Preservation of Viable Platelets by Freezing. Effect of Plastic Containers[1] (37019)

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Pioneer work by Cohen and Gardner (1) demonstrated in 1961 that dog platelets could be frozen in plasma-glycerol and thawed in the presence of hypertonic dextrose. These platelets had a normal survival time when infused in recipient dogs. When, however, human platelets were frozen by the same method, only very few could recirculate and survive (2). The existence of large species differences for platelets and the lack of an *in vitro* laboratory method by which viability[2] of human platelets could be predicted with accuracy were probably the main factors which precluded significant progress in the study of platelet preservation by freezing (3–5).

Recently, the *in vitro* biphasic reaction of platelets to hypotonic stress (6) has been found to be a valuable indicator of platelet integrity in a variety of experimental conditions including freezing and thawing (7). Using this practical *in vitro* test we indicated that dimethylsulfoxide (DMSO) was preferable to glycerol and that the concentration of 5% DMSO was least damaging for platelet freezing. The present paper reports experiments demonstrating that: (a) when osmotic stress was minimized, human platelets could be frozen and thawed in 5% DMSO in plasma with only little damage to their viability; (b) results of the *in vitro* platelet reaction to osmotic stress paralleled viability data obtained *in vivo;* (c) the type of plastic container could grossly influence viability of the frozen platelets.

Materials and Methods. The platelet survival studies were carried out in healthy male and female volunteers 21 to 43 yr of age, using autologous platelets labeled with radioactive sodium chromate. Procedures used for separation and labeling of the platelets were as previously described (8), with the following modifications. Blood was collected in double blood packs (PA-20, Fenwal Laboratories, Morton Grove, Ill.) containing ACD anticoagulant. All centrifugations were done at 15° (International Refrigerated Centrifuge, PR-6, International Equipment Co., Needham Heights, Mass.). Between centrifugations, the blood was kept at room temperature. The platelet button was resuspended in platelet-poor plasma (PPP) by slow and gentle kneading of the plastic bag. Volume of the platelet concentrate was 15 ml. Five hundred microcuries of ^{51}Cr-sodium chromate (Amersham/Searle Corp., Arlington Heights, Ill.) were used for labeling. The labeled platelet concentrate was then transferred to one of two types of bags used for freezing and storage, either the "Unitag" bag (Abbott Laboratories, North Chicago, Ill.) or the "Hemoflex" bag (#2030-2, Union Carbide Corp., Chicago, Ill.). An equivolume of 10% DMSO (sterile, analytical grade; Fisher Scientific Co., Pittsburgh, Pa. or Crown Zellerbach Corp., Camas, Wash.) in PPP was very slowly and gradually added at the rate of about 1 ml per min to the labeled platelet concentrate, while the bag was maintained in motion by continuous, slow tilting. The platelet concentrate, now 30 ml in volume and containing 5% DMSO, was then cooled at the rate of 1° per min to —35° in a controlled slow freezing bath (Model 11-200,

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[2] Viability is here defined as the property of the platelets to reappear and survive in the circulation upon infusion.

Virtis Co., Gardner, N. Y.). Temperature in the platelet concentrate was indirectly recorded using a thermocouple inserted into a simulator bag containing a similar amount of PPP and DMSO and placed in the cooling chamber together with the platelet bag. The frozen platelet concentrate was then stored at —79° in a dry-ice-ethanol bath for about 24 hr. Rapid thawing (usually within 1 min) was done in a 37° water bath, then 120–150 ml PPP were gradually added to the platelet concentrate. Special care was used in adding the first 60 ml of PPP and this was done slowly while maintaining the bag in motion. The first 30 ml PPP were added at the rate of 1.5 ml per min, then, after a 10 min waiting period, the next 30 ml were added at the same rate. After the rest of the PPP was added at the rate of 3 ml per min, the platelet button was separated again by centrifugation at 3200 rpm (3000*g*) for 25 min at 15°, and finally resuspended in 22 ml PPP. The pH of the concentrate was 6.7. Twenty milliliters of the platelet concentrate was infused in the donor. Two milliliters was used for determination of platelet number, radioactivity, amount of residual DMSO, bacterial culture, and for the study of the platelet response to osmotic stress. After infusion, platelet survival was determined by following platelet radioactivity in multiple blood samples (8). These samples (10.8 ml blood collected in 1.2 ml EDTA in saline) were taken 5 min, 2 hr, 4 hr post-infusion and daily thereafter for 6 to 9 days depending on the length of the platelet survival. Platelet viability *in vivo* was judged from the value of maximum recovery, from the platelet survival time and the shape of the survival curve (8). To combine the three parameters in a single value useful for comparative purposes, the "viability index" was calculated as previously described (2), by measuring the area under the curve and expressing it in percentage of the mean value obtained with normal, freshly prepared platelets.

Special care was taken to avoid bacterial contamination. An aliquot from each platelet concentrate was cultured in veal broth, tryptic soy agar blood plate and thioglycollate broth. No bacterial growth was observed for a week in both aerobic and anaerobic cultures.

The platelet reaction to hypotonic stress was determined before adding DMSO and after freezing and thawing and removal of the DMSO. An equivolume of distilled water was added to 0.5-ml platelet suspension (0.4 to 0.5 $\times$ 10^9 platelets) and changes in light absorbancy were measured in a recording spectrophotometer (Model 2000, Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at 420 nm wave length, at 22°. After a sudden decrease in light absorbancy, there was a gradual recovery phase, hereupon called "reversal reaction," with a return of light absorbancy towards normal. A plateau was usually reached within 7 min. The rise in light absorbancy occurring during the first 2 min of the recovery phase was the value used for comparison and expressed in percent of the control value. The aliquot of platelet concentrate withdrawn before the addition of DMSO was used as control.

Residual DMSO in the final platelet suspension was measured by gas chromatography (Automatic Preparative Chromatograph, Series 105, Pye & Co. Ltd., Cambridge, England). For each determination, 0.5 ml platelet concentrate was extracted with 0.5 to 1.5 ml acetone. Efficiency of the acetone extraction for DMSO was 70% in preliminary experiments. Only trace amounts of DMSO were found in the platelet concentrates after the single washing with 120–150 ml PPP. The 20-ml platelet concentrate infused contained 45 mg (21 to 78 mg) of residual DMSO. None of the recipients complained of unpleasant smell, nausea, vomiting or pain at the site of infusion. These side effects have been described only with the infusion of large amounts of DMSO (4).

Platelet counts in the blood samples were done by phase contrast microscopy (9). A Coulter counter, model B (Coulter Electronics, Inc., Hialeah, Fla.) was used for platelet counts in PRP and in platelet concentrates.

Results. Viabiltiy of platelets frozen in "Unitag" bags. In five experiments (Fig. 1), the maximum recovery values varied from

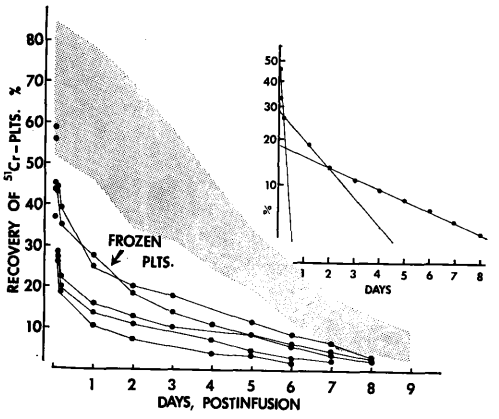

FIG. 1. Survival curves of human platelets frozen in "Unitag" bags. To simplify graphic representation, the first recovery values obtained 5 min post-infusion were not connected with rest of the respective curves. Shadowed area representing survival values of fresh human platelets (8) is reported for comparison. The insert represents a visual analysis of the mean survival curve of frozen platelets on semilog paper suggesting the existence of 3 cell populations with different survival rates.

37% to 59%, mean 49%, and were observed immediately (5 min) after infusion. Corresponding normal values obtained in previous experiments with freshly prepared human platelets (8) were 52% to 85%, mean 66%. Survival curves of the frozen platelets showed a sharp initial drop, then a more gradual exponential slope. The $t_{½}$ was 0.9 to 2.2 days (normal 3.5–5.2 days). The viability index was 30% (16% to 46%). Results of the five experiments were averaged and plotted on semilog paper. Visual analysis indicated that about 45% of the surviving platelets had a very short lifespan ($t_{½}$ of 4 hr), about 20% showed a moderately short lifespan ($t_{½}$ of 1.7 days), while the remaining 35% had a normal lifespan ($t_{½}$ of 3.6 days).

Viability of platelets frozen in "Hemoflex" bags. The results of 7 experiments are shown in Fig. 2. Peak recovery values of platelet radioactivity were obtained between 5 min and 4 hr post-infusion and varied between 46% and 71%, mean 63%. Some of the curves showed a very small initial drop. Visual analysis on semilog paper (Fig. 2) indicated that only 8% of the platelets recirculating after infusion had a $t_{½}$ of about 14 hr, while most of the platelets survived almost normally with a $t_{½}$ of 2.6 to 3.8 days, mean 3.1 days (normal 3.5–5.2 days). Two curves were linear and fell within the normal range (Fig. 2); the others were moderately curvilinear. The mean viability index was 69% (50% to 94%). When in other experiments not reported in Fig. 2, addition of the PPP after thawing (*i.e.*, removal of the DMSO) was done less gradually, the recovery values were constantly lower (44% to 52%, mean 48%).

Relationship between in vivo *platelet survival and* in vitro *reversal reaction.* Values of reversal reaction were correlated with the values of viability index obtained in the 16 survival experiments with frozen platelets (Fig. 3). The platelet samples for measurement of the reversal reaction were taken just before infusion *in vivo* of the labeled platelets. Correlation between viability index values obtained *in vivo* and values of *in vitro* reversal reaction was significant with a relation coefficient of 0.95. Values of reversal reaction were different betwen platelets frozen in Unitag and those frozen in Hemoflex bags with

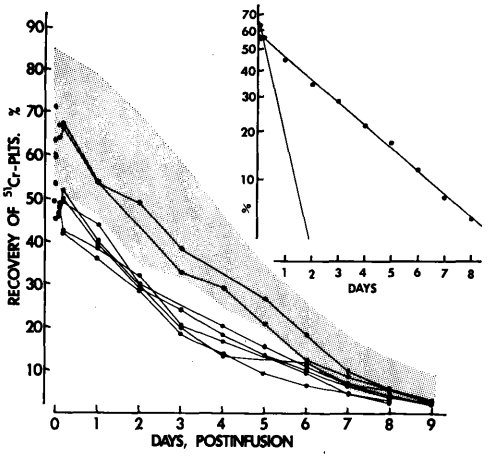

FIG. 2. Survival curves of human platelets frozen in "Hemoflex" bags. To simplify graphic representation, the first recovery values obtained 5 min post-infusion were not connected with rest of the respective curves. The mean survival curve reported on semilog paper in the insert suggests the existence of only a very small fraction of platelets with short life span. Shadowed area in chart represents survival curves of normal, fresh human platelets (8).

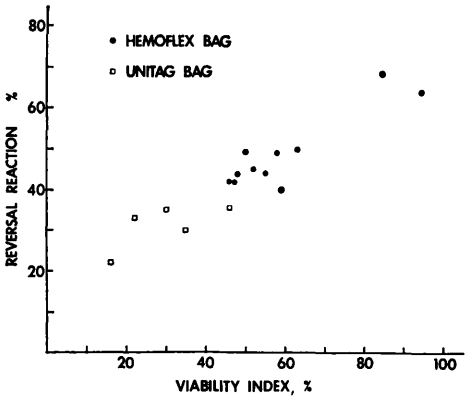

FIG. 3. Correlation between *in vivo* viability index values of frozen platelets and values of *in vitro* platelet reaction to osmotic stress (reversal reaction).

higher values in the latter preparations. The mean values were 31 ± 2.7% in Unitag bags and 48.8 ± 3.8% in Hemoflex bags. The difference was highly significant ($p < 0.005$).

Changes in platelet number caused by freezing and thawing. Freezing and thawing caused destruction of a certain percentage of platelets. The platelet loss was greater in Unitag than in Hemoflex bags. The Unitag platelet concentrates before freezing had a platelet count of 46×10^9 to 88×10^9 with a mean of 68×10^9. After freezing and thawing the counts varied between 35×10^9 and 52×10^9 with a mean of 42×10^9. The Hemoflex concentrates had a platelet count of 48×10^9 to 84×10^9 with a mean of 72×10^9. After freezing and thawing, the mean count was 64×10^9, varying between 43×10^9 and 84×10^9. Freezing and thawing produced a 38% mean platelet loss in Unitag bags and a 11% loss in the Hemoflex container. The difference between the two groups was statistically significant ($p < 0.0005$).

Cooling rate and heat of fusion. Recording of the cooling rate showed that the heat of fusion developed between −14° and −16°. Temperature rose suddenly to −3° to −5° and it took 6 to 7 min to eliminate the heat of fusion. Attempts at preventing the heat of fusion by rapid introduction of more cooling fluid into the chamber were unsuccessful. The heat wave could only be shortened, not eliminated.

Discussion. Platelet volume changes caused by variation in osmolarity of the suspending medium can be studied by recording the changes in light absorbancy of the suspension in a spectrophotometer. By this technique Fantl (6) could demonstrate in 1968, that the platelet response to hypotonic stress was biphasic: platelet swelling first occurred causing a decrease in light absorbancy which was followed by a gradual increase presumably reflecting a reactive platelet volume contraction. This recovery phase causing a reversal of light absorbancy, here called "reversal reaction," was energy dependent. It was inhibited by sulfhydryl reagents and by a combination of sodium cyanide and iodoacetate. We (7) and others (10) could demonstrate that platelet storage at 4° or at 22° produced depression of the platelet ability to recover from hypotonic stress. The loss of platelet reversal reaction paralleled the loss of platelet survival *in vivo*. In the course of these experiments, we could furthermore demonstrate (7) that freezing of the platelets with DMSO or glycerol caused inhibition of the reversal reaction which was more pronounced with glycerol than with DMSO. Inhibition was less with 5% DMSO than with other concentrations. The least amount of inhibition was seen when addition and removal of the DMSO were done very gradually and confirmed, indirectly, previous experience with red cell freezing (11).

More than one factor may have played a role in producing improvement in results by the use of the freezing method described above. Among these are the increased experience in handling platelets, the pH of the suspending medium and, probably more important than others, the reduced osmotic stress obtained by the gradual addition and removal of the cryoprotective agent. Although DMSO is very permeable across the cell membrane and osmotic gradients can be less critical with DMSO than with other cryoprotective agents (12), our experiments indicate that in our method, the major technical difference with previous, less successful techniques was the carefully graded addition and removal of DMSO. As for other cells, platelet damage during slow freezing is known to

be due to osmotic changes. However, platelets have a greater "resistance" to osmotic stress than red cells (13) and it was rather surprising in the past that a valid method of platelet freezing could not be obtained. In our studies, the platelet reversal reaction was instrumental in devising our successful method. The direct relationship between viability index values *in vivo* and values of reversal reaction *in vitro* (Fig. 3) shows that this simple *in vitro* technique may be an accurate indicator of platelet viability in experiments of this type. In fact, in our current studies, we have seen a similar relationship also with human platelets preserved at 4° or at room temperature.

Our results showed a gross difference between viability values of platelets frozen in the two different plastic containers. It has been long known that plastic surfaces can affect platelet integrity (14, 15). The reason for this is not yet clear. We speculate that this may occur because of the action of phthalate ester plasticizers which have recently been demonstrated in blood and solutions stored in disposable polyvinyl chloride plastic blood packs (16). Di-2-ethylhexylphthalate (D.E.H.P.) is lipophilic and it is conceivable that it becomes concentrated in the platelets as it does in other body tissues and causes functional damage. The main reason for the better results obtained with the Hemoflex bags may have been the fact that this plastic film of polyolefin contains no plasticizer.

If one assumes that platelets which disappeared immediately after infusion had little or no hemostatic function while those which survived a normal or nearly normal length of time could arrest hemorrhage as much as fresh, normal platelets, the recovery value two hours post-infusion was more significant in terms of hemostatic effectiveness than the value immediately after infusion. The two hour mean recovery value was 53% in Hemoflex bags (Fig. 2), that is within the normal range and only moderately lower than our corresponding mean value with fresh platelets (66%). With an 11% loss of platelets occurring during freezing, thawing and washing of the concentrate, the total loss was only of moderate magnitude. It is conceivable that with further improvement in technique this loss can be reduced. Studies to this end are now in progress.

Summary. The *in vitro* biphasic reaction of platelets to hypotonic stress measured in a spectrophotometer (reversal reaction) was used as an indicator of platelet integrity in a series of experiments on preservation of human platelets by freezing. Following these results as a guideline, human platelets were cooled at 1° per min to —35° using 5% DMSO in plasma as the cryoprotective agent. Addition and removal of the DMSO was done very gradually to minimize osmotic stress. Survival studies after labeling with radioactive sodium chromate demonstrated nearly normal viability of the frozen–thawed platelets. Platelets frozen in polyvinyl chloride bags had lower recovery values than platelets frozen in polyolefin bags. Correlation between *in vivo* viability of the frozen platelets and values of *in vitro* reversal reaction was highly significant further suggesting that this practical *in vitro* test may be a valid indicator of platelet viability in experiments of this type.

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1. Cohen, P., and Gardner, F. H., "Blood Platelets," p. 485. Little, Brown and Co., Boston (1961).

2. Baldini, M. G., Costea, N., and Damashek, W., Blood **16**, 1669 (1960).

3. Cohen, P., and Gardner, F. H., New Engl. J. Med. **274**, 1400 (1966).

4. Djerassi, I., Farber, S., Roy, A., and Cavins, J., Transfusion **6**, 572 (1966).

5. Baldini, M. G., Cryobiology **5**, 49 (1968).

6. Fantl, P., J. Physiol. **198**, 1 (1968).

7. Kim, B. K., and Baldini, M. G., Fed. Proc. **29**, 717 (1970).

8. Morrison, F. S., and Baldini, M. G., Vox Sang. **12**, 90 (1967).

9. Brecher, G., and Cronkite, E. P., J. Appl. Physiol. **3**, 365 (1950).

10. Handin, R. I., Fortier, N. L., and Valeri, C. R., Transfusion **10**, 305 (1970).

11. Valeri, C. R., New Engl. J. Med. **284**, 81 (1971).

12. Morrison, F. S., Cryobiology **5**, 29 (1968).

13. Lundberg, A. S., Meryman, H. T., and Estwich, N., Amer. J. Physiol. **222**, 1100 (1972).

14. Ozge, A., Baldini, M. G., and Goldstein, R., J. Lab. Clin. Med. **63**, 378 (1964).

15. Marcus, A. J., and Zucker, M. G., "The Physiology of Blood Platelets," p. 104. Grune and Stratton, New York (1965).

16. Jaeger, R. J., and Rubin, R. J., Science **170**, 460 (1970).

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