The Cryopreservation of Colony-forming Cells from the Bone Marrow of Children with Acute Lymphocytic Leukemia

A. H. Ragab, E. Gilkerson, and S. C. Choi

Division of Pediatric Oncology, The Edward Mallinckrodt Department of Pediatrics [A. H. R., E. G.], and the Division of Biostatistics [S. C. C.], Washington University, St. Louis, Missouri 63110

SUMMARY

Bone marrow cells from 15 children with acute lymphocytic leukemia in remission were preserved in liquid nitrogen for periods of up to 16 weeks. Although a substantial reduction in the number of myeloid cells was noted after cryopreservation, the colony-forming cells were still capable of proliferation. Dimethyl sulfoxide was demonstrated to be superior to glycerol as a cryoprotective agent for human marrow cells.

INTRODUCTION

Attempts have been made to preserve autologous bone marrow cells from patients with malignant disorders at low temperatures and then reinfuse them immediately after chemotherapy has been administered. The results to date have been equivocal (7, 10, 14). Many tests have been used to determine the viability of the cryopreserved cells after thawing. These tests include dye exclusion tests, supravital acridine orange staining, and observation for mobility of cells and thymidine incorporation (3). The most important cells in any bone marrow transplantation are the stem or progenitor cells that will repopulate the marrow. Since these stem cells are not morphologically identified and since these tests do not measure their proliferative potential, they are of very limited value. In mice, the cryopreservation of bone marrow stem cells may be measured by their ability to restore hemopoietic function when administered to lethally irradiated syngeneic recipients (5) or by the spleen colony assay (11, 12). Such tests cannot be used in humans.

An in vitro culture system is now available for the quantitation of granulopoietic progenitor cells in human bone marrow samples. When bone marrow cells are cultured in a semisolid medium [agar (17) or methylcellulose (19)], with an appropriate source of colony-stimulating factors, colonies are formed that are composed of granulocytes and macrophages. Each colony arises from a granulocytic progenitor cell or a CFC.2 Quantitation of the colonies observed gives an estimate of the number of CFC in a bone marrow sample. Recently, Gray and Robinson (9) have used the agar culture technique to measure the viability and proliferative potential of human marrow CFC after cryopreservation in a Revco freezer (West Columbia, S. C.) at -79°C. Marrow samples from 6 normal individuals were studied, and they noted that more than 40% of CFC per 2 x 10^6 nucleated cells plated were recovered after 14 days of cryopreservation. The number of total nucleated cells recovered was not mentioned and therefore the absolute number of CFC recovered was not estimated.

In this study we have attempted to define a method by which bone marrow cells from children with acute lymphocytic leukemia in remission can be preserved in liquid nitrogen. The viability and proliferative capacity of the CFC after cryopreservation were measured by their ability to form colonies in agar. The morphology of the bone marrow samples before and after cryopreservation were compared to determine which cells were damaged by this procedure. Also, DMSO and glycerol were compared for efficacy as cryoprotective agents for human marrow cells.

MATERIALS AND METHODS

Bone marrow cells were obtained from 15 children with acute lymphocytic leukemia in remission. All the bone marrow samples had a normal differential with less than 5% lymphoblasts; 12 of the samples were normocellular and 3 were hypocellular. All children were receiving maintenance chemotherapy which consisted of one of the following agents: 6-mercaptopurine, methotrexate, or cyclophosphamide.

The 1st bone marrow sample was aspirated for morphological evaluation. Then another sample (3 to 4 ml) was aspirated and transferred to a heparinized test tube and left to sediment for 1 hr. The cell-rich plasma was aspirated and centrifuged, and the cells were washed twice in McCoy's Medium 5A (Grand Island Biological Co., Grand Island, N. Y.) and counted in an electronic particle counter. A smear of the cells was prepared and stained with Leishman's stain. An aliquot of these cells was immediately plated in agar on underlayers prepared from the peripheral blood cells of normal individuals according to the method of Robinson and Pike (17). Cultures from each bone marrow sample were performed in duplicate at a concentration of 10^6 nucleated cells/plate. The rest of the cells were diluted to a...
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The percentage of CFC recovery for each bone marrow sample was calculated according to the following formula:

\[
\% \text{ CFC recovery} = \frac{(\text{CFC} / 10^8 \text{ nucleated cells after freezing})}{(\text{CFC} / 10^8 \text{ nucleated cells before freezing})} \times \% \text{ cell recovery}
\]

The mean percentage of CFC recovery after 1 week in liquid nitrogen was 51.8 (median, 40.0), while after 12 weeks in liquid nitrogen it decreased to 30.7 (median, 24.5) (Table 1).

Most of the data presented in Table 1 are characterized by positive skewness. The possible difference of the percentage of CFC recovery values in 1, 4, and 8 weeks was compared by the t test described in the “Appendix.” The test was performed on the logarithmically transformed data. The data for 12 and 16 weeks were not analyzed because of paucity of the sample. The difference between 1 and 4 weeks was not significant (based on the paired differences). The percentage of CFC recovery value after 8 weeks was significantly smaller than that after 1 week (p < 0.01). The t test applied to data for 4 and 8 weeks indicated that the difference is significant at p < 0.05. It therefore appears that the greatest loss of CFC occurs in the process of freezing and thawing, although there is continuous loss of CFC after many weeks in liquid nitrogen (Chart 1).

We have also compared the effectiveness of 10% DMSO and 10% glycerol as cryoprotective agents. Bone marrow cells from 10 children with acute lymphocytic leukemia in remission were cultured in agar. The remaining cells from each bone marrow sample were divided into 2 aliquots; one aliquot was cryopreserved with 10% DMSO and the other aliquot was cryopreserved with 10% DMSO. After 1 week in liquid nitrogen, both aliquots were thawed and the percentages of cell recovery and CFC were compared. DMSO was demonstrated to be superior to glycerol as a cryoprotective agent (p < 0.001) (Table 2).

DISCUSSION

In our present study we have demonstrated that CFC from the bone marrow of children with acute lymphocytic leukemia can be maintained in a viable and proliferative state for many weeks in liquid nitrogen. With the application of the in vitro culture technique for CFC as a monitor, the freezing and thawing processes may be optimized until the maximum yield of CFC is obtained. It is obvious from the present study that DMSO is a better cryoprotective agent for bone marrow cells than glycerol. This is in accord with the observation of some investigations (1, 2, 4) although this is disputed by others (9).

Certain factors may account for the variation in the number of CFC observed from the bone marrow of the leukemic children studied. We have previously demonstrated that the number of CFC derived from the bone marrow of children with acute lymphocytic leukemia in remission is significantly less than those derived from the bone marrow of “control” children (15, 16). This reduction in the number of CFC is due to the leukemic process or to the effect of the chemotherapeutic agents on the CFC. We have also demonstrated that the colony-stimulating activity of the peripheral blood cells of normal individuals, when used as underlayers in the agar culture, is subject to some...
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Table 1
CFC recovery from remission bone marrow after cryopreservation

<table>
<thead>
<tr>
<th>Patient</th>
<th>% cell recovery after freezing</th>
<th>CFC/10^4 cells before freezing</th>
<th>CFC/10^4 cells after freezing</th>
<th>CFC/10^4 after freezing / CFC/10^4 before freezing</th>
<th>% CFC recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>56 ± 6.5</td>
<td>47.1 ± 5.7</td>
<td>40.1 ± 5.7</td>
<td>35.0</td>
<td>27.3 ± 7.5</td>
</tr>
<tr>
<td>2</td>
<td>11 ± 3.0</td>
<td>8.1 ± 1.5</td>
<td>6.3 ± 1.0</td>
<td>3.0 ± 0.5</td>
<td>2.2 ± 0.7</td>
</tr>
<tr>
<td>3</td>
<td>25 ± 4.0</td>
<td>12.7 ± 2.5</td>
<td>10.3 ± 2.0</td>
<td>8.3 ± 1.5</td>
<td>6.7 ± 1.3</td>
</tr>
<tr>
<td>4</td>
<td>36 ± 5.0</td>
<td>18.4 ± 2.5</td>
<td>15.3 ± 2.0</td>
<td>12.3 ± 1.5</td>
<td>9.5 ± 1.3</td>
</tr>
<tr>
<td>5*</td>
<td>48 ± 6.0</td>
<td>24.7 ± 3.0</td>
<td>20.7 ± 3.0</td>
<td>16.7 ± 2.5</td>
<td>13.3 ± 2.0</td>
</tr>
<tr>
<td>6*</td>
<td>43 ± 7.0</td>
<td>19.4 ± 2.5</td>
<td>15.4 ± 2.0</td>
<td>11.4 ± 1.5</td>
<td>9.2 ± 1.3</td>
</tr>
<tr>
<td>7</td>
<td>86 ± 9.0</td>
<td>42.8 ± 4.0</td>
<td>34.8 ± 4.0</td>
<td>27.8 ± 3.5</td>
<td>22.3 ± 3.0</td>
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<tr>
<td>8</td>
<td>73 ± 8.0</td>
<td>36.5 ± 3.5</td>
<td>28.5 ± 3.5</td>
<td>22.5 ± 2.5</td>
<td>18.8 ± 2.3</td>
</tr>
<tr>
<td>9</td>
<td>88 ± 9.5</td>
<td>49.7 ± 4.5</td>
<td>41.7 ± 4.5</td>
<td>33.7 ± 3.5</td>
<td>27.2 ± 3.0</td>
</tr>
<tr>
<td>10</td>
<td>49 ± 7.5</td>
<td>23.4 ± 2.5</td>
<td>18.4 ± 2.5</td>
<td>14.4 ± 2.0</td>
<td>11.2 ± 1.7</td>
</tr>
<tr>
<td>11</td>
<td>35 ± 6.0</td>
<td>17.2 ± 1.5</td>
<td>13.2 ± 1.5</td>
<td>10.2 ± 1.0</td>
<td>8.1 ± 0.8</td>
</tr>
<tr>
<td>12</td>
<td>30 ± 5.0</td>
<td>13.5 ± 1.0</td>
<td>10.5 ± 1.0</td>
<td>8.5 ± 0.8</td>
<td>6.4 ± 0.7</td>
</tr>
<tr>
<td>13</td>
<td>33 ± 6.5</td>
<td>18.0 ± 1.5</td>
<td>14.0 ± 1.5</td>
<td>11.0 ± 1.0</td>
<td>9.0 ± 0.9</td>
</tr>
<tr>
<td>Mean ± S.E.</td>
<td>39.5 ± 3.7</td>
<td>28.5 ± 4.5</td>
<td>23.5 ± 4.0</td>
<td>18.5 ± 3.5</td>
<td>14.7 ± 3.1</td>
</tr>
<tr>
<td>Median</td>
<td>42.0</td>
<td>27.0 ± 1.5</td>
<td>22.0 ± 1.5</td>
<td>17.0 ± 1.0</td>
<td>13.0 ± 1.0</td>
</tr>
</tbody>
</table>

* Hypocellular bone marrow.

variation (A. H. Ragab and E. Gilkerson. Variability in the Colony Stimulating Activity Derived from the Peripheral Blood Cells of Normal Individuals. Submitted for publication).

When the morphology of the bone marrow cells before and after cryopreservation was compared, it was apparent that there was a substantial loss of mature granulocytes and myelocytes whereas lymphocytes, lymphoblasts, and nucleated red cells were well preserved. Myeloid cells have previously been demonstrated to be destroyed by cryopreservation (6, 13). In mice and monkeys the CFC has been shown to be a mononuclear cell which resembles lymphocytes by light microscopy but is distinguishable by electron microscopy (8, 18, 20). Zucker-Franklin et al. (21) have also recently demonstrated that the human CFC, present in the peripheral blood of normal subjects, are a subclass of

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of the paired observations computed by subtracting the 2nd from the 1st. Further, let \( x_1 \) and \( x_2 \) be the sample mean and standard deviation for the unpaired \( i \)th group. Then, under the null hypothesis of equal means a test statistic

\[
t = \frac{d + (\bar{x}_1 - \bar{x}_2)}{\sqrt{\frac{\sum (x_i - \bar{x}_1)^2}{n_1} + \frac{\sum (x_i - \bar{x}_2)^2}{n_2}}}.
\]

Table 2

CFC recovery from remission bone marrow using 10% DMSO and 10% glycerol as cryoprotective agents

<table>
<thead>
<tr>
<th>Patient</th>
<th>% cell recovery AF (DMSO)</th>
<th>% cell recovery AF (glycerol)</th>
<th>CFC/10^4 AF BF</th>
<th>CFC/10^4 AF (DMSO)</th>
<th>CFC/10^4 AF (glycerol)</th>
<th>(CFC/10^4 AF)/(CFC/10^4 BF) x 100</th>
<th>(CFC/10^4 AF)/(CFC/10^4 BF) x 100</th>
<th>% CFC recovery (DMSO)</th>
<th>% CFC recovery (glycerol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42</td>
<td>13</td>
<td>23</td>
<td>44</td>
<td>14</td>
<td>191</td>
<td>61</td>
<td>80</td>
<td>8</td>
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<tr>
<td>2</td>
<td>55</td>
<td>8</td>
<td>58</td>
<td>15</td>
<td>6</td>
<td>26</td>
<td>10</td>
<td>14</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>5</td>
<td>63</td>
<td>72</td>
<td>14</td>
<td>114</td>
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<td>40</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>2</td>
<td>46</td>
<td>62</td>
<td>27.5</td>
<td>135</td>
<td>44</td>
<td>9</td>
<td>1</td>
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<tr>
<td>5</td>
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<td>17</td>
<td>7</td>
<td>19</td>
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<td>5</td>
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<tr>
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<td>31</td>
<td>3</td>
<td>20</td>
<td>40</td>
<td>22.5</td>
<td>201</td>
<td>112</td>
<td>62</td>
<td>3</td>
</tr>
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<td>44</td>
<td>14</td>
<td>59</td>
<td>61</td>
<td>6</td>
<td>103</td>
<td>10</td>
<td>45</td>
<td>1</td>
</tr>
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<td>69</td>
<td>66</td>
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<td>43</td>
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<td>9</td>
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<td>43.5</td>
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<td>150</td>
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<td>27.5</td>
<td>2.5</td>
<td>131</td>
<td>12</td>
<td>59</td>
<td>1</td>
</tr>
</tbody>
</table>

Mean ± S.E.

<table>
<thead>
<tr>
<th>% cell recovery AF (DMSO)</th>
<th>% cell recovery AF (glycerol)</th>
<th>CFC/10^4 AF BF</th>
<th>CFC/10^4 AF (DMSO)</th>
<th>CFC/10^4 AF (glycerol)</th>
<th>(CFC/10^4 AF)/(CFC/10^4 BF) x 100</th>
<th>(CFC/10^4 AF)/(CFC/10^4 BF) x 100</th>
<th>% CFC recovery (DMSO)</th>
<th>% CFC recovery (glycerol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37.6 ± 3.5</td>
<td>7.0 ± 1.3</td>
<td>39.7 ± 6.8</td>
<td>44.8 ± 6.4</td>
<td>10.9 ± 2.7</td>
<td>116.6 ± 19</td>
<td>37.0 ± 11.4</td>
<td>46.8 ± 7.4</td>
<td>2.3</td>
</tr>
</tbody>
</table>

* AF, after freezing; BF, before freezing.

In comparing the means of 2 populations, suppose that the situation is such that \( m \) sets of observations are paired but \( n_1 \) and \( n_2 \) observations are not. Let \( d \) and \( s_0 \) denote the mean and standard deviation of difference \( |d| \) mononuclear cells distinguishable from lymphocytes by electron microscopy. In our study the mononuclear cells were fairly well preserved by the process of cryopreservation, and the number of CFC per 10^5 nucleated cells plated did not decrease substantially in the majority of cases after cryopreservation.

The applicability and usefulness of the process of autologous bone marrow reinfusion must await trials in which cryopreserved cells are given back to the same patients after the administration of chemotherapeutic agents or when the patient is neutropenic. The time required for the CFC to give rise to mature granulocytes may take a few days, although this period may be considerably shortened when the patient is neutropenic. Although the administration of autologous remission marrow cells back to leukemic patients will contain a few leukemic cells, this may be justified in life-threatening bacterial infections when the patient is neutropenic.

APPENDIX

In comparing the means of 2 populations, suppose that the situation is such that \( m \) sets of observations are paired but \( n_1 \) and \( n_2 \) observations are not. Let \( d \) and \( s_0 \) denote the mean and standard deviation of difference \( |d| \) the test is applicable as long as \( m > 1 \), \( n_1 > 1 \), and \( n_2 > 1 \). If \( m = 1 \), then one may desire to use the unpaired \( t \) test, discarding the paired observations. Similarly, if \( n_1 = 1 \) or \( n_2 = 1 \), then the paired \( t \) test can be used discarding the unpaired sample.

REFERENCES

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