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ASSOCIATION EURATOM - T.N.O.
European Atomic Energy Community
and
Toegepast Natuurwetenschappelijk Onderzoek

# THE EFFECTIVENESS OF DIFFERENT FREEZE STORAGE TECHNIQUES FOR MOUSE BONE MARROW CELL SUSPENSIONS

by

L. M. VAN PUTTEN (T.N.O.)

1963


Work performed under the Euratom Contract N° 004-59-12 BIAN

Paper presented at the Conference
on Modification of Radiation Injury by Bone Marrow Transplantation
and Chemical Protection
New York, 1-3 December 1962

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survival. The following variables in the technique were investigated : ACD versus Tyrode's solution as a diluent, comparative effectiveness of glycerol and dimethyl sulfoxyde to protect during freezing and three different methods of handling the suspensions after thawing. The percentage recovery of protecting ability of the suspensions after storage varied with different techniques between 72 % and less than 10 %. Best results were obtained when glycerol-protected cells were slowly diluted with Tyrode's solution before injection. Application of these techniques to the storage of rhesus monkey bone marrow suspensions indicated that there are marked inter-species differences in storage efficiency for a given technique.

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## SUMMARY

The effectiveness of different freeze-storage techniques for mouse bone marrow cell suspensions was quantitatively investigated. As the criterium for effective storage, the ability of the suspensions to protect lethally irradiated mice was estimated. The fresh suspensions as well as the suspensions that had been frozen and thawed by different methods were tested in serial twofold dilutions in irradiated mice to estimate the 50% effective cell dose for 30-day survival. The following variables in the technique were investigated: ACD versus Tyrode's solution as a diluent, comparative effectiveness of glycerol and dimethyl sulfoxyde to protect during freezing and three different methods of handling the suspensions after thawing. The percentage recovery of protecting ability of the sus-

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Many studies have been made of methods for storing living cells at low temperature and these studies have been applied to bone marrow cells used for injection in lethally irradiated mice[1-3], rabbits[4] and dogs[5]. Similarly use has been made of these methods for application in the storage of human hemopoietic cells[6, 7, 8]. In many instances attempts have been made at a quantitative evaluation of these storage techniques by estimation of for instance oxygen uptake, incorporation of radioactive phosphorus or tritiated nucleotides, by vital staining techniques or motility studies of the cells recovered after thawing. Discrepancies between the results with different techniques have made it desirable to attempt a more accurate quantitative estimate of the most important parameter relevant to stored hemopoietic cells, i.e. their ability to protect lethally irradiated animals. This report describes a comparative study of the ability of mouse bone marrow cells, fresh or after storage by different methods, to protect lethally irradiated mice. The study was designed to make the results directly comparable to those obtained in rhesus monkeys in our institute[9]. In these animals poor conservation of protective effect had been found when testing the effectiveness of stored autologous bone marrow. The technique involved the procurement of bone marrow from the living anesthesised monkey and this

caused a large admixture of peripheral blood with the bone marrow cell suspension.

Since this admixture of blood and the presence of heparin might have influenced the effectiveness of storage of monkey bone marrow, in mice the effect of a similar admixture of heparinised blood was investigated. By testing bone marrow cell suspensions in serial twofold dilutions in irradiated mice an accurate estimate of the protective ability of these suspensions was attempted.

## MATERIALS AND METHODS

C57BL/Rij females were used as bone marrow donors; the hosts were $F_1$ (C57BL/Rij x CBA/Rij) females. In this combination no appreciable secondary disease occurs when bone marrow alone is injected[10]. To prevent a possible graft-versus-host reaction when in addition peripheral blood is given, this blood was irradiated in vitro with 1000 to 2000 r. Two to 6 hours before the injection of cell suspensions the hosts received 910 r of total body X-irradiation from a Maxitron 250 kVp, 30 mA X-ray machine at a dose rate of 70 r/min (HVL 2 mm Cu). The donor marrow was suspended in Tyrode's solution and the number of nucleated eosin resistant cells was counted. The cell suspension was then adjusted to a concentration of $24 \times 10^6$ cells per ml; in many experiments at this stage heparinised C57BL-blood was added to an equal volume of bone marrow suspensions containing $48 \times 10^6$ cells/ml. Immediately before cooling, the suspensions were further diluted with an equal volume of 30% glycerol or 30% dimethyl sulfoxyde (DMS) in Tyrode's; in one experiment ACD (Acid Citrate Dextrose) solution was substituted for Tyrode's in the 30% glycerol or DMS solutions. The resulting suspension of $12 \times 10^6$ cells per ml in 15% glycerol or 15% DMS was sealed in 2 ml ampoules. The ampoules were cooled slowly from room temperature to $-20^\circ$ to $-30^\circ$ C

at the speed of $1^{\circ}$ C/minute. After this they were rapidly cooled to $-79^{\circ}$ C, the temperature of dry ice in alcohol, and after equilibration for a few minutes at this temperature they were transferred to liquid air at a temperature of $-196^{\circ}$ C at which they were stored for periods of a week to a few months. From each suspension to be stored a small amount was taken before the addition of glycerol or DMS for direct injection in irradiated mice in a number of twofold serial dilutions to measure the comparable effectiveness of the fresh bone marrow suspension.

Before use the stored ampoules were rapidly warmed in a $38^{\circ}$ C waterbath until completely thawed. After thawing the contents were either injected without dilution or diluted by the Sloviter[11] or Drašil[12] methods. The undiluted suspensions were injected by microsyringe in twofold serially increasing volumes varying from 10 to 83 µl representing $1/8 \times 10^6$ to $10^6$ cells. The Sloviter technique consisted of dilution with a half volume of 50% glucose, followed 10 minutes later by three volumes of Tyrode's. The Drašil method was essentially a slow dilution technique performed by adding at three minute intervals quantities of Tyrode's calculated to lower the concentration of glycerol by 0.3 M per interval, until after 24 minutes a sixfold dilution was obtained. These diluted suspensions were serially further diluted with Tyrode's to permit the injection of $\frac{1}{2}$ ml per mouse. The amounts injected in irradiated mice were not based on numbers of cells recovered after thawing, but on the number of cells frozen.

The endpoint of the test was 30-day survival. As controls a few irradiated mice were always left untreated; they uniformly died within the test period. Two experiments were excluded from the estimate because significant mortality occurred before the 10th day after irradiation due to Proteus septicemia. Early mortality in the other experiments was below 5%.

## RESULTS

The results of single experiments in which at each cell dose 5 to 10 mice were used, proved to vary too widely to give dependable information; variations by a factor of 2 were encountered. Therefore each method was tested in a number (2-7) of separate experiments. Within the small range of storage times studied no correlation was found between recovery of protective effectiveness and time of storage at -196°C.

The combined results of a large series of experiments on storage of bone marrow without blood are shown in figure 1, where survival is plotted against cell numbers for fresh and for glycerol-frozen slowly diluted (Drašil) bone marrow suspensions. Estimates for cell dose efficiency by the method of Spearman and Karber gave the following results:

The $ED_{50}$ and its 95% confidence limits for the fresh suspensions were $3.5 \times 10^5$ cells ($3.1 - 4.0 \times 10^5$) and for the frozen suspensions $4.9 \times 10^5$ ($4.2 - 5.7 \times 10^5$). Thus the recovery of the protective effect averaged 72%.

With the suspensions to which heparinised blood had been added the different storage methods and dilution techniques were compared by combining in each separate experiment cell suspensions treated with different techniques. The results are presented in table I.

## DISCUSSION

The earlier results with bone marrow without blood are probably not comparable with the others and the difference in $ED_{50}$, found between fresh bone marrow with and without blood is possibly partly due to seasonal or long-term variation in the test system.

It is evident that for mouse bone marrow different methods seem to permit a satisfactory recovery of therapeutic effectiveness after storage. Direct intravenous injection of undiluted suspensions may give

inferior results. This is more clearly the case when glycerol is used than when DMS is the protective agent. Surprisingly the Sloviter method gives very poor results when used with DMS and finally ACD solutions are inferior to a balanced salt solution like Tyrode's.

These data show that in the technique of sorting cells at low temperature, the avoidance of osmotic shock is at least as important as the avoidance of temperature shock. In addition attention is drawn to the fallacy of comparing the relative usefulness of glycerol and dimethyl sulfoxyde as protective agents when the suspensions are not diluted before injection. In this situation DMS appears to be highly superior to glycerol, but this study demonstrates that the protective ability of these substances during freezing is nearly the same. However it seems that for mouse bone marrow cells their osmotic effects are different.

The most important conclusion from these studies follows from a comparison of these data with the disappointing results in rhesus monkeys: data from one species evidently cannot be extrapolated to another. This implies that for human studies it will be necessary to determine the most effective freezing technique by indirect methods. Nevertheless it is felt that further animal studies may throw some light on the cellular differences between different species, which are the cause of these variations.

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## TABLE I

THE EFFECTIVENESS OF FRESH AND STORED BONE MARROW CELLS IN PREVENTING DEATH OF IRRADIATED MICE AS FOUND WITH DIFFERENT METHODS OF PREPARING FROZEN CELL SUSPENSIONS

| | effective cell dose ($ED_{50}$) | relative effectiveness | 95% confidence limits | number of mice used |
|---|---|---|---|---|
| Fresh bone marrow with blood | $1.5 \times 10^5$ | 100 | $1.3 - 1.8 \times 10^5$ | 146 |
| Stored with glycerol and slowly diluted (Drašil) | $2.5 \times 10^5$ | 60 | $2.1 - 3.1 \times 10^5$ | 59 |
| " " " " glucose diluted (Sloviter) | $3.0 \times 10^5$ | 50 | $2.6 - 3.5 \times 10^5$ | 65 |
| " " " " undiluted | $10.8 \times 10^5$ | 14 | $8.1 - 14.4 \times 10^5$ | 85 |
| Stored with DMS and slowly diluted (Drašil) | $3.1 \times 10^5$ | 48 | $2.5 - 3.9 \times 10^5$ | 105 |
| " " " " glucose diluted (Sloviter) | $> 15 \times 10^5$ | $< 10$ | ? (no survivors) | 50 |
| " " " " undiluted | $4.3 \times 10^5$ | 34 | $3.1 - 6.1 \times 10^5$ | 45 |
| Stored with DMS or glycerol but with ACD solution instead of Tyrode's and slowly diluted (Drašil) | $> 15 \times 10^5$ | $< 10$ | ? (no survivors) | 60 |

## RELATIVE EFFECTIVENESS OF FRESH AND STORED BONE MARROW

| | relative effectiveness | number of mice used |
|---|---|---|
| Fresh bone marrow with blood | 100 | 146 |
| Stored with glycerol and slowly diluted (Drašil) | 60 | 59 |
| " " " " glucose diluted (Sloviter) | 50 | 65 |
| " " " " undiluted | 14 | 85 |
| Stored with DMS and slowly diluted (Drašil) | 48 | 105 |
| " " " " glucose diluted (Sloviter) | <10 | 50 |
| " " " " undiluted | 34 | 45 |
| Stored with DMS or glycerol but with ACD solution instead of Tyrode's and slowly diluted (Drašil) | <10 | 60 |

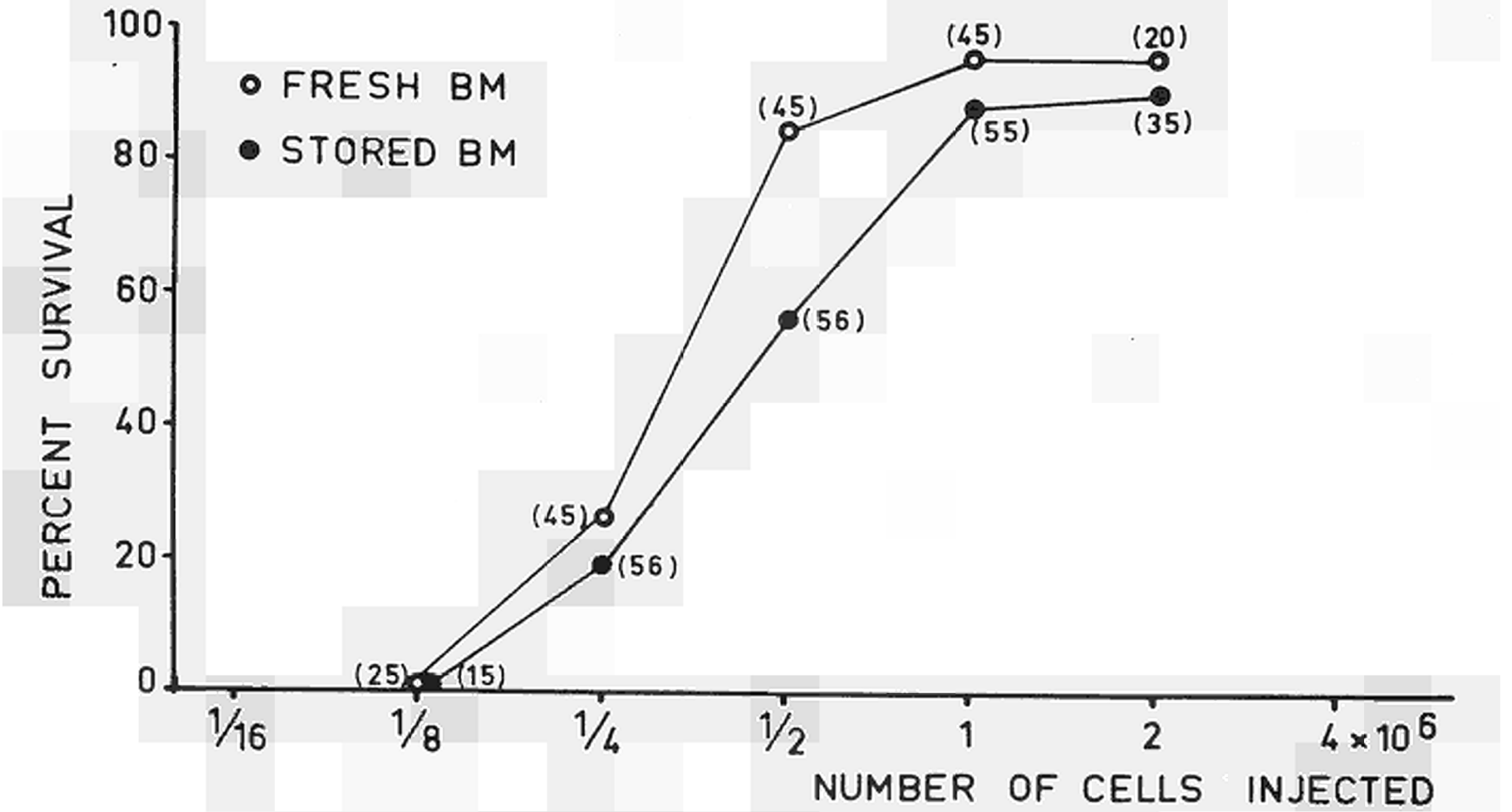


Figure 1 - Percentage 30-day survival of mice after total-body irradiation with 912 r and injection of fresh or stored bone marrow cells

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