Effect of Cell Concentration on Bone Marrow and Peripheral Blood Stem Cell Cryopreservation

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The effects of cell concentration during cryopreservation on bone marrow (BM) or peripheral blood (PB)-derived hematopoietic progenitor cells have not been described. The much greater numbers of cells harvested for autologous PB stem cell (PBSC) transplantation requires that the cells be frozen at higher cell concentrations, or in much greater volumes, compared with BM. We cryopreserved 108 PBSC collections from 30 patients at an average (±SD) cell concentration of 3.7 ± 1.9 × 10^6 nucleated cells per mL in 127 ± 45 mL. The proportion of mononuclear cells was 52.9% ± 27.2%. The products also contained 2.9 ± 2.1 × 10^6 platelets/mL and an average red cell proportion of 12.9% ± 7.2%. The nucleated cell recovery after thawing was 75.4% ± 13.0%. The nucleated cell concentration during freezing was not predictive for the postthaw recoveries of nucleated cells (P = .38), granulocyte-macrophage colony-forming unit (P = .06) or CD34+ cells (P = .54), or for the viability of mononuclear cells (P = .81). The platelet and red cell concentrations similarly were not predictive for these endpoints. Samples (3 BM, 7 PBSC) from 10 patients were simultaneously cryopreserved at two-fold, and from 5 additional patients (PBSC) at 6- to 24-fold differing cell concentrations. A lower recovery of erythroid burst forming unit was found for samples frozen at higher cell concentrations (P = .04), but no significant differences were found in the other endpoints listed above. The average cell concentration during freezing for each patient’s PBSC collections (n = 34 patients) did not predict time to achieve a PB count of >500 granulocytes/μL (P = .51) or platelet transfusion independence (P = .39). Patients achieved these endpoints of engraftment at medians of 12 and 13 days, respectively. The infusion of these products was generally well tolerated. Similarly, the cell concentration at which BM cells were frozen did not predict for the duration of granulocyte (P = .63) or platelet (P = .36) aplasias for 54 patients undergoing autologous BM transplantation. These data suggest that PBSC or BM cells collected for transplantation may be cryopreserved at very high cell concentrations without loss of engraftment potential or undue infusion-related toxicity.

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Submitted July 9, 1993; accepted December 23, 1993.
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0006-4971/94/8309-0014$3.00/0

Cryopreservation of hematopoietic stem cells (HSC) is a routine aspect of autologous bone marrow (BM) transplantation. Progressive loss of HSC viability over time, beyond that associated with the freezing and thawing procedures, does not appear to occur if storage conditions are adequate. Thus, cryopreservation allows the administration of conditioning regimens requiring multiple days, as well as storage of HSC for future use. Although no standard technique is accepted by all centers, variations in techniques are generally minor.1 Virtually all centers performing autologous BM transplantation cryopreserve cells in dimethyl sulfoxide (DMSO). Furthermore, engraftment failure or delay has not been attributed to variations in technique, although optimal conditions such as concentrations of cells or protein, and storage temperatures have not been defined for human HSC.

Recently, several centers have reported the transplantation of peripheral blood stem cells (PBSC) collected after mobilization by granulocyte–colony stimulating factor (G-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF).2,3 Compared with collection during rebound after chemotherapy, large quantities of cells (frequently exceeding 5 × 10^6 cells) are collected during each apheresis. These cell quantities exceed by several-fold the quantity of cells usually harvested for BM transplantation. Cryopreservation of these cells at the cell concentrations generally used for BM (commonly, 2 to 4 × 10^7 nucleated cells/mL) generates large product volumes containing large quantities of DMSO. Reinfusion of these cells may be associated with considerable toxicity during infusion, unless cryopreservation and reinfection techniques are modified in response to the quantity of cells harvested.6,7 Options include washing and concentrating the cells after thawing, or freezing at higher cell concentrations.

We concentrated PBSC collected after G-CSF or GM-CSF mobilization in minimal volumes resulting in high cell concentrations during cryopreservation, and prospectively studied HSC recovery after thawing. No consistent detrimental effect of nucleated cell, platelet, or red cell concentrations during cryopreservation could be shown. Furthermore, no effect on engraftment kinetics could be determined.

MATERIALS AND METHODS

Patient selection and transplantation procedures. Patients eligible for PBSC transplantation underwent stem cell mobilization using either G-CSF (Amgen Inc, Thousand Oaks, CA) or GM-CSF (Immunex Corp, Seattle, WA).3 G-CSF (16 μg/kg/d subcutaneously for 5 or 6 days) was administered during steady-state hematopoiesis without chemotherapy rebound for most patients (patients with unique patient numbers [UPNs] 7262, 7364, 7902, and 7923 listed in Table 3 received G-CSF after cyclophosphamide or cyclophosphamide plus etoposide administration, with PBSC collection during recovery from neutropenia). PBSC collections were performed for either 3 or 4 days starting on the fourth day of G-CSF administration. GM-CSF (250 μg/m2) was administered daily to a limited number of patients treated for multiple myeloma during the recovery phase from cyclophosphamide-induced marrow hypoplasia, with collection of PBSC after PB white cell count exceeded 1 × 10^5/μL. All patients underwent 12 L blood volume leukapheresis daily using a COBE Spectra (COBE BCT, Lakewood, CO) as previously described.3 During apheresis, patients were anticoagulated with acid-citrate dextrose formula A (ACD-A; Fenwal, Deerfield, IL) and heparin (5,000 U/500 mL ACD-A). In addition, 20 to 40 mL of ACD-A was added.
to the collection container before the start of the apheresis run to decrease the risk of cell clumping. BM was collected under general or regional anesthesia from the iliac crests using techniques previously described and without stimulation with G-CSF or other cytokines. Informed consent under Institutional Review Board-approved protocols for collection and transplantation of BM and/or PBSC was obtained for all patients.

**Cryopreservation technique.** Excess plasma was removed from PBSC products by centrifugation in 600-mL blood-transfer packs in a Sorvall RC-3 centrifuge (Du Pont Co, Wilmington, DE) at 3,000 rpm for 10 minutes. The volume of the residual cell pellet was adjusted with autologous plasma as necessary, and 15- to 30-mL aliquots were added to the number of freezing bags necessary. A volume of cryoprotectant solution (see below) equal to the volume of the cells was added and the cells cooled at 1°C/min to −40°C, and then 10°C/min to −80°C using a rate-controlled freezer (CryoMed, New Baltimore, MD) before transfer into the vapor phase of nitrogen at −180°C or below. BM cells were similarly cryopreserved in a minimum of two bags (50 to 60 mL each). BM cells were processed before cryopreservation by either collection of buffy coat cells or separation of light-density cells (specific gravity < 1.078 g/mL) on Ficoll-Hypaque gradients (LSM, Organon Teknika, Durham, NC) using a COBE 2991 Blood Cell Washer (COBE BCT). Fresh cryopreservation solution consisting of 20% DMSO (CryoServ, Research Industries, Salt Lake City, UT) and 40% autologous plasma in TC199 ( Gibco, Grand Island, NY) was prepared for each PBSC or BM product. This was added to the cell products at equal volume to achieve a final concentration of 10% DMSO and 20% plasma. The cryoprotectant solution was generally chilled to 4°C before use, but the cells were not chilled before addition.

Before reinfusion, the cells were rapidly thawed in a 37°C water bath at the patient’s bedside. A volume of ACD-A equal to 20% of the bag volume was added to prevent cell clumping. Samples for analysis after thawing were obtained after the addition of ACD. Each bag of cells was infused over 5 to 10 minutes through a large bore, intravenous catheter. All patients were hydrated and medicated with diphenhydramine, mannitol, and hydrocortisone immediately before cell infusions.

**Cell counts.** Nucleated cell and platelet counts, and hematocrits were obtained for fresh PBSC products using a Sysmex E2500 (Toa, Inc, Chicago, IL). Nucleated cell counts for BM products before cryopreservation and for both BM and PBSC products after thawing were obtained using a Coulter ZM (Coulter, Inc, E). The proportion of mononuclear cells (defined as lymphocytes and monocytes) was obtained using a Coulter ZM (Coulter, Inc, E) and myeloid (Cm-GM) colonies were identified after 14 days of culture on methylcellulose supplemented with Iscove’s Modified Dulbecco’s medium (IMDM; Gibco, Grand Island, NY), 1% fetal bovine serum (FBS; Hyclone, Logan, UT), 1% bovine albumin (Boehringer Mannheim Corp, Indianapolis, IN), 10⁻⁴ mol/L 2-mercaptoethanol (Sigma Chemical Co, St Louis, MO), 10⁻⁴ mol/L methylprednisolone sodium succinate (Upjohn Co, Kalamazoo, MI), 30 U/mL GM-CSF (Amgen), 100 U/mL interleukin-3 (Amgen), and 1 U/mL erythropoietin (Amgen). Erythroid burst-forming unit (BFU-E) and myeloid (CFU-GM) colonies were identified after 14 days of culture in a fully humidified 5% to 6% CO₂ in air atmosphere. Samples (1 mL) of thawed cells were obtained after the addition of ACD-A, serially diluted in 5 steps to 36 mL by addition of equal volumes of phosphate-buffered saline containing 1% FBS, and washed twice in IMDM before culture as described above. The total quantity of progenitor cells after freezing was calculated from the number of colonies enumerated, the seeding density of the culture dishes, and the total number of cells frozen. The quantity of progenitor cells after thawing was similarly calculated, but with adjustment for the recovery of the nucleated cells after serial dilution and washing.

**Quantification of viable CD34⁺ cells.** Red blood cells (RBCs) from samples of cells obtained before cryopreservation or after thawing and serial dilution were removed by hypotonic lysis using ammonium chloride. The cells were then stained with a fluorescein isothiocyanate or phycoerythrin-conjugated CD34 antibody (8G12; Becton Dickinson, San Jose, CA) or an irrelevant isotype control, washed, and counterstained with propidium iodide (PI; Becton Dickinson) or 7-aminoactinomycin D (7AAD; Sigma Chemical Co). After an additional wash and within 2 hours of staining, the proportions of viable (PI or 7AAD excluding) cells, mononuclear cells, and CD34⁺ cells in the fresh and thawed specimens were determined by flow cytometric analysis (FACScan, Becton Dickinson). CD34⁺ cells showed fluorescence greater than 99.8% of isotype control-stained cells. The quantities of these cell populations in each product before and after freezing were determined as described above.

**Statistical analysis.** The relation of the cell concentration during cryopreservation to the recovery of nucleated cell recovery, mononuclear cell viability, and the recoveries of myeloid (CFU-GM), erythroid (BFU-E), and CD34⁺ cells was evaluated by linear regression analysis and calculation of Pearson’s correlation coefficient. The significance of the correlation parameters was tested by Student’s t-test. The relationship of samples frozen simultaneously at two cell concentrations was evaluated by the Wilcoxon signed-rank test. The prognostic importance of cell concentration in predicting engraftment (censored for death) was assessed using the proportional hazards regression model of Cox. For all evaluations, time refers to the interval between cell infusion (day 0) and day of event (engraftment or death). No adjustments for multiple comparisons were made in calculating the reported P values. For this reason, P values between .01 and .05 should be viewed as suggestive and not conclusive evidence of a difference.

**RESULTS**

**Effect of cell concentrations during freezing on HSC recovery.** To determine the effect of cell concentration during cryopreservation on the recovery of hematopoietic progenitor cells, we studied 108 PBSC products harvested from 30 patients (Table 1). We subsequently collated data on 57 of these products from 22 patients after thawing. The average PBSC collection contained 4.8 ± 3.4 × 10⁹ cells (mean ± SD; range, 0.6 to 14.9 × 10⁹) cryopreserved at an average cell concentration of 3.7 ± 1.9 × 10⁶ nucleated cells/mL (range, 0.4 to 8.0 × 10⁶). Large quantities of platelets and RBCs were also cryopreserved (Table 1). The nucleated cell recovery after thawing was 75.4% ± 13.0%. Nucleated cell concentration during cryopreservation did not predict nucleated cell recovery or mononuclear cell viability as determined by PI dye exclusion after thawing (Fig 1, A and B). Although the cell concentration during freezing was borderline (P = .06) and poorly (r = .29) predictive for the recovery of CFU-GM progenitors after thawing (Fig 1D), it did not predict the recovery of viable CD34⁺ cells (Fig 1C) or erythroid progenitors (r = .17, P = .27, Fig not shown).

The proportion of mononuclear cells contained in these products was determined by light microscopy and ranged from 10.5% to 100% of the nucleated cells. To determine if the presence of granulocytes that predominately composed the remainder of the nucleated cells of these products affected the recovery of hematopoietic progenitors after cryopreservation, we similarly attempted to correlate, specifically, the concentration of mononuclear cells, and separately,
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A limited number of BM or PBSC products were split for freezing at 2-fold or greater different cell concentrations. The cell concentrations of the light-density cells isolated so that the final protein and DMSO concentrations, and the volume contained in each freeze bag were the same. We adjusted the cell concentration of PBSC products with autologous plasma collected during apheresis to again maintain identical concentrations of DMSO and plasma in each bag. After thawing and anticoagulation with ACD-A, samples were obtained from each bag for serial dilution and analysis. We found no consistent differences based on the cell concentration during freezing on the recovery of nucleated cells, mononuclear cell viability, or the recoveries of viable CD34+ cells or CFU-GM (Table 3). The recovery of BFU-E was lower for samples frozen at higher cell concentrations. This difference and a similar trend for the recovery of CFU-GM and CD34+ cells may be artifacts of the dilution process because samples frozen at the higher cell concentrations were more likely to clump after the wash step, and the recovery of nucleated cells after washing was entered into the calculation of progenitor cell recovery. CFU-GM–derived colonies per 5 × 10⁶ cells plated averaged 45.4 ± 58.7 (±SD) for samples frozen at the higher concentrations and 46.0 ± 61.2 for samples frozen at the lower concentrations (P = .88). Although the number of samples analyzed was limited, analysis of the five PBSC samples frozen at 6- to 24-fold differences in cell concentration showed no significant difference in any of these parameters of HSC survival (P > .19 for all analyses).

Effect of cell concentration during cryopreservation on engraftment kinetics. A total of 34 patients were transplanted with PBSC alone. These patients were treated for breast cancer (n = 14), non-Hodgkin's lymphoma (n = 10), multiple myeloma (n = 5), and a variety of other solid tumors (n = 5). Although 1 patient underwent only two collections and 4 patients required six to nine collections in two series of G-CSF mobilization to achieve adequate numbers of mononuclear cells for infusion, most patients underwent either three (n = 22) or four (n = 7) aphereses while receiving G-CSF. Patients were conditioned with busulfan and cyclophosphamide with (n = 25) or without (n = 4) total body irradiation (TBI) with lung and liver shielding, or cyclophosphamide, TBI, and etoposide (n = 3). One patient each was conditioned with etoposide, BCNU, and cyclophosphamide, or etoposide, thiotepa, and cyclophosphamide. Six patients received G-CSF, 5 μg/kg/d, and another 6 patients received GM-CSF, 250 μg/m²/d, starting the day of PBSC infusion. The cell concentration during cryopreservation for the total cells collected was calculated for each patient and averaged (±SD) 3.8 ± 1.9 × 10⁸ nucleated cells/mL, with a range 0.2 × 10⁸ to 7.4 × 10⁸ cells/mL. These 34 patients reached greater than 500 granulocytes/μL at a median of 12 days (range, 8 to 15 days), and platelet-transfusion independence at a median of 13 days (range, 7 to 73 days). We assessed the prognostic importance of cell cryopreservation in predicting time to these two engraftment endpoints. The average cell concentration during cryopreservation did not predict either time to achieving greater than 500 granulocytes/μL (P = .51) or time to platelet-transfusion independence (P = .40) by univariate analysis. Adjusting for diagnosis and growth factor administration did not alter this conclusion. The limited range in granulocyte aplasia duration also suggests that cryopreservation of PBSC at these cell concentrations did not deleteriously affect the survival of cells responsible for hematologic recovery after reinfusion.
rine monoclonal antibodies and rabbit complement were used for purging using a panel of B-cell or T-cell directed murine monoclonal antibodies and rabbit complement were used for purging. Light-density cells were collected after density-gradient separation and immunologic purging using a panel of B-cell or T-cell directed murine monoclonal antibodies and rabbit complement were used for purging. Light-density cells were collected after density-gradient separation and immunologic purging using a panel of B-cell or T-cell directed murine monoclonal antibodies and rabbit complement were used for purging.

Similarly, a total of 54 patients received cryopreserved BM (without supplementation with PBSC). Light-density cells collected after density-gradient separation and immunologic purging using a panel of B-cell or T-cell directed murine monoclonal antibodies and rabbit complement were used for purging. Light-density cells were collected after density-gradient separation and immunologic purging using a panel of B-cell or T-cell directed murine monoclonal antibodies and rabbit complement were used for purging. Light-density cells were collected after density-gradient separation and immunologic purging using a panel of B-cell or T-cell directed murine monoclonal antibodies and rabbit complement were used for purging. Light-density cells were collected after density-gradient separation and immunologic purging using a panel of B-cell or T-cell directed murine monoclonal antibodies and rabbit complement were used for purging.

Table 2. Effect of Platelets and Erythrocytes on Cryopreservation of PBSC

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<td>Mononuclear cell viability (%)</td>
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* Shown are the correlation coefficient (r) and significance of correlation (P) for the effects of platelets and RBCs on various measures of hematopoietic cell recovery after cryopreservation. Samples were obtained before and after cryopreservation from 57 products from 22 patients. The numbers available for analysis of the various parameters are shown (n).

Fig 1. Mononuclear cell viability (B) and recoveries after thawing of (A) nucleated cells, (C) viable CD34+ cells, and (D) CFU-GM. Correlation coefficients and the linear regression equation are shown for each curve. None of the slopes differ significantly from 0, as shown by the P values for each equation. Cell concentration is $10^9$ per mL.

cryopreserved for 30 patients; the other patients received unpurged buffy-coat cells separated by centrifugation using a COBE 2991 Cell Washer. The patients were transplanted for the treatment of a variety of malignancies including non-Hodgkin’s lymphoma (n = 24), Hodgkin’s disease (n = 13), acute lymphoblastic leukemia (n = 6), multiple myeloma (n = 4), breast cancer (n = 4), and other solid tumors (n = 3). Most patients received hematopoietic cytokines (GM-CSF, G-CSF, or IL-3) after marrow infusion. The cell concentration at which the light-density cells were frozen ranged from $1.03 \times 10^7$ to $3.46 \times 10^9$ nucleated cells/mL (median, $7.14 \times 10^7$). Buffy-coat cells were frozen over a range of $2.79 \times 10^7$ to $1.96 \times 10^9$ nucleated cells/mL (median, $9.50 \times 10^7$). These patients achieved greater than 500 granulocytes/µL at medians of 12 days (range, 9 to 100 days) and 15 days (range, 10 days to 36 days) for recipients of buffy-coat and light-density cells, respectively. Median time to platelet-transfusion independence was 27 days (range, 10 to 100 days) and 23.5 days (range, 5 to 278 days), respectively. The cell concentration at which these cells were frozen did not predict time to achieving greater than 500 granulocytes/µL (P = .63) or last platelet transfusion (P = .36) in univariate analysis stratified by initial marrow processing. When a number of possibly clinically relevant variables, including age, diagnosis, use of growth factors, cryopreservation cell concentration, and PB counts on the day of marrow harvesting were entered into multivariate analysis, only platelet count on day of harvesting (median, $257 \times 10^3/µL$; range 26 to $800 \times 10^3/µL$) remained prognostic for duration of granulocyte (P = .001) and platelet aplasia (P = .03).
DISCUSSION

This study found no consistent detrimental effects from the cryopreservation of PBSC products at varying, but relatively high cell concentrations. Similarly, the freeze concentration of BM cells at varying, but lower cell concentrations was not predictive for the kinetics of engraftment after transplantation. These findings are in agreement with the previously described uniform survival of murine spleen colony-forming unit (CFU-S) cryopreserved over the range of 5 to 210 × 10^6 cells/mL. However, these conclusions are based primarily on the recoveries of nucleated cells, viable CD34⁺ cells, and hematopoietic progenitors at varying, but relatively high cell concentrations. Similarly, the murine studies were limited to the detection of CFU-S, not engraftment success after transplantation. In this study, there was no discernible effect upon engraftment kinetics or durability, however, suggesting a major effect on the survival of primitive and committed hematopoietic stem cells is unlikely. Also, only the effects of relatively high cell concentrations during cryopreservation were investigated. The previously described murine studies found a significant deterioration of CFU-S survival when marrow cells were cryopreserved at concentrations less than 5 × 10^6 cells/mL, a situation that may occur when CD34⁺ cells are highly enriched for human transplantation, for example. A wide range of cryopreservation cell concentrations are used by the various autologous transplant programs. It had been previously recommended that BM cells not be cryopreserved at high cell concentrations, with 2 × 10⁷ nucleated cells/mL suggested as a reasonable concentration. Thus, the large quantities of PBSC after cytokine mobilization would require cryopreservation in volumes of about 7 L for patients in this study (total for three collections), resulting in infusions of over 10 g of DMSO per kilogram of patient weight. Although the lethal dose of DMSO for humans has not been determined, the lethal dose for 50% of animals (LD₅₀) is 3.1 to 9.2 g/kg for mice, and 2.5 g/kg for dogs. Therefore, postthaw washing or infusion over several days would be required if such concentrations are used. Another practical consideration that affects laboratory decisions is the desire to split a product into two bags instead of one for freezing. This would lower the cell concentration for some products, but would primarily be of concern if small cell quantities are being stored.

The infusions were generally well tolerated. Infusion-related toxicities have been reported by a number of centers. These complications appear volume related and could result from the quantity of DMSO, the quantity of cells infused, or both. Cryopreservation of PBSC at the cell concentrations used in this study resulted in total volumes of products that were usually less than 10 mL/kg of recipient weight containing less than 1 g of DMSO per kilogram recipient weight. Although we did not specifically quantify patient symptoms during infusion, infusion of these large quantities of cells in relatively small volumes were generally well tolerated, suggesting that the amount of DMSO, not the quantity of cells, may be the primary cause of the previously reported infusion-related toxicities. One patient, who concomitantly received both BM (purged with murine antibodies and rabbit complement) and PBSC, developed pulmonary decompensation requiring ventilatory support 4 hours after the infusion. Bronchospasm is occasionally observed in patients receiving immunologically purged BM cells at this center and others.

Abbreviations: ND, no data; SD, standard deviation.

* BM (UPNs 7043, 7055, and 7117) or PBSCs (all other patients) were cryopreserved at twofold or up to 24-fold different cell concentrations.

**Thawing (quantity before freezing × 100) was determined. Statistical evaluation of differences between bags 1 and 2 was performed using Wilcoxon signed-rank test.
Although the severity of the reaction was probably greatly increased by the large quantity of cells infused. A second patient with preinfusion vascular instability became hypoxic after each of two infusions, separated by several hours, of 168 and 262 mL (2.0 and 3.2 mL/kg). Subsequent infusions, each separated by several hours on the following day, of 69, 138, and 141 mL were tolerated without complaint. Similar events have not been observed in over 105 patients to date with the infusion of PBSC alone, PBSC in combination with unpurged BM, or PBSC infused on a different day than infusion of immunologically purged marrow cells.

A major concern about freezing large numbers of highly concentrated cells was the risk of cell clumping occurring immediately before freezing or after thawing. These products required secondary centrifugation after collection to concentrate the cells for cryopreservation, and also contained large and variable quantities of mature blood cells. The freeze process was initiated within 1 hour of this secondary centrifugation and obvious cell clumping did not occur. A single product appeared gelatinous after concentration and additional ACD (10% vol/vol) was added immediately to prevent clumping. None of the products clumped after thawing, perhaps because of the routine addition of ACD before infusion.

Since the development of effective cryoprotectants, many aspects of BM and PBSC cryopreservation and storage have not been defined. Current techniques appear to be adequate in preserving sufficient quantities of HSC for successful reconstitution of the recipient’s hematopoietic function after marrow- lethal conditioning regimens. However, what may not be evident is the possible loss of HSC that may affect engraftment kinetics. Improved cryopreservation techniques may improve the acceptability of cell infusion to the patients who frequently develop low-grade toxicities. The data reported in this study suggested that cryopreservation and infusion of autologous peripheral blood stem cells. Bone Marrow Transplant 5:25, 1980 (suppl 1)

**ACKNOWLEDGMENT**

We are indebted to Beth MacLeod, Kale Slechta, JoAnn Dalton, David Yadock, and, especially, Daphne McDonald for their expertise in processing these cell samples, CD34 analysis, hematopoietic progenitor cell culture, and data collation.

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