Stored Human Platelets Retain Full Aggregation Potential in Response to Pairs of Aggregating Agents

By Giovanni DiMinno, Melvin J. Silver, and Scott Murphy

Human platelet concentrates stored at 22°C for transfusion purposes progressively lose their in vitro sensitivity to single aggregating agents such as adenosine diphosphate (ADP). It is known that very small amounts of epinephrine, collagen, or arachidonic acid can markedly enhance the aggregation of platelets in fresh platelet-rich plasmas in response to concentrations of ADP that do not cause aggregation when used singly. Therefore, we investigated the effects of pairs of aggregating agents on eight platelet concentrates on the day of preparation and after 3 and 5 days of storage at 22°C. We studied platelet aggregation and secretion with a lumi-aggregometer and thromboxane B_2 formation using a radioimmunoassay. When aggregation was tested in response to single agents, stored platelets were completely unresponsive to epinephrine, markedly insensitive to ADP, and moderately insensitive to collagen and arachidonic acid. In spite of this, there was no statistically significant loss of sensitivity to aggregating agents after 5 days of storage when the agents were used in pairs. There was no loss of sensitivity to ADP (2 μM) or epinephrine (2 μM) when they were used as a pair or when either was used to potentiate aggregation induced by low concentrations of collagen or arachidonic acid. In addition, the patterns of aggregation and secretion in response to pairs of agents were similar in fresh and 5-day stored platelet concentrates. Compared to fresh platelet concentrates, platelets stored for 5 days formed less thromboxane-B_2 at all concentrations of collagen or arachidonic acid tested. However, the amount of thromboxane-B_2 formed in response to low concentrations of pairs of agents was unchanged after 5 days of storage. Retention of in vitro sensitivity of stored platelets to pairs of aggregating agents may help explain the apparent discrepancy between poor aggregation in vitro and adequate function in vivo of stored platelets, since in vivo aggregation may well occur in response to combinations of agents.

P LATELETS STORED at 22°C for 3 days, although functionally useful in vivo, progressively lose their ability to respond in vitro to aggregating agents such as ADP, collagen, epinephrine, and thrombin. This apparent discrepancy has been a puzzle. It is known that the addition of very small amounts of arachidonic acid (AA) to platelet-rich plasma (PRP) can markedly enhance the aggregation produced by low concentrations of ADP or other aggregating agents. Recently more detailed studies have been reported on the synergistic action of aggregating agents in fresh PRP and in PRP stored for 3 days. We report that stored platelet concentrates (PC) aggregate, secrete nucleotides, and form thromboxane-B_2 in response to low concentrations of aggregating agents used in pairs, like fresh PC. We have made observations both at 3 and 5 days of storage, since a recent report indicates that storage can be extended to 5 days.

MATERIALS AND METHODS

Four normal volunteers, who denied taking any drug for at least 10 days, donated blood for PC preparation on two occasions, once for collection in citrate-phosphate-dextrose (CPD) and once for collection in citrate-phosphate-dextrose-adenine (CPD-A). Fresh PRP and PC were prepared as previously reported. PC were stored at 22°C in commercially available transfer packs constructed of plastic (Fenwal Corp., Morton Grove, Ill.) on a horizontal agitator for 5 days. Platelet-poor plasma (PPP) was obtained by centrifugation of fresh PRP at 2000 g for 15 min, stored in small amounts at −20°C, and thawed at 37°C immediately before each series of experiments. Platelet-free plasma (PFP) was prepared by centrifugation of PPP in an Eppendorf 3200 centrifuge (Brinkmann Instruments, Westbury, N.J.) at 12,000 g for 5 min. Platelet counts were obtained using a Coulter Counter as previously described.

The platelet count of fresh PC was adjusted, with PPP, to 1000-1200 x 10^9/liter to prevent a fall in pH during storage. pH was measured at 22°C as previously described. After 5 days of storage, the lowest pH observed was 6.95. PC suspensions were prepared immediately before each series of studies by diluting PC samples in autologous PPP to count in the range of 300-350 x 10^9/liter. Platelet counts in fresh PRP were also within this range.

Aggregating agents employed were ADP (adenosine-5-diphosphate, sodium salt) Sigma Co., St. Louis, Mo.; epinephrine (epi) (epinephrine hydrochloride, isotonic solution) Elkins-Sinn Inc., Cherry Hill, N.J.; collagen (Col) (Kollagen Reagents Horm), Hormon Chemie, München; arachidonic acid (AA) (>99% pure), Nuchek Prep, Elysian, Minn. Solutions of sodium arachidonate were prepared as described elsewhere. Dilutions of agents were in Tris buffer, 0.15 mM, pH 7.4.

Platelet aggregation was determined in a Lumi-aggregometer (Chronolog Co., Havertown, Pa.) that also records the luminescence resulting from the interaction of released ATP (secreted simultaneously with ADP) with firefly luciferase and luciferin (Chronolume 395, Chronolog Corp., Havertown, Pa.). The sensitivity was such that a concentration of released ATP as little as 0.1 μM could be detected. The apparatus was adjusted so that PFP and PRP produced 10% and 90% light transmittance, respectively. An aggregating agent or an equal volume of vehicle was added in microliter
Table 1. EC50 for ADP, Epinephrine, Collagen, and AA for Aggregation of PRP or PC Suspensions at Different Times of Storage (Mean ± SEM of 8 Different PC Suspensions)

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>PRP</th>
<th>Fresh PC</th>
<th>3</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP (μM)</td>
<td>2.3 ± 1.2</td>
<td>6.2 ± 0.9*</td>
<td>40.1 ± 26.0†</td>
<td>80.0 ± 20.1‡</td>
</tr>
<tr>
<td>Epi. (μM)</td>
<td>6.4 ± 2.3</td>
<td>&gt;200*</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>Col. (μg/ml)</td>
<td>0.5 ± 0.1</td>
<td>1.1 ± 0.1*</td>
<td>2.5 ± 0.5†</td>
<td>5.0 ± 0.2$</td>
</tr>
<tr>
<td>AA (mM)</td>
<td>0.31 ± 0.01</td>
<td>0.43 ± 0.02*</td>
<td>0.59 ± 0.01†</td>
<td>0.62 ± 0.04†</td>
</tr>
</tbody>
</table>

*p < 0.05, significant difference between PRP and fresh PC.
†p < 0.05, significant difference between stored and fresh PC.
‡p < 0.01, significant difference between stored and fresh PC.
§p < 0.005, significant difference between stored and fresh PC.

amounts to 0.5 ml of platelet suspension that had been stirred at 1000 rpm and 37°C for 1 min.

Thromboxane-B2 (TXB2) levels were measured by radioimmunoassay (RIA) in aliquots of the supernatant solution after completion of tests for platelet aggregation and secretion. The sensitivity was such that as little as 0.5 pmole/ml TXB2 could be detected. Preliminary experiments showed that the reagents used for the measurement of ATP secretion did not interfere with the RIA of TXB2. Highly specific antibodies for TXB2 were kindly provided by Dr. J.B. Smith, Cardeza Foundation, Philadelphia, Pa.

EC50 was defined as the lowest concentration of an agent added to a platelet suspension that caused more than 50% light transmittance within 3 min.

Statistical analysis was performed using the Student's t test for paired comparison.

Informed consent was obtained from the donors after approval of the local Human Investigation Committee in accord with an assurance filed with and approved by the H.S.S.

RESULTS

Preliminary Data

CPD and CPD-A PRP showed aggregation and ATP secretion patterns similar to those already reported for citrated PRP. Collection of blood from the same donor into CPD or CPD-A showed that fresh and stored PC suspensions gave both qualitatively and quantitatively similar results for both anticoagulants. Therefore, the CPD and CPD-A data were pooled in later studies. Suspensions prewarmed in plastic tubes at 37°C for 60 min before starting the experiments were found to be more responsive to aggregating agents than those prewarmed for shorter periods. After 60 min prewarming, the EC50 for each agent was unchanged for about 3 hr. All tests were started after 60-min incubation at 37°C and continued up to 2 hr thereafter. Appropriate controls were performed repeatedly during and at the end of each series of experiments.

Decreased Sensitivity to Single Aggregating Agents After Storage

Table 1 shows that the concentrations of ADP, collagen, and AA required to cause 50% light transmittance were significantly greater (p always <0.05) in fresh PC suspension than in autologous PRP.
Decreases in sensitivity to aggregating agents occurred during storage of PC. For example, a 14 times higher concentration of ADP was required on day 5 of storage to obtain aggregation similar to that seen with fresh PC suspensions. Sensitivity to AA and collagen was also reduced but to a lesser extent. In addition, the maximal extent of aggregation in response to high concentrations of ADP (100 \( \mu M \)), collagen (10 \( \mu g/\)ml), and arachidonic acid (1 \( mM \)) was also reduced after 5 days of storage by 20\%, 10\%, and 10\%, respectively (data not shown). Epinephrine did not induce aggregation, even in suspensions from fresh PC, at 30 times the concentrations causing aggregation in autologous PRP.

**Relationship Between Aggregation and Secretion in Fresh and Stored PC in Response to Single Aggregating Agents**

Figure 1 shows typical aggregation and secretion recordings obtained after 5 days of storage. The patterns and the relationships observed between aggregation and secretion were the same as for fresh citrated PRP, although larger concentrations of aggregating agents were required to obtain a given response.

**TXB\(_2\) Formation in Fresh and Stored PC in Response to Single Aggregating Agents**

The mean concentration of TXB\(_2\) was 5.9 ± 0.9 pmole/ml in PFP prepared from fresh PC, 13.2 ± 0.2 pmole/ml in PFP from fresh PC suspensions, and 13.8 ± 0.4 pmole/ml in PFP from suspensions of PC stored for 5 days. Thus, PC preparation but not storage caused TXB\(_2\) formation. There was less TXB\(_2\) formation after storage in response to a given concentration of collagen or AA when compared to fresh PC (Fig. 2). However, the EC\(_{50}\) concentration of each agent stimulated the same amount of TXB\(_2\) production before and after storage. At concentrations of ADP as high as 100 \( \mu M \), the amount of TXB\(_2\) formed by suspensions of fresh PC and PC stored for 5 days was only 21.9 ± 1.2 pmole/ml and 18.2 ± 0.4 pmole/ml, respectively. Likewise, 200 \( \mu M \) epinephrine only produced 23.4 ± 6.4 pmole/ml TXB\(_2\) in fresh PC suspensions and 21.7 ± 5.3 in suspensions from PC stored for 5 days.

**Enhancement of Aggregation in PC Suspensions by Pairs of Agents**

When pairs of agents were used, maximal aggregation and secretion were achieved when the agents were added within 15 sec of each other. At intervals greater than 15 sec, the enhancement diminished progressively and was no longer discernible at 50 sec. Therefore, all further experiments were carried out with a 15-sec delay between addition of the agents. Arachidonic acid and collagen, when used at concentrations that did not cause aggregation when tested alone, caused considerable aggregation when used in combination with 2 \( \mu M \) ADP or 2 \( \mu M \) epinephrine. The concentrations of agents necessary to induce similar aggregation before and after storage of PC were not significantly different (\( p > 0.05 \) in all cases, Table 2).

**Relationship Between Aggregation and Secretion in Response to Pairs of Agents**

The enhancement of aggregation of PC suspensions induced by low concentrations of pairs of aggregating agents was accompanied by secretion (Fig. 3). The patterns of aggregation and secretion for fresh and 5-day-old PC suspensions were similar.
Table 2. Minimal Concentrations of Aggregating Agents, Tested in Pairs, Which Produce 50% Aggregation in Suspensions From Fresh and 5 Day Stored PC (Mean ± SEM of 8 Different PC Suspensions)

<table>
<thead>
<tr>
<th>ADP (μM)</th>
<th>Epi (μM)</th>
<th>Col (μg/ml)</th>
<th>AA (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F 5</td>
<td>F 5</td>
<td>F 5</td>
<td>F 5</td>
</tr>
<tr>
<td>ADP (2 μM)</td>
<td>3.1 ± 1.1</td>
<td>5.3 ± 1.2</td>
<td>0.29 ± 0.04</td>
</tr>
<tr>
<td>Epi (2 μM)</td>
<td>3.6 ± 0.8</td>
<td>6.2 ± 1.8</td>
<td>0.21 ± 0.07</td>
</tr>
</tbody>
</table>

F, Fresh PC; 5, PC stored for 5 days at 22°C.

TXB₂ Formation in Response to Pairs of Aggregating Agents

After 5 days of storage, the enhancement of aggregation induced by ADP (2 μM) and epinephrine (2 μM) in combination was not accompanied by potentiation of the formation of TXB₂ (Fig. 4). Even the combinations of 100 μM ADP and 200 μM epinephrine produced only 40.6 ± 9.8 pmole/ml TXB₂, which was equal to the sum of the amounts formed when the agents were tested alone (see above). In contrast, potentiation of TXB₂ formation was observed when 2 μM ADP or 2 μM epinephrine were tested in combination with collagen or AA. Increasing the concentrations of ADP up to 100 μM or epinephrine up to 200 μM did not further increase the amounts of TXB₂ formed (data not shown). This pattern of TXB₂ response to pairs of aggregating agents was similar to the pattern observed with fresh PC suspensions.

DISCUSSION

There have been several reports demonstrating a decrease in sensitivity of platelets to single aggregating agents after storage of PC at 22°C.³⁻⁵ We confirmed this for ADP and collagen, and also found reduction of sensitivity to AA. The change was minimal in degree for AA, intermediate for collagen, and marked for ADP. There was no aggregation in response to epinephrine in fresh or stored PC, even at a final concentration of 200 μM.

Exogenous AA causes platelet aggregation and secretion by being metabolized, via cyclo-oxygenase, to cyclic endoperoxides and in turn, via thromboxane synthetase, to thromboxane-A₂. We determined that the EC₅₀ for AA increased from 0.43 ± 0.02 mM to 0.62 ± 0.04 mM after 5 days of storage. This relatively small change suggests that cyclo-oxygenase and thromboxane synthetase are affected little by storage. Collagen is known to cause release of AA from platelet phospholipids.²⁰⁻²¹ The decreased response of stored PC to collagen could be due, in part, to impaired AA release. Figure 2 shows that five times as much collagen was required in stored PC than in fresh PC to stimulate the production of about 100 pmole/ml of TXB₂. On the other hand, only a small increment in the amount of exogenously added AA sufficed to produce about 300 pmole/ml of TXB₂ in stored PC as compared to that needed in fresh PC. These results are
consistent with a decrease in the ability of stored platelets to release endogenous AA in response to collagen. Collagen, like thrombin, may produce aggregation by mechanisms other than AA release, so other pathways may be affected by storage as well.

The decreased sensitivity to ADP as a single agent after storage was particularly striking. In spite of this, there was no loss of sensitivity when low concentrations of ADP were used in combination with epinephrine, collagen, or AA in fresh and stored PC suspensions. This responsiveness was particularly impressive in combination with epinephrine, which by itself did not induce aggregation at concentrations as high as 200 μM in fresh or stored PC suspensions. Similarly, epinephrine retained its ability to potentiate aggregation with collagen or AA through 5 days of storage. We found that ADP or epinephrine also potentiated the formation of TXB₂ when combined with collagen or AA in fresh PC suspensions. This effect was also retained for 5 days of storage (Fig. 4).

The concentration of TXB₂ present in PFP prepared from fresh PRP was 5.9 ± 0.9 pmole/ml, whereas when the PFP was prepared from fresh PC suspensions, the TXB₂ concentrations were 2–3 times higher. This indicates that during preparation of PC, small amounts of TXB₂ are formed. No further formation of TXB₂ occurred during the 5 days of storage. This suggests that, although AA metabolism is known to be triggered by many stimuli, none of the events occurring during storage such as decrease in pH, agitation, and contact of platelets with the container wall induces further formation of TXB₂.

After 5 days of storage, the patterns of secretion in relation to aggregation were typical for each agent or pair of agents employed and similar to the response of fresh platelets. However, as with aggregation, higher concentrations of single agents were required to produce a given response. This is similar to the response of TXB₂ production. Higher concentrations of collagen and AA were required to produce 50% aggregation after storage, but at these EC₅₀ concentrations, the amount of TXB₂ produced by stored platelets was similar to fresh platelets (Fig. 2). Thus, the three responses that we have examined in this study—aggregation, secretion, and TXB₂ formation—appear to be intact after 5 days of storage.

Our findings may explain the apparent discrepancy between poor in vitro aggregation and the effectiveness of stored platelets in vivo, since it is likely that platelet aggregation in vivo occurs at sites of injury in response to combinations of aggregating agents.

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