Induction of In Vitro Graft-Versus-Leukemia Activity Following Bone Marrow Transplantation for Chronic Myeloid Leukemia

By Stephen Mackinnon, Jill M. Hows, and John M. Goldman

We studied the in vitro effects of lymphokine-activated killer (LAK) cells from the peripheral blood of chronic myeloid leukemia (CML) patients after allogeneic and syngeneic bone marrow transplantation (BMT). LAK cells were generated by incubating peripheral blood mononuclear cells from patients post-BMT with recombinant interleukin-2 (IL-2) (500 U/mL) in 10% AB serum for 7 days. They were phenotyped and tested for activity in a standard 4-hour $^{51}$Cr release assay ($n = 37$) and in a CFU-GM assay ($n = 24$). We found that the LAK cells were mainly activated natural killer cells, but some were CD3$^+$ T cells. In the $^{51}$Cr release assay LAK cells from 20 of 33 (61%) allogeneic and 2 of 4 syngeneic recipients killed recipient CML cells and in 22 of 37 (60%) cases also killed the HLA disparate CML cells. In the CFU-GM assay the LAK cells incubated together with the CML cells in liquid culture before plating inhibited ($P < .05$) colony growth in 16 of 22 allogeneic and 2 of 2 syngeneic recipients. Cell–cell contact was necessary for optimal effect. There was little or no inhibition of proliferation of donor marrow CFU-GM. This in vitro graft-versus-leukemia (GVL) effect could also be demonstrated after LAK effectors were depleted of CD3$^+$ T cells. It was inducible in recipients of both T cell–depleted and T cell–replete donor marrow and in recipients with or without graft-versus-host disease. These results suggest that a major histocompatibility complex–unrestricted GVL effect is inducible following allogeneic and syngeneic BMT. The use of IL-2/LAK cells after BMT could reduce the risk of relapse.

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Materials and Methods

Patients. All patients studied were in complete hematologic and cytogenetic remission after BMT for CML. Thirty-seven were studied using a standard 4-hour $^{51}$Cr release cytotoxic assay. Twenty-six patients had received allogeneic marrow from HLA-identical siblings, seven allogeneic marrow from HLA-matched unrelated donors, and four syngeneic marrow from identical twin donors. Twenty-four of these patients were studied using a clonal assay to assess GVL activity. Fifteen patients had received allogeneic marrow from HLA-identical siblings, seven allogeneic marrow from HLA-matched unrelated donors, and two syngeneic marrow from identical twin donors. Pretransplant all patients were conditioned with cyclophosphamide (60 mg/kg/d × 2) and total body irradiation ($6 \times 2$ Gy). Some patients also received in vivo antilymphocyte monoclonal antibodies (MoAbs), additional chemo-

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therapy, total lymphoid irradiation, or splenic irradiation. GVHD prophylaxis consisted of T-cell depletion of the donor marrow with the rat monoclonal antibody Campath-1 or postgraft immunosuppression with cyclosporine with or without methotrexate. Patients were studied at intervals ranging from 4 weeks to 10 years post-BMT. In addition, we generated LAK cells from the peripheral blood of eight bone marrow donors pretransplant to assess whether they were capable of mediating GVL activity. Each patient and donor gave informed consent for these studies.

**Generation of LAK Cells.** Venous blood was collected from the donors pretransplant and from the recipients posttransplant. PBMCs were prepared by centrifugation on Lymphoprep (Nyegaard, Oslo). The low-density cells were collected and washed twice with Ca²⁺, Mg²⁺-free Hanks' balanced salt solution (HBSS) (GIBCO, Paisley, Scotland) and resuspended in RPMI 1640 (Flow, Irvine, Scotland) supplemented with 2 g/L sodium bicarbonate, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L L-glutamine, and 10% heat-inactivated human AB serum (RPMI AB).

For generation of LAK cells, PBMCs at a concentration of 1 to 2 × 10⁶/mL were incubated for 7 days in 5% CO₂ at 37°C in RPMI AB containing 500 U/mL recombinant IL-2 (kind gift of Cetus Corporation, Emeryville, CA). The cells were tested for cytotoxic activity after washing (see below).

**Elimination of T-lymphocyte subpopulations.** Washed LAK cells were incubated for 60 minutes on ice with an anti-CD3 monoclonal antibody (UCHT1, kindly provided by Dr Peter Beverley, ICRF, London) at saturating concentration. The cells were washed and centrifuged, resuspended, and incubated with sheep antimouse IgG-coated magnetic beads (Dynal, Oslo) on ice for 40 minutes at a bead:cell ratio of 20:1. Following the addition of 5 mL RPMI 1640, the resuspended preparation was placed in a magnetic field for 5 minutes. The cells in the supernatant were then withdrawn for use in the cytotoxicity assay.

**Surface marker analysis.** Phenotypic analysis was performed by indirect and two-color direct immunofluorescence using standard techniques. Samples were analyzed on a Becton Dickinson FACS-CALIBER flow cytometer. MoAbs were purchased from Becton Dickinson (Mountain View, CA) and were directed against CD3 (Leu-4), CD4 (Leu-3), CD8 (Leu-2), CD16 (Leu-11), and CD56 (Leu-19) antigens.

**Target cells.** Target cells used in the cytotoxic assay were (1) the NK-sensitive cell line K562; (2) an NK-resistant LAK-sensitive Epstein-Barr virus–transformed B-lymphoblastoid cell line (LCL); and (3) cryopreserved leukemia cells from the transplant recipients and from 37 different third-party HLA-disparate patients with CML. Target cells in CFU-GM assays were cryopreserved CML mononuclear cells from the transplant recipients and cryopreserved donor marrow mononuclear cells. Recipient leukemic cells were collected from the peripheral blood or bone marrow pretransplant. Normal human bone marrow was obtained after informed consent from the donors at the time of the bone marrow harvest by aspiration from the posterior iliac crests. The cryopreserved target cells were thawed for 1 minute at 37°C in a water bath, diluted in HBSS, and centrifuged on Lymphoprep. The low-density cells were then collected, washed twice in HBSS, and resuspended in RPMI AB.

**Cytotoxicity assay.** Details of these assays have been described previously. All cells were labeled by incubation with 250 µCi ⁵¹Cr (Na₂CrO₄) (Amersham, England) for 60 minutes. The target cell suspension at 5 × 10⁵ cells/mL was dispensed in 100 µL volumes into U-shaped microtest plate wells. The effector cells were then added in a series of five different effector:target ratios, each in triplicate. The cells were incubated for 4 hours, after which the supernatant was removed and ³¹Cr release quantitated on a LKB Wallac 1260 Multigamma counter (Wallac, Turku, Finland). The percentage specific lysis for each target was calculated as follows:

\[
\text{Percent Specific Lysis} = \frac{\text{Experimental Release} - \text{Spontaneous Release}}{\text{Maximum Release} - \text{Spontaneous Release}} \times 100\%
\]

where the maximum release was obtained from target cells exposed to 1% Triton X-100 (New England Nuclear, Boston, MA) and the spontaneous release was that observed when the target cells were incubated in RPMI AB. Some results were expressed in lytic units defined as the number of effector cells per 10⁶, resulting in specific lysis of 60% of 5,000 target cells. The spontaneous release was always less than 10% for the cell line targets and between 15% and 25% for the cryopreserved CML targets. Cytotoxic activity was considered positive when the value of experimental ³¹Cr release at an effector:target ratio of 50:1 was significantly above that of spontaneous ³¹Cr release according to Student's t-test.

**Assay for granulocyte-macrophage progenitor cells (CFU-GM).** Mononuclear cells, 1 × 10⁵, in RPMI AB either of normal donor marrow or recipient CML origin were preincubated with or without the LAK cells at an effector:target ratio of 10:1 for 18 hours in liquid culture. After incubation the cells were resuspended and subsequently cultured in triplicate for CFU-GM in a methylcellulose culture system as described previously. In experiments designed to establish the role of effector–target cell–cell contact and to assess the possibility of nonspecific inhibition or stimulation of progenitor cell growth due to the presence of LAK cells or LAK-derived cytokines in the semisolid culture medium, LAK cells at the same effector:target ratio were added to the progenitor cells immediately before plating. All LAK cells were irradiated (5 Gy) before use to prevent them from forming colonies. CFU-GM colonies were defined as granulocytic, monocytic, or mixed aggregates containing more than 20 cells. They were scored on day 14 under an inverted microscope. The number of colonies cultured from 10⁵ normal marrow or CML mononuclear cells cultured in the absence of LAK cells was taken as 100%. In the cultures containing LAK cells, the percentage of surviving progenitor cells was calculated by dividing the total number of colonies by the number of colonies obtained in cultures free of LAK cells × 100%.

**Statistical methods.** Differences in cytotoxic activity or phenotypic analysis were analyzed using Student's paired t-test.

### Table 1. Phenoypype of LAK Effector From 29 Patients

<table>
<thead>
<tr>
<th>Time From BMT/Cytotoxicity</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>CD16</th>
<th>CD56</th>
<th>CD3/8</th>
<th>CD3/56</th>
<th>CD8/56</th>
<th>CD4/8</th>
<th>CD16/56</th>
</tr>
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<tbody>
<tr>
<td>&lt;3 mo (n = 8)</td>
<td>41 ± 7</td>
<td>15 ± 6</td>
<td>40 ± 18</td>
<td>8 ± 6</td>
<td>56 ± 30</td>
<td>23 ± 21</td>
<td>9 ± 13</td>
<td>21 ± 17</td>
<td>1 ± 1</td>
<td>9 ± 6</td>
</tr>
<tr>
<td>1 y (n = 13)</td>
<td>40 ± 22</td>
<td>12 ± 13</td>
<td>39 ± 15</td>
<td>8 ± 5</td>
<td>53 ± 27</td>
<td>24 ± 17</td>
<td>8 ± 10</td>
<td>18 ± 14</td>
<td>1 ± 1</td>
<td>9 ± 5</td>
</tr>
<tr>
<td>&gt; 1 y (n = 16)</td>
<td>52 ± 23</td>
<td>10 ± 8</td>
<td>50 ± 17</td>
<td>12 ± 9</td>
<td>48 ± 19</td>
<td>36 ± 20</td>
<td>12 ± 6</td>
<td>15 ± 8</td>
<td>2 ± 2</td>
<td>12 ± 9</td>
</tr>
<tr>
<td>Cytotoxic LAKs (n = 17)</td>
<td>47 ± 25</td>
<td>13 ± 13</td>
<td>43 ± 19</td>
<td>8 ± 4</td>
<td>46 ± 24</td>
<td>31 ± 21</td>
<td>9 ± 9</td>
<td>15 ± 12</td>
<td>1 ± 1</td>
<td>8 ± 4</td>
</tr>
<tr>
<td>Noncytotoxic LAKs (n = 12)</td>
<td>47 ± 22</td>
<td>9 ± 6</td>
<td>48 ± 13</td>
<td>14 ± 10</td>
<td>53 ± 20</td>
<td>32 ± 19</td>
<td>11 ± 8</td>
<td>19 ± 9</td>
<td>2 ± 2</td>
<td>14 ± 10</td>
</tr>
</tbody>
</table>

Results shown are for single and two-color fluorescence expression as means ± 1 SD. Studies are subdivided on both time from BMT to time of assay and whether the patients' LAK cells were or were not able to kill their own pretransplant leukemia cells.
RESULTS

Surface markers. The phenotype of the effector LAK cells is shown in Table 1. The predominant phenotype of the LAK cell population was CD56+, CD8+, CD4−, with a variable number of CD3+ cells. The CD8+ fraction was usually split between the CD3+/CD8bright and CD3−/CD8dim populations, with the CD8dim cells coexpressing the CD56 antigen (Fig 1). Following culture in IL-2 there was an increase in the expression of the CD56 antigen concurrent with a reduction in the expression of the CD16 antigen (Fig 2) with the CD56 population being either CD16− or CD16dim (Fig 1). Although we observed an increasing number of CD3+ T cells and a reduced number of CD56+ NK cells with time from transplant, these changes were not statistically significant.

![Flow cytometric analysis of day 7 LAK cells from a single patient.](image)
Surface Phenotype Targets

Fig 2. Surface marker analysis of peripheral blood mononuclear cells from seven patients pre- and post-IL-2 incubation expressed as means ± 1 SD. Following culture with IL-2 we saw a reduced number of T cells with an increased number of NK cells, although these differences were not significant. There was a reduction \((P < .01)\) in the proportion of CD56 cells that coexpressed the CD16 antigen following IL-2 incubation.

significant. Furthermore, there was no significant difference in the phenotype of the effectors that were able to lyse the recipients' leukemic cells and those that were not killers (Table 1).

Activity of effectors against cell line targets. The effectors generated after 7 days' culture in IL-2 always caused significant lysis of the NK-sensitive cell line K562 and the NK-resistant LCL in 15 consecutive studies. Killing of the cell line targets did not predict killing of the patients pretransplant leukemia cells. Figure 3 demonstrates the lytic activity against the NK-resistant LCL cell line targets of LAK cells from seven patients where the effectors caused significant lysis of their pretransplant CML cells compared with the LAK cells from eight patients that did not kill recipient leukemic cells. There was no significant difference in the cell line lytic potential between LAK cells that were or were not CML lytic, indicating that CML cells appear to be more resistant to lysis than the cell line targets. Figure 3 also shows that the lytic activity against the recipient CML cells is significantly less than that against the LCL cell line.

Cytotoxicity of effectors against CML cells. In the \(^{51}\)Cr release assay LAK cells from 20 of 33 (61%) allogeneic and two of four syngeneic recipients killed recipient CML cells and in 22 of 37 (59%) cases also killed the HLA disparate CML cells. The data in Fig 4 indicate the mean specific lysis for the leukemic targets in the 22 positive assays. In 9 cases (2 syngeneic) where we demonstrated killing of the recipients' CML cells, the effector cells were depleted of CD3+ T cells and assessed for GVL activity in the same experiment. In all experiments the T-cell-depleted effectors lysed the recipients' and the third-party CML cells with a slight though not significant increase in specific lysis when compared with the nondepleted LAK cells (Fig 5).

LAK cell efficacy versus leukemic cell susceptibility. We analyzed the data to look for variability in both the LAK cell lytic potential and the leukemic target cell susceptibility to killing among the 37 patients (Table 2). Patients were categorized into four groups. Group 1 produced LAK cells with good lytic potential that killed more than one susceptible leukemic target. Group 2 failed to lyse either target, but...
INDUCTION OF GVL ACTIVITY FOLLOWING BMT

Fig 5. Results from all nine patients whose LAK cells were T-cell depleted and who demonstrated significant lysis of the CML targets. Unmanipulated and CD3-depleted LAK cells were added to recipient CML and allogeneic CML cells at E:T ratios of 80, 25, 12, 6, and 3 to 1. Each E:T ratio was set up in triplicate and the points plotted are the mean % Cr release. The graph shows that T-cell depletion of the effectors does not reduce their ability to lyse the leukemic cells. There is no significant difference in the killing of the recipients' and the allogeneic CML cells.

because these targets were not killed by effectors from any other patient, we cannot determine whether this group represents poor effector lytic ability or resistant target cells. The LAK cells of the patients in group 3 were able to lyse one of the two CML targets. The targets that were resistant to lysis were not susceptible to killing from other effectors used in separate experiments. This suggests a differential sensitivity of the leukemic cells to lysis in different patients. The LAK cells from patients in group 4 were unable to kill at least one leukemic target that had been susceptible to lysis when tested with effectors from other patients, indicating that the LAK cells from group 4 patients were less active. These data suggest that both effector and target cell factors are important in determining the outcome of an assay.

Correlation of clinical and in vitro data. There was no correlation between the ability of the patients' LAK cells to lyse their pretransplant cryopreserved CML cells and (1) the interval from transplant to the time of assay, (2) whether the donor marrow was T-cell depleted or T-cell replete, or (3) the incidence and severity of acute and chronic GVHD (Table 3). None of the patients relapsed during the study period, so we can make no comment about the relationship between in vitro GVL activity and the likelihood of relapse.

**CFU-GM assay.** We generated LAK cells from PBMC of 24 transplant recipients. The effect of LAK cell–derived diffusible factors in the CFU-GM cultures was evaluated by comparing cultures grown in the absence of LAK cells with those cultures in which the LAK cells were not in contact with the CML progenitors during the liquid phase. We found that the LAK cells significantly increased colony numbers in eight assays, significantly decreased colony numbers in 10 assays, and made no difference in six assays in the absence of effector–target cell contact (Fig 6).

To assess the influence of effector–target cell contact independent of the effects of LAK cell cytokine release we compared experiments in which LAK cells were or were not in cell–cell contact with the CML progenitors in liquid culture before plating in the methylcellulose. These two sets of data are presented with the results expressed as percentages of the colony numbers obtained in cultures without LAK cells. We found significant inhibition of CFU-GM proliferation from CML cells in 18 (2 syngeneic) of the 24 (75%) experiments (Fig 6). The mean ± SEM inhibition in these 18 studies was 47% ± 5% (range, 19% to 87%). No significant difference was seen on three occasions and significant stimulation of colony growth in three assays (Fig 6).

We then compared the activity of LAK cells cultured from one syngeneic and five HLA-identical sibling transplant recipients incubated simultaneously with either donor marrow progenitors or recipient CML progenitors. Recipient LAK cells inhibited leukemic CFU-GM proliferation to a significant degree in all six experiments (mean ± SEM: 49% ± 9%, range 19% to 85%) (Fig 7A), whereas proliferation of donor marrow CFU-GM was significantly inhibited only in two experiments (31% and 33%) (Fig 7B).

Similarly, we compared the activity of LAK cells generated from one syngeneic and seven sibling allogeneic bone marrow donors. Of the eight donors, seven were males or nulliparous females with no previous blood transfusions. We again used both donor marrow and recipient CML cells as concurrent targets. Donor LAK cells inhibited recipient leukemia CFU-GM proliferation to a significant degree in
six of eight experiments (mean ± SEM: 52% ± 7%, range 38% to 86%) (Fig 8A), whereas proliferation of donor marrow CFU-GM was significantly inhibited (36%) in only one experiment (Fig 8B).

DISCUSSION

Adoptive immunotherapy with LAK cells and IL-2 is a new approach in the treatment of malignant disease. For hematologic malignancies this strategy has been proposed for purging autologous bone marrow and as remission maintenance after conventional chemotherapy or BMT. Although there is good evidence for the existence of enhanced NK and LAK cell activity after both autologous and allogeneic BMT, previous studies have largely used cell line targets rather than the recipient leukemia cells, and it has not been demonstrated that LAK cells can mediate in vitro GVL activity.

In the early phase of immune reconstitution post-BMT, CD3⁺, CD16⁺, and CD56⁺ large granular lymphocytes with NK activity form up to 45% of the lymphocyte compartment and CD3⁺ cells are slow to reappear. Therefore, we might have expected to generate LAK cells with a predominantly NK phenotype from the patients studied during the first 3 months post-BMT. In practice we produced LAK cells with surface markers not usually present in significant numbers in the peripheral blood of normal individuals, ie, CD8⁺dim/CD3⁻, CD8⁺dim/CD56⁺, CD56⁺/CD16⁻, and CD56⁺/CD16⁺dim, phenotypes that have been described following culture of lymphocytes in IL-2 and in BMT recipients. Although a greater percentage of CD56⁺/CD3⁻ cells was found in patients studied within 3 months of BMT, there was no significant difference when compared with the LAK cell phenotype of patients studied at longer intervals from transplant. Our inability to demonstrate phenotypic differences in...
the LAK cells that were or were not capable of mediating in vitro GVL activity could reflect the possibility that the GVL activity resides in a small subpopulation that we have not fully characterized. Alternatively, it could reflect the variation in leukemic target sensitivity that we saw in different patients (Table 2).

Conventionally, LAK activity after BMT is tested using NK-resistant tumor cell line targets. Previous studies indicated that whereas fresh leukemia cells are more resistant to killing by LAK cells than cell line targets, there was a correlation between lysis of cell line targets and fresh leukemia cells. However, our data indicate that LAK cells can in some cases kill substantial numbers of cell line target cells but induce no detectable lysis of the cryopreserved CML cells (Fig 3); thus, correlating results using cell line targets and leukemia cells is not always possible.

The main new finding in this study is that LAK cells generated from BMT recipients in remission can kill recipient leukemic cells in vitro in over half of the patients studied. Because immune reconstitution after BMT results in replacement of the recipients' NK and T cells with those of donor origin, this represents an inducible in vitro GVL effect. Because the effectors used in this study were donor-derived and the targets were either autologous (donor marrow) or allogeneic (HLA-identical sibling donors pretransplant) the mechanisms underlying this antileukemic activity with complete or relative sparing of normal marrow remain poorly characterized.

In the CFU-GM assay we demonstrated stimulation as well as inhibition of colony growth when LAK cells were added to the cultures immediately before plating in methylcellulose but not in cell–cell contact with the progenitor cells (Fig 6). These effects mediated by LAK-derived diffusible factors that we presume to be the cytokines reported by a number of investigators. These apparently contradictory findings can be explained by the fact that LAK cells produce colony-stimulating factors whose growth-promoting activities may be masked by colony-inhibiting activity due to concurrent release of TNF. Therefore, by comparing cultures containing LAK cells that were or were not in cell–cell contact with the target cells, we were able to define the role of LAK–target cell contact because paired cultures were subject to identical cytokine effects.

The experiments performed with preincubation of LAK cells and target cells together in liquid culture allowed us to determine the consequences of this cell–cell contact. Although we showed significant inhibition of colony growth of the majority of the recipients' CML progenitors (Figs 6, 7A, and 8A), we saw a similar effect in only a minority of cultures where normal donor marrow was the target, and then in an attenuated form (Figs 7B and 8B). The mechanisms underlying this antileukemic activity with complete or relative sparing of normal marrow remain poorly characterized. Possible mechanisms include enhanced LAK cell recognition of leukemic targets with resultant triggering of perforin-mediated lysis or local release of cytokines such as TNF or interferon, which are known to affect leukemic cells more
than normal cells.\textsuperscript{50-52} In the chromium-release cytotoxicity assay, we have shown that the LAK cells are capable of lysing CML targets, suggesting that the reduction in colony numbers seen may be a reflection of progenitor cell death rather than an inhibition of cell division.

Previous work in BMT recipients failed to show any correlation between LAK activity and the age of the patient,\textsuperscript{18} but may increase the risk of leukemic relapse. Bone Marrow Transplantation for the treatment of recipients of T-cell depleted marrow with recipients of conventional marrow grafts. Blood 73:1340, 1989

Our data also fail to demonstrate any association between the presence or absence of in vitro GVL activity and time from BMT to assay, GVHD prophylaxis, or the presence of acute or chronic GVHD. It could be argued that the lack of correlation results from culturing PBMC in supraphysiologic concentrations of IL-2, which produces cells with activities that have little in vivo relevance. On the other hand, it is possible that in vivo this could provide a mechanism for enhancing MHC-unrestricted GVL activity. As the CD3\textsuperscript{+} LAK cells kill the leukemia cells, these effectors could in theory be exploited to reduce leukemia relapse without the morbidity and mortality of GVHD.

In conclusion, even if LAK cells do have some inhibitory activity that prevents normal bone marrow cell growth, the in vitro data presented here indicate that the susceptibility of donor marrow is considerably less than recipient CML progenitors. This might allow IL-2/LAK cell therapy after BMT to enhance antileukemic activity without compromising engraftment. In the context of syngeneic or autologous BMT, GVL activity could perhaps be generated without the risk of initiating or enhancing GVHD. However, in allogeneic BMT the use of LAK cell therapy with in vivo IL-2 could promote the growth of donor alloreactive T cells with resultant GVHD. Animal data suggest that this might be avoided by depleting the donor marrow of T cells pretransplant.\textsuperscript{15} Whether this in vitro GVL activity can be exploited in the clinic is at present unclear, but preliminary studies in vivo using IL-2 postautografting indicate that LAK activity is indeed enhanced.\textsuperscript{19}

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