

# Long-term Hematopoietic Culture-initiating Cells Are More Abundant in Mobilized Peripheral Blood Grafts Than in Bone Marrow But Have a More Limited Ex Vivo Expansion Potential

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**ABSTRACT:** Mobilized peripheral blood hematopoietic progenitor cells obtained from cancer patients treated with high-dose cyclophosphamide (7g/m<sup>2</sup>) followed by G-CSF, GM-CSF, IL-3, PIXY321, or combinations of these cytokines have been successfully used for autologous stem cell transplantation. We investigated the ability of hematopoietic progenitor cells (HPC) derived from mobilized peripheral blood (PB) to undergo ex vivo expansion in short term cultures by enumerating numbers of de novo generated CD34<sup>+</sup> cells, assayable progenitor cells, and the frequency of long-term hematopoietic culture-initiating cells (LTHC-IC). These parameters were examined in CD34<sup>+</sup> cells generated in culture through the use of cell tracking with the membrane dye PKH2. Fresh isolated mobilized CD34<sup>+</sup> cells contained 0.49 ± 0.36% LTHC-IC. However, due to the high number of total CD34<sup>+</sup> cells in mobilized PB, the absolute number of LTHC-IC was higher than that contained in a bone marrow (BM) harvest. Mobilized CD34<sup>+</sup> cells were stained with PKH2 and incubated with SCF, IL-3, and IL-6. After 5 to 6 days, numbers of total CD34<sup>+</sup> cells and clonogenic progenitors increased 1.4- and 2.2-fold, respectively. Numbers of total progenitors continued to increase such that 10 to 12 days after the initiation of cultures a 6.4-fold increase was demonstrable. However, between days 5 and 7 of culture, the frequency of LTHC-IC in CD34<sup>+</sup>PKH2<sup>bright</sup> cells (cells which did not divide) was less than 50% of that determined for fresh cells, while the frequency among CD34<sup>+</sup>PKH2<sup>dim</sup> cells (cells that had divided) was very low or undetectable. However, moderately higher frequencies of LTHC-IC were detected following expansion for 48 hours only. In similar assays, both BM and cord blood cells were capable of generating LTHC-IC in CD34<sup>+</sup>PKH2<sup>dim</sup> cells but not to expand the overall number of these progenitors. These observations suggest that although mobilized PB CD34<sup>+</sup> cells contain large numbers of LTHC-IC, these cells might not be capable of further ex vivo expansion and generation of additional LTHC-IC in vitro. Furthermore, these data indicate that mobilized PB CD34<sup>+</sup> cells may have undergone maximal "in vivo expansion" such that additional ex vivo expansion of primitive progenitor cells may not be possible.

**Keywords:** mobilized peripheral blood CD34<sup>+</sup> cells, ex vivo expansion

## INTRODUCTION

Mobilized peripheral blood progenitor cells (MPBPC) have been used successfully as grafts capable of regenerating marrow hematopoietic

activities following myeloablative therapy both in the autologous (1-3), and recently, in the allogeneic (4,5) settings. A regimen in which high-dose cyclophosphamide (HD-CTX) at 7 g/m<sup>2</sup>, followed by treatment with G-CSF,

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GM-CSF, IL-3, PIXY321, or combinations of these cytokines results in a marked increase in the number of CD34<sup>+</sup> cells mobilized into the circulation (3,6,7). Recent studies have demonstrated that peripheral blood (PB) of such patients contained a substantially higher number of total CD34<sup>+</sup> cells (8,9). Whereas some laboratories identified in the PB of such patients a significantly higher content of subsets of CD34<sup>+</sup> cells traditionally identified as primitive hematopoietic progenitor cells (HPC), such as CD34<sup>+</sup> HLA-DR<sup>-</sup> cells (8, 9), others have reported however, a lower number of cells with similar phenotypic properties (10). However, regardless of the number of such cells present in PB, functional properties of these cells have been previously shown to be very similar to those described for their bone marrow (BM) counterparts (8,10).

Utilization of mobilized PB cells as a source of transplantable stem cells offers several advantages over the use of BM grafts. In addition to eliminating invasive procedures for the collection of stem cells and their utility as optimal vehicles for retroviral mediated gene transfer (11), perhaps the most important and clinically relevant property of MPBPC, is their ability to initiate a more rapid granulocyte and platelet recovery than is currently possible with BM grafts (2,5-7,12,13). This remarkable ability of MPBPC to shorten the period of cytopenia following autologous stem cell transplantation may result from the large content of HPC in these grafts (14,15), or to the ability of primitive HPC present in mobilized PB to undergo massive proliferation and generation of committed progenitor cells in a short period of time (8-10). Alternatively, this may also be due to the fact that mobilized PB contains large numbers of stem cells with short-term marrow repopulating potential. Recent studies indicated that indeed a large number of committed progenitor cells identified as CD34<sup>+</sup> HLA-DR<sup>+</sup> cells could be found in mobilized PB (8) and that higher or comparable numbers of HPC present in mobilized PB displayed an equivalent proliferative capacity to BM CD34<sup>+</sup> cells (9,10).

The putative ability of mobilized PB-derived

primitive HPC to generate large numbers of committed progenitors can be easily examined using a number of ex vivo expansion methodologies. A number of laboratories (9,10,16,17), including ours (8), have recently begun to study the behavior of MPBPC in vitro. These studies investigated the kinetics of mobilization (17) and primitive HPC content (8,17) of PB as well as the production of mature colony forming units (CFU) such as the CFU-granulocyte/macrophage (CFU-GM) in stromal cell-based or suspension long-term hematopoietic cultures (LTHC). However, the examination of hematopoietic activities associated with primitive HPC in ex vivo expanded MPBPC has been limited (16) or has focused on phenotypic characterization of these cells (18). In this report, we utilized novel approaches for the examination of the primitive HPC content of ex vivo expanded MPBPC as well as their content of committed CFU. To follow the proliferation of cells in culture, cell tracking via the membrane dye PKH2 was employed to isolate newly formed CD34<sup>+</sup> cells which were then assayed for functional activity in biological assays. In order to examine the primitive HPC content of ex vivo expanded CD34<sup>+</sup> cells, the frequency and number of LTHC-initiating cell (LTHC-IC) were determined among de novo generated CD34<sup>+</sup> cells. Our results indicate that mobilized PB CD34<sup>+</sup> cells have a restricted capacity for ex vivo expansion of HPC, especially the more primitive LTHC-IC, relative to other hematopoietic tissues.

## MATERIALS AND METHODS

### *Patients and Treatment Protocol*

Mobilized PB samples were obtained from breast cancer (stage II with more than 9 involved axillary ipsilateral lymph nodes), lymphoma, glioblastoma, or germ cell cancer patients all evaluated in Milan, Italy. These patients did not receive any chemotherapy prior to their enrollment in a peripheral blood stem/progenitor cell mobilization protocol which included HD-CTX

chemotherapy followed by the administration of HGF. Absence of BM infiltration by tumor cells in these patients was determined by morphologic analysis of marrow aspirates and biopsies. Informed consent from these patients was obtained according to the guidelines outlined by the Istituto Nazionale Tumori which adheres to the principles of the Declaration of Helsinki. HD-CTX (7 g/m<sup>2</sup>) was administered in five divided doses, each dose being administered i.v. for 1 hour every 3 hours. Beginning 1 day after HD-CTX, patients received intravenous infusions of HGF according to the dosages and treatment schedule outlined in Table 1. Recombinant

human IL-3 was provided by Sandoz (Basel, Switzerland); recombinant human GM-CSF was provided jointly by Sandoz and Schering Plough (Milan, Italy), and recombinant GM-CSF/IL-3 fusion protein (PIXY321) was provided by Immunex (Seattle, WA). Collection of MPBPC was carried out by leukapheresis, starting as soon as CD34<sup>+</sup> cells were at least 50 per microliter in the peripheral blood, and continued until the dose of  $\geq 8 \times 10^6$  CD34<sup>+</sup> cells per Kg body weight was achieved. Cytokine administration was discontinued after the end of the last leukapheresis procedure.

Table 1. Clinical characteristics, type and schedule of treatment, and number of PB cells harvested from patients entered in these studies

UPN	Disease	Type of Chemotherapy <sup>*</sup>	Cytokines used after Chemotherapy <sup>†</sup>	Number of total cells collected <sup>‡</sup> (x10 <sup>9</sup> )	Number of CD34 <sup>+</sup> cells/Kg <sup>‡</sup> (x10 <sup>6</sup> )
1	Breast cancer	HD-CTX	IL3-G-CSF	33.74	10.2
2	Glioblastoma	HD-CTX	PIXY321 (500 µg/m <sup>2</sup> )	21.7	7.9
3	Breast cancer	HD-CTX	IL3-GM-CSF	14.7	7.5
4	Breast cancer	HD-CTX	GM-CSF	44.4	10.5
5	Breast cancer	HD-CTX	IL3-GM-CSF	61.0	30.7
6	Lymphoma	HD-CTX	PIXY321 (750 µg/m <sup>2</sup> ) + G-CSF	153.3	6.0
7	Lymphoma	HD-CTX	PIXY321 (1000 µg/m <sup>2</sup> ) + G-CSF	29.2	30.7
8	Breast cancer	HD-CTX	IL3-GM-CSF	12.8	9.5
9	Breast cancer	HD-CTX	IL3-G-CSF	ND	ND
10	Breast cancer	HD-CTX	IL3-G-CSF	12.0	12.0
11	Germinal cell cancer	PEI	G-CSF	20.1	20.1
Mean				45.4 ± 40.9	14.5 ± 9.4

\* Chemotherapy received by these patients consisted of either high dose cyclophosphamide (HD-CTX) given at 7 g/m<sup>2</sup> in five divided doses, each dose being administered i.v. for 1 hour every 3 hours or Ifosfamide, etoposide, cisplatin combination (PEI) given at 1.2 g/m<sup>2</sup>, 100 mg/m<sup>2</sup>, and 20 mg/m<sup>2</sup>, respectively for 5 days.

† IL3 was given at the dose of 5 µg/Kg/day by s.c. route for 7 days, followed by GM-CSF or G-CSF for an additional 5 to 7 days (until completion of apheresis). GM-CSF or G-CSF, either alone or in combination, were given at the dose of 5 µg/Kg/day, either by s.c. or iv route. PIXY321 was given at the indicated dose by s.c. route, either alone or in combination with G-CSF.

‡ Number of total nucleated cells (x 10<sup>9</sup>) collected by leukapheresis. Values reported as mean ± S.D.

§ Number of CD34<sup>+</sup> cells (x 10<sup>6</sup>) per Kg body weight collected by leukapheresis and determined by flow cytometric analysis. Mean is reported as mean ± S.D.

ND Not determined

### *Cell Separation*

Hematopoietic cells used in these studies were isolated from marrow aspirates obtained from normal adult volunteers, from CB, and from MPB collected from patients described above. MPB samples collected in Milan, Italy were shipped by overnight courier to Indianapolis such that samples were processed within 48 hours of their collection. Human BM aspirates were obtained from normal adult volunteers after informed consent was obtained according to guidelines established by the Human Investigation Committee of the Indiana University School of Medicine. CB samples were destined to be discarded and therefore obtaining informed consent was deemed unnecessary. Low density cells were separated over Ficoll/Hypaque (Pharmacia, Piscataway, NJ) and then fractionated by immunomagnetic selection to obtain CD34<sup>+</sup> cells as previously described (19). For immunomagnetic selection cells were first incubated on ice with 0.5 µg 9C5 (an IgG<sub>1</sub> murine anti-human CD34 monoclonal antibody) per 10<sup>6</sup> cells for 30 min, washed three times, and then incubated for 30 min with sheep anti-mouse IgG<sub>1</sub> conjugated to paramagnetic micro spheres. Bead and bead-cell complexes were attracted to and retained against a permanent magnet allowing for the removal of non rosetted cells (CD34<sup>-</sup>). After washing the beads and bead-cell complexes 3 times, the rosetted CD34<sup>+</sup> cells were enzymatically cleaved from the beads by incubation for 15 min at 37°C with 200 pKat/mL chymopapain. The enzymatic reaction was stopped by the addition of cold media, and the released CD34<sup>+</sup> cells were removed after collecting the beads using a permanent magnet. All reagents for the immunomagnetic separation procedure were kindly provided by Baxter Healthcare, Santa Ana, CA.

### *Immunofluorescence Staining and Flow Cytometric Cell Sorting*

Immunomagnetically enriched CD34<sup>+</sup> cells were stained on ice for 20 min with

FITC-conjugated anti-CD34 (8G12 mAb, kind gift from Baxter Healthcare, Santa Ana, CA). Control monoclonal antibodies consisted of fluorochrome-conjugated, isotype matched nonspecific myeloma proteins. Cells were washed and resuspended for flow cytometric cell sorting in PBS supplemented with 1% human serum albumin. Immediately after staining, cells were sorted as previously described (20) on a FACStar<sup>plus</sup> (BDIS) flow cytometer. For limiting dilution analysis (LDA) assays, the exact number of cells required for a given experiment were sorted into a collection tube, centrifuged, and utilized as described below. Viability and purity of sorted cells always exceeded 98% and 95%, respectively.

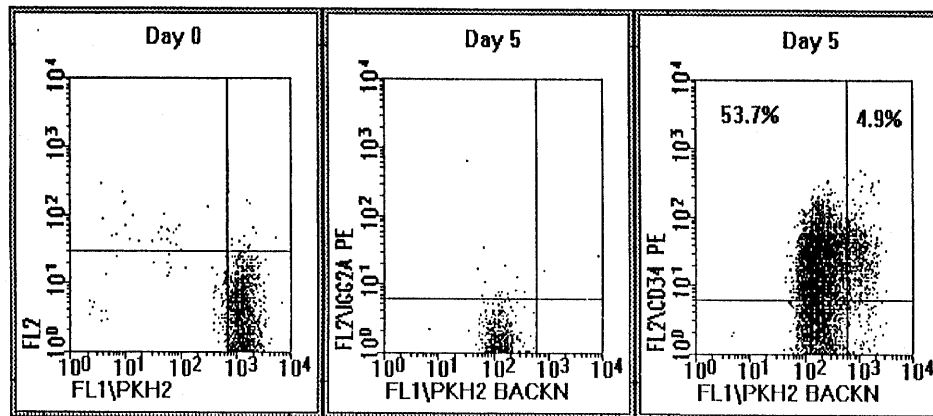
When needed, sorted CD34<sup>+</sup> cells were stained with PKH2 (Sigma ImmunoChemicals, St Louis MO) prior to their use in short-term culture as per manufacturer's instructions. Briefly, cells were suspended in 1 mL of diluent (Sigma ImmunoChemicals) and immediately transferred into a polypropylene tube containing 1 mL of 4 x 10<sup>-6</sup> mol/L PKH2 in diluent at room temperature. Following five minutes of incubation with frequent agitation, 2 mL of FCS (Hyclone, Logan, Utah) were added to the suspension for 1 min. The total volume was brought up to 8 mL with Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS, L-glutamine and antibiotics (complete medium) and the cells were washed 3 times in complete medium. All of the complete medium ingredients (except for FCS) were obtained from BioWhitaker, Walkersville, MD. Antibiotics consisted of penicillin and streptomycin which were used at 100 U/mL and 100 µg/mL, respectively. Following the first wash, cells were transferred to a new tube. After the third wash, cells were resuspended in complete medium and cultured with cytokines as described below.

### *Short-Term Cell Cultures*

On day zero, up to 10<sup>5</sup> cells/mL previously stained with PKH2 as described above were seeded in individual wells of 48-well tissue culture plates in complete medium and incubated at 37°C

in 100% humidified 5% CO<sub>2</sub> in air. At initiation and every 48h thereafter, cultures were supplemented with 200 ng/mL SCF, 100 ng/mL IL-3, and 100 ng/mL IL-6. Care was taken to avoid cell densities exceeding 10<sup>6</sup>/ml. Whenever such densities were reached, cultures were split and fresh medium and cytokines were added. Cells were removed as needed, counted, washed, and used for cell sorting, immunofluorescence flow cytometric analysis, or in clonogenic assays. Cells intended for cell sorting were stained with PE-conjugated CD34 for 20 min on ice. A sample of cultured cells not stained with any fluorochrome (except the original PKH2 from day zero) was used to determine the background PKH2 staining and to apply adequate compensation. Another sample was stained with PE-conjugated, isotype matched nonspecific myeloma protein and used to establish PE positivity. PKH2 fluorescence of cultured cells was compared to that of a sample fixed in 1%

paraformaldehyde on day 0 immediately after staining of fresh sorted CD34<sup>+</sup> cells in order to determine the green fluorescence intensity corresponding to cells that had not divided. This comparison allowed for the localization, within a dual parameter histogram (Figure 1, panel C), of the group of CD34<sup>+</sup> cells that have remained quiescent and are therefore CD34<sup>+</sup>PKH2<sup>bright</sup>. After determining the relative size of this group of cells, a group of approximately equal size of CD34<sup>+</sup> cells displaying low intensity PKH2 fluorescence was identified as CD34<sup>+</sup>PKH2<sup>dim</sup> (Figure 1, panel C). In all experiments, CD34<sup>+</sup>PKH2<sup>bright</sup> cells constituted less than 5% of total cells (Figure 1, panel C) and therefore both populations of CD34<sup>+</sup>PKH2<sup>dim</sup> and CD34<sup>+</sup>PKH2<sup>bright</sup> were smaller than 5% of total cultured cells. Flow cytometric analysis was done on samples stained with FITC-conjugated CD34 and PE-conjugated HLA-DR (BDIS) as previously described (20).



**Fig. 1.** Cell tracking of cells cultured for 5 days using the membrane dye PKH2. Fresh sorted mobilized peripheral blood CD34<sup>+</sup> cells were stained with PKH2 and analyzed on day 0 (left panel) then cultured in a short-term culture maintained with SCF, IL-3, and IL-6 as described in Materials and Methods. On day 5 cells were removed and stained with PE-conjugated isotype control antibodies (middle panel) or PE-conjugated CD34 (right panel) and analyzed. The PE signal of cells appearing in the panel on the left is background fluorescence from freshly sorted cells. Each two parameter histogram displays the fluorescence of PKH2 measured along the X axis and the indicated PE signal measured along the Y axis. The placement of the vertical cursor in the left panel was used as a reference to track the movement of cells to the left and to identify CD34<sup>+</sup> cells that remain to the right of this vertical (right panel) as those which have failed to respond to cytokine stimulation (CD34<sup>+</sup>PKH2<sup>bright</sup>). In this particular experiment 4.9% of the cultured cells were identified as CD34<sup>+</sup>PKH2<sup>bright</sup> cells on day 5. Arbitrarily chosen sorting windows were established for these cells along with an equal size population of CD34<sup>+</sup>PKH2<sup>dim</sup> cells for cell sorting.

### *Limiting Dilution Analysis (LDA) for the Determination of LTHC-IC*

Limiting dilution assays were performed as previously described (20). Briefly, fresh isolated CD34<sup>+</sup> cells, as well as cultured CD34<sup>+</sup>PKH2<sup>dim</sup> and CD34<sup>+</sup>PKH2<sup>bright</sup> cells were seeded at 64, 32, 16, 8, 4, and 2 cells/well in 100  $\mu$ l of complete medium supplemented with 200 ng/mL SCF, 25 ng/mL IL-3, 25 ng/mL IL-6, 10 ng/mL GM-CSF, and 2 U/mL erythropoietin into the wells of flat-bottomed 96-well plates. Depending on the type of cells used, only four of the cell dilutions mentioned above (starting at 64, 32 or 16) were delivered by hand into individual wells of culture plates using a multi-channel pipette. For every cell dilution a total of 96 wells were prepared. Plates were incubated at 37°C in 100% humidified atmosphere containing 5% CO<sub>2</sub> and fed with another 100  $\mu$ l of complete medium supplemented with cytokines on day 7. After 14 days, a total of 120  $\mu$ l of medium were removed from each well followed by the addition of 150  $\mu$ l of a mixture consisting of 3 parts FCS and 4 parts 3.3% methylcellulose and containing at a final concentration 5 x 10<sup>-5</sup> mol/L 2-mercaptoethanol, 100 ng/mL SCF, 25 ng/mL IL-3, 25 ng/mL IL-6, 25 ng/mL GM-CSF, and 2U/mL erythropoietin. Cells were mixed into the semisolid medium by gentle vortexing and the plates were incubated as described above. After 14 days the plates were scored under an inverted microscope for the presence of burst forming unit-erythroid (BFU-E), colony forming unit-granulocyte-macrophage (CFU-GM) or colony forming unit-mixed (CFU-GEMM) in every well. Wells were considered positive, indicating the presence of at least one LTHC-IC in the original cell inoculum deposited into the well, only if one or more hematopoietic colonies per well were detected. The number of negative wells per cell dose input was then calculated and the data used in a chi-minimalization assay (21) to calculate a frequency of LTHC-IC in a given test cell population. The ability of this modified assay to measure cells similar or identical in nature to the long-term culture-initiating cells assayed by the

methodology of Sutherland and coworkers (22) has been previously demonstrated (20).

### *Hematopoietic Progenitor Cell Assays*

Fresh isolated CD34<sup>+</sup> cells (500 to 10<sup>3</sup>/mL) or cultured cells (2 x 10<sup>3</sup> to 5 x 10<sup>3</sup>/mL) were suspended in duplicates in plastic 35-mm tissue culture dishes containing 1 mL 30% FBS, 5 x 10<sup>-5</sup> mol/L 2-mercaptoethanol, cytokines as indicated above, and 1.1% methylcellulose in IMDM. Cultures were incubated in 100% humidified 5% CO<sub>2</sub> in air at 37°C. After 14 days BFU-E, CFU-GM and CFU-GEMM were enumerated using an inverted microscope according to previously reported criteria (23).

### *Statistical Analysis*

All results are reported, where applicable, as mean  $\pm$  standard error of the mean. Statistical analysis on groups of data was performed using two-sided *t*-test or the non parametric Mann-Whitney test for independent measurements.

## RESULTS

### *Determination of the Frequency and Number of LTHC-IC in Mobilized Peripheral Blood Grafts*

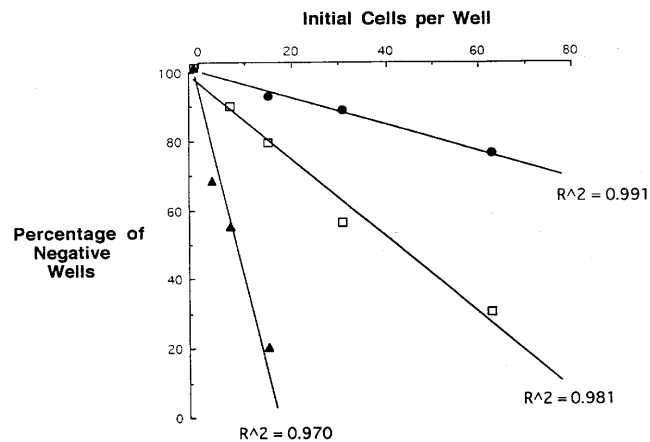
The frequency of LTHC-IC in freshly isolated mobilized PB cells was estimated from selected total CD34<sup>+</sup> cells. Figure 2 depicts plots generated from data obtained from the limiting dilution analysis of MPB, BM, and CB CD34<sup>+</sup> cells using a stromal cell-free suspension assay. In these representative experiments, the frequency of LTHC-IC among BM CD34<sup>+</sup> cells was 1.81% (with 1.42 and 2.19% as the 95% confidence limits) while that detected in CB CD34<sup>+</sup> was 10.39% (with 9.15 and 11.62% as the 95% confidence limits). A lower frequency of LTHC-IC calculated to be 0.35% (with 0.24 and 0.46% as the 95% confidence limits) was detected in mobilized PB cells. From similar additional experiments it was calculated that the average

frequencies of LTHC-IC among MPB, BM, and CB CD34<sup>+</sup> cells were  $0.49 \pm 0.36\%$  (n=4),  $2.01 \pm 0.98\%$  (n=6), and  $7.56 \pm 2.48\%$  (n=8), respectively.

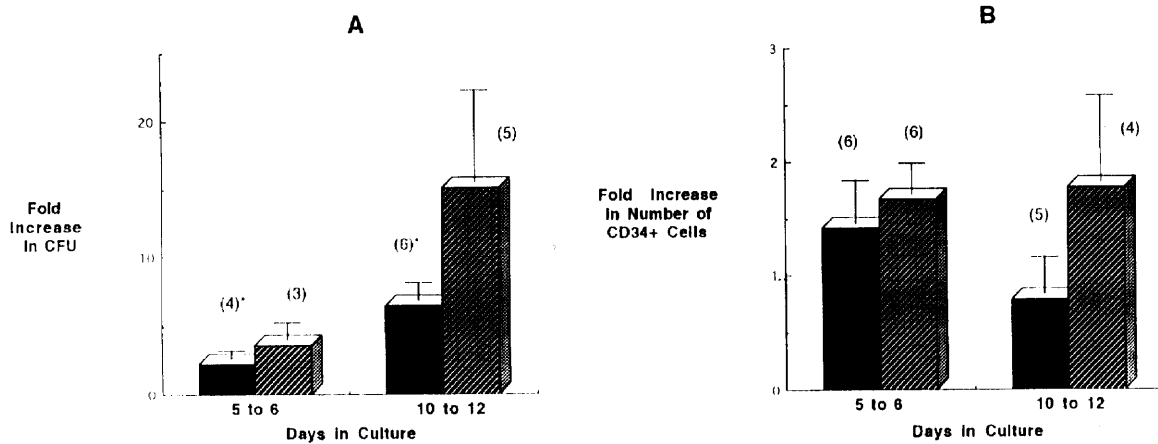
### *Ex vivo Expansion*

Low density unfractionated or CD34<sup>+</sup> selected mobilized PB cells were expanded in vitro for up to 12 days in the presence of SCF, IL-3, and IL-6 and two parameters were examined to assess the expansion potential of these cells at two different time points. Production of both clonogenic progenitor cells and CD34<sup>+</sup> cells were assayed 5 to 6 and 10 to 12 days after the initiation of short-term suspension cultures (Figure 3). Cultures initiated with either low density cells or

with selected CD34<sup>+</sup> cells contained a higher number of assayable progenitor cells 5 to 6 days later and were capable of sustaining an additional increase in the number of these cells at 10 to 12 days. Of interest is that a greater increase in the number of clonogenic cells was detected in cultures initiated with low density PB cells than with selected CD34<sup>+</sup> cells at both time points examined (Figure 3, panel A). However, a modest increase in the number of CD34<sup>+</sup> cells present in short-term cultures was detected 5 to 6 days later followed by a decline in the number of CD34<sup>+</sup> cells after 10 to 12 days especially in cultures initiated with selected CD34<sup>+</sup> cells (Figure 3, panel B).



**Fig. 2.** Limiting dilution analysis of data from three representative experiments in which decreasing numbers of fresh isolated mobilized peripheral blood (solid circles), bone marrow (open squares), and cord blood (solid triangles) CD34<sup>+</sup> cells were plated in suspension cultures in flat bottomed 96 well plates. After 14 days, all the wells were overlaid with semi-solid medium as described in Materials and Methods. The percentage of negative wells was then calculated two weeks later after all individual wells were scored for the presence of hematopoietic colonies. In these experiments the LTHC-IC frequency among mobilized PB CD34<sup>+</sup> cells was 0.35% (with 0.24 and 0.46% as the 95% confidence limits), that calculated for BM CD34<sup>+</sup> cells was 1.81% (with 1.42 and 2.19% as the 95% confidence limits), and among CB CD34<sup>+</sup> cells the LTHC-IC frequency was 10.39% (with 9.15 and 11.62% as the 95% confidence limits). The R<sup>2</sup> values for each line is shown next to it. Similar values were obtained from 3, 5, and 7 additional experiments using MPB, BM, and CB cells, respectively.



**Fig. 3.** Fold increase in the number of assayable colony forming units (panel A) and cultured CD34<sup>+</sup> cells (panel B) in short-term cultures initiated with selected CD34<sup>+</sup> (solid bars) or low density (hatched bars) mobilized peripheral blood cells. These parameters were measured 5 to 6 days and 10 to 12 days following culture initiation. Short-term suspension cultures were maintained by the addition of SCF, IL-3, and IL-6 every 48 hr as described in Materials and Methods. CFU refers to the total sum of erythroid, granulocytic/macrophage, and mixed colonies detected at every time point. The number of experiments in every group is indicated in parentheses above each bar. A statistically significant difference ( $p < 0.05$ ) was detected between groups identified with an asterisk.

**Table 2.** Frequency of LTHC-IC among fresh and cultured mobilized peripheral blood (MPB), bone marrow (BM), and cord blood (CB) CD34<sup>+</sup> cells

Phenotype of Cells*	Frequency of LTHC-IC per 100 cells								
	MPB			BM			CB		
	Exp.1	Exp.2	Exp.3	Exp.1	Exp.2	Exp.3	Exp.1	Exp.2	Exp.3
Day 0 CD34 <sup>+</sup>	0.45	0.35	1.00	0.85	6.71	2.07	12.40	21.55	10.39
Day 2 CD34 <sup>+</sup> PKH2 <sup>bright</sup>	2.19	1.01	ND <sup>¶</sup>	ND	ND	ND	ND	ND	ND
Day 2 CD34 <sup>+</sup> PKH2 <sup>dim</sup>	1.40	0.46	ND	ND	ND	ND	ND	ND	ND
Day 5 -7 CD34 <sup>+</sup> PKH2 <sup>bright</sup>	0.32	0.08	0.35	2.02	4.44	2.64	1.62	2.01	5.77
Day 5 -7 CD34 <sup>+</sup> PKH2 <sup>dim</sup>	0.14	<0.01 <sup>†</sup>	<0.01	6.62	4.34	4.38	0.88	0.22	7.99

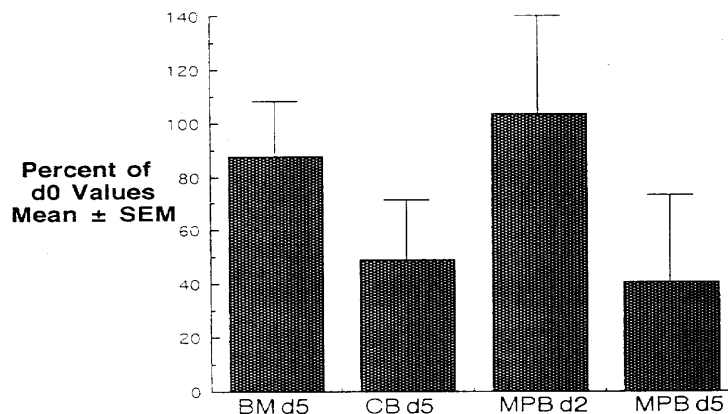
\* Isolated CD34<sup>+</sup> cells were stained with PKH2 on day 0 and cultured for 5 to 7 days in suspension cultures as described in Materials and Methods. Frequencies of LTHC-IC were calculated for CD34<sup>+</sup> cells on day 0 and for CD34<sup>+</sup> PKH2<sup>bright</sup> and CD34<sup>+</sup> PKH2<sup>dim</sup> cells isolated on day 2 or between days 5 and 7.

† Frequencies of LTHC-IC for these groups were undetectable.

¶ Not determined.

We next examined *ex vivo* expanded mobilized PB CD34<sup>+</sup> cells for their LTHC-IC content. In order to be able to focus our analysis on *de novo* generated CD34<sup>+</sup> cells, we used the membrane dye PKH2 to track cells in culture and to allow for the isolation of CD34<sup>+</sup> cells which have divided and therefore have a "dim" PKH2 fluorescence relative to that displayed by PKH2-stained cells at time 0. Figure 1 depicts one experiment in which PKH2 staining at time 0 is compared to that seen 5 days later and demonstrates how newly formed CD34<sup>+</sup> cells were identified for isolation and examination. Along with CD34<sup>+</sup>PKH2<sup>dim</sup> cells, CD34<sup>+</sup>PKH2<sup>bright</sup> cells (these represent cells which are resistant to cytokine stimulation (20) and therefore fail to proliferate) were isolated. Following 5 to 7 days in culture, both PKH2<sup>dim</sup> and PKH2<sup>bright</sup> fractions of cultured CD34<sup>+</sup> cells displayed a reduced frequency of LTHC-IC relative to fresh cells (Table 2). This behavior was similar to what was observed with CB cells where lower frequencies

of LTHC-IC were detected after 5 to 7 days in culture. However, the behavior of mobilized PB CD34<sup>+</sup> cells in culture was not similar to that of BM CD34<sup>+</sup> cells utilized in similar experiments (Table 2). We therefore measured the frequency of LTHC-IC of mobilized PB cells after 2 days in culture. Newly formed PB CD34<sup>+</sup> cells present in culture after 48 hours, contained a higher frequency of LTHC-IC than that detected among freshly isolated cells (Table 2). Given the minimal increase in cell number in cultures of CD34<sup>+</sup> cells and the decline in the percentage of CD34<sup>+</sup> cells present in culture, the increased frequency of LTHC-IC did not translate into an overall significant increase in the absolute number of LTHC-IC present among peripheral blood CD34<sup>+</sup> cells expanded *in vitro* for 2 days (Figure 4). In addition, in excess of 50% loss of LTHC-IC activity was detected following 5 days of *ex vivo* expansion of peripheral blood CD34<sup>+</sup> cells (Figure 4).



**Fig. 4.** Percent recovery of total numbers of LTHC-IC present in bone marrow, cord blood, and mobilized peripheral blood CD34<sup>+</sup> cells expanded *in vitro* for 2 or 5 days. Each bar depicts the total number of LTHC-IC detected in culture at a given day represented as a percentage of that enumerated in the culture on day 0. Short-term suspension cultures were maintained by the addition of SCF, IL-3, and IL-6 every 48 hr as described in Materials and Methods.

Table 3. Total number of LTHC-IC present in a typical T-cell depleted allogeneic bone marrow graft, an autologous marrow graft, and a mobilized peripheral blood graft

Source of Cells	Values per Kg body weight				
	Average number of MNC ( $\times 10^6$ )	Percent CD34 <sup>+</sup>	Total number of CD34 <sup>+</sup> ( $\times 10^5$ )	LTHC-IC Frequency	Total number of LTHC-IC
Allogeneic* BM	41.12 $\pm$ 0.29	1.93 $\pm$ 0.25	6.9	2.01	13,869
Autologous† BM	62.37 $\pm$ 0.49	1.78 $\pm$ 0.27	10.7	2.01	21,507
MPBSC‡	648.8 $\pm$ 583.7	2.82 $\pm$ 2.65¶	145.5	0.49	71,050

\* Values for allogeneic BM grafts were calculated from 25 patients receiving T-cell depleted grafts. T cell depletion of these grafts was achieved by soybean agglutination and E-rosetting with sheep erythrocytes using a modified procedure of that previously described by Reisner et al (24,39)

† Values for autologous BM grafts were calculated from 10 patients.

‡ Values for MPBSC were obtained from 10 of the 11 patients receiving high dose cyclophosphamide succeeded by sequential treatment with IL-3 followed with either GM-CSF (n=4) or G-CSF (n=7).

¶ Percentage of CD34<sup>+</sup> cells in mobilized peripheral blood was calculated as the average of that detected in 22 leukapheresis products. As such, this percentage is not in agreement with the total number of CD34<sup>+</sup> cells/Kg since this figure was derived from the actual number of CD34<sup>+</sup> cells contained in the grafts of the 11 patients described in Table 1.

The rapid increase in the frequency of LTHC-IC among cultured MPB CD34<sup>+</sup> cells, suggested that a large fraction of mobilized PB CD34<sup>+</sup> cells may be metabolically active residing in S or G2 + M phases of cell cycle. However, flow cytometric analysis of the cell cycle status of freshly isolated mobilized PB CD34<sup>+</sup> cells revealed that in excess of 96% of these cells were in G0/G1, an observation reminiscent of CB CD34<sup>+</sup> cells (20).

The low frequency of LTHC-IC among mobilized PB CD34<sup>+</sup> cells raised the question of how many of these primitive hematopoietic progenitor cells were present in a typical graft of PB stem cells and whether such numbers were comparable to those detected in BM grafts. Using the mean values of the frequencies of LTHC-IC derived from the present studies, we calculated the overall number of LTHC-IC contained within a typical T-cell depleted allogeneic bone marrow graft, an autologous marrow graft, and a mobilized PB graft (Table 3). Allogeneic marrow grafts used for these calculations were T cell depleted as previously described by Reisner and coworkers (24) with minor modifications using soybean

agglutination and rosetting with sheep RBC. Despite the lower frequency of LTHC-IC detected among mobilized PB CD34<sup>+</sup> cells, the high number of CD34<sup>+</sup> cells collected following HD-CTX plus growth factor mobilization, resulted in a total number of LTHC-IC per Kg body weight, exceeding up to 5 and 3 times that calculated for allogeneic and autologous BM grafts, respectively (Table 3).

## DISCUSSION

Mobilized peripheral blood hematopoietic progenitor cells have been extensively used in the clinic as primary autologous stem cell grafts or for supportive therapy following myelosuppressive treatments. Infusion of MPBPC results in early engraftment and a reduction in the duration of cytopenia associated with ablative conditioning regimens (25, 26). Harvests of MPBPC have been traditionally conducted by pheresing peripheral blood in order to collect an adequate number of leukocytes. This method yields large numbers of cells, such that, in general, sufficient numbers of CD34<sup>+</sup> cells required to generate

clinically suitable grafts are collected. Consequently, the need to exercise *ex vivo* expansion of MPBPC is of little clinical interest, except in the small minority of patients who are poor mobilizers or if such an approach is deemed essential for efficient retroviral mediated gene transfer. Recently, MPBPC have been successfully used for allogeneic stem cell transplantation demonstrating the practicality and adequacy of these cells in more than just the autologous setting.

We have previously examined MPB CD34<sup>+</sup> cells and their subsets and compared the functional activities of these cells to their BM counterparts using a number of *in vitro* assays (8). Subsets of mobilized PB CD34<sup>+</sup> cells, namely CD34<sup>+</sup> HLA-DR<sup>-</sup> cells, were found, in our previous studies (8), to support long-term *in vitro* hematopoiesis as measured by the cumulative production of assayable clonogenic cells (27, 28). However, production of committed progenitor cells *in vitro* may not be a suitable assay for the evaluation of *ex vivo* expansion potential of primitive HPC. To better understand the *ex vivo* expansion capability of mobilized PB CD34<sup>+</sup> cells, we investigated the production of primitive HPC *in vitro* by focusing on the frequency of LTHC-IC among *de novo* generated CD34<sup>+</sup> cells. To that end, we chose to expand mobilized PB CD34<sup>+</sup> cells with SCF, IL-3, and IL-6, a cytokine combination previously proven effective as an *in vitro* priming regimen for retroviral mediated gene transfer into primitive HPC (29,30).

It has been previously demonstrated that MPB contains cells capable of sustaining long-term *in vitro* hematopoiesis in stromal cell-supported cultures (31). We therefore began these studies by determining the frequency of LTHC-IC using our modified limiting dilution analysis methodology (20) which revealed that the frequency in mobilized PB CD34<sup>+</sup> cells was very similar to that reported by Henschler and coworkers (16). In their studies, Henschler and coworkers (16) used a stromal cell-based LDA assay to examine PB CD34<sup>+</sup> cells derived from patients receiving a different chemotherapy regimen than those utilized in our study.

Although the read-out of the LDA assays of Henschler et al. (16) was the cobblestone area formation, as described by Breems et al. (32), very similar LTHC-IC frequencies were obtained in our studies. These results indicate that our methodology measures identical or very similar primitive HPC as those described by Sutherland et al. (22) or by Breems and coworkers (32). Furthermore, akin to our results, long-term culture-initiating cells were not expanded *in vitro* in the report of Henschler et al (16), although a concomitant increase in cell number and assayable progenitor cells was achieved. In a recent study, Carlo-Stella and coworkers (33) examined the *ex vivo* proliferative potential of MPB cells and reported that even with more purified populations of primitive HPC (CD34<sup>+</sup> CD45RA<sup>-</sup>), only "maintenance or moderate decrease" in the number of long-term culture-initiating cells was possible after 7 to 10 days of *in vivo* culture.

Our results indicate that the frequency of LTHC-IC among MPB CD34<sup>+</sup> cells is much lower than that detected in cells possessing the same phenotypic make-up isolated from BM and CB. Although in agreement with other studies indicating that MPB have a lower frequency of these primitive HPC than BM, our results differ from those of Udomsakdi et al (34) in the degree of these differences. While the frequency of LTHC-IC in BM CD34<sup>+</sup> cells was approximately 6-fold higher than that detected in MPB CD34<sup>+</sup> cells in our studies, Udomsakdi and coworkers (34) reported a frequency of long-term culture-initiating cells in peripheral blood which was 100-fold lower than that observed in BM. However, in the studies reported by the group from Vancouver (34) steady state PB cells were examined rather than cells mobilized into the periphery with chemotherapy and growth factor treatment, a variable which may very well explain the differences between the two studies. It is important to point out however, that given the large number of CD34<sup>+</sup> cells contained in MPB, the absolute number of LTHC-IC contained in a typical graft of peripheral blood HPC exceeds that normally found in a BM graft (Table 3).

It is essential that conclusions drawn from

these studies be viewed with some caution. Although patients in this study were untreated, the effects of cancer on the expansion potential of their HPC remains unknown. This is especially important since the expansion potential of these cells was compared to normal BM and CB cells. The deleterious effects of chemotherapy on the ability of MPB to undergo expansion has been already established (35). However, since all patients included in the present studies were chemotherapy-naive, such a concern may not be valid. Whereas fresh CB and BM samples were utilized in these studies, a lag period of 48 hr separated the collection of MPB cells and their actual processing. Although the expansion potential of MPB CD34<sup>+</sup> cells may have been compromised by this delay, such an event is probably unlikely. We have previously examined MPB obtained under these same conditions using the long-term production of assayable clonogenic cells as a measure of ex vivo expansion potential (8). These studies demonstrated similar functional properties between phenotypically matched fractions of BM and MPB CD34<sup>+</sup> cells (8) suggesting that the additional 24 hr delay in the processing of MPB cells may be inconsequential. Finally, the variation in the mobilization regimens preclude our ability to confirm whether the limited expansion potential of MPB is universal or whether our observations are restricted to the regimens employed in these studies. However, we have previously shown that changes in progenitor cell compartments in the MPB of patients receiving HD-CTX were similar regardless of the growth factor(s) used (14).

Since we have previously shown that mobilized PB CD34<sup>+</sup> cells were very similar in their behavior to BM cells, we hypothesized that differences observed in the frequencies of LTHC-IC between ex vivo expanded BM and PB CD34<sup>+</sup> cells may reflect differences in the kinetics of ex vivo proliferation of these cells. This hypothesis suggested to us two possible alternatives. First, it is possible that mobilized PB CD34<sup>+</sup> cells may reside in active phases of cell

cycle. Cell cycle analysis of these cells demonstrated however, that they were quiescent. In excess of 96% of these cells were in G<sub>0</sub>/G<sub>1</sub> phases of cell cycle, a finding similar to what we previously reported for CB CD34<sup>+</sup> cells (36). To and coworkers (9) have recently shown that mobilized PB CD34<sup>+</sup> cells have a low uptake of the mitochondrial dye rhodamine 123, an observation consistent with our reported high percentage of cells in G<sub>0</sub>/G<sub>1</sub> phases of cell cycle. However, Danova et al. (37) have recently demonstrated that following chemotherapy and growth factor treatment, a higher percentage of MPB cells in S phase of cell cycle as well as cells with a higher labeling index were detected compared to steady state values. Second, the rate of loss of LTHC-IC activity may be different between cultured BM and mobilized PB CD34<sup>+</sup> cells such that trends of behavior similar to those observed with BM cells may be detected if mobilized PB cells were examined at earlier time points following initiation of cultures. Indeed, when LTHC-IC frequencies were estimated 2 days following ex vivo expansion instead of 5 to 6, a modest increase in the frequency of mobilized PB LTHC-IC, relative to day 0 values, was observed.

When considered collectively, these data suggest that the ability of mobilized PB CD34<sup>+</sup> cells to undergo ex vivo expansion may be compromised by the extensive "in vivo expansion" required to generate the massive numbers of nucleated cells and CD34<sup>+</sup> cells collected after mobilization. Preliminary results from our laboratory (38) indicate that loss of LTHC-IC activity among ex vivo expanded CD34<sup>+</sup> cells correlates with the number of divisions cells go through (data not shown). Given these experimental findings, it might be possible that mobilized PB CD34<sup>+</sup> cells may have undergone maximal "in vivo expansion" such that ex vivo expansion of primitive progenitor cells, which requires additional cellular proliferation, may not be possible. The ramifications of these results on the areas of gene transfer and other related applications are yet to be investigated.

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