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Correction of Radiation-Induced Thrombocytopenia and Bleeding Tendency by Preserved Platelets.* (27603)

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The hemorrhagic tendency associated with thrombocytopenia is susceptible to modification only by administration of freshly collected platelets in suitable amount(1,2). Pharmaceutical compounds with alleged hemostatic properties have been found ineffective in *in vivo* experiments with thrombocytopenic animals(2). Processing of concentrated platelets by various methods for the purpose of storage has been associated with loss of the property of these blood elements to correct the hemorrhagic diathesis of thrombocytopenic patients(1) or animals(2,3). On the other hand, long-term storage of glycerol-suspended frozen RBC is possible(4,5) and this technic has been applied to preservation of concentrated platelets which, reconstituted and administered to dogs, remained present in the circulating blood in sizable number for at least 24 hours(6,7). The activity of these platelets against a bleeding tendency, however, has not been demonstrated(2,7). The practicality of preservation of blood elements by suspension in glycerol is also limited by the fact that the alcohol has to be separated from the cells by cumbersome technics before administration(5). The purpose of this report is to describe a technic by means of which platelets can be preserved indefinitely by quick freezing at liquid nitrogen temperature. The suspension fluid described by Meryman(4), which can be safely injected in the recipient, is used to minimize freezing injury to the cells. Sterility is maintained by processing the cellular suspension in a special receptacle. Platelets thus preserved, upon reconstitution, maintain their morphologic integrity and antihemorrhagic activity.

Materials and methods. The technics for quantitative estimation of bleeding tendency of irradiated thrombocytopenic rats have been described(1,2) and have been used to evaluate the efficacy of allegedly hemostatic agents in thrombocytopenic patients(8) or animals(2).

Blood for platelet separation was collected from the abdominal aorta of ether-anesthetized 400 to 500 g rats (discarded breeders). The blood was mixed with 3.8% sodium citrate in proportion of 1 to 10 and centrifuged immediately in a refrigerated International Centrifuge, Model PR-11. Brief (25 to 120 seconds) spurts of centrifugation at 3,000 r.p.m. yield plasma containing more platelets with less WBC and RBC contamination than plasma obtained after the usual 10-minute centrifugation at the moderate speed of 1000 r.p.m. The platelet-rich plasma was separated from the RBC, transferred to graduate centrifuge tubes and spun down for 30 minutes at 3500 r.p.m. The clear supernatant plasma was removed and 2 volumes of it mixed with one volume of the Meryman fluid (dextrose 29.2%, sodium citrate 1.28%, citric acid 0.4%)(4). An appropriate amount of this mixture was then added to the sediment to obtain a 20% concentration (v/v) of platelets. A homogeneous suspension was made by gentle mixing with a waxed applicator. No clumping was noted. The suspension was introduced into polyethylene tubing 0.125 inch in internal diameter.† The tub-

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† Intramedic Polyethylene Tubing PE 350, Clay-Adams, Inc., New York.

TABLE I. Platelet Counts in Controls and in Rats Given Infusions of Preserved Platelets.

Exp. No.	No. of animals		Avg platelet count						
			Before infusion	Following infusion of preserved platelets*					
	Treated	Controls		1 hr	3 hr	6 hr	18 hr	24 hr	72 hr
1	7		39,571	230,300			206,250		
1		2	32,500	36,250			28,750		
2	6		28,416	186,875			113,500		
2		2	29,250				19,750		
3	4		41,666	186,750			143,750		97,500
3		2	30,500	29,250			25,000		33,750
4	2		30,750		128,750		62,500		
4		2	32,750				32,500		
5	8		42,250			159,750			
5		2	43,750			45,000			
6	4		38,750	219,250		186,250		50,000	

* Difference in storage time did not affect results.

ing had been previously sterilized by ethylene oxide gas exposure using commercially available apparatus and the technic recommended by the manufacturer.‡ Two aluminum seals§ were applied to the tubing in the vicinity of the ends of the fluid column and the section filled with the suspension cut off. This tubing section was then coiled around a wire frame and plunged into liquid nitrogen contained in an insulated vessel.

The platelets were stored in liquid nitrogen for periods varying from 24 hours to 37 days. At the moment of use the plastic tubing was removed from the liquid nitrogen, quickly immersed in water at 45°C and kept submerged for 30 seconds.

The reconstituted platelets were administered to ether-anesthetized rats intravenously in the tail vein directly from the tube which was connected at one end with a hypodermic needle and at the other with a parenteral administration set attached to a bottle containing physiologic solution which exerted gravity on the column of platelets (Fig. 1). Alternatively, when strict sterility was not to be observed, the material was transferred to a syringe and administered intravenously from it. Special conditions in control experiments are described below.

Results. 1) *Persistence in circulation of preserved-reconstituted platelets.* One and one-half ml of a 20% concentration of preserved-reconstituted platelets, corresponding to approximately 1.5×10^{10} elements, were administered to each of 31 rats in 6 different experiments. A substantial increase of circulating platelets was obtained (Table I). The elements in circulation, however, represent only about ¼ of the increment expected by calculation and about ⅓ of the increment obtained when fresh (non-stored) platelets were administered(2). After 18 hours, 63.9% of the transfused platelets were still circulating (Fig. 2).

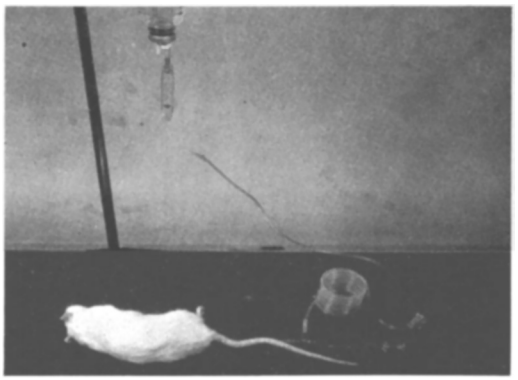
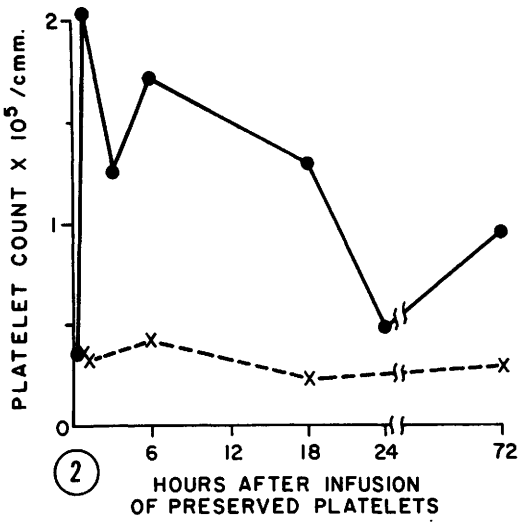


FIG. 1. Administration of preserved-reconstituted platelets to a rat directly from tubing used to freeze and store cells.

2) *Antihemorrhagic activity of preserved-reconstituted platelets.* Bleeding time and platelet count were determined in different groups of irradiated thrombocytopenic animals at 1, 6, or 18 hours after administration

‡ Ben Venue Sterilizer, Ben Venue Laboratories, Inc., Bedford, Ohio.

§ Clips, hand sealer, Fenwal Laboratories, Morton Grove, Ill.



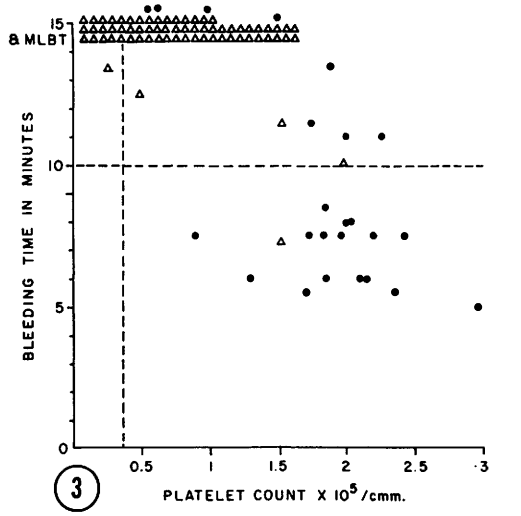


FIG. 2. Persistence in circulation of preserved-reconstituted platelets. Avg figures from treated animals (●——●) and controls (×– – –×) indicated in Table I.

FIG. 3. Correlation between platelets present in circulation of irradiated rat and bleeding time. ● = Animals which received infusion of preserved-reconstituted platelets. △ = Irradiated, untreated animals at 5th to 7th day after exposure to 850 rep. Vertical broken line indicates avg platelet count in all animals before infusion of preserved-reconstituted platelets. Bleeding times below 10 min. (horizontal broken line) are considered indicative of efficient hemostasis.

of preserved-reconstituted platelets. Bleeding time was below 10 minutes in 66.6% of the treated animals (Fig. 3). According to our method(2) this indicates a "good" correction of the hemorrhagic tendency. Four rats (16.6%) demonstrated "moderate" correction of the bleeding tendency (bleeding time between 10 and 15 minutes). A third group of 4 animals (16.6%) had bleeding times of 15 minutes or more. This is conventionally called "maximum length bleeding time" (MLBT) and is considered an indication of failure to correct the hemorrhagic tendency of thrombocytopenia. The triangular symbols of Fig. 3 show the results of bleeding time determination in a group of 68 untreated irradiated thrombocytopenic animals. Approximately 93% of these had MLBT. Fig. 3 indicates also the relationships between platelet count attained by transfusion and length of bleeding time. It appears, however, that fewer circulating platelets are necessary to shorten bleeding time in the present experiments than in those described earlier.

(3) *Miscellaneous experiments and observations.* (a) Platelets were prepared and administered to 6 irradiated thrombocytopenic rats under the same conditions as above except that they stood at +6°C for 6 hours following separation from the whole blood. They were ineffective in correcting the bleeding tendency of the animals. In fact, all 6 recipients demonstrated MLBT and their platelet count increased only to an average of 36,666 from a pretreatment average of 26,666.

(b) Platelets suspended in the usual medium were cooled at the rate of 1°C per minute and kept at −20°C for 72 hours before thawing. They induced no correction of bleeding tendency and no significant increase of platelet counts of the recipients.

(c) Rapid administration of preserved-reconstituted platelets is injurious to the recipient animals. We observed immediate death by cardiac arrest and respiratory paralysis when animals were infused suddenly with as little as 0.10 ml of preserved-reconstituted platelets. We considered this a reaction to vasoconstrictive materials in platelets(9). This reaction was not observed when comparable amounts of fresh platelets were infused. The infusion rate of 0.3 ml per minute of the 20% platelet concentrate has been found safe.

(d) Approximately one-third of the reconstituted frozen platelets observed by phase microscopy demonstrated swelling, grouping

of the granules at one pole of the cell, and loss of the characteristic dendrons. This morphologic abnormality seems identical to that observed by Feissly in the platelets suspended in a cocain solution(10).

Discussion. Preservation of viable biological material for an indefinite period has been possible for a number of years but application of the technics used for storage of tissues and biological fluids to preservation of human blood and blood derivatives has been slow. Contributing to this delay are probably the numerous conditions required to apply the known theoretical principles of cryogenics to the practical use of transfusions of reconstituted-frozen blood. Glycerol, which delays crystallization of water during freezing and thus reduces cellular damage, cannot be safely injected into the circulation. Removal of this compound from the cells requires expensive apparatus which can be operated only in specialized laboratories(5). The use of glucose solution and the droplet spray technic(4) has been precluded from wide application by the difficulties encountered in processing large amounts of blood and preserving sterility by this method.

With the technic described here the preservation of platelets for an indefinite period has been realized. The condition of cells stored at liquid nitrogen temperature can be considered stable and such cells preserved for "biological eternity"(11). The polyethylene tubing allows the freezing and thawing of the material in a closed, sterile environment while keeping the size of the column small enough for immeditae vitrification upon immersion in liquid nitrogen and immediate reconstitution upon immersion in warm water. On the other hand, the electrolyte solution used as suspending fluid can be safely injected, thus avoiding further manipulations of the reconstituted cells.

With the exception of fresh platelets, platelets prepared according to the technic here described have been the only material among the numerous compounds and biological preparations tested with our system which corrected the bleeding tendency of the thrombocytopenic animals(2). *Of paramount importance for preserving the viability of platelets is rapidity of separation from whole blood and resuspension of the cells in glucose before freezing.* An interval longer than 3 hours from collection to processing for storage is associated with loss of antihemorrhagic activity of the platelets.

Administration of preserved platelets to humans is being presently attempted. Pilot experiments for preservation of whole blood and bone marrow with a slightly modified technic have been carried out. Nucleated elements of the bone marrow demonstrate no visible morphologic changes. Up to 80% of RBC remain intact upon reconstitution after preservation. The amount of free hemoglobin liberated in the suspension fluid is, however, still considered too great to allow safe administration.

Summary. Rat platelets suspended in a glucose-sodium citrate-citric acid solution plus plasma have been introduced in polyethylene tubing of 0.125 inch in diameter and rapidly frozen by immersion of the whole container in liquid nitrogen. The material was stored in the same environment. The material was thawed by immersing the whole container in water at 45°C. Administration of these platelets to thrombocytopenic animals was associated with average increase of platelet counts from 36,900 to 205,793 in one hour. Eighteen hours after administration, 63.9% of these platelets were detectable in the circulation. Satisfactory correction of the bleeding tendency was observed in 66.6% of the recipients.

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Effect of Catecholamines on the Individual Free Fatty Acids of Plasma.* (27604)

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Previous studies from this laboratory dealt with the removal and mobilization of individual free fatty acids in normal and diabetic dogs(1,2). The present report extends these observations to include the effects of administration of epinephrine and norepinephrine on plasma individual free fatty acids.

Methods. Normal dogs of both sexes were employed after fasting for 16 hours. All experiments were carried out under nembutal anesthesia. Blood was sampled from the femoral artery through a cannula in a small side branch. After a control sample, epinephrine or norepinephrine was infused intravenously at the rate of 10 μg/kg/min. A second sample was withdrawn after 20 minutes of infusion. Total concentration of free fatty acids (FFA) was determined by Dole's method(3), free fatty acids were separated from other lipid fractions on silicic acid and ion exchange columns and the individual fatty acids analyzed by gas liquid chromatography, as described previously(1).

Results. The effect of epinephrine administration on arterial individual FFA is shown in Fig. 1. Total arterial concentration of FFA increased (mean increase = .268 meq/l) due to a rise in concentration of most major fatty acids. The percentage composition of the elevated FFA was also different; oleic and linoleic acids represented a significantly higher percentage. Although the percentage concentration of palmitic and stearic acids decreased, these changes were not statistically significant.

The arterial change in individual FFA after administration of norepinephrine is illustrated in Fig. 2. The rise in total arterial concentration of FFA (mean increase = .611 meq/l) is accompanied by an elevated concentration of the major 5 fatty acids, while the percentage concentration of oleic acid increased and that of stearic acid decreased significantly. Thus, it appears from the above findings that catecholamine-induced mobilization of FFA from the adipose tissue is characterized by a relative increase of oleic acid (and possibly linoleic acid) in the plasma. Since catecholamines have also been assumed to regulate the release of FFA from adipose tissue under physiological conditions, it could be of value to demonstrate a correlation between the normal arterial FFA level and its fractional content of oleic acid. The existence of such a correlation (which has a correlation coefficient of .618 and is significant at $p < .01$ level) is shown in Fig. 3. Each of the 38 points on the scattergram represents a different normal dog under anesthesia. No such relationship could be shown for palmitic or linoleic acids.

A similar scattergram for 29 alloxan-diabetic dogs is shown in Fig. 4. This slope also indicates a significant positive correlation (correlation coefficient = .555, $p<.01$). Thus, as the blood level of FFA rises, oleic acid comprises an increasing percentage of the total FFA.

Discussion. Previous studies have indi-

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