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DISCUSSION ON THE CURRENT STATUS OF PLATELET PRESERVATION*

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Studies on platelet preservation have increased in intensity during the past few years. This investigative urgency was prompted by the realization that: 1) Bleeding due to severe thrombocytopenia can be effectively treated with platelet transfusions. 2) To be effective on hemostasis, platelets must be "viable," i.e. capable of recirculation and survival. Lyophilized or disintegrated platelets cannot correct thrombocytopenic bleeding.[16, 24] 3) Blood platelets cannot withstand storage at 4°C for more than a few hours. It was indeed surprising, when first demonstrated in 1960, to see that viability of human platelets was almost completely lost within 48 hrs of storage at 4°C.[5] (4) The increasing need of platelets for transfusion purposes cannot be adequately filled by the supply of fresh platelets that a regular blood bank can make available by the presently adopted methods.

The present discussion is meant to summarize some of the prevailing concepts of platelet preservation on the basis of recent experience and to indicate possible guidelines for studies in the immediate future.

DEFINITION OF PLATELET VIABILITY

For the purpose of platelet transfusion, one could define as viable those platelets which have lost none of their hemostatic functions. It derives that the ideal test for a given platelet preparation would be the one which measures its hemostatic effectiveness in a thrombocytopenic recipient. The hemostatic effect of the infused platelets, however, cannot be reliably expressed by their *in vivo* effects on parameters of blood coagulation. Prothrombin consumption may return to normal upon infusion of lyophilized platelets which are known to have no effect in arresting hemorrhage due to thrombocytopenia. Tests for capillary fragility are also not reliable indicators of platelet efficacy since they can be influenced by secondary factors which do not promote hemostasis.[26] Unfortunately, most techniques presently available for the measure of hemostasis lack precision and reproducibility. The bleeding time can be almost reliably standardized on the ear of a rabbit or on the tail of a rat or mouse, but it is indeed much less significant for quantitative data in humans.

It has been widely accepted, in recent years, to equate platelet viability (and their effectiveness on hemostasis) with their property to recirculate and survive upon infusion. Studies in thrombocytopenic rabbits have shown a significant correlation between capacity of the platelets to

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survive in the circulation after infusion and their effect on the bleeding time. Platelets stored for various intervals at 4°C had less capacity to survive in the circulation and less effect in reducing the bleeding time in thrombocytopenic rabbits.† In human subjects, the measure of platelet survival is at present the most reliable method for assessing the efficacy of platelet transfusions.

The question may arise whether in no circumstance platelets which have lost some of their hemostatic properties because of storage or other type of stress will ever be capable to survive in the circulation. Exceptions to this rule may be possible as shown by Arnaud et al.,[2] and the ultimate assessment of function for a given platelet preparation will have to be by its effect on hemostasis.

The Measure of Platelet Survival

Platelet survival in the circulation can be measured by two methods: 1) by enumeration after infusion into suitable thrombocytopenic subjects, and 2) by radioactive labeling prior to infusion. The latter method is more practical and precise in human subjects. Of a few radioactive compounds which are available for survival studies of transfused platelets (DFP^{32}, tritiated DFP, P^{32}, S^{35}, C^{14}-labeled serotonin), Cr^{51}-sodium chromate is at present the most suitable for labeling platelets.[1, 4] For all practical purposes, this radioactive label does not elute from the platelets during their survival in the circulation, although a small portion of the label elutes from the platelets immediately after infusion.[4] This can also be held valid for platelets which have been damaged by storage at 4°C. We found no gross discrepancies in thrombocytopenic rabbits when survival of the stored platelets was simultaneously determined by enumeration and by Cr^{51} labeling. Rabbit platelets which had been stored at 4°C exhibited reduced stability of the Cr^{51} label *in vitro*. In fact, with protracted washings in plasma-saline more than 50% of the label could be removed. However, these stored platelets did not demonstrate loss of label after infusion into thrombocytopenic animals.[14] Studies in humans on this subject are only few. Those of Flatow and Freireich[17] confirm our experience in rabbits. Gardner and Cohen,[18] however, do not agree with these results when using frozen platelets. It is possible that the lesion produced by freezing in plasma-glycerol is not completely reversible and is such that the Cr^{51} label is partially eluted in the circulation. Data of platelet survival with Cr^{51} after freezing may have to be regarded, therefore, as rather inaccurate for the measure of platelet viability in the circulation.

Unlike stored red cells, the capacity for survival of stored platelets does not recover completely after infusion and recirculation. The value of immediate survival (recovery value) is the only important parameter in measuring viability of stored red cells since those red cells which are capable of recirculation will, practically always, survive a normal length of time. This is not true for stored platelets. Two parameters are important for the measure of platelet viability *in vivo:* 1) platelet recovery, i.e., the percentage of platelets which will reappear in the circulation after infusion,

† Ozge, A., and Baldini, M. The effect of fresh and stored platelets on the bleeding time of thrombocytopenic rabbits. In preparation.

and 2) platelet survival time, that is, the time required for the recirculating platelets to disappear. Storage of blood platelets at 4°C and also freezing cause a reduction in either or both these parameters. Immediate survival as well as long-term survival of blood platelets can, therefore, be affected by storage. It is known, however, that freezing in plasma-glycerol usually affects more the value of platelet recovery than the survival time, while storage at 4°C affects both parameters.[4] This implies that the measure of platelet viability after storage will always have to take into consideration both parameters (recovery value and survival time) and will be more cumbersome than the measure of viability of stored red cells.

Indices of Platelet Viability

The difficulty of finding adequate numbers of volunteers for platelet survival studies and the fact that the technique of measuring platelet survival is lengthy and cumbersome have stimulated the search for an *in vitro* method. Research on platelet coagulation factors as related to problems of platelet preservation has been of little value. These seem to be independent to a great extent of the energy metabolism. They withstand long-term preservation at 4°C and lyophilization and are not affected by inhibitors of glycolysis and respiration. Prothrombin conversion,[25] thromboplastin generation,[11] and the clot retraction test have been utilized. Influence on prothrombin conversion and thromboplastin generation can be obtained also with lyophilized platelets, which have no effect on hemostasis. These tests are, therefore, unsuitable for the measure of platelet viability. The clot retraction test is probably more truthful although platelets preserved in plasma for more than 1 week still had good clot retraction property,[22] while platelet viability was lost within 48 hrs in similar conditions.[5]

Other attempts have been made more recently. A number of tests have been proposed: oxygen consumption, isocitric dehydrogenase activity, glucose utilization, lactate production, ATP concentration, and serotonin binding.[15, 21, 35] However, none of these parameters decreased as rapidly as viability. Platelets from blood stored for several days at 4°C showed little impairment by these tests, yet they failed to circulate when transfused. Those parameters which change more rapidly during storage at 4°C are supposed to be more reliable because it is known that platelet viability is lost mostly during the first 24 to 48 hrs. This, however, does not necessarily prove that the particular function being tested is strictly connected with platelet viability. This can be said also for the level of phosphoglyceraldehyde dehydrogenase (PGADH) and glucose 6-phosphate dehydrogenase (G-6-PD). The platelet level of these two enzymes drops to 10 to 20% in 4 days with storage at 4°C.[28] Whether this drop is causally correlated with loss in viability has not as yet been demonstrated. The same could be said also for the determination of platelet esterases recently proposed by Kilburn and Firkin (personal communication). The release of other enzymes (nucleotide diphosphokinase, 3-phosphoglycerate kinase, and enolase) has also been proposed for the detection of platelet damage.[30] Enzyme release was reduced by lowering the pH or by addition of adenosine and was greater when the platelets in the concentrates were clumped. How much

the release of these enzymes from the platelets can be correlated with their *in vivo* viability still remains to be demonstrated. The loss in the ability to produce lactate upon thrombin stimulation is also a phenomenon which is rapidly influenced by storage of the platelets at 4°C.[10] Further studies on this phenomenon are also needed.

One could even speculate that the idea that the measurement *in vitro* of one single platelet component or function could assess quantitatively platelet viability may only reflect our ingenuity. It is entirely conceivable that the capacity of the platelets to survive and function after infusion may basically depend on several unrelated metabolic and structural properties so that the evaluation of only one platelet function chosen at random may not always reflect the damage produced to platelet viability. There is some evidence to support this idea: platelet viability upon storage at 4°C is lost with both reduction in recovery value (immediate survival) and simultaneous shortening of survival time (long-term survival). Addition of inosine and adenine to the plasma medium improves the recovery value but not the survival time.[14] This result indicates that immediate and long-term survival may not always be interdependent and are probably influenced by different platelet functions.

Methods for Improving Viability of Stored Platelets

Studies on the possibility of extension of the storage period at 4°C have been only a few. Presently, the problem of platelet preservation in the liquid state is still complex, and very few results of practical importance have so far been obtained in this field.

The choice of the anticoagulant is important in preserving blood platelets at 4°C. It is known that ethylenediaminetetraacetate (EDTA) exerts a deleterious effect on the platelets although its mechanism is not clear. Platelets collected with EDTA demonstrate a much lower recovery value than when acid-citrate-dextrose (ACD) is used.[3, 29] The survival time, on the other hand, is equal with either anticoagulant (8 to 10 days). In addition to the reduction in immediate survival (recovery value) of infused platelets seen with EDTA, there is an associated *in vitro* "sphering effect," [36] a marked reduction in the incorporation *in vitro* of P^{32} into the platelet phosphonucleotides,[34] and a rapid decline of serotonin uptake.[32] EDTA has profound effects on platelet glycolysis,* and the study of these changes may disclose the reason for its deleterious effect on viability. It is important to emphasize here that platelet concentrates prepared with ACD-plasma can withstand storage better than EDTA-platelets.[29]

The effect of the container surface on viability of stored platelets has also been studied. While some plastic containers are known to alter a few platelet properties, including their capacity to promote clot retraction, they do not seem to damage the capacity of the platelets to survive in the circulation.[32, 33]

A number of investigations on the glycolytic metabolism of the platelets have recently appeared in the literature.[15, 20, 21, 28, 34] These emphasize

* Kim, B. K., and Baldini, M. Effect of anticoagulants and storage on non-enzyme components of the Embden-Myerhoff pathway in human platelets. In preparation.

that glycolysis is very active in blood platelets and plays an important role in the energy metabolism of these cells. This information has suggested that the preservation of glycolysis may be a determining factor for the maintenance of platelet viability during storage. Studies on this subject have been concentrated so far on determination of enzyme activities, glucose, and oxygen consumption and of end products such as pyruvate and lactate or CO_2. When compared to the rapid loss of platelet viability, these results have not provided a useful index for the *in vitro* measure of it.

The study of these compounds alone may not be necessarily expected to provide quantitative conclusions on the effective substrate transformations in such a complex enzyme system because the concentration of metabolites, coenzymes, and ions can also determine the rate of flow of such transformations. Data on nonenzyme components of the Embden-Meyerhoff pathway in fresh and stored platelets are still lacking to a large extent. The study of these compounds, i.e., of the glycolytic intermediates and end products, may be fruitful and could also disclose important parameters for the *in vitro* assessment of platelet viability. Preliminary studies by Kim (in preparation) have shown that glycolysis of human platelets is greatly influenced by the anticoagulant used: in the presence of EDTA, glycolysis is greatly enhanced in comparison to that of platelets collected with ACD. Storage at 4°C produces accumulation of trioses in large amounts. 3-PG becomes sharply reduced during the first 48 hrs of storage. The ratio between lactic acid production and glucose utilization increases progressively during storage rather than remaining constant. This suggests that during storage increasing portions of lactic acid may derive from sources other than the glucose of the suspending medium. More studies on this subject are being conducted in our laboratory in close correlation with the *in vivo* viability of platelets stored in various preservation media.

Glycolysis in platelets exceeds oxidative metabolism.[20] Under aerobic conditions only ⅕ of the glucose is recovered as CO_2. The hexokinase is the rate-limiting enzyme for glycolysis as in other blood cells. The importance of respiration in blood platelets has not been adequately studied with reference to the maintenance of platelet integrity and function. Recent experiments seem to rehabilitate this metabolic pathway, thought to be of little importance in the platelets.[20] However, incubation of platelets in an oxygen atmosphere results in a somewhat lower ATP concentration than incubation in air.[20] Accumulation of lipid peroxides damaging mitrochondrial and other platelet structures could be thought of as the cause of this phenomenon. Lipid peroxides may also produce damage to a number of enzymes. —SH groups of proteins and amino acids are destroyed by the lipid peroxides.[27] Peroxide formation in red cells has been linked with hemolysis both *in vitro* and *in vivo*.[13]

We have determined lipid peroxides in human platelets preserved as concentrates in ACD-plasma at 4°C.* These preliminary experiments have shown a rapid accumulation of these compounds in the platelets. Gross has proposed the addition of tocopherol to the suspending medium for platelet

* Okuma, M., Steiner, M. and Baldini, M. Lipid peroxides in stored human platelets. In preparation.

preservation.[20] This substance is an antioxidant and may not only protect the sensitive lipid structures of the mitochondria but also stimulate the oxidative metabolism by activating phosphorylation.[7] In the presence of tocopherol, glucose utlization is improved and the level of ATP in the platelets is higher. Correlation of these results with the problem of preservation of platelet viability is still lacking. However, it is possible that tocopherol may prove to be an important compound for the preservation of blood platelets at 4°C.

During the past 12 years, a number of investigations have reported on the ability of purine nucleosides to extend the preservation of human red cells stored at 4°C in acid-citrate-dextrose. Nakao and associates[31] have also shown that when blood which had been previously stored for 8 to 10 weeks was incubated with both the purine adenine and the purine nucleoside inosine, alterations in shape of the red cells improved considerably and regeneration of adenosine triphosphate took place.

These results prompted parallel studies on blood platelets preserved as concentrates in plasma medium at 4°C. Utilizing rabbit platelets, we showed that the addition of adenine and inosine to the plasma medium produced a definite improvement of platelet viability after storage at 4°C for various intervals.[6] This beneficial effect could also be shown with human platelets when these were collected using EDTA-anticoagulant.[14] The effect was at times striking, but almost exclusively exerted on the recovery value (immediate survival), while the survival time (long-term survival) remained practically unchanged. When, more recently, we added the same amounts of inosine and adenine to platelet concentrates suspended in ACD-plasma,* no improvement above the controls could be obtained with storage for 24 to 72 hrs at 4°C. Viability of platelets preserved in ACD-plasma was greatly improved when compared to the viability of preserved EDTA-platelets, but no more improvement could be obtained with the addition of the purine metabolites. However, when these compounds were added to the platelet concentrates at the end of the storage period (24, 48, or 72 hrs), a definite, but small increase in the recovery value of about 10 to 20% was constantly found. The reasons for this effect are still obscure and are now under study.

One aspect of platelet physiology that has received limited attention is the function of the platelet membrane in transport processes, particularly with regard to electrolyte transport. Although platelets are more resistant than red cells to osmotic rupture, alterations in the electrolyte and water contents may influence metabolic processes, particularly those occurring at the membrane level, and therefore injure viability. Evidence has been presented for the existence of transmembrane gradients of sodium and potassium[23] and transmembrane cation flux.[19] However, no quantitative data have as yet been presented with reference to the changes that these parameters undergo following platelet preservation.

A final consideration on the problem of platelet preservation at 4°C may be indicated. The normal life span of human platelets is less than one-tenth

* Cottanco, N., Ciucci, A. and Baldini, M. Further studies on the effect of adenine and inosine on the maintenance of platelet viability during storage at 4°C. (In preparation.)

of that of the red cells. One cannot reasonably expect that even the ideal method would preserve platelets in the liquid state for lengthy intervals. However, the loss of platelet viability with the present conditions of storage is so rapid that any improvement would assume practical importance.

Preservation of Blood Platelets in the Frozen State

It is obvious that this would be the ideal method for platelet preservation because is it conceivable that in the frozen state platelet viability can be maintained indefinitely. Platelet freezing is, however, still in its infancy and much work will be needed to make this procedure truly useful for platelet preservation. Pioneer work by Gardner[8] demonstrated that dog platelets could be frozen in plasma-glycerol and thawed in the presence of hypertomic dextrose. These platelets had normal survival time when infused in recipient dogs. We demonstrated that when the same method of freezing was applied to human platelets, the recovery value for the infused platelets was only about 20% of the normal value obtained with fresh platelets while the survival time of the platelets which recirculated was relatively long and, at times, normal.[3] More recent experiments have obtained slightly better results.[9] A method using slow freezing in liquid nitrogen and a preservative medium containing 5% dextrose and 5% dimethyl sulfoxide has been utilized by Djerassi.[12] The average *in vivo* yield of these platelets was only 30% of the yield observed with fresh material.

These results demonstrate an important point, i.e., that platelets can be frozen. The excessive loss obtained with the current methods makes this procedure of little value for large scale preparation of frozen platelets. There is no reason, however, to think that the yield of viable platelets cannot be increased with improved methods of freezing and thawing. Basic physiology of platelet freezing is still obscure. Metabolic and functional studies of such platelet preparations are very few. The relationship between additive concentration and freezing rate is not as yet completely known for human platelets. This has been an important point in freezing red cells: optimum additive concentrations were observed with each freezing rate.

Much work will be needed before progress can be made in this field. This is slowed greatly by the lack of *in vitro* tests by which viability of the frozen-thawed platelets can be accurately quantitated.

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