Platelet Storage at 22°C: Effect of Type of Agitation on Morphology, Viability, and Function In Vitro

By Stein Holme, Kalpana Vaidja, and Scott Murphy

Recovery in vivo after 51Cr labeling, platelet morphology, and platelet aggregation were studied with platelet concentrates (PC) stored for transfusion under carefully controlled conditions. PC were prepared to a final volume of 50 ml from whole blood anticoagulated with citrate-phosphate-dextrose (CPD). The platelet count was kept between 0.8 and 1.6 x 10^12 platelets/liter. The PC were stored in bags constructed of polyvinylchloride (PVC) or polyethylene (PE) at 22°C for 72 hr. The bags were placed on a horizontal shaker or a ferris wheel for agitation during storage. No significant changes in pH or platelet count were observed during storage. PC stored on the wheel showed moderate loss of viability and a marked deterioration of platelet morphology and aggregation compared to the shaker. PC stored on the shaker in bags made of PE showed better aggregation with ADP and thrombin but had the same viability and morphology as PC in bags constructed of PVC. Maintenance of normal platelet morphology as determined by phase-contrast microscopy, extent of shape change response, and the size distribution according to the Coulter Counter correlated with recovery in vivo.

There is at present considerable controversy concerning the optimal technique for storing platelet concentrates (PC) for transfusion. Some of the disappointing results obtained with storage at 22°C (room temperature) may result from inadequate attention to technical detail. For example, some form of gentle agitation is required. In addition, we have emphasized the importance of preventing extreme changes in pH if storage is to be successful. These changes result from unfavorable conditions for exchange of CO2 and O2 between the PC and the surrounding air and may be prevented by controlling the volume and platelet count of the PC and by agitating the PC within containers constructed of materials with increased permeability for gas exchange. In this study we will show that with maintenance of constant pH the type of agitation is itself an important variable in the maintenance of platelet viability and function in vitro. Previous studies have indicated that maintenance of normal discoid morphology during storage may be essential for maintenance of viability. If pH falls below 6.0 or rises above 7.6 a disc-to-sphere transformation of the platelets takes place, resulting in marked loss of recovery in vivo upon transfusion. PC prepared with EDTA as anticoagulant or stored at 4°C show only spherical platelets and also have reduced survival in vivo. In this study we examined the
relationship between maintenance of normal discoid morphology and viability after infusion under conditions of storage where pH did not vary significantly, temperature was 22°C, and citrate-phosphate-dextrose (CPD) was used as anticoagulant.

To do this we developed methods that quantitatively reflected the morphologic integrity of the platelets. We found that the extent of shape change response as measured by light transmission studies and the dispersion of the platelet size distribution according to a Coulter Counter correlated with maintenance of normal discoid shape as judged by phase-contrast microscopy and with viability.

Marked decrease in function in vitro has been reported after storage. In this study we will show that the loss of responses in vitro to aggregating agents during storage at 22°C varies with the conditions of storage.

MATERIALS AND METHODS

PC were prepared to a final volume of 50 ml as previously described except that CPD was used as primary anticoagulant. PC were stored in two types of containers: (1) polyethylene (PE) (Hemoflex bags from Union Carbide, Chicago, Ill.), measuring 9 x 22 cm, 0.003 inches thick, and (2) polyvinylchloride (PVC) (plastic PL-146 from Fenwal Laboratories, Morton Grove, Ill.), measuring 12 x 15 cm, 0.015 inches thick. PC kept in PE bags were stored in an atmosphere of 10% CO2 in air, while storage in PVC containers was carried out in air only.

The temperature of storage, 22°C, and the CO2 atmosphere were controlled as described previously. During storage two types of agitators were used: (1) a motor-driven ferris wheel (wheel) as used in previous studies (rate of agitation, 5 cycles/min), and (2) a platform horizontal shaker (Eberbach, Ann Arbor, Mich.) (shaker); the containers were placed on a rack to allow more gas exchange with the surrounding air and to prevent absorption of heat generated from the motor of the shaker, and the speed of agitation was 90 cycles/min (this system is identical to the one described by Slichter and Harker).

PC platelet count was determined by phase-contrast microscopy and by Coulter Counter (Model F). The settings of the Coulter Counter were: attenuation, 1/4; aperture current switch, D = 64. A standard 50-μm aperture tube was used. At the completion of storage, platelet morphology was observed by oil-phase microscopy and the percentage of disc forms estimated prior to Coulter Counter, aggregometer, and viability studies. One hundred consecutive platelets in random fields were classified as discoid or spherical. A platelet was classified as a disc when it rolled over readily, presenting a generally circular or ellipsoid appearance observed face-on and a rodlike appearance when viewed edge-on. Otherwise, the platelets were classified as spherical. Platelet size distributions with the Coulter Counter were obtained by counting the percentage of platelets with size greater than seven arbitrarily chosen windows. The number of particles with sizes above window 6 was considered to be 100%, because it represented the midpoint of the plateau for normal fresh PRP. The percentages of particles with size greater than window numbers 10, 13, 20, 30, 40, 50, and 60 were plotted on log-normal probability paper (Fig. 1). A straight line was obtained in all cases, indicating that the distributions were log-normal. Median platelet size was determined as the window number at 50th cumulative frequency, and dispersion (geometric standard deviation) was defined as window number at 50th cumulative frequency divided by window number at 84.13th cumulative frequency. The dispersion reflects the slope of the line. Viability was measured as percentage 51Cr recovery in vivo and r/2 survival. PC stored for 3 days were labeled with 51Cr and reinfused into the original normal volunteer as previously described.

Previous studies have shown that disc-to-sphere transformation of platelets may be reversed when the factor inducing shape change is removed. In order to allow for maximal recovery of discoid shape and aggregation response after storage, some precautions were taken. The PC was diluted with autologous platelet-poor plasma (PPP) (stored undisturbed at room temperature from the day of phlebotomy) to a platelet count of 0.3 x 10^12/liter. This sus-
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Fig. 1. Size analysis, Coulter Counter (see Materials and Methods). Typical results for fresh PC and for PC stored 3 days on wheel and shaker. Calculated dispersions in parentheses. Standard latex particle with volume of 4.3 μm³ peaks at window number marked by arrow.

Pension (PCS) was well aerated and kept at 37°C for 1 hr prior to the light-transmission studies. The pH of the PCS ranged from 7.2 to 7.4 and was comparable in all studies because little PC pH change was observed during storage and all PC were diluted in autologous PPP. For aggregation studies with thrombin (Parke, Davis, Detroit, Mich.) platelets were separated from plasma by gel filtration of PC on a Sepharose 2B column as described by Lages et al.21 Platelets were eluted with calcium-free Tyrode buffer containing 0.1% dextrose and 0.35% bovine albumin (Sigma Chemical, St. Louis, Mo.). The pH of the buffer was 7.6. The gel filtered platelets were collected and diluted with the buffer to obtain a platelet suspension with a count of 0.3 x 10^12/liter.

Shape change response and aggregation were measured by light-transmission studies using a Payton Dual Channel Aggregation Module (Payton Associates, Scarborough, Canada) as described previously.20 They were quantitated as follows (Fig. 2):

Platelet shape change response (SC) (Fig. 2A). EDTA 0.1 ml (final concentration 5 mM) was added to a continuously stirred suspension of 0.9 ml PCS, producing an increase in light transmission (diluting effect), a. Then, 15 sec later, ADP (final concentration 10 μM) was added, giving a maximal decrease in light transmission, b, in the time Δt. Extent of shape change is given by b/a. This ratio, which reflects a disc-to-sphere transformation of the platelets, was independent of the settings of the aggregometer and the inherent light transmission of the PCS. Complete SC response was assured by observing the light-transmission tracing until maximal extent of response was reached. A tracing without oscillations reflected the presence of spherical platelets only.22

Platelet aggregation (Fig. 2B). The signals from PCS and PPP were adjusted to 2 and 20 mV, respectively. The increase in light transmission D during the 75 sec after the addition of the aggregating agent was measured. In preliminary studies we had observed that PCS from various donors differed in light transmission even when all of them were standardized to a platelet count of 0.3 x 10^12/liter. This was due not only to variations in light transmission of the respective PPP but also to differences in light scattering and adsorbance properties among various platelet populations themselves. Thus when the inherent light transmissions of various PCS differ it is questionable whether or not a given increase in light transmission reflects the same “degree of aggregation.” As a primary standard for evaluation of aggregation response of various PCS we instead chose to compare the light transmission of the aggregated samples with those of serial dilutions of the nonaggregated PCS in autologous PPP. Dilutions of the control suspensions (PCS count, percent) were plotted against the respective light-transmission values (in mV) (Fig. 2C). D was then converted into D', giving the percentage platelet dilution equivalent to the increase in light transmission produced by the aggregation.
Fig. 2. Determination of extent of platelet shape change response and rate of aggregation (see Materials and Methods). (A) Platelet shape change response. Extent of shape change given by $b/a$, where $a$ is diluting effect of 0.1-mI EDTA addition. (B) Platelet aggregation. Increase in light transmission during 75 sec, $D$, was measured. (C) Light transmission (in mV) measured for serial dilutions of PCS in autologous PPP. $D$ is then converted to $D'$, giving percentage of platelet dilution equivalent to increase in light transmission produced by aggregation. (D) Rate of aggregation, $D'$, measured at four to six different ADP concentrations versus log concentration of ADP, giving a logarithmic dose-response curve. $ED_{50}$ estimated as concentration of ADP ($\mu M$) producing 50% of maximal response.

This additional effort seemed justified because the variability of the aggregation results from donor to donor was greatly reduced by this standardization. With freshly made PC, $D$ had a mean of 44 ± 13 (SD) (arbitrary chart units), while the mean of $D'$ was 61 ±, with a smaller SD of 7, when maximal response was measured.

To get additional information about the character of the deteriorating aggregation response during storage, rate of aggregation was determined with four to six different ADP concentrations. Maximal response was obtained when a fivefold increase in ADP concentration failed to give an increase in response. Rate of aggregation $D'$ was plotted against logarithmic concentration of ADP, giving a logarithmic dose-response curve (Fig. 2D). $ED_{50}$ was estimated as the concentration of ADP (in $\mu M$) producing 50% of a maximal response.

Osmotic reversal response was measured as described by Valeri et al.23

Informed consent was obtained from all normal donors and participants after approval of the local Human Investigation Committees in accord with an assurance filed with and approved by the Department of Health, Education, and Welfare.
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RESULTS

The freshly made PC had a platelet count of 0.8–1.6 × 10^{12} liter and a pH of 7.0–7.2. We did not observe any significant change in platelet count during storage. The pH of the PC after 3 days of storage ranged from 6.8 to 7.4. There were no significant differences in the final pH for PC stored under the four conditions studied.

Table 1 shows percentage recovery in vivo and survival t\(\frac{1}{2}\) of fresh PC and PC stored for 72 hr under varying conditions. A statistically significant decrease in percentage recovery in vivo was observed after storage. PC stored on the shaker yielded recovery results superior to the wheel when the results for PE and PVC on the same agitator were combined. When PE was compared with PVC either on the shaker or on the wheel, no significant differences were seen. For survival t\(\frac{1}{2}\) no significant differences were observed using an unpaired t test. For storage in PE on the shaker versus the wheel, six pairs of studies were carried out in the same donor so that comparison of the two methods could be made within the same individual volunteer (Fig. 3). This type of analysis showed the superiority of the shaker most clearly. Statistically significant differences were obtained with the paired t test: percentage recovery in vivo (p < 0.01), t\(\frac{1}{2}\) survival (p < 0.05), maximal rate of aggregation (p < 0.005), ED_{50} (p < 0.01). For individual donors, there was a high correlation between the result on the shaker and the result on the wheel: r = 0.86 and 0.94 for recovery in vivo and survival t\(\frac{1}{2}\), respectively. This suggests that the platelets from some individuals are better suited for storage than those of others.

The percentage of platelets that underwent disc-to-sphere transformation during storage was estimated by oil-phase microscopy and by the extent of shape change response as observed with the aggregometer. In a preliminary experiment, it was found that the extent of shape change could be used to reflect

<table>
<thead>
<tr>
<th>Table 1. Recovery In Vivo, t(\frac{1}{2}) Survival, and Maximal Rate of Aggregation and ED_{50} With ADP for Fresh and Stored PC</th>
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<tr>
<td>Recovery (%)</td>
</tr>
<tr>
<td>Fresh</td>
</tr>
<tr>
<td>3-day storage</td>
</tr>
<tr>
<td>Shaker</td>
</tr>
<tr>
<td>PE</td>
</tr>
<tr>
<td>PVC</td>
</tr>
<tr>
<td>PE + PVC</td>
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<tr>
<td>Wheel</td>
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<tr>
<td>PE</td>
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<tr>
<td>PVC</td>
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<td>PE + PVC</td>
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Mean ± 1 SEM, number of studies in parentheses. Statistically significant differences (Student’s t test): recovery in vivo: Fresh PC vs 3-day-stored PE + PVC, shaker (p < 0.05), PE + PVC, shaker vs PE + PVC, wheel (p < 0.025). Maximal rate of aggregation, D': Fresh PC vs 3-day-stored PE + PVC, shaker (p < 0.005), PE + PVC, shaker vs PE + PVC, wheel (p < 0.005). PE, shaker vs PVC, shaker (p < 0.05); ED_{50}: Fresh PC vs 3-day-stored PE + PVC, shaker (p < 0.005), PE, shaker vs PVC, shaker (p < 0.05). No results for ED_{50} could be obtained with PC on the wheel, since many of the PC showed no aggregation.
the percentage of discoid platelets in a given sample of platelet-rich plasma (Fig. 4). This was confirmed in practice when we found a high correlation between percentage of discs in stored PC as estimated by phase microscopy and the extent of shape change response (Fig. 5B). Figure 1 shows typical changes in the size distribution of platelets in PC stored for 72 hr when plotted on log-probability paper. PC stored for 3 days showed an increase in dispersion. Again, there was a good correlation between this measurement and the microscopic estimate of percentage of discs (Fig. 5C), although the shape change correlation was better. The osmotic reversal reaction showed the least correlation (Fig. 5A). The results for all four measurements were significantly superior for the shaker as opposed to the wheel (percentage of discs, \( p < 0.05 \);
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Fig. 5. Platelet morphologic integrity as determined by observations by phase microscopy and quantitatively reflected by osmotic reversal reaction, extent of shape change, and dispersion with Coulter Counter. All three of the latter measurements correlated significantly with percentage of discs by phase microscopy as reflected by correlation coefficients $r$.

osmotic reversal, $p < 0.05$; extent of shape change, $p < 0.01$; dispersion, $p < 0.01$).

There was a good correlation between the three tests in vitro, Coulter Counter dispersion, extent of shape change and osmotic reversal reaction, and the viability in vivo results (Fig. 6). The highest correlation ($r = 0.83$) was found between the Coulter Counter dispersion and the percentage recovery in vivo. Median platelet size as reflected by the median window number decreased during storage: $17\% \pm 2\%$ (±1 SEM) for PC on the wheel, $11\% \pm 2\%$ for PC on the shaker. The differences were statistically significant ($p < 0.01$).

Fig. 6. Correlation between three tests in vitro: osmotic reversal, extent of shape change and dispersion with Coulter Counter, and percentage recovery in vivo. All tests correlated with viability, with dispersion having highest correlation coefficient $r$. 
Table 2. Rate of Aggregation With Thrombin After Gel Filtration of Fresh and Stored PC

<table>
<thead>
<tr>
<th>Thrombin (U/ml)</th>
<th>No Storage, PE (6)</th>
<th>3-day Storage</th>
<th>Shaker</th>
<th>Wheel</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>70 ± 8</td>
<td>35 ± 12</td>
<td>19 ± 11</td>
<td>4 ± 4</td>
</tr>
<tr>
<td>0.5</td>
<td>61 ± 9</td>
<td>59 ± 3</td>
<td>52 ± 6</td>
<td>17 ± 8</td>
</tr>
<tr>
<td>1.0</td>
<td>62 ± 9</td>
<td>59 ± 4</td>
<td>55 ± 5</td>
<td>18 ± 8</td>
</tr>
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</table>

Mean ± 1 SEM; number of studies in parentheses. In either container the aggregation results obtained with PC on the shaker were superior to the wheel, p < 0.01. No statistically significant difference was found between storage in PE or PVC with either agitator.

0.05). Significant correlation was also found between the percentage decrease in median size and percentage recovery in vivo (r = 0.57). None of the tests in vitro correlated with θ/2 survival. No comparative studies in vitro for PC stored in PVC on the wheel were done. The recovery data in vivo reported for these storage conditions were obtained prior to the institution of the studies in vitro.

Marked influence of the various conditions of storage on platelet aggregation was observed (Table 1, Fig. 3). PC stored in PE on the shaker showed the best maintenance of aggregation response to ADP. Maximal rate of aggregation was reduced 48%, and, as shown by the ED₅₀ results, a 2.3-fold greater concentration of ADP (0.40 increase in log dose) was needed to obtain 50% of maximal response when compared with fresh platelets. PC stored in PVC on the shaker had significantly poorer aggregation when compared with PE, while storage on the wheel had a marked deleterious effect on the aggregation response in either container. Even under optimal and identical conditions of storage, there was a marked variability of the aggregation results. For example, PC stored in PE on the shaker showed a mean of 32% ± 16% (SD) (22 studies) for maximal rate of aggregation, compared to 61% ± 7% (15 studies) for freshly made PC. At present, we have no explanation for such variation.

Aggregation studies with thrombin showed results similar to those for ADP (Table 2). Aggregation responses were superior after storage on the shaker as opposed to the wheel. During storage of platelets in PE bags on the shaker the sensitivity to thrombin was decreased when compared with fresh platelets, but there was no significant change in maximal rate of aggregation.

DISCUSSION

In this study we were able to show that not all methods of agitation are equivalent and that agitation technique may be an important variable in the storage of platelets for transfusion at 22°C. Specifically, we showed that PC stored on a ferris wheel had moderate loss of viability and a marked loss of function in vitro compared to a horizontal shaker. We related the deterioration of platelet quality during storage to the morphologic changes we observed microscopically and attempted to quantitate these changes objectively.

These morphologic changes may have been induced in two different ways. First, it has been well documented that platelets are activated by foreign surfaces. When PC are agitated during storage, the platelets are likely to
collide with the walls of the plastic container. This interaction may lead to a disc-to-sphere transformation of the platelets with extension of pseudopods, and platelet constituents may be released. Various investigators have shown that the fluid dynamics within a system exert a strong influence on observed platelet-surface interactions. In our study the flow patterns of the PC are clearly different in the two types of agitation. On the shaker the flow of the PC is to and fro in a linear fashion. Takano et al. showed that for oscillatory flow in a tube there are forces present that drive particles away from the tube walls. Similar mechanisms may be present in PC with agitation on the shaker, keeping the platelets from interacting with the plastic wall. With PC on the wheel, however, the direction of flow changes continuously with respect to the walls of the container. This may lead to more platelet-plastic wall collisions, resulting in greater loss of shape and function. Another possible explanation is that the platelets are subjected to greater shear stress during storage on the wheel. It has been shown that platelet shape change, lysis, release, and development of refractoriness to aggregating agents may take place under high shear forces.

As a practical consideration, there may be devices better for agitating platelets than the horizontal shaker as we used it. Our study does show that other forms of agitation may be worse. Since the method we used is identical to that used by Slichter and Harker in obtaining excellent results with storage at 22°C, it would seem unwise to use other methods until they have been proven to be of equivalent efficacy. Considerable variability was found from donor to donor in recovery in vivo even under the same conditions of storage (Fig. 3). However, there was a very good correlation between results obtained on the shaker and on the wheel in each individual with the PE containers. Therefore differences between donors play a significant role in studies of this type. Paired studies may be necessary to detect small differences between storage techniques.

It has been reported that the uptake of serotonin and the osmotic reversal response are useful tests in vitro to predict viability in vivo. It has been our subjective impression, and Kunicki et al. have shown, that maintenance of normal discoid morphology, as judged by phase microscopy, correlated with viability in vivo. Our studies add further confirmation for this concept. We showed that the degree of maintenance of discoid shape could be quantitated by the shape change response to ADP and that shape change response correlated with viability. Recently, we have developed a more sensitive and standardized test for measuring the disc/sphere ratio in a platelet suspension. This method is based on the difference in optical properties of suspensions of discoid and spherical platelets when stirred. It does not depend on the capacity to respond to ADP, as does the shape change response.

The dispersion of platelet size distribution appeared to be the best measure for predicting viability (Fig. 6). The observed increase in dispersion after storage suggests an increased proportion of small and large platelets, actually voltage pulses, registered by the Coulter Counter as the platelets pass through the orifice. The height of the pulse is not only a function of the size of the platelet but also of its shape and specific resistance. Disc-to-sphere transformation of an entire population of platelets causes an increase in the height of all pulses with no change in the dispersion. A possible explanation for our results is that
during injurious storage a certain proportion of the platelets become smaller from release of constituents, resulting in an increased number of small pulses, while other platelets become spherical only, resulting in an increase in large pulses recorded by the Coulter Counter.

No test in vitro adequately documents the viability of stored platelets. However, in serial studies of the multiple variables that might affect the outcome of storage it is costly and time-consuming to perform studies in vivo each step of the way. We propose this battery of studies in vitro for developmental work. Major conclusions can then be verified by study in vivo.

It is clear that the degree to which platelet aggregation in vitro deteriorates varies markedly with the conditions of storage at 22°C. Aggregation after storage on the wheel was very poor. PC stored on the shaker in bags constructed of PE showed superior aggregation response to PC stored in PVC bags. The relevance of function studies in vitro to hemostatic function in vivo has been appropriately questioned. In a recent study, Slichter and Harker showed that the bleeding time of thrombocytopenic recipients was corrected equally by fresh PC and PC stored for 3 days at 22°C. If this is correct, studies in vitro may seem irrelevant. However, the Slichter and Harker studies were not confirmed by Aster et al. Furthermore, the bleeding time may not reflect all platelet functions in vivo. Finally, there may be a time interval after infusion during which the defects in vitro are corrected. In bleeding patients, hemostasis during this interval may be critical. Until the nature of the refractory state that develops after storage and the mechanisms of recovery in vivo are better understood, it seems wisest to maintain the function of stored platelets, as measured in vitro, as close to fresh platelets as possible.

These studies were carried out with blood anticoagulated with CPD, whereas previous experience had been with acid-citrate-dextrose (ACD). The major problem with storage in PVC, rapidly falling pH in PC with high platelet count, is just as significant a problem with PC from CPD blood as it is for ACD blood. The use of plastics such as PE that are permeable to gases eliminates pH fall during storage in PC from both CPD and ACD blood. However, we do not yet know whether it will be possible to incorporate such plastic into plateletpheresis equipment or whether the requirement for an external 10% CO₂ atmosphere will continue when these plastics are used with less traumatic forms of agitation such as the shaker. In any event, it is clear that for a given PC four variables—the platelet count in PC, the type of agitator, the type of container, and the external gas atmosphere—all interact to determine the success or failure of storage.

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