Autologous Bone Marrow Transplantation Using Unfractionated Cells Cryopreserved in Dimethylsulfoxide and Hydroxyethyl Starch Without Controlled-Rate Freezing

By Patrick J. Stiff, Alan R. Koester, Mary K. Weidner, Kristen Dvorak, and Richard I. Fisher

To develop a simplified method of bone marrow (BM) cryopreservation, changes were made in the standard method in three areas: the cryoprotectant, the method of cell freezing, and the storage temperature. Unfractionated BM cells from 60 patients were cryopreserved in 300-mL aliquots in both dimethyl sulfoxide (DMSO) and hydroxyethyl starch (HES), a combination known to preserve granulocytes successfully. The cells were frozen without rate-controlled freezing by simple immersion into a -80°C freezer where they remained until the time of reinfusion. The 60 patients underwent 72 autologous transplants after three high-dose chemotherapy regimens: 30 received high-dose carbamustine in combination, five received high-dose busulfan and cyclophosphamide, and 37 received high-dose aziridinylbenzoquinone. The BM was infused for more than 30 minutes after rapid thawing at 37°C. The mean postthaw nucleated cell recovery was 96% ± 11.6%, and Trypan blue dye exclusion was 82.2% ± 9.2%. The mean postthaw CFU-GM and BFU-E recoveries were 81.9% ± 39.0% and 90.5% ± 41.2%. Complete count recovery occurred in 68 of 72 transplants. Median times to a WBC count >1,000/µL, a granulocyte count >1,000/µL and a platelet count >20,000/µL were 15, 21, and 15 days, respectively. Risk factors for delayed recovery were not found. Unfractionated BM cells can be successfully cryopreserved in the DMSO/HES mixture rapidly and inexpensively, without rate-controlled freezing or storage at liquid nitrogen temperatures.© 1987 by Grune & Stratton, Inc.

HIGH-DOSE CHEMOTHERAPY with or without total body irradiation, combined with the autotransplantation of cryopreserved bone marrow (BM), is being increasingly used with promising results to treat patients with lymphomas, solid tumors, and even leukemias.1-9 Methods of BM cryopreservation have been standardized. The cells are frozen in 10% dimethyl sulfoxide (DMSO) in a rate-controlled freezer set to cool at a constant 1°C per minute and are then stored until the time of thawing in either the liquid or vapor phase of liquid nitrogen.10,11,12 To prevent clinical problems due to the severe clumping of granulocytes postthaw, many groups use cell separators to remove them from the BM before freezing, as first described by Weiner et al.13 Although effective, current cryopreservation techniques are time-consuming as well as expensive, requiring cell separators, rate-controlled freezers, and large liquid nitrogen freezers.

To develop an inexpensive, rapid, yet effective marrow cryopreservation technique we investigated the cryoprotectant combination of DMSO, an intracellular cryoprotectant, and hydroxyethyl starch (HES), an extracellular cryoprotectant that had been previously reported to reduce postthaw granulocyte lysis and clumping.10-15 On the basis of data from previously reported cryopreservation studies, we also questioned the need for rate-controlled freezing4,16 and storage at liquid nitrogen temperatures.17-20 In preliminary small- aliquot studies, we showed that the postthaw nucleated cell and colony-forming unit–granulocyte-macrophage (CFU-GM) recovery of unfractionated BM cells stored in the vapor phase of liquid nitrogen was higher for cells in 5% DMSO and 6% HES than for those in 10% DMSO alone.21 The postthaw CFU-GM recovery for BM in either DMSO or DMSO/HES was similar to that reported by others using rate-controlled freezing.22-24 Despite the fact that the BM in our study was frozen by simple immersion in a -80°C freezer. Additional samples frozen in the DMSO/HES mixture without rate-controlled freezing and stored at -80°C for 12 to 16 months gave a postthaw CFU-GM recovery of 105% ± 39%,25 which suggests that storage at liquid nitrogen temperatures was also not a requirement for successful BM cryopreservation.

We present here our initial clinical report of a simple cryopreservation technique in 72 autologous transplants done on 60 patients with refractory solid tumors and lymphomas. In this study unfractionated BM cells were cryopreserved in 5% DMSO and 6% HES by simple placement into a -80°C freezer where they were stored until the time of autotransplantation.

MATERIALS AND METHODS

Patients. This study involves 60 consecutive patients who underwent a total of 72 autologous BM transplants between March 1982 and October 1986. Of these, 24 had malignant lymphomas and 36, solid tumors. The median age was 49 (18 to 70), and the male-to-female ratio was 1.3:1. Requirements for entry into the study included an absence of tumor cells in bilateral posterior iliac crest BM aspirates and biopsy samples and normal pelvic x-rays and bone scan results. In addition, the entry WBC, hemoglobin, and platelet counts had to be >4,000/µL, 10.0 g/dL, and >150,000/µL, respectively, except for documented cases of anemia of chronic disease. All patients had to provide written informed consent for this institutional review and approved study.

Cryoprotectant preparation. One hundred thirty-eight milliliters of Normosol-R in 5% dextrose (pH 5.2; Abbott Laboratories, North Chicago, IL) and 42 g of low–molecular weight HES powder (mol wt, 150,000; American Critical Care, McGaw Park IL) were
placed into a sterile 500-mL bottle. The mixture was autoclaved for 15 minutes to dissolve the HES and sterilize the mixture. After cooling the mixture to room temperature, 100 mL of 25% human serum albumin, used to stabilize granulocyte membranes postthaw, and 70 mL of 50% DMSO (Rimso-50; Research Industries Corp., Salt Lake City) were added. The final volume of the mixture was 350 mL, and the concentrations of DMSO, HES, and human albumin were 10%, 12%, and 8%, respectively. The cryoprotectant was usually made within 24 hours of use. It was stored in glass bottles and kept at 4°C before use.

**BM cryopreservation.** BM was collected from the anterior and posterior iliac crests and occasionally the sternum as previously described. Cell counts were done intraoperatively to ensure that a minimum of 2 × 10^6 cells/kg body weight were harvested. The cells were mixed with heparinized RPMI 1640 media (M. A. Bioproducts, Walkersville, MD) with a final heparin concentration of 6 to 7 U/mL marrow. No further heparin was added during the processing procedure. After filtering, the entire marrow volume was placed into a single 2,000-mL plasma transfer bag (Fenwal Labs, Deerfield, IL). The subsequent processing was done as a closed-system technique.

To minimize nucleated BM cell loss, the red cells were initially sedimented from the BM by using 6% HES (Hespan; American Critical Care) at 1 vol to 8 vol of BM. The RBCs were allowed to settle for 60 minutes at room temperature. The sedimented RBCs and the cell-rich plasma were each transferred to 600-mL transfer bags and then centrifuged for ten minutes at 400 g at 4°C. The buffy coat cells from the sedimented RBC layer were added to those from the cell-rich plasma and the volume of the combination adjusted to 300 mL with RPMI 1640 tissue culture media. One hundred fifty milliliters of both the cells and the cryoprotectant was simultaneously added to two polyolefin freezing bags (4403-2; Delmed, Canton, MA). The bags were sealed, placed into aluminum freezing frames, and placed horizontally into a −80°C freezer where they remained until reinfusion. Cell counts were recorded before and after the initial processing of the BM and after thawing the cells. Trypan blue dye exclusion testing was done postthaw, and committed stem cell assays were done both before and after the cryopreservation procedure, with the postthaw handling of the BM as previously described.

The cryopreserved BM was thawed at the patients’ bedside in a 37°C water bath. Each 300-mL bag was infused through a central venous catheter, without filtering, for more than 30 minutes.

**Preparative regimens.** The preparative regimens varied depending primarily on the tumor being treated. In 30 cases high-dose Carmustine (BCNU; 900 mg/m²) was used, usually in combination with VP-16-213 (500 to 750 mg/m²), cyclophosphamide (4,000 mg/m²) and cisplatin (100 mg/m²) or VP16-213 (1,000 mg/m²) and methotrexate (1,500 mg/m²). In five cases high-dose busulfan (16 mg/kg) and cyclophosphamide (120 mg/kg) were given as previously described. In 37 cases high-dose aziridinylbenzoquinone (AZQ; 50 to 125 mg/m² as a single dose given intravenously over a period of 24 hours) was given. AZQ was being given as a phase I study, with the entry dose being twice the conventional solid tumor dose for this drug.

**Committed stem cell assays.** CFU-GM and burst-forming unit-erythroid (BFU-E) assays were done precryopreservation and postcryopreservation as previously described with minor modifications to assess the efficacy of the freezing technique. For the CFU-GM assays, 1 × 10^6 unfractionated BM cells were cloned in McCoy’s media in 1% methylcellulose by using a feeder layer of 1 × 10^6 normal human peripheral blood leukocytes immobilized in 0.5% agar as the source of colony-stimulating activity. The plates were incubated in a 6% CO₂ atmosphere at 37°C. Quadruplicate assays were performed on each specimen, and the plates were counted by using an inverted microscope (Leitz, Rockleigh, NJ). An aggregate of 40 or more cells after ten days in culture was considered to be a colony.

For the BFU-E assays, 1 × 10^6 unfractionated BM cells were cloned in Iscove’s media in 1% methylcellulose by using 2 units of sheep Step III erythropoietin (Connaught Laboratories, Swiftwater, PA) per plate. Ten percent phytohemagglutinin-conditioned media was used as the source of burst-promoting factor. The plates were incubated in a 5% CO₂ atmosphere at 37°C. A single aggregate or multiple aggregates consisting of 200 or more red cells after 14 days in culture were considered to be a burst.

The percent postthaw nucleated cell, CFU-GM, and BFU-E recoveries were calculated as percentages of the total number of these cells frozen.

**RESULTS**

The nucleated BM and stem cell recovery data for the 72 transplants are presented in Table 1. There was only a 9% nucleated cell loss during the initial processing of the unfractionated cells in the 60 harvests. After thawing the 300-mL aliquots there was a negligible cell loss, with approximately 82% of the cells excluding Trypan blue. The BM storage times varied from 1 week to 4 months, although the majority of the transplants were done within the first several weeks after cryopreservation. Prethaw and postthaw CFU-GM and BFU-E assays were available for 63 of 72 and 37 of 72 transplants, respectively. Technical problems with the prefreeze CFU-GM assay prevented the analysis of percent recovery after thawing in nine of the transplants. This was also the case in seven instances for the BFU-E assay. In the remainder, this assay was not attempted prefreeze or postthaw. Percent CFU-GM recovery data from three transplants were censored from the analysis of postthaw recovery because these values were significantly greater than 200% of the pretransplant values.

<table>
<thead>
<tr>
<th>Recovery (%)</th>
<th>Mean Cell Dose</th>
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<tbody>
<tr>
<td>Nucleated cell recovery during initial processing</td>
<td>91.2 ± 16.7 (60)*</td>
</tr>
<tr>
<td>Nucleated cell recovery after thawing</td>
<td>96.7 ± 11.6 (71)</td>
</tr>
<tr>
<td>Trypan blue viability after thawing</td>
<td>82.2 ± 9.2 (65)</td>
</tr>
<tr>
<td>CFU-GM recovery after thawing</td>
<td>81.8 ± 39.0 (63)</td>
</tr>
<tr>
<td>BFU-E recovery after thawing</td>
<td>90.5 ± 41.2 (37)</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD.

*Number of harvests or transplants.
Of the 72 BM reinfluosions, no clinical toxicity was seen in 45. The most common side effect noted was a transient, slight substernal chest tightness, usually noted at or just after the infusion of the 300-mL aliquots (20.8%). It usually lasted five minutes or less and was not associated with significant dyspnea, cough, fever, nausea, or vomiting. A single episode of nausea and vomiting occurred in ten cases (13.9%), characteristically at the time the patients first experienced the taste of the DMSO. Six patients experienced a mild temperature elevation, usually after the infusion was complete and usually <38°C (8.3%). One patient developed truncal pruritis during the infusion that responded to parenteral diphenhydramine hydrochloride. No abnormalities in the proteins or partial thromboplastin times that were done after the infusions were noted.

The nucleated BM cell and CFU-GM/CFU-E doses transplanted are shown in Table 1. Twelve patients received two transplants each. All were transplanted with $>10^3$ CFU-GM/kg, recently suggested to be a requirement for successful engraftment. The hematopoietic engraftment data are shown in Table 2. Engraftment was considered to have occurred if the total WBC and granulocyte counts were $>1000/\mu L$ and the platelet count remained $>20000/\mu L$ for two consecutive days without transfusions. For all patients, the median time to engraftment was 21 days, with a median of 26 days for the 30 high-dose BCNU transplants, 24 days for the five high-dose busulfan transplants, and 21 days for the 37 high-dose AZQ transplants. Platelet count recovery was more rapid for those receiving high-dose AZQ ($P < .01$) as compared with the others. Although 12 of 37 AZQ transplants were done on patients who had not received any prior myelosuppressive therapy, eliminating these from the analysis did not change the differences in the platelet count recovery ($P < .01$).

There were four deaths before complete engraftment, or 5.6% of the total number of transplants. Two deaths occurred at 12 days posttransplant without any signs of engraftment. One each died of progressive disease and cardiac/noncardiac pulmonary edema with sepsis. The other two died at 24 and 12 days posttransplant without any signs of engraftment. Both had engraftment of granulocytes to $>500/\mu L$. Because three of four of these patients received transplants with $<1.8 \times 10^4$ CFU-GM/kg body weight, we analyzed CFU-GM cell dose as well as several other factors as potential risk factors for slow engraftment. As shown in Table 2, the 12 patients who received transplants with $<1.8 \times 10^4$ CFU-GM/kg body weight as well as those who received transplants with $<1.0 \times 10^4$ nucleated cells/kg and those with a CFU-GM recovery $<50\%$ of prefreeze values had an engraftment pattern similar to the entire group.

**DISCUSSION**

Because of the expense and complexity of the standard BM cryopreservation method, we decided to investigate all aspects of the freezing process. The technique reported here involves significant changes in the standard method in three areas: the cryoprotectant, the method of cell freezing, and the storage temperature of the frozen cells.

Since Lovelock and Bishop first described the cryoprotective effect of DMSO on bovine RBC in 1959 it has become the standard cryoprotectant agent for animal and human bone marrow cells. Unlike glycerol, another effective agent, DMSO does not need to be diluted or washed from thawed cells, which permits rapid reinfusion after thawing. Although 10% has been established as the optimum concentration of DMSO for the preservation of hematopoietic stem cells, terminally differentiated granulocytes are not successfully preserved. They lyse after thawing and release nucleoprotein and lysosomal enzymes, which causes macroscopic clumping and a gel formation.

Although many groups remove these cells before freezing to prevent this problem, we decided to investigate the combination of 6% HES with 5% DMSO, which successfully cryopreserves granulocytes. Initially reported by Lionetti et al., the recovery of human granulocytes frozen in this mixture ranges from 72% to 101%, with Trypan blue dye exclusion rates of 84% to 89%. Our initial in vitro data confirmed the effectiveness of this two-drug mixture for unfractionated BM, with nucleated cell recovery after thawing of 102% ± 13% and Trypan blue dye exclusion of 70% ± 5% for cells stored at −80°C for 12 to 16 months. In addition, we noted no postthaw macroscopic cell clumping or gel formation.

The use of rate-controlled freezing at a constant 1°C/min is based on early studies of Polge and Lovelock and Lewis et al. The effectiveness of the freezing rate was determined based on early studies of Polge and Lovelock and Lewis et al.

**Table 2. Hematopoietic Recovery After Intensive Chemotherapy and the Autotransplantation of BM Cryopreserved in 5% DMSO and 6% HES With Examination of Possible Risk Factors**

<table>
<thead>
<tr>
<th>Days to</th>
<th>Total White Cells $&gt;1000/\mu L$</th>
<th>Granulocytes $&gt;500/\mu L$</th>
<th>Platelets $&gt;20000/\mu L$</th>
<th>$&gt;50000/\mu L$</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients (72)*</td>
<td>15</td>
<td>18</td>
<td>21</td>
<td>15</td>
</tr>
<tr>
<td>High-dose BCNU (30)</td>
<td>16</td>
<td>20</td>
<td>26</td>
<td>22</td>
</tr>
<tr>
<td>High-dose busulfan and cyclophosphamide (5)</td>
<td>12</td>
<td>11</td>
<td>13</td>
<td>24</td>
</tr>
<tr>
<td>High-dose AZQ (37)</td>
<td>14</td>
<td>18</td>
<td>21</td>
<td>12†</td>
</tr>
<tr>
<td>Transplant $&lt;10^5$ nucleated cells (8)</td>
<td>16</td>
<td>20</td>
<td>23</td>
<td>12</td>
</tr>
<tr>
<td>Transplant $&lt;1.8 \times 10^4$ CFU-GM (12)</td>
<td>15</td>
<td>21</td>
<td>25</td>
<td>17</td>
</tr>
<tr>
<td>Transplants with $&lt;50%$ recovery of CFU-GM postthaw (14)</td>
<td>18</td>
<td>21</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>

Values are expressed as the median time in days to engraft.

*Number of patients receiving transplants.

†$P < .01$ compared with those who received transplants after high dose BCNU and busulfan-cyclophosphamide.
The latter group used the colony-forming unit-spleen (CFU-S) assay to assess optimal freezing rates for marrow cells cryopreserved in 12% glycerol. In their study only the duration of the postfreezing plateau and the rate of cooling after the postfreezing plateau affected the CFU-S recovery after thawing. Increasing the postfreeze plateau from 0 to 4 and 0 to 16 minutes, however, decreased the CFU-S recovery only 16% and 28%, respectively. Increasing the cooling rates after the postfreeze plateau from 1 to 3°C/min decreased the CFU-S recovery by only 8%. Other groups have investigated a more rapid freezing technique for human BM cells without a dramatic drop in CFU-GM recovery. In fact, Wells et al compared the CFU-GM recovery of unfractionated human marrow cells cryopreserved in 10% DMSO at 1°C/min with those frozen by simple immersion in liquid nitrogen and found that the CFU-GM recovery was 64.8% ± 14.8% for the programmed freezing method vs 50.6% ± 15.2% for the immersion method. Because of these modest differences and the fact that the cryopreservation of cells by simple placement into a −80°C freezer occurs at a rate of approximately 3°C/min, we decided to use this as our freezing method. This technique has also been used successfully to freeze platelets as well as granulocytes. Thus, although there may be an optimal freezing rate for human BM, there appears to be some flexibility in this rate without causing a severe impairment in the reconstitutive potential of the frozen cells. Our small-aliquot study seemed to verify this. CFU-GM assays done on cells that had been frozen by simple placement into a −80°C freezer and stored for 6 months at liquid nitrogen temperatures showed an excellent postthaw CFU-GM recovery for cells in both 10% DMSO alone (78%) and DMSO/HES (110%).

Early animal studies suggested that the storage of cryopreserved marrow at −79°C was sufficient to give excellent postthaw stem cell growth and was able to reconstitute animals after irradiation. With the subsequent ability to freeze at liquid nitrogen temperatures, most groups have chosen to store marrow at the lower temperature, with excellent CFU-GM recovery after several years of storage. In reexamining storage at −80°C, we found that for cells stored in the DMSO/HES mixture for periods of 12 to 16 months postthaw CFU-GM recovery was comparable to that of cells stored at liquid nitrogen temperatures.

The results of the present study demonstrate that marrow cryopreservation can be simple, rapid, reproducible, yet inexpensive. The total time to cryopreserve the 700 to 2,200 mL of BM obtained from a typical harvest procedure took as little as two hours, including the 60 minutes needed to sediment the red cells. Cells are concentrated to a volume of 200 mL or less with <10% loss of total nucleated cells. No clumping is seen after thawing, which permits us to freeze the cells in 300-mL aliquots, and there are only minor side effects associated with the infusion of these cells. Most importantly, hematopoietic reconstitution occurred as quickly in our patients as it did for those whose BM cells are cryopreserved in 10% DMSO using rate-controlled freezing and storage at liquid nitrogen temperatures.

Although several studies have demonstrated the value of CFU-GM assays in predicting the engraftment of cryopreserved marrow, these assays may not necessarily be predictive of pluripotent stem cell numbers. The only proof of true engraftment is the demonstration of hematopoietic reconstitution after lethal marrow damage. Although it is assumed that the busulfan-cyclophosphamide combination used in this study is myeloablative, neither the AZQ nor the BCNU combinations have been definitely shown to be marrow lethal. The BCNU dose used here is, however, a minimum of 50% higher than other recently published studies using this agent in drug combinations with autologous BM rescue. Although such treatments may not be marrow lethal, autologous transplants do lead to a rapid restoration of blood counts posttransplant, thereby decreasing the potential morbidity, mortality, and the costs associated with the prolonged pancytopenia resulting from such treatments.

We saw no differences in engraftment rates and times among the three preparative regimens used in this study. The more rapid platelet count recovery seen in the group receiving AZQ was surprising and appeared not to be due to either prior therapy or dose because patients in the ongoing study receiving as much as 150 mg/m² of the drug demonstrated the same phenomena. It is possible that this effect was due to less marrow microenvironmental damage than that caused by BCNU and busulfan.

Although our small-aliquot study demonstrated the viability of CFU-GM after storage for 12 to 16 months at −80°C, the autotransplants done in this study were performed within 4 months of the harvest procedure. Because studies using liquid nitrogen storage temperatures demonstrate that the majority of cell damage occurs during the freezing and thawing of the cells and not the storage time, it is possible that long-term storage at −80°C will be as useful. In an early canine study, the autotransplantation of BM cryopreserved in glycerol and stored at −80°C for 14 months led to rapid reconstitution after lethal irradiation. Longer periods, ie, years, have not been investigated. Studies are in progress to determine whether our small-aliquot data are correct in predicting that hematopoietic reconstitution using cells stored at −80°C for longer than 4 months will be prompt. Although this method can only be currently recommended for the short-term storage of cryopreserved BM, the majority of transplants performed at our and most other centers are done within this time period.

This new method has permitted us to expand our autotransplantation program with a minimal increase in cost and support personnel. It should allow for a wider application of high-dose chemotherapy regimens being developed to treat patients with resistant neoplasms.

ACKNOWLEDGMENT

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Autologous bone marrow transplantation using unfractionated cells cryopreserved in dimethylsulfoxide and hydroxyethyl starch without controlled-rate freezing

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