Assay of Lymphokine-Activated Killer Activity Generated From Bone Marrow Cells of Children With Acute Lymphoblastic Leukemia

By Muxiang Zhou, Harry W. Findley, Jr, Rogena Davis, and Abdelsalam H. Ragab

We recently reported that low molecular weight B-cell growth factor (LMW-BCGF) plus recombinant interleukin-2 (rIL-2) synergistically induced lymphokine-activated killer (LAK) activity from the bone marrow (BM) cells of children with acute lymphoblastic leukemia (ALL). The kinetics of cell growth, antigenic phenotype, and lytic activity of the generated effector cells were further analyzed in this study. BM cells from ALL patients with active disease and in complete remission (CR) were cultured with a combination of LMW-BCGF and rIL-2. Monoclonal antibodies (anti-CD3 and anti-Leu 19) and immunomagnetic beads were used to separate LAK cells into three subsets: CD3+/Leu 19+, CD3+/Leu 19+, and CD3-/Leu 19+. Cytotoxicity assays with different subsets were performed versus K562, Raji, and autologous leukemic cells, using a 3-hour 51Cr release test. There was a significant cell expansion of 54-fold (mean value) for CD3+ cells and 15-fold for Leu 19+ cells in culture with LMW-BCGF plus rIL-2 for 7 to 14 days, whereas no cell expansion was observed in culture with rIL-2 alone. Although NK activity (K562) was generated from leukemia BM cells in culture with rIL-2 alone, it is only about one third of that generated in culture with rIL-2 plus LMW-BCGF. Analysis of lytic activity of cells generated in the latter cultures demonstrated that CD3+/Leu 19+ cells expressed highest lytic activity against NK-sensitive K562 cells as well as against NK-resistant Raji cells. CD3+/Leu 19+ cells showed weak cytotoxicity, and CD3-/Leu 19− cells mediated only minimal cytotoxic activity. Also, lytic activity of CD3−/Leu 19+ cells against autologous leukemic blasts was noted in patients with active disease. Our results demonstrate that LAK activity generated from BM cells by LMW-BCGF and rIL2 is mediated mainly by two types of Leu 19+ cells: CD3+/Leu 19+ NK cells and CD3−/Leu 19+ T cells. Although CD3+ T cells (both Leu 19+ and Leu 19−) mediated less antitumor cytotoxicity than CD3+/Leu 19+ cells, the former cells were the major expanding cell population in culture with LMW-BCGF and rIL-2. The new culture system may be effective in generation of cells with LAK activity for therapeutic use.

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ELLS WITH lymphokine-activated killer (LAK) activity can be generated from peripheral blood lymphocytes (PBL) in the presence of interleukin-2 (IL-2). These cells are able to lyse a wide variety of target cells, including natural killer (NK)-resistant tumor cells in a non-major histocompatibility complex (MHC) restricted fashion.1 LAK activity in PBL is predominately mediated by CD3−/Leu 19+ (CD56) cells,2 which is evidence that LAK activity is primarily an expansion and activation of NK cells. However, there is also evidence that LAK activity can be generated from T lymphocytes derived both from peripheral blood and lymphoid tissues devoid of NK cells, such as the thymus.3−13 Effector cells with LAK activity generated from T lymphocytes are NKH-1 (Leu 19+ T cells).3,5,11,13 In addition, bone marrow (BM) cells that harbor T and NK precursors are able to generate cytolytic activity,14,17 but the characteristics of the effector cells are unclear.

Although both NK and T cells are able to generate LAK activity, their activation requirements are different. NK cells are activated merely by IL-2, and accessory cells or additional factors are apparently not required.4,15 Conversely, purified peripheral blood T cells do not develop LAK function when cultured with IL-2 alone,8,18 having a strict requirement for accessory cells for the development of lytic activity.11,18,19 Many studies have also shown that the generation of cytotoxic T lymphocytes (CTL) from resting BM precursors or thymocytes requires both IL-2 and a soluble factor that is present in the supernatants of Con-A or PHA-activated T cells, and is distinct from IL-2.20−22 Takatsu et al23 demonstrated that IL-5/BCGF-2, a T-cell-derived B-cell differentiation factor, also induced CTL in the presence of IL-2.

We recently reported that LMW-BCGF (12 Kd), purified from lectin-stimulated peripheral blood mononuclear cell-conditioned medium,24 plus recombinant IL-2 (rIL-2) synergistically induced the expansion of CD3+ cells accompanied by LAK activity from the BM cells of children with acute lymphoblastic leukemia (ALL).25 To investigate whether this LAK activity was mediated by the expanded CD3+ T cells or by CD3− NK cells that were simultaneously generated by the combined lymphokines, the antigenic phenotype and lytic activity of the generated cells were further analyzed in this study.

MATERIALS AND METHODS

Patient material. Ten pediatric patients (under 18 years of age) with non-T, non-B ALL who were treated at Emory University (Atlanta, GA) were included in this study. Informed consent was obtained according to institutional guidelines. Six patients had active disease (diagnosis N = 2, relapse N = 4), and four patients

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were in complete remission (CR). After informed consent was obtained, BM cells were aspirated from the iliac crest. The diagnosis of leukemia was based on the French-American-British cooperative group designation and the surface marker profiles of the leukemic blasts (as determined by standard methods of immunophenotyping and flow cytometry). Selected clinical and immunologic data of the patients entered into this study are summarized in Table 1.

Preparation of cells with LAK activity. BM mononuclear cells were isolated by centrifugation on Ficoll-Hypaque (1.077 g/mL). In all cases monocytes were removed by adherence to plastic Petri dishes in which BM cells were incubated at 37°C for 1 hour, and mature CD3+ T lymphocytes were depleted by an immunomagnetic separation procedure.25,26 CD3− cells (10^6/mL) were suspended in RPMI 1640 containing rIL-2 (100 U/mL) (Amgen, Thousand Oaks, CA) and/or low molecular weight B-cell growth factor (LMW-BCGF) (0.1 U/mL) (Cellular Product Inc, Buffalo, NY), and incubated in tissue culture flasks. Cells were counted every 2 to 4 days and refed with fresh medium, rIL-2, and/or LMW-BCGF. Cell concentration was readjusted to 10^6/mL. Cultures were kept in 5% CO₂ at 37°C. After 7 to 14 days, cells were harvested for surface-marker determination and separation.

Immunomagnetic bead separation. CD3+ cells and Leu 19+ cells were positively selected by use of immunomagnetic beads.27 Cells that expanded in BM culture with rIL-2 plus LMW-BCGF were divided into two parts and pelleted by centrifugation in two tubes. Cells in each were mixed with anti-CD3 and anti-Leu 19 antibodies, respectively. The mixture was incubated at 2 to 8°C for 30 minutes and washed twice. Cells treated with the primary antibodies were then incubated with immunomagnetic beads coated with sheep anti-mouse immunoglobulin G (Dynabeads M-450, Dynal AS, Oslo, Norway) for 30 minutes at 2 to 8°C. The rosette-positive fraction was washed 2 or 3 times by the above procedure. At this time, anti-Leu 19 antibody was added to CD3+ cells, and anti-CD3 antibody was added to Leu 19+ cells. The beads-to-target cell ratio was 20:1. Finally, three subsets of LAK cells (CD3+/Leu 19−, CD3+/Leu 19+, and CD3−/Leu 19+) were obtained. The purity of separated cells in the second step was usually over 90% by reanalysis.

Cytotoxicity assay for different subsets. A 3-hour ^51Cr release test was used to test the cytotoxicity of unseparated and separated effector cells after culture with rIL-2 plus LMW-BCGF. Targets were K562, Raji (Burkitt’s lymphoma), and autologous leukemic blasts that were stored in liquid nitrogen and thawed before labeling. For the labeling of the target cells, 100 μCi ^51Cr was incubated with 10^6 pelleted cells for 1 hour and then washed three times. Different effector-target ratios (E:T) were used. Cells were incubated in microculture wells in triplicate. After 3 hours of incubation, the supernatants were harvested and radioactivity was determined with a gamma counter. Cytotoxic activity was calculated by the following formula:

\[
\text{Experimental Release (CPM)} = \frac{\text{Maximum Release (CPM)} - \text{Spontaneous Release (CPM)}}{\text{Maximum Release (CPM)}} \times 100\%
\]

Lytic units (LU) were calculated according to the formula of Pross et al.28 One LU was defined as the number of cells needed to lyse 33% of the targets and calculated per 10^7 effector cells. LU per culture were calculated using the total number of cells at the end of culture.

RESULTS

Generation of cells with LAK activity from BM. BM mononuclear cells with less than 3% of CD3+ T cells were directly cultured, and those with more than 3% of T cells were cultured after depletion of mature (CD3+) T cells. A comparison of proportions of CD3+ and Leu 19+ cells from preculture and postculture with rIL-2 plus LMW-BCGF is shown in Table 1. All tested samples expressed increased percentages of CD3+ cells either in CR or in active disease. Leu 19+ cells, including both CD3+ and CD3− cells, increased in 9 of the 10 cases. The total number of cells in samples with leukemic blasts was not expanded at the end of

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Diagnosis</th>
<th>BM Blastic (%)</th>
<th>Phenotype (%)</th>
<th>Pre-culture</th>
<th>Post-culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>c-ALL</td>
<td>98</td>
<td>NT</td>
<td>CD10 CD19 CD3+ Leu19</td>
<td>CD3</td>
</tr>
<tr>
<td>2</td>
<td>N-ALL</td>
<td>83</td>
<td>89</td>
<td>5 78</td>
<td>0 2 1</td>
</tr>
<tr>
<td>3</td>
<td>N-ALL</td>
<td>79</td>
<td>84</td>
<td>0 75</td>
<td>3 2</td>
</tr>
<tr>
<td>4</td>
<td>N-ALL</td>
<td>86</td>
<td>92</td>
<td>0 93</td>
<td>2 1</td>
</tr>
<tr>
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<td>88</td>
<td>76</td>
<td>59 75</td>
<td>6 3</td>
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<tr>
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<td>42</td>
<td>49 81</td>
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<td>7</td>
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<td>NT NT</td>
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<td>ALL(CR)</td>
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<td>1 1</td>
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<tr>
<td>10</td>
<td>ALL(CR)</td>
<td>0</td>
<td>NT</td>
<td>NT NT</td>
<td>2 2</td>
</tr>
</tbody>
</table>

The immunologic surface markers of cells were detected by immunofluorescence and flow cytometry. T-depletion procedure was performed in patients 6, 7, 8, and 10 (more than 3% of CD3+ cells). Abbreviations: c-ALL, common ALL; N-ALL, null ALL; blast, lymphoblast; NT, not tested.

*Post−T-cell depletion.
culture, except for one case (patient 5) in which there was a threefold expansion of cells, and two- to fivefold expansion of cells was obtained in four samples in CR. However, there was a 54-fold (mean value) expansion of CD3+ cells in five tested cases with active disease, and a 15-fold expansion of Leu 19+ cells in 4 of 5 samples in culture with LMW-BCGF plus rIL-2 (Table 2). No cell expansion in either total cell number or number of T cells was observed in cultures with rIL-2 alone in these five cases (data not shown).

Lytic activity of unseparated cultured cells. Cells generated from five patients with active disease in culture with a combination of rIL-2 and LMW-BCGF were harvested directly on days 7 or 14 to determine their lytic activity against K562, Raji, and cryopreserved autologous blasts. All five cases expressed LAK activity against K562 and Raji cells, and four cases showed lytic activity against autologous leukemic blasts (Table 2).

To compare the cytolytic activity of cells in culture with rIL-2 plus LMW-BCGF with those in culture with rIL-2 alone, cells from three samples of each culture were harvested on day 7, and their cytotoxicity against K562 assayed. In addition to the absence of cell expansion in culture with rIL-2 alone, the cytotoxicity of these cells was much lower than that of cells in culture with rIL-2 plus LMW-BCGF. No lytic activity against K562 was detected in culture with LMW-BCGF alone (Fig 1).

Lytic activity of different subsets of generated cells. Generated cells in culture with rIL-2 plus LMW-BCGF from four cases (two with active disease, two in CR) were chosen for separation. Immunobeads and anti-CD3 and anti-Leu 19 antibodies were used in a two-step separation procedure to isolate these cells in three groups. Table 3 shows the cytotoxicity of three subsets against K562 and Raji on a per-cell basis. CD3−/Leu 19+ cells expressed the highest lytic activity against NK-sensitive K562 cells as well as NK-resistant Raji cells. CD3+/Leu 19+ cells showed median cytotoxicity, and CD3+/Leu 19− cells mediated only minimal cytotoxic activity. LAK activity against both K562 and Raji in each subset of cells from patients in CR was higher than that of those with active disease, as shown in Table 3. CD3−/Leu 19+ cells in two patients with active disease expressed lytic activity against autologous leukemic blasts, while no cytotoxicity was detected in either CD3+/Leu 19− or CD3+/Leu 19+ subsets.

In addition to a comparison of different subsets of cells, the cytotoxicity of fresh BM cells and unseparated cells that had been generated from the same sample were also tested in patient 8. As shown in Fig 2, minimal cytotoxicity against K562 and Raji was detected in fresh BM. CD3−/Leu 19+ cells isolated from the same samples express higher lytic activity than the cytotoxicity mediated by unseparated cells.

**Table 2. Expansion and Lytic Activity of Cells From BM Cells of ALL Patients in Culture With LMW-BCGF (0.1 U/mL) Plus rIL-2 (100 U/mL) for 7 to 14 Days**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Days in Culture</th>
<th>CD3+</th>
<th>Leu19+</th>
<th>K562</th>
<th>Raji</th>
<th>ALB</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>72</td>
<td>12</td>
<td>141</td>
<td>117</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>44</td>
<td>0</td>
<td>187</td>
<td>94</td>
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<td>10</td>
<td>60</td>
<td>20</td>
<td>70</td>
<td>62</td>
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<td>9</td>
<td>54</td>
<td>15</td>
<td>98</td>
<td>43</td>
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<td>5</td>
<td>14</td>
<td>42</td>
<td>26</td>
<td>243</td>
<td>196</td>
<td>63</td>
</tr>
</tbody>
</table>

Expansion of CD3+ and Leu19+ cells was calculated based on the percentage of positive cells and total cell number in pre- and postcultures. Cytotoxicity is expressed as lytic units of specific 51Cr release from labeled target cells, using a 3-hour assay. Abbreviation: ALB, autologous leukemic blasts.

**DISCUSSION**

NK cells lyse a broad spectrum of tumor-cell lines and virus-infected cells in a non-MHC restricted manner, while CTL have fine antigen specificity and lytic function is MHC-restricted. Both NK and some T cells in the peripheral blood are able to develop LAK activity. Unlike their progenitors, cells with LAK activity have an ability to lyse a wide variety of NK-resistant targets, including cell lines and fresh tumors without MHC-restriction. Further study of the immunologic surface markers of cells with LAK activity demonstrate that not all cells activated by IL-2 have this function; only CD3−/Leu 19+ NK cells and CD3+/Leu 19+ T cells can mediate appreciable lytic activity.
different. NK cells are activated merely by IL-2, while T cells need accessory cells or additional factors for their induction.\textsuperscript{4,8,11}\textsuperscript{19}

Cytotoxicity against various types of neoplasias, including leukemia and solid tumors, can be generated from BM cells after culture with IL-2.\textsuperscript{12-17} These cells have been characterized as NK cells, not T cells.\textsuperscript{19} In this report we used LMW-BCGF with IL-2 to generate cells with antileukemic activity from the BM cells of children with ALL (non-T, non-B). We previously found that LMW-BCGF and rIL-2 together are able to generate CD3\textsuperscript{+} cytotoxic T cells with LAK activity from CD3\textsuperscript{-} precursors in BM.\textsuperscript{25} Purified LMW-BCGF did not contain detectable amounts of IL-1, IL-2, IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), or gamma interferon (\(\gamma\)-IFN) as determined by previously described standard bioassays, but was able to induce proliferation of activated normal B cells.\textsuperscript{26,31} The mechanism by which LMW-BCGF and IL-2 together are able to develop LAK activity from BM cells devoid of mature T cells and NK cells is unclear. The action of LMW-BCGF in inducing CTL proliferation and maturation is similar to IL-5, which induces expression of IL-2 receptors on CTL precursors and stimulates their growth in the presence of IL-2.\textsuperscript{27} This inducing action is different from that of IL-4 and IL-6, which are able to generate CTL activity from mature peripheral blood T cells rather than from immature T cells.\textsuperscript{32-34} It is also different from that of anti-CD3 antibody, which, with IL-2, enhances expansion of cells with LAK activity from mature lymphocytes in the peripheral blood in long-term culture. In the latter case, it is possible that anti-CD3 works via activation of the CD3/T-cell receptor complex.\textsuperscript{35,36} There is no evidence that LMW-BCGF induces expression of the T cell receptor on immature T cells, but it does induce expression of the IL-2 receptor on these cells.\textsuperscript{35} In this study we investigated the antigenic phenotype and lytic characteristics of LAK cells generated from the BM cells of children with ALL after culture of these cells with LMW-BCGF and rIL-2. We found that cultures from all patients studied generated primarily CD3\textsuperscript{+}/Leu 19\textsuperscript{-} cells. Smaller numbers of CD3\textsuperscript{+}/Leu 19\textsuperscript{+} and CD3\textsuperscript{-}/Leu 19\textsuperscript{+} cells were also induced. We also found that cytotoxicity against fresh leukemic cells and cell lines is mediated mainly by Leu 19\textsuperscript{+}/CD3\textsuperscript{-} NK cells. A lower level of cytotoxicity is expressed by Leu 19\textsuperscript{+}/CD3\textsuperscript{+} cells, which have been termed "T cells with NK activity."\textsuperscript{39} Our results are similar to those of Phillips and Lanier,\textsuperscript{4} who found that LAK activity generated from PB cells was primarily due to activated CD3\textsuperscript{-}/Leu 19\textsuperscript{+} NK cells and secondarily to CD3\textsuperscript{+}/Leu 19\textsuperscript{+} T cells. Our results are also consistent with those of Lotzova and Savary.\textsuperscript{13} They found that incubation of normal BM cells with IL-2 generated LAK activity, and that the effector cells were CD3\textsuperscript{-}/Leu 19\textsuperscript{+} NK cells, with only a minor contribution from CD3\textsuperscript{+} cells. In our study, BM cells were incubated with both IL-2 and LMW-BCGF, and we noted several effects from the addition of LMW-BCGF to the cultures: (1) A rapid expansion of CD3\textsuperscript{+} cells, which became the major cell type by 10 to 14 days. (2) A subpopulation of CD3\textsuperscript{+} cells expressing the Leu 19 marker showed LAK activity. Although the activity per cell of the CD3\textsuperscript{+} cells (both Leu 19\textsuperscript{+} and Leu 19\textsuperscript{-}) was lower than that of the CD3\textsuperscript{-}/Leu 19\textsuperscript{+} cells, the total number of the former cells was expanded a mean value of 54-fold in the cultures with LMW-BCGF and IL-2, compared with less than 15-fold for the latter cells. (3) We generated this LAK activity from the BM cells of leukemic patients with active disease, and this activity was comparable with that obtained from BM cells of an ALL patient in CR. Another interesting observation is that CD3\textsuperscript{-}/Leu 19\textsuperscript{+} cells in these cultures require the presence of CD3\textsuperscript{+} cells for proliferation; isolated CD3\textsuperscript{-}/Leu 19\textsuperscript{+} cells are unable to grow with LMW-BCGF and rIL-2, although they are able to do so in the presence of CD3\textsuperscript{+} cells (unpublished observa-

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**Table 3. Cytotoxicity of Three Subsets of Cells With LAK Activity Generated in Culture With rIL-2 Plus LMW-BCGF for 7 to 14 Days Against K562, Raji, and Autologous Leukemic Blasts Using BM Cells From Four Patients**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Disease</th>
<th>Period (weeks)</th>
<th>E: T Ratio</th>
<th>CD3\textsuperscript{+}/Leu19\textsuperscript{-}</th>
<th>CD3\textsuperscript{+}/Leu19\textsuperscript{+}</th>
<th>CD3\textsuperscript{-}/Leu19\textsuperscript{+}</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>AD</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>16</td>
<td>9</td>
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<tr>
<td>5</td>
<td>AD</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>8</td>
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<td>8</td>
<td>CR</td>
<td>9</td>
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<td>CR</td>
<td>6</td>
<td>8</td>
<td>NT</td>
<td>38</td>
<td>37</td>
</tr>
</tbody>
</table>

Cytotoxicity is expressed as percentage of specific \(^{31}Cr\) release from labeled target cells in a 20:1 E:T ratio. In these experiments the purity of the separated cells was over 90%.

Abbreviations: E, effector; T, target; AD, active disease; ALB, autologous leukemic blasts.
tion, October 1988). This finding seems contrary to Silvennoinen et al's report that NK cells were activated merely by IL-2. It is consistent with previous reports that to maintain NK function in clones of NK cells, these cells required the continuous presence of IL-2 and feeder cell monolayers derived from cells such as K562 or lymphoblastoid cell lines. It is possible that the proliferation of CD3+/Leu 19+ cells requires other lymphokines in addition to LMW-BCGF and IL-2, and these additional factors are secreted by CD3+ cells.

In conclusion, LMW-BCGF plus IL-2 can generate three subsets of lymphocytes: CD3+/Leu 19+, CD3+/Leu 19+, and CD3+/Leu 19− from BM cells. Of these, CD3+/Leu 19+ cells mediated the highest LAK activity but were unable to proliferate without CD3+ cells. CD3+/Leu 19+ cells had less LAK activity, and CD3+/Leu 19− cells had the least LAK activity. This finding may be clinically relevant. Because these cells with LAK activity have been shown to mediate antileukemic activity, activation of autologous BM cells with LAK activity using lymphokines may improve not only conventional chemotherapy salvage treatment, but also BM transplantation results.

REFERENCES


