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Cryopreservation of Erythrocytes in Small Aliquots for Isozyme Electrophoresis

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INTRODUCTION

A standard technique developed for preserving erythrocytes at blood banks (see Walter, 1984, for review) involves a piece of tubing as an integral part of the donor blood bag made of polyvinyl chloride (PVC). The tubing is filled with erythrocytes suspended in a cryopreservative and heat-sealed into small segments, each of which contains an aliquot of the sample. The samples in the segments are used for antigenic typing, cross-matching, and other assays. A variation of the tubing storage method was developed by Cheng *et al.* (1986) for storing large numbers of serum or plasma samples frozen in small aliquots. In addition to eliminating repeated freezing and thawing of samples, this method is particularly convenient for cataloging and retrieving samples, prolongs the shelf life of samples, and is economical in the use of freezer space.

We have examined the feasibility of storing cryopreserved erythrocytes by the storage method of Cheng *et al.* (1986) and compared the efficacy of two different freezing solutions. One of these is a citrate–ethylene glycol solution used by one of us (JLV) since 1970 for preserving erythrocytes from many species (VandeBerg and Johnston, 1977), and the other is a buffered glycerol solution (American Association of Blood Banks, 1974) that we now use in preference to the citrate–ethylene glycol solution for some (but not all) species. This communication presents the results obtained from erythrocytes

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of two primate species, the olive baboon (*Papio hamadryas anubis;* see VandeBerg and Cheng, 1986) and the rhesus macaque (*Macaca mulatta*).

MATERIALS AND METHODS

Blood Sample Collection. Blood samples from olive baboons and rhesus macaques were collected into Vacutainers containing acid–citrate–dextrose (Becton–Dickinson and Co., Rutherford, N.J.). Plasma was separated from erythrocytes by centrifugation and removed. Erythrocytes were washed in phosphate-buffered saline (PBS; 10 mM sodium phosphate, 0.15 M NaCl, *p*H 7.4) several times until no hemolysis was visible in the supernatant.

Erythrocyte Preservation. Washed erythrocytes were preserved in the following two freezing solutions: (1) citrate ethylene glycol—200 mM trisodium citrate and 400 ml ethylene glycol (certified grade, Fisher Scientific Co.) in 1000 ml, *p*H 8.4; and (2) buffered glycerol—33 mM sodium phosphate (monobasic), 33 mM sodium phosphate (dibasic), 106 mM tripotassium phosphate, and 400 ml glycerol in 1000 ml. One volume of packed erythrocytes was mixed with 2 vol of freezing solution and incubated at 4°C for 30–60 min. The Tygon tubing (i.d. 3.97 mm, o.d. 5.56 mm; R-3603, Norton Co., Akron, Ohio) was cut into 20-cm lengths, and each was attached to a 5-ml disposable syringe. The erythrocyte suspension was mixed thoroughly by gently inverting each tube several times before 4 ml of the mixture was drawn into a piece of tubing. Each piece of tubing containing erythrocytes was then heat-sealed into approximately 1.5-cm intervals with a Hematron dielectric sealer. Each tubing segment contained approximately 250 μl of the erythrocyte suspension. Each series of attached tubing segments was inserted into a protective sleeve—a cellulose acetate butyrate tube (CAB tube; i.d. 0.95 cm, o.d. 1.11 cm., length 50 cm; Thermoplastics Inc., Wayne, N.J.). Both ends of the CAB tube were capped with Capsicles (Intermed, Denmark). To reduce friction between the Tygon tubing and the CAB tube, the inner wall of the CAB tube was prelubricated with mineral oil. After an inventory number had been assigned to each specimen and labels affixed on the CAB tube near both ends as well as on the Capsicles, each CAB tube was placed in a three-tiered aluminum rack (Cheng *et al.*, 1986) in a 9-ft^3 constant-temperature chest freezer (Sears), maintained at −20°C.

Recovery of Erythrocytes for Isozyme Electrophoresis. Since neither ethylene glycol (VandeBerg and Johnston, 1977) nor glycerol (see Results and Discussion) affects electrophoretic mobility or histochemical staining procedures, it is not necessary to remove either cryoprotectant from erythrocytes. Each Tygon tubing segment was severed at the seal, placed in a prelabeled vessel (e.g., microfuge tube), and warmed to room temperature. One corner of the tubing segment was cut open with an incision scissor and centrifuged for

10 sec in a Beckman microfuge to sediment erythrocytes. The supernatant was removed by aspiration, and the erythrocytes were lysed in deionized water or appropriate lysing solution for electrophoresis.

Assessment of Hemolysis of Cryopreserved Erythrocytes. The extent of hemolysis of cryopreserved erythrocytes during storage was evaluated by the amount of cyanmethemoglobin present (Eilers, 1967) in the supernatant thawing (freezing) solution in comparison with that in the remaining intact erythrocytes at intervals of 3, 6, and 12 months. The hemoglobin assay kit was obtained from Sigma Chemical Co. (St. Louis, Mo.). Drabkin's solution and cyanmethemoglobin standard solution were prepared according to the manufacturer's instructions. Tygon tubing segments containing cryopreserved erythrocytes were warmed for 1 min in a 37°C water bath. The contents of two segments from each animal cryopreserved in the same freezing solution were pooled and spun for 5 min at 15,000*g* in a referigerated centrifuge. The supernatant was removed and transferred into a borosilicate disposable culture tube (75 × 12 mm). Ten microliters of the supernatant was mixed with 990 μl of Drabkin's solution in a borosilicate disposable culture tube. The packed erythrocyte pellet was lysed in 1 ml of Drabkin's solution in a borosilicate disposable culture tube. A 10-μl aliquot of the lysate was mixed with 990 μl of Drabkin's solution in a separate tube. Further dilution of this lysate in Drabkin's solution was frequently needed for accurate spectrophotometric measurement of cyanmethemoglobin in the solution. The tubes were allowed to stand at room temperature for 15 min before being read spectrophotometrically at a wavelength of 540 nm. The absorbance values were then converted to actual total amounts of hemoglobin as measured by cyanmethemoglobin present in the thawing supernatant and in the lysate of intact erythrocytes. The proportion of erythrocytes hemolyzed during storage in Tygon tubing segments was computed as $[T/(P + T)]$, where T is the amount of cyanmethemoglobin in the thawing supernatant, and P is the amount of cyanmethemoglobin in the lysate of intact erythrocytes. Comparison of the effects of the freezing solutions on the survival of erythrocytes of baboons or rhesus macaques over a period of 1 years was performed by analysis of variance.

Electrophoresis and Enzyme Staining Techniques. Lysates of cryopreserved erythrocytes and of freshly drawn blood were compared electrophoretically. Baboon erythrocyte adenine phosphoribosyl transferase (APRT; EC 2.4.2.7) isozymes were resolved following the method of Mowbray *et al.* (1972) using starch gel electrophoresis and stained with a solution containing ^{14}C-labeled adenine to develop an autoradiogram. Glucose phosphate isomerase (GPI; EC 5.3.1.9) isozymes of baboon erythrocytes also were resolved by starch gel electrophoresis and stained by the procedure of Harris and

Hopkinson (1976). However, the *p*H of the gel and bridge buffers was adjusted to 8.9 with NaOH to increase the anodal mobility of the baboon GPI isozymes. Erythrocyte mannose phosphate isomerase (MPI; EC 5.3.1.8) isozymes were separated by cellulose acetate electrophoresis in Helena Titan III plates (Helena Laboratories, Beaumont, Tex.) in 0.025 M Tris–0.192 M glycine, *p*H 8.35, and stained according to Harris and Hopkinson (1976).

RESULTS AND DISCUSSION

Hemolysis of Cryopreserved Erythrocytes. Table I shows the percentages of hemolysis of erythrocytes of four baboons and four rhesus macaques cryopreserved in citrate–ethylene glycol solution or in buffered glycerol solution and stored in small aliquots in heat-sealed Tygon tubing segments. Analysis of variance indicated significant differences in the extent of hemolysis of erythrocytes due to (1) different freezing solutions; (2) the species origin of erythrocytes; and (3) the duration of storage. Both rhesus macaque and baboon erythrocytes cryopreserved in buffered glycerol had substantially less hemolysis than those cryopreserved in citrate–ethylene glycol ($F_{1,12} = 450.28$; $P < 0.001$). Consistently more hemolysis occurred in baboon erythrocytes than in rhesus erythrocytes ($F_{1,12} = 205.28$; $P < 0.001$); however, the magnitude of difference was much greater for erythrocytes cryopreserved in citrate–ethylene glycol than for those cryopreserved in buffered glycerol. For instance, at the end of the 12-month period, the mean percentage hemolysis was 31 for rhesus and 74 for baboon erythrocytes cryopreserved in citrate–ethylene glycol. In contrast, the mean percentage hemolysis was 15 for rhesus

Table I. Percentages of Hemolysis of Erythrocytes of *Macaca mulatta* (MMA) and *Papio hamadryas anubis* (PHA) Cryopreserved in Citrate–Ethylene Glycol or Buffered Glycerol

	Ethylene Glycol			Glycerol		
	3 mo[a]	6 mo	12 mo	3 mo	6 mo	12 mo
MMA 1	11.5	32.4	32.6	7.5	15.0	16.0
MMA 2	21.6	26.2	26.6	2.7	12.9	14.2
MMA 3	21.2	27.0	28.5	2.5	6.3	6.6
MMA 4	25.4	33.6	36.0	11.4	20.7	21.4
Mean	19.9	29.8	30.9	6.0	13.7	14.6
PHA 1	66.3	68.4	68.1	10.6	13.4	14.4
PHA 2	50.9	77.2	76.8	7.8	23.4	24.1
PHA 3	56.1	78.2	78.5	6.7	19.8	21.0
PHA 4	54.6	72.5	73.0	7.9	20.1	20.9
Mean	57.0	74.1	74.1	8.3	19.2	20.1

[a] Months.

and 20 for baboon erythrocytes cryopreserved in buffered glycerol. The difference in percentage hemolysis for both species after 6 and 12 months of storage was not significant ($F_{1,24} = 0.214$; $P > 0.05$), but the difference between 3 months and the weighted mean of 6 months and 12 months was highly significant ($F_{1,24} = 40.6$; $P < 0.001$). Increasing hemolysis in the first 6 months may be due to initial shocks (e.g., changes in osmosis of erythrocyte membranes in freezing solutions, changes in temperature due to freezing, trauma induced by the heat-sealing process). After 6 months of storage, the rate of hemolysis of erythrocytes of both species cryopreserved in either freezing solution was negligible.

Isozyme Patterns. The electrophoretic zymograms of fresh erythrocytes and erythrocytes cryopreserved for 18 months or longer in buffered glycerol for three representative baboon erythrocyte enzyme systems, APRT, GPI, and MPI, are shown in Fig. 1. Comparable enzyme activities, judged from histochemical staining intensity and electrophoretic mobilities, were observed for the two types of erythrocytes. Furthermore, there was no increase in the number of subbands, indicating that enzymes were stable in erythrocytes stored in buffered glycerol.

VandeBerg and Johnston (1977) reported that ethylene glycol has no apparent effect on the mobility or activity of red kangaroo or rhesus macaque erythrocyte enzymes subjected to electrophoresis in starch or cellulose acetate media. We have confirmed this observation in rhesus macaques (data not shown) and extended it to baboons.

Effect of PVC on Erythrocytes. Tygon tubing is made of PVC and contains approximately 40% (w/w) of the plasticizer, di(2-ethylhexyl)phthalate (DEHP). DEHP is leached from the tubing and enters the red cells during storage. The potential toxicity of DEHP in laboratory animals has been of considerable concern. However, it also has been shown that DEHP facilitates the survival of erythrocytes during storage at low temperatures by decreasing their osmotic fragility (Horowitz *et al.*, 1985).

General Discussion. Our primary objectives for developing this storage method were to prolong the shelf life of stored erythrocytes, to protect the structural integrity and activity of enzymes, to eliminate contamination of erythrocyte samples, to ease the complexity in sample cataloging, and to reduce the labor involved in retrieving samples from and returning samples to freezers.

Our experimental results indicate that the stability of erythrocytes in citrate–ethylene glycol or buffered glycerol is species specific. This conclusion is reinforced by long-term observations on many thousands of cryopreserved erythrocyte samples in our laboratory. In fact, we initially were prompted to explore the use of glycerol as a cryopreservative when we noticed that

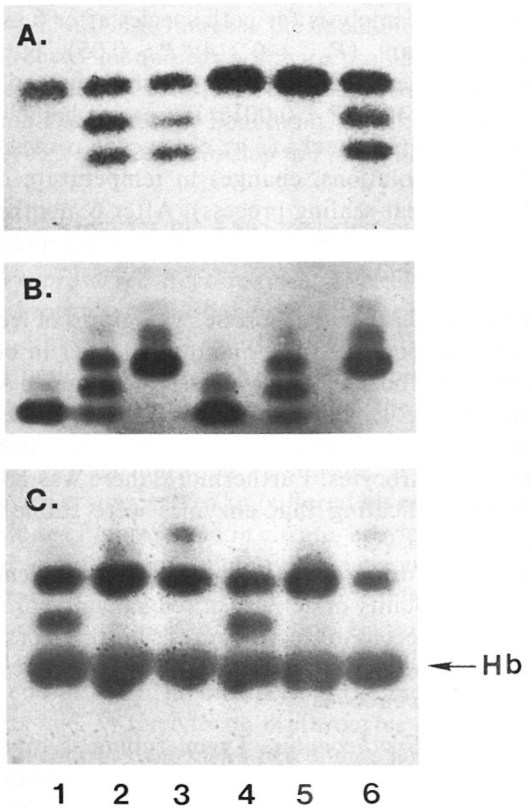

Fig. 1. Zymograms of three baboon erythrocyte enzymes. The anode is at the top of each zymogram. In all three zymograms, channels 1–3 contained lysates of glycerized baboon erythrocyte samples stored in small aliquots in Tygon tubing segments kept in an 18°C freezer, and channels 4–6 contained lysates of freshly collected baboon erythrocyte samples. A shows an autoradiogram of adenine phosphoribosyl transferase (APRT) isozymes separated by starch gel electrophoresis. The phenotypes, from left to right, are A, AB, AB, A, A, and AB. B illustrates glucose phosphate isomerase (GPI) isozymes also separated by starch gel electrophoresis. The phenotypes, from left to right, are B, AB, A, B, AB, and A. C shows mannose phosphate isomerase (MPI) isozymes separated by cellulose acetate electrophoresis. The phenotypes, from left to right, are BC, B, AB, BC, B, and AB. No differences in the electrophoretic mobilities or staining activities of the enzymes derived from stored and fresh erythrocytes were detected. Hb, hemoglobin.

erythrocytes from rats, mice, and rhesus macaques had undergone very little hemolysis during 10 years of storage in citrate–ethylene glycol, whereas erythrocytes of baboons [not one of the species investigated by VandeBerg and Johnston (1977)] were quite labile when stored under identical conditions. When we explored the use of glycerol as an alternative, we observed that erythrocytes of squirrel monkeys formed gummy aggregates when mixed with the buffered glycerol solution but could be adequately preserved in the citrate–ethylene glycol solution. Clearly, it is important to identify a cryoprotectant that is compatible with the erythrocytes to be preserved in preliminary experimental trials.

Based on our experience, we suggest several other precautions, although none has been rigorously evaluated in controlled experiments: (1) fragile erythrocytes in freshly collected blood should be removed by several washes in PBS before mixing with cryopreservative; (2) the ratio of packed erythrocytes to freezing solution should be no less than 1:1.5; and (3) the buffered glycerol- or citrate–ethylene glycol-preserved erythrocytes should be maintained above the freezing temperature of the cryopreservative, because the formation of ice can damage erythrocyte membranes and cause hemolysis; a household freezer maintained at −20°C is satisfactory.

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