Distinct Characteristics of Lymphokine-Activated Killer (LAK) Cells Derived From Patients With B-Cell Chronic Lymphocytic Leukemia (B-CLL). A Factor in B-CLL Serum Promotes Natural Killer Cell-Like LAK Cell Growth

By Frances Santiago-Schwarz, Catherine Panagiotopoulos, Arthur Sawitsky, and Kanti R. Rai

We show that lymphokine-activated killer (LAK) cell precursors derived from patients with B-cell chronic lymphocytic leukemia (B-CLL) and cultured in the presence of recombinant interleukin-2 and normal human serum (NHS), develop into primarily NK cell-like (CD57+) LAK cells, whereas identically prepared LAK cell precursors from normal subjects develop into mainly T cell-like (CD3+, CD8+) LAK cells. B-CLL LAK cells exhibited greater proliferative capacity than did normal LAK cells. When normal LAK cells were grown in B-CLL serum instead of NHS, their proliferation increased; NK cell levels also increased to those found in B-CLL LAK cells, suggesting that B-CLL serum contains a factor that promotes NK cell-like growth. LAK cells derived from normal or B-CLL patients demonstrated similar lytic activity toward K562 and Raji cells. Growth in B-CLL serum did not reduce their lytic potential. Thus, the altered phenotype and growth exhibited by B-CLL LAK cells and normal LAK cells grown in B-CLL serum does not lead to abnormalities in their cytolytic functions. We propose instead that the predominance of NK-like cells in B-CLL LAK cell populations and the presence of an NK cell-like growth factor in B-CLL serum reflect abnormalities related to NK cell-mediated B-cell regulation; ie, either inhibition of normal B-cell growth and/or growth stimulation of the leukemic clone in B-CLL.

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EFFECCTOR AND target cells are equally important participants in natural cytotoxicity.1-5 In certain diseases, specific abnormalities in either cell type have been identified or suggested.6-10 With respect to B-cell chronic lymphocytic leukemia (B-CLL), some impaired natural killer (NK) cell functions have been attributed to decreases in the number of azurophilic granules present within NK cells.11 Interleukin-2 (IL-2) increases the number of lytic granules to normal levels and reverses defective natural cytotoxicity in B-CLL, as demonstrated by in vitro lysis of K562 target cells.12 Notwithstanding, there is little or no lysis of leukemic B-CLL targets by lymphokine-activated killer (LAK) cells.13 It has been speculated that tumor cells escape lysis because of their relative immaturity, and efforts to augment lysis have included pretreating the tumor targets with agents that induce differentiation or proliferation.14,15

In this study, rather than focusing on the leukemic cell as the target, we investigate whether other distinctions exist in the LAK cell populations generated from B-CLL patients (B-CLL LAK) that may alter their effector functions. We question whether B-CLL LAK cell precursors exhibit differences in proliferation, phenotype, and lytic function when compared with normal LAK cells. In addition, we investigate whether B-CLL serum, which contains abnormally high levels of immune modulators such as soluble IL-2 receptors (sIL-2R), affects LAK cell development and function. Our results illustrate inherent differences between normal and B-CLL LAK cells that may reflect peculiarities in their B-cell regulatory functions rather than in their cytolytic functions. Additionally, we show that B-CLL serum contains a component that is a potent inducer of NK-like LAK cell growth and differentiation.

MATERIALS AND METHODS

Study population. Heparinized blood was collected from a total of 17 B-CLL patients for cellular studies. Of these, 10 were studied on multiple occasions (2 to 4) and 11 were male. Rai classification of these patients was as follows: stage 0, two patients; stage I, five patients; stage II, six patients; stages III and IV, four patients. Serum samples were obtained from 25 B-CLL patients, of which 16 were male. Three of the patients were in Rai stage 0, eight in stage I, seven in stage II, two in stage III, and five in stage IV. A total of nine normal individuals were studied; of these, four were studied on multiple occasions (2 to 4) and four were male.

Mononuclear cell (MNC) isolation. Heparinized blood from normal individuals or B-CLL patients was diluted 1:1 with RPMI 1640 (GIBCO Lab, Grand Island, NY) and centrifuged through a pyrogen-less lymphoprep gradient (Nyegaard, Norway) for mononuclear cell isolation. The resulting MNC were washed twice and placed on two consecutive nylon wool (NW) columns for the isolation of the nonadherent LAK cell precursors.16

LAK cell generation. NW nonadherent cells were reseeded at a concentration of 1 x 10⁶ cells/mL in RPMI 1640 containing 2 mmol/L L-glutamine, 10 mmol/L HEPES, 50 IU/mL penicillin, 50 µg/mL streptomycin, 0.25 µg/mL amphotericin B, 200 U/mL IL-2 (generously provided by Dr Daniel Levitt, Hoffman-La Roche, Nutley, NJ), and either pooled normal human serum (NHS) (GIBCO Lab) or B-CLL serum (prepared from patients with B-CLL) at a final concentration of 10%. The cells were then placed in a humidified 5% CO₂ incubator at 37°C with no subsequent feeding during the culture period. The decision to use IL-2 at 200 U/mL and 10% serum was based on prior dose-response experiments that determined these as optimal doses. IL-2 was tested at 20, 200, and 2,000 U/mL, and 200 U/mL was found to induce maximal LAK cell proliferation and cytolytic potential (as detailed below). Serum was similarly tested, at 5% and 10%, concentrations commonly used in the preparation of LAK cells. Comparison between a particular LAK cell preparation grown in NHS and B-CLL serum was always concurrent.

From the Division of Hematology/Oncology, Long Island Jewish Medical Center, New Hyde Park, NY.

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Address reprint requests to Frances Santiago-Schwarz, PhD, Rheumatology Research, Winthrop-University Hospital, Mineola, NY 11501.

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**LAK cell proliferation.** Nonadherent cells placed in culture for LAK cell generation were analyzed for proliferation after 4, 8, and 14 days by the uptake of \(^{3}H\) thymidine, and by manual counts using a hemacytometer. Viability was assessed by trypan blue dye exclusion. For thymidine uptake, 0.5 \(\mu\)Ci of \(^{3}H\) thymidine (specific activity 25 Ci/mmol/L, Amersham, Arlington, IL) was added to 100 \(\mu\)L of cells in microtiter plates. After 5 hours, cells were collected onto glass fiber filters using an automated sample harvester and counted in a liquid scintillation counter. Results are presented as the mean of triplicate counts.

**LAK cell cytotoxicity.** The cytotoxic capacity of the LAK cells was determined between days 7 and 12 using a standard chromium release assay. Target K562 (NK-sensitive) or Raji (NK-resistant) cells (greater than 80% viable) were incubated with 100 \(\mu\)Ci \(^{51}Cr/10^7\) cells/mL (Dupont, New England Nuclear, Wilmington, DE) for 1.5 hours at 37°C. Target cells were then washed twice in RPMI 1640, adjusted to 5 \(\times\) 10^4 cells/well, and added to round bottom 96-well microtiter plates at 5 \(\times\) 10^4 cells/well. Effector cells were then added at the ratios specified in the text. Results, expressed as percent-specific cytotoxicity, represent the mean of triplicate samples and were calculated as follows: 

\[
\% \text{ specific cytotoxicity} = \left( \frac{E - S}{T - S} \right) \times 100,\text{ where } E = \text{mean cpm obtained in the presence of effector cells}, \ S = \text{spontaneous release}, \text{ and } T = \text{total release, mean cpm obtained as a result of complete cell lysis with 1% NP40 in double distilled water.}
\]

**LAK cell surface marker analysis.** Reactivity was detected by immunofluorescence using multiwell slides (Carlson Scientific, Peotone, IL) as previously described. Monoclonal antibodies (MoAbs) toward CD57 (HNK-1, leu 7), CD3, CD4, and CD8 were obtained from the American Tissue Culture Collection (Rockville, MD); MoAb toward CD16 (leu 11b) was obtained from Becton Dickinson (Mountain View, CA); and toward CD56 (NKH-1) from Coulter Immunology (Hialeah, FL). The corresponding isotypic controls (Coulter Immunology) were compared in each assay and always resulted in less than 5% reactivity.

**Detection of sIL-2R in B-CLL sera.** Sera from normal individuals and B-CLL patients was collected and stored at -20°C, and analyzed for sIL-2R levels using a multistep enzyme immunoassay that uses two MoAbs with specificities toward different IL-2R epitopes (T Cell Sciences, Cambridge, MA).

**Statistics.** Where indicated, data was analyzed using Student t-tests or Mann-Whitney tests.

**RESULTS**

**Growth of LAK cell precursors in B-CLL or normal human serum.** Figure 1 demonstrates the proliferative capacity of LAK cell precursors derived from B-CLL patients and grown in either NHS, autologous B-CLL serum, or allogeneic B-CLL serum, as detected by the uptake of \(^{3}H\) thymidine. Peak proliferation of leukemia-derived LAK cells occurred around day 8, irrespective of serum source. Although LAK cell precursors grown in B-CLL serum exhibited greater uptake of \(^{3}H\) thymidine, this increase was not statistically significant (t-test, \(P > .05\)). Manual cell counts using a hemacytometer also showed cell proliferation, but no differences in cell numbers between the leukemia-derived LAK cell cultures (data not shown). Viability of the B-CLL LAK cells, as assessed by trypan blue dye exclusion, was not affected by the serum source, and during peak proliferation viability was greater than 80%.

Peak proliferation of normal LAK cells also occurred around day 8 (Fig 2); however, the proliferative capacity of these cells was much lower than that exhibited by B-CLL LAK cells (Fig 1). Growth of normal LAK cells in B-CLL serum increased proliferation as measured by \(^{3}H\) thymidine uptake (t-test, \(P = .035\)). Manual cell counts also showed that culture of normal LAK cells in B-CLL serum induced almost a twofold increase in total cell content (data not shown).

**Cell surface analysis of fresh nonadherent cells.** The surface antigen profile of fresh normal and leukemia-derived nonadherent cells containing the LAK cell precursors is compared in Table 1. The most notable distinctions are the increased numbers of CD57 and CD8 positive cells, and the low CD4/8 ratio in the nonadherent B-CLL cells. These data are consistent with previous studies concerning the non-B lymphocyte cell content in B-CLL, and reflect the predomi-

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Fig 1. \(^{3}H\) thymidine incorporation in nonadherent LAK cell cultures obtained from patients with B-CLL (n = 9 to 12) and grown in either pooled NHS or B-CLL serum, and IL-2. (●), NHS; (○), autologous B-CLL serum; (□), allogeneic B-CLL serum. Results represent the mean ± SE.

Fig 2. \(^{3}H\) thymidine incorporation in nonadherent LAK cell cultures obtained from normal individuals (n = 11 to 14) and grown in either pooled NHS or B-CLL serum. (●), NHS; (○), B-CLL serum. Results represent the mean ± SE.
B-CLL serum promotes NK cell-like growth

Table 1. Surface Markers of Fresh Nonadherent Cells

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<tr>
<th>Cluster</th>
<th>Normal</th>
<th>B-CLL</th>
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<tbody>
<tr>
<td>CD57</td>
<td>15.0 ± 4.5</td>
<td>42.0 ± 5.0</td>
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<tr>
<td>CD56</td>
<td>6.8 ± 0.5</td>
<td>4.3 ± 1.0</td>
</tr>
<tr>
<td>CD16</td>
<td>11.0 ± 2.3</td>
<td>11.6 ± 3.0</td>
</tr>
<tr>
<td>CD3</td>
<td>89.0 ± 2.3</td>
<td>82.0 ± 6.0</td>
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<tr>
<td>CD8</td>
<td>38.0 ± 5.0</td>
<td>63.0 ± 8.0</td>
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<tr>
<td>CD4</td>
<td>77.0 ± 6.0</td>
<td>52.0 ± 4.0</td>
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</table>

NW nonadherent cells were obtained as described in Materials and Methods, and adjusted to 1 x 10⁶ cells for immunofluorescence cell surface analysis. The values are expressed as percent of cells and represent the mean ± SE of four to seven experiments.

Table 1. Surface Markers of Fresh Nonadherent Cells

Nw nonadherent cells were obtained as described in Materials and Methods, and adjusted to 1 x 10⁶ cells for immunofluorescence cell surface analysis. The values are expressed as percent of cells and represent the mean ± SE of four to seven experiments.

Table 1. Surface Markers of Fresh Nonadherent Cells

Cell surface analysis of LAK cells grown in B-CLL or normal serum. The LAK cell-associated antigen distribution on day 8 leukemia-derived LAK cells cultured in normal, autologous, or allogeneic B-CLL sera showed a predominance of CD57⁺ CD8⁺ cells (Fig 3). The serum source in which the cells were grown in did not drastically alter this distribution. LAK cells derived from normal donors and grown in NHS were primarily T cell-like (CD3⁺ CD8⁺) with few CD57⁺ cells present (Fig 3). The difference between CD3 and CD57 expression on the normal and leukemia-derived LAK cells was statistically significant (Mann-Whitney tests; CD3, P < .05; CD57, P < .01).

However, growth of normal LAK cells in B-CLL serum resulted in a greater percentage of CD57⁺ cells and some increase in CD8⁺ cells (Fig 3). Moreover, the expression of class II antigens (detected with MoAb L243) increased from 25% to 43% when normal LAK cells were grown in B-CLL serum (data not shown).

NK cell-associated antigen content in LAK cell populations. To accurately assess the NK cell content in normal LAK cells grown in NHS or B-CLL serum, MoAbs specific for three distinct NK-cell-associated antigens (CD56, CD57, and CD16) were used. The data in Fig 4 illustrate the absolute NK cell content as determined by reactivity with these MoAbs after 8 days in culture. Normal LAK cells grown in NHS contained few CD57⁺, CD56⁺, or CD16⁺ cells. The absolute number of cells in the LAK cultures expressing these NK-cell-associated markers increased greater than threefold when cells were grown in B-CLL serum. After 2 weeks of in vitro culture, normal LAK cells grown in NHS were still primarily T cell-like (CD3⁺, CD8⁺), and normal LAK cells grown in B-CLL serum were still primarily NK cell-like (CD16⁺ CD56⁺ CD57⁺) (data not shown).

Analysis of NK-cell-associated markers on leukemia-derived LAK cells grown in B-CLL serum also showed increases in the absolute number of NK-like cells after 8 days.
in culture, as reflected by increases in NK-cell–associated antigen expression, but the absolute number of CD57+ cells was greater (Fig 4). Leukemia-derived NK-like LAK cells grown in NHS retained their original CD57+ CD56− CD16− cell surface profile.

There was no proliferation of B cells in any of the LAK cell cultures (less than 1% reactivity with MoAb CD20), excluding the possible effect of B-cell–derived factors such as soluble IL-2 receptors (especially in the leukemia-derived cultures) on LAK cell generation.

Cytotoxicity of LAK cells grown in normal or leukemic serum. In Table 2 we compare the lytic activity of leukemia and normal-derived LAK cells that were grown in either NHS or B-CLL serum toward K562 and Raji cell targets. The growth of normal or leukemia-derived LAK cells in B-CLL serum does not decrease their lytic potential toward either target. On the contrary, LAK cells obtained from both normal and leukemic patients grown in B-CLL serum demonstrated slightly greater lysis toward Raji cell targets.

sIL-2R levels in B-CLL sera. When sera from 22 B-CLL patients were analyzed for sIL-2R levels, only one exhibited normal levels (less than 500 U/mL) of the receptor. Of the remaining 21 patients, 6 exhibited an average of 1,200 U/mL and 15 exhibited greater than 1,600 U/mL of sIL-2R.

**DISCUSSION**

We demonstrate that in the presence of NHS and IL-2, B-CLL–derived LAK cell precursors develop into cells expressing primarily an NK cell-like phenotype, whereas normal LAK cell precursors under identical conditions develop into LAK cells expressing primarily a T cell-like phenotype. Growth in B-CLL serum, instead of NHS, affected the phenotypic outcome of normal LAK cells by dramatically increasing the NK cell content to levels similar to those found in the leukemia-derived LAK cell populations. The increase in NK cell content was reflected by increases in the absolute number of CD56+, CD57+, and CD16+ antigens. Increases in class II molecules, which are indicative of cell activation/proliferation, and CD8 also occurred when normal LAK cells were grown in B-CLL serum. These results imply that there is a factor in B-CLL serum, which in the presence of rIL-2, induces NK cell-like growth. This factor was present in all B-CLL sera tested, regardless of the patient’s stage, and was active on all of the LAK cells prepared from normal individuals, indicating that it is not MHC-restricted. In addition, the factor is heat stable, since incubation at 56°C for 1 hour did not alter its ability to induce the NK cell phenotype (data not shown).

Most of the normal-derived NK-like LAK cells grown in B-CLL serum exhibited markers characteristic of mature NK cells (CD56, CD16), although a substantial number did exhibit an immature NK cell phenotype (CD57+ CD56− CD16−) (Fig 4). This was in marked contrast to normal LAK cells grown in NHS, in which the number of cells expressing all three markers was similar. When leukemia-derived LAK cells were grown in B-CLL serum, increases in mature NK cells were also noted, but the immature phenotype was still predominant (Fig 4).

Because of the predominance of the immature NK cell phenotype, the dramatic increase in the absolute number of CD57+ NK cells, and the increase in cell growth of the normal cells grown in leukemic serum, we propose that the NK cell growth factor in B-CLL serum is primarily an inducer of immature NK cells. Subsequent NK cell maturation may involve other factors such as IL-2 or CD16, as previously suggested. Clearly, further studies are required before these differentiation events can be accurately described. Because relatively little is known about NK cell maturation, a factor such as that described in these studies may be crucial in providing information regarding NK lineage and function.

We showed, as others have, that LAK cells derived from B-CLL patients are capable of normal cytolytic activity toward K562 and Raji cell targets. This has been attributed to increases in mature NK cells (CD16+, CD56−) in the B-CLL LAK cell populations, which exhibit greater lytic potential than the predominantly immature (CD57+ CD56− CD16−) NK cells present in freshly isolated B-CLL cells. Generating normal or leukemia-derived LAK cells in B-CLL serum did not alter their cytolytic effector functions (Table 2). This may be explained by the presence of a critical number of recycling mature NK-like cells in the distinct LAK cell populations.

We propose that the abnormal increase in NK-like LAK cells in B-CLL reflects abnormalities in noncytolytic functions of NK cells, such as the regulation of B-cell differentiation or stem cell proliferation. Indeed, in recent studies it has been demonstrated that NK cells derived from patients with B-CLL excessively inhibit normal B-cell differentiation, which may explain the low number of normal B cells in

<table>
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<th>Table 2. Lytic Activity of LAK Cell Populations</th>
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<td><strong>E:T ratios:</strong></td>
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<tr>
<td><strong>Serum:</strong></td>
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<tr>
<td>NHS</td>
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<td>CLL</td>
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<td><strong>Normal LAK v Raji</strong></td>
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<tr>
<td>NHS</td>
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<td>CLL</td>
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Results are expressed as percent cytotoxicity and represent the mean ± the SE of three to five experiments.
B-CLL. Another consequence of the increase in immature NK cell stems from recent studies which show that CD57⁺ CD16⁻ NK cells produce a B-cell growth factor on activation.²⁸ Such a growth factor may contribute to the proliferation of the leukemic B cells in B-CLL, as previously proposed by Kimby et al.²⁹

IL-2 receptors are present at abnormally high levels in the serum of patients with various malignancies, including B-CLL.¹⁷,²⁸,³⁰ In these instances high levels correlate with progressive disease. Although the mechanism by which high levels of sIL-2R contribute to pathogenesis is unclear, it is possible that such a factor would interfere with a variety of IL-2-dependent immune responses, such as T- and NK-cell-mediated natural cytotoxicity, as well as IL-2-dependent B-cell proliferation. Despite the excessively high levels of sIL-2R in the majority of B-CLL sera we tested, we could not establish an inhibitory role of leukemic sera in either in vitro LAK cell generation or cytolytic function. On the contrary, some B-CLL LAK cells grown in B-CLL serum with high sIL-2R levels exhibited even greater lytic activity toward K562 and Raji cells than did the parallel LAK cells grown in NHS (Table 2).

In summary, the observations reported here reflect the complexity inherent in B-CLL serum. The description of a novel component in B-CLL serum that promotes NK cell growth may not only be of benefit in establishing a model for normal NK cell differentiation, but also gives insight into how the abnormal increase in immature NK cells in B-CLL is achieved.

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