

Changes in Platelet Shape and Structure After Freeze Preservation

By James P. Crowley, Anthony Rene, and C. Robert Valeri

About 60% of the platelets freeze preserved in 6% dimethylsulfoxide retained the discoid shape characteristic of fresh platelets. The remainder were spheroid. Platelet spheroiding occurred during freezing and thawing and was not related to the

addition or removal of the cryoprotective agent. Platelets that retained the disc shape had a well-preserved ultrastructure, but spheroid platelets showed extensive ultrastructural changes.

IN 1966 Djerassi, Farber, and co-workers¹ found that human platelets remained intact following freezing and thawing in plasma to which dimethylsulfoxide had been added. When these platelets were infused, however, most were removed rapidly from the circulation.^{1,2} Improved methods of addition and removal of DMSO increased the in vivo recovery substantially,² but, nevertheless, a large proportion of freeze-preserved platelets are cleared from the bloodstream shortly after infusion.²⁻⁴ We examined platelets that had been freeze preserved for structural alterations⁵⁻⁷ that might explain this observation.

MATERIALS AND METHODS

Platelet Concentrate Preparation

Approximately 450 ml of blood was collected into 67.5 ml of acid-citrate-dextrose (ACD) for each study. All processing before freezing was performed at 22°C. We separated the whole blood into platelet-rich plasma and red cell fractions by a 3-min centrifugation at 4500 g. The platelet-rich plasma was transferred to a 300-ml transfer pack and centrifuged at 4500 g for 5 min to concentrate the platelets. The platelet button was left undisturbed for 30 min, then gently resuspended in 30 ml of autologous plasma.⁸ The recovery of platelets using this technique was 60%-70% of those in the original unit.

Freeze-preservation Technique

The method of freeze preservation has been previously reported.⁴ To the resuspended platelet concentrate, an additional 30 ml of 12% DMSO plasma (w/v) was added at a rate of 1 ml/min. The final volume of the platelet suspension was 60 ml, and the final DMSO concentration was 6%. After transfer to a polyolefin plastic freezing container (Union Carbide, N.Y.), the platelets were frozen to -80°C at an approximate rate of 2 Ci/min.⁴ Twenty-four hours after freezing, the platelets were thawed by immersion in a 37°C water bath, transferred to a 300-ml transfer pack, and then diluted with 100 ml of 2% DMSO in plasma followed by 16 ml of ACD solu-

From the Naval Blood Research Laboratory, Chelsea, Mass. 02150 and the Armed Forces Radiobiological Research Institute, Bethesda, Md. 20014.

Submitted January 11, 1974; revised March 26, 1974; accepted April 12, 1974.

Address for reprint requests: Officer-in-Charge, Naval Blood Research Laboratory, Chelsea, Mass. 02150.

Direct correspondence to Dr. James P. Crowley, Peter Bent Brigham Hospital, Boston, Mass. 02115.

Supported by the U.S. Navy.

The opinions or assertions contained herein are those of the authors, and are not to be construed as official or reflecting the views of the Navy Department or Naval Service at large.

© 1974 by Grune & Stratton, Inc.

tion. After centrifugation at 4500 *g* for 5 min, the supernatant was removed and the platelets resuspended in 30 ml of autologous plasma.

Platelet Morphology by Phase Contrast Microscopy

The presence of platelet discoid shape⁹ was determined with a Leitz Dialux microscope fitted with phase contrast equipment. Samples were obtained for examination (1) after platelet concentrate preparation, (2) after DMSO addition, (3) after freezing and thawing, and (4) after washing and resuspension.

As reported by Zucker and Borelli,¹⁰ considerable variation was encountered if the temperature was not rigidly controlled. Sample, siliconized glass slides, cover slips, and microscope were incubated at 37°C for 1 hr prior to the enumeration of the percentage of discs. Then a 10- μ l sample was pipetted to the center of the glass slide and covered with a glass cover slip. After an additional 2 min at 37°C to minimize random platelet motion, 200 platelets were examined with an oil-immersion phase-contrast objective (100 \times , N.A. 1.30) and the per cent of platelets in the discoid configuration determined. Samples were examined in duplicate.

Platelet Morphology by Electron Microscopy

Platelets from three different donors were examined with an electron microscope before and after freeze preservation. Samples were prepared for examination by a modification of the Behnke procedure⁶ and were subjected to the temperature control described above. One milliliter of the platelet concentrate to be examined was added to 1 ml of 2.6% glutaraldehyde buffered to pH 7.3 with 0.05 *M* cacodylate. After 10 min of incubation at 37°C, an additional 8 ml of fixative was added, and the sample centrifuged at 3000 rpm for 10 min. The supernatant was removed and 5 ml of fresh fixative gently layered over the platelet button. After washing in 0.05 *M* phosphate-buffered 0.9% NaCl solution, the platelets were postfixed in veronal-buffered osmic acid, pH 7.3, and then embedded in Epon 812 and thin ultratome sections cut and treated with uranyl acetate and lead citrate. The sections were examined with a Siemens Elmiskop I electron microscope.

RESULTS

Approximately 58% of the previously frozen washed platelets were in the discoid shape following freezing and thawing (Fig. 1). Changes in platelet shape were not induced by DMSO addition alone, and the per cent of platelets spherized was not increased by the handling following thawing (Fig. 1).

Previously frozen platelets examined before and after washing (Fig. 1) exhibited a similar appearance with an admixture of discoid and spherized forms. Platelets that were spherized showed a loss of ground substance, degranulation, and no discernible microtubular or canalicular system (Figs. 2 and 3). Previously frozen platelets in the discoid configuration had discrete granules and

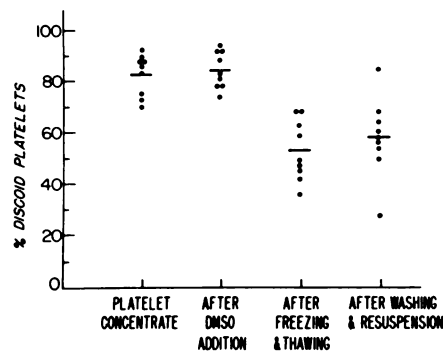


Fig. 1. The per cent of platelets in the discoid form after (1) isolation and concentration, (2) the addition of DMSO to a final concentration of 6%, (3) freezing and thawing, and (4) removing the DMSO by dilution centrifugal washing.

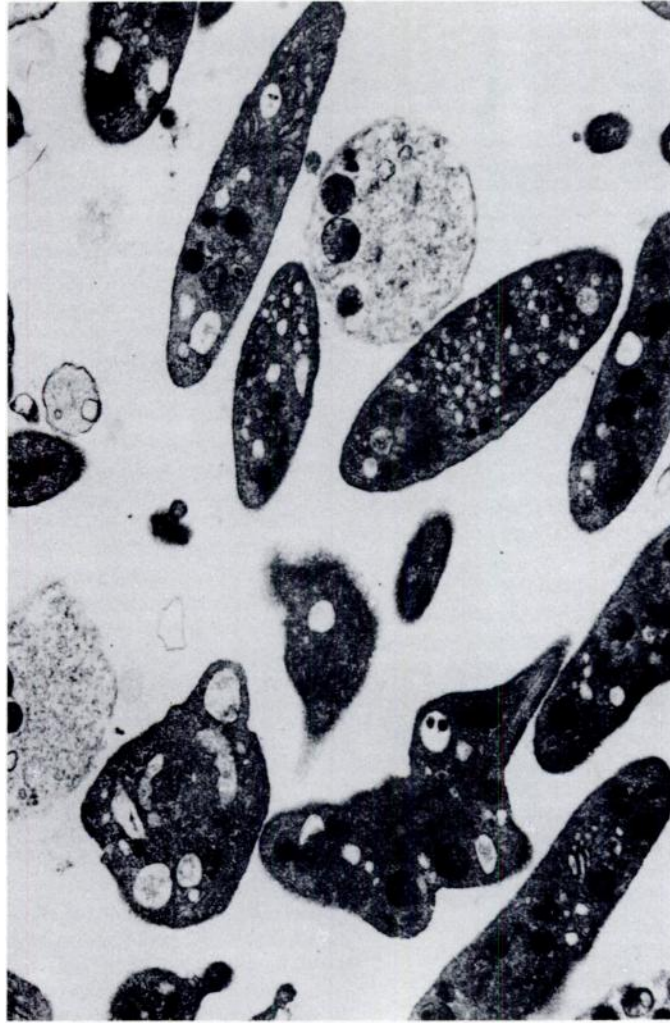


Fig. 2. The ultrastructural appearance of platelets frozen with 6% DMSO and stored at -80°C for 24 hr that were thawed, washed, and resuspended in autologous plasma. An admixture of discs and spheres is evident. ($\times 15,000$)

identifiable canalicular and microtubular elements (Fig. 3). In no instance were spheroid, *degranulated* forms seen in fresh platelet concentrates that were fixed and handled in an identical manner to the previously frozen platelets.

DISCUSSION

The results confirmed that there are more damaged platelets in freeze-preserved concentrates than in fresh platelet concentrates.² Whether the lower percentage of disc forms explains entirely the early rapid removal of many of the freeze-preserved platelets is a matter for conjecture, but the extensive internal alterations in platelet ultrastructure that accompanied spheroiding (Fig. 3) support the concept that these spheroid platelets would be rapidly re-

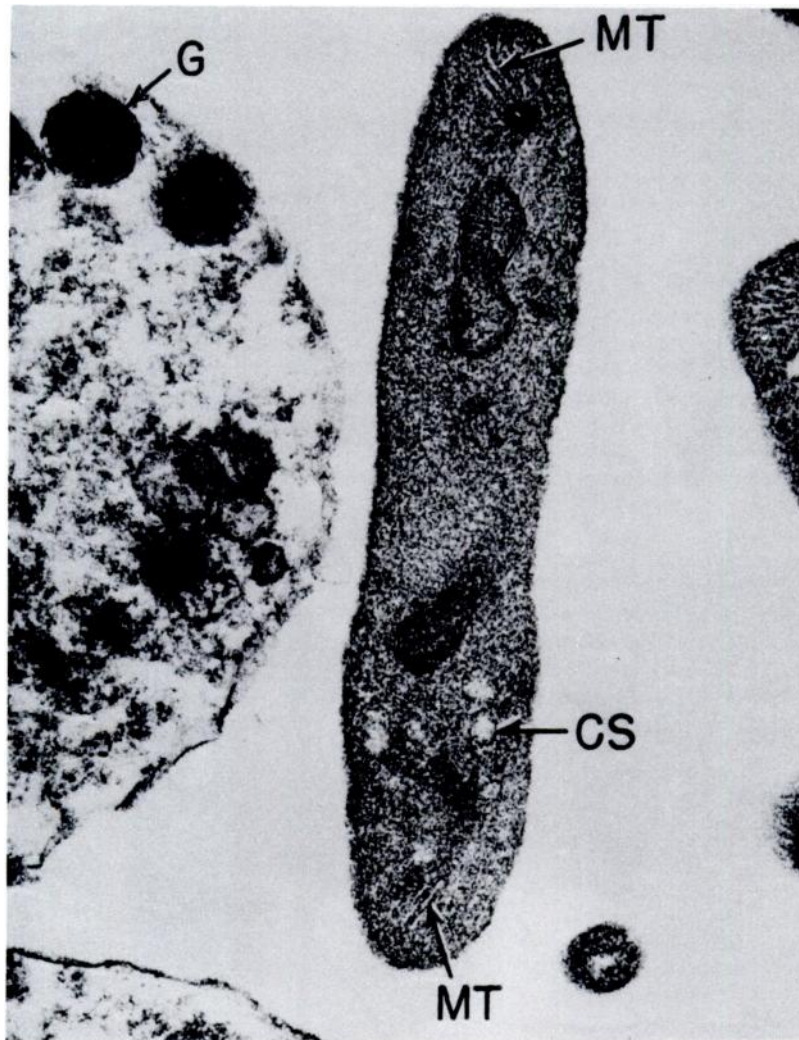


Fig. 3. The ultrastructure of freeze-preserved platelets. A discoid platelet is seen in the center of the field with intact microtubules and open canalicular system. To the left is a spheroid platelet in which there is no discernible microtubular or canalicular elements, and the granules appear to be discharging to the periphery ($\times 60,000$; G, granules; MT, microtubules; CS, canalicular system).

moved from the circulation when they are reinfused.⁶ Our observation that the per cent of platelets in the disc form was about 70% of the fresh platelet value (Fig. 1) is of interest in this regard since it coincides with the finding that washed frozen platelets have an *in vivo* recovery that is approximately 70% of fresh platelets.²

The chemical and physical changes that cause damage to living cells when they are frozen are not well understood, but they are generally held to arise from the osmotic effects of dehydration and the mechanical effects of ice crystal formation during freezing. Freeze preservation depends upon the avoidance of

these effects by the introduction of a cryoprotective compound and the control of the rate of freezing so that osmotic effects are gradual and ice crystal formation minimized.¹¹ The simplest explanation for damage to a portion of the platelet population with sparing of the remainder would be adverse local conditions in certain areas of the freezing container. Further studies of the process of heat transfer in different parts of the freezing containers may shed light on the causes of platelet sphering and possibly give some insight into a means of preventing its occurrence.

REFERENCES

1. Djerassi I, Farber S, Roy A, Cavins J: Preparation and in vivo circulation of human platelets preserved with combined dimethylsulfoxide and dextrose. *Transfusion* 6:572, 1966
2. Handin RI, Valeri CR: Improved viability of previously frozen platelets. *Blood* 40:509, 1972
3. Kim BK, Baldini MG: Preservation of viable platelets by freezing. Effect of plastic containers. *Proc Soc Exp Biol Med* 142:345, 1972
4. Valeri CR, Feingold H, Marchionni LD: A simple method for freezing human platelets using 6% dimethylsulfoxide and storage at -80°C . *Blood* 43:131, 1974
5. Sixma JJ, Molenaar I: Microtubules and microfibrils in human platelets. *Thromb Diath Haemorrh* 26:153, 1966
6. Behnke O: Some possible practical implications of the lability of blood platelet microtubules. *Vox Sang* 13:502, 1967
7. White JG, Krivit W: An ultrastructural basis for the shape changes induced in platelets by chilling. *Blood* 30:625, 1967
8. Mourad N: A simple method for obtaining platelet concentrates free of aggregates. *Transfusion* 8:48, 1968
9. Tocantins LM: Historical notes on blood platelets. *Blood* 3:1073, 1948
10. Zucker MB, Borelli J: Reversible alterations in platelet morphology produced by anticoagulants and by cold. *Blood* 9:602, 1954
11. Meryman HT: The interpretation of freezing rates in biological materials. *Cryobiology* 2:165, 1966