A considerable number of patients with malignancies who are treated with high-dose therapy and hematopoietic stem cell transplantation subsequently relapse. Analyses of peripheral blood stem cell (PBSC) harvests obtained from 49 cancer patients showed that the PBSC harvest contained precursors for antitumor effector cells. Ex vivo manipulation of these harvests to maximize the antitumor effector cell activity may provide a new therapeutic approach to decrease or eliminate any minimal residual disease that remains after high-dose therapy. Characterization of PBSC from consecutive collections determined the collections best suited for ex vivo augmentation of antitumor cytotoxic effector cells. We report the results of a functional and phenotypical characterization of PBSC obtained from six consecutive collections from 18 cancer patients receiving granulocyte-macrophage colony-stimulating factor (GM-CSF) for hematopoietic stem/progenitor cell mobilization. The PBSC were evaluated for their cytotoxicity using the 51Cr-release assay. The frequency

HIGH-DOSE THERAPY followed by autologous blood stem cell transplantation (PBSC) has been used to treat a variety of advanced cancers.16-18 PBSC facilitates the reconstitution of the lymphohematopoietic system in cancer patients treated with high-dose myeloablative therapy.19 In a retrospective study, the clinical outcome of patients with aggressive non-Hodgkin's lymphoma who received PBSC was reported to be superior to that of similar patients who received autologous bone marrow transplantation.2 One possible explanation for this finding is that, in addition to hematopoietic reconstituting ability, PBSC may have in vivo antitumor therapeutic effects.

Many patients treated with high-dose therapy later relapse from minimal residual disease (MRD). These residual tumor cells are often resistant to conventional therapies. Therefore, one approach to control/eliminate MRD is to enhance the cells are often resistant to conventional therapies. Therefore, one approach to control/eliminate MRD is to enhance the antitumor therapeutic effects. Thus, our results show that PBSC obtained from advanced cancer patients do contain considerable levels of precursor cells for the generation of LAK cell populations. These results suggest that cells from the earlier collections are best suited for ex vivo manipulation to augment the antitumor effects.

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MATERIALS AND METHODS

Patient population. PBSC were procured as described below from 49 cancer patients, 25 males and 24 females. Their mean age was 44 years with a range of 10 to 66 years. PBSCs from the first 31 patients studied were used to measure cytotoxicity against target tumor cells. Twenty-six patients had non-Hodgkin's lymphoma, 2 had Hodgkin's lymphoma, 1 had multiple myeloma, and 2 had breast cancer. PBSC were obtained from consecutive collections of an additional 18 patients. Thirteen of these patients had non-Hodgkin's lymphoma, 3 had Hodgkin's lymphoma, 1 had Ewing's sarcoma, and 1 had medulloblastoma. In addition, PBSC were obtained from 8 normal healthy donors and used in the cytotoxicity studies. All patients and normal donors gave written informed consent to participate in the study.

Peripheral stem cell harvest. PBSC were collected from cancer patients or normal donors at the University of Nebraska Medical Center using the COBE Spectra (COBE BCT Inc, Lakewood, CO) or the Haemonetics Model V-50 (Haemonetics, Braintree, MA) apheresis machine. The procedure for PBSC collection using the Haemonetics Model V-50 apheresis device was previously described by Bishop et al.30 Briefly, apheresis of PBSC was performed over a 4-hour time period. For each pass, stem cell collection began after half of the platelet band was discharged and was completed when 80 mL of the red blood cell band was collected. After each pass, the stem cells were returned to the Latham bowl and the apheresis procedure was again repeated. Each apheresis procedure consisted of 10 to 12 passes. Stem cell collections from each patient were

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performed no more than five times per week. After the sixth collection, the plasma flow rate was adjusted during each procedure to maintain the color of the collect line between 3% to 5% on the manufacturer’s colorogram scale. ACD Formula A (Baxter Healthcare Corp, Deerfield, IL) was used as the anticoagulant during stem cell collections using either the Haemonetics Model V-50 or COBE Spectra apheresis machine. A maximum of five apheresis procedures was performed each week for any patient. A minimum of 6.5 × 10^6 MNCs/kg patient weight were collected per patient. The minimum number of MNCs and apheresis procedures was determined based on previous studies at the University of Nebraska Medical Center using PBSC from nonmobilized patients for hematopoietic rescue after high-dose chemotherapy. All patients received a continuous 24-hr intravenous infusion of GM-CSF (Immunex, Seattle, WA) at a dose of 125 μg/m² or 250 μg/m² to mobilize stem/progenitor cells. PBSC apheresis was achieved. After the sixth collection, PBSCs from some patients were collected using the COBE Spectra apheresis device with program revision 3.6. The single-stage white blood cell (WBC) channel was used and the target cells were mixed in round-bottom 96-well microtiter plates. Triplicate samples were incubated for 4 hours at 37°C. Spontaneous and total release controls were prepared for each assay. At the end of the incubation period, the supernatant was collected using a Titertech supernatant harvester (Flow Laboratories, United Kingdom) or the plates were centrifuged for 10 minutes at 800 rpm and 150 μL of supernatant was removed from each well. Radioactivity released was counted using a Beckman 5500 gamma counter (Beckman Instruments, Irvine, CA). The percentage of target cells lysed was calculated as described below.²¹

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

where experimental CPM includes effector and target cells; spontaneous CPM includes target cells and medium; and maximum CPM (total release) includes target cells and 1% Triton-X 100.

To normalize the results from the cytotoxicity assay using different effector:target (E:T) cell ratios, the percent cytotoxicity was converted to lytic units (LU) using a computer program developed by L. Broekhoven (Queen’s University, Kingston, Ontario, Canada). One lytic unit is defined as the number of effector cells required for 20% lysis of 5,000 tumor target cells. Calculation of LU was based on the following exponential fit equation described by Pross et al.²²

\[
y = A(1 - e^{-kt})
\]

where \(y\) is the percentage of chromium release, \(A\) is the maximum value for target cell lysis (100), \(x\) is the E:T ratio, and \(k\) is equivalent to the negative slope of the survival curve for the target cells as determined by plotting \(\ln(A - y)\) versus \(x\).

**Immunophenotyping analysis.** Cell surface immunophenotyping of patient PBSCs or normal peripheral blood mononuclear cells (PBMCs) was performed by flow cytometric analysis using the Coulter Profile II flow cytometer. Three hundred thousand cells were suspended in 200 μL of phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin and 0.05% NaN₃ (fluorescence buffer [FB]). Cells were incubated on ice for 30 minutes with 10 μL of fluorescein isothiocyanate (FITC) or 5 μL of phycoerythrin (PE)-labeled monoclonal antibody (MoAb) specific for the following cell surface markers: CD2, CD3, CD4, CD8, CD14, CD19, CD20, CD25, CD34, CD56, and HLA-DR (Dako, Glostrup, Denmark). After incubation, the cells were washed with 1 mL FB, centrifuged, and resuspended in 800 μL of PBS for analysis.

**Differential cell counts.** PBSCs were layered over a glass slide and allowed to air dry. The slides were stained for 2 minutes with Wright’s stain followed by a 5-minute staining period in staining buffer (6.25% Wright’s stain, 4.72 mmol/L sodium phosphate dibasic, and 3.57 mmol/L potassium phosphate monobasic, Curtin Matheson Scientific, Inc.). The slides were rinsed gently with tap water and dried. Differential counts were performed under oil immersion using a light microscope.

**Colony-forming unit granulocyte-macrophage (CFU-GM) assay.** To quantitate the stem/progenitor cell content in the individual PBSC collections, an in vitro CFU-GM colony forming assay was performed. Cells were stimulated by a combination of cytokines which consisted of GM-CSF, G-CSF, and IL-3, all at a concentration of 200 units/dish in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 10% FCS. The IMDM medium was heated to 34°C followed by the addition of a known volume of melted agar for a final agar concentration of 0.3%. After the medium/agar tube had cooled to 37°C in a water bath, cells were added to the medium/agar tube for a final cell concentration of 3 × 10⁵ cells/mL. One milliliter of the cells-medium-agar mixture was added to each 35-mm culture dish to which 0.1 mL of CSFs was previously added. The cultures were allowed to solidify at room temperature and then were placed into an incubator at 37°C for 14 days.

**Characterization of Blood Stem Cell Harvests**

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Fig 1. In vitro cytotoxicity of PBSC obtained from cancer patients against K562 (□) or Raji (●) tumor target cells was determined using the 51Cr-release assay immediately after PBSC apheresis (Baseline) or after culturing for 3 days in medium alone (Control) or in medium supplemented with IL-2 (IL-2 Treated). LYTIC units are expressed as the mean ± SEM. The cytotoxicity of PBSC after in vitro activation with IL-2 was significantly greater (P < .05) than control or baseline cytotoxicity. N = 31 for PBSC cytotoxicity against K562, N = 12 for PBSC cytotoxicity against Raji.

After incubation, the cultures were stained with Wright’s stain and CFU-GM colonies were counted using an inverted microscope.

Statistics. For each subject, samples were analyzed from several PBSC collections. Thus, repeated measurements were obtained for each subject. The correct method to account for these repeated observations is the repeated measures analysis of variance. This technique was used from the generalized models procedure from the Statistical Analysis System software (SAS Institute Inc, Cary, NC). The pairwise comparisons were made by Scheffe’s method where P ≤ .05 was considered statistically significant.

RESULTS

Cytotoxicity levels in patient PBSC. The antitumor cytotoxicity levels in PBSCs from cancer patients before and after in vitro activation with IL-2, determined using the 51Cr-release assay immediately after PBSC apheresis (Baseline) or after culturing for 3 days in medium alone (Control) or in medium supplemented with IL-2 (IL-2 Treated). Values represent mean lytic units ± SEM. The cytotoxicity of IL-2-activated PBSC versus K562 is significantly greater (P < .05) than control or baseline cytotoxicity. N = 8.
In vitro cytotoxicity of PBLs obtained from normal healthy donors was determined using the ⁵¹Cr-release assay immediately after PBL collection (Baseline) or after culturing PBLs for 3 days in medium alone (Control) or in medium supplemented with IL-2 (IL-2 Treated). PBL cytotoxicity was determined using K562 as the tumor target cell. Values represent mean lytic units ± SEM. The cytotoxicity of IL-2-activated PBLs is significantly greater (P < .05) than control or baseline cytotoxicity.

was significantly greater compared with the cytotoxicity of IL-2-activated normal PBSCs.

Immunophenotyping of PBSC. To determine the proportion of the various lymphocyte subset populations present in the mononuclear cell population of the PBSC harvest, immunophenotyping was performed using flow-cytometric techniques. The relative lymphocyte subset distributions in PBSC harvests and normal PBMCs were similar (Table I). To determine the effects of IL-2 on the phenotypic expression of PBSC, immunophenotyping studies were performed on PBSC after their activation with IL-2 (data not shown). Findings from seven samples indicate that exposure to IL-2 did not significantly change the percentages of cells positive for T-cell markers CD2, CD4, CD8, or CD8, the monocyte antigen CD14, HLA-DR, or the B-cell activation antigen CD20. Also, the percentage of CD56⁺ cells did not change significantly after IL-2 activation. The immunophenotypic analysis showed that the majority of the cells present in PBSC harvests were T cells and NK cells.

These studies showed that daily PBSC collections contained a significant number of precursor cells for LAK cells with tumoricidal activity in vitro (Fig 1). These results also suggested that the PBSC are an ideal cell source for ex vivo manipulation to augment antitumor properties of these effector cells. Because some of the ex vivo manipulation procedures may differentiate the hematopoietic stem/progenitor cells, only a portion of the PBSC should be used for ex vivo manipulation and the remaining PBSC harvest must be transplanted into the patients for hematologic reconstitution. Therefore, to determine which stem cell collections are most suitable for ex vivo augmentation of cytotoxic effector cell functions and which stem cell collections are best suited for hematologic reconstitution, we characterized PBSC from consecutive collections from 18 cancer patients.
Cytotoxicity levels of patient PBSC obtained from consecutive collections. Figure 4 shows the cytotoxicity levels of PBSC from consecutive collections before (baseline cytotoxicity) and after in vitro activation with or without IL-2. There was no difference in the baseline cytotoxicity levels of PBSC obtained from consecutive collections. As observed with the baseline cytotoxicity results, the cytotoxicity levels of PBSC from different collections against K562 target cells after in vitro culturing without IL-2 (control) was not significantly different between the consecutive collections. However, there was a significant increase in the cytotoxicity levels of the same cells after their activation with IL-2 against the K562 targets. The levels of cytotoxicity in the earlier collections was significantly greater when compared with the cytotoxicity levels seen in the later collections, i.e., collection 2 versus 5 (P < .05), and tending towards significance in collection 3 versus 5 (P = .055). This suggests that the earlier collections contain more precursors cells for LAK cell generation.

Immunophenotypic analysis of patient PBSCs obtained from consecutive collections. The phenotypic characterization of mononuclear cells in the six consecutive PBSC collections was performed using flow-cytometric techniques. The frequency of lymphocyte subsets in each collection was determined by staining the cells with FITC-conjugated or PE-conjugated MoAbs that were specific for CD2, CD3, CD4, CD8, CD20, or CD56 cell surface antigens and analyzed with the flow cytometer. Figure 5 illustrates the frequency of different lymphocyte subpopulations in consecutive PBSC collections from 11 patients. These patient samples were the same samples that were used to determine the cytotoxicity levels against tumor target cells. The earlier collections contained a greater number of lymphocytes expressing CD2, CD3, and CD4 cell surface antigens. For CD2+ lymphocytes, the percentage of positive cells in collections 1 (65.35% ± 6.2%) and 2 (64.26% ± 5.8%) was greater than collection 3 (39.30% ± 10.1%). Also, for both CD3+ and CD4+ lymphocytes, the percentage of positive cells in collection 1 (31.55% ± 5.5% for CD3 and 22.08% ± 4.3% for CD4) was greater than collection 3 (15.73% ± 3.9% for CD3 and 9.07 ± 3.2% for CD4). For CD56-positive lymphocytes, the percentage of positive cells in collection 5 (20.99% ± 3.8%) was significantly greater than collection 3 (10.23% ± 3.2%) (P < .05). However, the phenotypic profile for the CD56+ cells did not correlate with the functional cytotoxicity profile described in Fig 4.

The mean percentage of lymphocytes determined from the differential cell counts in collection 1 (35.8% ± 3.6%) was significantly greater than collection 4 (25.30% ± 3.0%) and 5 (26.00% ± 2.5%) (P < .05).

The composition of CFU-GM progenitor cells from consecutive PBSC collections was determined using an in vitro colony-forming assay. Figure 6 illustrates the mean number

![Graph showing cytotoxicity levels](image-url)

**Fig 5.** Immunophenotypic analysis of PBSCs obtained from consecutive collections was performed by staining the cells with FITC or PE-labeled antibodies specific for various lymphocyte subset markers and analyzing them using the flow cytometer. Values represent the mean percent positive cells ± SEM. Values that are different are indicated by single (*) versus double asterisk (**) as described in the text.

![Graph showing CFU-GM progenitor cells](image-url)

**Fig 6.** The frequency of hematopoietic progenitor cells in consecutive PBSC collections was determined using the CFU-GM assay. Values are expressed as the mean number of CFU-GM colonies formed per 3 x 10^5 PBSCs. *Significantly different from the first collection, P < .05.
of CFU-GM colonies formed per $3 \times 10^5$ cells from each of the six consecutive PBSC collections. The earlier stem cell collections contained fewer CFU-GM precursors compared with the later collections. The number of CFU-GM from collection 1 was significantly lower than the CFU-GM from collection 5 ($P < .05$).

Our analysis for CD34+ cells in consecutive collections obtained from 33 patients showed that, in general, the CD34+ cell peak preceded the CFU-GM peak by 24 hours or one collection. On average then, the CD34 peak occurred in collection 4. We found the mean CD34+ cells/apheresis from 291 collections to be $0.15\% \pm 0.11\%$, representing $19.9 \pm 13.2 \times 10^6$ cells. Because the frequency of CD34+ cells is very low in number, to get a better representation of the CD34+ cell content, these results have included additional patients for the CD34+ cell analysis.

The percentage of monocytes, banded neutrophils, and segmented neutrophils in consecutive PBSC collections was determined based on the differential cell counts performed on each PBSC collection. There was no significant difference in the number of monocytes present between the different collections. Although the percentage of banded neutrophils appeared to be lower in the earlier collections compared with later collections, this difference was not statistically significant. Although the percentage of segmented neutrophils was higher in the first collection, lower in collections 2 through 5, and again higher in collection 6, these differences were not statistically significant.

**DISCUSSION**

Our results show that PBSC from cancer patients, even after several cycles of conventional therapies, contain a considerable level of LAK cell precursors (Fig 1). The main cellular component of PBSCs are T cells and NK cells as shown in Table 1. Furthermore, the results from our study using PBSCs from consecutive collections show that PBSCs from the earlier collections have a greater number of cells with antitumor cytotoxic potential (Fig 4) compared with the cells from later collections that contained a greater number of cells with CFU-GM activity (Fig 6). The immunophenotypic analysis of the cells obtained from each consecutive collection showed a decrease in CD56+ cell populations in collection 3, whereas our results from the cytotoxicity studies show that collection 3 contained a significantly higher LAK precursor cell population. Therefore, these results indicate that the cytotoxicity observed in collection 3 could be caused by not only CD56+ cells, but also some other cytotoxic effector cell population. The exact reason for this observation is not known. These results need further investigations to determine the type of cytotoxic effector cell populations in the IL-2-activated cells from collection 3. Our findings describing the increase in CFU-GM progenitors in the later PBSC collections is supported by the results described by Bishop et al showing a relative increase in CFU-GM numbers in the later apheresis products from cancer patients treated with GM-CSF for stem cell mobilization. In an ideal situation, after high-dose therapy, patients could be transplanted with PBSC obtained from later collections containing higher numbers of CFU-GM progenitors for hematopoietic reconstitution. Once engraftment occurs, the earlier PBSC collections could be transplanted followed by a low to moderate dose of cytokine administration in vivo to augment the antitumor effects of the effector cells. Because MRD in cancer patients continues to be one of the major causes of death after high-dose therapy and hematopoietic stem cell transplantation, this strategy might result in improved long-term disease-free survival for these patients or lead to the elimination of residual tumor cells. Alternatively, the effector cell populations could be ex vivo manipulated to enhance their antitumor immune response.

Because the in vitro activation of peripheral stem cell harvests with IL-2 is known to enhance their antitumor cytotoxicity, evaluation of the effects of cryopreservation on the cytokine-induced antitumor activity of these cells would be important. Ideally, if one could cryopreserve the cytokine-activated killer cells without abrogating their cytolytic activity, the cells could be thawed and transplanted in conjunction with nonactivated peripheral stem cells. In a preliminary study, we attempted to cryopreserve IL-2–activated PBSC using two different cryomedia, namely 10% dimethyl sulfoxide (DMSO) or 5% DMSO plus 6% hydroxyethyl starch (HES). Our results showed that recovery of IL-2–activated effector cells after freezing in 6% HES/5% DMSO was much better (49%) than effector cells recovered after freezing in 10% DMSO (27%), showing that effector cells can be cryopreserved. Similar results were also reported by Schmidt-Wolf et al regarding the cryopreservation of LAK cells from patients with Hodgkin’s disease. Controlled-rate freezing was not used in our studies. If controlled freezing is used, the percent recovery of effector cells cryopreserved in 10% DMSO might be increased significantly. Studies to determine the optimal freezing method are currently under way.

Unfortunately, chemotherapeutic protocols used to eradicate malignant disorders are often unable to eliminate all tumor cells. To determine if residual tumor cells can be destroyed, Slavin et al designed a set of experiments to study the response of residual tumor to in vivo IL-2 treatment. BALB/c mice were injected intravenously with low doses of B-cell lymphoma (BCL1) cells to induce MRD. IL-2 treatment was successful in preventing residual tumor growth. However, mice that were injected with higher concentrations of BCL1 cells failed to respond to IL-2 treatment. Results from studies in our laboratory showed an effective immunotherapy by in vivo administration of IL-2 against an aggressive murine large cell lymphoma. Induction of cytokine-activated antitumor immune responses in humans that can suppress the growth of residual tumor cells may be feasible. The results presented in this report lay the foundation for future studies to augment an effective antitumor response of effector cells from PBSC harvests.

PBSCs from cancer patients cultured with IL-2 resulted in the augmentation of cytotoxic effects against K562 and Raji tumor target cells. When the baseline cytotoxicity levels of patient effector cells were compared with the baseline of normal effector cells, the cytotoxic activity was significantly lower in the patient samples. The loss of functionally active NK cells has been reported by other investigators in patients...
with leukemia, lymphoma, and preleukemic disorders. In this report, we have shown that PBLs that express no cytotoxic activity can be activated with IL-2. Moreover, in vitro generated cytotoxic cells from patient samples express levels of cytotoxicity that were similar to levels seen with IL-2–activated lymphocytes from the apheresis product from normal, healthy donors. Recently, Neubauer et al. showed that the lymphocytes present in the stem cell harvest show LAK cell activity before and after autologous peripheral blood stem cell transplantation. These results as well as those from our study, support the hypothesis that PBSC contain antitumor effector cells that mediate an antitumor effect that ultimately may lead to the improvement in the disease-free status of patients. Furthermore, effector cells obtained from the earlier PBSC collections might be ideal cells for ex vivo manipulations to enhance antitumor activity.

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Functional and phenotypic characterization of human peripheral blood stem cell harvests: a comparative analysis of cells from consecutive collections

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