Effect of Immunotherapy with Allogeneic Lymphokine-activated Killer Cells and Recombinant Interleukin 2 on Established Pulmonary and Hepatic Metastases in Mice

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ABSTRACT

The adoptive transfer of lymphokine-activated killer (LAK) cells in conjunction with the systemic administration of recombinant interleukin 2 (RIL-2) results in the regression of established pulmonary and hepatic micrometastases from a variety of immunogenic and nonimmunogenic murine tumors in syngeneic C57BL/6 mice. Recent studies have shown that this therapeutic approach can mediate the regression of cancer in humans as well. Because of the practical difficulties in obtaining syngeneic or autologous LAK cells for the therapy of cancer in humans, we have now evaluated the antitumor efficacy of allogeneic LAK cells generated from different strains of mice. The in vitro lysis of fresh tumor targets by LAK cells is not a major histocompatibility complex-restricted phenomenon since LAK cells of BALB/c-H-2k, DBA/2-H-2b, and C3H-H-2k origin all exhibited lytic activity when tested against allogeneic MCA-102-H-2b targets in short term 51Cr release assays. In vivo, the i.v. transfer of allogeneic LAK cells combined with i.p. injections of RIL-2 reduced the number of established pulmonary metastases induced by either MCA-105 or MCA-101 tumors which are syngeneic to C57BL/6 hosts. The extent of reduction of these pulmonary metastases by the allogeneic LAK cells was directly dependent upon the dose of RIL-2 given; increasing doses of systemically administered RIL-2 resulted in increasingly greater reduction in the numbers of established 3-day pulmonary sarcoma metastases. In dose titration experiments, adoptive transfer of at least 2 doses of 10^8 allogeneic LAK cells was necessary to achieve significant antitumor effect in vivo. Allogeneic LAK cells were also successful in mediating significant regression of hepatic micrometastases. Again, the i.v. transfer of allogeneic LAK cells had a smaller therapeutic benefit compared to i.p. transfer of syngeneic LAK cells. When allogeneic LAK cells were injected intraportally, however, they were as effective as syngeneic LAK cells. Allogeneic LAK cells had little, if any, therapeutic effect on established pulmonary and hepatic metastases when administered to recipients previously immunized to the histocompatibility antigens on the donor cells. Taken together, our results indicate that allogeneic LAK cells from several strains of mice are effective in lysing fresh MCA-102 tumor in vivo and that when given i.v. in sufficient numbers, in conjunction with RIL-2, they can mediate significant reduction in the number of established pulmonary and hepatic micrometastases in nonalloimmunized C57BL/6 mice. Direct intraportal administration of allogeneic LAK cells is more effective than i.v. injection in mediating regression of hepatic metastases in C57BL/6 mice.

INTRODUCTION

Murine splenocytes or human peripheral blood lymphocytes on incubation in vitro in high concentrations of the lymphokine IL-2 acquire the capacity to lyse a variety of fresh syngeneic murine and autologous human tumor cells in short term 51Cr release assays. We have further shown that these LAK cells are distinct from natural killer cells and classical cytotoxic T-cells and are lytic to allogeneic tumor targets from murine and human sources as well (1–3). The adoptive transfer of syngeneic LAK cells in conjunction with the systemic administration of relatively low doses of RIL-2 markedly reduced the number of established pulmonary and hepatic metastases from murine tumors of different histological types (4–7). Recent clinical studies have demonstrated that adoptive transfer of LAK cells and RIL-2 can mediate the regression of advanced cancer in humans as well (8). A major difficulty in the application of the principles learned from our animal models to the treatment of human cancer has been the availability of sufficient numbers of autologous lymphoid cells for LAK cell generation and reinfusion. The number of LAK cell precursors obtained from peripheral blood of cancer patients by leukopheresis is limited; based on our mouse models, larger number of cells lead to a greater antitumor therapeutic effects (9). One potential solution to this problem is the use of LAK cells generated from the lymphocytes of pooled buffy coats from normal blood donors. Theoretically, those lymphocytes would serve as a large source of LAK cell precursors for IL-2 activation in vivo. We therefore undertook a series of in vitro and in vivo experiments to explore the antitumor efficacy of allogeneic LAK cells against murine tumors. We demonstrated that LAK cells generated from three mouse strains (DBA/2, BALB/c, and C3H) can lyse allogeneic target cells of the MCA-102 tumor which is a tumor in C57BL/6 mice. In addition, we have evaluated the ability of allogeneic LAK cells to mediate the regression of established 3-day pulmonary and hepatic metastases of MCA-105 and MCA-101 murine sarcomas in immunized and nonimmunized C57BL/6 hosts.

MATERIALS AND METHODS

Animals. Female C57BL/6 (B6) mice (at least 12 weeks of age) and female B6, C3H, BALB/c, or DBA/2 retired breeders were obtained from the Animal Production Colonies of the NIH, Bethesda, MD. The mice were fed standard laboratory chow and water ad libitum.

Splenocytes. Spleens were removed aseptically. After the spleens were crushed with the hub of a syringe in HBSS (Biofluids, Rockville, MD), the cell suspension and spleen fragments were passed through a single layer of 100-gauge nylon mesh (Nitec; Lawshe Industrial Co., Bethesda, MD). The cells were centrifuged at 400 × g for 5 min and the erythrocytes were osmotically lysed by resuspension of the pellet in 10% buffered ammonium chloride solution for 2 min. The cells were then centrifuged and washed 3 times with HBSS.

RIL-2. As previously described, the gene for human IL-2 was isolated from a high producer Jurkat cell line and expressed in Escherichia coli (10). The resulting IL-2 which was purified to apparent homogeneity has a specific activity of 3–4 × 10^4 units/mg. The endotoxin level in the purified preparation was less than 0.1 mg/10^6 units of RIL-2 as measured in a standard Limulus assay. This human RIL-2 was kindly supplied by the Cetus Corp. (Emeryville, CA).

Generation of LAK Cells. Fresh splenocytes (5 × 10^6 cells prepared as described above), were placed in 175-cm² (750-ml) flasks containing...
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175 ml of CM and 175,000 units of RIL-2. CM consisted of RPMI 1640 (Biofluids) with 10% heat-inactivated fetal calf serum (Biofluids), 0.1 mm nonessential amino acids, 0.1 μM sodium pyruvate (all from Microbiological Associates, Walkersville, MD), 5 × 10^{-3} M 2-mercaptoethanol (Aldrich, Milwaukee, WI), 100 streptomycin (100 μg/ml), penicillin (100 units/ml), 0.03% fresh glutamine (all from the NIH Media Production Section, Bethesda, MD), gentamycin (50 μg/ml; Schering, Kenilworth, NJ), and Fungizone (0.5 μg/ml; Flow Laboratories, McLean, VA). The flasks were incubated supine for 72 h at 37°C in a moist atmosphere with 5% CO_2. The LAK cells were then harvested, passed over Ficoll (Lympholyte-M; Cedarlane Laboratories, Hornby, Ontario, Canada) to remove dead cells, and washed 3 times with HBSS before resuspending in HBSS for i.v. injection. Aliquots of LAK cells were tested for cytotoxicity in vitro in a standard 4-h 51Cr release assay against the fresh MCA-102 sarcoma target as described previously.

Tumors. The tumors used in our experiments were MCA-101 and MCA-105 sarcomas which were syngeneic to B6 mice. These sarcomas were induced by the i.m. injection of 0.1 ml of 1% MCA in sesame oil as described previously (11). A large number of vials of MCA-101 and MCA-105 from the first passage generation were cryopreserved. After thawing from storage at −70°C, the tumors were injected s.c. into B6 mice and were used for serial passage within the first 7 transplant generations. Single cell suspensions of the MCA-101 and MCA-105 sarcomas were prepared from fresh tumors that were excised, minced with scissors, and stirred in a triple enzyme solution of DNase, hyaluronidase, and collagenase (Sigma Chemical Co., St. Louis, MO) for 3 h at room temperature. The cell suspension was then filtered through 100-μm nylon mesh (Nitinex), washed 3 times in HBSS, and resuspended at the appropriate cell concentration for injection in HBSS.

For induction of pulmonary metastases, B6 mice were given i.v. injections via the lateral tail vein of 1 ml of a single cell suspension containing 3–4 × 10^6 MCA-101 or MCA-105 cells using a 27-gauge needle.

Adoptive Immunotherapy Model of Pulmonary Metastases. For treatment of micrometastases, LAK cells were suspended at 1 × 10^7, 3 × 10^7, or 1 × 10^8 cells in 1 ml of HBSS and injected into the lateral tail vein on days 3 and 6 after tumor injection. From days 3 through 8, mice were given i.p. injections of either HBSS or RIL-2 in HBSS, 0.5 ml per injection every 8 h. These injections were given from days 3 through 10 for doses ≤25,000 units and days 3 through 7 for doses >25,000 units. At least six mice were included in each treatment group. At 14 days after tumor injection, the mice were ear tagged and randomized. They were then given a tail vein injection consisting of 0.5 ml of a 15% solution of India ink (Higgins black No. 4417; A. W. Faber Castell) in phosphate buffered saline rendering the liver black. The mice were then killed by cervical dislocation, and their livers were harvested and bleached in Fekete’s solution (14) allowing the metastases to become easily countable as they formed discrete white nodules on the surface of the liver. Nodules were counted in a blinded fashion. Liver metastases too numerous to count were given an arbitrary value of 250. After all data were recorded, the code was broken.

Selective Intraportal Infusion of LAK Cells. LAK cells were selectively infused into the liver as described previously (7). Briefly, on day 3 after tumor induction, mice were lightly anesthetized with pentobarbital (Somnifer), prepared with ethanol, and positioned supine. A transverse abdominal incision was made through skin and muscle. The portal vein was isolated and 1 × 10^7 LAK cells were suspended in 1 ml of HBSS without calcium and magnesium was injected into this vessel by means of a 30-gauge needle (American Hospital Supply) over a period of 10–15 s. The needle was removed 5 s after the injection was completed, hemostasis was secured with a sterile gauze, and the abdominal incision was closed with 9-mm Autoclip wound clips. The animals were then randomized to their respective treatment group.

Immunization. B6 mice (H-2b) were immunized weekly by repetitive i.p. injection of either BALB/c-H-2b splenocytes or cells of the P815 tumor (a plasmacytoma syngeneic to DBA/2). Immunized animals were given injections of tumor cells to induce pulmonary metastases 2 weeks after the last immunization challenge. Three mice from each group were also bled at this time and the sera were later examined for the presence of specific antibody to the relevant H-2 haplotype(s) of the donor cell challenge by antibody-mediated cytotoxicity assay as described elsewhere (15). Nonimmunized age-matched B6 mice were also bled and were used as a negative control group in the assay. Another group of B6 mice were given i.p. injections of C3H splenocytes, 1 week later they were challenged with the same number of cells, and 4 weeks after the first immunization they were given tumor cell injections transplentrically to induce hepatic metastases. B6 mice not immunized to C3H MHC were used as a control group.

Fresh Tumor Targets. Fresh tumor cells prepared from a natural killer cell-resistant methylcholanthrene-induced sarcoma (MCA-102) passed s.c. were used as 51Cr-labeled target cells. Fresh tumor cells were prepared for each assay from s.c. tumor nodules by debridement of necrotic tissue, fat, and skin and then minced with scissors. Tumor fragments were then dispersed with 0.25% trypsin in Dulbecco’s phosphate-buffered saline without Ca^2+ and Mg^2+ (NIH Media Unit) at room temperature for 5 min; trypsinization was then repeated twice. The supernatant containing released tumor cells was removed and washed 3 times in HBSS, treated with ammonium chloride solution before resuspending the tumor cells in CM. EL-4, which is a chemically induced lymphoma syngeneic to B6 mice and passed as ascites, was also used in some in vitro assays. Cells of this tumor were obtained from ascites, washed 3 times in HBSS, treated with ammonium chloride solution to remove erythrocytes, and resuspended in CM.

Chromium Release Assay. An aliquot of 10^4 target cells was labeled with 200 μCi of Na^51CrO_4 (Amersham Corp., Arlington Heights, IL).
in 1 ml of CM for 1 h at 37°C, washed three times, and recounted before dilution and incubation in sextuplicate with effector cells in 96-well round bottomed microtiter plates (Costar). LAK cells were used as effectors. The effector and target cells were mixed in varying ratios and plates were centrifuged at 180 × g for 5 min and then incubated at 37°C in 5% CO₂ for 4 h. The plates were then spun at 400 × g for 5 min and the culture supernatants were harvested with the SCS harvesting frames and Macrowell strips (Skatron, Inc., Sterling, VA). Spontaneous release was measured after incubation of labeled tumor cells with CM only while maximal isotope release was obtained by incubation of the target cells with 0.1 N HCl. The spontaneous release was <35% of maximal release in all data presented. The percentage of specific lysis was calculated as

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

Cytotoxicity was expressed in lytic units per 10⁶ effector cells. A lytic unit is the number of effector cells that lyse 30% of ³¹Cr labeled target cells (normalized for 10⁴ target cells, assuming a constant percentage of cells/ml); 2 ml of the cell mixture were seeded in 16-mm-diameter 24-well plates (Costar, Cambridge, MA). The plates were incubated at 37°C with 5% CO₂ and 4 days later the cells were harvested.

Anti-MHC Antibody Blocking of Cytotoxicity. Four-day C3H and DBA/2 LAK cells were tested for cytotoxicity in vitro in a standard 4-h ³¹Cr release assay against fresh MCA-102- H-2* target cells. Four anti-MHC murine sera (a gift from Dr. David H. Sachs, NIH, National Cancer Institute, Bethesda, MD) were diluted 1:10 by adding 10 µl of sera to 100 µl of ³¹Cr-labeled target cells (10⁴ cells/well). After 30 min of incubation at 37°C 100 µl of LAK effector cells (at varying concentrations) were added. The plates were then spun, incubated for 4 h, and harvested as described above. The C3H and DBA/2 4-day ML CMC cells were tested for cytotoxicity using fresh EL-4-H-2* cells as a target. The anti-MHC sera used in these blocking assays were: (a) AB.Y anti-A/J (anti-H-2a); (b) A/J anti-AB.Y (anti-H-2b); (c) A/J anti-B10 (anti-H-2d); (d) A/J anti-B10A (anti-H-2*).

Statistical Analysis. The significance of differences in numbers of lung metastases between groups was determined by the Wilcoxon rank sum test (16). Two tailed P values are presented in all experiments.

RESULTS

Allogeneic LAK Cells Lyse Fresh MCA-102 Targets in Vitro. Syngeneic LAK cells of B6-H-2* mice and allogeneic LAK cells of C3H-H-2* DBA/2-H-2* and BALB/c-H-2* mice were tested for in vitro cytotoxicity against fresh MCA-102-H-2* targets in 4-h ³¹Cr release assays. The cell yields of splenocytes obtained from B6, BALB/c, and DBA/2 mice after 3 days of incubation in RIL-2 were similar and ranged from 38 to 43%. We have also generated LAK cells by coincubation of fresh splenocytes from different strains in RIL-2 and we have termed these cells “pooled” LAK cells. These “pooled” allogeneic LAK cells were also tested in vitro for cytolysis. An illustration of a typical experiment is shown in Fig. 1 in which allogeneic LAK cells of C3H and DBA/2 mice were compared to the syngeneic B6 LAK cells for cytolytic activity. All 3 LAK cell groups exhibited similar lysis of the MCA-102 target. We performed multiple in vitro cytotoxicity assays using various allogeneic LAK cells and have summarized the results in Table 1 and Fig. 2. Allogeneic fresh splenocytes incubated with RIL-2 either alone or as “pools” were effective in lysing fresh MCA-102 tumor cells in vitro. Minor differences existed in the lytic capacity of LAK cells from various mouse strains. It is of interest that “pooled” allogeneic LAK cells generate a lower cytolytic activity that one would expect based on the cytotoxicity of each haplotype alone. The combination of B6 and C3H splenocytes incubated together in IL-2 generated, for example, less cytotoxicity than either haplotype alone (Table 1). The possibility exists that bidirectional MLC may be responsible for the reduced cytotoxicity of “pooled” allogeneic LAK cells. Results of preliminary experiments in vivo showed, however, no difference in antitumor efficacy of “pooled” versus single allogeneic LAK cells (data not shown).

LAK Cytotoxicity Is Not Blocked by Antisera to MHC Determinants. To test whether or not LAK cell recognition of targets in vitro is MHC restricted we used specific antisera to H-2 determinants expressed on the targets in an attempt to block cytolyis. As shown in Fig. 3, the cytotoxicity of both DBA/2-H-2* and C3H-H-2* tumor cells was diminished by preincubating the targets with specific antibodies against the MHC of H-2* before the addition of LAK cells (Fig. 3, A and B). However, the cytotoxic activity of allogeneic CTL from the same mouse strains as the LAK cells (generated by a 5-day MLC of DBA/2 or C3H splenocytes and irradiated B6 stimulators) against EL-4 targets (H-2d) was blocked almost completely by the antibodies (Fig. 3, C and D). These results showed that the recognition of targets by LAK cells is not via MHC determinants.

Allogeneic LAK Cells Mediate Significant Reduction of Established Pulmonary Metastases but Less Effectively Than Syngeneic LAK Cells. The adoptive transfer of allogeneic LAK cells of C3H, DBA/2, and BALB/c mice in conjunction with the systemic administration of RIL-2 resulted in a significant reduction of established 3-day pulmonary metastases in C57BL/6 mice. In a representative experiment presented in Fig. 4, the i.p. injection of 7500 units of RIL-2 alone every 8 h for 5 consecutive days, had no effect on the number of pulmonary metastases when compared to HBSS alone. However, the addition of 1 × 10⁶ LAK cells, either syngeneic or allogeneic, to the dose of RIL-2 resulted in significant reductions in pulmonary metastases in all 3 treated groups (B6, P < 0.005; BALB/c, P < 0.005; DBA/2, P < 0.01). The antitumor effect of the transfer of allogeneic LAK cells plus RIL-2 was reproducible and was seen in 13 of 17 experiments.

Syngeneic LAK cells of B6 mouse origin, however, demonstrated a greater capacity to diminish MCA-105 pulmonary metastases than allogeneic LAK cells of either BALB/c or DBA/2 mice. In a few experiments allogeneic LAK cells failed to show significant therapeutic benefit in vivo when compared to an equivalent number of transferred syngeneic LAK cells.

Fig. 1. Four-h ³¹Cr release assay of LAK cells generated in vitro from 3 different mouse strains against a fresh MCA-102 sarcoma target from the C57BL/6 strain. The syngeneic (B6) LAK cells and the allogeneic (C3H and DBA/2) LAK cells show similar cytolytic activity.
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Table 1: Cytolytic activity of syngeneic and allogeneic LAK cells to MCA-102 tumor cells

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<th>Mouse strain (no. of experiments*)</th>
<th>Effector:target ratio</th>
<th>C57BL/6 (28)</th>
<th>BALB/c (21)</th>
<th>DBA/2 (13)</th>
<th>C3H (5)</th>
<th>Pool:BALB/c + C3H (3)</th>
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* LAK cells were generated by the in vitro incubation of fresh splenocytes in RIL-2 for 3-4 days.

** Percentage of specific lysis (mean ± SE) in a 4-h ¹¹Cr release assay.

Fig. 2. Mean in vitro cytotoxicity against a fresh MCA-102 sarcoma target by LAK cells generated from 4 separate strains of mice. The results of multiple assays are shown here. N, number of experiments performed for each mouse strain.

Fig. 3. Effects of anti-H-2 antibodies on the cytotoxicity of LAK cells and specific CTL. Cytolytic activity of allogeneic (DBA-H-2(+) and C3H-H-2(−)) LAK cells against MCA-102 (H-2b) targets was not inhibited by antibodies directed against the MHC of H-2(+) as shown in A and B. The same antibodies induced a marked decrease in the ability of specific CTL of the DBA/2 and C3H mouse strains (sensitized in a mixed lymphocyte culture against H-2(+) splenocytes) to lyse EL-4 (H-2b) targets in vitro. Anti-H-2(+) antibodies (anti-A/BJ) failed to inhibit the specific cytotoxic cells (C and D), S, sensitized.

which demonstrated significant reduction of pulmonary metastases in all the experiments (Table 2). Moreover, the mean percentage of reduction of metastases in mice that received syngeneic LAK cells was significantly greater than in those mice treated with either allogeneic BALB/c or DBA/2 LAK cells (87.3 versus 68.2 and 57.5%, P < 0.05). Two therapy experiments with transferred allogeneic C3H LAK cells plus RIL-2 resulted in a mean reduction of 75%, albeit not statistically conclusive because of the too small number of experiments; the therapeutic effect is again reduced in comparison to the syngeneic LAK cells (87.3%).

Dose Titration of LAK Cells in Vivo. We next tested the ability of varying numbers (1 x 10⁷, 3 x 10⁷, and 1 x 10⁸) of syngeneic and allogeneic LAK cells administered i.v. on days 3 and 6 after tumor injection to mediate the reduction of pulmonary metastases. As shown in Fig. 5, adoptive transfer of 1 x 10⁷ LAK cells in conjunction with 7500 units of RIL-2 resulted in significant reduction of tumor in the 3 treatment groups (B6, 93%, P < 0.005; BALB/c, 85%, P < 0.005; and DBA/2, 71%, P < 0.01). Syngeneic but not allogeneic LAK cells at 3 x 10⁷ cells significantly decreased the number of
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Fig. 5. Dose titration of the percentage of reduction of MCA-105 pulmonary metastases treated with RIL-2 plus increasing numbers of LAK cells. RIL-2 (7500 units) was given i.p. every 8 h beginning on day 3 after tumor injection. C57BL/6, BALB/c, or DBA/2 LAK cells were injected i.v. at various numbers (1 x 10^7, 3 x 10^7, and 1 x 10^8) on days 3 and 6. Each point represents the mean percentage of reduction in a group of 6 mice compared to mice treated with RIL-2 alone.

Fig. 6. Dose titration of the percentage of reduction in the number of MCA-101 pulmonary metastases in B6 mice given BALB/c LAK cells and increasing doses of RIL-2. Each point represents the mean percentage of reduction in a group of 6 mice compared to LAK cells given with HBSS. Each animal was given an i.v. injection of 3 x 10^5 MCA-101 cells and given either HBSS or RIL-2 at a dose ranging from 3,000 to 30,000 units every 8 h for 6 days. Allogeneic LAK cells (1 x 10^8) generated from BALB/c mice were given i.v. injections on days 3 and 6 after tumor injection. Increasing doses of RIL-2 led to an increase in the percentage of reduction in the number of lung metastases.

pulmonary metastases (50%, P < 0.05); 1 x 10^7 LAK cells caused less than 20% reduction in all 3 groups when compared to RIL-2 alone (P > 0.05). Similar results were obtained in 4 additional experiments and demonstrated that allogeneic LAK at a dose of 1 x 10^5 cells plus RIL-2 were effective in achieving significant reductions in the number of pulmonary metastases but that at limiting doses syngeneic cells were somewhat more effective than allogeneic cells in vivo.

Dose Titrations of RIL-2 in Vivo. The reduction in the number of pulmonary metastases resulting from the administration of 1 x 10^5 allogeneic LAK cells varied depending on the dose of RIL-2 administered. LAK cells (1 x 10^5) alone resulted in no therapeutic benefit and elevation of the RIL-2 dose administered systemically resulted in increasingly greater reductions in the number of metastases (Fig. 6: HBSS and 3,000 units RIL-2, NS; 7,500 units RIL-2, P > 0.05; 15,000 and 30,000 units RIL-2, P < 0.005). These results were reproducible in 3 additional experiments in which allogeneic LAK cells from various mouse strains were given in conjunction with RIL-2.

Allogeneic LAK Cells Injected i.v. Mediate a Less Significant Reduction of Hepatic Micrometastases Than Syngeneic LAK Cells but Selective Intraperitoneal Infusion Increases Their Antitumor Activity. Mice with established day 3 liver metastases were treated with varying doses of RIL-2 alone or in combination with allogeneic (BALB/c-H2k) or syngeneic (C57BL/6-H2k) LAK cells. LAK cells were given i.v. on days 3 and 6 and RIL-2 was given i.p. every 8 h from days 3 through 10 as described in "Materials and Methods." Results of three characteristic experiments are shown in Table 3. For all experiments performed, the systemic injection of RIL-2 given alone at doses of 25,000 units did not significantly reduce the number of metastases over that seen with therapy with HBSS alone (Experiment 1: HBSS, 250; 25,000 units RIL-2, 250). Similarly, the i.v. or intraportal injection of allogeneic LAK cells combined with HBSS failed to reduce the number of metastases significantly (Experiment 1: HBSS, 250; allogeneic LAK cells i.v. plus HBSS, 250; allogeneic LAK cells intraportally plus HBSS, 241; P NS). Combining the i.v. injection of allogeneic LAK cells with RIL-2, although reducing the number of metastases, failed to reduce the number of metastases to a level comparable to that obtained with syngeneic LAK cells given i.v. (Experiment 1: allogeneic LAK cells plus 25,000 units RIL-2, 200; syngeneic LAK cells plus 25,000 units RIL-2, 126; P < 0.05. Experiment 2, 143 versus 72; P < 0.01). However, when allogeneic LAK cells were injected intraportally with a RIL-2 dose that by itself had no effect, a significant reduction in the number of metastases was now obtained which was not statistically different than that seen with syngeneic LAK cells given intraportally (allogeneic LAK cells intraportally plus 25,000 units RIL-2, 32 and 39, versus syngeneic LAK cells intraportally plus 25,000 units RIL-2, 11 and 48 in experiments 1 and 3, respectively; P NS). This antitumor effect was highly reproducible regardless of the H-2 histocompatibility complex of the donor strain of allogeneic LAK cells and in 3 further experiments the addition of either C3H (in 2 experiments) or DBA/2 (in 1 experiment) LAK cells to 25,000 units RIL-2 induced a marked reduction in the number of hepatic metastases from means of 241, 241, and 219 to means of 37, 20, and 19, respectively (P < 0.005).
Allogeneic LAK Cells Are Not Effective in Immunized Mice. All B6 mice that had received repeated i.p. injections of allogeneic splenocytes or tumor cells (as described in “Materials and Methods”) and were tested by the antibody-mediated cytotoxicity assay (14) were highly immunized against the donor cells (data not shown). Allogeneic LAK cells did not have a significant therapeutic effect in vivo when injected into immunized mice. While $1 \times 10^8$ BALB/c LAK cells plus 7500 units of RIL-2 significantly diminished pulmonary metastases from MCA-105 nonimmunized B6 recipients, the same treatment did not affect liver metastases in immunized animals, even when immediately transferred LAK cells. Our laboratory has shown previously that the adoptive transfer of syngeneic LAK cells in conjunction with RIL-2 can mediate a significant reduction in the number of hepatic micrometastases as syngeneic LAK cells have found previously that allogeneic LAK cells lyse fresh murine tumor cells in vitro (1) and can also mediate a significant decrease in liver metastases (19 ± 4). It appears therefore that allogeneic LAK cells are not effective against either pulmonary metastases or hepatic metastases in mice immunized against the MHC antigens on the transferred LAK cells.

DISCUSSION

Our laboratory has shown previously that the adoptive transfer of syngeneic LAK cells in conjunction with RIL-2 can markedly reduce established pulmonary and hepatic metastases in B6 mice (4–7). We have recently reported encouraging preliminary results for the systemic administration of allogeneic LAK cells plus RIL-2 to patients with advanced cancer (8). A major limitation to the application of the animal therapeutic model to the treatment of human cancer has been the availability of adequate numbers of allogeneic lymphoid cells to be activated in vitro with RIL-2. The numbers of LAK cell precursors obtained from peripheral blood by leukopheresis are limited, especially in many patients who have leukopenia secondary to radiotherapy or prolonged administration of chemotherapy agents. Furthermore, leukopheresis is an expensive method which demands special equipment and trained staff and is time consuming.

Our observations in mouse tumor models have been accurate predictions of the results of our human studies; both murine and human LAK cells have a wide range of in vitro antitumor efficacy against different histological tumors (1, 2), implying the existence of a shared determinant on tumor cells that is recognized by LAK cells even on allogeneic tumors. Indeed we have found previously that allogeneic LAK cells lyse fresh murine tumor cells in vitro (1) and can also mediate the regres-
cells. Each group contained 5 to 10 mice. Livers and lungs were removed 13 or 14 days after tumor injection and metastases were counted blindly.

Blood donors for generating allogeneic LAK cells may be useful for the clinical treatment of human cancers. We have therefore performed a series of experiments in order to evaluate the therapeutic parameters necessary for successful therapy. We began our studies by examining the in vitro cytotoxicity of allogeneic LAK cells from 3 different mouse strains against fresh MCA-102 tumor cells. We were able to repeatedly demonstrate that either allogeneic LAK cells generated from a single strain or "pooleled" allogeneic LAK cells (in various combinations) were capable of lysing tumor cells in vitro (Table 1). Those results led us to another set of experiments in order to evaluate the therapeutic effectiveness of murine allogeneic LAK cells and study the parameters necessary for successful therapy.

We began our studies by examining the in vitro cytotoxicity of allogeneic LAK cells from 3 different mouse strains against fresh MCA-102 tumor cells. We were able to repeatedly demonstrate that either allogeneic LAK cells generated from a single strain or "pooleled" allogeneic LAK cells (in various combinations) were capable of lysing tumor cells in vitro (Table 1). Those results led us to another set of experiments in which we tested whether the in vitro cytotoxicity of allogeneic and syngeneic LAK cells could be blocked by specific antibodies directed against the MHC antigens present on the tumor cells. It was found that the activity of the LAK cells was not affected by those anti-MHC antibodies in contrast to classical allogeneic cytotoxic T-cells which were inhibited by those antibodies (Fig. 3). The LAK phenomenon thus appeared to be non-MHC restricted. The process by which LAK cells do identify their targets is still obscure and is under investigation.

Preliminary studies in our laboratory had shown that allogeneic LAK cells generated from splenocytes of DBA/2 mice, when combined with RIL-2, were capable of mediating the regression of established pulmonary metastases upon i.v. transfer (9). The primary success of this approach stimulated us to further investigate the therapeutic benefit of allogeneic LAK cells in mice. In order to elucidate the antitumor efficacy of allogeneic LAK cells from various strains of mice and in the therapy of hepatic as well as pulmonary metastases we have conducted multiple experiments using allogeneic LAK cells generated from 3 different mouse strains (DBA/2, BALB/c, and C3H) against pulmonary and liver metastases and we have also performed studies with i.v. and intraportal injection of allogeneic LAK cells.

The first issue we addressed was whether various allogeneic LAK cells were effective against 3-day established pulmonary metastases. Successful therapy of pulmonary metastases was seen when allogeneic LAK cells generated from 3 different mouse strains (BALB/c, DBA/2, and C3H) were administered i.v. in conjunction with RIL-2. We have shown previously that destruction of allogeneic LAK cells seems to be responsible for the decreased capacity of allogeneic LAK cells to induce regression of pulmonary as well as hepatic metastases.

### Table 5: Ability of allogeneic LAK cells to mediate a significant reduction in the number of hepatic and pulmonary metastases in nonimmunized recipients

<table>
<thead>
<tr>
<th></th>
<th>Hepatic metastases</th>
<th>Pulmonary metastases</th>
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<tbody>
<tr>
<td></td>
<td>RIL-2 alone</td>
<td>Allogeneic LAK cells (i.v.) + RIL-2</td>
</tr>
<tr>
<td></td>
<td>(A)</td>
<td>(C) Immuno-</td>
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<td></td>
<td>(C)</td>
<td>(D) Nonimmuno-</td>
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<td>Experiment</td>
<td>Injected tumor</td>
<td>(A)</td>
</tr>
<tr>
<td>1</td>
<td>MCA-105</td>
<td>250</td>
</tr>
<tr>
<td>2</td>
<td>C3H</td>
<td>219</td>
</tr>
<tr>
<td>3</td>
<td>BALB/c</td>
<td>244</td>
</tr>
<tr>
<td>4</td>
<td>DBA/2</td>
<td>19</td>
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* Statistical significance was as follows. Experiment 1: A versus B, C, not significant: (NS); D, P < 0.005. Experiment 2: E versus F, G, NS; H versus I, NS; J, P < 0.05, H versus J, P < 0.05. Experiment 3: E versus F, P < 0.05; F versus G, NS; H versus I, P < 0.005; J, P < 0.001; I versus J, P < 0.05.

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after i.v. administration as compared to syngeneic cells. Increased doses of RIL-2 administered i.p. to the tumor-bearing mice overcame the decreased therapeutic efficacy of the allogeneic LAK cells (Fig. 6). However, the toxicity associated with IL-2 administration in clinical trials might limit the application of this approach in cancer patients. Allogeneic LAK cells appeared to be more effective in reducing pulmonary metastases than liver metastases. The first capillary bed after i.v. injection is the lungs and, as we have previously shown (23), only after several h are lymphoid cells redistributed to the liver and spleen. The number of LAK cells that circulate to the liver may therefore not be large enough to mediate optimal antitumor activity. Injection of allogeneic LAK cells directly to the liver through the portal vein would be expected to overcome this problem of LAK cell traffic to the liver. Indeed, when we injected LAK cells intraportally, both syngeneic and allogeneic LAK cells when given together with RIL-2 had substantial therapeutic effect with no visible difference in their efficacy.

Prior immunization to the histocompatibility antigens on the allogeneic cells caused a dramatic change in the pattern of effectiveness of LAK cell therapy and led to a complete elimination of the antitumor activity of the allogeneic LAK cells against both lung and liver metastases. This factor may pose a major obstacle to the use of repeated cycles of therapy as may be required in treating humans with cancer.

Injection of large numbers of parental T-lymphocytes in normal F1 mice results in GVHR of variable intensity depending upon the strain combination used. This phase of GVHR is of short duration because during the course of the reaction host B-cells stimulated to produce alloantibodies that eliminate the transferred allogeneic cells. This process results in a rapid self-cure of the GVHR and finally in little morbidity (24). During the 14-day observation period in our experiments treating pulmonary or hepatic metastases there was no increased mortality in the animals treated by allogeneic LAK cells plus IL-2 as compared to the tumor-bearing mice treated by syngeneic LAK cells. We also have not noted visible signs of graft versus host disease in these therapy experiments. Since the mice we used in our experiments were immunocompetent, we did not expect graft versus host disease to occur. It is possible, however, that allogeneic LAK cells may cause the disease in mice that are immunosuppressed (e.g., by sublethal total body irradiation).

We have shown in this paper that allogeneic LAK cells from different strains lyse in vitro tumor cells. The ability to recognize and kill tumor target cells is based on a recognition mechanism which is not MHC restricted. We have also shown that injection of allogeneic LAK cells plus IL-2 can mediate tumor regression in the lungs and in the liver, although therapy with allogeneic LAK cells is less effective than with syngeneic LAK cells. Intraportal administration of allogeneic LAK cells caused a marked reduction in liver metastases in nonimmunized mice. Immunized recipients, however, show no tumor regression, either in the lungs (after i.v. injection) or in the liver (after i.p. administration).

The experiments in mice reported here were done to address the possibility of using allogeneic LAK cells in conjunction with RIL-2 in the therapy of pulmonary and liver metastases in humans. Although reduction of established pulmonary and hepatic metastases was achieved by this approach, the decreased effectiveness of allogeneic compared to syngeneic LAK cells and the lack of efficacy of LAK cell therapy in immunized hosts may, however, limit the success of this approach in humans.

REFERENCES


Effect of Immunotherapy with Allogeneic Lymphokine-activated Killer Cells and Recombinant Interleukin 2 on Established Pulmonary and Hepatic Metastases in Mice


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