Lymphokine-activated Killer Activity Induced by in Vivo Interleukin 2 Therapy: Predominant Role for Lymphocytes with Increased Expression of CD2 and Leu19 Antigens but Negative Expression of CD16 Antigens

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ABSTRACT

The phenotype and function of lymphocytes from cancer patients treated with repetitive weekly cycles of continuous i.v. infusions of recombinant interleukin 2 (IL-2) were examined. Peripheral blood lymphocytes (PBL) obtained after IL-2 therapy showed an increased percentage of cells bearing the CD16 and CD19 markers which are associated with natural killer cells. These PBL meditated significantly increased levels of IL-2-dependent lymphokine-activated killer (LAK) activity against the Daudi cell line. Depletion of CD16+ cells from PBL obtained after in vivo IL-2 caused only slight inhibition of their LAK activity or their proliferative response to IL-2 in vitro. This indicates that CD16+ cells are involved but play only a minor role in these responses. In contrast, depletion of leu19+ cells, from PBL activated in vivo with IL-2, virtually abrogated their LAK activity and their proliferative response to IL-2. Two-color flow cytometry studies showed that a leu19+/CD16− population was expanded by in vivo IL-2 therapy and was responsible for the majority of LAK activity by in vivo-activated PBL. Moreover, this CD16− population showed an increased density of leu19 and CD2 (E rosette receptor) antigens when compared to the resting PBL obtained prior to IL-2 treatment. These data show that the predominant population mediating in vivo LAK activity, induced by in vivo IL-2 therapy, consists of activated natural killer cells with a high density of leu19 and CD2 antigens but negative for the CD16 antigen.

INTRODUCTION

Culture of murine or human lymphocytes with IL-2, a glycoprotein produced by activated T-cells, can augment or induce a variety of lymphocyte responses (1, 2) including the LAK phenomenon (3–5). The therapeutic potential of cells with LAK activity is based on in vitro studies showing preferential lysis of fresh tumor cells and tumor cell lines (3, 4) with lower levels of cytotoxicity against normal cells (6). Antitumor effects of IL-2 treatment, either alone or in combination with in vitro activated LAK cells, have been documented in many murine tumor models (7–14). Several clinical approaches utilizing IL-2 have been tested in cancer patients resulting in tumor regression in some of the patients (15–20). LAK activity has been induced in vivo IL-2 therapy in several of these clinical studies (20–23). We have reported the clinical and immunological effects of IL-2 measured in a Phase I trial for patients who received four repetitive weekly cycles of 4 days of IL-2 by continuous i.v. infusion (19, 20). Following IL-2 therapy, circulating lymphocytes mediated significant IL-2-dependent LAK activity (20). The phenotype of these cells was determined in this study.

Several laboratories have investigated the phenotype of in vitro IL-2-activated human PBL that mediate LAK activity. Initial studies using IL-2 obtained from culture supernatants of phytohemagglutinin-activated lymphocytes suggested that cells with LAK activity express T-cell markers (24). More recent studies, in which PBL were activated with purified recombinant IL-2, demonstrate that cells with LAK activity are derived from phenotypically heterogeneous lymphocyte subpopulations (25–31). Most of these reports agreed that the majority of LAK activity is mediated by IL-2-activated NK cells (26–31). Both precursor and effector cells with LAK activity were characterized by expression of the NK cell markers CD16 (leu11) and leu19 (NKH1) (26–31). The involvement of T-cells in the LAK phenomenon remains controversial. This may be due to differences in the cell separation techniques, the degree of purification achieved and the culture conditions used to generate the population of LAK cells. Separation of cells by sorting in a flow cytometer resulted in generation of LAK activity only from NK cells expressing the leu19 or CD16 NK markers and lacking the mature T-cell marker CD3 (22, 23, 28, 30). In contrast, by panning or E rosette separations, T-lymphocytes (CD3+, CD16−) incubated in IL-2 exhibited some LAK killing although at a lower level and with slower kinetics than IL-2-activated NK cells (25, 26, 29). Some of these studies have also documented the expression of low levels of CD8 antigen on LAK cells (23, 25, 26, 30). In vivo-activated PBL obtained from patients following IL-2 or combined “IL-2 + LAK” therapy demonstrate LAK activity that is also primarily mediated by cells with NK features (leu19+, CD3−) (22, 23).

Over 90% of the leu19+ cells mediating NK or LAK activities were reported to coexpress the CD16 antigen (26, 28, 32). More recently, McMannis et al. (23) found that LAK cells were heterogeneous in regard to expression of CD16; both CD16+ and CD16− populations mediated LAK activity. However, Phillips et al. (22) noticed a decrease in CD16 expression on NK cells following in vitro or in vivo IL-2 activation.

In this study, we have further evaluated the role of cells expressing either CD16 or leu19 markers in the IL-2-dependent LAK activity induced by IL-2 therapy. We used complement mediated lysis to deplete the leu19+ or CD16+ cells from PBL obtained after 4 cycles of IL-2 therapy. The phenotype and function of the depleted populations were determined. In parallel, PBL obtained from the same patients before starting IL-2 therapy were activated with IL-2 in vitro and then tested for LAK activity following similar depletion procedures. Following in vivo or in vitro treatment with IL-2, a major component of LAK activity was mediated by cells expressing the leu19 but not the CD16 determinant. This subpopulation showed an increase in intensity of fluorescence staining with antibodies to the leu19 and CD2 markers, indicating an increased surface density of these molecules on the IL-2-activated cells.

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4 The abbreviations used are: IL-2, interleukin-2; LAK, lymphokine-activated killer; PBL, peripheral blood lymphocytes; NK, natural killer; PBS, phosphate-buffered saline; LU, lytic units; FITC, fluorescein isothiocyanate; E/T, effector to target ratio; MHC, major histocompatibility complex.
**MATERIALS AND METHODS**

Patient PBL. Cancer patients were treated in this Phase 1 clinical trial with four repetitive cycles of 4 days of continuous i.v. infusion of IL-2 followed by 3 days of observation (19, 20). PBL, from four male patients out of 29 entered in this Phase 1 trial, were used for the *in vitro* studies reported here. Patient 8 (37 years old) had renal carcinoma with lung, bone, and intraabdominal metastasis (status postnephrectomy). Patient 11 (29 years old) had melanoma with metastatic large upper thorax soft tissue mass. Patient 14 (50 years old) had renal carcinoma with pulmonary metastasis (status postnephrectomy). Patient 26 (59 years old) had melanoma with pulmonary, pleural, lymph nodes, and s.c. metastasis. None of these patients had previous chemotherapy. Three of these patients (11, 26, 8) received continuous infusion of IL-2 at 3 x 10⁶ units/ml/day while one patient (Patient 14) received a lower dose of 1 x 10⁶ units/ml/day with half of each daily dose as a bolus and the remainder as a continuous infusion. All patients treated in this protocol signed consent forms for *in vivo* and *in vitro* studies approved by the University of Wisconsin Committee for the Protection of Human Subjects. Patient PBL obtained before IL-2 therapy and 24 h after the last day of IL-2 therapy (Day 27) were separated from heparinized blood by Ficoll-hypaque density gradient centrifugation. The cells were cryopreserved by controlled rate freezing in 10% dimethylsulfoxide, stored in liquid N₂, and thawed on the day of the experiment. Surface phenotype was not affected by the freezing procedure and LAK activity was retained by these samples (21, 33).

Cryo preserved PBL, obtained before therapy from the same patients, were thawed, washed, and incubated *in vitro* with IL-2 (100 units/ml) for 5–6 days; these *in vitro* IL-2-activated cells were tested in parallel to the freshly thawed PBL obtained and cryopreserved after IL-2 therapy (on Day 27).

Recombinant human IL-2 for both *in vivo* and *in vitro* testing was provided through the National Cancer Institute Biological Response Modifiers Program (NCI-BRMP) via Hoffmann-La Roche, Inc., Nutley, NJ. All IL-2 units listed are based on the NCI-BRMP standard IL-2 unit determination.

Cell Depletion. Specific cell subpopulations were selectively depleted by complement-mediated lysis. The CD mononuclear antibodies used in this technique were anti leu 11b (IgM Ab from Becton Dickinson, Mountain View, CA) and anti NKH1A (IgM Ab equivalent to anti leu 19 which was generously provided by Dr. Jerome Ritz, Harvard University, Boston, MA). PBL at a concentration of 10–15 x 10⁶/ml were washed with PBS and incubated with a saturating dose of monoclonal antibodies for 30 min at 4°C. Rabbit complement (Pel-Freez, Brown Deer, WI) was added at a final dilution of 1:2.5 and cells were incubated further for 40 min at room temperature on a rotator. Then, cells were washed with PBS and the depletion was repeated to increase efficiency. Using this double-depletion procedure, 95–99% of the positive cells were depleted as assessed by immunofluorescence (see below).

Cells were washed in PBS, resuspended in RPMI 1640 supplemented with 25 mM HEPES buffer, l-glutamine, penicillin/streptomycin and 10% heat-inactivated pooled nontransfused male human serum (HS-RPMI; Pel-Freez). Cells were counted and plated in microtiter plates for proliferative, cytotoxicity, and immunofluorescence assays in medium supplemented with 200 units/ml of IL-2. In previous studies we have observed that patients' PBL obtained following *in vivo* IL-2 therapy mediated greater levels of LAK activity if IL-2 was added to the 4-h ⁵¹Cr release assay (21). This activity was markedly enhanced following overnight incubation in IL-2. *In vivo*-activated PBL were found to lose their activity after culture in the absence of IL-2 (showing low levels of LAK activity such as 30–70 LU/10⁷ PBL), suggesting that IL-2-activated PBL are IL-2-dependent. Therefore, following antibody depletion, cells were cultured overnight in medium containing IL-2 (200 units/ml) prior to phenotypic and functional assessment. Overnight incubation in IL-2 did not change the phenotype of the cells but enhanced their LAK cytotoxic activity enabling us to compare more readily the different subpopulations tested.

**Immunofluorescence for Phenotypic Markers.** One day after depletion, unseparated and depleted cells were washed with PBS and labeled with fluorescent monoclonal antibodies for 30 min at 4°C, in a standard direct immunofluorescence assay. Cells were washed twice and the lymphocyte fraction was analyzed on a Coulter Epics C flow cytometer (Coulter; Hialeah, FL). All antibodies were purchased from Becton Dickinson (Mountain View, CA): anti-leu19 conjugated to phycoerythrin (which reacts with protein with a 220,000 molecular weight expressed on NK cells), anti-leu11-FITC (which reacts with CD16, the Fc receptor for IgG expressed on NK cells), anti-leu4-FITC (directed against the CD3 component of the T-cell receptor complex on T-cells) and anti-leu5b-FITC (directed against CD2, the E rosette receptor).

Cell-mediated Lysis Assay. Following the depletion procedure, unseparated and depleted cells were seeded in U-bottomed microplates at 0.6 x 10⁶ cells per well and serially diluted. IL-2 at 200 units/ml was added to the wells and the plates were incubated overnight. The next day, target cells were labeled with 250 μCi ⁵¹Cr for 2 h at 37°C in 5% CO₂, washed and resuspended in HS-RPMI. The "NK resistant" Daudi cells (Burkitt's lymphoma cell line) were used as a LAK target and in parallel, the "NK sensitive" K562 target (erythroleukemia line) was tested. Both targets were obtained from ATCC (American Type Culture Collection, Rockville, MD). Labeled target cells (5 x 10⁴) in 0.1 ml HS-RPMI were added to triplicate wells resulting in E/T ratios of 6, 3, 1.5, and 0.75 to 1, with a final concentration of 100 units/ml of IL-2.

Following incubation at 37°C for 4 h, the plates were processed, the percentage cytotoxicity was calculated using the mean of the three triplicate wells, and L were determined using the three to four effector/target ratios tested, as described previously (21). One lytic unit was defined as the number of effector cells causing 20% lysis of 5 x 10⁶ target cells; lytic units are expressed as LU/10⁷ effector cells.

Proliferative Assay. Following depletion, unseparated and depleted cells, at 5 x 10⁶ per well in 0.2 ml, were incubated in HS-RPMI alone or supplemented with 200 units/ml IL-2 in U-bottom microplates. After 3 days at 37°C in 5% CO₂, cells were pulse labeled with 1 μCi [³H]thymidine (New England Nuclear, Boston, MA) for 6 h. Cultures were harvested with a Mash harvester (Otto Hiller; Madison, WI) and counted by liquid scintillation. Mean cpm (±SD) of triplicate samples are reported.

**RESULTS**

Phenotypic Analysis of leu19 or leu11 Depleted Populations

Analysis of Post-IL-2 Therapy PBL. Table 1 shows the phenotypic analysis of PBL from four patients obtained before IL-2 therapy and 24 h after the 4th 4-day cycle of IL-2 therapy (Day 27). Following IL-2 therapy, unseparated PBL populations from the four patients show a wide range for the percentage of leu19+ cells (18 to 81%). Patient 11 (45% leu19+) was representative of most of the patients treated with this IL-2 regimen. [On Day 27, the mean percentage of leu19+ cells for 21 patients treated in this protocol was 44% (34).] In the four patients studied here, PBL bearing the leu19 antigen could be distinguished into two populations, one which coexpressed the leu11 antigen (leu11+/leu19+) and one which did not (leu19+/ leu11−), as monitored by two color flow cytometry analysis. The majority of leu11+ cells coexpressed the leu19 antigen and were not markedly expanded by *in vivo* IL-2 therapy (except for Patient 8). However, the percentage of cells with the leu19+/leu11− phenotype was increased by *in vivo* IL-2 therapy. These *same in vivo*-activated PBL specimens from Patients 11, 8, and 14 were also depleted of leu11 or leu19 bearing cells and analyzed in parallel to the unseparated cells for all the same markers. Following the leu19 depletion, few leu11+ cells remained (2%, 14%, and 1% for Patients 11, 8, and 14, respectively) while most residual cells were CD3+ T-cells (81–96%) (Table 1). In contrast, depletion of leu11+ cells eliminated only the PBL subpopulation coexpressing the leu11 and leu19 mark-
er (leu 19+/leu 11+) leaving a significant number of residual PBL with the leu 19+/leu 11+ phenotype, as expected (Table 1).

Following in vivo IL-2 treatment, a decrease in the percentage of PBL with the CD3+ phenotype was noted, as described previously (20, 23). Very few of the CD3+ T-cells also expressed the leu 19 marker (2–5%). Thus, the small fraction of leu 19+/CD3+ cells, previously described in resting peripheral lymphocytes as able to mediate a low level of NK activity (32), was not markedly expanded by in vivo IL-2 therapy. An analysis of in vitro-activated PBL. PBL obtained prior to in vivo IL-2 therapy from all four patients were activated with IL-2 in vitro for 5–6 days and tested in parallel to the in vivo-activated PBL generated by incubation of pretherapy PBL with IL-2 for 5–6 days.

In vivo- or in vitro-activated PBL were stained with fluorochrome-conjugated monoclonal antibodies to leu 19 (anti-leu 19-PE), leu 11 (anti-leu 11-FITC) or CD3 (anti-leu 4-FITC). Control antibodies (mouse IgG1-PE or IgG1-FITC) were used to control for nonspecific binding. For two-color analysis, cells were double labeled with anti-leu 19-PE and anti-leu 11-FITC or anti-leu 19-PE and anti-leu 4-FITC. Gates were set for nonspecific binding using cells labeled with mouse IgG1-PE and mouse IgG1-FITC. To control for nonspecific binding in depleted populations, cells pretreated with C’ alone and incubated overnight in IL-2 (similarly to cells treated with antibody and C’) were labeled with the control antibodies. All cells were analyzed by flow cytometry and results are expressed as the percentage of cells expressing the individual marker for single marker analysis, or percentage of total cells expressing both markers for two-color analysis.

### Table 1: Surface phenotype of patient PBL following in vivo or in vitro treatment with IL-2

<table>
<thead>
<tr>
<th>Samples</th>
<th>Separation</th>
<th>leu 19</th>
<th>leu 11</th>
<th>leu 19+/leu 11+</th>
<th>leu 19+/leu 11-</th>
<th>CD3</th>
<th>leu 19+/CD3+</th>
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<td></td>
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</tr>
<tr>
<td>Pre-IL-2</td>
<td>uns</td>
<td>10</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>64</td>
<td>1</td>
</tr>
<tr>
<td>In vivo IL-2a</td>
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<td>18</td>
<td>10</td>
<td>7</td>
<td>9</td>
<td>80</td>
<td>NT</td>
</tr>
<tr>
<td>In vitro IL-2b</td>
<td>uns</td>
<td>72</td>
<td>10</td>
<td>7</td>
<td>9</td>
<td>80</td>
<td>NT</td>
</tr>
<tr>
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<td></td>
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</tr>
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<td>5</td>
<td>NT</td>
<td>NT</td>
<td>82</td>
<td>NT</td>
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<tr>
<td>In vivo IL-2</td>
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<td>18</td>
<td>10</td>
<td>7</td>
<td>9</td>
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<td>NT</td>
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<tr>
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<td>uns</td>
<td>72</td>
<td>10</td>
<td>7</td>
<td>9</td>
<td>80</td>
<td>NT</td>
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<tr>
<td><strong>Patient 14</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Pre-IL-2</td>
<td>uns</td>
<td>14</td>
<td>10</td>
<td>8</td>
<td>5</td>
<td>77</td>
<td>4</td>
</tr>
<tr>
<td>In vivo IL-2</td>
<td>uns</td>
<td>40</td>
<td>17</td>
<td>16</td>
<td>24</td>
<td>63</td>
<td>6</td>
</tr>
<tr>
<td><strong>Patient 26</strong></td>
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<tr>
<td>Pre-IL-2</td>
<td>uns</td>
<td>30</td>
<td>7</td>
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<td>25</td>
<td>62</td>
<td>2</td>
</tr>
<tr>
<td>In vivo IL-2</td>
<td>uns</td>
<td>40</td>
<td>17</td>
<td>16</td>
<td>24</td>
<td>63</td>
<td>6</td>
</tr>
</tbody>
</table>

* In vivo IL-2, in vivo-activated PBL obtained on Day 27 of IL-2 therapy.

b In vitro IL-2, in vitro-activated PBL generated by incubation of pretherapy PBL with IL-2 for 5–6 days.

c Partially depleted population.

d Leu 19- or Leu 11-, PBL depleted of leu 19+ cells (leu 19-) or leu 11+ cells (leu 11-).

LAK Activity of Unseparated and Depleted Populations

LAK activity was monitored using the NK-resistant Daudi cell line as a target, with IL-2 present during the 4-h CML assay. Fig. 1 shows the percentage cytotoxicity on Daudi targets by PBL activated in vivo or in vitro with IL-2 (from Patients 11, 8, and 14, corresponding to the populations shown in Table 1 before and after cell depletion). These data, as well as cytotoxicity on K562 targets, are expressed as LU in Table 2. As shown previously (20, 21), in vivo IL-2 therapy generated significant IL-2-dependent LAK activity against Daudi cells. Cytotoxicity was ≥50% in Patients 11 and 8 at an E/T ratio of 3:1 (Fig. 1). PBL from Patient 14, who received a lower dose of IL-2, had an unusually low percentage of leu 19+ cells (18%) and mediated low-level LAK activity (20% at 6:1 E/T ratio). In all three patients, depletion of leu 19+ cells from in vivo-activated PBL virtually abrogated killing of Daudi cells by the remaining lymphocytes (Fig. 1, Table 2). Since the leu 19-depleted population consisted primarily of T-cells (Table 1), this result indicates that leu 19- T-cells were not activated by IL-2 therapy to mediate LAK activity. This was confirmed in a separate experiment by the minimal LAK activity of CD3+ T-cells obtained at 98% purity following depletion of both leu 19+ and leu 11+ cells from posttherapy PBL. These T-cells did not mediate LAK activity against Daudi targets following overnight incubation in IL-2 (only 10 LU) while purified leu 19+ cells (isolated by CD3+ T-cell depletion) mediated high LAK activity (1903 LU) comparable to the unseparated population.
CD2 AND leu19 BUT NOT CD16 ON IL-2-ACTIVATED PBL

In contrast to leu19 depletion, depletion of leu11+ cells minimally affected LAK killing (Fig. 1, Table 2). In this leu11 depleted population, a residual leu19+/leu11- fraction is still present in addition to T-cells. Since leu19- T-cells were unable to mediate LAK killing, the high LAK activity observed in the leu11- depleted population can be attributed to the leu19+/leu11- cell fraction. Therefore, it seems that the leu11+ population coexpressing leu19 (i.e., the leu11+/leu19+ cells) is not essential for mediating LAK activity, although it does mediate some degree of LAK activity (Table 2).

Similarly to in vivo-activated PBL, cytotoxic activity against Daudi targets by PBL activated in vitro with IL-2 was slightly diminished following leu11 depletion while leu19 depletion nearly abrogated this LAK activity (Fig. 1). Nevertheless, a low level of cytotoxic activity was retained in the PBL depleted of leu19+ cells (see lytic units in Table 2) which primarily consisted of T-cells (see Table 1). Thus some T-cells may have been activated to mediate LAK function by IL-2 in vitro.

Cytotoxicity was also measured on K562 target cells and showed a pattern similar to that seen on Daudi target cells (Table 2). This confirms that the majority of cells mediating LAK and NK activities express the leu19 determinant; these may well represent the same cell populations (26-28). Exceptions to this pattern have been observed with IL-2-activated PBL clones (36).

Table 2 Cytotoxicity of unseparated and depleted populations against Daudi and K562

<table>
<thead>
<tr>
<th>Patient</th>
<th>In vivo IL-2*</th>
<th>In vitro IL-2*</th>
<th>In vivo IL-2</th>
<th>In vitro IL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT 11</td>
<td>4,505 ± 2,606</td>
<td>76 ± 40</td>
<td>2,774 ± 965</td>
<td>3,752 ± 138</td>
</tr>
<tr>
<td>PT 8</td>
<td>10,888 ± 6,759</td>
<td>711 ± 402</td>
<td>7,761 ± 3,076</td>
<td>3,076 ± 1,538</td>
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<td>PT 14</td>
<td>2,131 ± 2,246</td>
<td>15 ± 19</td>
<td>1,538 ± 1,620</td>
<td>4,716 ± 2,246</td>
</tr>
</tbody>
</table>

*a Leu19- or Leu11-, PBL depleted of leu19+ cells (leu19- or) or depleted of leu11+ cells (leu11-).
*b In vivo IL-2, in vivo-activated PBL generated by incubation of pretherapy PBL with IL-2 for 5-6 days.
*c In vitro IL-2, in vitro-activated PBL generated by incubation of pretherapy PBL with IL-2 for 5-6 days.

Table 3 IL-2-induced proliferation of unseparated and depleted populations

Following leu19 or leu11 depletion from in vivo or in vitro IL-2-activated PBL, cells were cultured at 5 x 10⁶ well in IL-2 for 3 days and then labeled with [3H] thymidine for 6 h and harvested. Mean cpm of triplicates samples ± SD are reported. Treatment of cells with C' alone did not influence their proliferative response to IL-2. As a control, cells were incubated in medium without IL-2, these cells did not proliferate (~50-400 cpm).

<table>
<thead>
<tr>
<th>Patient</th>
<th>In vivo IL-2</th>
<th>In vitro IL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT 11</td>
<td>2,367 ± 272</td>
<td>734 ± 72</td>
</tr>
<tr>
<td>PT 8</td>
<td>10,492 ± 560</td>
<td>7,643 ± 737</td>
</tr>
<tr>
<td>PT 14</td>
<td>5,073 ± 673</td>
<td>641 ± 159</td>
</tr>
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</table>

*a Leu19- or Leu11-, PBL depleted of leu19+ cells (leu19-) or depleted of leu11+ cells (leu11-).
*b In vivo IL-2, in vivo-activated PBL generated by incubation of pretherapy PBL with IL-2 for 5-6 days.
*c In vitro IL-2, in vitro-activated PBL generated by incubation of pretherapy PBL with IL-2 for 5-6 days.

affected by leu11 depletion (paired t test analysis for the three patients of leu19- cells versus leu11- cells gave a P value of 0.038). This suggests that cells bearing the leu19 marker account for more of this response than do the cells expressing the leu11 marker. The in vitro-activated PBL showed a strong proliferative response to IL-2, but in contrast to in vivo-activated PBL, the response by in vitro-activated PBL was only slightly and similarly reduced by either leu19 or leu11 depletion (Table 3) (P = 0.73 by analysis of leu19- cells versus leu11- cells for the three patients). Thus, following IL-2 activation in vitro, the leu19- PBL population, consisting primarily of T-cells, was capable of proliferating in response to IL-2.

Phenotype and activity of in vivo-activated PBL Further cultured in IL-2

We have shown that in vitro-activated PBL, once depleted of leu19+ cells are enriched in T-cells but did not mediate LAK activity (Fig. 1). We then tested whether these T-cells could be induced to mediate LAK activity by further incubation with IL-2 in vitro for 8 more days. For this study Patient 14 was selected since his LAK cytotoxic activity was low (Fig. 1) and his leu19 depleted PBL consisted of 96% CD3+ T-cells (Table 1). Culture of unseparated PBL from this patient in IL-2 for 8 more days caused a marked increase in LAK killing from 380 to 7500 LU (Table 4). In this culture, the leu19+ cells were expanded from 18 to 62% by IL-2 treatment (Table 4). Less than half of these leu19+ cells coexpressed the leu11 marker (leu19+/leu11+ phenotype) (Table 4). Thus, the major PBL fraction expanded in vitro by IL-2 (increasing from 9 to 43%) carried the phenotype leu19+/leu11-, consistent with data shown in Table 1. In contrast, further culture in IL-2 of the leu19 depleted cells generated only minimal killing (from 0 to 339 LU on Days 1 and 9, respectively) (Table 4). This low level of killing may be due to the small expansion of the remaining fraction of leu19+ cells from 0.7 to 5%. Alternatively, the T-cells present in this culture, might have been activated by in vitro IL-2 and mediated this low level of LAK activity, comparable to the leu19 depleted population from in vitro-activated PBL (Fig. 1). In a separate experiment, T-cells purified by depletion of both leu19+ and leu11+ cells from posttherapy patient PBL, were cultured in IL-2 for 3 and 6 days. These CD3+ T-cells (leu19-) generated very low LAK activity, only 56 and 186 LU on Days 3 and 6, respectively. These data confirm that little or no LAK function is generated by peripheral blood T-cells isolated from patients following IL-2 therapy, even after further in vitro activation with IL-2.

Following leu11 depletion, further in vitro culture in IL-2 of the remaining cells caused an expansion of the leu19+ population from 7 to 43% while there was virtually no recovery of leu11+ cells (from 0.3 to 1.6%). Thus, this leu19+/leu11- population, obtained following leu11 depletion of in vivo-activated PBL, preserved the same phenotype and mediated high LAK activity (8014 LU, comparable to the unseparated population, Table 4) after further expansion by IL-2 in vitro.

Increased intensity of Leu19 and CD2 expression

Appearance of leu19+ bright cells following IL-2 therapy. Following in vivo IL-2 therapy, leu19+ PBL can be separated into cells showing dim and bright leu19 fluorescence staining. Fig. 2 shows the analysis of leu19+ cells from a representative patient (Patient 11). Before therapy, 27% of the cells stained positively for leu19 marker, of which 21% showed dim fluorescence while only 6% stained in the “bright” fraction (Fig. 2A). Following IL-2 therapy, 45% of the PBL stained for leu19 of which 24% showed the leu19+ bright phenotype (Fig. 2B). This increase in the percentage of cells expressing a high density of leu19 antigen (from 6 to 24% for Patient 11) was observed following in vivo and in vitro IL-2 activation for all patients.
tested (data not shown). Interestingly, the depletion of leu11+ cells from in vivo-activated PBL from Patient 11 eliminated the leu19+ dim population (a decrease from 21% before leu11 depletion to 2% after) while the majority of cells with bright expression of leu19 remained (a change from 24 to 16%) (Fig. 2C). Therefore, the leu11 depletion of in vivo-activated PBL resulted in a population consisting predominantly of cells with bright leu19 expression and no CD16 expression, which mediated the majority of both LAK cytotoxicity (Fig. 1) and proliferation to IL-2 (Table 2).

Appearance of CD2+ Bright Cells following IL-2 Therapy. The expression of the E rosette receptor molecule (CD2), involved in some effector-target cell adhesive interactions, was also influenced by IL-2 therapy. Data for Patient 11 are shown in Fig. 3. Before therapy, the majority of PBL (82%) showed expression of the CD2 marker, but most of them (77%) showed dim fluorescence intensity (Fig. 3A). Following IL-2 therapy, the percentage of PBL with bright CD2 staining increased from 5 to 29% (Fig. 3B). Because the majority of PBL express the CD2 marker (91% for Patient 11), it is not surprising that the majority of leu19+ cells (89%) coexpress CD2 (45% of PBL are leu19+ for Patient 11 and 40% of the PBL are CD2+/leu19+). However, depletion of the leu19+ cells from in vivo-activated PBL selectively eliminated the PBL showing bright rather than dim CD2 expression (Fig. 3C). This leu19 depletion also eliminated LAK activity (Fig. 1). Thus, it seems that most CD2+ bright cells are leu19+ and are contained within the cell fraction responsible for LAK activity. This increase in CD2 antigen density was observed with in vivo-activated PBL from the three other patients as well as with their pretreatment PBL activated in vitro with IL-2 (data not shown).

Appearance of leu19+ Bright/CD2+ Bright Cells. Double marker analysis of PBL activated in vivo with IL-2 from Patient 11 documented the appearance of a cell population expressing a high density of both leu19 and CD2 antigens (Fig. 4). Before IL-2 therapy, most leu19+ circulating lymphocytes coexpressed CD2 antigen but these cells stained dimly for both leu19 and CD2 antigens (Fig. 4A). After IL-2 therapy, cells with bright expression of both leu19 and CD2 could be distinguished from
the PBL showing dim expression of these two molecules (Fig. 4B). The percentage of PBL staining brightly for both leu19 and CD2 increased from 1 to 20% following in vivo activation with IL-2. Interestingly, depletion of leu11+ cells, from these in vivo IL-2-activated PBL, depleted most of the cells with dim coexpression of the leu19 and CD2 markers; the remaining leu19+ cells were predominantly bright for both leu19 and CD2 markers (Fig. 4C). Thus most leu19+ and CD2+ bright cells are leu11−, suggesting that the increase in leu19 and CD2 antigen density induced by in vivo IL-2 (Figs. 2 and 3) occurs primarily on leu19+/leu11− cells as a result of activation with IL-2. This population with bright expression of both leu19 and CD2 was also observed in PBL activated in vitro with IL-2 (not shown).

Biological Activity of PBL with Bright Expression of Both leu19 and CD2

In order to determine the function of the cells with bright expression of both leu19 and CD2 markers, these cells were sorted by flow cytometry and tested for cytotoxic function. PBL obtained after IL-2 therapy from two patients (Patients 11 and 8) were double labeled with anti-leu19-PE and anti-CD2-FITC fluorescent monoclonal antibodies (as shown in Fig. 4) and sorted. Cells with bright staining for both leu19 and CD2 were isolated, as were cells with positive but dim, expression of both markers. These separate cell populations were incubated overnight in IL-2 and then tested for cytotoxicity against Daudi targets (Fig. 5). Unlabeled cells and cells labeled with both antibodies but not sorted were also tested in parallel to the sorted populations. Following overnight incubation in IL-2, these cells did not clear all surface antibodies and still showed some residual fluorescence. Nevertheless, in both patients, the antibody labeled unsorted cells showed the same level of killing against Daudi as did the unsorted cells which were not antibody labeled (Fig. 5). Thus, presence of residual leu19 and CD2 antibodies did not influence the LAK activity in these assays. The sorted population with bright expression of leu19 and CD2 mediated significantly higher LAK activity against Daudi than did the unsorted cells (Fig. 5) (P = 0.004 for Patient 11 and 0.002 for Patient 8). In contrast, the population with dim expression of leu19 and CD2 mediated much lower cytotoxic activity than the bright cells (P < 0.001) or the unsorted cells (P < 0.001). These data were similar for in vivo-activated PBL from both patients tested. The destruction of K562 targets (not shown) by these same PBL populations showed the same pattern presented in Fig. 5 on the Daudi target. The proliferation induced by in vitro IL-2 of these same sorted cell populations was also tested with PBL from Patient 8. The bright cells proliferated more in response to IL-2 in vitro for 3 days (5489 cpm 3H TdR incorporation) than did the dim cells (1031 cpm).

DISCUSSION

Cells with LAK activity seem to be derived from phenotypically heterogeneous lymphocyte subpopulations (25–31). Nevertheless, the majority of LAK activity has been found to be mediated by IL-2-activated NK cells on the basis of expression of the NK cell markers leu19 and CD16 (26–31).

The present study was designed to assess the role of cells expressing either CD16 or leu19 antigens in the IL-2-dependent LAK activity induced by in vivo IL-2 therapy. The leu19 antigen (equivalent to NKH1) is a glycoprotein with 220,000 molecular weight expressed on 15% of freshly isolated PBL (32, 38), on most IL-2-dependent clones that mediate non-MHC restricted cytotoxicity (38) as well as on most IL-2-dependent cytotoxic T-lymphocyte lines (39). The CD16 (leu11) antigen is a M, 50,000–70,000 protein associated with the Fc receptor for IgG present on NK cells and neutrophils (32, 40). In this study, PBL obtained from cancer patients treated with four repetitive weekly cycles of IL-2 (19, 20) mediated significant IL-2-dependent LAK activity and were tested in parallel to pretreatment PBL-activated in vitro with IL-2. We found that depletion of leu19+ cells from in vivo-activated PBL virtually abrogated LAK cytotoxic activity. Thus, cells expressing the leu19 marker are of major importance for mediation of the LAK activity induced by in vivo IL-2 therapy.

Following leu19 depletion, or both leu19 and leu11 depletion, the remaining population consisting mainly of CD3+ T-cells did not mediate LAK activity, indicating that leu19− T-cells were not significantly activated by this regimen of IL-2 in vivo to mediate non-MHC restricted cytotoxicity. Two recent studies, analyzing PBL from patients who received IL-2 therapy have also shown that LAK activity is mediated by cells expressing the leu19 NK marker with no involvement of T-cells (22, 23). Leu19 depletion of in vitro-activated PBL nearly abrogated LAK activity but a low level of cytotoxicity and a high proliferative response to IL-2 were retained by the residual leu19− cells (Table 3) consisting mainly of T-cells. Thus, in addition to the leu19+ cells activated by IL-2 in vivo and in vitro, some T-cells may also have been activated by IL-2 in vitro. This may be due to the higher concentration of IL-2 used in vitro (100 units/ml), while in vivo the steady state concentration of IL-2 in the patient serum was approximately 30 units/ml (18). Low levels of non-MHC restricted cytotoxicity mediated by T-cells activated with IL-2 in vitro have been previously reported in some studies (25, 26, 29, 30) but not in others (28). Culture conditions may account for this discrepancy as recently shown in a study where significant LAK activity was generated from T-cells cultured in autologous serum and in the presence of monocytes (41).

Two-color flow cytometry analysis of PBL activated by IL-2 in vivo or in vitro showed that the leu19+ cells could be divided...
into the leu19+/CD16+ and the leu19+/CD16− subpopulations. Depletion of CD16+ cells, from in vivo-activated PBL, results in a population consisting of leu19+/CD16− cells and T-cells that mediated significant LAK activity. Since T-cells were found to be unable to mediate LAK killing, the high LAK activity observed in the CD16 depleted population can be attributed to the leu19+/CD16− subpopulation. It seems, therefore, that the CD16+ cells (coexpressing leu19) are not essential for mediation of LAK activity although they are slightly expanded by the in vivo IL-2 therapy or by in vitro IL-2 treatment and can mediate some LAK activity. These leu19+/CD16+ cells have been previously described as the major lymphocyte component of fresh PBL-mediating NK activity (32). The precursor and effector cells for LAK activity generated in vitro with IL-2 were also defined as CD16+ cells or leu19+ cells (26–28). However, it was reported that 90% of the leu19+ cells coexpressed the CD16 antigen (26, 28). The minor fraction of leu19+ cells not expressing CD16 antigen (leu19+/CD16−) was also shown to mediate LAK activity (28).

In contrast, our findings indicate that the majority of LAK effector cells activated in vivo with this regimen of IL-2 or in vitro with 100 units/ml for 5–6 days, are primarily CD16− and leu19+ with only a minor role for the cells coexpressing the leu19 and CD16 antigens. Recently, Phillips et al. (22) noted a decrease in CD16 expression on NK cells following IL-2 treatment. However, McManns et al. (23) have shown that comparable levels of LAK activity could be measured in cells positive or negative for CD16 from patients undergoing IL-2 therapy according to a protocol involving higher doses of IL-2 than those used in our protocol. This same research team has recently published that the increase in the percentage of positive cells coexpressing leu19 and CD16 (Leu19+/CD16+) following IL-2 therapy was more substantial than the increase seen in the leu19+/CD16− subpopulation (42). In contrast, using a separate IL-2 treatment regimen, our data indicate a greater increase in leu19+/CD16− than the leu19+/CD16+ cells, by single color analysis of 16 patients’ PBL (the percentage of leu19+ cells was 1.5–2-fold higher than the percentage of CD16+ cells) (34), and by the two color analysis shown here.

In this present study, the leu19+/CD16− subpopulation was further analyzed and its function determined. These cells, which represented a small fraction of the resting PBL isolated from the patients before IL-2 therapy, were expanded by IL-2 therapy and then mediated substantial LAK activity. This leu19+/CD16− subpopulation stained brightly with fluorescent anti-leu19 antibody, indicating a high density of leu19 antigen on the cell surface. The leu19+/CD16+ cells stained dimmer for leu19 antigen. McManns et al. (23) also noted that leu19+ cells developing in patients receiving IL-2 comprised two subpopulations, dim leu19+ and bright leu19+ with a predominant expansion of bright leu19+ cells (42). Our data corroborate these reports and in addition demonstrate that most of the bright leu19+ cells do not express the CD16 antigen. Lanier et al. (32) have described a small cell subset of leu19+/CD16−/CD3− cells in resting PBL that are capable of mediating NK activity; these cells also stained brightly for leu19 antigen. It could be that these leu19+ bright/CD16− cells present in resting PBL are the cells which are preferentially expanded by IL-2 therapy to constitute the major component of LAK activity. This is also confirmed by the finding that these leu19+/CD16− cells, obtained following CD16 depletion, gave a strong proliferative response to IL-2. When leu19+/CD16−/CD3− cells were sorted by flow cytometry and cultured in IL-2 in vitro, they remained leu19+/CD16−/CD3− and mediated LAK cytotoxic activity (28). This is corroborated by our findings showing that leu19+/CD16− cells, obtained from in vivo-activated PBL depleted of CD16+ cells, were considerably expanded by further culture in IL-2 with no induced expression of CD16 antigen; they mediated high levels of LAK cytotoxic activity. This indicates that these leu19+/CD16− cells are highly sensitive to IL-2, maintain their leu19+/CD16− phenotype, mediate LAK activity, and play a major role in the IL-2-induced generation of cells with LAK activity.

Lanier and Phillips (28, 32) have suggested that this leu19+/CD16− subpopulation may represent an early stage of NK cell differentiation before acquisition of CD16 Fc receptors. Our data suggest that some leu19+/CD16− cells, particularly those obtained after IL-2 activation, might represent a later stage of NK cell differentiation in which the CD16 Fc receptors are lost (or masked by acquisition of other molecules) and are more readily responsive to IL-2. Alternatively, the leu19+/CD16− cells could also represent a separate lineage of highly IL-2-responsive cells with NK activity that never express the CD16 marker. Further studies are underway to clarify this issue.

The E rosette receptor, CD2, is expressed on most NK cells (32). The CD2 antigen is involved in effector-target cell adhesive interactions through the LFA-3 molecule (43). This study showed that CD2 is expressed on most PBL, including the leu19+ cells, both before and after IL-2 therapy. Furthermore, a population expressing a high density of CD2 antigen was expanded by IL-2 therapy and coexpressed a high density of the leu19 antigen by double marker analysis. This result confirms the recent report by Ellis et al. (42) that there is an expansion of CD2 bright/leu19 bright cells following IL-2 therapy. Furthermore, our study demonstrates that these leu19+ bright/CD2+ bright cells mediated both higher LAK activity and proliferative responses to IL-2 than did the leu19+ dim/CD2+ dim cells. Since the leu19+ bright cells were found to lack the CD16 antigen, these results suggest that the functional phenotype of LAK cells generated by in vivo IL-2 therapy is predominantly leu19+ bright/CD2+ bright/CD16−. The high density of CD2 antigen on these cells could be involved in more efficient binding of these cells to their targets, consistent with their more potent LAK cytotoxic activity.

These results suggest the development of strategies which attempt to facilitate better binding of these IL-2-activated leu19+/CD2+ cytotoxic cells to tumor cells in vivo. One potential approach could involve bispecific monoclonal antibodies, such as heterocross-linked antibodies (44, 45), able to simultaneously bind to the leu19+/CD2+ effector cells and to the tumor cells.

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3687


Lymphokine-activated Killer Activity Induced by *in Vivo* Interleukin 2 Therapy: Predominant Role for Lymphocytes with Increased Expression of CD2 and Leu19 Antigens but Negative Expression of CD16 Antigens
