The Response of Human Platelets to Hypotonic Stress: Direct Measurement of Volume Change

MICHAEL H. SUMMERER, M.D., PETRINA V. GENCO, Ph.D., and ALFRED J. KATZ, M.D.

Department of Laboratory Medicine, University of Connecticut School of Medicine, Farmington, CT 06032
and Connecticut Red Cross Blood Center, Farmington, CT 06032

ABSTRACT

Human platelets stored at 22°C were subjected to standard hypotonic stress at intervals up to five days after collection. Changes in light absorbance were recorded during the reaction, and size distribution was measured directly using a Coulter Model ZBI Counter and Channelyzer. Recovery following osmotic stress was measured by change in absorbance, as well as by change in platelet size distribution. Significant correlation was demonstrated between the two methods. By both measures, fresh platelets demonstrated substantial recovery following hypotonic stress, but platelets stored for five days had lost this ability. The requirements for intact sulphydryl groups and the lack of effect of colchicine were confirmed.

Introduction

It is known that blood platelets, when exposed to hypotonic stress, show a transient decrease in light absorption. With fresh platelets this decrease in absorbance rapidly reverses over 15 min. The reversal reaction appears to be a metabolic process which can be suppressed by metabolic inhibitors and SH-blockers. Stored or damaged platelets show a diminished capacity for this reversal reaction. Storage conditions, particularly temperature and pH, have a marked effect on the reaction, the optimum temperature reported to be 22°C and the optimum reaction pH range 5.5 to 7.5. Platelets stored below pH 6.0 will not react. Therefore, there has been some interest in this phenomenon as a possible predictor of platelet viability for transfusion. Kim and Baldini showed a correlation between the reversal reaction and a post-transfusion viability index. Valeri et al showed a correlation between the reaction and the number of transfused platelets circulating two hours post infusion, but they failed to demonstrate a relation with the 51-Chromium T-½ of preserved platelets.

It has been assumed that the change in optical density is due to hypotonic swell-
ing of platelets. This optical change however, may be due to shape change, volume change, change in platelet opacity, and/or change in platelet number owing to osmotic lysis. In 1972, Lundberg et al showed that, under equilibrium situations, the packed platelet volume permanently increases at approximately one half iso-osmolarity. With less reduction in osmolarity, platelet volume is maintained at equilibrium by the extrusion of intracellular solute, namely potassium. To date, however, change in platelet volume during the disequilibrium conditions of acute hypotonic stress has not been directly measured.

This study, therefore, was designed to measure directly the size distribution of the platelet population during the reversal reaction, and to compare the results with changes in absorbance by the same platelet sample. In addition, observations were made on the effect of several chemical agents on the reversal reaction, as measured by both methods.

Materials and Methods

Platelet concentrates were prepared at the Connecticut Red Cross Blood Center.* These were prepared from standard whole blood units collected from volunteer donors into blood packs containing citrate phosphate dextrose anticoagulant. Quality control samples were used for this study; they were stored in PL-146 plastic transfer packs on a Meddev platelet rotator at 22°C. Sampling from the bags was done with sterile precautions.

Before spectrophotometry, a portion of each sample of platelet concentrate was diluted with type AB platelet-poor plasma to achieve a platelet count of about 4 × 10⁶ per μl (measured range 3.5 to 4.2 × 10⁶). Two ml of this mixture were added to one ml of distilled water in a cuvet and the light absorption at 610 nm was then recorded for the next 12 min in a spectrophotometer† attached to a recorder.† In each case, an isotonic saline control was also run, substituting 1 ml Isoton® (azide-free) for the water.

Within one hour of the spectrophotometry, another portion of each sample was subjected to hypotonic stress, and size distributions were determined. Exactly 0.2 ml of undiluted platelet concentrate was added to 0.1 ml of particle free distilled water in a plastic cup, and the mixture was gently agitated. The mixture was sampled at selected intervals with a 3.33 microliter pipet and diluted in 20 ml of a 2:1 mixture of Isoton® and particle-free water. The aperture tube§ was filled with the identical Isoton®-water mixture. These cell suspensions were each placed in turn in an electronic model‖ attached to a channelyzer‖ and X-Y plotter for the generation of size distributions. A 100-channel distribution, reflecting absolute volume of 0.79 to 8.72 μ³ was generated; maximum cell count in any channel was 1000. The sizing procedure lasted between 10 and 40 seconds in each case and generated reproducible curves. Repeated sizing of several samples showed that the Isoton® halted the reversal reaction so that size spectra were stable for a minimum of eight minutes.

The Channelyzer allowed integration below selected parts of the curve. Total counts under the curve in Channel numbers 40 to 99 (3.96 μ³ to 8.72 μ³) were selected for analysis. Size distributions of commercially prepared platelet controls** were generated daily. These failed to show any instrument variability during the course of the experiment. Isotonic

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* Farmington, CT.
† Turner Associates #350 spectrophotometer.
‡ Perkin Elmer #56 recorder.
§ Coulter aperture tube (50/60/s).
‖ Coulter Electronics Model ZBI.
¶ Coulter channelyzer.
** Coulter.
controls were prepared for sizing each platelet sample by adding 0.2 ml of platelet concentrate to 0.1 ml of Isoton®. The suspensions were sampled at 30° only, using the same technique as described previously with the exception that the diluting fluid was undiluted Isoton®. Several experiments showed that the isotonic spectrum was stable for seven hours. To compare changes in absorbance with change in platelet size, 10 separate platelet concentrates were examined at times from zero to five days, generating a total of 22 sets of paired data.

To study the effects of chemical additives on recovery from hypotonic stress, platelets stored for less than 24 hours were used. Samples for spectrophotometry were diluted with type AB platelet poor plasma as before; samples for sizing were not diluted. In both cases control runs were performed, then agents were added to the samples and recovery measurements were repeated. Reagents† obtained were of the highest commercial grade available. Except for the addition of these chemicals, the samples were handled exactly as described earlier. Platelet samples were incubated with 5 mM mersalyl acid at 22°C and measurements were taken after 30 min of exposure. Precisely 10 mM of dithiothreitol (DTT) were added to the 30 min mersalyl acid sample and the effect of the addition of DTT was immediately measured. Other samples were exposed to either 1 mM colchicine or 10 mM sodium flouride, and tests were performed immediately after the addition of the chemical and after one hour of incubation. The osmolarity of samples was not altered by the addition of these agents. Five separate samples were studied with additives in each method. Results from the spectrophotometry and sizing were not paired, as separate platelet concentrates were used in each case.

† Sigma Chemical.

**Figure 1.** A sample response of optical density changes in platelet concentrates subjected to osmotic stress. Measurements were taken of the differences between control and stressed samples at 30° and 4°. Recovery was calculated using the following equation:

\[
\frac{30° \Delta \text{OD} - 4° \Delta \text{OD}}{30° \Delta \text{OD}}
\]

**Figure 2.** Regression analysis of platelet function as a measurement of percent recovery from osmotic stress at 4° versus platelet age. Recovery is calculated as a function of the change in optical density after exposure to osmotic stress. The relationship was shown to be significant, \( r = -0.6796; p = 0.0005 \).
This reflects the movement of cells in and out of a size window of 3.96 $\mu^3$ to 8.72 $\mu^3$ (Channel numbers 40 to 99).

Results

A sample response of optical density of platelet concentrate to osmotic stress is shown in figure 1. As shown in figure 2, there was a significant relationship between the percent recovery from osmotic stress at four min and the number of days of platelet storage. The computer-generated regression equation was: Percent recovery at four min equals $-7.49 \times (\text{age in days}) + 56.35$. ($r = -0.6796, p = 0.0005$). From this relationship, the predicted recovery of fresh platelets from osmotic stress at four min would be 56.4 percent, decreasing to 19.2 percent after five days of storage.

A representative platelet size distribution experiment is presented in figure 3. In figure 4 is presented the regression equation: Size recovery at four min equals $-12.87 \times (\text{age in days}) + 80.4$. ($r = -0.7445, p = 0.0005$). The predicted recovery of fresh platelets is 80.4 percent, dropping to 16.2 percent after five days of storage.

Twenty-two pairs of recovery data, using spectrophotometry and sizing
showed a correlation of 0.5054, \( p = 0.01 \). This relationship is shown in figure 5. In addition, paired absolute changes in optical density, and the absolute changes in counts within the size window, were subjected to linear regression analysis. No significant correlation was demonstrated.

Results of studies with chemical additives are shown in tables I and II. In table I are shown the effects of mersalyl acid, dithiothreitol and colchicine on platelets. The spectrophotometry data show that a 30 min exposure to 5 mM of mersalyl acid inhibits the reversal reaction and that the addition of DTT was associated with a small increase in the recovery. Colchicine, however, showed no major effect on the reversal reaction when compared to the control. The sizing data reinforces both observations. After exposing platelets to 10 mM sodium fluoride (table II) for one hour, the reversal reaction as measured by spectrophotometry was reduced to 33 percent of the control. Platelet counts were observed to be reduced by more than 90 percent after this exposure to sodium fluoride. The sizing study confirmed these observations by showing a 67 percent decrease in the number of platelets in the size window under consideration.

**Discussion**

The light absorbance reversal reaction of human platelets subjected to hypotonic stress has been considered due to an immediate swelling of the platelets with a subsequent gradual decrease of volume with the extrusion of water. The present

### TABLE I

Effect of Various Chemical Agents on Platelet Recovery

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>30' 5mM MA*</th>
<th>*</th>
<th>Fresh 10mM DDT†</th>
<th>1 Hour 1mM Colchicine</th>
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</thead>
<tbody>
<tr>
<td><strong>Absorbance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mean percent recovery</td>
<td>44.9</td>
<td>0.28</td>
<td>7.36</td>
<td>37.0</td>
<td>37.2</td>
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<tr>
<td>Range</td>
<td>(40.9-48.0)</td>
<td>(-9.1-10.9)</td>
<td>(-7.9-18.2)</td>
<td>(29.0-42.8)</td>
<td>(27.5-44.7)</td>
</tr>
<tr>
<td>Mean, expressed as</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>percent of control mean</td>
<td>0</td>
<td>16</td>
<td>82</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td><strong>Sizing</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean percent recovery</td>
<td>65</td>
<td>-8.4</td>
<td>16</td>
<td>54.1</td>
<td>70.1</td>
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<tr>
<td>Range</td>
<td>(43.0-89.3)</td>
<td>(-17-3.4)</td>
<td>(-2.0-35.4)</td>
<td>(-5.6-139)</td>
<td>(61.9-75.7)</td>
</tr>
<tr>
<td>Mean, expressed as</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>percent of control mean</td>
<td>-12.9</td>
<td>25</td>
<td>83</td>
<td>108</td>
<td></td>
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</tbody>
</table>

*Mersalyl acid  †Dithiothreitol

### TABLE II

Effect of 10mM Sodium Fluoride on Platelet Recovery

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Fresh 1mM Sodium Fluoride</th>
<th>1 Hour 1mM Sodium Fluoride</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Absorbance</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mean percent recovery</td>
<td>57</td>
<td>47</td>
<td>19</td>
</tr>
<tr>
<td>Range</td>
<td>(50.0-61.0)</td>
<td>(42.0-52.0)</td>
<td>(17.0-39.0)</td>
</tr>
<tr>
<td>Mean, expressed as</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>percent of control mean</td>
<td>82.0</td>
<td></td>
<td>33.0</td>
</tr>
<tr>
<td><strong>Sizing</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mean percent recovery</td>
<td>81.63</td>
<td>34.32</td>
<td></td>
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<tr>
<td>Range</td>
<td>(63.6-95.0)</td>
<td>(19.4-45.1)</td>
<td></td>
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<tr>
<td>Mean, expressed as</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>percent of control mean</td>
<td>42.04</td>
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authors have been able to observe directly size distribution of platelets during the stress period and demonstrate a similar reversal reaction. Further, there is significant correlation between the percent recoveries calculated by the two methods. It was not possible, however, to demonstrate a correlation between the absolute physical changes measured in the two procedures. It is known that the optical density of platelet solutions decreases when the platelets sphere after the addition of adenosine diphosphate (ADP).\(^1\) Light absorbance of a platelet suspension may well be a complex function of cell density, cell shape, light scattering from the cell surface, as well as cell volume.

Our results with chemical agents confirmed earlier work.\(^4\) Measurements were similar whether made by light absorbance or volume changes. Colchicine had essentially no effect on the reaction, even after one hour, indicating that microtubules are not required. Mersalyl acid completely abolished the reaction after 30 minutes of exposure, but dithiothreitol restored some recovery. This confirmed the requirement of active sulfhydryl groups for the reaction to take place.\(^2,4\)

Kim and Baldini have reported that sodium fluoride is an inhibitor of the platelet reversal reaction.\(^4\) Marcus and Zucker have also documented the inhibitory effect of sodium fluoride on specific platelet functions.\(^6\) From the results of our study, it appears that a 60 min incubation with 10 mM sodium fluoride causes platelet lysis. The 90 percent reduction in platelet concentration and the 67 percent decrease in platelets in the size window under consideration support the theory that sodium fluoride at this concentration acts as a lytic agent. From the data gathered in this study, it cannot be determined if sodium fluoride does act as an inhibitor of the platelet reversal reaction or if the reported inhibitory effect is due to the lytic action of sodium fluoride on platelets.

The authors have been able to demonstrate the usefulness of platelet sizing as another method to investigate the process of reversal from osmotic stress. In addition, the ability to measure platelet size distribution during a kinetic process was apparent, and this method may be applied in other investigations involving variations in the sizes of cell populations.

Acknowledgment

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References