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Quantitative aspects of the storage of bone marrow cells for transplantation

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Abstract

Quantitative studies are presented of the effect of different storage techniques on mouse and monkey bone marrow cell suspensions. The ability to produce 30-day survival in lethally irradiated hosts was used as an endpoint. The following results were obtained:

(1) Optimal protection of mouse bone marrow cell suspensions was found after addition of 10% polyvinylpyrrolidone (PVP) and with the combinations 10% PVP, 10% glycerol and 10% PVP, 10% dimethyl-sulphoxide (DMSO). For isologous mouse bone marrow optimal storage efficiency approximates 100%, for foetal mouse liver cells 40%.
(2) For autologous monkey bone marrow storage methods based on the addition of glycerol or DMSO were found to be ineffective. Poor protection was afforded by addition of 10% PVP alone or by a 10% PVP, 10% DMSO mixture. Optimal preservation during storage (up to 50% effectiveness) was found for monkey bone marrow with a 10% glycerol, 10% PVP mixture.

It is concluded that although species differences in storage effectiveness preclude dependable recommendations for storage of human bone marrow, a careful trial of the glycerol–PVP combination in man seems indicated.

Résumé

Une étude quantitative de la conservation de la motile osseuse de souris et de singe à basses températures a été fait afin de comparer l'efficacité de différentes methodes. Le critère d'efficacité était la possibililé pas des suspensions de cellules médullaires defaire survivre des souris irradiées à doses léthales. Les résultats suivants ont été obtenus:

(1) La meilleure protection des suspensions de cellules médullaires de souris a été observee par I'adjonction de 10% de polyvinylpyrrolidone (PVP) ou par les combinaisons de 10% de PVP avec 10% de glycerol et de 10% de PVP avec 10% de dimethyl-sulfoxide (DMSO). Pour les transplantations isologues de moelle osseuse, Vefficacite maxima, aprés congélation, était 100%; pour les cellules hépatiques foetales 40%.
(2) Pour la conservation de la moelle osseuse autologue de singe, Vaddition de glycerol ou de DMSO était sans effet. Une protection assez faible a été observee par l'addition de 10% de PVP et par la combinaison de 10% de PVP et 10% de DMSO. La combinaison de 10% de PVP et 10% de glycerol donnait, au contraire, une bonne protection jusqu' à 50% d'efficacité.

Ainsi il y a des variations importantes, selon l'espèce, dans la technique optimale de conservation de moelle osseuse. Bien qu'il soil impossible de formuler des indications precises quant a la conservation de la moelle osseuse humaine, il semble que l'application de la combinaison PVP et glycérol merite une étudeclinique.

Zusammenfassung

Verschiedene Methoden zur Aufbewahrung von Knochenmarkzellen von Mäusen und Affen bei niedrigen Temperaturen wurden vergleichungsweise quantitativ geprüft. Das Knterium des erfolgreichen Aufbewahrens war die Fähigkeit der Zellsuspensionen, letal bestrahlte Tiere ins Leben zu erhalten. Für Mäusezellen wurde eine optimale Konservie-rung nach Zusatz folgender schutzenden Substanzen gefunden: 10% Polyvinylpyrrolidon (PVP) oder eine Mischung von 10% PVP mit 10% Glyzerin oder 10% PVP mit 10%

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Dimethylsulfoxyd (DMSO). Die maximale Aufbewahrungseffizienz für isologe Knochenmarkzellen war 100%, für foetale Leberzellen nur 40%.

Zusatz von ausschliesslich Glyzerin oder DMSO erwies sichfiir autologes Knochen-mark bei Affen als unwirksam. Eine schwache Schutzwirkung wurde gefunden nach einfrieren der Zellen mit 10% PVP oder mit 10% PVP und 10% DMSO. Optimale Effizienz bei der Aufbewahrung (bis 50% Wirksamkeit) wurde beobachtet bei Suspen-sionen denen eine Mischung von 10% PVP mit 10% Glyzerin zugefügt war.

Diese Befunde weisen auf Unterschiede zwischen den optimalen Aufbewahrungs-methodenfur verschiedene Tierarten. Eine Empfehlung einer optimalen Aufbewahrungs-methode menschlicher Knochenmarkzellen ist demnach nicht möglich. Trotzdem ist eine behutsame klinische Prufung der Brauchbarkeit von der PVP – Glyzerin – Misschung als Konservierungsmethode für menschliche Zellen durchzufürhen.

1. Introduction

The data on the storage of human bone marrow cells for transplantation have so far only provided suggestive evidence for the effectiveness of the storage methods used [1–6]. In experimental animal studies a wide choice of storage techniques has been demonstrated as qualitatively effective (see the review by Phan The Tran and Bender [7]). Often a quantitative evaluation of a proposed storage technique has not been presented or a preference was based on the observation of better survival of irradiated animals after the injection of cells stored by one method than by another. Dependable evaluation is only possible from dose–effect curves and knowledge of the reproducibility of such curves [8–10]. Indirect methods for the evaluation of the repopulating capacity of haemopoietic cells have been widely used [9,11–13]. These are useful as guides for choosing preservation methods but cannot be depended upon for direct application without confirmation by studies of the actual repopulating potential [8,10,12].

In an earlier report [14] on the quantitative analysis of storage effectiveness it was shown that the method giving best results for mouse bone marrow storage was completely ineffective in the Rhesus monkey. These negative results – which were obtained although every effort was made to simulate in the mice the conditions prevailing in primate studies where the aspiration of bone marrow from a living donor causes admixture of blood and heparin with the bone marrow cells – led to the conclusion that there are marked species differences in the effectiveness of bone marrow storage.

The present report describes further variations in mouse bone marrow and foetal liver cell storage techniques, in which use was made of polyvinylpyrrolidone, either alone or in a combination with glycerol or dimethyl-sulphoxide as additives during freezing. The methods showing best results were again tested in the monkey and the combination of glycerol and PVP gave satisfactory results for monkey bone marrow storage. In contrast, some methods used for clinical bone marrow transplantation were found ineffective.

2. Material and methods

The irradiation of mice and the methods for preparing mouse bone marrow cell suspensions as well as the system for testing the effectiveness of storage methods have been described previously [14]. Foetal liver cell suspensions were prepared from C57BL or (C57BL × CBA)F_1 hybrid foetuses between the 14th and 17th days of pregnancy. Twofold serial dilutions of fresh and stored cell suspensions were injected into groups of (C57BL × CBA)F_1 hybrid mice that had been irradiated with a supralethal dose of 910 r (875 rad). Survival at 30 days after irradiation was the endpoint. An equal volume of heparinized mouse blood was added to the mouse bone marrow cell suspensions to simulate the conditions encountered in the storage of autologous bone marrow in monkeys and man. The addition of protective agents, glycerol, dimethylsulphoxide (DMSO) or polyvinylpyrrolidone (PVP) (Subtosan, Specia MW 37,000.) was performed by adding to the blood–bone marrow cell suspension an equal volume of a solution containing the protective agent in Tyrode's solution. Before these substances were added, in each case a portion of the suspension was set apart for testing the therapeutic capacity of the fresh cells. During freezing, cell concentrations for mouse bone marrow were 12×10^6/ml and for foetal liver $12–48 \times 10^6$/ml.

A standard method of freezing was used in all experiments: glass ampoules of a capacity of 2 or 5 ml were cooled in a slow freezing apparatus together with a similar ampoule in which a thermocouple was immersed in the suspending medium. Thus, the temperature of the suspension was recorded at each freezing session. The temperature was lowered by 1 °C/min from +20 to about −40 °C. Thereafter, the ampoules were rapidly cooled and stored in liquid air at −196 °C. The storage times varied from 1 week to 3 months. Within the range tested in these experiments no influence of storage time upon recovery was noted. Immediately before use the ampoules were brought from liquid air into a waterbath kept at 38 °C, where they were shaken to thaw the frozen solution as rapidly as possible. After thawing, the suspensions were handled in one of three different ways:

1. They were step-wise slowly diluted [15];
2. They were rapidly diluted 10 min after addition of hypertonic glucose [16];
3. They were used without dilution or, if necessary for adjustment of the cell dose, quickly diluted with Tyrode's solution without special precautions. The suspensions were then injected as soon as possible into irradiated hosts in volumes containing the desired number of bone marrow cells as calculated from the pre-freezing counts. The injections were made 1–6 h after irradiation. No cell counts were made on the stored suspensions.

Primate studies were performed in young Rhesus monkeys (*Macaca mulatta*), weighing 2–4 kg. Autologous marrow was obtained before irradiation by puncture and aspiration of the femoral shaft through the knee-joint. The irradiation and post-irradiation treatment of the monkeys was similar to that described previously [17]. The duration of monkey marrow storage varied between 1 and 3 months. Supralethal radiation doses of 800–900 r equal to 770–865 rad, were given to preclude spontaneous haemopoietic recovery as an alternative to re-population from the injected material. In earlier studies it was found that after irradiation with 600 r (575 rad) no indication of recovery of the peripheral blood cell counts occurred before the 20th day in animals that had been kept alive with platelet transfusions. The highest radiation dose after which a single control animal survived with symptomatic treatment alone was 650 r (624 rad). No blood or platelet transfusions were given in the present study.

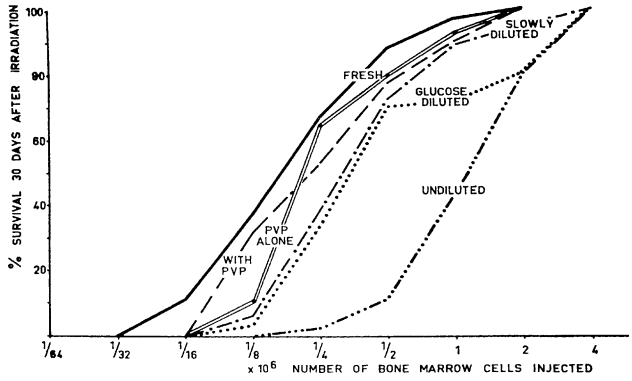

Fig. 1. Dose–effect-curves of fresh and stored C57BL bone marrow cells in supralethally irradiated (C57BL × CBA)F_1 hybrids. Storage with glycerol.

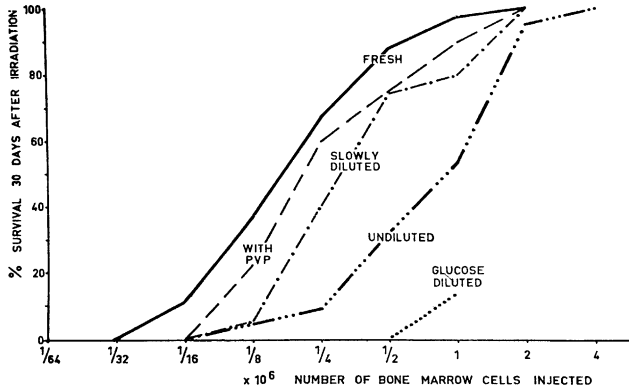

Fig. 2. Dose–effect-curves of fresh and stored C57BL bone marrow cells in supralethally irradiated (C57BL × CBA)F_1 hybrids. Storage with DMSO.

3. Results

3.1. Studies in mice

The survival of hybrid mice injected with the parental cell suspensions are plotted in Figs. 1 and 2 for bone marrow and in Fig. 3 for foetal liver cells. The relative effectiveness of the various storage procedures and the statistical accuracy of the data are presented in Table 1. As was noted previously [14] there is a marked influence of the dilution technique used after thawing on the ultimate efficiency of the suspensions. A major improvement seems the combination of PVP with glycerol or DMSO. Not only does the addition of PVP make the precautions in the dilution technique unnecessary, it also improves average recovery over that obtained with optimal dilution methods without this substance. The observation of Persidsky and Richards [18] that PVP alone gave good protection, prompted us to use PVP alone. In mice it evidently gives similar protection as the PVP–glycerol mixture. Fig. 4 shows that in isologous combinations 100% effectiveness is obtained and obviously further improvements are unnecessary.

From Fig. 3 it is evident that storage efficiency for foetal haemopoietic cells is lower than for adult bone marrow cells. The curve for stored C57BL foetal liver in Fig. 3 has a distinctly lower slope than the others. This may have been caused by toxic effects of the large amounts of PVP and protective agents injected; at the highest cell dosages 30–40 mg pf PVP with the same amount of glycerol or DMSO were injected intravenously in mice weighing about 20 g.

3.2. Studies in monkeys

The 30-day survival of individual monkeys injected with fresh or stored autologous bone marrow suspensions after supralethal irradiation has been plotted in Fig. 5. The minimal effective number of fresh bone marrow cells is about 0.4×10^8/kg body weight. Monkey bone marrow cell suspensions stored with 15% glycerol or 15% DMSO that were kept for 5 min at room temperature after thawing, or diluted by the slow dilution or glucose dilution techniques showed appearance of a sticky viscous material in the suspension, presumably

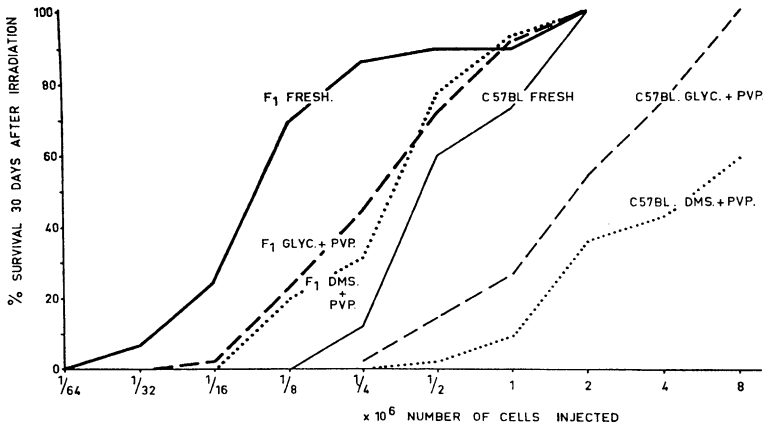

Fig. 3. Dose–effect-curves of fresh and stored C57BL and isologous foetal liver cells in supralethally (C57BL × CBA)F_1 hybrids.

Table 1
Effectiveness cell of suspensions after different storage and dilution procedures

Cell suspension	Additives and dilution procedure[a] after thawing	Number of mice used	ED_{50}[b] and 95% confidence limits (10^5 cells)	Effectiveness of stored suspension (% of fresh material)
C57BL bone marrow	Fresh	705	1.76 (1.58–1.95)	–
	15% Glycerol S	285	3.51 (3.03–4.07)	50
	15% Glycerol G	179	4.71 (3.65–6.07)	37
	15% Glycerol U	168	11.01 (8.14–14.88)	16
	10% Glycerol, 10%PVP U	324	2.46 (2.99–4.15)	72
	15% DMSO S	275	3.52 (2.999–2.90)	50
	15% DMSO G	101	>20	<10
	15% DMSO U	189	7.38 (6.16–8.85)	24
	10% DMSO U, 10% PVP U	130	2.50 (2.00–3.05)	71
	10% PVP U	277	2.54 (2.16–2.98)	69
Isologous (F_1) bone marrow	Fresh	431	0.38 (0.32–0.45)	–
	10% Glycerol, 10% PVP U	341	044 (0.37–0.52)	87
	10% PVP U	169	0.38 (0.31–0.46)	101
C57Bl foetal liver	Fresh	201	5.16 (4.31– 6.17)	–
	10% Glycerol, 10% PVP U	219	16.94 (12.7–21.0)	30
	10% DMSO, 10% PVP U	208	>40	<13
Isologous (F_1) foetal liver	Fresh	297	1.11 (0.93–1.33)	–
	10% Glycerol, 10% PVP U	268	2.79 (2.38–3.27)	40
	10% DMSO, 10% PVP U	179	3.04 (2.49–3.54)	37

[a] S, slow dilution according to Drasil [15]; G, glucose dilution according to Sloviter [16]; U, undiluted or rapid dilution with Tyrode's without special precautions.
[b] ED_{50}, cell number which produced 50% survival in lethally irradiated female (C57BL × CBA)F_1 hybrid mice.

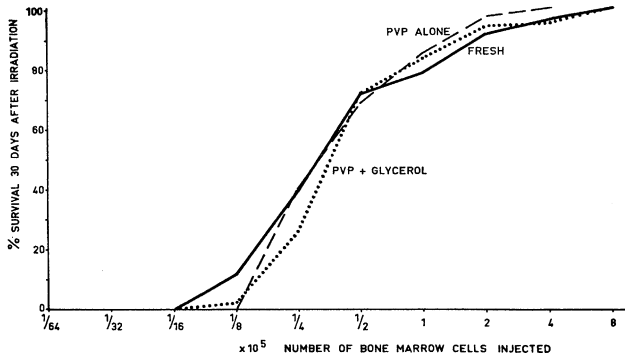

Fig. 4. Dose–effect-curves of fresh and stored isologous bone marrow cells in supralethdly irradiated (C57BL × CBA)F_1 hybrids.

DNA, as has been described for thawed human marrow suspensions [13]. No attempts were made to inject these viscous suspensions in monkeys. If similar suspensions were not diluted but injected immediately after rapid thawing, the irradiated monkeys failed to show haemopoietic recovery and died with aplasia (Fig. 5). These methods which are widely used for storing human bone marrow, were thus completely ineffective in monkeys. The use of 10% PVP alone or in combination with DMSO resulted in the survival of some monkeys but frequent unexplained failures made these methods less dependable. A regular good protection was obtained with the use of a mixture of 10% PVP with 10% glycerol, although the storage efficiency of about 50% is somewhat lower than for mouse bone marrow cells.

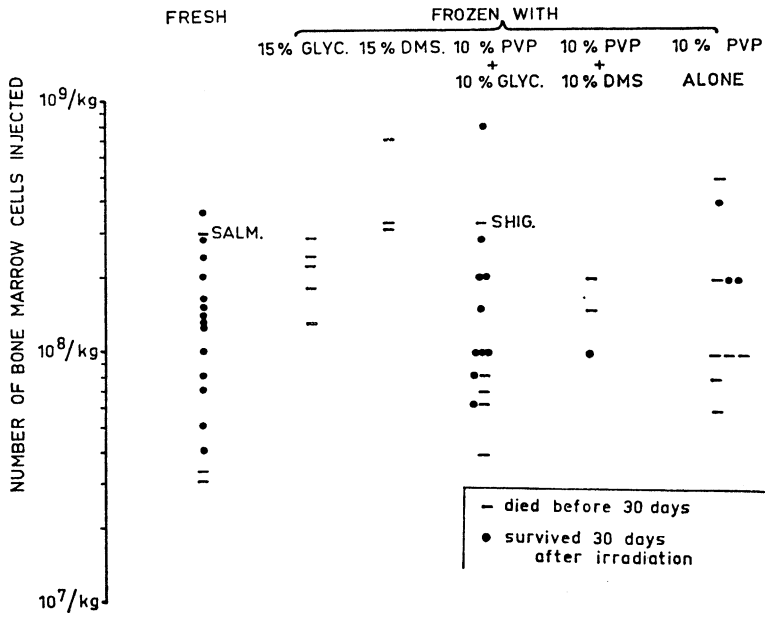

Fig. 5. Diagram showing the fate of supralethally irradiated monkeys injected with different numbers of autologous bone marrow cells. Fresh suspensions are compared with suspensions frozen with different additives.

4. Discussion

These studies confirm the usefulness of quantitative comparison of freeze storage techniques. Only in this way the best technique of storage, both for mice and monkeys, could be established. The test system that was used initially in mice: C57BL bone marrow injected into irradiated (C57BL × CBA)F_1 hybrids seems to differ from other parent → F_1 systems. More C57BL cell are needed for successful transplantation than in other P → F_1 combinations [19,20] or even more than in homologous transplantations of C57BL cells [21] The cause for this anomalous behaviour is not clear at present. Nevertheless, the results seem to indicate that the P → F_1 system reacts similarly to the isologous combination, although these show a slightly higher storage efficiency.

Since the report by Bender *et al.* [22] that 7% PVP gave no protection during freezing and the routine use of 3% PVP in procedures for testing other protecting agents [7], the good protection with PVP alone as reported by Persidsky and Richards, was unexpected. Even more surprising are the present quantitative results which show PVP to be one of the best agents for protecting mouse bone marrow cells during freezing. Differences in the molecular weight of the PVP preparations used may perhaps explain these discrepancies.

Evidently, marked differences exist between the species regarding the case with which their cells may be recovered after freezing. These differences are not only quantitative but also qualitative, notably the 10% PVP addition which ranks among the best methods in the mouse, seems to be far less effective in the monkey. Although many aspects of bone marrow transplantation in monkeys and man seem to be similar, this finding makes it impossible to recommend with certainty any technique as suitable for storage of human bone marrow.

A pertinent suggestion from these studies would be to test the glycerol–PVP mixture in the clinic since untoward effects are unlikely and infusion of limited quantities of PVP in man has been well tolerated.

One other important conclusion may be drawn from these data. Whatever number of cells is recommended for effective autologous or isologous transplantation in man, it seems necessary to double this number if stored cells are to be used, since a 50% loss of effectiveness is to be expected.

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