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Usefulness of Lymphokine-activated Killer Cells Generated from Bone Marrow Mononuclear Cells for the Purging of Residual Tumor Cells in Peripheral Blood Stem Cell Graft

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Lymphokine-activated killer (LAK) cells were generated from bone marrow mononuclear cells (BM), and the usefulness of the BM-LAK for purging of residual tumor cells in autologous peripheral blood stem cell (PBSC) graft was determined. The BM and peripheral blood lymphocytes (PBL) were obtained from the same bone marrow donors. The BM-LAK and PBL-LAK were generated by incubation with interleukin-2 for 7 days. The BM-LAK demonstrated higher killer activity against a lymphoma cell line Raji than the PBL-LAK. The BM-LAK also had a higher percentage of $CD4^-CD8^-CD16^+$ cells than the PBL-LAK, which suggests that their high killer activity is related to these cells. The BM-LAK did not show any killer activity against the PBSC graft. However, they killed tumor cells which contaminated the PBSC graft, and in particular, killed chimeric bcr/abl messenger RNA-positive residual leukemic cells. These results suggest that the BM-LAK may be applicable for purging. As the BM-LAK possess higher killer activity than the PBL-LAK, they may be more useful than the PBL-LAK.

Key words: Bone marrow mononuclear cells — LAK activity — Purging — PBSC — Chimeric bcr/abl mRNA

Recently, it has been stated that lymphokine-activated killer (LAK) cells generated from peripheral blood lymphocytes (PBL-LAK) are useful not only for adoptive immunotherapy,[1,2] but also for the purging of residual tumor cells before autologous bone marrow transplantations.[3,4] These LAK cells are not harmful to normal hematopoietic progenitor cells.[5,6] Therefore, their application for purging is reasonable. However, it is necessary to use LAK cells which possess high killer activity.[7,8]

In this study, we determined the LAK activity of bone marrow mononuclear cells (BM), and found that the BM-LAK possess very high killer activity compared to the PBL-LAK. Hence, the BM-LAK were applied to the purging of residual tumor cells in peripheral blood stem cell (PBSC) grafts. As residual tumor cells, we used chimeric bcr/abl messenger RNA (mRNA)-positive leukemic cells, and a polymerase chain reaction (PCR) was applied to detect these leukemic cells. In this report, the characteristics of the BM-LAK and the usefulness of these cells for purging of tumor cells are described.

MATERIALS AND METHODS

Generation of LAK cells from BM and PBL Bone marrow cells were obtained by suction from allogeneic bone marrow donors (6–32 years old, average age 25.5, 7 male donors, 1 female donor) and samples of them were used for experiments after informed consent had been obtained. The mononuclear cells (BM) were separated by Ficoll-Hypaque gradient centrifugation. Adherent cells were removed by adhesion to a plastic flask. Nonadherent cells were suspended at a concentration of 2×10^6 cells/ml in a medium (RPMI 1640+10% fetal calf serum) supplemented with 400 IU/ml of recombinant interleukin-2 (IL-2, Shionogi Pharmaceutical Co. Ltd., Osaka). Five ml of this suspension was added to a 25 cm^2 plastic culture flask. Incubation for 7 days at 37°C in a 5% CO_2 atmosphere afforded the BM-LAK. PBL were separated from peripheral blood of the same donors. The PBL-LAK were generated by the same method.

Determination of natural killer (NK) and LAK activities NK and LAK activities were determined by a 4-h ^{51}Cr-release assay. A myeloid leukemia cell line K562 and a lymphoma cell line Raji were used as the target cells for the NK and LAK determinations, respectively. The ^{51}Cr-labelled target cells, both alone and with the effector cells, were added to a microtiter plate and incubated for 4 h at 37°C. Then the radioactivity of the supernatants was determined. Maximum release was produced by the target cells incubated with 1% Nonidet P40. Spontaneous release was that produced after incubating in the medium alone. Experimental release was that produced from the target cells killed by the effector cells. The ^{51}Cr-release rate was determined by use of the following equation:

$$\frac{\text{CPM experimental release} - \text{CPM spontaneous release}}{\text{CPM maximum release} - \text{CPM spontaneous release}} \times 100\ (\%)$$

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Isolation of $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ subsets The BM and PBL were separated into $CD4^+$ and $CD8^+$ subsets by using commercially available capture flasks coated with either anti-$CD4^+$ or anti-$CD8^+$ antibody (Micro CELLector, Applied Immune Sciences, Inc., Menlo Park, CA). The $CD4^-CD8^-$ subset was the non-captured cells in both of these flasks. These isolations were performed at a purity level of over 97%. Surface markers of the BM and PBL were determined by using an Ortho Cytoron (Ortho Diagnostic Systems, Raritan, New Jersey) and OK series antibodies (Ortho Diagnostic Systems).

Collection of PBSC grafts PBSC grafts were collected by using a cell separator (AS104, Fresenius Co., Oberursel, Germany). A sample of them (non-frozen fresh sample, viability of over 98%) was used for experiments after informed consent had been obtained. Contaminating red cells were removed after separating them by Ficoll-Hypaque gradient centrifugation. For experiments, these PBSC grafts were incubated with the BM-LAK in a plastic tube (7 mm in diameter) instead of a microtiter plate.

Isolation of $CD34^+$ cells from the PBSC grafts $CD34^+$ cells were isolated from the PBSC grafts by using a commercially available cell-separation kit (Isolex 50, Baxter Healthcare Co., Irvine, CA). Briefly, the PBSC grafts were incubated with a murine anti-human CD34 antibody at 4°C for 30 min. They were washed and incubated with Dynal paramagnetic beads at 4°C for 30 min. The $CD34^+$ cells were then separated from the remaining cells using a magnetic particle concentrator. The linkage between the $CD34^+$ cells and the beads was cleaved with Chymo Cell-R. Then the $CD34^+$ cells were separated. The CD34 marker was determined by using an anti-CD34 monoclonal antibody (Becton Dickinson, San Jose, CA). In a preliminary experiment, the mean concentration of the isolated $CD34^+$ cells increased from 0.8 to 90.3% (n=8) with a mean harvest of 75%.

Colony formation of the $CD34^+$ cells For colony formation, a commercially available kit (Stem Cell CFU Kit, Baxter Healthcare Co.) was used. The $CD34^+$ cells were adjusted to 1×10^3 cells in 1 ml of the "Dilution Medium" of the kit. This cell suspension was then mixed with 3 ml of the "CFU Culture Medium" of the kit. One ml of the mixture was vortexed and plated into each of three 35 mm plastic dishes. After 14 days of culturing, the number of colonies (CFU-GM) was counted.

Detection of chimeric bcr/abl mRNA by PCR The primers were synthesized according to the method of Hariharan and Adams[9] and Fainstein *et al.*[10] (Table I and Fig. 1). RNA was isolated from the above cell samples by acid guanidinium thiocyanate-phenol-chloroform extraction. The isolated RNA was subjected to reverse transcriptase (RT) reaction to synthesize complementary DNA. One μg of the RNA sample, 25 pmol of primer ABL-1, 200 U of Moloney murine leukemia virus RT (Gibco/BRL, Breda, the Netherlands), 2.5 mmol of each deoxynucleoside triphosphate (dNTP) and 20 U of RNAsin (RNASE inhibitor) were mixed, incubated for 60 min at 37°C, and then heated at 99°C for 5 min (denaturing). After this, two series of PCR were performed. In the first series, the RT product (27 μl) was mixed with 25 pmol of primers (BCR-3 and ABL-1), 2.5 mmol of each dNTP, 1.5 U of Taq polymerase (Gibco/BRL) and an appropriate PCR buffer (Gibco/BRL). PCR consisting of 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 65°C and extension for 90 s at 72°C, followed by a final incubation for 10 min at 72°C, was then performed. As an internal control, the primers ABL-3 and ABL-1 were used. In the second series, the nested primers BCR-4 and ABL-2 were used.

Table I. Primers Used for the Detection of Chimeric bcr/abl Messenger RNA by Polymerase Chain Reaction.

Primer name	Sequence (5′–3′)	Base
ABL 1	5′-GGCCCATGGTACGAGCAGTG-3′	520–539
ABL 2	5′-GTTTCTCCAGACTGTTGACTG-3′	500–520
ABL 3	5′-CATCTGACTTTGAGCCTCAG-3′	248–267
ABL 4	5′-GGTGTGAGTGAAGCCGCTCG-3′	268–287
BCR 1[a]	5′-GCTTCTCCGTGACATCCGTG-3′	3208–3227
BCR 2[a]	5′-GGAGCTGCAGATGCTGACCAAC-3′	3227–3248
BCR 3[b]	5′-CGCATGTTCCGGGACAAAAGC-3′	1677–1697
BCR 4[b]	5′-CGCTCTCCCTCGCAGAACTC-3′	1698–1717

These primers were synthesized according to the methods of Hariharan and Adams[9] and Fainstein *et al.*[10]

a) Primer of major bcr for CML.

b) Primer of minor bcr for ALL.

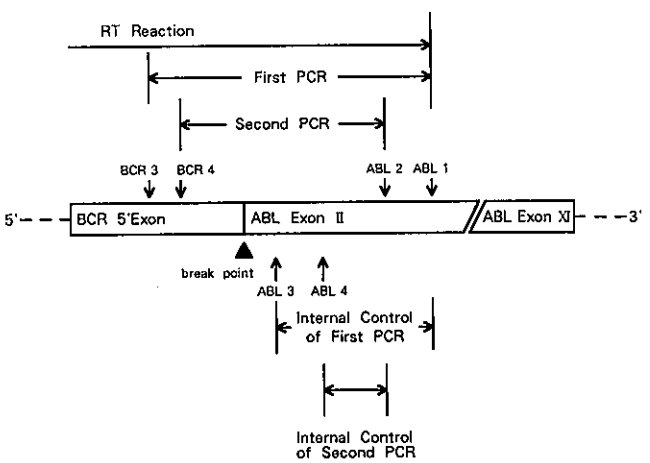

Fig. 1. The range of nested PCR.

The first series product (4 μl), 25 pmol of both primers and the same reagents as used in the first series were mixed together. Reaction cycles were the same as in the first series. As the internal control of the second series, the primers ABL-4 and ABL-2 were used. All reagents were checked in order to avoid contamination. Each reaction was performed in a different room. Ten μl of the second series product was analyzed by electrophoresis on a 2% agarose gel and bands were visualized by ultraviolet fluorescence after staining with ethidium bromide.

The sensitivity of the PCR for bcr/abl mRNA detection was confirmed by using a myeloid leukemia cell line K562 (which is positive for chimeric major bcr/abl mRNA). The PCR was able to detect one K562 cell in 5 $\times 10^5$ normal PBL.

Statistical analysis The results are given as mean$\pm$SD, and the statistical significance of differences was calculated using a Student's *t* test.

RESULTS

NK and LAK activities of the BM and PBL The NK activities of the BM and PBL were determined. As shown in Fig. 2a, the NK activities of the BM were very low. Most of the samples did not exhibit any killer activity at all. The PBL of the same donors did show some NK activities, at similar levels to our routine determinations.

The LAK activities were also determined. When the BM were cultured in the presence of IL-2, proliferation started on the 4th day of culture and progressed rapidly. Killer activities appeared on about the 5th day. The killer activities of the BM-LAK and PBL-LAK on the 7th day are shown in Fig. 2b. The BM-LAK demonstrated significantly higher killer activity than the PBL-LAK.

Relation of surface markers and killer activities The surface markers of the PBL, PBL-LAK, BM and BM-LAK were determined. As shown in Table II, the BM exhibited low percentages of $CD3^+$, $CD4^+$, $CD8^+$ and $CD16^+$ compared to the PBL. The BM-LAK demonstrated an increase in these markers, especially $CD16^+$.

By using the Micro CELLector, the $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ subsets were isolated from the PBL and BM, and $CD4^+$-LAK, $CD8^+$-LAK and $CD4^-CD8^-$-LAK were generated from each subset. Then, the surface markers and killer activities of these LAK cells were examined. As shown in Table III, the $CD8^+$-LAK and the $CD4^-CD8^-$-LAK exhibited various levels of killer activity. In particular, the $CD4^-CD8^-$-LAK of the BM showed an extremely high killer activity. This subset also had a high percentage of $CD16^+$ cells. These results suggest that the high killer activity of the BM-LAK may be related to this high percentage of $CD4^-CD8^-CD16^+$ cells. The percentage of the $CD4^-CD8^-$ subset in the total BM-LAK was 48.4%, whereas that in total PBL-LAK was 9.5%. It is not practicable to use the PBL-$CD4^-CD8^-$ cells clinically, because it is difficult to prepare a large number of the cells from the PBL.

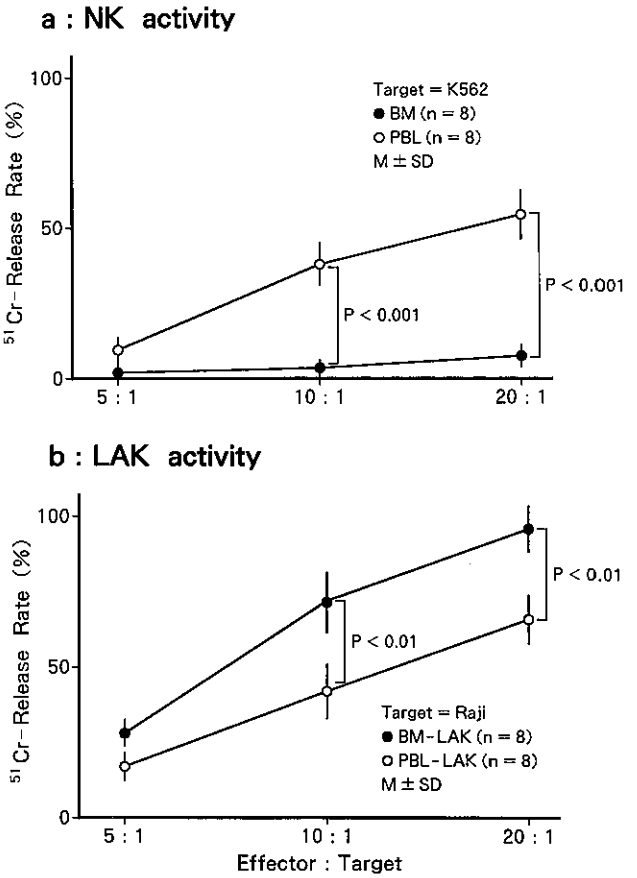

Fig. 2. NK and LAK activities of the BM and PBL. The BM and PBL were obtained from the same bone marrow donors. The LAK cells were generated by culturing in the presence of recombinant IL-2 for 7 days.

Table II. Determination of Surface Markers in PBL, PBL-LAK, BM and BM-LAK (n=8, mean$\pm$SD)

	Surface marker (%)			
	CD3	CD4	CD8	CD16
PBL	72.6±5.6	38.6±3.0	33.8±2.5	10.3±0.8
PBL-LAK	90.5±7.8	46.4±3.6	43.6±3.6	16.6±1.3
BM	36.2±2.8	17.7±1.1	18.5±1.2	3.3±0.2
BM-LAK	49.1±4.2	20.5±1.5	28.4±3.0	46.8±3.1

The surface markers were determined by using an Ortho Cytoron. The PBL-LAK and BM-LAK were generated by culturing in the presence of recombinant IL-2 for 7 days.

Table III. Surface Markers and Killer Activities of LAK Cells Generated from PBL and BM Subsets (n=8, mean±SD)

	Surface marker (%)				LAK activity against Raji (%)	
	CD3	CD4	CD8	CD16	(E:T=10:1)	(E:T=20:1)
PBL						
$CD4^+$-LAK	98.5±7.8	98.4±5.4	3.6±0.5	0.4±0.0	1.3±0.2	1.5±0.2
$CD8^+$-LAK	96.2±5.1	3.2±0.2	98.5±6.2	14.7±2.4	35.4±3.1	48.8±4.9
$CD4^-CD8^-$-LAK	4.9±0.3	2.5±0.2	2.3±0.1	60.2±4.8	66.2±5.6[a)]	76.2±6.7[a)]
BM						
$CD4^+$-LAK	96.2±7.8	94.4±5.5	1.6±0.1	0.3±0.1	1.2±0.2	1.2±0.2
$CD8^+$-LAK	93.3±4.8	4.6±0.3	90.6±6.2	26.8±2.1	43.7±5.6	58.4±6.3
$CD4^-CD8^-$-LAK	4.2±0.4	2.4±0.2	2.6±0.2	77.6±5.8	75.6±7.7	94.4±8.6

a) n=5.
The $CD4^+$ and $CD8^+$ subsets were isolated from the PBL and BM by using commercially available capture flasks (Micro CELLector). The $CD4^-CD8^-$ subset was the noncaptured cells in these flasks. The difference between BM-$CD4^-CD8^-$-LAK and PBL-$CD4^-CD8^-$-LAK at a ratio of E:T=20:1 is significant ($P<0.05$).

Table IV. Effects of the BM-LAK on ^{51}Cr-Labelled PBSC Grafts (n=5, mean±SD)

BM-LAK: PBSC graft	^{51}Cr-Release rate of PBSC graft (%) Incubation time (h)				
	1	2	3	4	6
1:1	1.3±0.1	2.5±0.1	3.2±0.2	3.3±0.2	3.7±0.6
0.5:1	1.2±0.1	2.3±0.1	3.2±0.2	3.2±0.2	3.5±0.6
0.25:1	1.2±0.1	2.3±0.1	3.0±0.2	3.2±0.2	3.4±0.5

The allogeneic BM-LAK and ^{51}Cr-labelled PBSC graft (2×10^6) were added to a tube, and incubated for several hours. Then the ^{51}Cr-release rate was determined.

Effects of the BM-LAK on PBSC grafts Next, the effects of the BM-LAK on residual tumor cells in PBSC grafts were determined.

First, the effect on the PBSC grafts was tested. The PBSC grafts were obtained from patients at autologous PBSC transplantations. In plastic tubes, the allogeneic BM-LAK were mixed with the ^{51}Cr-labelled PBSC grafts (2×10^6) at ratios of 1:1, 0.5:1 and 0.25:1, and incubated at 37°C for 1 to 6 h. Then the ^{51}Cr-release rates were determined. As shown in Table IV, after incubation for 6 h or less, the ^{51}Cr-release rate did not exceed 3.7%. This finding suggests that the BM-LAK scarcely killed the PBSC grafts within 6 h. We did not perform the experiment using ratios of BM-LAK to the PBSC grafts higher than 1:1, because such high ratios are not routinely practical.

Killer activity against Raji cells mixed with the PBSC graft The killer activity of the BM-LAK against Raji cells mixed with the PBSC graft was determined. The BM-LAK (or PBL-LAK) were added to a mixture of ^{51}Cr-labelled Raji cells (1×10^5) and the PBSC graft (2×10^6) using the following ratios; BM-LAK (or PBL-LAK):Raji:PBSC graft = 20:1:20 and 10:1:20. The mixture was incubated at 37°C for 1 to 6 h. It was stirred gently at 30 min intervals, in order for the LAK cells to be sufficiently in contact with the target cells. Then the ^{51}Cr-release rate was counted. In Fig. 3, adhesion of the BM-LAK (single donor's BM-LAK) to the Raji cells is shown. Fig. 3a shows the mixture of the Raji cells and PBSC graft. Fig. 3b shows the adhesion of the BM-LAK to the Raji cells 20 min after admixture. The BM-LAK adhered to the Raji cells within 20 min. The ^{51}Cr-release rates (Table V) reached the peak within 3 h in the experiment with BM-LAK, whereas the peak was late in the experiment with PBL-LAK. These findings suggest that the BM-LAK can kill most Raji cells within 3 h.

Effects on the isolation of $CD34^+$ cells from the PBSC grafts The effects of the BM-LAK on the isolation of $CD34^+$ cells from the PBSC grafts were determined. The BM-LAK (or PBL-LAK) and the PBSC graft (2×10^6) were mixed at ratios of 1:1 and 0.5:1, and preincubated at 37°C for 5 min (control), 3 h, 6 h and 24 h. Then, the $CD34^+$ cells were isolated. As shown in Table VI, the $CD34^+$ cells were sufficiently isolated from both groups preincubated up to 6 h, although the isolation rate decreased after preincubating for 24 h. These results suggest that the BM-LAK and PBL-LAK do not have any effect on the isolation of $CD34^+$ cells within 6 h.

Killer activity against chimeric bcr/abl mRNA-positive cells The killer activity against a chimeric bcr/abl mRNA-positive PBSC graft was determined. The graft was obtained from a 12-year-old patient. He was diagnosed with Philadelphia (Ph^1) chromosome-positive acute lymphocytic leukemia (FAB:L2). PCR test for this abnormal mRNA was positive (Fig. 4a). As a hematologically matched donor could not be found, his PBSC were collected repeatedly. The PBSC grafts which were collected after the first high-dose chemotherapies showed

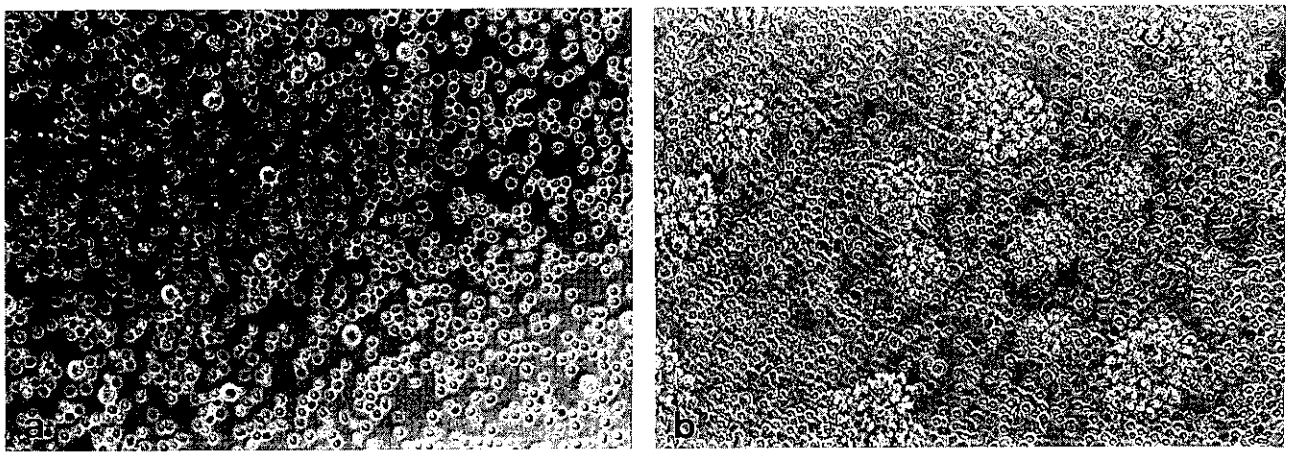

Fig. 3. Adhesion of the BM-LAK to Raji cells. The BM-LAK were obtained from a bone marrow donor and the PBSC graft was obtained from a patient. a: mixture of Raji cells and the PBSC graft (the large, round cells are the Raji). b: adhesion state of the BM-LAK to the Raji cells 20 min after mixing. This figure shows that the BM-LAK can adhere to the Raji cells within 20 min.

Table V. Killer Activities of BM-LAK and PBL-LAK against ^{51}Cr-Labelled Raji Cells Mixed with the PBSC Graft (n=5, mean±SD)

	^{51}Cr-Release rate of Raji cells (%)				
	Incubation time (h)				
	1	2	3	4	6
BM-LAK:Raji:PBSC graft					
20:1:20	53±4	94±7	96±8	96±7	96±9
10:1:20	35±3	88±6	95±7	95±7	96±9
PBL-LAK:Raji:PBSC graft					
20:1:20	13±2	33±3	45±5	66±7	68±7
10:1:20	7±2	26±3	38±4	53±6	56±7

The BM-LAK (or PBL-LAK) were added to a mixture of ^{51}Cr-labelled Raji (1×10^5) and the PBSC graft (2×10^6). They were incubated for several hours, followed by gentle stirring at 30 min intervals, in order for the BM-LAK (or PBL-LAK) to sufficiently come into contact with the target cells. Then the ^{51}Cr-release rate was determined.

Table VI. Effects of BM-LAK and PBL-LAK on the Isolation of CD34$^+$ Cells from the PBSC Grafts (n=4, mean±SD)

	Isolation rate of CD34$^+$ cells (%)			
	Preincubation time			
	5 min (control)	3 h	6 h	24 h
BM-LAK:PBSC graft				
1:1	100	98±9	96±9	73±9
0.5:1	100	98±6	97±9	84±9
PBL-LAK:PBSC graft				
1:1	100	98±8	95±8	67±8
0.5:1	100	98±7	97±8	82±9

The allogeneic BM-LAK (or the PBL-LAK) were mixed with the PBSC graft (2×10^6) and incubated for several hours. Then the CD34$^+$ cells were isolated using Isolex 50. The isolation rate is shown compared to the control.

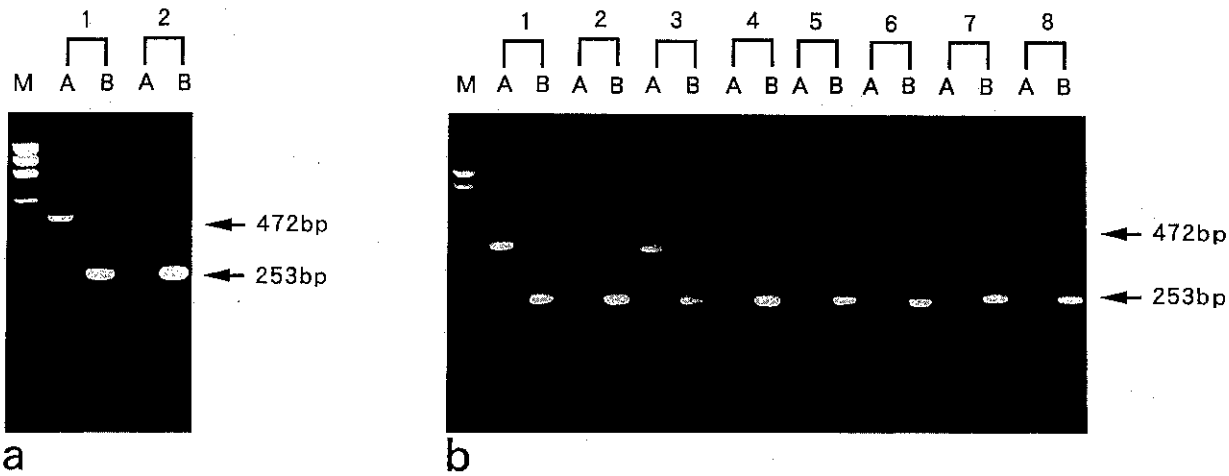

Fig. 4. Detection of chimeric bcr/abl messenger RNA by PCR in a Ph^1 chromosome-positive acute lymphocytic leukemia patient. M, size marker ($\phi \times 174/HaeIII$); A, bcr/abl mRNA; B, abl mRNA (internal control). a: examination of the bone marrow sample (1, the patient; 2, a donor). b: reexamination of the patient's PBSC graft after treatment with the donor's BM-LAK for 3 h at 37°C (1, control PBSC; 2, treated donor's BM (BM-LAK:BM=0.1:1); 3, 4, 5, treated patient's PBSC (BM-LAK:PBSC=0.05:1); 6, 7, 8, treated patient's PBSC (BM-LAK:PBSC =0.1:1)).

Table VII. Reexamination of PCR Positivity in PBSC Grafts Treated with BM-LAK or PBL-LAK, Isolation of $CD34^+$ Cells from the Treated PBSC Graft, and Recovery Rate and Colony Formation of the $CD34^+$ Cells

	Reexamination of PCR		No. of isolated $CD34^+$ cells ($\times 10^3$)	% recovery /control	No. of colony /plated cells[a)]
	Positive	Negative			
Control PBSC graft	3	0	148.8	100	103.4±9.8
Treated PBSC graft					
BM-LAK:PBSC graft					
0.1:1	0	3	119.5	80.3	101.6±10.1
0.05:1	1	2	128.1	86.1	105.0±9.5
0.025:1	2	1	137.6	92.5	104.7±9.7
PBL-LAK:PBSC					
0.1:1	2	1	—	—	—
0.05:1	3	0	—	—	—
0.025:1	3	0	—	—	—

a) Average of nine dishes in three experiments, mean±SD.
The PBSC grafts (2×10^6 cells) were treated with BM-LAK (or PBL-LAK) for 3 h at 37°C. After PCR reexamination, the $CD34^+$ cells were isolated from the treated PBSC graft. The number of $CD34^+$ cells was adjusted to 10^3 cells/ml, then they were cultured for 14 days and the number of colonies (CFU-GM) was counted.

negative in PCR. After 6 months, his disease relapsed. The bone marrow taken after the relapse contained 47.7% blastic cells. After second high-dose chemotherapy, this percentage decreased to 2.3%. At that time, blastic cells were not detectable microscopically in the peripheral blood. Therefore, the second PBSC collections were performed. However, three grafts which were collected during 3 days were positive in PCR. As these PCR-positive grafts could not be used for transplantation, one of them was used for the following experiments. This PBSC graft contained $CD34^+$ cells amounting to 8.0% of the total.

The BM-LAK (or PBL-LAK) were added to this PBSC graft (2×10^6) at ratios of 0.1:1, 0.05:1 and 0.025:1, and incubated at 37°C for 3 h. After washing three times, the positivity in PCR was reexamined. (As a

Table VIII. Effects of BM-LAK on the PBSC Graft Obtained from a Chronic Myelocytic Leukemia Patient

	Reexamination of PCR		No. of isolated $CD34^+$ cells ($\times 10^3$)	% recovery /control	No. of colony /plated cells[a)]
	Positive	Negative			
Control PBSC graft	3	0	131.5	100	97.6±8.5
Treated PBSC graft					
BM-LAK:PBSC graft					
0.5:1	0	3	26.8	20.4	94.5±8.2
0.25:1	1	2	37.3	28.4	96.1±8.4

a) Average of nine dishes in three experiments, mean±SD.
The PCR was performed using the major primers shown in Fig. 1. The PBSC graft (2×10^6 cells) was treated with the BM-LAK for 3 h using the same method as described in Table VII.

control, the PBSC graft was incubated for 175 min without the BM-LAK, and then incubated for a further 5 min with the BM-LAK at a ratio of 0.1:1.) Three samples were used for each determination. As shown in Table VII, the reexamination by PCR demonstrated negative conversion in all three samples of the BM-LAK at a ratio of 0.1:1. In Fig. 4b, a typical result of the PCR test using the BM-LAK (single donor's BM-LAK) is shown. These findings suggest that the BM-LAK can kill the abnormal mRNA-positive leukemic cells.

Colony formation of the isolated $CD34^+$ cells The $CD34^+$ cells were isolated from the above treated PBSC grafts, and colony formation (CFU-GM) was counted. As shown in Table VII, sufficient colony formation was observed in each sample. These findings suggest that the BM-LAK spare the hematopoietic progenitor cells.

Table VII also shows the number of isolated $CD34^+$ cells and their recovery rates. When the PBSC graft was treated with the BM-LAK at a ratio of 0.1:1, the recovery rate was 80.3% compared to the control PBSC. This result seems to suggest that this PBSC graft contained approximately 20% of $CD34^+$ leukemic cells and 80% of normal progenitor cells.

Effects of the BM-LAK on another PBSC graft Finally, the effect of the BM-LAK on another PBSC graft was examined. The PBSC graft was obtained from a 45-year-old patient with Ph^1-positive chronic myelocytic leukemia. The patient showed a positive reaction in PCR using the major primers shown in Fig. 1. After chemotherapy, the percentage of blastic cells in the bone marrow decreased from 60% to 10% and that in the peripheral blood from 45% to 2%. Then PBSC collections were performed. The PBSC graft contained 0.9% $CD34^+$ cells. The graft was treated with the BM-LAK by the same method as described above, and reexamined by PCR. As shown in Table VIII, when the PBSC graft was treated with the allogeneic BM-LAK (BM-LAK:PBSC graft = 0.5:1 and 0.25:1) for 3 h, the PCR test showed negative conversion in all three experiments at a ratio of 0.5:1. When the $CD34^+$ cells were isolated from these treated PBSC grafts, they still exhibited colony formation. This finding also suggests that the BM-LAK can kill $CD34^+$ leukemic cells.

DISCUSSION

While LAK cells are heterogenous, NK cells are considered to be the main effector cells.[11, 12] After having been released from the bone marrow, most of the NK precursor cells circulate in the peripheral blood. Their maturation is regulated by several cytokines in the blood.[13, 14] However, it is not certain whether or not these precursor cells can differentiate entirely in the bone marrow. Consequently, it is of interest to examine the killer activity of the BM.

In this study, we determined the killer activity of the BM. The BM tested were obtained from donors at allogeneic bone marrow transplantations. Although the BM may contain small numbers of PBL, they are different from the PBL. The findings were as follows: NK activity of the BM was very low, whereas LAK activity was very high. We compared the killer activity of the BM-LAK to that of the PBL-LAK in the same donors, and found that the BM-LAK had higher killer activity than the PBL-LAK. There have been several reports concerning the generation of BM-LAK.[15-17] van den Brink *et al.*[15] stated that the BM had no NK activity, but the killer activity appeared after culturing with IL-2. Migliorati *et al.*[13] also stated that the BM had no NK markers, but the markers appeared after culturing with IL-2. Although few authors have compared the killer activities of BM-LAK and PBL-LAK, Agah *et al.*[16] stated that murine bone marrow-derived LAK cells exhibited higher killer activity than the murine spleen-derived LAK cells.

It is interesting to consider why the NK activity is very low, and why the LAK activity is very high in the BM. The BM included a large number of $CD4^-CD8^-$ cells, but only a few $CD16^+$ cells. However, when cultured in

the presence of IL-2, the $CD4^-CD8^-CD16^+$ cells increased. These results suggest that the high killer activity of the BM-LAK may be related to these cells. The NK cells in the bone marrow seem to be not entirely differentiable. After culturing with IL-2, they may differentiate more rapidly than other types of bone marrow cells.

In this study, we applied the BM-LAK for the purging of tumor cells. First, the effects of the BM-LAK on the PBSC grafts were determined. At ratios of the BM-LAK to the PBSC graft of 1:1 or less, the BM-LAK showed no killer activity against the grafts. When the BM-LAK were added to a mixture of the ^{51}Cr-labelled Raji cells and the PBSC graft, the BM-LAK adhered to the Raji cells within 20 min. The peak of the ^{51}Cr-release rate appeared within 3 h. This finding suggests that the BM-LAK can kill Raji cells within 3 h. The BM-LAK had no effect on the isolation of $CD34^+$ cells from the PBSC graft preincubated for 6 h (Table VI). The isolation rate decreased after preincubation for 24 h, which may be due not only to the cell killing, but also to some inhibiting factor(s) produced by LAK cells.[18, 19]

In this study, in particular, the effects of the BM-LAK on the chimeric bcr/abl mRNA-positive PBSC graft were determined. The chimeric bcr/abl mRNA was detected by a PCR technique, because the PCR is very sensitive.[20, 21] After treatment with the BM-LAK for 3 h at 37°C, the PCR-positive PBSC graft exhibited negative conversion. Furthermore, when $CD34^+$ cells were isolated from the treated PBSC graft and cultured for 14 days, colony formation was still observed. These results suggest that the BM-LAK can kill the chimeric bcr/abl mRNA-positive leukemic cells, while sparing the hematopoietic progenitor cells.

In conclusion, BM-LAK may be applicable for tumor cell purging. As BM-LAK possess higher killer activity than the PBL-LAK, they may be more useful than the PBL-LAK.

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