Low Molecular Weight B-Cell Growth Factor and Recombinant Interleukin-2 Are Together Able to Generate Cytotoxic T Lymphocytes With LAK Activity From the Bone Marrow Cells of Children With Acute Lymphoblastic Leukemia

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We are reporting here that low-mol wt B-cell growth factor (LMW-BCGF) and recombinant interleukin-2 (rIL-2) are together able to induce CD3+ cytotoxic T lymphocytes (CTL) with lymphokine-activated killer cell (LAK) activity from the bone marrow (BM) cells of children with acute lymphoblastic leukemia (ALL). Ficoll-Hypaque (FH)-separated BM cells were obtained from patients with active disease (at diagnosis N = 13, in relapse N = 15) and in complete remission (CR; N = 12). CD3+ cells were removed by Leu-4 antibody and immunobeads. Cells were cultured (10^5 cells/mL) in semisolid media with rIL-2 (100 μ/mL), LMW-BCGF (0.1 μ/mL), and the combination of rIL-2 plus LMW-BCGF, respectively, for seven to ten days. Pooled colonies were harvested for phenotyping. LMW-BCGF plus rIL-2 induced large numbers of CD3+ colonies from CD3− precursors. rIL-2 alone did not induce colony formation. In addition, cells were cultured in liquid media with LMW-BCGF, rIL-2, and the combination of LMW-BCGF plus rIL-2, respectively, for seven to 21 days. They were harvested for phenotyping, and cytotoxicity assays were performed *v* K562, Raji, and autologous leukemic cells. LMW-BCGF plus rIL-2 induced significant expansion of CD3+ cells from CD3− precursors, and these cells were activated to kill autologous leukemic cells in addition to Raji and K562 cell lines. LMW-BCGF or rIL-2 alone did not induce significant expansion or activation of cytotoxic CD3+ cells. Our hypothesis is that LMW-BCGF plus rIL-2 stimulates the proliferation and activation of CD3− precursors from the BM cells of children with acute leukemia to become CD3+ cells that have LAK activity. This finding may have therapeutic implications.

INTERLEUKIN-2 (IL-2) is a lymphokine necessary for the proliferation of human activated peripheral-blood T lymphocytes, but it alone does not induce cytotoxic T lymphocytes (CTL) from cytolytically inactive precursors. Proliferation of these precursors requires a soluble factor that is present in supernatants of concanavalin A (ConA) or phytohemagglutinin (PHA)-activated T cells.[1-6] This soluble factor is referred to as killer-helper factor (KHF),[6] previously known as T-cell replacing factor (TRF).[7,8] It induces CTL to develop from thymocyte precursors in the presence of IL-2. Harada et al[9] and Kinashi et al[10] demonstrated that TRF is identical to B-cell growth factor-2 (BCGF-2, also known as IL-5[11]) and acts on activated B cells as a B-cell differentiation factor.

Recently, additional human BCGFs have been purified from lectin-stimulated, peripheral-blood mononuclear cell-conditioned medium. LMW-BCGF[12] (12 Kd) and high mol wt (HMW) BCGF[13] (60 Kd) react not only with activated B cells[14,15] but also with neoplastic B cells.[16] Little is known about the action of these human BCGFs on T cells and T-cell precursors.

The aim of the present study was to evaluate the effects of purified human LMW-BCGF and rIL-2 on the growth of normal and neoplastic lymphocyte progenitors derived from the bone marrow (BM) of children with ALL in active disease and in complete remission (CR). We have used a clonogenic assay and cell culture in liquid medium to evaluate cell growth. Two-color immunofluorescence techniques and the ^{51}Cr release assay were also used to study the phenotype and cytotoxicity of the cultured cells.

MATERIALS AND METHODS

Patient material. After informed consent was obtained, 40 samples of BM cells were collected from children with ALL. Of these, 28 were from patients with active disease (diagnosis N = 13, relapse N = 15), and 12 were from patients in CR. Blast cells and mononuclear cells were isolated by centrifugation on Ficoll-Hypaque (FH; 1.077 g/mL), washed twice in phosphate-buffered saline (PBS), and resuspended at 10^6/mL in RPMI 1640 containing 15% fetal bovine serum (FBS). The cells were incubated on plastic petri dishes for one hour at 37°C. The nonadherent cells were obtained by gently washing the dishes.

T-cell depletion. An immunomagnetic separation procedure[17] for depletion of T cells from BM was used. Briefly, nonadherent mononuclear cells from the samples obtained from children in CR and from samples with more than 3% CD3+ cells were incubated with anti-CD3 (Leu-4) monoclonal anti-T-cell antibody for 30 minutes at 2°C to 8°C and washed twice. The cells treated with the primary antibody were then incubated with immunomagnetic beads coated with sheep antimouse IgG (Dynabeads M-450, Dynal AS, Norway) for 30 minutes at 2°C to 8°C. The immunobeads-to-target cell ratio was 40:1 for negative selection. Separation was performed by placing the culture tube in a specially designed magnet (Dynal) for one to two minutes. During this time the CD3+ cells (rosetted with immunomagnetic beads) were attracted to the wall of the test tube by the magnetic field. Nonrosetted cells suspended in the supernatant were harvested for use in the following tests.

Colony assay for BM progenitor cells. BM mononuclear cells obtained by gradient separation and depleted of adherent cells and CD3+ cells were assayed for in vitro colony growth. Cells (10^5/mL) were suspended in alpha-minimum essential medium (MEM) supplemented with 0.9% methylcellulose, 30% FBS, 1% MEM vitamin

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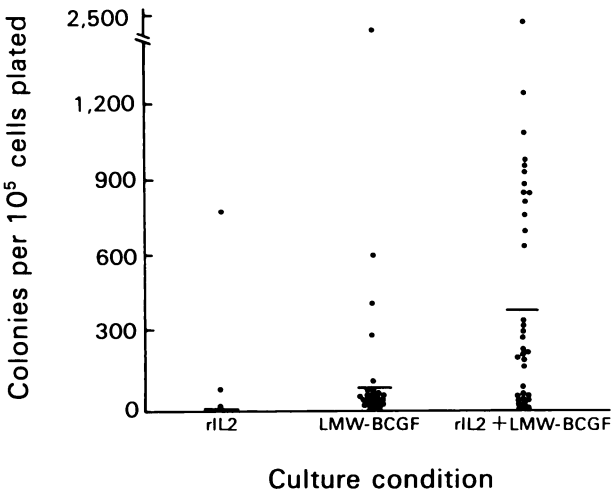

Fig 1. Number of colonies from BM cells of children with ALL in culture with rIL-2, LMW-BCGF, and a combination of rIL-2 and LMW-BCGF. Data are expressed as a mean value per 10^5 cells in replicate cultures. Each point represents data for individual patients. Patients with no colony growth (rIL-2 alone, N = 37; LMW-BCGF alone, N = 14; rIL-2 plus LMW-BCGF, N = 5) are not shown. Bar represents mean colony numbers for all patients in each group.

solution, 0.5% MEM amino acids solution, 2 mmol/L L-glutamine, and 50 μmol/L 2-mercaptoethanol. LMW-BCGF (0.1 μ/mL; Cellular Products Inc, Buffalo, NY) and/or rIL-2 (100 μ/mL; Amgen, CA) were also added. Replicate 1-mL cultures were plated in 35-mm petri dishes at 37°C in a humidified 5% CO_2 atmosphere. On day 7, colonies containing more than 20 cells were counted using an inverted-phase microscope.

Cells cultured in liquid medium. BM mononuclear cells obtained as previously described were suspended at 10^6/mL in RPMI 1640 containing LMW-BCGF (0.1 μ/mL) and/or rIL2 (500 μ/mL) and incubated in tissue-culture flasks. Cells were counted every two to four days and refed with fresh medium, LMW-BCGF, and rIL-2. The cell concentration was readjusted to 10^6/mL. Cultures were kept in 5% CO_2 at 37°C.

Surface marker analyses. Monoclonal antibody (MoAb) staining and flow cytometry were used for a surface-marker assay. A series of reagents (Becton Dickinson, San Jose, CA), including Leu-4 (CD3) and Leu-19 for T lineage; common acute lymphoblastic leukemia antigen (CALLA) (CD10), B4 (CD19), and HLA-DR for leukemic blasts; and Tac (anti–IL-2 receptor, CD25) were used to phenotype fresh BM cells and cultured cells. Cell-staining procedure and flow cytometry were carried out as described[18] by using a fluorescence-activated cell sorter (FACS, Becton Dickinson). A mouse monoclonal antihuman cell antibody IgG-fluorescein isothiocyanate (FITC) served as a control.

Cytotoxicity assay for expanded cells. A three-hour ^{51}Cr release test was used to test the cytotoxicity of expanded cells in culture with different growth factors. Cells that were cultured for ten to 14 days and whose viability was more than 80% (by trypan blue) were used as effectors. Targets were K562, Raji (Burkitts lymphoma), and autologous leukemic blasts. Unlike K562, Raji and autologous leukemic cells are resistant to nonactivated natural killer (NK) cell killing and are killed effectively only by lymphokine-activated killer (LAK) cells.[19] Autologous leukemic BM cells were stored in liquid nitrogen and thawed prior to labeling. Thawed cells were centrifuged over FH to remove dead cells. For the labeling of the target cells, 100 μCi ^{51}Cr was incubated with 10^6 pelleted cells for one hour and then washed three times. The effector-to-target ratio (E:T) was 20:1, and cells were incubated in microculture wells in triplicate. After three hours of incubation, the supernatants were harvested and radioactivity determined with a gamma counter. Cytotoxic activity was calculated by the following formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental release (cpm)} - \text{Spontaneous release (cpm)}}{\text{Maximum release (cpm)} - \text{Spontaneous release (cpm)}} \times 100\%$$

RESULTS

Colony assay with BM from children with ALL. The nonadherant BM mononuclear cells from common ALL (c-ALL) with less than 3% of CD3+ T cells (N = 8) and from T-ALL (N = 5) were cultured directly in methylcellulose. BM specimens from children with c-ALL with more than 3% T cells (N = 13), from children with B-ALL (N = 2), and from children in complete remission (CR; N = 12) were cultured after depletion of mature T (CD3+) cells. LMW-BCGF plus rIL-2 supported colony formation by BM cells from all patients with c-ALL and B-ALL and in CR. LMW-BCGF alone stimulated the majority of cases (26/35) in these patients to form colonies. rIL-2 alone did not induce colony formation from the BM cells of any patient with active disease (28 cases). There was colony formation in only two of 12 BM samples in CR. LMW-BCGF and rIL-2, either singly or in combination, failed to stimulate T-ALL colony formation. Figure 1 shows the data for colony formation from individual BM samples using different culture conditions. More colonies were seen in cultures containing a combination of LMW-BCGF and rIL-2 than in those with

Table 1. Number and Phenotype of Colonies From BM Cells of ALL in Culture With LMW-BCGF (0.1 μ/mL) and LMW-BCGF (0.1 μ/mL) Plus rIL-2 (100 μ/mL)

		LMW-BCGF				LMW-BCGF Plus rIL-2				
Case	Diagnosis	Colonies	CD3 (%)	CD10 (%)	CD19 (%)	Colonies	CD3 (%)	CD10 (%)	CD19 (%)	Tac (%)
1	c-ALL	130	9	8	83	249	82	NT	7	NT
2	c-ALL	39	15	65	71	208	90	7	NT	7
3	c-ALL	40	0	NT	93	408	91.6	NT	13.7	56.1
4	c-ALL	610	20	NT	44.5	933	69.8	4.9	44.5	NT
5	B-ALL	2,400	1	NT	40.8	2,500	32.2	NT	34	11.6
6	ALL (CR)	52	NT	NT	NT	845	93.8	NT	NT	NT

Abbreviations: NT, not tested due to limited number of cells.

Cells from day-7 pooled colonies were stained with MoAbs and detected using FACS.

Table 2. Phenotype of Expanded Cells From BM Cells of ALL Patients Cultured in Liquid Medium With LMW-BCGF (0.1 μ/mL) Plus rIL-2 (500 μ/mL) for Ten to 14 Days

		Phenotype (%)							
		Preculture			Postculture				
Case	Diagnosis	CD3	CD10	CD19	CD3	CD3/Leu-19	Tac	CD10	CD19
1	c-ALL	2	5	93	39.3	9.3	20.7	0	0
2	c-ALL	2	90	62	91.6	4.2	45.3	3.8	2.2
3	c-ALL	8	76	NT	80	7	32	6	7
4	c-ALL	18	0	78	73.8	14	25.9	0	0
5	B-ALL	27	0	56	54.2	6	19.3	9.4	5.4
6	ALL (CR)	14	NT	NT	79	15	41	NT	NT
7	ALL (CR)	11	NT	NT	94.5	34	11.6	NT	NT
8	c-ALL	5	47	89	75	21	27.4	NT	2.3
9	c-ALL	0	89	93	64.5	8	24.5	4	7
10	ALL (CR)	13	NT	NT	65	16	35.6	NT	NT

The immunologic surface markers of cells were detected by FACS. T-depletion procedure was carried out in patients with more than 3% of CD3+ cells in preculture.

LMW-BCGF alone. In addition, cell viability was greater in cultures with both LMW-BCGF and rIL-2.

Immunologic surface markers of colonies and expanded cells in culture with LMW-BCGF plus rIL-2. Pooled colonies of BM cells from six ALL patients cultured in methylcellulose with LMW-BCGF and LMW-BCGF plus rIL-2 were harvested for phenotyping (Table 1). Cells from pooled colonies were from 32% to 94% CD3+ in culture with LMW-BCGF plus rIL-2. In these culture conditions, cells with leukemic phenotypes (CD10 and/or CD19) from four of five samples with active disease were decreased. In contrast, LMW-BCGF alone gave primarily leukemic colonies. Samples of BM cells of patients in CR demonstrated colonies that were primarily myeloid. Identical results were also obtained from the expanded cells of ten patients, including the six cases phenotyped for colony cells, which were incubated with LMW-BCGF plus rIL-2 in liquid media (Table 2). All ten samples expressed increased CD3+ cells and CD3+ Leu-19+ cells. The expression of CD8 and CD4 antigens on the generated cells was detected from case 2 (CD8, 25%; CD4, 72%) and case 7 (CD8, 65%; CD4, 21%). Cells from all these cases expressed increased rIL-2 receptors (Tac). In all seven samples from patients with active disease, less than 10% of cells expressed CD10 and CD19 following culture. There was no significant cell expansion or increased CD3+ T cells in culture with either LMW-BCGF or rIL-2 alone, even at increased concentrations of lymphokine. Table 3 shows the phenotype of cultured cells from case 2 in liquid medium with these agents.

Table 3. Phenotype of Cultured Cells From BM Cells of Case 2 in Liquid Medium With LMW-BCGF, rIL-2, and LMW-BCGF Plus rIL-2 for Seven Days

	Preculture	LMW-BCGF*	rIL-2†	LMW-BCGF + rIL-2‡
CD3 (%)	2.03	9.40	1.87	71.02
Tac (%)	1.32	29.41	3.40	69.40
CD10 (%)	90.12	60.16	63.00	33.74
CD19 (%)	62.00	79.85	66.51	27.40

Concentration: *LMW-BCGF, 0.2 μ/mL; †rIL-2, 1,000 μ/mL; ‡LMW-BCGF, 0.1 μ/mL; rIL-2, 500 μ/mL.

Figure 2 shows the kinetics of CD3+ T cell and CD10+ leukemic cell growth in liquid culture from case 2. During the 14-day incubation period with LMW-BCGF and rIL-2, there was a progressive decrease in CD10+ cells and increase in CD3+ cells.

Lytic activity of expanded cells in culture with LMW-BCGF plus RIL-2. BM mononuclear cells from four patients with active disease and one in CR were incubated with either LMW-BCGF alone, rIL-2 alone, or the combination of the two in liquid medium for ten to 14 days and then harvested to determine their lytic activity against K562, Raji, and cryopreserved autologous blasts. Preculture cells and cells cultured with LMW-BCGF alone showed no or very little lytic activity against K562 or Raji. Cells in culture with rIL-2 alone showed slightly increased lytic activity against K562 and Raji, including two cases that showed activity against autologous leukemic cells. Cells cultured with combined LMW-BCGF and rIL-2 mediated significantly increased lytic activity against these three target cells (Table 4).

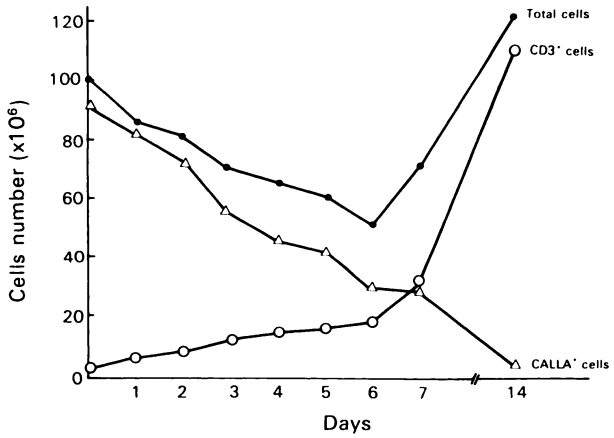

Fig 2. The kinetics of CD3+ and CD10+ cell growth in liquid culture with LMW-BCGF (0.1 μ/mL) plus rIL-2 (500 μ/mL) from case 2. The cell number was counted daily, and surface markers were detected using FACS.

Table 4. Lytic Activity of BM Cells From ALL Patients in Culture With LMW-BCGF (0.1 μ/mL), rIL-2 (500 μ/mL), and a Combination of LMW-BCGF (0.1 μ/mL) and rIL-2 (500 μ/mL) Against K562, Raji, and Autologous Leukemic Cells

		E:	Preculture		LMW-BCGF		rIL-2			LMW-BCGF + rIL-2		
Case	Diagnosis	T:	K562	Raji	K562	Raji	K562	Raji	A.L.	K562	Raji	A.L.
1	c-ALL		2.4	NT	2.8	0	24	17	9	53	70	14
2	c-ALL		0	0	4.6	NT	19	16	0	44	22	23
4	c-ALL		3	2	4.3	0	7	6	0	59	72	15
8	c-ALL		5.6	3.1	7.8	2.6	16	13	4	60	79	16
10	ALL (CR)		7	0	12	4	27	19	NT	82	67	NT

Abbreviations: E, effector; T, target; AL, autologous leukemic cells.

Cytotoxicity is expressed as percentage of specific ^{51}Cr release from labeled target cells in a 20:1 E:T ratio using a three-hour assay.

DISCUSSION

Previous studies have shown that cytotoxic T lymphcoytes and LAK activity can be generated from T-cell precursors in human BM.[20-22] Our results using a combination of LMW-BCGF and rIL-2 have supported this finding. The generation of CTL is thought to involve an antigen signal as well as signals delivered by soluble lymphokines. Many studies[1-5] have indicated that a soluble lymphokine present in supernatants of Con-A or PHA-activated T cells was required for the generation of CTL. This lymphokine has been identified as a 30- to 60-Kd polypeptide called IL-5 (also known as BCGF-2). IL-5 is required in the early period of culture, whereas IL-2 is required at a later stage of culture for stimulating CTL.[11,23]

Our results provide the first evidence that LMW-BCGF together with rIL-2 is able to induce growth of CD3+ CTL with LAK activity from BM cells of children with ALL. Thus LMW-BCGF and IL-5 have similar activities in inducing CTL proliferation and maturation. Furthermore, both lymphokines require the presence of IL-2 for maximum proliferation of BM-derived CTL. 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.[24-26]

Mononuclear cells separated from the BM cells of children with ALL (either in active disease or in remission) contain immature normal and neoplastic lymphoid progenitors. LMW-BCGF probably induced expression of IL-2 receptors on these cells and stimulated their growth. The colonies in culture with LMW-BCGF alone usually emerged in four to six days of culture and expanded for up to seven to ten days. These pooled colony cells expressed primarily leukemic surface markers (CD10 and/or CD19+), as has been reported by Uckun et al.[16] However, colonies grown in the presence of both LMW-BCGF and rIL-2 were primarily CD3+ T cells. These colonies were more numerous and larger than those in LMW-BCGF alone and continued to proliferate for up to 3 weeks. Cells proliferated indefinitely in flasks fed with fresh media, LMW-BCGF, and rIL-2. Furthermore, the CD3+ T cells expanded in liquid culture expressed LAK activity against autologous leukemic cells. In cultures depleted of T cells, the cells in the colonies were probably not derived from residual mature T lymphocytes remaining after the initial T-cell depletion, since there was no colony formation in cultures with rIL-2 alone. This contrasts with the ability of rIL-2 to stimulate the growth of T-cell colonies from mature peripheral-blood T cells and is consistent with a previous report that rIL-2 alone does not induce CTL from T precursors.[2,5,11,22]

All C-ALL and B-ALL BM samples tested showed CD3+ T-cell colony formation in semisolid media with LMW-BCGF plus rIL-2, even following T-cell depletion (as detected by FACS). Moreover, in liquid media the leukemic cells (CD10 and/or CD19+) disappeared rapidly as the CD3+ cells expanded. This may have been due to lysis of the blast cells by the expanding CTL, which we have found to exhibit LAK activity. CD3+ colony formation and expansion were also observed in cultures of BM cells from ALL patients in complete remission. However, no CD3+ colony formation was observed in cultures from five BM samples from children with T-ALL. The reason for the failure of normal CD3+ cells to proliferate in these cases is not clear.

In our experiment we found that cytotoxicity is not necessarily correlated with the number of CD3+ cells in culture. For example, case 2 shows a high percentage of CD3+ cells and low lytic activity. Also, cells in culture with rIL-2 alone expressed some cytotoxicity, although there was no expansion of CD3+ cells in this culture. There is much previous evidence that cytotoxicity against tumor cells is mainly due to Leu-19+ cells, including CD3+/Leu-19+ T cells and CD3− /Leu-19+ NK cells.[27-29] We are further investigating the role of these subsets of effector cells in generating cytotoxicity for leukemic cells in our BM cultures. Our data indicate that the CD3− Leu-19+ and CD3+ Leu-19+ cells account for all the LAK activity in these cultures (manuscript in preparation).

On the basis of these results, our hypothesis is that LMW-BCGF induced the expression of IL-2 receptors on immature T cells in the BM samples of children with ALL in both active and remission phases of the disease. In the presence of IL-2, these induced cells then differentiate and proliferate to become CTL with LAK activity. The ability of LMW-BCGF and rIL-2 to expand BM derived CTL with cytolytic activity against autologous leukemic cells may have implications for the therapy of ALL with these agents.

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