UREA AS A PRESERVATIVE FOR FROZEN BONE MARROW CELLS

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A new property of urea has been found, by virtue of which it will preserve a higher percentage of bone marrow stem cells than glycerol. It is unnecessary to wash the urea from the thawed-out cells before using them for transplantation.

KEY WORDS: *cryopreservatives; glycerol; urea; bone marrow.*

The unique combination of properties of urea — reversible denaturation of proteins and nucleic acids, high solubility and marked ability to lower the freezing point of aqueous solutions, good permeability through cell membranes, and rapid elimination from the body — has served as the basis for the suggestion that urea can be used as an effective protective agent and preservative for use during the freezing of bone marrow cells.

To test this suggestion the protective action of urea and glycerol was compared by studying the degree of absorption of eosin by bone marrow cells, the number of colonies in the spleen of lethally irradiated mice after transplantation of thawed bone marrow cells previously frozen with these two preservatives, and the action of the two preservatives on incorporation of thymidine-H^3 into DNA of human bone marrow cells.

EXPERIMENTAL METHOD

Experiments were carried out on bone marrow cells of man, dogs, and CBA mice. Bone marrow obtained from donors or animals was diluted with ZF solution [1] or in medium No. 199 with the addition of heparin [2]. Erythrocytes were separated by standing and myelokaryocytes by centrifugation at 500 g for 15 min. The myelokaryocytes thus obtained were suspended in 100 ml of the supernatant, filtered to remove fat and clots, then treated with 30 ml of the preservative (30 ml glycerol or 30 ml 15% or 30% urea solution in 10% glucose solution), 20 ml of group ABO serum, and 50 ml of supernatant obtained after separation of the myelokaryocytes. The final concentration of urea in the incubation medium was 2.25 or 4.5%.

Freezing was carried out in corrugated containers with a capacity of 170 ml in accordance with the following program: at the rate of 1 deg per minute down to −13°C, then at the rate of 10 deg per minute to −196°C. Thawing was carried out in a water bath at 38 or 39°C for 1 min.

DNA was extracted by the method of Schmidt and Thannhauser and its content estimated spectrophotometrically (2 ml medium contained 4×10^6 cells and 5 μCi thymidine-H^3). Radioactivity was measured on a Nuclear Chicago Mark II (USA) scintillation counter.

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TABLE 1. Number of Colonies in Spleens of Lethally Irradiated (900 R) Mice after Receiving Injection of 10^5 Thawed Homologous Bone Marrow Cells Previously Frozen in ZF Medium Containing 15% Glycerol (group A) or 4.5% Urea (group B)

Experiment No.	Group and number of animals (n)	Number of colonies in spleens of surviving mice														Mean number of colonies in each group (M ± m)
1	Control (bone marrow not injected) $n=15$	1	0	0	0	0	1	0	0	0	0	—	—	—	—	0,18±0,1
	Group A, glycerol (15%), $n=20$	10	8	4	8	6	4	9	4	10	9	5	5	5	10	6,9±0,64
	Group B, urea (4.5%) $n=20$	6	14	10	15	8	2	7	7	10	9	11	10	8	—	9,0±0,89
2	Control (bone marrow not injected) n = 5	0	0	0	0	—	—	—	—	—	—	—	—	—	—	0
	Group B, urea (4.5%) $n=10$	14	10	14	6	5	—	12	6	13	—	—	—	—	—	10,0±1,36

TABLE 2. Number of Colonies in Spleens of Lethally Irradiated (900 R) Mice after Receiving Injection of Thawed Homologous Bone Marrow Cells Previously Frozen in Medium No. 199 with the Addition of Heparin

Group and number of animals (n)	Number of colonies in spleens of surviving mice										Mean number of colonies in each group (M ± m)
Control (bone marrow not injected), n = 10	3	0	0	1	4						1,6±0,57
1 (nonfrozen bone marrow), n = 12	21	8	18	15	11	17	10	26	23	9	15,8±1,84
2 (bone marrow frozen with 15% glycerol), n = 12	6	6	7	5	4	5	9				6,0±0,57
3 (bone marrow frozen with 4.5% urea), n = 12	25	6	9	7	7	9	6	4			9,0±2,25
4 (bone marrow frozen with 15% polyethylene oxide), n = 12	5	4	10	7	4	2	1				4,7±1,07

EXPERIMENTAL RESULTS

Since the object of preserving and freezing the bone marrow cells was to preserve the largest number of stem cells, the principal criteria of the effectiveness of the preservatives and protectives were given by experiments to study cloning of the stem cells in the spleen of lethally irradiated mice.

The optimal concentrations of urea and the optimal programs of freezing with it were not determined; nevertheless, relative to the basic index — the number of colonies in the spleen of lethally irradiated mice — urea proved to be the best preservative compared with glycerol and polyethylene oxide (Tables 1 and 2). The study of the cellular composition of the colonies in the spleen revealed no difference from the composition of the colonies in the spleens after injection of freshly obtained bone marrow. It will be noted that in all three of the cloning experiments bone marrow

TABLE 3. Action of 15% Concentration of Glycerol and 4.5% Concentration of Urea on Incorporation of Thymidine-H^3 into DNA of Human Bone Marrow Cells (M ± m)

Experiment No.	Incubation time (in h)	Incorporation of thymidine-H^3 into DNA (counts/min/μg DNA)			
		without addition	glycerol (15%)	urea (4.5%)	Polyethylene oxide (15%)
1	1	44,8±2,2	26,6±3,9	34,8±8,4	38,5±9,7
	18	214±32,7	133±16,5	230±24,8	118±20,6
2	15	1483±275	—	1517±103	—

cells frozen with 4.5% urea were injected into the mice immediately after thawing, without washing them to remove urea, whereas the glycerol, which is usually used for this purpose, had to be removed for it has a toxic action. This advantage of urea is of great practical importance.

The proliferative power of the bone marrow is a very valuable indicator of the viability and normal functioning of bone marrow cells. As Table 3 shows, the addition of glycerol to the incubation medium in ZF solution reduced the incorporation of thymidine-H^3 into DNA by half during incubation with the labeled precursor for 1 and 18 h. Addition of 4.5% urea to ZF incubation medium did not reduce the incorporation of thymidine-H^3 into DNA of bone marrow cells (Table 3), whereas cells frozen in medium containing 2.25% urea incorporated several times more thymidine-H^3 into DNA after thawing than the same cells when frozen with 15% glycerol.

On the basis both of the number of colonies in the spleen of the lethally irradiated mice and of the intensity of incorporation of thymidine-H^3 into DNA, the use of urea as preservative before freezing thus gives better results than the use of glycerol. Unlike glycerol, urea is able to preserve bone marrow cells (using absorption of eosin and morphological integrity as criteria) for at least 5 days at 4°C. A great advantage of this method of preservation is that there is no need to remove the preservative after thawing the bone marrow cells. This fact, together with the properties of urea already mentioned, make the use of urea solutions a promising method also for organ perfusion with aim of preserving them at temperatures close to 0°C.

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