Generation of Lymphokine-activated Killer Cells in Strain 2 Guinea Pigs and Their Use in the Therapy of L2C, an Acute B-Cell Leukemia

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ABSTRACT

Lymphokine-activated killer (LAK) cells were induced by incubating strain 2 guinea pig splenocytes or lymph node-derived cells in recombinant human interleukin-2 (IL-2) for 3–5 days. These effector cells had the morphology of lymphoblasts and were able to lyse murine P815 tumor cell targets. Fresh, unstimulated, guinea pig effector cells were not capable of lysing these targets.

The therapy of the L2C leukemia, an acute B-lymphoblastic leukemia of strain 2 guinea pigs, using LAK cells and recombinant IL-2 was examined. Antitumor effects were demonstrated by premixing LAK and tumor cells prior to intradermal injection in Winn type assays and then measuring the growth of local tumor and survival of the animals. In further experiments i.p. administration of LAK cells, 4 h following tumor cell inoculation by the i.p. route, prolonged the survival of treated animals. The best results in this i.p. therapy model were obtained with a 10-fold excess of LAK cells over tumor cells plus additional treatment with 1000 units of IL-2 for 20 days. This resulted in a 10-day increase in median survival of treated animals. Despite these in vivo antitumor effects, lytic activity of LAK effector populations against L2C targets could not be demonstrated in vitro.

The potential synergy between LAK cells, IL-2, and a monoclonal antibody directed against the idiotype of the neoplastic cell surface immunoglobulin was also investigated. In these experiments enhanced survival of the combined treatment group, beyond that of either singly treated group, was not found.

This study shows that LAK cells are useful agents in the therapy of a widely disseminated, aggressive, B-cell lymphoblastic leukemia. The use of such effectors, even in cases where in vitro lysis of the target tumor cell cannot be demonstrated, is encouraged by these results.

INTRODUCTION

A minor population of mononuclear cells from thymus, bone marrow, lymph node, spleen, thoracic duct lymph, and peripheral blood respond to IL-2 in vitro without prior stimulation, causing the induction of cells with a broad spectrum of lytic activity against tumor cell targets in vitro (1, 2). These IL-2-activated effectors are known as LAK cells. The availability of large quantities of recombinant IL-2 has facilitated the study of their induction (3).

Adoptive transfer studies have shown that LAK cells clearly have antitumor effects in vivo. In the murine B16 melanoma model the number of lung metastases was significantly decreased and the overall survival of tumor-bearing animals increased by an infusion of LAK cells after tumor inoculation (4, 5). This effect was most notable when exogenous IL-2 was also administered to tumor-bearing animals. In the majority of these studies using LAK cells and IL-2 infusions, solid, metastatic tumors have been used as models and little work has been performed on leukemic models where the neoplastic cells are widely disseminated very early in the course of the disease.

The L2C leukemia arose in an inbred strain 2 guinea pig more than 30 yr ago (6). It is an acute B-cell leukemia, the neoplastic cells expressing surface IgM, the receptor for C3b and Fc molecules (7, 8). This leukemia has been the subject of many immunological studies and has been used in several immunotherapeutic models. For example, preimmunization of guinea pigs with irradiated L2C cells or membrane extracts, in complete Freund’s adjuvant, prevents the growth of tumors on subsequent challenge, indicating the presence of a tumor-specific transplantation antigen (9). Adoptive transfer of lymphocytes from immunized animals also prevents tumor growth when transferred cells are given before or up to 2 days after tumor inoculation (10). Chemotherapy combined with immunotherapy was also shown to be effective in this model (11). Antibody therapy, directed at the idiotype of the L2C cell surface IgM molecule, has also been successfully used by Stevenson et al. (12, 13).

The purpose of this study was to determine whether LAK cells could be generated in guinea pigs and to evaluate their therapeutic potential, together with recombinant IL-2, in this aggressive and well-studied leukemic model. Finally, combination therapy using LAK cells, IL-2, and a monoclonal antibody, directed against the idiotype of the L2C cell surface IgM, was performed.

MATERIALS AND METHODS

Media, DMEM (10% heat inactivated fetal calf serum, Biofluids, Rockville, MD; 2 mM glutamine, Biofluids; 1 mM sodium pyruvate, Flow Laboratories, McLean, VA; penicillin (100 units/ml) plus streptomycin (100 μg/ml), Biofluids; amphotericin B (2.5 μg/ml), Serva Chemicals, New York, NY; all dissolved in DMEM, Biofluids) was used throughout this study. Whenever cells were required free from fetal calf serum, they were washed three times in PBS (Biofluids) or HBSS (Biofluids).

Animals. Inbred strain 2 guinea pigs, 300–500 g in weight, of either sex, were supplied by the animal production facilities, NIH. Animals were fed NIH guinea pig chow and given unrestricted access to water. All experiments were performed in accordance with NIH guidelines for use and care of experimental animals.

Antibodies. A murine monoclonal antibody M6-3D10, of γ1 subclass, directed against the idiotype of the surface immunoglobulin of the L2C leukemia cells (14), was provided by Dr. M. J. Glennie, Tenovus Research Laboratory. Other murine monoclonal antibodies which react with determinants expressed on subsets of guinea pig leukocytes were provided by Dr. E. M. Shevach (Laboratory of Immunology, National Institute of Allergy and Infectious Diseases). The specificity of, the distribution of staining with, and the characterization of these monoclonal antibodies is cited in the appropriate tables.

Tumor Cells. The L2C leukemia has been maintained in this laboratory for 15 yr by serial passage in strain 2 animals and by cryopreservation. Other tumor cells and lines described below were used exclusively for in vitro testing of LAK cell cytotoxic activity.

The line 10 hepatocarcinoma of strain 2 guinea pigs was obtained from Dr. John Weinstein, National Cancer Institute, and also main-
tained by serial passage as an ascitic tumor in strain 2 animals. The RT273 fibrosarcoma of strain 2 guinea pigs was obtained from Dr. Berton Zbar and maintained in tissue culture by weekly subculture following Trypsin Versene (Biofluids) treatment for 15 min at 37°C. The murine tumor cell lines P815 and YAC-1 and the human tumor cell line K562 were provided by Dr. Elizabeth Benson (Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases). These cell lines were maintained in tissue culture by splitting every 2-3 days, when the cell concentration approached 1 x 10^6/ml. A methylcholanthrene-induced murine fibrosarcoma (MCA102) was provided by Dr. J. Mulé (National Cancer Institute).

**Tumor Model.** Inoculation of 1-3 x 10^7 washed L2C cells, >90% viable by trypan blue exclusion into strain 2 guinea pigs by the intradermal route, in a volume of 0.1 ml HBSS, causes the development of a local tumor mass within 8 days which increases in size until death. This number of cells is more than 1000 times the lethal dose and is used to ensure uniform death in control animals.

The tumor cells rapidly become widely disseminated; for example, on day 10 leukemic lymphoblasts, detected with antidiotype, comprise up to 10% of the mononuclear cells obtained from the spleen. Death occurs between 12-16 days at which time the animals have a very large tumor burden, with infiltration of all organs (15). The peripheral WBC count at death is between 1-3 x 10^5/ml (normal WBC count 4-10 x 10^5/ml), of which >95% are leukemic lymphoblasts. Inoculation of cells i.p. gives a similar clinical course without the growth of a local tumor mass. There is not a pronounced ascitic phase after such an inoculation.

**Generation and In Vitro Testing of LAK Cell Activity.** Lymph node enlargement was induced by the injection of 0.1 ml complete Freund's adjuvant/PBS emulsion in each footpad. After 10 days the animals were sacrificed with CO_2 gas and the popliteal, inguinal, axillary, submaxillary, cervical, and mesenteric lymph nodes were excised. The spleen was also removed. Spleens from animals which had not been induced with complete Freund's adjuvant were also used as a source of LAK precursors. Mononuclear cells were prepared by chopping these tissues into small fragments and then mashing them through wire mesh in HBSS at 4°C. After one wash the cells were layered onto lymphocyte separation medium (Litton Bionetics, Kensington, MD) and centrifuged at 400 x g for 20 min at room temperature. Cells at the interface were washed twice in DMEM and resuspended at a density of 1 x 10^6/ml in DMEM in upright 50-250 ml tissue culture flasks (Falcon) to which varying amounts of highly purified recombinant IL-2 from *Escherichia coli* (3, 16) (Cetus Corp., Emeryville, CA) were added. The cultures were then incubated at 37°C in a humidified atmosphere containing 7% CO_2 for 1-4 days.

After various time periods in culture, indicated in tables and figures, LAK effector cells were washed twice in DMEM and counted and their viability checked with 0.2% trypan blue. Aliquots of all of the LAK cells thus generated were then tested for in vitro cytotoxicity as described below.

The cell concentration was adjusted to give the desired effector-to-target ratio in cytotoxicity assays. Four-h Cr release assays were used to determine cytotoxic capability of such effectors. The assays were performed as described previously (17) with slight modifications. Briefly, the target cells, in 50 µl HBSS containing ~0.5% fetal calf serum, were labeled for 1 h at 37°C with 100 µCi of sodium chromate (specific activity, 1 mCi/ml in PBS, ICN, Irvine, CA). After this incubation the cells were washed twice in DMEM also at 37°C and resuspended at a density of 1 x 10^6/ml in DMEM containing 7% CO_2 for 15 min at 37°C in DMEM. After a further wash 10^4 target cells were added to the wells of a microtiter plate in a volume of 10 µl. Effector cells were added in appropriate numbers to the targets in the microtiter plates and the final volume adjusted to 200 µl with DMEM. Plates were sealed with adhesive sealers (Flow Laboratories) and centrifuged at 200 x g for 5 min. They were then incubated for 4 h at 37°C in a humidified atmosphere of 7% CO_2. The supernatants were harvested using a supernatant collection system (Skatron, Sterling, VA) and cytotoxicity was calculated as shown:

\[
\frac{\text{Test cpm} - \text{spontaneous cpm}}{\text{Maximum cpm} - \text{spontaneous cpm}} \times 100 (A)
\]

% of specific lysis

The maximum Cr release was obtained with 0.1 N HCl for each target and spontaneous release was determined by incubation of targets in medium alone during the assay. All points were performed in triplicate. Effector were used at three different ratios for each Cr-labeled target.

**Formulation of IL-2.** Highly purified recombinant IL-2 from *Escherichia coli* (3, 16) was supplied lyophilized by the Cetus Corporation and reconstituted with sterile water to give a concentration of 1 x 10^6 units/ml. This concentrated stock was stable when stored for up to 4 wk at 4°C. Any stock not used in this period of time was discarded. Dilutions were made from stock into DMEM for *in vitro* work or into 5% guinea pig serum in HBSS for *in vivo* administration, this added protein being necessary to prevent loss of material due to nonspecific adsorption. Such diluted preparations were used within 1 h, although bioassay indicated their stability for at least 2 wk at 4°C.

Samples of excipient were also supplied lyophilized. This material was prepared by Cetus as a control for recombinant IL-2 but lacked the biologically active material. This material was diluted and handled in a similar manner to IL-2 whenever it was used.

Throughout this study the units of IL-2 used are those quoted by Cetus for lyophilized IL-2 stock, with appropriate dilution.

**Phenotypic Analysis of LAK Cell Populations.** Viable cells were analyzed for the expression of surface markers using fluorescein antibody staining techniques. Fluorescein isothiocyanate-conjugated rabbit antibody was used to detect guinea pig immunoglobulin and monoclonal antibodies were used in a two-step assay with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Becton Dickinson, Mountain View, CA) used as the detecting antibody. The anti-mouse immunoglobulin was passed over an immunosorbent column of Sepharose 4B (Pharmacia, Piscataway, NJ) coupled to guinea pig IgG prior to use.

LAK cells for this phenotypic analysis were generated by incubation of guinea pig splenocytes at 1 x 10^6/ml in DMEM containing IL-2 (1000 units/ml), for 3 days. The cells were washed once in DMEM at 37°C and layered onto lymphocyte separation medium (Liton Biotechnica). Cells collected from the Ficoll interface were washed twice in PBS and split into aliquots of 1 x 10^6 cells. These cells were pelleted and 20 µl of diluted antibody were added to the pellet. Following a 30-min incubation on ice the cells were washed three times with PBS containing 10 mM sodium azide at 4°C. When a two-step procedure was used, cell pellets were incubated for a further 30 min at 4°C with the second antibody and the washing repeated. After all staining and washing was completed, cells were resuspended in 0.5 ml PBS/azide and analyzed using a fluorescence-activated cell sorter analyzer (Becton Dickinson). Ten thousand cells were counted for each antibody. The proportion of cells staining with greater fluorescence intensity than 99% of cells stained with an appropriate negative control is reported. Rabbit anti-sheep immunoglobulin was used as a control for the rabbit anti-guinea pig immunoglobulin and class-matched monoclonal antibodies of irrelevant specificity were used as controls in the first step of the two-step procedure.

**Wiss Assay.** L2C tumor cells were washed twice in HBSS and mixed, immediately prior to intradermal injection at a ratio of 1:10, with freshly generated LAK cells or fresh mononuclear spleen cells, both also washed twice in HBSS. The final injection volume was 0.1 ml and the cells were injected through a 25-gauge needle. The site of injection was marked and additional injections of 1000 units IL-2 or excipient (buffer control for IL-2) were given directly into this site, also in a volume of 0.1 ml, once daily for 20 days. An additional group of animals received tumor cells alone, followed by 1000 units of IL-2 directly into this site, also for 20 days.

**LAK Cell Therapy i.p.** Tumor cells (1 x 10^6) were given by the i.p. route in 1 ml HBSS. The specified number of LAK cells, generated for 4 days as described above, were also given by the i.p. route after a delay of 4 h. IL-2 was given i.p. at the same time as the LAK cells and additionally for 5 or 20 days, as specified in the appropriate sections. Combined LAK Cell and Antiidiotype Antibody Therapy. Tumor cells, LAK cells, and IL-2 were given as described for the i.p. LAK cell therapy and monoclonal antibody with specificity for the idiotype of the IgM expressed on the L2C cell surfaces, described above, was given by the i.p. route 24 h after the tumor cells. The monoclonal antibody
was purified from ascites by ammonium sulfate precipitation, ion-exchange chromatography, and sizing gel separation as described (14). One mg (approximately 2.5 mg/kg) of this purified antibody was given per animal.

RESULTS

Generation of Lymphokine-activated Killer Cells in Vitro. At the initiation of this study we were unaware of any descriptions of the generation of LAK cells in guinea pigs. T-cells from guinea pigs, however, had been reported to respond to partially purified human IL-2 (18), suggesting that the guinea pig IL-2 receptor was similar to the murine and human IL-2 receptor and thus we felt that such an attempt was feasible. The morphology of cells obtained after a 3-day induction with 100–10,000 units of IL-2 was examined after Giemsa staining of cytospin preparations. A large proportion of cells were lymphoblastic in character with many cytoplasmic granules. In Table 1 the specific lysis of 51Cr-labeled targets using LAK cells generated in vitro with different concentrations of recombinant human IL-2 is shown. In these initial studies only the murine tumor cell line, P815, and a sarcoma of strain 2 guinea pigs (RT273) were lysed. The strain 2 guinea pig L9S leukemia cell line and line 10 hepatoma cell targets were not lysed by LAK cells in vitro. Spleen- and lymph node-derived LAK cells were both equally effective at causing the lysis of P815 and therefore no distinction has been made between them (data not shown).

Although it is generally desirable to use a tumor target syngeneic to the effector cells in cytotoxicity assays, the RT273 tumor has only recently been isolated (19) and may not be phenotypically stable. This tumor also grows as an adherent cell line in vitro and must be trypsin treated to prepare cell suspensions. Such treatment may render the cells more susceptible to lysis by a variety of reagents. We have chosen, therefore, to define guinea pig LAK activity in terms of lysis of the P815 mastocytoma cell line. This tumor was initially included in the panel for Table 1 because it is widely used as a target for allospecific cytotoxic cells in murine systems and appears to be resistant to NK cells from that species. The lysis of this target by IL-2-induced guinea pig effector (LAK) cells shows these effectors are not restricted to lysis of targets expressing identical major histocompatibility molecules.

In order to demonstrate that the lysis of P815 targets was due to lymphokine-induced effectors and not uninduced NK-like effectors, these targets were tested for susceptibility to lysis using unstimulated splenic effectors. Such guinea pig effector cells have been demonstrated to have NK activity in the studies of other workers (20). In addition, the YAC-1 tumor cell targets, which are sensitive to murine NK cells but not to guinea pig NK cells (21) and the K562 tumor cell targets which are susceptible to guinea pig NK cells were tested for lysis using guinea pig LAK effectors.

Table 2 shows a comparison of 51Cr release obtained in the presence of either fresh, unstimulated guinea pig effectors, or LAK effectors. It is apparent that, even after 18 h, the maximum specific 51Cr release from P815 targets using unstimulated effectors (13%) is considerably lower than the value obtained in a 4-h LAK assay (44%). This observation strongly suggests that NK cells were not responsible for the lysis observed by the LAK effectors. The YAC-1 and K562 tumor cell targets were not lysed by guinea pig LAK cells although a low degree of lysis of K562 targets was observed using fresh splenic effector cells.

In further studies the kinetics of induction of LAK cells, capable of mediating the lysis of P815 targets, was examined. Table 3 shows that the lytic activity of these effectors increased linearly during 69 h of culture in the presence of IL-2. Significantly, lysis of the P815 targets was seen at the highest effector to target ratio after only 21 h in such a culture.

Phenotypic Analysis of Guinea Pig LAK Cells. Further characterization of guinea pig LAK cell populations was performed. The expression of markers, defined by the monoclonal antibodies 8BE6 and 5C3 and by rabbit antibody to guinea pig immunoglobulin, on the surface of viable, Ficoll enriched, LAK cells is shown in Table 4. The monoclonal antibody 8BE6 reacts with guinea pig T-cells and precipitates a molecule which is thought to be the guinea pig analogue of murine Lyt1, with "pan" T-cell reactivity (22). 5C3 reacts with a molecule which is involved in the IL-2 driven proliferation of guinea pig T-cells but is distinct from the IL-2 receptor (18). In two separate experiments a large proportion of the population consisted of surface immunoglobulin positive (B-) cells and together with the cells expressing the marker defined by the monoclonal antibody 8BE6, accounted for almost all of the cells in these populations.

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Table 1: Effect of IL-2 concentration guinea pig LAK cell generation in vitro

<table>
<thead>
<tr>
<th>Target</th>
<th>Species</th>
<th>Concentration (units/ml)</th>
<th>Specific Lysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCA 102 Mouse</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>P815 Mouse</td>
<td>400</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>YAC-1 Mouse</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>LC2 Guinea pig</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>L10 Guinea pig</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>RT273 Guinea pig</td>
<td>2,500</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* Values represent the mean percentage of cytotoxicity and the figures in parentheses are the number of separate assays in which each test was performed.

Table 2: Comparison of LAK and unstimulated splenic effector cells

<table>
<thead>
<tr>
<th>Assay length</th>
<th>Effector cells</th>
<th>Target</th>
<th>(E/T) ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 h LAK</td>
<td>P815</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td></td>
<td>YAC-1</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>18 h LAK</td>
<td>P815</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>4 h Fresh spleen</td>
<td>P815</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>K562</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>YAC-1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>18 h Fresh spleen</td>
<td>P815</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>K562</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>YAC-1</td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>

* Whole spleen mononuclear cells were used as effectors for these assays.

Table 3: Kinetics of guinea pig LAK cell induction

<table>
<thead>
<tr>
<th>Incubation with IL-2 (h)</th>
<th>Percentage of specific 51Cr release of P815 targets at E/T ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td>69</td>
<td>0</td>
</tr>
</tbody>
</table>

* Values shown are the mean of triplicate assay points.

Table 4: Phenotypic Analysis of Guinea Pig LAK Cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Marker</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAK cells</td>
<td>8BE6</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td>5C3</td>
<td>Positive</td>
</tr>
</tbody>
</table>

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725
To examine whether the 8BE6 and 5C3 markers were expressed by the same cells, simultaneous staining with these monoclonal antibodies was performed. There was no additive increase in the number of cells staining and therefore these markers were expressed on the same cells. Thus our LAK populations consisted mainly of B-cells and cells expressing markers normally associated with activated guinea pig T-cells.

Testing of Guinea Pig LAK Cells in Vivo. The reproducible lysis of the P815 targets observed using IL-2 induced guinea pig effectors showed that guinea pig LAK cells were generated in vitro. Despite the failure of these effectors to lyse L2C targets in vitro and because it is difficult to demonstrate lysis of these targets using any guinea pig effector populations in vitro (23), it was decided to examine the effects of LAK cells on L2C cells in vivo. This initial in vivo examination was performed by mixing LAK effectors and target cells immediately prior to intradermal injection as described by Winn (24). The modification of injecting IL-2 directly into the tumor site was taken from Forni et al. (25). Due to the wide dissemination of the L2C cells both the growth of a local tumor and the survival of the animals was measured.

Fig. 1 shows the growth of the local tumor for the groups of animals given L2C cells admixed with LAK and given additional exogenous IL-2 or excipient. The death of each animal is also indicated with a vertical line. The increase in the local tumor size and time of death of an untreated animal given 3 x 10^5 tumor cells is shown in Fig. 1A and is representative of more than 100 animals thus treated, in this laboratory. Fig. 1, parts B-F show the local tumor growth in animals given 3 x 10^5 tumor cells mixed with 3 x 10^6 LAK cells and given excipient into the tumor site for 20 days (or until death). In this group two animals did not develop a local tumor (Fig. 1, E and F). In another group of animals given L2C cells mixed with LAK cells and daily injections of IL-2 (Fig. 1, G-K), 2/5 animals (I, J) did not develop a local tumor mass and in a further two animals (G, H) the local mass regressed. These local effects, i.e., an 80% absence or regression of tumor, were superior to the group in which daily injections of LAK cells plus excipient were given, where only a 40% absence of local tumor growth was noted. The prevention of local tumor growth in the majority of animals where LAK and exogenous IL-2 were given and in some of the animals where LAK and excipient were given, clearly shows antitumor actions of the admixed LAK cells. In the Winn assays in which normal splenocytes were mixed with L2C cells, local tumors grew progressively, similar to the animal shown in Fig. 1A (data not shown).

In another group of animals given tumor cells alone and then daily injections of IL-2 into this site, all of the animals developed a local tumor mass although in two out of five animals these regressed during the course of treatment (data not shown).

The survival curves for all of the groups used in this experiment are shown in Fig. 2. Significantly increased survival was seen in groups in which tumor cells were mixed with LAK cells when these groups were compared to animals in which tumor cells were mixed with normal splenocytes. This shows a direct inhibitory effect of LAK cells on L2C cells, in contrast to the negative in vitro findings in a 4-h 51Cr release assay. Injection of IL-2 at the site of these Winn assays did not significantly enhance the survival of animals in either admixed effector group, when compared to the groups treated with cells alone. However, when IL-2 was injected locally into the site where L2C cells were given without additional effector cells, an increase in survival was also seen. The death of all of the animals
LAK CELL THERAPY OF A GUINEA PIG B-CELL LEUKEMIA

Fig. 2. Survival curves for groups of animals inoculated intradermally with tumor cells mixed with different populations of effector cells. 3 x 10^6 L2C cells mixed with: (−Δ−) 3 x 10^6 normal splenocytes and given excipient, directly into the tumor site, for 20 days (N = 6); (−□−) 3 x 10^6 normal splenocytes and given 1000 units IL-2, directly into the tumor site, for 20 days (N = 6); (−O−) 3 x 10^6 LAK cells and given excipient, directly into the tumor site, for 20 days (N = 5); (−Φ−) 3 x 10^6 LAK cells and given 1000 units IL-2, directly into the tumor site, for 20 days (N = 5); (−×−) no admixed cells but given 1000 units IL-2, directly into the tumor site, for 20 days (N = 5); (−A−) 3 x 10^6 normal splenocytes and given excipient, directly into the tumor site, until death (N = 6); (−X−) no admixed cells but given 1000 units IL-2, directly into the tumor site, until death (N = 6); (−A−) 3 x 10^6 normal splenocytes and given excipient, directly into the tumor site, until death (N = 6); (−O−) 3 x 10^6 LAK cells and given excipient, directly into the tumor site, until death (N = 6); (−Φ−) 3 x 10^6 LAK cells and given 1000 units IL-2, directly into the tumor site, until death (N = 6); (−×−) no admixed cells but given 1000 units IL-2, directly into the tumor site, until death (N = 6).

Data on animals treated with admixed LAK cells is also shown in Fig. 1. Statistical analyses using two-tailed Mann-Whitney test: A vs. O, P < 0.05; A vs. Φ, P < 0.05; A vs. A, P > 0.10; O vs. Φ, P > 0.10.

Table 5 Combined antiidiotypic antibody therapy and LAK cell plus IL-2 therapy

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumor^a</th>
<th>LAK^b</th>
<th>IL-2^b</th>
<th>Anti-Id^c</th>
<th>Median (days)</th>
<th>Range (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>13</td>
<td>13–15</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>37</td>
<td>25–41</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>16</td>
<td>15–28</td>
</tr>
<tr>
<td>D</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>18</td>
<td>16–29</td>
</tr>
<tr>
<td>E</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>25</td>
<td>23–29</td>
</tr>
<tr>
<td>F</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>25</td>
<td>23–29</td>
</tr>
</tbody>
</table>

^a 1 x 10^6 L2C cells given i.p. (time 0). Five animals per group.
^b 1 x 10^6 LAK cells given i.p. together with IL-2, 4 h after tumor cells, i.p.
^c LAK cells were generated for 4 days in vitro as described in "Materials and Methods."

The beneficial therapeutic effects obtained when only IL-2 was administered into the tumor site were surprising, especially in view of the poor effect observed with normal splenocytes plus recombinant IL-2 although it is possible that these cells absorbed most of the IL-2 and prevented its antitumor effects. The effects of systemic IL-2 therapy of the L2C leukemia were pursued and the results of such a study are described elsewhere.

Having demonstrated an effect of LAK cells on the L2C leukemia in vivo when the effectors and targets had been pre-mixed, an attempt was made to use LAK cells in a therapeutic model in which the tumor targets were more widely disseminated. In these experiments, LAK cells plus IL-2 were given i.p. 4 h after inoculation with tumor cells, also i.p. Two different treatment groups were used; in one group a 10-fold excess of LAK cells over tumor cells was given together with 1000 units of IL-2 and this amount of IL-2 was given once daily for 20 days. In the second group a 70-fold excess of LAK cells over tumor cells was given together with 100,000 units of IL-2 and this dose of IL-2 was given once daily for a total of 5 days. In Fig. 3 the survival curves for these groups of animals are shown. In both of the treatment groups a significant prolongation of survival, compared with the untreated L2C bearing, guinea pigs was observed. The best increase in survival was seen in the group treated with a 10-fold excess of LAK cells plus 20 additional doses of 1000 units of IL-2. In this group a 10-day increase in median survival over control animals, was found. There was, however, no statistical significance between the two therapy groups. Thus, as increasing the number of effector cells and giving a much higher dose of IL-2 for a short period of time did not further enhance survival, this possibility represents the maximum effect which can be obtained with LAK cells under the conditions used.

Absence of Synergy with Antidiotopic Antibody Therapy. The potential synergy between LAK cell plus IL-2 therapy combined with administration of a murine monoclonal antibody directed against an idiotypic determinant on the surface immunoglobulin expressed by the L2C cells was examined. It was possible that these two therapies could synergize by recruiting different effector functions. The results of such attempts are shown in Table 5. Individually, all of these agents caused a modest increase in survival of treated animals. Antidiotype alone gave the best results in this particular experiment, causing a 24-day increase in the median survival compared to untreated animals. This was a particularly good result using antidiotype alone as in other, similar, larger trials of this monoclonal antibody, only a 6-day increase in median survival compared to untreated animals was seen (data not shown). The increased survival obtained with LAK cells and IL-2 treatment was similar to that described in earlier sections (Fig. 3).

Statistical analysis clearly showed that the treatment groups could be split into two categories, those animals receiving monoclonal antidiotype therapy, alone or in combination with...
LAK plus IL-2 therapy, and those animals not receiving monoclonal antiidiotype. Statistical comparison of the antiidiotype treatment alone with the antiidiotype and LAK plus IL-2 combination therapy showed that these modes of therapy could not be distinguished from each other. Thus, there was no demonstration of additive or synergistic actions between these two modes of therapy.

DISCUSSION

This study has demonstrated that LAK cells can be generated from strain 2 guinea pig lymphoid populations and are cells with a lymphoblastic appearance and granules in the cytoplasam, capable of causing the lysis of murine P815 tumour cell and the guinea pig RT273 sarcoma targets in vitro. Although lysis of L2C tumor cells by LAK cells could not be demonstrated in vitro, LAK cells were shown to mediate antitumor effects in vivo. This in vivo action was first demonstrated in Winn assays, where the LAK and tumor cells were premixed, and subsequently in a systemic therapeutic model where LAK cells were given i.p. 4 h after tumor cells. In the most successful therapeutic regimen a 10-day increase in median survival, compared to untreated animals, of animals treated with a 10-fold excess of LAK cells and once daily injections of 1000 units of IL-2 for 20 days, was seen. It should be stressed that these relatively modest increases in survival are highly significant in this tumor model, considering the aggressive nature of the tumor with a doubling time of approximately 20 h and its wide dissemination. It was calculated that a 3-day increase in median survival represents a 90% kill of inoculated tumor, in this model (13). Thus the 10-day increase in median survival observed in one of the present experiments possibly represents a 99.9% elimination of tumor.

Human and murine LAK cells have T-cell lineage markers but they can be distinguished from CTL or secondary CTL by the markers expressed on the precursors of these respective cell types (2, 3, 5) and their ability to lyse non-major histocompatibility complex matched target cells. LAK effector cells also have a broader spectrum of lytic activity in vitro than NK cells and depletion of NK cells from the induction cultures using a mixture of monoclonal antibodies does not prevent subsequent LAK effector generation (2). In addition, strains of mice deficient in NK activity are capable of generating LAK cells, further dissociating these cell types (26).

Examination of guinea pig LAK populations showed them to consist of B-cells and cells expressing activated T-cell markers. In a recent study of the phenotype of murine LAK cells and their precursors (27) a large number of Thy1.2 negative cells were present in unsorted LAK induction cultures. Although the murine LAK precursor is Thy1.2 negative, depletion of surface immunoglobulin positive cells from the induction cultures did not prevent LAK generation. It seems probable that the surface immunoglobulin positive cells in guinea pig LAK populations are not therefore responsible for their lytic activity, suggesting that the cells expressing markers of activated T-cells are the lytic effectors. We were unable to exclude an NK lineage of such cells due to the limited number of markers available for guinea pig subsets. For example, antiisialo GM1 antiserum does not react with guinea pig cells (21) and T-cell subset markers equivalent to the human CD4 and CD8 markers have not yet been defined. The inability to assign an unequivocal cellular lineage to guinea pig LAK cells is not surprising in view of the limited number of markers available, especially as many groups have been unable to resolve this issue in human and murine systems for which a large number of subset markers are available.

When the functional activity of guinea pig NK and LAK cells was compared in the current study, differences were found. The murine P815 tumor cell targets were not lysed by NK effectors but were consistently lysed by LAK effector cells whereas the converse was true for the human K562 tumor cell targets.

In the studies of other workers, the demonstration of LAK activity was first made by in vitro testing against fresh tumor cells (1, 2) and these effectors were subsequently shown to have therapeutic effects against animal tumors (4, 5). In the present study we were unable to show in vitro cytotoxicity of LAK cells against L2C targets. Nevertheless, IF and IF-C cells were mixed in vitro and this mixture injected intradermally into strain 2 guinea pigs, a clear inhibition of local tumor growth was observed and this was associated with prolonged survival of these animals. These in vivo actions were subsequently confirmed in a more demanding model in which more than 1000 times the lethal dose of L2C cells were given and then the LAK cells were administered as a therapy, separated by 4 h from the tumor inoculation.

The in vivo antitumor effects of LAK cells in the current study, despite the failure to demonstrate lysis of tumor cells in vitro, suggest that LAK cells may have actions, detrimental to tumor cell growth, other than direct tumoricidal ones. It is, however, possible that the 4-h 51Cr release assay is unsuitable for demonstrating the potential lytic activity with in vivo relevance of guinea pig LAK cells.

In other studies in guinea pig (28) and murine (29, 30) tumor models in which specifically sensitized T-cells were used similar discrepancies between the in vitro lysis of tumor targets and the in vivo effectiveness of these populations have been described. This phenomenon has not been previously documented for LAK cells. Regardless of the reason for this discrepancy, the results encourage the therapeutic use of LAK cells, even in conditions where in vitro tumor lysis cannot be demonstrated.

In previous studies it has been difficult to demonstrate in vitro lysis of L2C targets even when the T-cell effectors were obtained from allogeneic mixed lymphocyte cultures with both class I and class II major histocompatibility differences between stimulators and responders. Only when clones of T-lymphocytes reactive with the Ia molecules expressed on strain 2 pig cells were prepared from such cultures was the lysis of strain 2 concanavalin A blast cells and L2C targets observed (23). Thus L2C targets can be lysed by guinea pig cellular effectors in vitro although the demonstration of this phenomenon is more difficult than in many murine systems.

The therapy of the L2C leukemia with LAK cells and IL-2, although giving encouraging results, did not cure any animals in any of the groups tested. Thus we attempted to improve the survival of treated animals by combining this mode of therapy with antiidiotypic antibody therapy, which has shown promise in this model (12, 31). It was possible that these separate therapeutic regimens would recruit different effector functions and show a synergistic action. However, we did not observe any increased benefit due to the combination of these modes of therapy.

The studies in this report were designed to determine the conditions for LAK cells generation in strain 2 guinea pigs with the intention of testing their antitumor actions in an aggressive, widely disseminated leukemic model. Consistent with the findings in murine and human systems, lytic effectors were induced by culture of lymphoid populations in recombinant IL-2. The in vivo antitumor actions of these cells, demonstrated in this
leukemia model, encourages the further use of LAK cell plus IL-2 therapy.

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Generation of Lymphokine-activated Killer Cells in Strain 2 Guinea Pigs and Their Use in the Therapy of L2C, an Acute B-Cell Leukemia

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