Generation of Human Lymphokine-activated Killer Cells following Brief Exposure to High Dose Interleukin 2

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ABSTRACT

Current laboratory lymphokine-activated killer (LAK) cell activation procedures require culture of peripheral blood mononuclear cells (PBMC) in the presence of 1000–1500 units/ml of interleukin 2 (IL-2) for 3–7 days. However, we have observed that a brief exposure (15 min–1 h) of PBMC to a high concentration of IL-2 results in the maturation of LAK precursor cells to cytolytic effector cells over the course of 1–3 days. These IL-2-pulsed LAK cells express cytolytic activity comparable to that of nonpulsed PBMC (cultured continuously in IL-2) at 3 days of culture. The acquisition of cytolytic activity followed the same kinetics for both pulsed and nonpulsed mononuclear cells and was maintained when tested at day 7. The pulsed LAK cells were capable of significantly lysing 11 different tumor targets tested and flow cytometric analysis revealed that pulsed LAK cells were phenotypically similar to nonpulsed LAK cells. Serum obtained from cancer patients undergoing IL-2/LAK cell therapy did not inhibit the maturation of the pulsed mononuclear cells into LAK cells. Interestingly, only PBMC obtained from cancer patients receiving in vivo IL-2 infusions could be induced to generate the same levels of cytolytic activity as those in nonpulsed cells using this pulse procedure. PBMC obtained from healthy, normal donors could not be pulsed to the same levels of activation as nonpulsed LAK cultures. Our study demonstrates that for the generation of maximum LAK cell cytolytic activity, LAK cell precursors must be primed in vivo with IL-2. Implementation of this procedure could eliminate the high cost of cell culture which normally accompanies IL-2/LAK cell therapy. Such an approach could make IL-2/LAK cell therapy more accessible for cancer patients.

INTRODUCTION

The in vitro culture of human PBMC1 with IL-2 results in the activation of a subset of PBMC that is capable of lysing nonspecifically fresh human tumor cells (1). These activated cells have been termed LAK cells. Although this cell population is heterogeneous in nature, the cells believed to be responsible for the cytolytic activity are Leu-19* CD3- cells (2). The therapeutic efficacy of LAK cells has been shown by both West et al. (3) and Rosenberg et al. (4, 5). These studies used LAK cells accompanied by the infusion of recombinant IL-2 in the treatment of a variety of human cancers with responses most notably in patients with melanoma and renal cell carcinoma.

The kinetics of LAK cell activation was originally described by Grimm et al. (1). Peripheral blood leukocytes obtained from cancer patients were incubated at a culture density of 1 × 10^6 cells/ml in the continuous presence of T-cell growth factor. They found that a low level of tumor cell lysis was evident by day 2, continued to increase until day 5, and persisted until day 7. However, they were not able to detect any cytolytic activity of the LAK cells at day 1. Recently, however, using recombinant IL-2, Owen-Schuab et al. (6) showed that mononuclear cells obtained from normal donors could be pulsed for 30 min with doses of 2.5 × 10^1–1 × 10^4 units IL-2/10^6 cells and acquire a low level of cytolytic activity that was detectable at both 24 and 96 h. We examined whether this was true in cancer patients receiving in vivo IL-2 and studied whether continuous exposure to IL-2 was absolutely necessary for the acquisition of significant cytolytic activity.

In this report, we show that mononuclear cells obtained from cancer patients receiving IL-2/LAK cell therapy could be pulsed with IL-2 for a short period of time and attain significant levels of cytolytic activity against tumor cell targets. Interestingly, we were not able to generate the same levels of cytolytic activity in pulsed LAK cells as in nonpulsed LAK cultures generated from PBMC obtained from normal donors. This emphasizes the necessity for priming the cancer patients with high dose IL-2 in vivo in order to generate LAK cell precursors capable of responding maximally to a brief pulse of high dose IL-2. Utilized clinically, an approach such as this in which mononuclear cells are pulsed with high concentrations of IL-2 for a short period of time could significantly reduce the cost and risk to cancer patients undergoing IL-2/LAK cell therapy.

MATERIALS AND METHODS

Mononuclear Cell Collections. Mononuclear cells were obtained from cytopheresis products collected from either normal donors or from cancer patients receiving IL-2/LAK cell therapy at The Williamson Medical Center, Franklin, TN. The cytopheresis procedure and cell washing were done as described previously (7). In brief, between 10 and 12 liters of whole blood were processed at a flow rate of 50 ml/min using a Fenwal CS-3000 Automated Blood Cell Separator (Fenwal Laboratories, Deerfield, IL). The mononuclear cell fraction (>90% lymphocytes, >5% monocytes, and <5% granulocytes) were washed free of residual platelets in the CS-3000 collection chamber using 2 liters of sterile saline and an automated wash procedure we developed.

IL-2 Pulsing Procedure. Mononuclear cells obtained from cytopheresis products were counted and assessed for viability using trypan blue exclusion. Without changing the diluent (saline) in which the cells had been following cytopheresis, aliquots of approximately 1 ml were placed in Costar (Cambridge, MA) 15-ml centrifuge tubes to which were added 1000 units of IL-2/3 × 10^6 cells. Following a 15-min to 1-h incubation in a 5% CO₂ in air atmosphere at 37°C, the cells were diluted with 10 ml of IL-2 free HL-1, serum-free medium (Ventrex, MA). The cells were centrifuged at 250 × g for 10 min. After centrifugation, the supernatant was removed to the level of the cell pellet which was resuspended in 10 ml of fresh IL-2-free HL-1. This wash procedure was repeated for a total of 3 times in order to remove unbound exogenous IL-2. Following the wash procedures, the cells were adjusted to a concentration of 3 × 10^6 cells/ml in IL-2-free medium in Falcon T25 tissue culture flasks. Control cultures consisted of nonpulsed mononuclear cells cultured under the same conditions in the presence or absence of 1000 units/ml IL-2. Following the 3-day incubation period, the cells were dislodged from the flasks with gentle scraping using Falcon cell scrapers. The cells were assessed for viability and assayed for cytotoxicity against tumor cell targets.

In some experiments, the pulsed mononuclear cells were subjected to an IL-2-stripping procedure designed to remove any exogenous IL-2.
2 bound to the cell following the IL-2 pulse. The procedure used was a modification of that described by Tsudo et al. (8). In brief, following a 15-min to 1-h pulse procedure, the cells were resuspended for 15 s in medium adjusted to pH 4 with 2 N HCl. The cells were then centrifuged for 10 min at 250 × g and washed 3 times in IL-2-free medium as described above.

Collection and Preparation of Autologous Serum. Approximately 10 ml of whole blood were collected from the cancer patients or normal donors by venipuncture prior to the cytopheresis procedure. The blood was placed in a silicone-coated Vacutainer tube without additives (red top tube) (Becton-Dickinson, Rutherford, NJ). The serum was allowed to clot for a minimum of 1 h. The blood was centrifuged for 20 min at 250 × g to separate the serum from the remaining blood components. The serum was removed from the tube, centrifuged once again for 10 min at 250 × g to remove any residual cells, and then filtered through a 0.45-μm filter and used fresh.

Culture Media. Complete HL-1 serum-free medium contained 100 units/ml of penicillin, 10 μg/ml of streptomycin, 2 mm of L-glutamine, and 50 μg/ml of gentamicin sulfate. In some experiments HL-1 was supplemented with varying concentrations of autologous serum.

Recombinant Human Interleukin 2. The recombinant IL-2 used in this study was kindly provided by the Cetus Corporation (Emeryville, CA). The IL-2 had a specific activity of 3.0 × 10^6 units/mg of protein. The IL-2 was reconstituted to 10^6 units/ml with sterile water.

Target Cells. The natural killer cell-sensitive human erythroleukemia cell line, K562, and the LAK-sensitive human B-lymphoblastoid cell line, Daudi, were used as tumor targets in most 4-h 51Cr release assays. In some studies, the following tumor types known to be sensitive to lysis by standard LAK cells were used as tumor targets: ACHN (renal cell carcinoma); A375 (melanoma); BXR-LU5 (lung carcinoma); BT20 (breast); BRX-ORI (ovarian); HT29 (colon carcinoma); JY (lymphoblastoma); BRX-SR2 (sarcoma); and BRX-PRI (prostate). The tumor cell lines were Mycoplasma free as determined by routine screening assays (9). They were maintained at densities between 5 × 10^6 and 1 × 10^6 cells/ml of RPMI 1640 plus 10% heat-inactivated fetal bovine serum and 2 mm L-glutamine. For cytotoxicity assays, cells were washed, counted, and labeled with 51Cr for 2 h.

51Cr Release Microcytotoxicity Assay. All 51Cr release assays were performed as described previously (10). Briefly, 2.5 × 10^5 51Cr-labeled target cells were incubated with varying numbers of effector cells in a final volume of 150 μl of RPMI 1640 plus 5% fetal bovine serum. The cells were incubated in V-bottomed microtiter plates at 37°C. After 4 h, 100 μl of culture supernatant were removed and counted for 51Cr using an LKB Gamma Counter (Gaithersburg, MD). Maximal 51Cr release was determined in the presence of 2 N HCl. The percentage of specific 51Cr release was equal to

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

In some cases cytotoxicity is expressed in terms of LU. The number of LU was obtained by determining the number of effector cells necessary to give 33% lysis of 2.5 × 10^6 target cells in a 4-h assay and dividing that number into 1 × 10^6 effector cells.

Assessment of Cell Number and Viability. All cytopheresis products and activated cell cultures were assessed for viability by standard trypan blue exclusion analysis. Cell numbers were obtained through manual counts using a hemacytometer and a microscope. All counts were done in duplicate and repeated if the values were not within 10% of one another.

Flow Cytometric Analysis. For cell surface phenotype analysis, cells were adjusted to a concentration of 1.0 × 10^7/ml and incubated for 30 min at 4°C with saturating dilutions of mouse anti-human monoclonal antibodies. The antibodies and their specificity were: CD4-FITC, helper/inducer; CD8-FITC, cytotoxic/suppressor; T4-1-RDI, activated T-cell; NKH-1-RDI, natural killer cell; CD25-FITC, IL-2 receptor (Coulter Immunology, Hialeah, FL). Following incubation, the cells were washed three times in ice cold Hanks' balanced salt solution that did not contain magnesium, calcium, or phenol red. The cells were then resuspended in a volume of 0.5 ml of Hanks' balanced salt solution and analyzed on an Ortho Diagnostics System Cytofluorograf 11s. Results are presented as the percentage of cells expressing each particular cell surface marker of interest.

RESULTS

Demonstration of LAK Activity following IL-2 Pulsing of PBMC Obtained from Cancer Patients. Cancer patients' PBMC were pulsed with high concentrations of IL-2, extensively washed, and immediately tested for cytotoxicity against the LAK-sensitive target cell line Daudi. A slight increase in lytic activity was observed for pulsed PBMC over nonpulsed PBMC at high E:T cell ratios (100:1-12:1) (Fig. 1). Pulsed PBMC generated 30% lysis of Daudi at an E:T ratio of 50:1 compared to only 2% lysis by the nonpulsed PBMC. At lower E:T ratios, there was no increase in cytolitic activity.

When pulsed PBMC were placed in culture in the absence of IL-2, a significant increase in cytolytic activity was observed at day 3 (Table 1). In 3 of 9 cases (patients 3, 6, and 7) LU were greater in the cultures containing pulsed LAK cells than in cultures containing nonpulsed LAK cells which received continuous exposure to IL-2. In another 3 of 9 cases (patients 1, 4, and 9) the lytic activity was higher in the nonpulsed cultures, while in the final 3 of 9 cases (patients 2, 5, and 8) the differences in LU between the 2 groups was less than 10%.

When a modified IL-2-stripping procedure designed to remove cell-bound IL-2 was performed using low pH medium (patients 2, 3, and 9), lytic activity of the IL-2-stripped pulsed LAK cells was not significantly different from the control cultures after 3 days. Taken together, there was no significant difference between the mean LU generated between the two treatment groups [nonpulsed LAK cells, 6374 ± 1125 (SEM) LU; pulsed LAK cells, 6352 ± 1038 LU]. Cells not exposed to IL-2 at any time in vitro developed little if any cytolytic activity. When the kinetics of LAK cell development was followed over a period of 3 days, we observed that nonpulsed LAK cells exposed to IL-2 continuously as well as the pulsed LAK cells exhibited a significant amount of cytolytic activity by day 1 of culture which continued to increase to day 3 (Fig. 2). On day 1, the nonpulsed LAK cells generated 72% lysis of the Daudi tumor cell target at an E:T ratio of 12:1 compared to 68% lysis of Daudi by pulsed LAK cells at the same E:T ratio. By day 2, nonpulsed cells generated 77% lysis as compared to 76% lysis of Daudi by the pulsed cells at an E:T ratio of 12:1. Likewise, on day 3, nonpulsed and pulsed LAK cells exhibited equivalent levels of cytotoxicity at an E:T ratio of 12:1. These groups yielded values of 79% and 78% lysis, respectively, against the
### Table 1 Effect of IL-2 pulse on PBMC obtained from cancer patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Procedure</th>
<th>Control&lt;sup&gt;a&lt;/sup&gt; (0 unit/ml IL-2)</th>
<th>Nonpulsed&lt;sup&gt;b&lt;/sup&gt; (1000 units/ml IL-2)</th>
<th>Pulsed&lt;sup&gt;c&lt;/sup&gt; (0 unit/ml IL-2)</th>
<th>Pulse/strip&lt;sup&gt;d&lt;/sup&gt; (0 unit/ml IL-2)</th>
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<tr>
<td>1</td>
<td>C1</td>
<td>9</td>
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<td>3701</td>
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<td>C1</td>
<td>46</td>
<td>5755</td>
<td>6196</td>
<td>9437</td>
</tr>
<tr>
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<td>5799</td>
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</tr>
<tr>
<td>6</td>
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<tr>
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<td>C5</td>
<td>23</td>
<td>8129</td>
<td>6228</td>
<td>5448</td>
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</tbody>
</table>

Mean ± SD<sup>e</sup>  

<sup>a</sup> Measured in a 4-h 51Cr release assay as described in “Materials and Methods.”

<sup>b</sup> All patients were undergoing IL-2/LAK cell therapy (3).

<sup>c</sup> Procedures refer to PBMC obtained via cytopheresis at different times after the cessation of IL-2 infusion: C1, 24 h; C5, 36 h; C6, 60 h.

<sup>d</sup> Cells were pulsed with IL-2 as described in “Materials and Methods.”

<sup>e</sup> Cells were pulsed, stripped of IL-2 as described in “Materials and Methods.”

<sup>f</sup> P < 0.05, Student’s t test.

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**Effects of Increasing IL-2 Concentration and IL-2 Pulse Time on Development of LAK Activity.**

Since only low levels of cytotoxic activity were obtained immediately following the IL-2 pulse procedure, we wanted to determine if this level could be increased significantly by increasing the concentration of IL-2 with which the cells were pulsed. In these experiments, 1,000, 5,000, or 10,000 units of IL-2/3 x 10⁶ cells were used (Fig. 4). In all experiments, there was an increase in the amount of cytotoxicity displayed immediately following the pulse procedure. However, the increase was not considered significant. In additional experiments, the time of exposure to IL-2 was reduced to 15 min. A substantial amount of cytotoxicity was generated with a pulse of 15 min followed by culture in the absence of IL-2 for 3 days. However, the cytolytic activity generated was less than the cytolytic levels that occurred when the cells had been pulsed with IL-2 for 1 h (Fig. 5).

**Effects of IL-2 Pulsing on the Expression of Cell Surface Markers on Mononuclear Cells Obtained from Cancer Patients.**

Cells obtained from cytopheresis procedures of cancer patients given IL-2 in vivo were examined for the expression of certain T-cell surface antigens. Observe in the representative experiment presented in Table 2 that the majority of cells (cytopheresis product) expressed either CD4 or CD8 with a larger percentage of NKH-1-positive cells than usually observed in cyto-

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**Fig. 2.** Kinetics of LAK cell activation following a 1-h pulse with high concentrations of IL-2. Cancer patients' mononuclear cells were briefly exposed to high concentrations of IL-2, then incubated for the indicated time, and assessed for cytotoxicity against Daudi targets in a 4-h 51Cr release assay (A). The controls, 0 unit/ml IL-2 (O), and nonpulsed cells, 1000 units/ml IL-2 (●), were also tested for lysis against the Daudi target. Data are represented as means ± SEM (bars) of 6 experiments.

**Fig. 3.** Generation of cytolytic activity at day 7 of culture. In this representative experiment (of 3 experiments) cells were either incubated in the absence of IL-2 (C) or in the presence of 1000 unit/ml IL-2 (●) or pulsed and then incubated in the absence of IL-2 (●). The target cells used were K562 (top) and Daudi (bottom). The data are presented as percentage of specific 51Cr release values at various effector:target cell ratios.
either continuously or pulsed, the percentage of CD4-positive cells was lower while the percentage of CD8-positive cells increased and was higher than the control cultures not exposed to IL-2 \textit{in vitro}. The percentage of cells expressing NKH-1 was maintained at the same levels as found in the cytopheresis product. Interestingly, the cultures exposed to IL-2 contained an increased percentage of cells coexpressing NKH-1 and the T-cell activation marker TAI as compared to the cytopheresis product and the control cultures not exposed to IL-2 \textit{in vitro}. In summary, there was no phenotypic difference after 3 days between cells exposed to IL-2 continuously or by a 1-h pulse procedure.

Ability of Pulsed LAK Cells to Lys e a Variety of Different Tumor Cell Targets. Pulsed LAK cells obtained from cancer patients who were primed with \textit{in vivo} infusions of IL-2 were tested for their ability to lyse a variety of different tumor cell targets. In these experiments, both K562 and Daudi were lysed equally well by either pulsed LAK cells or nonpulsed LAK cells (data not shown). In addition, tumor cell lines representing 9 different tumor types were lysed equally well over a range of E:T ratios from 100:1 to 0.75:1 by both pulsed LAK cells and nonpulsed LAK cells (Fig. 6).

Generation of LAK Activity in the Presence of Autologous Serum. To examine the effect of cancer patient serum on the ability of the IL-2-pulsed cells to mature into LAK cells, we cultured the cells in the presence of 10% (v/v) autologous serum. Serum was obtained from cancer patients receiving continuous IL-2 infusion (3 × 10^6 units/m^2/day) in a standard IL-2/LAK cell therapy protocol (2). In these experiments, incubation of the pulsed cells for 3 days in the presence of autologous serum was not inhibitory to the generation of cytolytic effector cells (Fig. 7). To the contrary, the presence of up to 10% autologous serum appeared to enhance the cytolytic activity of both pulsed LAK precursor cells and those LAK cells exposed to IL-2 continuously for 3 days.

Effects of IL-2 Pulsing on Mononuclear Cells Obtained from Normal Donors. In an effort to determine if \textit{in vivo} IL-2 priming is a necessary step to allow pulsed mononuclear cells to mature into cytolytic effector cells, we examined the PBMC obtained from normal donors not previously exposed to IL-2. In these studies, summarized in Fig. 8, LAK cells generated from normal donor mononuclear cells (pulsed or nonpulsed) were not as cytotoxic against Daudi target cells as LAK cells obtained from IL-2-primed cancer patients. As noted before, however, there was no difference observed in the ability of pulsed or nonpulsed LAK cells obtained from IL-2-primed cancer patients to lyse Daudi. In contrast, differences were observed using PBMC obtained from normal donors not primed \textit{in vivo} with IL-2. Univariate analysis of variance showed that ^{51}Cr release values for pulsed normal donor LAK cells were significantly lower than nonpulsed normal donor LAK cells at all E:T ratios except 0.75:1. Multivariate analysis was used in a confirmatory study

![Table 2. Phenotypic analysis of cancer patient cells following exposure to high concentrations of IL-2](image.png)

<table>
<thead>
<tr>
<th>Procedure</th>
<th>CD4</th>
<th>CD4/TA1</th>
<th>CD8</th>
<th>CD8/TA1</th>
<th>NKH-1</th>
<th>NKH-1/TA1</th>
<th>CD25</th>
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</thead>
<tbody>
<tr>
<td>Cytopheresis product</td>
<td>38</td>
<td>3</td>
<td>34</td>
<td>2</td>
<td>19</td>
<td>1</td>
<td>ND</td>
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<td>52</td>
<td>4</td>
<td>31</td>
<td>1</td>
<td>5</td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td>Nonpulsed (1000 units/ml IL-2)</td>
<td>28</td>
<td>6</td>
<td>36</td>
<td>5</td>
<td>20</td>
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<td>Pulsed (0 unit/ml IL-2)</td>
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<td>6</td>
<td>35</td>
<td>2</td>
<td>19</td>
<td>12</td>
<td>22</td>
</tr>
</tbody>
</table>

* Patient cells were tested for the coexpression of certain T-cell antigens, for instance: CD4/TA1, CD8/TA1, or NKH-1/TA1.
* Flow cytometric analysis was performed on the same day on which the cytopheresis product was obtained from the patient.
* Cells were cultured for 3 days in the presence of 1000 units/ml IL-2.
* Cells were pulsed with IL-2 as described in "Materials and Methods" and then cultured for 3 days in the absence of IL-2.

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Fig. 6. Ability of pulsed LAK cells to lyse a variety of tumor cell targets. In these experiments (representative of 3 experiments performed) cancer patient mononuclear cells were either incubated in the presence of 1000 units/ml IL-2 (C) or pulsed with IL-2 (•) and then incubated in the absence of IL-2. On day 3 of culture, the LAK cell activity was determined in a 4-h 51Cr release assay. The data are presented as percentage of specific 51Cr release values at various effector:target cell ratios.

Fig. 7. Effect of cancer patients’ autologous serum on the generation of LAK activity. In this representative experiment (of four experiments performed) cells were either incubated in the presence of 1000 units/ml IL-2 for 3 days (no pulse) or pulsed with IL-2 as described in “Materials and Methods” and then cultured for 3 days in the absence of IL-2 (pulsed). Both groups were cultured in the presence or absence of 10% autologous serum for 3 days. Cytotoxicity assays were done as described in “Materials and Methods.” The data are presented as percentage of specific 51Cr release values at various effector:target cell ratios.

Fig. 8. Effect of IL-2 pulsing on mononuclear cells obtained from normal donors. In this figure (a summary of 21 cancer patients and 9 normal donors) PBMC obtained from unprimed normal donors (A, □) and primed cancer patients (C, ▪) were either incubated in the presence of 1000 units/ml IL-2 (C, △) or pulsed with IL-2 and then cultured in the absence of IL-2 (P, ▲). On day 3 of culture, the LAK cell activity generated was determined in a 4-h 51Cr release assay. The target cells used were the LAK-sensitive cell line Daudi. The data are presented as the mean ± SEM (bars) of the percentage of specific 51Cr release values at various effector:target cell ratios.

to allow for the effects of repeated measures and to consider each E:T ratio simultaneously. For cancer patients primed with IL-2, there was no effect of pulsing (P = 0.35); while for unprimed normal donors, the difference between cytolytic activity of pulsed LAK cells and nonpulsed LAK cells was significant (P = 0.009). In summary, the results of our studies suggest that LAK cell precursors receive a signal obtained during in vivo priming that increases their ability to respond maximally to an IL-2 pulse and become cytolytic effector cells in vitro.

DISCUSSION

Previous studies of LAK cell activation examined the ability to generate these nonspecific cytotoxic cells following contin-
uous exposure of PBMC to high concentrations of IL-2 (1, 7). In these studies, either natural or recombinant IL-2 was used. It was determined that LAK cell activity appeared approximately 24 h after the establishment of cell culture and increased significantly between days 3 and 5 (1, 6). In the present study, we report that in some cases continuous exposure of PBMC to high concentrations of IL-2 is not necessary to obtain LAK cells with significant cytolytic activity. Instead, a brief exposure (15 min-1 h) of PBMC to high concentrations of IL-2 results in the generation of cytolytically active LAK cells within 1–3 days. This procedure works very well using PBMC obtained from cancer patients who were primed in vivo with r-IL-2. Although PBMC from normal donors could also be pulsed, they could not be pulsed to the same level of cytotoxicity as normal donor PBMC exposed continuously to IL-2 for 3 days.

In a previous report, Owen-Schuab et al. (6) showed that normal donor PBMC could be pulsed with IL-2 to become cytolytically active LAK cells. The levels of cytotoxicity were within 6–57% of the control, which consisted of nonpulsed PBMC cultured continuously in the presence of IL-2 for 1–4 days. In their report, 1 x 10^6 Tac-negative PBMC were pulsed with either 2,500, 5,000, or 10,000 Cetus units of IL-2. In the present report we have confirmed these results, using unpurified PBMC obtained from normal donors, that were pulsed with 1000 Cetus units IL-2/3 x 10^6 cells for 1 h. It is interesting to note that in either study, pulsed normal donor mononuclear cells were not as cytotoxic as the nonpulsed LAK cells when tested at days 3 or 4.

We have extended the findings of Owen-Schuab et al. to include cancer patients' PBMC. In these cases, however, the PBMC were obtained as cytopheresis products from cancer patients who had received continuous infusion IL-2 in an IL-2/LAK cell protocol. In our studies, all cytopheresis products were collected at least 36 h after the cessation of IL-2 infusion to the cancer patients. In ^51^Cr release studies, slight cytolytic activity against the LAK target, Daudi, was seen immediately following the removal of PBMC from the cancer patients. The cytolytic activity increased slightly following the IL-2 pulse (1000 units IL-2/3 x 10^6 cells) procedure. Additional time in cell culture (1–3 days) was required for the acquisition of full cytolytic activity by the pulsed LAK cells which was equivalent to the cytolytic activity of nonpulsed LAK cells.

A question that still remains is whether or not the cytotoxic activity exhibited by the pulsed LAK cells is indeed the result of a signal delivered by IL-2 in less than 1 h as suggested by the present report. Alternatively, this activity, detectable by 24 h, could be the result of continuous exposure of the PBMC to IL-2 carried over in soluble form from the pulse procedure. Our analysis of 24 experiments using IL-2-primed cancer patients PBMC and 9 experiments using normal donor PBMC argues against this possibility. These experiments were done in 15-ml conical centrifuge tubes and used only the number of PBMC required for subsequent cytotoxicity analysis. Thus, in each individual experiment, a different number of PBMC were contained in a 1-ml volume to which was added between 20,000 and 62,000 Cetus units of IL-2 (1,000 units/3 x 10^6 PBMC). After the 1-h pulse procedure, the cells underwent extensive washes which involved removing >95% of the medium containing IL-2 and in each case replacing it with 10 ml of fresh IL-2-free medium. After the 3 washes, the cells were resuspended in 30 ml of IL-2-free medium used for cell culture. Calculations show that only small amounts of soluble IL-2 could be carried over to cell culture (1.7 ± 1.4 units IL-2/3 x 10^6 IL-2-primed cancer patient PBMC; 1.1 ± 0.4 units IL-2/3 x 10^6 normal donor PBMC). We have confirmed these levels using a standard lymphocyte proliferation assay as described previously (11). In one study, after pulsing 4.2 x 10^10 IL-2-primed cancer patients' PBMC with 14 x 10^6 units of IL-2 in a volume of 200 ml, following the wash procedure, we detected 1.2 units of IL-2/ml of saline containing 210 x 10^6 PBMC. Thus, when diluted to the correct cell concentration, the pulsed PBMC began cell culture in the presence of 81 unit of IL-2/3 x 10^6 cells. Dose-response studies done in our laboratory (data not shown) as well as in other laboratories (6, 12) have clearly shown that these amounts of IL-2 are not enough to generate the levels of cytotoxicity observed in the control cultures using either IL-2-primed or normal donor PBMC exposed continuously to 1000 units IL-2/3 x 10^6 PBMC for 3 days. Another possibility, aside from soluble IL-2 carryover, is IL-2 remaining bound to cells specifically to the IL-2 receptors or nonspecifically to the cell surface. However, our low pH IL-2 strip procedure, developed by other investigators (8) and routinely used in receptor binding studies (13–17), followed by the extensive washing as described above, would have removed and washed away the bound IL-2. Still as demonstrated in these experiments, the same levels of cytotoxicity were attained. Thus, our feeling is that IL-2 carryover is not the primary reason why PBMC develop into LAK cells over the course of 3 days following a 1-h pulse with IL-2. Instead, we think that it is the result of a signal delivered early in the pulse process which takes between 1 and 3 days to be realized as a change in functional and phenotypic properties. Regardless, the present study clearly shows that in vitro culture of PBMC in the continuous presence of 1000 units IL-2/3 x 10^6 cells is not necessary for the generation of LAK cells particularly using PBMC from IL-2-primed cancer patients.

An interesting observation made in the present report is the difference in the ability to make pulsed LAK cells from PBMC obtained from IL-2-primed cancer patients as opposed to unpulsed normal donors. We found that PBMC obtained from cancer patients before IL-2 priming could also not be pulsed to the same levels of cytolytic activity as PBMC cultured continuously in the presence of IL-2 (data not shown). We feel that the in vivo IL-2 priming is an important step in allowing the LAK precursors to respond maximally to the IL-2 pulse procedure. We have noted similar differences in the ability to make LAK cells from PBMC obtained from IL-2 primed cancer patients and from unpulsed normal donors using interleukin 4. Although the mechanism explaining such differences remains unknown, it is important to consider the differences in activation potential between these two cell populations when studying the in vitro activation of PBMC using IL-2.

It is not known whether the pulsed PBMC obtained from cancer patients will differentiate in the in vivo environment as they do in vitro using the described culture conditions. To address this, we have cultured the cells following the pulse in culture medium containing 10% (v/v) serum obtained from cancer patients undergoing IL-2/LAK cell therapy. In each of these experiments, no inhibition of the activation process, as detected by cytotoxicity at day 3, was observed. Thus, on the basis of our in vitro studies, we are optimistic that the activation process will proceed in the cancer patients.

A distinct advantage of using pulsed cells in IL-2/LAK cell therapy is supported by the observation made by Dupere and O'Connor^4^ and others (18) describing the release of lymphokines and cytokines from LAK cells during the culture period.

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4 M. Jadus and J. R. Yannelli, unpublished observation.
5 S. Dupere and T. O'Connor, personal communication.
These potent soluble mediators are being released into culture media contained in the bags or flasks. The cells are routinely harvested and washed before reinfusion into the cancer patient; thus, the soluble mediators are discarded. We believe that the pulse procedure would allow these cells to release their lymphokines/cytokines inside the cancer patient where they have the potential to exert their maximum effect by boosting the patients’ immune response against the tumor.

We believe that reinfusion of IL-2-pulsed PBMC into cancer patients offers other advantages as well. We and other investigators have noted that LAK cell populations undergo dramatic morphological and associated changes such as adhesiveness (19) to surfaces following extended culture in IL-2. These changes may provide an explanation for the relatively low motility of cultured LAK cells as described by Ratner and Heppner (20). In contrast to the 3–7-day-cultured LAK cells, we have found that the pulsed mononuclear cells maintain their normal morphology following the 1-h pulse. Thus, since these pulsed cells still maintain their size and normal morphology, they may circulate and better migrate to the tumor bed, than cells cultured for 3–7 days in the continuous presence of IL-2. On the basis of the findings in the present study, we speculate that once in the tumor bed, these PBMC will mature into cytolytic LAK cells. In addition, there is virtually no loss of cells following the pulse procedure, whereas there is generally a 10–50% loss of cells following cell culture for 3–7 days.

The concept of using pulsed mononuclear cells in IL-2/LAK cell therapy is currently being tested by the National Biotherapy Study Group. In addition to the advantages listed above, this approach offers a less costly method of cancer therapy using LAK cells. Thus, we believe that this new methodology, if successful, could make LAK cell therapy more accessible to cancer patients and reduce the dependency of the treatment on the availability of a large scale culture facility.

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Generation of Human Lymphokine-activated Killer Cells following Brief Exposure to High Dose Interleukin 2

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