Studies of the Irradiation Protection Effect of Fetal Liver in Mice. II. Storage by Freezing

By John H. Githens, Paul N. Tschetter, Giovannella Moscovic and William E. Hathaway

Recent reports have suggested that fetal liver or spleen may have certain immunologic advantages over bone marrow in hematopoietic transplants. Therefore, the present study was undertaken to determine whether some of the technics described for the preservation of bone marrow by freezing might also be applied to the preservation of liver tissue from fetal donors. It has been demonstrated in several animal species and in man that the irradiation protection effect of bone marrow can be preserved by slow freezing in glycerol. It has also been shown that mouse bone marrow can be frozen successfully in media that contain appropriate concentrations of certain amino acids or inorganic compounds.

Only a few investigators have applied this technic to the preservation of liver or spleen. Barnes and Loutit have shown that the irradiation protection effect of adult mouse spleen can be preserved by freezing in glycerol at -70 C. Ferrebee et al. demonstrated that human fetal liver and spleen cells remained viable and retained their ability to synthesize DNA after being frozen in glycerol and serum.

In the present investigation the preservation of the irradiation protection effect of frozen fetal liver was studied in vivo by evaluating the ability of the thawed cells to promote recovery in lethally irradiated mice.

Materials and Methods

The irradiated recipients and the donors were Webster strain mice. The grafts were considered to be homologous rather than isologous since the animals were not bred to be genetically identical and since all attempts at cross skin grafting within the colony had been unsuccessful. The recipients were females 12 weeks of age. The donor fetal liver tissue was obtained from fetuses of 16 and 17 days gestation. The full gestational period for these mice is 21 days.

The recipients were given total body irradiation in a dose of 775 r, measured in air at 50 cm. A 220 KV, 15 MA Maximair therapy unit was used. The filter was 0.5 mm. copper and 1.0 mm. aluminum with a half-value layer equivalent to 1.35 mm. of copper.

The hematopoietic tissue to be transplanted was fetal liver. The tissue was divided into single cells by passage through stainless steel screens according to the method of Ferrebee et al. The liver tissue was prepared for freezing within one hour of its removal from the donor fetuses.

Freezing was performed by placing the vials of tissue in a mixture of dry ice and alcohol. The slow freezing (at 1-2 C. per minute) followed the Polge-Smith-Parkes technic with...
the temperature being reduced at a rate of 1 C. per minute to −20 and then at a rate of
2 C. per minute from −20 to −80 C.

The ability of stored fetal liver to protect lethally irradiated mice for 24 days was
determined for each of the methods of freezing. Donor cells in each experiment were stored
in the frozen state for two weeks before being transplanted. In all cases, approximately
20,000,000 thawed nucleated cells were injected intravenously into mice a few hours after
their irradiation. The concentration of glycerol in the final suspension that was injected
never exceeded 5 per cent because all of the liver preparations were diluted with Hanks
and calf serum, or by the Sloviter technic with concentrated glucose. There were es-
essentially no deaths that might have been attributed to the direct effect of the injection of
any of the liver preparations. Mortality in the injected animals did not proceed at a more
rapid rate than that of the uninjected irradiated controls.

RESULTS

Table 1 summarizes the effects of the rate of freezing, the storage tempera-
ture, and the rate of thawing on the preservation of the irradiation protection
effect of fetal liver tissue. The results are recorded as the number of animals
dead at 24 days over the original number in the experiment. The rate of freez-
ing was found to be important. Tissue frozen rapidly gave no protection while
that which was frozen at a rate of approximately 1–2 C. per minute showed
definite preservation of the irradiation protection effect. Equally good protection
was obtained with cells frozen slowly by the technic of wrapping the vial in
tissue paper and placing it in dry ice. Storage at −50 C. appeared to be as ef-
eective as storage at −80 C. Tissue thawed rapidly at 37 C. was much more
effective than that which was thawed slowly.

Table 2 summarizes the variations in the freezing mixture, in the method
of tissue preparation, in the dilution technic, and their effects on the preser-
vation of the irradiation protection of the frozen fetal liver. In all of these ex-

| Table 1.—Effects of the Rate of Freezing, Storage Temperature and Rate of Thawing on the Preservation of the Irradiation Protection Effect of Fetal Liver |
|---|---|---|---|
| Rate of freezing | Storage temperature | Rate of thawing | % Glycerol in mixture | 24-day mortality number dead/ original number |
| 1. Rapid to −80 C. | −80 C. | Rapid at 37 C. | 5 | 10/10 (100.0%) |
| 2. Rapid to −80 C. | −80 C. | Rapid at 37 C. | 15 | 10/10 (100.0%) |
| 3. Slow at 1–2 C./min. to −80 C. | −80 C. | Rapid at 37 C. | 5 | 22/40 (55.0%) |
| 4. Slow at 1–2 C./min. to −80 C. | −80 C. | Rapid at 37 C. | 15 | 28/58 (48.3%) |
| 5. Slow in tissue paper | −80 C. | Rapid at 37 C. | 15 | 9/18 (50.0%) |
| 6. Slow at 1–2 C./min. at −80 C. | −50 C. | Rapid at 37 C. | 5 | 6/10 (60.0%) |
| 7. Slow at 1–2 C./min. to −80 C. | −50 C. | Rapid at 37 C. | 15 | 6/10 (60.0%) |
| 8. Slow at 1–2 C./min. to −80 C. | −80 C. | Slow at 1–2 C. min. | 5 | 9/10 (90.0%) |
| 9. Slow at 1–2 C./min. to −80 C. | −80 C. | Slow at 1–2 C. min. | 15 | 8/10 (80.0%) |
| 10. Uninjected irradiated controls | — | — | — | 40/40 (100.0%) |

*Tissue was stored at these temperatures for a period of two weeks.
|The basic solution for freezing and dilution in all experiments in the above table was Hanks' with 10 per cent calf serum.
### Table 2.—Effects of the Freezing Mixture, Method of Preparation of the Tissue, and Dilution Technic on the Preservation of the Irradiation Protection Effect of Frozen Fetal Liver

<table>
<thead>
<tr>
<th>Glycerol concentration</th>
<th>Serum concentration</th>
<th>Tissue preparation</th>
<th>Dilution technic</th>
<th>24-day mortality number/total number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 0%</td>
<td>10%</td>
<td>Single cell</td>
<td>Hanks’ &amp; 10% calf serum</td>
<td>10/10 (100.0%)</td>
</tr>
<tr>
<td>2. 5%</td>
<td>10%</td>
<td>Single cell</td>
<td>Hanks’ &amp; 10% calf serum</td>
<td>22/40 (55.0%)</td>
</tr>
<tr>
<td>3. 15%</td>
<td>10%</td>
<td>Single cell</td>
<td>Hanks’ &amp; 10% calf serum</td>
<td>28/58 (48.3%)</td>
</tr>
<tr>
<td>4. 25%</td>
<td>10%</td>
<td>Single cell</td>
<td>Hanks’ &amp; 10% calf serum</td>
<td>10/10 (100.0%)</td>
</tr>
<tr>
<td>5. 15%</td>
<td>50%</td>
<td>Single cell</td>
<td>Hanks’ &amp; 10% calf serum</td>
<td>18/36 (50.0%)</td>
</tr>
<tr>
<td>6. 15%</td>
<td>10%</td>
<td>Minced</td>
<td>Hanks’ &amp; 10% calf serum</td>
<td>11/18 (61.1%)</td>
</tr>
<tr>
<td>7. 15%</td>
<td>50%</td>
<td>Minced</td>
<td>Hanks’ &amp; 10% calf serum</td>
<td>8/18 (44.4%)</td>
</tr>
<tr>
<td>8. 5%</td>
<td>10%</td>
<td>Single cell</td>
<td>Sloviter</td>
<td>12/20 (60.0%)</td>
</tr>
<tr>
<td>9. 15%</td>
<td>10%</td>
<td>Single cell</td>
<td>Sloviter</td>
<td>9/20 (45.0%)</td>
</tr>
<tr>
<td>10. Uninjected, irradiated controls</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>40/40 (100.0%)</td>
</tr>
</tbody>
</table>

The freezing mixture in all experiments consisted of Hanks’ solution with the amounts of glycerol and serum listed in the table. In all experiments the freezing was done slowly at 1–2 C. per minute to –80 C.; storage was at –80 C.; thawing was rapid.

Experiments the rate of freezing was slow at 1–2 C. per minute to –80 C. The storage was at –80 and thawing was done rapidly in a water bath at 37 C. The basic freezing mixture was Hanks’ solution with varying amounts of glycerol and serum. A freezing mixture containing 5 to 15 per cent glycerol in Hanks’ electrolyte solution gave the best protection. Varying the amount of serum in the freezing mixture from 10 per cent to 50 per cent did not influence the 24-day mortality.

Tissue frozen in the minced state was preserved as well as that which had been broken down to single cell size prior to freezing. Dilution by the Sloviter technic did not influence the irradiation protection effect and would appear to be unnecessary.

**DISCUSSION**

These studies suggest that the optimum conditions for preservation of the irradiation protection effect of fetal mouse liver by freezing in glycerol are very similar to those that have been previously described for bone marrow. Since it would appear to be important for the tissue to be frozen slowly, it is of interest that good results were obtained when the vials containing the liver in the freezing mixture were wrapped in tissue paper and placed in a thermos of dry ice. This method has been recommended by Ferrebee et al. for the preservation of human fetal liver. The purpose of wrapping the vials in tissue paper is to slow the rate of freezing and it presents the advantage of being technically more simple than the Polge-Smith-Parkes technic in which the rate
of fall of the temperature is carefully controlled in the freezer. The temperature in the wrapped vials dropped at a rate of 3°C per minute until it reached 5°C and then decreased only 0.5°C per minute to −80°C.

These in vivo studies also support the recommendation of Ferrebee et al. for freezing fetal liver tissue in the minced state rather than as single cells. Preparation of the tissue by this method is less complicated and the preservation of the irradiation protection effect would appear to be comparable.

The fact that rapidly thawed fetal liver gave better protection than that which was thawed slowly is in accord with the findings of Tran and Bender for mouse bone marrow, although Ferrebee et al. have reported good results with slowly thawed marrow.

Dilution of the thawed cells was carried out with concentrated glucose as well as with the usual diluting fluid. This method was tried because Sloviter has shown that red blood cells can be preserved successfully by freezing in glycerol only if the dilution is done in this manner. The concentrated glucose in the diluting fluid equalizes the osmotic pressure of the glycerol within the cells and thereby prevents hemolysis. This procedure appears to be unnecessary in the dilution of hematopoietic cells of fetal liver which have been frozen in glycerol.

The 45 to 55 per cent mortality in these irradiated Webster mice injected with frozen fetal liver (stored under optimum conditions) compares favorably with an early mortality of 37 per cent in 179 mice of the same strain injected in our laboratory with fresh fetal liver from donors of the same age.

Summary

Various methods of freezing have been evaluated for the preservation of the radiation protection effect in fetal mouse liver. The best results were obtained with tissue frozen slowly in glycerol to −80°C, stored at −50°C or lower, and thawed rapidly. Other variables such as the size of the tissue particles, the amount of serum in the freezing mixture, and the method of dilution did not influence the results.

Summary in Interlingua

Varie methodos de congelation esseva evalutate pro le preservation del effecto radio-protectori de murin hepate fetal. Le melior resultatos esseva obtenite con tissu congelate lentemente in glycerol usque a −80°C, magasinate a −50 o plus basse, e disgelate rapidemente. Altere variabiles—le dimensiones del particulas de tissu, le quantitate de sero in le mixtura de congelation, e le methodo de dilution—non influentiava le resultatos.

References

4. Tschetter, P. N., Githens, J. H., and


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