Uncontrolled-rate freezing of peripheral blood progenitor cells allows successful engraftment by sparing primitive and committed hematopoietic progenitors

CAMILLO ALMICI, PIERO FERREMI, ARNALDA LANFRANCHI, EMILIO FERRARI, ROSANNA VERARDI, MIRELLA MARINI, GIUSEPPE ROSSI

Background and Objectives. Uncontrolled-rate freezing (URF) techniques, which are fast and easy, could represent an attractive alternative to controlled-rate cryopreservation procedures which are time consuming and require high-level technical abilities. It was the aim of the present study to evaluate, on a routine basis, whether URF might spare primitive hematopoietic progenitors and maintain engrafting capacity.

Design and Methods. One-hundred and nineteen peripheral blood progenitor cells (PBPC) collections from 104 patients with hematologic malignancies were cryopreserved in bags, with an URF procedure, in a cryoprotectant solution consisting of PBS, HSA and 10% DMSO and stored in liquid nitrogen. PBPC bags were tested before cryopreservation and at thawing for primitive (LTC-IC) and committed hematopoietic progenitors (CFU-Mix, BFU-E, CFU-GM) by means of long- and short-term culture assays, respectively. In addition, PBPC bags were evaluated for $CD34^+$ cell numbers.

Results. Although thawing was associated with a statistically significant reduction of the absolute number of nucleated cells, recovery of LTC-IC, CFU-Mix, BFU-E, CFU-GM and $CD34^+$ cells was not affected by the freezing/thawing procedures. No adverse effects were reported at thawing and only mild transient reactions were recorded in 22 patients during reinfusion of cryopreserved PBPC. All the patients underwent myeloablative therapy followed by reinfusion of PBPC, and prompt and rapid hematopoietic recovery was obtained in all patients.

Interpretation and Conclusions. Our freezing procedure is fast and easy, and allows rapid hematopoietic recovery after myeloablative therapy by sparing primitive and committed hematopoietic progenitors. Our study strongly supports technical improvements aimed at cost reduction and feasibility of routine freezing procedures.

Key words: uncontrolled-rate freezing, stem cell transplantation, peripheral blood progenitor cells, LTC-IC, $CD34^+$ cells.

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From the Department of Hematology, BMT Unit (CA, GR); Blood Bank (PF, EF, MM) and 3° Servizio Analisi, Institute of Chemistry, (AL, RV), Spedali Civili di Brescia, University of Brescia, Brescia, Italy.

Correspondence: Camillo Almici, MD, Department of Hematology, BMT Unit Spedali Civili di Brescia, pz.le Spedali Civili 1, 25125 Brescia, Italy. E-mail: almici@osp.unibs.it

The expanding indications for high-dose chemotherapy followed by peripheral blood stem cell (PBSC) transplant are associated with an exponentially growing request for PBPC collection, cryopreservation and storage. According to current therapeutic strategies, cryopreservation of hematopoietic progenitor cells is required for virtually all autologous and some allogeneic blood cell transplants.[1-3] Freezing and storage conditions capable of limiting the functional damage of hematopoietic progenitor cells are crucial pre-requisites to guarantee the maintenance of progenitor cell reconstituting activity on a long-term basis.[4] Hematopoietic progenitor cells are cryopreserved in a cryoprotective solution supplemented with dimethylsulfoxide (DMSO), as an intracellular cryoprotectant, plus either human serum albumin (HSA) or plasma, with or without hydroxyethylstarch, as extracellular cryoprotectants.[5] The standard procedure for PBPC cryopreservation includes controlled-rate freezing and subsequent storage in liquid nitrogen at –196°C;[5] however, this procedure is time-consuming and requires a high level of technical expertise and expensive computer-assisted devices. The technique of uncontrolled-rate freezing (URF), which is fast and easy, could represent an attractive alternative aimed at cost reduction and facilitation of routine freezing procedures.[6,7]

In the present study, we report our experience of URF of PBPC, in a cryoprotectant solution consisting of phosphate buffered saline (PBS), HSA and DMSO. To evaluate the potentially detrimental effects of the URF procedure, quality control tests involved analysis of primitive and committed progenitors, by means of long- and short-term culture assays, respectively. In addition, the capacity of PBPC to reconstitute hematopoiesis was evaluated in 104 consecutive patients undergoing high-dose therapy for their hematologic malignancies.

Design and Methods

Patients, mobilization and collection of PBPC

Between March 1997 and August 2001 PBPC were mobilized and collected from 104 patients (59 males and 45 females; median age 47 years, range 16-71) with hematologic malignancies (25 acute myeloid leukemia, 20 non-Hodgkin's lymphoma (NHL), 1 NHL with immunodeficiency virus (HIV), 12 HD, 2 HD-HIV and 44 multiple myeloma). Informed consent was obtained from all patients according to the regulations of the local ethics committees. Depending on the dif-

ferent diagnoses and therapeutic schedules, PBPC mobilization was accomplished with different chemotherapy regimens (Table 1) followed by granulocyte colony-stimulating factor (G-CSF) (5-10 μg/Kg/day). Apheretic procedures were started when the circulating $CD34^+$ cell count was >20/μL, by using a Fresenius AS 204 cell separator (Bad Homburg, Germany) and completed when at least $3\text{-}4\times10^6$/Kg $CD34^+$ cells had been collected, in relation to disease and conditioning regimen.

Cryopreservation and thawing procedures

The leukapheresis products were washed twice (2500 rpm, 15 minutes) with phosphate-buffered saline without Ca^{++} and Mg^{++} (PBS, Gibco Laboratories, Grand Island, NY, USA) and resuspended in a 20% solution of ice-cold HSA (Immuno, Vienna, Austria). Cells were counted and resuspended in an ice-cold cryoprotectant solution consisting of PBS (80%, vol/vol), HSA (10%, vol/vol) and DMSO (10%, vol/vol) (Tera Pharmaceuticals, Buena Park, CA, USA) at a final cellular concentration ranging from 40×10^6 to 200×10^6 per mL. Cell suspensions, rapidly transferred into freezing bags (DF-700, Gambro, Hechingen, Germany), were placed at –80°C for 24-48 hours and subsequently stored in liquid nitrogen until reinfusion.

Cryopreserved PBPC were thawed by rapid immersion into a 37°C water bath. Cells were reinfused into patients via a central venous catheter. Aliquots of thawed cells, collected to be assayed for progenitor cell contents, were slowly diluted stepwise over 10 minutes with 10 times the volume of Iscove's modified Dulbecco medium (IMDM) supplemented with 10% human albumin. Afterwards, cells were washed twice and resuspended in appropriate medium for short- and long-term culture assays. Cell viability was assessed by the trypan blue dye exclusion test.

Immunofluorescence analysis

Fresh and thawed cells were counted, their concentration adjusted to 0.5×10^6/mL and their surface antigen phenotype determined by immunofluorescence analysis, using phycoerythrin-conjugated anti-CD34 antibody (HPCA-2). Phenotypic analysis was performed with a FACScan (Becton Dickinson, Mountain View, CA, USA) flow cytometer.

Progenitor colony-forming assay

The assays for CFU-Mix, BFU-E and CFU-GM were performed as described in detail elsewhere.[8] Briefly, fresh and thawed nucleated cells (5×10^4) were plated in 35-mm Petri dishes in 1 mL aliquots of IMDM containing 30% FBS, 10^{-4} M 2-mercaptoethanol, and 1.1% (w/v) methylcellulose (Stem Cell Technologies, Vancouver, Canada). Cultures were stimulated with a combination of cytokines (interleukin-3 (IL-3) 10 ng/mL, Sandoz, Basel, Switzerland; granulocyte colony-stimulating factor 10 ng/mL, Amgen, Thousand Oaks, CA, USA; granulocyte-macrophage colony-stimulating factor 10 ng/mL, Sandoz; and erythropoietin 3 U/mL, Amgen). Progenitor cell growth was evaluated after incubation (37°C, 5% CO_2) for 14 days in a humidified atmosphere. Four dishes were set up for each individual data point per experiment. CFU-Mix, containing both erythroid and granulocytic cells, BFU-E containing >500 cells, and CFU-GM containing >40 cells were all scored from the same dishes.

Table 1. Characteristics of patients undergoing PBPC collection and a high-dose therapy program.

Patients	104
Sex (male/female)	59/45
Age (years) (range)	47 (16-71)
Diseases	
AML	25
NHL	20
NHL-HIV	1
HD	12
HD-HIV	2
Multiple myeloma	44
Mobilization regimen	
High dose ara-C	25
Cyclophosphamide 7 g/m^2	39
Cyclophosphamide 4 g/m^2	33
Other	7
PBPC collection	
Total number of aphereses	119
Patients undergoing one aphereses	89
Patients undergoing two aphereses	15
Mean processed blood (mL) (range)	8940 (4400-12300)
Mean collected volume (mL) (range)	183 (110-253)
Mean freezing/thaw interval (days) (range)	54 (27-298)
Conditioning regimens	
BU/Cy 200 mg/kg	19
BEAM	21
CBV	14
Mel 200 mg/m^2	41
Other	9
$CD34^+$ cells$\times10^6$/kg infused (range)	6.3 (3.1-12)
Hematopoietic recovery (days)	
ANC (>0.5×10^9/L) (range)	10 (8-14)
Plt (>20×10^9/L) (range)	11 (7-15)
Plt (>50×10^9/L) (range)	19 (10-77)

Table 2. Absolute recovery of primitive and committed progenitor cells (mean±SD) after uncontrolled-rate freezing of apheretic products.

	Numbers	*Pre-freezing*	*Post-thawing*	*p*
Nucleated cells ($\times10^9$)	119	21±4	17±2	<0.004
CD34$^+$ cells ($\times10^6$)	96	445±237	426±269	<0.6
LTC-IC ($\times10^5$)	45	17±4	18±3	<0.6
CFU-Mix ($\times10^5$)	63	12.3±3.1	9.2±2.3	<0.08
BFU-E ($\times10^5$)	63	143±57	131±27	<0.3
CFU-GM ($\times10^5$)	63	437±98	324±74	<0.1

Cell viability was assessed by the trypan blue dye exclusion test.

Long-term culture (LTC) assay

The LTC assay was performed on fresh and thawed nucleated cells according to methods previously described.[9] Briefly, test cell suspensions (5×10^6) were seeded into cultures containing a feeder layer of irradiated (8000 cGy) murine M2-10B4 cells (3×10^4/cm^2, kindly provided by Dr C. Eaves, Terry Fox Laboratories, Vancouver, Canada) engineered by retroviral gene transfer to produce human IL-3 and G-CSF. Test cells were resuspended in complete medium consisting of α-medium (Gibco) supplemented with 12.5% FBS, 12.5% horse serum, L-glutamine (2 mM), 2-mercaptoethanol (10^{-4} M), inositol (0.2 mM), folic acid (20 mM) and freshly dissolved hydrocortisone (10^{-6}M). At 7-day intervals, cultures were *demi-depopulated* by removal of half the culture volume followed by replacement with fresh complete medium. After 5 weeks in culture, adherent and non-adherent cells harvested by trypsinization were pooled, washed and assayed together for clonogenic cells in standard methylcellulose cultures. The total number of clonogenic cells present in 5-week-old LTC provides a relative measure of the number of LTC-IC originally present in the test suspension.[10] Absolute LTC-IC values were calculated by dividing the total number of clonogenic cells by four, which is the average output of clonogenic cells per LTC-IC, according to limiting dilution analysis studies reported by others.[10]

High-dose chemotherapy and PBPC reinfusion

After high-dose chemotherapy (Table 1) all patients underwent reinfusion of PBPC. The times to recover a neutrophil count $>0.5\times10^9$/L and platelet counts $>20\times10^9$/L and $>50\times10^9$/L were recorded (Table 1).

Statistical analysis

The Student's t-test for paired data was used to test for significance of changes in the comparison of data involving counts (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, California, USA).

Results

The effects of URF on PBPC was evaluated in 104 consecutive patients undergoing mobilization for therapeutic purposes. Eighty-nine patients (86%) underwent a single apheretic collection, while fifteen patients (14%) required a second procedure to obtain the targeted number of CD34$^+$/Kg cells in order to proceed to high-dose therapy. Therefore, a total of 119 collections were analyzed. The mean volume of blood processed during each apheretic collection was 8940 mL (range 4400-12300), and the mean volume collected was 183 mL (range 110-253). The freezing procedures required on average 1-1.5 hour to be completed. The mean storage time in liquid nitrogen was 54 days (range 27-298). The mean cell viability of thawed cells, determined by the trypan blue dye exclusion test, was 93±2%, with no significant differences in relation to the cellular concentration at freezing. The absolute numbers of nucleated cells were statistically significantly lower after thawing ($21\pm4\times10^9$ vs $17\pm2\times10^9$, $p \leq 0.004$). The effects of URF on primitive and committed progenitor cells were evaluated by means of long- and short-term culture assays, respectively; the numbers of CD34$^+$ cells were also recorded. Since progenitor cell recovery calculated on a *per number* basis, according to the number of cells plated, could be affected by a selective cell loss induced by freezing/thawing procedures and might not be a reliable parameter for

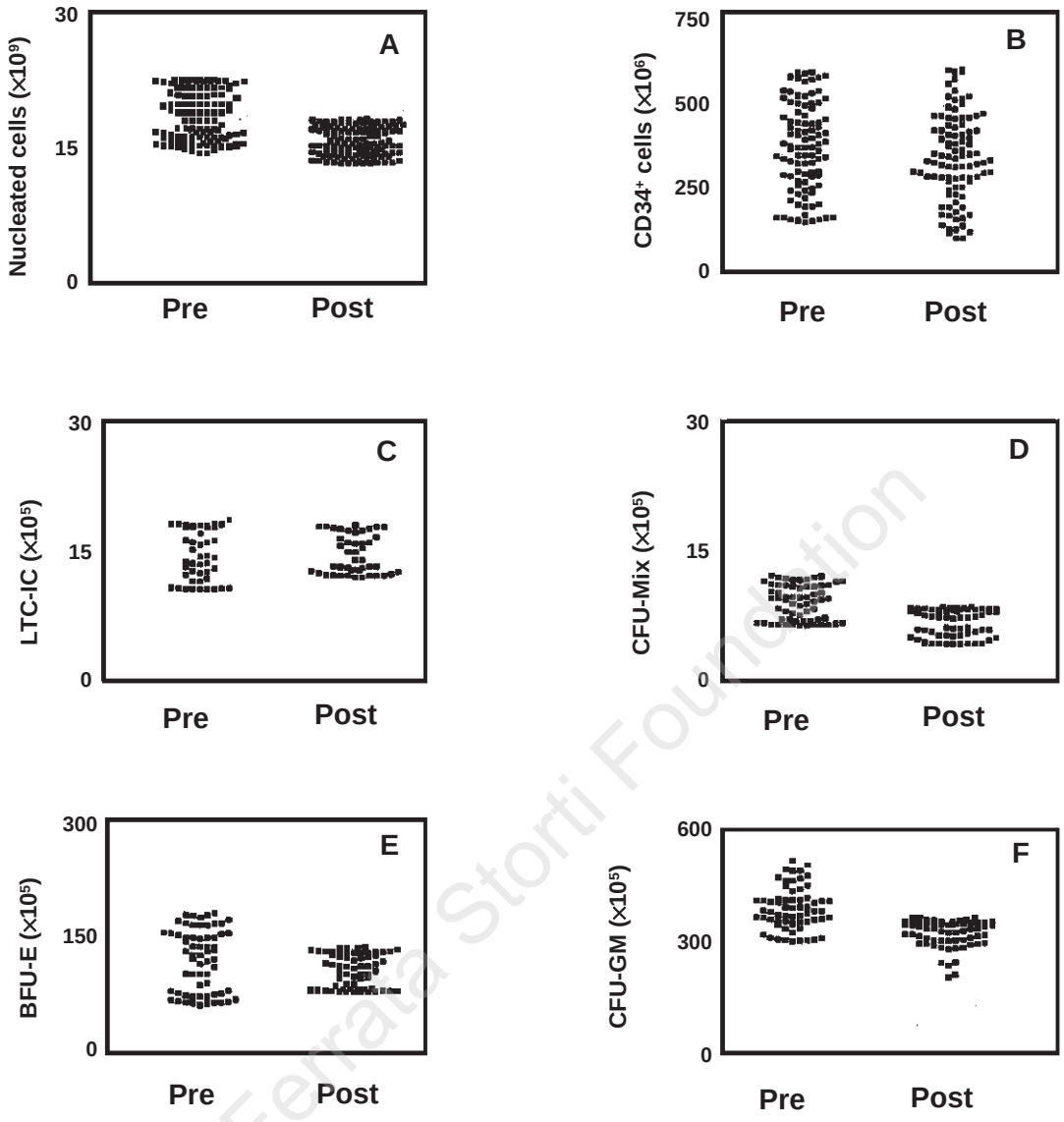

Figure 1. Absolute counts determined pre-freezing and post-thawing after URF procedures. The different panels show the single determinations performed for nucleated cells (A), CD34⁺ cells (B), LTC-IC (C), CFU-Mix (D), BFU-E (E) and CFU-GM (F).

clinical purposes, we compared absolute numbers of hematopoietic progenitor cells pre- and post-freezing (Table 2). Despite the significant reduction in nucleated cell numbers associated with thawing, the recovery of CD34⁺ cells, primitive (LTC-IC) and committed (CFU-Mix, BFU-E, CFU-GM) progenitor cells was not affected by the freezing/thawing procedure. Moreover, post-freezing recoveries were not influenced by storage duration or cellular concentration.

When the 104 patients underwent myeloablative chemotherapy followed by reinfusion of PBPC, no adverse events (e.g. clump formation) were reported at thawing and only mild transient reactions were recorded in 22 patients at reinfusion of the cryopreserved cells (flushing 11, headache 7, abdominal cramps 5, hypertension 3, shivering 2, fever 1). Hematopoietic recovery was documented in all patients with a mean time to neutrophil ($\geq 0.5\times10^9$/L) and platelet recovery ($\geq 20\times10^9$/L and $\geq 50\times10^9$/L) of 10 days (range 8-14), 11 days (range 7-15) and 19 days (range 10-77), respectively.

Discussion

Until the late 1980s, bone marrow (BM) was the source of progenitor cells routinely used to reconstitute hematopoiesis in patients undergoing chemo-radiotherapy for their hematologic malignancies. BM cells were cryopreserved, at a cooling rate of 1°C per minute, by using controlled-rate cryopreservation devices.[5] As our understanding of hematopoiesis grew,[11,12] alternative sources of cells capable of reconstituting hematopoiesis were identified and have since entered clinical practice. Mobilized PBPC[13,14] and umbilical cord blood (UCB) progenitor cells[15,16] are now commonly used sources of hematopoietic progenitors. Because PBPC and UCB progenitor cells differ from BM cells,[16-19] studies have been performed aimed at improving manipulation, and cryopreservation of PBPC and UCB.[6,7,18] Mobilized PBPC, in particular, are being increasingly used to reconstitute hematopoiesis in patients undergoing high-dose chemo-radiotherapy, both in the autologous and allogeneic settings.[1-3] Therefore, the request for PBPC collection, cryopreservation and storage has grown exponentially, making pressing demands for methodological improvements aimed at cost reduction and reproducibility of routine freezing procedures. Results obtained by a variety of investigators,[6,7,20-24] have indicated that PBPC can be cryopreserved using a mechanical freezer (-80°C) and that the cells subsequently support hematopoietic reconstitution. Recently, a prospective study comparing URF and controlled-rate freezing techniques of cryopreserving PBPC firmly confirmed that URF is a safe procedure allowing sustained long-term engraftment.[25] The authors reported a slower early engraftment after URF, although this did not increase risks and costs of transplantation in terms of days of hospitalization, transfusion requirement and days of antibiotic therapy.[25] Freezing and storage conditions capable of preventing or limiting the functional damage of hematopoietic progenitor cells are crucial prerequisites to guarantee the maintenance of progenitor cell reconstituting activity on a long-term basis.[4] As we previously reported, our freezing procedure has no detrimental effects on the cryopreservation of UCB progenitor cells since it does not impair their clonogenic capacity, immunophenotypic composition, feasibility of $CD34^+$ selection or *ex vivo* expansion capability.[18]

In the present study, we report our experience of routine URF of PBPC, in a cryoprotectant solution consisting of PBS, HSA, 10% DMSO, and subsequent storage in liquid nitrogen. Our freezing procedure was applied to bags containing up to 200×10^6 cells per mL. Freezing PBPC at higher concentrations has potential benefits, such as lower costs of processing and storage and fewer risks of the complications associated with the reinfusion of large component volumes and large quantities of DMSO.[26] Analysis of PBPC samples, pre- and post-cryopreservation, revealed that our URF procedure, even associated with a significant reduction of nucleated cells, had no detrimental effects on the recovery of primitive and committed progenitor cells, on the number of $CD34^+$ cells, or on the *in vivo* ability to reconstitute hematopoiesis. These findings suggest that the freezing/thawing procedure results in a selective loss of mature cells in the absence of substantial damage to the progenitor cell compartment. Other studies[6,7,20-22,24] reported the ability of URF to spare progenitor cells and guarantee hematopoietic recovery after high-dose chemotherapy, but clonogenic assays were limited to evaluating committed progenitors (CFU-GM, BFU-E), with no analysis of primitive progenitors (LTC-IC) and only occasionally of $CD34^+$ cells. More recently, Montanari *et al*.[25] confirmed that the URF procedure does not have detrimental effects on the recovery of $CD34^+$ cells and committed progenitors (CFU-GM), but no analysis was conducted on the recovery of more immature progenitor cells (CFU-Mix and LTC-IC). These authors hypothesized that URF could allow a minor recovery of only committed progenitor cells, sparing the more immature progenitor cells.[25] Since the structural and functional integrity of the hematopoietic system is maintained by a relatively small population of stem cells that undergo self-renewal,[11] and no *in vitro* assays for identifying pluripotential stem cells are available, surrogate assays include evaluation of the ability to initiate long-term culture (LTC-IC). Therefore, to evaluate the potentially detrimental effects of URF, in our study quality control tests involved analysis of primitive and committed progenitors by means of long- and short-term culture assays, respectively. Our results confirm a previous study[23] that reported a complete recovery of primitive and committed progenitors after URF, strongly suggesting that the procedure allows optimal freezing conditions for primitive hematopoietic cells with reconstituting capacity. This was further demonstrated by the rapid and long-lasting hematopoietic recovery achieved in all our patients undergoing myeloablative chemotherapy. Moreover, our study was conducted on a large number of samples and the freezing procedure was applied on a routine basis. A reduction of progenitor cells was noted after URF (starting six months post-cryopreservation), and was considered to be caused by storage at -80°C in a mechanical freezer or by lower concentrations of DMSO.[20, 27] These factors could have combined to accelerate the degradation of cell viability during storage to the point at which the loss limited the clinical use of the component.[28] The main disadvantage of URF might be the lack of records documenting the cooling rate. However, -80C° freezers can record the temperature over time and in this way any change of temperature inside the freezer remains recorded. On the other hand, the advantages of URF are that a controlled-rate device

is not needed, the technique is cheaper and it makes fewer demands on personnel; these characteristics translate into a marked reduction of work, costs and time needed for PBPC processing.

In conclusion, our freezing procedure allows optimal post-thawing recovery of committed and primitive progenitor cells and rapid hematopoietic recovery after myeloablative therapy. This procedure is fast and easy, and reproducible on a large scale and on a routine basis.

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Pre-publication report

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CA contributed to conception, design and interpretation of the study. Drafted the article and performed the clonogenic assays. PF and EF performed the PBPC collections. AL and RV performed the clonogenic assays and the flowcytometric analysis. GR contributed to the interpretation of the study. MM critically revised the manuscript. All the authors approved the final version of the manuscript.

Disclosures

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