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

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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 dimethylsulfoxide (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 carmustine 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% ± 11.8%, 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.

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.1-6 Methods of BM cryopreservation have been standardized. The cells are frozen in 10% dimethylsulfoxide (DMSO) in a rate-controlled freezer set to cool at a constant 1°C per minute and are then stored until the time of infusion in either the liquid or vapor phase of liquid nitrogen.1,2,5,9 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.10 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.11-13 On the basis of data from previously reported cryopreservation studies, we also questioned the need for rate-controlled freezing14,15 and storage at liquid nitrogen temperatures.16-20 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 freezing22-26 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%,21 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 institutionally reviewed and approved study.

Cryoprotectant preparation. One hundred thirty-eight milliliter- ters 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
were performed on each specimen, and the plates were counted by incubated in a 6% CO2 atmosphere at 37°C. Quadruplicate assays as the source of colony-stimulating activity. The plates were dose for this drug.25 CFU-GM assays, 1 x 106 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 1. BM Cryopreservation Using 5% DMSO and 6% HES: Nucleated Cell and Committed Stem Cell Recovery During Processing and After Cryopreservation and Mean Harvested/Transplanted Cell Doses |
|-------------------------------------------------|------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Recovery (%) | Mean Cell Dose |
|-------------------------------------------------|------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Nucleated cell recovery during initial processing | 91.2 ± 16.7 (60)* | — |
| Nucleated cell recovery after thawing | 96.7 ± 11.6 (71) | — |
| Trypan blue viability after thawing | 82.2 ± 9.2 (65) | — |
| BFU-GM recovery after thawing | 81.8 ± 39.0 (63) | — |
| BFU-E recovery after thawing | 90.5 ± 41.2 (37) | — |

Values are expressed as means ± SD.

*Number of harvests or transplants.
Of the 72 BM reinfections, 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%), characterized 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 protimes 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 >1.0 10^5 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 >1,000/μL and the platelet count remained >20,000/μL 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 causes and the other died of sepsis and multiorgan failure. A single death occurred at 12 days posttransplant of progressive disease and cardiac failure. A single death occurred at 12 days posttransplant of progressive disease and sepsis. Two deaths occurred prior to transplantation. One patient died of pneumocystis pneumonia and the other died of respiratory failure and shock. None of the deaths were related to the transplantation procedure. The data are shown in Table 2. Engraftment was considered to have occurred if the total WBC and granulocyte counts were >1,000/mm^3 and the platelet count remained >20,000/mm^3 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).

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 Engraftment</th>
<th>Total White Cells &gt;1,000/μL</th>
<th>Granulocytes &gt;500/μL</th>
<th>Platelets &gt;20,000/μL</th>
<th>&gt;50,000/μL</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/kg nucleated cells (8)</td>
<td>16</td>
<td>20</td>
<td>23</td>
<td>12</td>
</tr>
<tr>
<td>Transplant &lt;1.8 x 10^5 of 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.
and storage at liquid nitrogen temperatures. 

Cryopreserved in 10% DMSO using rate-controlled freezing quickly in our patients as it did for those whose BM cells are effects associated with the infusion of these cells. Most clumping is seen after thawing, which permits us to freeze the cells in 300-mL aliquots, and there are only minor side 200 mL or less with <10% loss of total nucleated cells. No red little as two hours, including the 60 minutes needed to inexpensive. The total time to cryopreserve the 700 to 1,200 mm 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 v 50.6% ± 15.2% for the immersion method.2 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,11,12,23 we decided to use this as our freezing method. This technique has also been used successfully to freeze platelets36 as well as granulocytes.11-13 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.21 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 post thaw 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 growth17,27 and was able to reconstitute animals after irradiation.16,17,18,20,31 However, 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.24 In reexamining storage at –80°C, we found that for cells stored in the DMSO/HES mixture for periods of 12 to 16 months post thaw CFU-GM recovery was comparable to that of cells stored at liquid nitrogen temperatures.21

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 1,200 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.1,12,4,8

Although several studies have demonstrated the value of CFU-GM assays in predicting the engraftment of cryopreserved marrow,25,38 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 myeloablatative,27 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.1,33 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,24 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.39 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.

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REFERENCES


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