In Vitro and in Vivo Assessment of the Viability of Dog Marrow after Storage

By John A. Mannick, Harry L. Lochte, Jr., E. Donnall Thomas and Joseph W. Ferree

Recent studies have indicated the usefulness of stored marrow in the repopulation of marrow spaces after lethal irradiation.\textsuperscript{1-4} The viability of marrow stored for this purpose is definitively demonstrated by survival of the irradiated recipient. A satisfactory in vitro test of viability, however, is essential for studies in man since opportunities for in vivo assay in patients are necessarily limited. In this laboratory deoxyribonucleic acid (DNA) synthesis in vitro has been used as an index of the ability of marrow cells to proliferate, since an increase in DNA is a prerequisite for cell division. In the studies on stored samples of dog marrow reported here, the relationship between DNA synthesis in vitro and cell viability in vivo was explored. The in vivo test used has been the ability of stored autologous marrow to induce survival in lethally irradiated dogs.

Materials and Methods

In Vitro Studies

Marrow was obtained from the long bones of dogs anesthetized with intravenous pentobarbital. The marrow was processed immediately through screens of graded porosity to prepare a cellular suspension.\textsuperscript{5} Supporting media were Hanks' solution and 10 per cent dog serum or plasma. The plasma contained 0.1 mg./ml. of heparin (Abbott). For storage at \(-79\) C. marrow was frozen in 15 per cent glycerol, as described previously.\textsuperscript{6} DNA synthesis was determined by measuring the incorporation of C\textsuperscript{14}-formate into the thymine isolated from DNA.\textsuperscript{7} Measurements on samples of frozen marrow were performed after thawing and removing the glycerol by a modification of the procedure of Sloviter and Tietze.\textsuperscript{5} One-half volume of 35 per cent dextrose in water was added to the marrow-glycerol suspension. After one to two minutes, one volume of 5 per cent dextrose in water was added. After one to two minutes, another volume of 5 per cent dextrose in water was added, making a final dilution of 1:6 and a glycerol concentration of 2.5 per cent. During this procedure deoxyribonuclease* was added to diminish the formation of clumps that frequently appeared because of DNA liberated by damaged or disrupted cells.\textsuperscript{6} All glassware was acid cleaned, siliconed, and kept free of contact with detergents.

In Vivo Studies

Beagle dogs of both sexes, between 5 and 18 months of age, were given lethal total-body irradiation by one of two methods: (1) 250 KVP x-ray therapy machine, dose rate 5 r/min. in air at mid-body line, HVL 2.2 mm. Cu, filters 1.0 mm. Al, 0.25 mm. Sn, 0.4 mm. Cu, 250 KVP, 10 ma, target distance 100 cm. (2) Double Co\textsuperscript{60} teletherapy unit, dose rate 1.8

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*Worthington Biochemical Corporation, Freehold, N. J.
r min., target distances 2.0 and 2.3 M. Radiation dosages ranged from 600 to 1500 r air
dose. The pre- and post-irradiation preparation and care, and the hematologic studies of
these dogs have been described in detail. No dog in this laboratory has survived 600 r
of total body irradiation unless given a post-irradiation infusion of marrow. Autologous
marrow for injection into irradiated dogs was obtained prior to irradiation by aspiration of
the femurs.

RESULTS

In Vitro

There was a partial loss of the ability to synthesize DNA by dog marrow
stored at 4 C. for 24 hours. This loss was independent of the presence or ab-

![DNA synthesis by dog marrow stored for 24 hours at 4 C.](image)

**Table 1.** The Effect of Duration of Storage on DNA Synthesis
by Marrow Kept at 4 C.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Duration of storage (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0  7,600</td>
</tr>
<tr>
<td></td>
<td>2  2,400</td>
</tr>
<tr>
<td></td>
<td>6  200</td>
</tr>
<tr>
<td></td>
<td>24 700</td>
</tr>
<tr>
<td>B</td>
<td>11,300</td>
</tr>
<tr>
<td></td>
<td>9,500</td>
</tr>
<tr>
<td></td>
<td>7,900</td>
</tr>
<tr>
<td></td>
<td>7,400</td>
</tr>
</tbody>
</table>

Incubation conditions were as described under figure 1. DNA synthesis is expressed
as counts per minute per micromole of recovered thymine.
Fig. 2.—DNA synthesis by dog marrow frozen in glycerol. The marrow was frozen to $-79^\circ$C. in 15 per cent glycerol and subsequently thawed at 37$^\circ$C. The glycerol concentration was reduced by the Sloviter procedure, the cells were separated by centrifugation at 200 g for 10 minutes and resuspended in the incubation medium. Incubation conditions were as described under figure 1.

The decrease in activity observed varied from dog to dog. Figure 1 illustrates this variation in several of the samples tested. It was usual to find at least 40 per cent of the initial ability to synthesize DNA preserved after storage at 4$^\circ$C. for 24 hours. Table 1 illustrates the progressive decrease in DNA synthesis with time in two marrow samples stored at 4$^\circ$C. Very low levels were reached at 96 hours.

Previous work with rabbit marrow and with human marrow has indicated that capacity for DNA synthesis decreases rapidly during storage at room temperature or at 37$^\circ$C. Measurements at these temperatures were, therefore, not repeated with dog marrow.

Figure 2 shows rates of DNA synthesis observed in samples of dog marrow that had been frozen to $-79^\circ$C. in 15 per cent glycerol and 5 per cent plasma. The values obtained were compared with those found in a sample of the same marrow, not frozen, but incubated immediately with C$^{14}$-formate. Between 40 and 50 per cent of the original ability to synthesize DNA was preserved in the frozen marrow samples.

In Vivo

Eighteen beagle dogs received supralethal total-body irradiation followed by intravenous infusions of autologous bone marrow. The quantities of marrow injected, the storage time, the storage temperature, the storage media, the radiation dosages, and the results obtained are summarized in table 2.

In general, the results of these in vivo assays were those anticipated from the in vitro studies. Eight irradiated dogs recovered after injection of autologous
Table 2.—Survival of Lethally Irradiated Dogs Given Intravenous Infusions of Stored Autologous Marrow

<table>
<thead>
<tr>
<th>Dog no.</th>
<th>Radiation dose (in r)</th>
<th>Marrow dose (in billions)</th>
<th>Storage media</th>
<th>Storage temp.</th>
<th>Storage time (hr.)</th>
<th>Survival after irradiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>65</td>
<td>600 250 KV</td>
<td>1.9</td>
<td>Hanks'</td>
<td>4 C.</td>
<td>3</td>
<td>yes</td>
</tr>
<tr>
<td>54</td>
<td>600 250 KV</td>
<td>1.4</td>
<td>Hanks'</td>
<td>4 C.</td>
<td>2½</td>
<td>yes</td>
</tr>
<tr>
<td>82</td>
<td>800 250 KV</td>
<td>3.2</td>
<td>Hanks'</td>
<td>4 C.</td>
<td>6</td>
<td>yes</td>
</tr>
<tr>
<td>83</td>
<td>800 250 KV</td>
<td>2.5</td>
<td>Hanks'</td>
<td>4 C.</td>
<td>24</td>
<td>yes</td>
</tr>
<tr>
<td>95</td>
<td>1000 Co&lt;sup&gt;60&lt;/sup&gt;</td>
<td>3.7</td>
<td>Hanks'</td>
<td>4 C.</td>
<td>23</td>
<td>yes</td>
</tr>
<tr>
<td>94</td>
<td>1200 Co&lt;sup&gt;60&lt;/sup&gt;</td>
<td>3.4</td>
<td>Hanks'</td>
<td>4 C.</td>
<td>20</td>
<td>yes</td>
</tr>
<tr>
<td>111</td>
<td>1500 Co&lt;sup&gt;60&lt;/sup&gt;</td>
<td>1.9</td>
<td>Hanks'</td>
<td>4 C.</td>
<td>22</td>
<td>yes</td>
</tr>
<tr>
<td>126</td>
<td>1300 Co&lt;sup&gt;60&lt;/sup&gt;</td>
<td>2.4</td>
<td>Hanks'</td>
<td>4 C.</td>
<td>24</td>
<td>yes</td>
</tr>
<tr>
<td>126</td>
<td>1000 Co&lt;sup&gt;60&lt;/sup&gt;</td>
<td>2.7</td>
<td>Hanks'</td>
<td>4 C.</td>
<td>96</td>
<td>no</td>
</tr>
<tr>
<td>125</td>
<td>1000 Co&lt;sup&gt;60&lt;/sup&gt;</td>
<td>4.5</td>
<td>Hanks'</td>
<td>4 C.</td>
<td>96</td>
<td>yes</td>
</tr>
<tr>
<td>113&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1000 Co&lt;sup&gt;60&lt;/sup&gt;</td>
<td>2.4</td>
<td>15% glycerol</td>
<td>-79 C.</td>
<td>26</td>
<td>no&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>116</td>
<td>1000 Co&lt;sup&gt;60&lt;/sup&gt;</td>
<td>1.0</td>
<td>15% glycerol</td>
<td>-79 C.</td>
<td>28</td>
<td>no</td>
</tr>
<tr>
<td>134</td>
<td>1000 Co&lt;sup&gt;60&lt;/sup&gt;</td>
<td>2.6</td>
<td>15% glycerol</td>
<td>-79 C.</td>
<td>25</td>
<td>yes</td>
</tr>
<tr>
<td>144</td>
<td>1000 Co&lt;sup&gt;60&lt;/sup&gt;</td>
<td>2.9</td>
<td>15% glycerol</td>
<td>-79 C.</td>
<td>100</td>
<td>yes</td>
</tr>
<tr>
<td>165</td>
<td>1000 Co&lt;sup&gt;60&lt;/sup&gt;</td>
<td>3.1</td>
<td>15% glycerol</td>
<td>-79 C.</td>
<td>72</td>
<td>yes</td>
</tr>
<tr>
<td>166</td>
<td>1000 Co&lt;sup&gt;60&lt;/sup&gt;</td>
<td>1.1</td>
<td>15% glycerol</td>
<td>-79 C.</td>
<td>120</td>
<td>no</td>
</tr>
<tr>
<td>167</td>
<td>1000 Co&lt;sup&gt;60&lt;/sup&gt;</td>
<td>3.6</td>
<td>15% glycerol</td>
<td>-79 C.</td>
<td>130</td>
<td>yes</td>
</tr>
<tr>
<td>168</td>
<td>1000 Co&lt;sup&gt;60&lt;/sup&gt;</td>
<td>4.0</td>
<td>15% glycerol</td>
<td>-79 C.</td>
<td>115</td>
<td>yes</td>
</tr>
</tbody>
</table>

<sup>*</sup>Satisfactory marrow recovery. Died of hepatitis.

bone marrow stored at 4 C. for 24 hours or less. These dogs received marrow stored under circumstances shown to preserve a rate of DNA synthesis of at least 30 to 40 per cent of control values. Two dogs similarly irradiated were given autologous marrow stored in vitro at 4 C. for 96 hours, a length of time sufficient to depress DNA synthesis markedly. One of these animals failed to recover from irradiation; the second recovered. The dog that recovered received an exceptionally large marrow dose, 4.5 billion nucleated cells. After 96 hours storage, enough viable cells apparently remained in this large marrow sample to induce repopulation of the marrow spaces.

After irradiation 8 dogs were given marrow that had been frozen in glycerol to -79 C. by the method shown above to preserve a rate of DNA synthesis of 40 to 50 per cent of the initial value. Two of the animals (dogs 113 and 116) received marrow in which, after thawing, the glycerol concentration was reduced to 2.5 per cent by step-wise dilution with 35 per cent dextrose in water and standard isotonic solutions (see Methods). Marrow function returned in one dog, but the animal died within 12 days of hepatitis. Autopsy demonstrated repopulation of marrow spaces. Marrow function did not return in the second dog. This animal received an exceptionally small infusion of marrow, 1.0 billion cells. Six dogs received frozen marrow in which the glycerol concentration after thawing was reduced to 10 per cent by addition of one-half volume of 35 per cent dextrose in water. Five of these animals regained marrow function and lived. The sixth dog failed to demonstrate return of marrow function and died. This animal received an exceptionally small infusion of marrow, 1.1 billion cells.

Frozen dog marrow was observed to have a greater tendency than rabbit or human marrow to form DNA slime and clumps during thawing and glycerol dilution. When the dilution was carried to a final glycerol concentration of...
2.5 per cent (see Methods), large clumps formed, and these clumps were not broken up by the DNAse added. Intravenous injection of such clumps killed a test animal because of pulmonary embolism. Therefore, in the experiments described, large clumps were screened out and discarded before the marrow suspensions were injected. Discarding these clumps caused a loss of about 3/4 of the nucleated cells. When frozen marrow samples were thawed and the glycerol concentration reduced to 10 per cent by the addition of one-half volume of 35 per cent dextrose in water, clumping due to DNA slime was minimal. It was found that such clumps as formed could be easily broken up by DNAse solution, leaving a smooth cell suspension for injection. No adverse clinical effect from the injection of these marrow suspensions with glycerol in 10 per cent concentrations was encountered in the six irradiated dogs that received them. Five of the six animals so treated promptly regained marrow function.

DISCUSSION

From the observations described it is evident that the capacity for DNA synthesis can be preserved moderately well for periods up to 24 hours in dog marrow cells stored in vitro at 4 C. Ability to synthesize DNA is also retained by dog marrow cells frozen to −79 C. in 15 per cent glycerol. The length of the period of viability of marrow stored at −79 C. is not known but is presumed to be of considerable duration. The essential trauma of the storage procedure lies in the processes of freezing, thawing, and glycerol extraction and not in the passage of time at −79 C. Experiments to determine the duration of viability at this temperature are in progress. In the results reported here, marrow samples stored for 17 days retained viability.

A quantitative correlation between in vivo and in vitro measurements of viability cannot be established by the small series of experiments presented. However, the survival of the irradiated animals given transplants of stored autologous marrow is consistent with the concept that the demonstrated ability of these marrow cells to synthesize DNA in vitro is reflected by their ability to proliferate in vivo, granted a congenial host environment. Indeed, there is evidence that marrow samples which have lost almost all their ability to synthesize DNA in vitro may still confer irradiation protection in vivo.² A limit to this growth potential, however, is shown in the present study by the death of the dog that received marrow stored for 96 hours at 4 C., i.e., long enough to lose almost completely its capacity for DNA synthesis in vitro. This animal succumbed to radiation effects without evidence of a successful marrow transplant.

In these studies dog marrow appeared to respond to freezing, thawing and glycerol removal in a manner similar to rabbit and human marrow, except that cell disruption and the subsequent formation of DNA slime and clumps of cells appeared to be more marked with dog marrow than with the marrow of other species. The sliming and clumping were within tolerable limits in samples that were thawed and the glycerol concentration before injection reduced to 10 per cent by the addition of one-half volume of 35 per cent dextrose in water. The associated osmotic trauma hemolyzed most of the red cells in the mar-
row samples so treated, but the ability of these samples to induce recovery in irradiated animals did not appear measurably affected, and the infusions were conveniently and safely performed.

**SUMMARY**

Assessment has been made of the ability of dog marrow cells to survive storage at 4 C. and at −79 C. Survival has been assessed in vitro by measurements of rates of DNA synthesis and in vivo by ability to restore marrow functions in lethally irradiated recipients.

Dog bone marrow cells synthesize DNA in vitro at values 30 to 80 per cent of normal after storage for 24 hours at 4 C. DNA synthesis is reduced to very low levels by storage for 96 hours at 4 C. Eight lethally irradiated beagle dogs (600 to 1500 r) survived acute irradiation effects and returned to normal health after receiving intravenous infusions of autologous bone marrow that had been stored at 4 C. for 24 hours or less. One dog, similarly irradiated, failed to survive when given marrow stored for 96 hours at 4 C. A tenth dog did survive when given an exceptionally large quantity of marrow stored for 96 hours at 4 C.

Dog marrow cells synthesize DNA in vitro at values 40 to 50 per cent of normal in samples stored at −79 C. in 15 per cent glycerol for periods up to 17 days. Five lethally irradiated beagle dogs (1000 to 1500 r) survived acute irradiation effects and returned to normal health after receiving intravenous infusions of autologous marrow that had been stored at −79 C. in 15 per cent glycerol. One other dog, similarly irradiated and treated, regained marrow function but died of hepatitis. Two other irradiated dogs similarly treated did not regain marrow function and died. These two had received exceptionally small infusions of marrow cells.

The observations demonstrate that dog marrow can be preserved satisfactorily at 4 C. for 24 hours and in glycerol at −79 C. for longer periods. The capacity of the stored marrow to synthesize DNA in vitro has correlated well with its ability to induce recovery in lethally irradiated autologous recipients. DNA synthesis in vitro appears to be a useful index of the ability of marrow cells to proliferate in vivo.

**SUMMARIO IN INTERLINGUA**

Esseva effectuate un evalutation del capacitate de medulla canin de super-viver immagasinage a 4 e a −79 C. Le superviventia del cellulas esseva evaluata in vitro per le mesuration del intensitate del synthese de acido disoxyribonucleic e in vivo per determinar le capacitate del cellulas de restaurar le functiones medullari in letalmente irradiate recipientes.

Cellulas del medulla ossee de canes synthetisa acido disoxyribonucleic a inter 30 e 80 pro cento del nivello normal post 24 horas de immagasinage a 4 C. Le synthese de acido disoxyribonucleic es reducita a basissime nivello per un immagasinage de 96 horas a 4 C. Octo letalmente irradiate canes del racia beagle (habente recipite 600 a 1500 r) superviveva le effectos acute del irradiation e retornava a un stato normal de valetude post reciper infusions
intraveneose de autologe medulla ossee que habeva essite immagasinate a 4 C durante 24 horas o minus. Un can, que esseva irradiate in les mesme maniera, non superviveva post recipier medulla immagasinate durante 96 horas a 4 C. Un decime can, del altere latere, superviveva post recipier un exceptionalmente grande quantitate de medulla immagasinate durante 96 horas a 4 C.

Cellulas de medulla canin synthetisa acido disoxyribonucleic in vitro a inter 40 e 50 pro cento del nivello normal in specimens immagasinate a -79 C in 15 pro cento de glycerol during periods de usque a 17 dies. Cinque letalemente irradiate beagles (habente recipite 1000 a 1500 r) superviveva le effectos acute del irradiation e retornava a un stato normal de valetude post recipier infusions intraveneose de medulla autologe que habeva essite immagasinate a -79 C in 15 pro cento de glycerol. Un altere can, similemente tractate e irradiate, reganiava su function medullari sed moriva de hepatitis. Duo altere canes que habeva recipite le mesme irradiation e le mesme tractamento non reganiava lor function medullari e moriva. Iste duo habeva recipite exceptionalmente micre infusions de cellulas medullari.

Le observationes demonstra que medulla canin pote esser preservate satisfactorimente a 4 C durante 24 horas e in glycerol a -79 C durante plus longe periodos de tempore. Le capacitate del immagasinate medulla de synthetisar acido disoxyribonucleic se ha monstrate ben correlationate con le capacitate del mesme medulla de inducer le restablimento de latelmente irradiate recipientes autologe. Le synthese de acido disoxyribonucleic in vitro pare esser un utile del capacitate de cellulas medullar de proliferar in vivo.

REFERENCES

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