Acquired Granular Pool Defect in Stored Platelets

By A. Koneti Rao, S. Niewiarowski, and S. Murphy

Platelets stored as concentrates (PC) for 72 hr at 22°C develop a functional defect. Alterations in adenine nucleotides of platelets have been shown to affect platelet function. Adenine nucleotide content of platelets was measured before and after storage and a decrease of 27.1 ± 1.7% (mean ± SE) in ATP and 39.1 ± 2.6% in ADP were found in 34 PC stored with final volume of 50 ml. In 11 PC with 30 ml volume, ATP and ADP decreased by 39.4 ± 3.2% and 49.4 ± 2.1%, respectively. The mean ATP to ADP ratio of stored platelets was significantly higher than of fresh platelets in both groups, suggesting a relatively greater decrease in granular than metabolic pool nucleotides. Levels of low affinity platelet factor 4 measured by radioimmunoassay in plasma increased from 0.86 ± 0.08 μg/ml in the fresh PC to 8.58 ± 0.39 μg/ml in stored PC, indicating a concomitant α-granular secretion. Labeling of metabolic pool with 14C-adenine revealed a mean decrease in the adenylate energy charge of 2.0 ± 0.4% in 12 of 16 stored PC, with a lower ATP and higher hypoxanthine labeling in stored as compared to fresh platelets. These observations suggest that stored platelets develop an acquired defect in both dense and α granules and in their ability to maintain ATP homeostasis.

STUDIES BY Filip and Aster,1 and Slichter and Harker2 have shown that the viability of platelet concentrates (PC) stored at 22°C is superior to that of PC stored at 4°C. However, even in PC stored at 22°C, in vitro function has been shown to be markedly reduced. Platelets stored at 22°C for 72 hr have shown a decrease in the maximal rate of aggregation and have required a greater concentration of ADP to elicit a response equivalent to fresh platelets.3,4 There is suggestive, but no conclusive evidence, that the correction of bleeding time of thrombocytopenic patients may not be optimal for the initial 1-2 hr after infusion of PC stored at 22°C for 72 hr.1,2 These facts suggest that a significant functional defect develops during storage. The mechanisms underlying this are unknown. It is also clear that further understanding of the biochemical changes occurring during storage would be useful to develop better methods for platelet storage.

Adenine nucleotides play an important role in the function of platelets. They exist as two different pools within the platelet,5 one in the cytoplasm and the other in the dense granules. A reduction in the content of adenine nucleotides within the dense granules (storage pool) has been shown to be associated with a significant defect of platelet function.6 On the other hand, reduction in the energy status of the platelets as induced by metabolic inhibitors also results in impairment of function.7 There are few studies that have measured the contents of ATP and ADP in platelets stored as concentrates at 22°C for 72 hr. Some investigators8,9 have examined the storage of platelet-rich plasma rather than concentrates, where additional factors such as fall in pH and close cell contact become important. In 1969, Murphy and Gardner10 reported no significant change in the level of total ATP and ADP in platelets stored as platelet rich plasma for 72 hr. More recently, Filip et al.11 studied PC stored for 72 hr and found that in 5 of the 10 PC stored at 22°C, the final ATP + ADP ranged between 10% and 34% of the values obtained prior to storage. However, a substantial number of concentrates were noted to have an unpredictable and precipitous fall in pH during storage.

In this study we report the effect of storage at 22°C for 72 hr on the adenine nucleotide content of human platelets stored as concentrates. The final pH was well maintained in all the PC. A decrease in the total contents of ATP and ADP was found in all concentrates with an increase in ATP to ADP ratio. We propose that these stored platelets develop a defect in the storage pool of adenine nucleotides. In addition, we also show that there is a concomitant release of the platelet-secreted protein, the low-affinity platelet factor 4 (LA-PF4) present in the α granules.12 This protein shares antigenic determinants with β-thromboglobulin but differs from it in having four additional amino acids.13

MATERIALS AND METHODS

Forty-five PC were prepared to a final volume of 50 ml or 30 ml as described previously.1 PC were stored in 2 types of containers: (1) Polyethylene (PE) (Hemoflex bags from Union Carbide, Chicago, Ill.) and Polyvinyl chloride (PVC) (Plastic PL-146 from Fenwal Laboratories, Morton Grove, Ill.). All bags were stored for 72 hr at

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22°C with constant agitation on a horizontal platform shaker as described previously. The final pH of the PC was measured at the end of 72 hr.

**Measurement of Adenosine Diphosphate (ADP) and Adenosine Triphosphate (ATP)**

The platelet suspensions were adjusted with autologous plasma to a count in the range 0.25-0.35 \times 10^11/liter. The total content of ATP and ADP was measured in the ethanolic extracts of the initial platelet-rich plasma, fresh PC, and stored PC by the firefly luciferase assay described by Holmsen et al. For the measurement of secretable ADP and ATP, aliquots of 10 PC obtained from 5 normal donors were gel-filtered using sepharose 2B (Pharmacia, Uppsala, Sweden) column as described by Lages et al. Platelets were eluted with calcium-free tyrode buffer, pH 7.3, containing 0.1% dextrose and 0.2% bovine albumin. The gel-filtered platelets were adjusted to a platelet count of approximately 0.3 \times 10^10/liter and incubated with 1 and 5 U/ml of bovine thrombin (Parke Davis, Detroit, Mich.) for 5 mm at 37°C. The resulting mixture was then placed on ice for 1 min and the contents spun down in an Eppendorf microcentrifuge at 12,000 g for 30 sec. The supernatant was extracted with equal volume of a mixture of 9 parts of ethanol and 1 part of 0.1 M ethylene diamine tetraacetic acid (EDTA) and the amounts of ATP and ADP measured by the firefly luciferase assay. Control experiments with normal saline were also performed.

Labeling of the adenine nucleotides of the metabolic (cytoplasmic) pool of platelets was accomplished using $^{14}$C-adenine (New England Nuclear, 43.95 mCi/m mole). To 1 ml aliquots of fresh and stored PC, 10 \mu M of $^{14}$C-adenine (455 \mu M) was added and incubated for 60 min at 37°C. The PC was then diluted with autologous platelet-poor plasma to a platelet count of 0.25 – 0.35 \times 10^10/liters and immediately extracted with the mixture of ethanol and EDTA as described above. The radioactive adenine nucleotides and their metabolites in the ethanolic extracts were separated by high voltage electrophoresis and counted as described by Holmsen et al. In this manner, the distribution of the radioactivity in ATP, ADP, AMP, IMP, hypoxanthine, and adenine was determined in platelets from 16 PC stored with final volume of 50 ml. From the first three values, the adenylate energy charge (AEC) was calculated using the formula:

$$\text{AEC} = \frac{2 \text{ATP} + \text{ADP}}{2(\text{ATP} + \text{ADP} + \text{AMP})}$$

The AEC reflects the overall energy potential in the metabolic pool of the adenine nucleotides in the platelets.

**Measurement of the Low-Affinity Platelet Factor-4 (LA-PF4)**

Aliquots (2.7 ml) of the initial platelet-rich plasma, the fresh PC, and the post storage PC from 9 studies were added to a cocktail containing 0.3 ml of 5.4 mg/ml theophylline in 10% EDTA and 15 \mu l of 1 mg/ml prostaglandin E, on ice and the platelet poor plasma (PPP) prepared by centrifuging at 2500 g for 1 hr at 4°C in RC-3 Sorval centrifuge. The levels of LA-PF, were measured in PPP by the radioimmunoassay described by Rucinski et al. The data was analyzed by the Student’s t test.

**RESULTS**

The platelet counts in the PC ranged from 0.7 \times 10^11/liter to 2.5 \times 10^12/liter. There was no significant change in the platelet count during storage. The final pH of all the PC was above 6.10.

In 36 of the 45 studies, simultaneous measurements of the total ATP and ADP contents of platelets from both the initial platelet-rich plasma and the fresh PC were made. The values obtained from the platelet-rich plasma were not significantly different from those of the fresh PC. Shown in Table 1 are the results of measurement of the total nucleotide content of the platelets from the platelet-rich plasma and the 72-hr stored platelets. There was a decrease in the total ATP and ADP content of the platelets in all the stored PC. The PC have been divided into two groups on the basis of their volume. In PC stored with a volume of 50 ml, the total ATP content fell from a mean of 6.6 ± 0.2 \mu moles/10^11 platelets (mean ± SE) to 5.1 ± 0.3 \mu moles/10^11 platelets ($p < 0.001$), a decrease of 27.1 ± 0.7%. The total ADP content fell from 3.5 ± 0.2 \mu moles/10^11 platelets to 2.1 ± 0.2 \mu moles/10^11 platelets containing 0.3 ml of 5.4 mg/ml theophylline in 10% EDTA and 15 \mu l of 1 mg/ml prostaglandin E, on ice and the platelet poor plasma (PPP) prepared by centrifuging at 2500 g for 1 hr at 4°C in RC-3 Sorval centrifuge.

<table>
<thead>
<tr>
<th>Table 1. Total ATP and ADP Content of Stored Platelets</th>
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<tbody>
<tr>
<td>ATP (\mu moles/10^11 Platelets)</td>
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<tr>
<td>Fresh</td>
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</tr>
<tr>
<td>PC: 50-ml volume</td>
</tr>
<tr>
<td>(n = 34)</td>
</tr>
<tr>
<td>PC: 30-ml volume</td>
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<tr>
<td>(n = 11)</td>
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</tbody>
</table>

Results are expressed as mean ± SE.

Fresh compared to stored: *$p < 0.001$, †$p < 0.05$.

<table>
<thead>
<tr>
<th>Table 2. Thrombin Secretable ADP and ATP</th>
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<tr>
<td>Fresh Concentrate</td>
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<tr>
<td>---</td>
</tr>
<tr>
<td>Total content ATP + ADP (\mu moles/10^11 platelets)</td>
</tr>
<tr>
<td>Secretable amount ATP + ADP (\mu moles/10^11 platelets)</td>
</tr>
<tr>
<td>Percent of total secreted</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE of 10 studies.
platelets ($p < 0.001$), a decrease of 39.1 ± 2.6%. The percent decrease in ADP and ATP was greater when the volume of the PC was 30 ml than when the volume of PC was 50 ml ($p < 0.05$ and $p < 0.01$ for ADP and ATP, respectively). In both groups, the percent decrease in ADP was greater than ATP ($p < 0.001$) and the ATP to ADP ratio was significantly higher after storage than in fresh platelets ($p < 0.001$ for 50 ml PC and $p < 0.05$ for 30 ml PC). Table 2 shows the thrombin secretable ATP + ADP content of platelets from 10 concentrates. As there was no significant difference in the amount of ATP and ADP released at 1 and 5 U of thrombin per ml, only the results with 5 µ/ml are shown. The stored platelets secreted lesser amounts of ATP + ADP on stimulation with thrombin than platelets from freshly prepared concentrates ($p < 0.001$). Figure 1 shows there was no correlation between the final pH of the PC and the percent decreases in ATP and ADP content of the platelets.

The results of the 16 studies involving labeling of the metabolic pool of adenine nucleotides in fresh and stored PC with $^{14}$C-adenine are shown in Table 3. The isotope distributed differently in the 2 preparations with 80.3 ± 3.3% in ATP of fresh and 66.4 ± 9.0% in ATP of stored platelets ($p < 0.001$); the relative incorporation in hypoxanthine was 11.0 ± 2.6% and 24.4 ± 8.2% in fresh and stored PC, respectively. There was no significant difference in the percent distribution of radioactivity in ADP, while slightly higher percentages were noted for IMP and AMP in stored than fresh PC. The AEC of the stored platelets was lower than that of the fresh PC in 12 of the 16 studies. The AEC in these 12 studies decreased from a mean of 0.953 ± 0.008 to 0.933 ± 0.020 ($p < 0.01$) corresponding to a mean decrease of 2.0 ± 0.4%. Irrespective of the AEC, the percent labeling of the ATP in the stored PC was decreased in all the 16 studies.

The results of the LA-PF$_4$ measurements are shown in Table 4. The level of LA-PF$_4$ rose from a mean of 0.11 ± 0.2 µg/ml (mean ± SE) in the platelet poor plasma from the initial platelet-rich plasma to 0.86 ± 0.08 µg/ml in the plasma from the fresh PC ($p < 0.001$). This increase in the plasma LA-PF$_4$ reflects the effect of the centrifugation and resuspension of

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**Table 3. Labeling of Metabolic Pool Adenine Nucleotides**

<table>
<thead>
<tr>
<th></th>
<th>AEC</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>IMP</th>
<th>Hypoxanthine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh platelets</td>
<td>0.952 ± 0.002</td>
<td>80.3 ± 0.8</td>
<td>6.9 ± 0.3</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>11.0 ± 0.6</td>
</tr>
<tr>
<td>Stored platelets</td>
<td>0.934 ± 0.005*</td>
<td>66.4 ± 2.3†</td>
<td>6.3 ± 0.4‡</td>
<td>1.4 ± 0.2§</td>
<td>1.5 ± 0.2§</td>
<td>24.4 ± 2.0†</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE of 16 studies.

*p < 0.02
†p < 0.001
‡p > 0.05
§p < 0.05
Calculation by the formula

\[ \text{higher than that of ATP} \]

ADP concentration in the dense granules of normal platelets is higher than that of ATP (ATP:ADP = 2:3). Hence, a larger decrease in the granular pool than in the metabolic pool during storage would be reflected by an increase in the ratio of total ATP to ADP. Such an increase in this ratio was noted in platelets on storage. Finally, the amount of secretable nucleotides is normally taken to reflect the content of the granular pool. Thrombin stimulation of stored platelets resulted in the release of lesser amounts of nucleotides than platelets from freshly prepared concentrates. For these reasons, our observations indicate that a distinctly larger decrease occurs in the granular pool than in the metabolic pool during storage of platelets. In the absence of a fall in the platelet counts of the PC, the decrease in the granular pool suggests that secretion of granular contents may be going on during storage of platelets. This conclusion is further supported by the large amount of LA-PF₄ detected in the plasma of the stored PC. The LA-PF₄ is present in the α-granules of the platelets and is a sensitive marker for release reaction. Thus, the present study provides evidence of release and depletion of both dense and α-granular material which may be related to the gentle but constant agitation that is required for successful storage at 22°C.

This study sheds light on the effect of centrifugation of the granular content of platelets. The total ATP and ADP content of the platelets from the platelet-rich plasma and the fresh PC were not significantly different suggesting that there is no substantial release of adenine nucleotides during the second centrifugation involved in the preparation of the PC. However, the levels of the LA-PF₄ in the plasma obtained from the fresh PC were several fold higher than in the plasma from the platelet-rich plasma indicating that
there is secretion of α-granular proteins during the processing. These observations may reflect a lower threshold for the release of LA-PF₄ as compared to that of ATP and ADP. It is equally possible that the current techniques enable us to pick up the release of a small fraction of the total LA-PF₄ content but not a similar decrease in the nucleotide content of platelets.

It is interesting to note that while gel filtered platelets from platelet rich plasma have been shown\(^9\) to release over 50% of their total ATP + ADP content on maximal stimulation with thrombin, platelets from even fresh PC released only 35% of their adenylate content. This may be related to the second centrifugation involved in preparing the PC from platelet-rich plasma. As there was no decrease in the adenine nucleotide content of platelets in the fresh PC when compared to the platelet-rich plasma, it may suggest the development of refractoriness of platelets to the action of thrombin. Likewise, as the stored platelets released only 28.7% of their content of adenine nucleotides, it is possible that after storage there are two factors operating—namely a depletion of the granular content and a decrease in response to the action of thrombin, to explain the reduction in the amount of nucleotides released. This aspect needs to be explored further.

Labeling experiments with \(^{14}\)C-adenine suggest that changes also occur in the metabolic pool adenine nucleotides of stored platelets. Incubation of platelets with \(^{14}\)C-adenine results in a uniform labeling of the various adenine nucleotides and hypoxanthine and the distribution of the label reflects their relative concentrations in the metabolic pool.\(^{20}\) When stored platelets were so labeled there was a significant reduction in the distribution of \(^{14}\)C in ATP with an increased contribution in the breakdown products, AMP and hypoxanthine, as compared to fresh platelets. There was no change in the labeling of ADP. Because of the fact that incorporation of labeled hypoxanthine into ATP is slow\(^{21}\) compared to incorporation of adenine\(^{22}\) and the period of incubation in our experiments was only 1 hr, the increased amount of radioactivity in hypoxanthine reflects a greater breakdown of ATP to hypoxanthine. Our data suggests that platelets develop a defect in their ability to maintain ATP homeostasis in the metabolic pool. The obvious question that arises is the significance of these findings relative to platelet functions. Available evidence indicates that platelet function depends more on the adenylate energy charge, which is an expression of the overall energy status of the cell, then on the ATP level. Using metabolic inhibitors such as antimycin and deoxyglucose, Holmsen and Robkin\(^{7}\) have shown that platelet shape change, primary aggregation, dense and α-granule secretion were unaffected by lowering of metabolic ATP provided the adenylate energy charge did not fall more than 5%. Our measurements show that in spite of the changes noted in the metabolic pool the mean decrease in A.E.C. of stored platelets is only 2.02%. This may indicate that it is unlikely that the altered function of platelets stored at \(22^\circ\)C is due to the changes in the metabolic pool of adenine nucleotides.

One of the major difficulties with storage of platelets at \(22^\circ\)C has been that the pH of some units of PC falls from the initial value of 7.0–7.2 to below 6.0.\(^{23}\) This fall is predominantly due to the production of lactic acid by platelet glycolysis and to a lesser extent to accumulation of CO\(_2\).\(^{24}\) As pH falls below 6.0, irreversible changes occur that render the platelets nonviable after infusion in vivo.\(^{25}\) In previously reported studies of Filip et al.,\(^{11}\) a precipitous and unpredictable fall in pH occurred in several of concentrates in association with drastically altered adenine nucleotide content. In the present study, the final pH was maintained above 6.10 in all the units. Further, no correlation was noted between the percent decrease in the total ATP and ADP content and the final pH of the PC, suggesting that the decrease in the nucleotide content is unrelated to the pH when it remains above 6.10.

Among the several unresolved issues regarding optimal conditions of platelet storage is the question of final volume of the concentrates. Currently final volumes of 30 ml to 50 ml are permitted by federal regulations. Our finding that the decrease in ATP and ADP is more pronounced in concentrates stored with final volume of 30 ml than of 50 ml may have an implication in the choice of the volume of PC. If it can be conclusively shown that the defective function of stored platelets is linked to the depletion of storage pool, then 50 ml final volume would be preferable. The greater depletion of total nucleotides in PC with 30 ml volume may be related to greater cell to cell or cell to wall contact in 30 ml units as compared to 50 ml units with the gentle agitation during storage.

With the increasing demand and use of platelet transfusion, there is continuing need to improve methods for storage. The current methods of storage are far from ideal. It would be helpful to have techniques that would allow storage beyond the current limit of three days. Detailed studies on the biochemical changes in platelets on storage would be important in formulating newer techniques that prevent or reverse the changes noted in this study.

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