2* cells. Therapy was initiated on day 3 after tumor inoculation according to the protocol described above. Mice from each of the treatment groups as well as a control tumor-bearing mouse were sacrificed on days 4, 6, 8, and 11 after tumor inoculation. Liver, lung, and spleen biopsies were performed, and the biopsy tissue was snap frozen in precooled isopentane and stored at −70°C until used.

Preparation and Staining of Tissue Sections. Frozen tissue sections (4 μm) were prepared in a Lipshaw cryostat under conditions of constant temperature (~25°C) and humidity (30%), air dried, acetone fixed, and washed with PBS, pH 7.4 (20). Phenotypic analysis of the tumor-infiltrating cells was made with rat MoAbs against murine Thy-1.2, L3T4, Lyt-2, and Mac 1. These were overlaid with affinity-purified, mouse serum-absorbed, FITC-F(ab')2; goat anti-rat IgG (Pel-Freeze Biologicals) and washed with PBS. Amplification was achieved by the use of a second FITC-conjugated reagent, affinity-purified, mouse serum-absorbed FITC-F(ab')2; rabbit anti-goat IgG (Cappel). After a final PBS wash, ethidium bromide was applied to stain nuclei, and p-phenylenediamide was added to retard fluorescence fading (21). In addition, tetraethylrhodamine isothiocyanate-conjugated rabbit anti-basement membrane antiserum was used in concert with certain of the above antibodies as a morphological probe to evaluate the relationship of reactive cells to vascular walls. Control sections of each tissue were prepared as above, omitting the primary antibody, and using appropriate second and third layers of FITC-labeled antibodies, ethidium bromide, and p-phenylenediamine. Sections were examined with a Zeiss Universal microscope equipped for epifluorescence.

Cell Depletions. A single-cell fresh splenocyte suspension was incubated with 1 μg/1 x 10^6 cells of either anti-Lyt-2 (500 μg/ml) or anti-L3T4 (500 μg/ml) for 30 min at 4°C. The cells were washed twice in HBSS. Goat anti-mouse IgG Fc-specific coated magnetic beads (Advanced Magnetics Inc., Cambridge, MA) were washed with PBS twice and incubated with PBS plus 10% fetal calf serum for 30 min. The beads were washed with PBS and mixed with the splenocytes at a bead:cell ratio of 20:1. The mixture of beads and cells was placed on a rotator at 4°C for 30 min and then separated on a magnetic separator tubes tray (Advanced Magnetics). The last step was repeated twice. Unbound cells were removed and washed twice with HBSS, activated with 2 μg of anti-CD3 MoAb/1 x 10^6 cells, and placed in TCM containing 100 units/ml of IL-2. These were labeled Lyt-2- or L3T4-depleted cell populations.

Analysis of purity of cell populations was performed on a Coulter
flow cytometer (Epics Profile Analyzer; Coulter Corp.). Cells were stained with Lyt-2 FITC, L3T4 PE, Thy-1.2 FITC (Becton Dickinson), NK 1.1 FITC, or control antibodies IgG2 FITC and mouse IgG2a PE (Becton Dickinson). Results consistently demonstrated less than 2% of the depleted cell population to be present. Further analysis of cell population purity was performed on days 5 and 9 of culture. Results demonstrated that the depleted cell subsets were always less than 4% of the cells present.

RESULTS

In pilot studies, 100% of mice developed liver metastases after splenic injection of 3.0 × 10^5 MCA-38 colon adenocarcinoma cells. Histological evaluation after hematoxylin-eosin staining detected liver micrometastases on day 3 and macrometastases on days 5 and 6. Untreated mice from all experiments done had 236 ± 30 liver metastases by day 11 and usually died between days 13 and 17. The therapeutic protocol followed in our experiments is presented in Fig. 1.

T-AK Cells and IL-2 Liposomes Have Therapeutic Efficacy against MCA-38 Hepatic Metastases. Experiments to evaluate the antitumor efficacy of IL-2 liposomes, free IL-2, or T-AK cells on MCA-38 hepatic metastases were performed. Daily doses of IL-2 liposomes or free IL-2 at 5 × 10^4 units were administered i.p. for 5 consecutive days. Mice in groups 3 and 5 received 5.0 × 10^7 T-AK cells i.v. on days 3 and 5 for a total of 1.0 × 10^8 cells during 1 week of therapy. Significant reduction in the number of hepatic metastases was not obtained with any of these single-arm regimens or once-daily free IL-2 + T-AK cells. However, when IL-2 liposomes were given in combination with T-AK cells, up to a 90% reduction in the number of hepatic metastases was achieved. Representative results are shown in Table 1. In all experiments performed, 10–20% of mice receiving this combination therapy had no hepatic metastases on day 11, but all animals had small identifiable primary tumors in the spleen. Additional experiments tested doses of IL-2 liposomes up to 1 × 10^5 units/day without showing any significant therapeutic effect when used alone (data not shown).

To determine the respective roles of the IL-2 liposomes and the T-AK cells in the therapeutic effect, titration experiments were performed. To test the dose response to IL-2 liposomes, mice received 1 × 10^4, 2.5 × 10^4, or 5 × 10^4 units i.p. once a day between days 3 and 7 and a constant number (5 × 10^7) of T-AK cells i.v. on days 3 and 5 after tumor inoculation. A clear dose response was observed, and the most significant decrease in the number of metastases was observed with the larger doses of IL-2 liposomes (Fig. 2). In a similar manner, while using a constant dose of IL-2 liposomes (5 × 10^4 units/day), T-AK cells were administered i.v. in numbers ranging from 1 × 10^7 to 5 × 10^7 cells/mouse on days 3 and 5 after tumor inoculation. As seen in Fig. 3 larger doses of cells resulted in significantly fewer hepatic metastases. Thus, dose response experiments clearly demonstrate the additive effects of both T-AK cells and IL-2 liposomes in the in vivo reduction in the number of hepatic metastases. High doses of free IL-2 (50,000 units twice a day), high doses of IL-2 liposomes (50,000 units twice a day), or T-AK cells alone did not induce a reduction in the number of metastases.

Empty Liposomes Are Not Effective with T-AK Cells against MCA-38 Metastases. It was possible that the therapeutic contribution of the IL-2 liposomes was a result of the lipid component of this preparation; therefore, we tested the individual components of the IL-2 liposome preparation in the same tumor model (Table 2). Similar to the results in Table 1, control mice had a mean of more than 200 metastases on the surface of the liver. "Empty" (HBSS loaded) liposomes resulted in a slight but not statistically significant increase in the number of metastases. When free IL-2 was mixed with empty liposomes, no reduction in the number of hepatic metastases was seen. Mice receiving empty liposomes mixed with free IL-2 and T-AK cells had a slight but not significant reduction in the number

![Fig. 1. Therapeutic protocol.](image1)

![Fig. 2. Dose response of IL-2 liposomes against MCA-38 hepatic metastases.](image2)

Table 1. Reduction in liver metastases produced by T-AK cells plus IL-2 liposomes

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of hepatic metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS control</td>
<td>Median, Mean, SE, SD, P</td>
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<tr>
<td>1. Free IL-2</td>
<td></td>
</tr>
<tr>
<td>2. Free IL-2 + T-AK cells</td>
<td></td>
</tr>
<tr>
<td>3. T-AK cells</td>
<td></td>
</tr>
<tr>
<td>4. IL-2 liposomes</td>
<td></td>
</tr>
<tr>
<td>5. Free IL-2 + T-AK cells</td>
<td></td>
</tr>
<tr>
<td>6. IL-2 liposomes + T-AK cells</td>
<td></td>
</tr>
</tbody>
</table>

![Fig. 3. Dose response of T-AK cells.](image3)
containing 15 mg DMPC in 0.2 ml HBSS i.p. on days 3-7. Mice in groups 3 and 4 received 5 x 10⁴ units IL-2 (33 ng) i.p. on days 3-7. Groups 4 and 5 received 5 x 10⁴ T-AK cells i.v. on days 3-7. Student’s unpaired t test was used to compare group x IO⁷ T-AK cells i.v. on days 3 and 5. Group 5 received 5 x IO⁴ units IL-2 cells, and the number of metastases was evaluated by measuring Thy-1.1 on their T-cells, which were inoculated with MCA-38 cells using congenic mice. T-AK cells were generated from spleens of B6:PL-Thy-la/Cy, which express Thy-1.2 on their lymphocytes of C57BL/6 mice that express Thy-1.1. Liver tissue was collected every 48 h beginning the day after therapy started (day 4 after tumor inoculation), and evaluated for the number of Thy-1.2*, Lyt-2*, L3T4*, and Mac 1+ cells infiltrating the hepatic metastases. Ethidium bromide was added to facilitate identification of the early micrometastases within the liver, and tetraethylrhodamine isothiocyanate antibody was used to allow differentiation of intravascular cells from infiltrating cells.

Mice treated with IL-2 liposomes alone demonstrated some host Lyt-2* cells infiltrating the metastases when first observed 48 h after injection (Fig. 4a). The number of infiltrating lymphocytes increased over the subsequent 48 h and remained constant thereafter. A much smaller number of Mac 1+ cells (including both polymorphonuclear leukocytes and monocytes) also infiltrated the metastases of these mice. Rare L3T4+ cells were noted. T-AK cells (Thy-1.2*) infused without IL-2 liposomes into Thy-1.1+ mice were noted to be infiltrating the tumor in small numbers. They appeared 2 days after initial injection (Fig. 4b). Rare Mac 1+ cells and no L3T4+ cells were observed.

When T-AK cells were administered with IL-2 liposomes, large numbers of Lyt-2* cells were noted; approximately 60% of the total cells were those cells which had been initially noted. T-AK cells (Thy-1.2*) infused without IL-2 liposomes into Thy-1.1+ mice were noted to be infiltrating the tumor in small numbers. They appeared 2 days after initial injection (Fig. 4b). Rare Mac 1+ cells and no L3T4+ cells were observed.

In four separate experiments, the ability of adoptively transferred T-AK cells to infiltrate MCA-38 hepatic metastases was evaluated using congenic mice. T-AK cells were generated from splenocytes of C57BL/6 mice that express Thy-1.2 on their lymphocytes. Mice of the congenic strain B6:PL-Thy-1*Cy, which express Thy-1.1 on their T-cells, were inoculated with MCA-38 and then treated with IL-2 liposomes alone, T-AK cells, or a combination of IL-2 liposomes and T-AK cells.

Liver tissue was collected every 48 h beginning the day after therapy was started (day 4 after tumor inoculation), and evaluated for the number of Thy-1.2*, Lyt-2*, L3T4*, and Mac 1+ cells infiltrating the hepatic metastases. Ethidium bromide was added to facilitate identification of the early micrometastases within the liver, and tetraethylrhodamine isothiocyanate antibody was used to allow differentiation of intravascular cells from infiltrating cells.

Mice treated with IL-2 liposomes alone demonstrated some host Lyt-2* cells infiltrating the metastases within 24 h of i.p. injection (Fig. 4a). The number of infiltrating lymphocytes increased over the subsequent 48 h and remained constant thereafter. A much smaller number of Mac 1+ cells (including both polymorphonuclear leukocytes and monocytes) also infiltrated the metastases of these mice. Rare L3T4+ cells were noted. T-AK cells (Thy-1.2*) infused without IL-2 liposomes into Thy-1.1+ mice were noted to be infiltrating the tumor in small numbers. They appeared 2 days after initial injection (Fig. 4b). Rare Mac 1+ cells and no L3T4+ cells were observed.

When T-AK cells were administered with IL-2 liposomes, large numbers of Lyt-2* cells were noted; approximately 60% Thy-1.2* represented adoptively transferred cells, while 40% were of host origin (Fig. 4c). These cells were apparent 48 h after therapy began. A moderate number of Mac 1+ cells were seen, but only rare L3T4+ cells were found. The cellular infiltrate was present up to day 11 of protocol (3 days after the end of therapy), when the mice were sacrificed.

Cytolytic Activity and Phenotype of T-AK Cells. The cytolytic activity of the T-AK cells was tested against a variety of syngeneic tumor targets as shown in Table 3. Cytolytic activity against the NK-sensitive target YAC-1 (H-2b) was consistently low, while activity against a variety of syngeneic NK-resistant tumor targets was high. To evaluate the lytic function of the different cell subpopulations within these cultures, cell depletion studies were performed. As described in “Materials and Methods,” fresh splenocytes were depleted of either Lyt-2* or L3T4* cells, activated with anti-CD3, and grown in IL-2 containing TCM. Cultures depleted of Lyt-2* cells had a low cytolytic activity compared to cultures depleted of L3T4* or unseparated cultures, suggesting that Lyt-2* cells are the primary cytolytic effectors in these cultures. Repeat phenotypic analysis of these cultures on days 5 and 9 demonstrated that <4% of the total cells were those cells which had been initially

Table 2 Empty liposomes do not produce metastases reduction

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of metastases</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>2. Empty liposomes</td>
<td>238</td>
</tr>
<tr>
<td>3. Empty liposomes + free IL-2</td>
<td>215</td>
</tr>
<tr>
<td>4. Empty liposomes + free IL-2+T-AK cells</td>
<td>194</td>
</tr>
<tr>
<td>5. IL-2 liposomes + T-AK cells</td>
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<table>
<thead>
<tr>
<th>Group</th>
<th>No. of metastases</th>
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<td>1. HBSS control</td>
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<tr>
<td>2. Empty liposomes</td>
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</tr>
<tr>
<td>3. Empty liposomes + free IL-2</td>
<td>220</td>
</tr>
<tr>
<td>4. Empty liposomes + free IL-2+T-AK cells</td>
<td>203</td>
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<tr>
<td>5. IL-2 liposomes + T-AK cells</td>
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Table 3 Cytolytic activity of T-AK cells and subpopulations

<table>
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<tr>
<th>Day of culture</th>
<th>Cell fold increase</th>
<th>YAC-1</th>
<th>MBL2</th>
<th>MCA-38</th>
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<td>Day 5</td>
<td>Unseparated*</td>
<td>11.4</td>
<td>30</td>
<td>313</td>
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<td></td>
<td>L3T4 depleted*</td>
<td>31</td>
<td>447</td>
<td>985</td>
</tr>
<tr>
<td></td>
<td>Lyt-2 depleted</td>
<td>24</td>
<td>50</td>
<td>88</td>
</tr>
<tr>
<td>Day 7</td>
<td>Unseparated</td>
<td>23.1</td>
<td>162</td>
<td>544</td>
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<td></td>
<td>L3T4 depleted</td>
<td>9</td>
<td>197</td>
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<td>Lyt-2 depleted</td>
<td>7</td>
<td>53</td>
<td>79</td>
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<td>Day 9</td>
<td>Unseparated</td>
<td>50.8</td>
<td>&lt;1</td>
<td>134</td>
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<td>331</td>
</tr>
<tr>
<td></td>
<td>Lyt-2 depleted</td>
<td>&lt;1</td>
<td>28</td>
<td>49</td>
</tr>
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* Unseparated splenocytes activated with anti-CD3 plus IL-2 (100 units/ml).

Fig. 4. A, Lyt-2* cells infiltrating hepatic metastases 48 h after infusion of IL-2 liposomes alone; B, Thy-2+ cells infiltrating metastases 48 h after infusion of Thy-1.2+ T-AK cells into congenic Thy-1.1 tumor-bearing mice; C, Lyt-2* cells infiltrating tumor 48 h after combination of IL-2 liposomes and adoptive immunotherapy; D, solitary Lyt-2* cell in untreated control mouse.
tumor regression in some patients (1, 2). It has been demon-
depleted. Similarly, NK cell populations (detected with mono-
cells to treat patients; they require extended periods of culture
accompanied by high NK function. The analysis of the lytic
cultures compared to LAK cultures. The isolation and identi-
cantly larger numbers of cells can be obtained from T-AK
cells, if present, exhibit little if any cytotoxicity (5, 6, 22).
function found in IL-2-stimulated lymphocyte cultures, while
strated that NK cells are primarily responsible for the lytic
ability to lyse normal, syngeneic targets was
less than 4% (data not shown). Therefore, the observed lytic
activity was not due to contaminating cell subsets.
Allogeneic tumor targets were tested to identify the extent of
non-MHC-restricted T-cell killing. As shown in Table 4, the
majority of cytolytic activity in these cultures is non-MHC
restricted. The ability to lyse normal, syngeneic targets was
tested by utilizing 48-h concanavalin A-stimulated syngeneic
lymphoblasts as targets. As seen in Table 4, very low levels of
autoreactive or allogeneic cytolytic activity were seen against
normal lymphoblasts.

### DISCUSSION

The combination of IL-2 infusion plus adoptive transfer of
IL-2 LAK cells has been tested in animal models and in patients
with cancer. These regimens have been shown to decrease the
number of metastases in animal models (22) and have induced
tumor regression in some patients (1, 2). It has been demonstrated
that NK cells are primarily responsible for the lytic
function found in IL-2-stimulated lymphocyte cultures, while
T-cells, if present, exhibit little if any cytotoxicity (5, 6, 22).
Imaging studies have shown that adoptively transferred LAK
cells do not home to tumor sites but accumulate in the lungs,
liver, and spleen (4). In addition, biopsy studies of responding
patients stained with immunoperoxidase do not show accumu-
lation of cells of NK phenotype but rather of T-cell phenotype.
Recent work has demonstrated that IL-2-activated and ex-
and T-cells for adoptive immunotherapy. While the
results utilizing TIL cells are exciting, significant technical
difficulties have made these trials cumbersome and expensive.
It is frequently difficult to obtain sufficient numbers of TIL
cells to treat patients; they require extended periods of culture
(30–45 days) and thus far have been obtained successfully only
from a limited number of tumor types.

We (9, 13, 26) and others (11, 27) have reported on the
utilization of anti-CD3 in combination with IL-2 as a stimulus to
generate large numbers of cells in culture which demonstrate
both in vitro cytotoxicity and antitumor effects in vivo. Signifi-
cantly larger numbers of cells can be obtained from T-AK
cultures compared to LAK cultures. The isolation and identi-
ification of the various subsets of cells developing non-MHC-
restricted lysis against different tumor targets have shown that
in addition to NK cells, CD3+Lyt-2+ T-cells develop lytic activ-
ity upon stimulation with anti-CD3 (11, 12).

It has generally been assumed that a high LAK activity is
accompanied by high NK function. The analysis of the lytic
function of the T-AK cultures demonstrates an apparent dis-
crepancy, with high lytic activity against NK-resistant targets
(high LAK) and low lytic function against YAC-1, an NK-
sensitive target. Previous reports have clearly established that
NK cells (CD3+) develop high lytic function in IL-2-stimulated
cultures (LAK). The present work confirms previous reports
which demonstrate that CD3+Lyt-2+ T-cells are responsible for
most of the lytic function in the cultures stimulated with anti-
CD3 and low concentrations (<100 units/ml) of IL-2. T-AK
cultures established with high concentrations (1000 units/ml)
of IL-2 show significant lysis of both NK-sensitive (YAC-1)
and NK-resistant (MCA-106) targets, suggesting the activation
of NK as well as T-cells (13).

Our results confirm the efficacy of T-AK cells and liposomal
IL-2 against MCA-38 and the ability of adoptively transferred
T-AK cells to infiltrate hepatic metastases. The tumor localiza-
tion and therapeutic efficacy of T-AK cells were greatly en-
hanced by the administration of IL-2 in liposomes to the tumor-
bearing mice. Free IL-2 or empty liposomes themselves mani-
fest no antitumor effect in this model. The mechanism for the
increased efficacy of IL-2 in liposomes when compared to free
IL-2 is uncertain. It is possible that since liposomes are prefer-
entially absorbed via the lymphatic system (28–30) and
clarified via the reticuloendothelial system, IL-2 liposomes
would be concentrated in the liver, thereby effectively increasing
the concentration of IL-2 at the site of the tumor. A second
possibility could be the prolonged elimination \( t_0 \) of IL-2 pro-
duced by encapsulation in liposomes. We have previously shown
that the elimination \( t_0 \) of i.p. free IL-2 is 24 min after injection
of 50,000 units of IL-2, while the elimination \( t_0 \) of liposomes
after i.p. injection is 4.3 h (15). It is also possible that macro-
phages are activated by IL-2 liposomes, becoming tumoricidal and
releasing chemo-attractive factors that could enhance the
migration of activated T-cells into the hepatic tissues. Biopsies
and immunofluorescence done in other organs such as the lung
and spleen did not show preferential traffic to those sites (data
not shown).

With this hepatic metastasis model, we have clearly demonstr-
ated the antitumor effect of T-AK cells in mice. These cells are
primarily Lyt-2+ T-cells which lyse targets in a non-MHC-restricted
fashion in vitro and are capable of localizing to metastatic
tumor sites after i.v. administration, thus sharing certain characteristics of TIL cells. However, some authors
have found TIL killing to be MHC restricted (31), although
this finding has not been universal (24), even when cells are
stimulated in the presence of autologous tumor (32). The ease
of generating large numbers of these cytolytic effector cells
makes T-AK cells promising candidates for future human clin-
ical trials.

### REFERENCES

1. Rosenberg, S. A., Lotze, M. T., Muul, L. M., Chang, A. E., Avis, F. P.,
Leitman, S., Linehan, W. M., Robertson, C. N., Lee, R. E., Rubin, J. T.,
Seipp, C. A., Simpson, C. G., and White, D. E. A progress report on the
treatment of 157 patients with advanced cancer using lymphokine-activated
killer cells and interleukin-2 or high-dose interleukin-2 alone. N. Engl. J.
2. Rosenberg, S. A., Lotze, M. T., Muul, L. M., Leitman, S., Chang, A. E.,
Etinghausen, S. E., Matory, Y. L., Skibber, J. M., Shiloni, E., Vetto, J. T.,
Seipp, C. A., Simpson, C. G., and Reichert, C. M. Observations on the systemic
administration of autologous lymphokine-activated killer cells and recombi-


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