Adaptive Immunotherapy of Human Cancer Using Low-Dose Recombinant Interleukin 2 and Lymphokine-activated Killer Cells

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ABSTRACT

The adoptive transfer of recombinant-methionyl human interleukin 2 (rIL-2)-activated autologous peripheral blood mononuclear lymphokine-activated killer (LAK) cells to cancer patients is being evaluated as an alternative to conventional cancer therapy. We have independently developed an alternative regimen to previously reported adoptive immunotherapy protocols using rIL-2 and LAK cells which features the prolonged administration of low-dose rIL-2 (30,000 units/kg) and an automated, entirely enclosed system of peripheral blood cell procurement, culture, harvest, and reinfusion of activated cells. The cell culture system was tested with a murine tumor model in which LAK cells generated in plastic culture bags were reinfused into tumor-bearing mice. Tumor regression was as effective with cells activated in the bags as in conventional culture flasks. Twenty-eight cancer patients were treated for 5 consecutive days with low-dose rIL-2, followed by leukapheresis, infusion of LAK cells, and prolonged IL-2 administration. At least 50% tumor regression was observed in 46% of all patients treated. These data imply that human peripheral blood mononuclear cells retain fully their capacity for rIL-2-induced activation and effector cell function under this alternative approach, and further, that a low-dose rIL-2 regimen with markedly reduced toxicities can be as effective as high-dose rIL-2 regimens if low-dose rIL-2 is given for a prolonged period of time following LAK cell infusion.

INTRODUCTION

The report of Grimm (1) first described the remarkable ability of IL-2 to induce in resting PBMC an antigen-nonspecific cytotoxicity uniquely different from NK activity. PBMC activated with IL-2 have been designated LAK cells and include as effectors, T-cells, non-T-cells, as well as potentiated NK activity, and, in the mouse, antibody-dependent cellular cytotoxicity (2-4). Several cancer research centers have instituted clinical protocols designed to exploit this unique observation for the treatment of certain tumors (5, 6).

Each protocol requires that cancer patients be subjected to systemic administration of rIL-2 and a series of consecutive leukaphereses and PBMC activation with rIL-2, each step, subject to microbial contamination. In addition, this generic approach imposes on the patient rIL-2-induced, dose-limiting side effects of vascular leak, hypotension, and significant mental status changes as well as dermatological changes (7, 8). The present protocol attempts to address some of these problems by reducing the dose of rIL-2 while prolonging the length of rIL-2 administration following LAK cell infusion. We report the clinical results of 28 patients treated on a low-dose protocol using rIL-2-activated PBMC which were collected, cultured, and harvested using a closed, automated system.

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2 Recipient of an American Cancer Society Career Development Award. To whom requests for reprints should be addressed, at Brigham and Women's Hospital, Department of Surgery, 75 Francis Street, Boston, MA 02115.
3 The abbreviations used are: IL-2, interleukin 2; LAK, lymphokine-activated killer; rIL-2, recombinant-methionyl human interleukin 2 (ala 125); PBMC, peripheral blood mononuclear cell; NK, natural killer.

MATERIALS AND METHODS

Low-Dose rIL-2 Protocol. Human peripheral blood mononuclear cells were obtained from cancer patients admitted to the Brigham and Women's Hospital Clinical Research Center following informed consent. Twenty-eight patients with evaluable tumor and no other therapeutic options were treated on a 19-day protocol. During Wk 1, the patients received 14 doses of rIL-2 (Ala 125; Ortho Pharmaceutical Corporation, Raritan, NJ) at a dose of 30,000 units/kg every 8 h. After a 2-day hiatus the patients underwent leukapheresis for 5 consecutive days. Following the leukapheresis on Day 12 the patient received the cells obtained on Days 8 and 9 along with rIL-2 every 8 h. Cells obtained on Day 10 were infused on Day 13, and cells obtained on Days 11 and 12 were reinfused on Day 15. rIL-2 was continued every 8 h through Day 19 for a total of 23 scheduled doses. Measurement of all evaluable tumor nodules was performed before and 2 wk and 6 wk after completion of the protocol. A complete response was scored if all measurable tumors disappeared. A partial response to treatment was scored if the sum of the products of the longest perpendicular diameters of all the measurable lesions decreased by more than 50%. If the decrease in tumor measurement was less than 50% but greater than 25%, a minor response was scored. One patient was retreated, yielding a total of 29 treatment courses.

Leukapheresis. Vascular access was accomplished by antecubital venipuncture, or if necessary, central venous lines with double- or single-lumen catheters. Leukapheresis was also successfully accomplished using a single peripheral vein by adapting the software to accommodate an intermittent, or discontinuous flow procedure following the same protocol used for mononuclear cell collection. Mononuclear cells were collected on a Fenwal CS-3000 continuous flow blood cell separator (Fenwal Laboratories, Deerfield, IL). Closed system apheresis software, with preattached transfer packs, 1-liter bags of both 0.9% sodium chloride and acid citrate dextrose (Solution A), and two butterfly needles, was utilized to reduce the risk of bacterial contamination to the patient and to the cultured cells. Erythrocytes, polymorphonuclear leukocytes, platelets, and plasma were returned to the patient through another antecubital line. After 10 liters of peripheral blood were processed, the contents of the collection container consisted of 200 ml of plasma plus platelets and of a pellet containing platelets and mononuclear cells. The pellet was resuspended by manual agitation and centrifuged for 3 min at 1000 rpm to separate the platelet-rich plasma from the mononuclear pellet. The platelet-rich plasma was siphoned into a clean transfer pack and reinfused to the patient. None of the patients experienced any adverse reactions requiring discontinuation of the procedure, and there was no evidence of infection in any of the cell cultures or at the venipuncture site.

The mononuclear cells that remained in the collection bag were then processed using a closed, automated protocol. The cells were resuspended by agitation without the addition of saline. Three hundred ml of Ficoll (lymphocyte separation medium; Litton Bionetics, Charleston, SC) were aseptically transferred to a 600-ml transfer pack and connected to the apheresis software via the injection site on the component-rich plasma line. Ficoll was underlaid at 4 ml/min without centrifugation to establish a density gradient until the collection container was full. The centrifuge was started and the supernatant plasma and platelets were collected into a waste bag until cells appeared in the supernatant line. Cells were then diverted into an empty transfer pack, and processing was continued until all of the Ficoll was used. The residue in the collection bag was washed out and directed to a waste bag. The purified PBMC were resuspended in 400 ml of normal saline, returned to the collection bag within the centrifuge chamber, and washed with 1 liter of normal saline. The PBMC were resuspended to 200 ml and...
transferred to a cell culture bag (PL 732 tissue culture flask, 330 cm², 1000 ml; Fenwal Laboratories) which was transported to the laboratory.

Cell Culture. A portion of washed PBMC were aseptically removed from the transfer pack and counted by trypan blue exclusion (Gibco Laboratories, Grand Island, NY). PBMC were suspended in RPMI-1640 (Whittaker, M. A. Bioproducts) supplemented with 2% Group AB serum, 50 units/ml of penicillin, 50 µg/ml of streptomycin, 2 mM l-glutamine, and 1000 units/ml of rIL-2. Cells and media were distributed into bags at a predetermined optimal density of 3 × 10⁶ cells/ml using a computerized auxiliary pump (Model SAV-EX2; Fenwal Laboratories) placed in a laminar flow hood. Bags were incubated at 37°C in 5% CO₂ without agitation. In some experiments PBMC were cultured in roller bottles at a predetermined optimal density of 1.5 × 10⁸ cells/ml and were incubated at 37°C in 5% CO₂ with rotation. After 3 to 4 days of incubation, plastic bag cultures were harvested and washed with the CS-3000. Cells were suspended in 200 ml of saline and 5% albumin (Massachusetts Public Health Biological Laboratories, Boston, MA) and received 75,000 units of rIL-2 before filtration (Model 4C2100 blood-component recipient set; Travelon Laboratories, Inc., Deerfield, IL).

Assessment of Cell Activation. Cells were evaluated at the end of the incubation period for LAK activity against the lymphoma cell line Daudi (American Type Culture Collection, Rockville, MD) and fresh frozen colon tumor targets. Natural killer cell activity was tested against the erythroleukemia K562 (American Type Culture Collection). Target cells were labeled with ¹¹⁵C (200 µCi; Du Pont NEN Research Products, Boston, MA) for 60 min at 37°C, washed with RPMI-1640, and incubated for an additional 30 min at 37°C. Target cells (5 × 10⁵/well) were incubated with various numbers of effector cells for 4 h at 37°C. The supernatant was collected with the supernatant collection system (Skatron, Inc., Sterling, VA). Samples were counted in a gamma counter (Gamma Trac 1191; TM Analytic, Elk Grove Village, IL). Results were converted to percent specific release.

% release = 100 × cpn (test) — cpn (background) cpn (total) — cpn (background)

In Vivo Evaluation of Murine LAK Cell Activity. Murine LAK cells, generated in tissue culture bags or tissue culture flasks (75 cm² culture flask; Becton Dickinson Labware, Oxnard, CA), were compared for the ability to mediate the regression of a lethal challenge of tumor using the murine MCA 105 fibrosarcoma model (9–11). C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were given 4 × 10⁸ tumor cells i.v. Concurrently, syngeneic spleen cells were cultured at 3 × 10⁶ cells/ml in RPMI-1640 plus 10% fetal calf serum (Gibco) and 1000 units/ml of rIL-2 (10) in either bags or tissue culture flasks. Three days later the LAK cells were harvested, and 10⁷ cells were adoptively transferred i.v. to tumor recipients along with i.p. rIL-2 (50,000 units, every 8 h for 5 days). Fourteen days after tumor injection, mice were killed. Their lungs were insufflated with 15% India ink and preserved in Fekete's solution (12) for subsequent enumeration. Multiple pairwise comparisons of treatments group were made with Bonferroni's inequality (13).

RESULTS

Evaluation of Tumor Cell Killing by LAK Cells Generated in Closed Culture Conditions. The characteristics of human PBMC, processed in an enclosed system of peripheral blood cell procurement, culture, and harvest, were assessed by measuring parameters such as cell yield, cytotoxicity against NK-resistant and NK-sensitive cell lines, fresh heterologous tumor, and cell surface phenotype, against conventional roller bottle cultures (14) of the same cell population. In agreement with others (15, 16) we found no significant difference in rIL-2-induced PBMC cytotoxicity between cells cultured and harvested using the automated bag system and conventional roller bottles (a representative experiment is shown in Table 1). There were no significant differences in any of the other parameters between cells cultured in roller bottles or cells cultured in bags at a variety of cell densities (from 1 × 10⁶ cells/ml to 3 × 10⁶ cells/ml) and harvested using the enclosed automated system (data not shown).

Evaluation of Murine Micrometastases in Response to Treatment with rIL-2 and LAK Cells Generated in Bags or Flasks. We adopted the murine MCA 105 pulmonary micrometastases model to compare the ability of LAK cells generated in the tissue culture bags or culture flasks to mediate the regression of tumor in vivo. A representative experiment is shown in which mice given injections of a single-cell suspension of MCA 105 developed extensive pulmonary tumor nodules by Day 14 (Fig. 1). Mice treated with rIL-2 alone had significantly fewer tumor nodules than controls. In contrast, mice treated with LAK cells in conjunction with rIL-2 had significantly less tumor than untreated and rIL-2-treated controls. However, pairwise comparison of the number of pulmonary metastases in treatment groups which received LAK cells plus rIL-2 uncovered no significant difference in tumor elimination by LAK cells generated in bags or flasks (Table 2).

Characteristics of Human LAK Cells Grown in Bags. The total numbers of cells infused and lytic units/10⁷ cells for all patients, as well as for responders and nonresponders, are shown in Table 3. While both of these parameters were higher for the responders, the differences were not statistically significant. LAK cells grown in bags demonstrated a high degree of cytotoxicity against Daudi, fresh tumor, and K562 after activation with rIL-2. Thus, significant numbers of LAK cells for infusion, possessing a high degree of cytotoxicity, were obtained using the automated bag system.

Regression of Evaluable Metastatic Disease in the Human Using LAK Cells Grown in Bags. Twenty-eight patients were evaluable, and 1 patient was retreated, yielding a total of 29 treatment courses. The distribution of responders according to diagnosis is shown in Table 4. Of the 28 initial treatment courses, 13 of the patients had responses exceeding 50%, an overall response rate of 46%. Of these 13 patients, 4 had complete responses (complete disappearance of all known metastatic disease). The first was a 44-yr-old male with retroperitoneal lymphoma causing bowel and biliary obstruction. His tumor mass at the start of the protocol measured 22 cm in the retroperitoneum and 18 cm in the pelvis. At follow-up laparotomy 1 mo posttreatment, all of the tumor was entirely destroyed, and the patient's pelvis was entirely normal. The second complete response was seen in a 44-yr-old female with ovarian carcinoma who had progressive disease, even after i.v. and then i.p. chemotherapy. At the time of entrance on the LAK cell protocol, the patient had extensive peritoneal and diaphragmatic disease as well as an increasing serum level of CA125 antigen. Following completion of the protocol, her CA125 antigen level decreased and was normal by 1 mo posttreatment. At 6 wk, she underwent a laparoscopy and biopsy of all suspicious areas. All biopsies were negative except for one

Table 1 Comparison of LAK cytotoxicity generated in 1-liter bags and roller bottles against allogeneic fresh tumor

<table>
<thead>
<tr>
<th>Tumor target</th>
<th>Culture vessel</th>
<th>% of specific release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daudi</td>
<td>Bag</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>RB</td>
<td>79</td>
</tr>
<tr>
<td>K562</td>
<td>Bag</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>RB</td>
<td>85</td>
</tr>
<tr>
<td>FT-1</td>
<td>Bag</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>RB</td>
<td>20</td>
</tr>
</tbody>
</table>

* RB, roller bottle.
Fig. 1. These are resected lungs from mice given injections of equal numbers of MCA 105 tumor cells. To provide contrast with the white tumor nodules, the lungs were insufflated with India ink. A, control (no treatment); B, treatment with rIL-2 alone; C, treatment with rIL-2 + LAK cells generated in tissue culture flasks; D, treatment with rIL-2 + LAK cells generated in plastic bags.

Table 2 Effects of culture vessel on LAK cell activation and elimination of established pulmonary tumor

<table>
<thead>
<tr>
<th>Group</th>
<th>Cells</th>
<th>Av no. of metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>A*</td>
<td>None</td>
<td>144 ± 12</td>
</tr>
<tr>
<td>B</td>
<td>None</td>
<td>235 ± 17</td>
</tr>
<tr>
<td>C</td>
<td>LAK, flask</td>
<td>35 ± 10</td>
</tr>
<tr>
<td>D</td>
<td>LAK, bag</td>
<td>53 ± 14</td>
</tr>
</tbody>
</table>

* Pairwise comparison (Bonferroni's inequality) between Groups A and B, A and C, A and D, B and C, and B and D is statistically significant (P < 0.05). Pairwise comparison between Groups C and D is not significant. ^ ND, not determined.

Table 3 Summary of cell culture data

<table>
<thead>
<tr>
<th>No. of cells infused</th>
<th>Normal responders</th>
<th>Nonresponders</th>
</tr>
</thead>
<tbody>
<tr>
<td>LUN/10^6 cells</td>
<td>4.3 ± 2.4</td>
<td>4.9 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>1.037 ± 906</td>
<td>1.042 ± 974</td>
</tr>
<tr>
<td></td>
<td>1.018 ± 843</td>
<td></td>
</tr>
</tbody>
</table>

* Number of cells infused (x 10^6).
* Mean ± SD.
* LUN, lytic unit; a lytic unit is defined as the number of cells required to obtain 40% specific lysis.

Table 4 Distribution of responders by diagnosis

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Responders/total treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal cell</td>
<td>5/10</td>
</tr>
<tr>
<td>Melanoma</td>
<td>5/9*</td>
</tr>
<tr>
<td>Colorectal</td>
<td>0/4</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>2/3*</td>
</tr>
<tr>
<td>Ovarian</td>
<td>1/1*</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>0/1</td>
</tr>
<tr>
<td>Renal (retr.)</td>
<td>1/1*</td>
</tr>
</tbody>
</table>

* Complete responder lasting 4+ mo, 2 mo, 5+ mo, and 9+ mo, respectively.

DISCUSSION

Our effort to improve upon existing protocols of adoptive immunotherapy included modifications of methods for PBMC procurement, cell culture, and harvest, as well as a meaningful reduction in toxicity. We have independently verified the results of Muul (15) and Yannelli (16) which demonstrated the safety and efficacy of LAK cell generation in bags as assessed by a battery of in vitro assays. Cell yields of rIL-2-activated PBMC cultured in bags were slightly but consistently higher than yields of PBMC cultured in roller bottles (data not shown). In addition, our data show that PBMC processed within the enclosed automated system generated a population of LAK cells that were indistinguishable from those generated in roller bottles as judged by their cell surface phenotype (data not shown) and their ability to kill chromium-labeled fresh tumor or Daudi. In addition, the enhanced natural killer activity against KS62 generated in bags was found to be equivalent to that generated in roller bottles.

Before this automated system of cell culture was implemented for use in our human LAK cell protocol, we assessed the ability of murine LAK cells, generated in bags or flasks, to mediate the regression of pulmonary metastases. We found significant differences in the number of pulmonary metastases between mice treated with rIL-2 alone and those treated with rIL-2 plus LAK cells generated in flasks or plastic bags. Consistent with
all other parameters examined, no significant difference in the reduction of the number of pulmonary metastases was observed between groups treated with rIL-2 plus LAK cells generated in bags or flasks. This indicates that murine LAK cells generated in bags were as effective as LAK cells generated in conventional culture flasks in mediating tumor destruction in vivo.

PBMCs were procured from patients using the automated CS-3000 cell separator and were purified by Ficoll density gradient centrifugation in the original cell collection bag. Cells were transported to the laboratory where a computerized auxiliary pump dispensed appropriate volumes of culture medium and cells into plastic tissue culture bags. Following the activation period with rIL-2, cells were harvested by the CS-3000. All of the required manipulations were carried out in a completely enclosed, aseptic system. Clinically significant numbers of cells were easily generated using the automated system, and in keeping with our conclusions from data presented earlier (Table 3), the in vitro cytotoxic activity was preserved. Although the number of cells infused into our patients was fewer than the numbers of cells infused in other studies, this is most likely attributable to a smaller lymphocytosis prior to leukapheresis rather than the automated cell culture technique. The reduced lymphocytosis is most likely the result of the low-dose rIL-2 regimen administered during Wk 1 of our protocol. We used a lower dose of rIL-2 during Wk 1 of the protocol than either Rosenberg (5) or West (6), and fewer leukaphereses were performed on our patients than those of West (6).

The response rates in humans treated with this low-dose rIL-2 and completely automated system are as good as previous published reports (5, 6). While the overall response rate is slightly higher here, this may partially reflect patient selection with regard to diagnosis (for example, only four patients with colon carcinoma were treated, all nonresponders). In addition, the patients treated on this protocol did not have overwhelming disease burdens; however, in data presented elsewhere, the patients responding were comparable with regard to age, amount of disease, location of disease, and duration of response, as well as diagnosis of metastatic disease. Another cardinal feature of this protocol, which may have an influence on the response rate, is the administration of rIL-2 for several days following LAK cell infusion. Patients tolerated the prolonged administration of low-dose rIL-2 such that for each patient, 100% of the scheduled 14 doses of rIL-2 were given during Wk 1 of the protocol and, on average, approximately 90% of the scheduled 23 doses following LAK cell infusion were administered (doses were withheld at the patient's request only, none were withheld for Grade III or Grade IV toxicity). This low-dose approach differs significantly from that of researchers at the National Cancer Institute (5) in which high-dose rIL-2 is given for up to 2 days following LAK cell infusion or until toxicity dictates holding additional doses. Studies in murine tumor models suggest that this limitation may curtail response rates, because the infused LAK cells are not consistently exposed to rIL-2 which is probably required for sustained LAK cell activity in vivo (9). The present protocol also differs from that of another study in which systemic rIL-2 was administered as a constant infusion in a 25- to 30-day treatment protocol in which patients were subjected to 2 rounds of leukapheresis and LAK cell infusion (6). Our data, in the case of the one treatment patient whose metastatic tumor burden completely resolved upon retreatment, imply the efficacy of multiple courses of low-dose rIL-2 and LAK cell immunotherapy.

Another advantage of this low-dose regimen is that toxicities are significantly minimized. In data submitted elsewhere, only 1 of 29 treatment courses required intensive care unit admission during or following completion of the protocol. The patients were managed by surgical house staff on a routine nursing unit. None of the patients has required vasopressors, none had hypotension, mental status changes have not been observed as with other protocols (7), and a total of 11 units of blood have been transfused during all 29 treatment courses. Similar reductions in toxicity associated with the constant infusion of rIL-2 have been reported (6).

In summary, we present a low-dose rIL-2 regimen, in conjunction with an automated system of PBMC collection, culture, and harvest. This automated system offers a simplified, less expensive, and safer alternative to previously described labor-intensive manual techniques. Our low-dose regimen with prolonged administration of rIL-2 following LAK cell infusion provides an alternative to other more toxic, longer regimens of rIL-2 immunotherapy. In the future we plan to retreat all responders at 3 mo and 6 mo after initial treatment in an effort to prolong the duration of response and further reduce disease burdens.

REFERENCES


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