Membranous microparticles (MP) appearing in the supernatant plasma of stored platelet concentrates (PC) were analyzed by flow cytometry. Two populations of MP were arbitrarily delineated by light scatter as larger or smaller than 0.5 μm fluorescent beads. An estimate of MP concentration was obtained by adding a known amount of fluorescent beads to each sample before analysis of a set number of counts on the flow cytometer. The addition of platelet activation inhibitors (prostaglandin E-1, theophylline, and aprotinin) to the anticoagulant during preparation of PC combined with a reduction in surface area of the storage container caused approximately a 40% reduction in the number of MP appearing during storage relative to donor-matched controls. In addition, the inhibited concentrates had 84% less platelet factor 3 (PF3) activity in the supernatant and 61% less released lactic dehydrogenase. A reduction in surface area of the container in the controls partially offset these differences. A significant correlation was found (r = .748) between PF3 levels and the concentration of larger MP. The inhibitors did not reduce the small number of MP found in stored platelet-poor plasma. Surface antigen analysis showed that the majority of MP in PC were platelet-derived; most were positive for glycoprotein (GP) IIb (73%) and/or for GPIb (43% to 48%). We conclude that procoagulant MP are released from platelets during storage as a result of platelet activation augmented by interaction of platelets with the bag wall.

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Vesiculation of Platelets During In Vitro Aging

By Arthur P. Bode, Susan M. Orton, Mary J. Frye, and Barry J. Udis

Vesiculation of Platelets has been observed under a variety of conditions. More than two decades ago, Wolf found a “coagulant material in minute particulate form...originating from platelets” in platelet-poor plasma and serum. Budding of vesicles from the platelet plasma membrane has been observed in electron micrographs of activated platelets, and was proposed to be the origin of platelet membrane material in the supernatant plasma. Sandberg et al. reported the release of small procoagulant vesicles from platelets stimulated with collagen. More recently, Sims et al demonstrated that sublytic treatment of platelets with the membrane attack complex of complement directly caused activation and vesiculation of platelets, resulting in the formation of microparticles (MP) that supported prothrombinase assembly as analyzed by flow cytometry.

George et al. have isolated and partially characterized membranous MP in supernatant preparations of banked plasma, serum, and platelet concentrates. They found a population of MP bearing platelet membrane glycoprotein (GP) IIb that increased over storage time in the supernatant plasma of platelet concentrates (PC) at a rate dependent on the type of agitation used. Solberg et al. observed membranous MP in PC by scanning electron microscopy and Coulter Counter studies, especially in samples from concentrates that showed an increase in pH over the storage period. We and Solberg et al. have shown that platelet membrane fragments in the cell-free supernate express a major fraction of the overall procoagulant platelet factor 3 activity (PF3) in PC. Although the presence of membranous MP in stored PC has been well demonstrated, direct enumeration and population analysis has not been possible until the application of flow cytometry.

In this report, we used the flow cytometer to identify light-scattering material smaller than platelets in supernatant plasma from PC and investigated its surface antigen profile as well as estimated the concentration of MP based on a “competitive counting assay” described herein. MP were discriminated from “background” light scatter by their uptake of the fluorescent hydrophobic dye 3,3'-Dihexyloxycarbocyanine iodide (DiOC6). With these techniques, we evaluated the effect of addition of a combination of platelet activation inhibitors (prostaglandin E-1 [PGE-1], theophylline, and aprotinin) on the generation of MP during storage of PC at 22°C over a 10-day storage period. It was previously demonstrated that these inhibitors and a reduced surface-to-volume ratio (S/V) of the storage container improve the preservation of platelet-cell integrity over extended storage periods. Now we present evidence that these optimized conditions retarded MP generation and inhibited release of PF3 and lactic dehydrogenase (LDH) into the supernatant plasma during the storage period.

Materials and Methods

Reagents. PGE-1 (synthetic hemisulfate), theophylline (anhydrous), aprotinin (10 to 20 trypsin-inhibiting U/mg) and DiOC6 were purchased from Sigma Chemical Co (St Louis, MO). Fluorescent latex beads (0.5-μm diameter) were obtained from Polysciences, Inc (Warrington, PA). Hematall, an azide-free, filtered isotonic diluent, was purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals and buffers were reagent grade or better.

Antibodies. M51G2A, a purified murine control antibody (1.0 μg/mL final concentration in incubations with MP) was purchased from Coulter Immunology (Hialeah, FL). Two murine IgG monoclonal antibodies (MoAbs) against human platelet GP Ib were investigated: AN-51 (1.0 μg/mL) and SZ-2 (1.0 μg/mL) were obtained from Dako Corp (Santa Barbara, CA) and Biodesign Inc (Kennebunkport, ME), respectively. 10E5 (1.0 μg/mL), a murine IgG MoAb against human GP Ib-IIIa complex, was a generous gift from Dr Barry Coller (Stoney Brook, NY). A rabbit anti-human thrombocyte polyclonal antisera (50 μg/mL) was obtained from Dako Corp. To detect leukocyte-derived MP a pan-leukocyte
antibody, HLE (1.0 μg/mL), was purchased from Coulter Immunology. Also, a murine IgG MoAb, D2-10 (1.0 μg/mL), specific for human glycophorin was obtained from AMAC, Inc (Westbrook, ME) to detect erythrocyte-derived MP. Nonimmune goat IgG (2 μg/mL) purchased from Sigma was used as a blocking reagent.

The secondary antibodies used were R-phycocerythrin (PE)-labeled goat antimonouse IgG (heavy chain specific) (10 μg/mL) and PE-labeled goat antirabbit Ig (polyvalent) (50 μg/mL), both purchased from Fisher Biotech (Orangeburg, NY).

Preparation of PC. To construct a paired experimental design to test the effect of inhibitors, two half-units of whole blood (250 mL) were collected in a single sitting from normal donors as previously described.15 The first half-unit was collected into 35 mL of CPDA-1 (Fenwal half-unit collection sets, No. 4R6004, Deerfield, IL) through a 17-gauge catheter. Immediately following attachment of a second collection set to the catheter, the other half-unit was collected into 35 mL of CPDA-1 supplemented with PGE-1 (delivered in 35 μL of absolute ethanol), theophylline, and aprotenin. The final concentrations of inhibitors in the whole blood were: 300 nmol/L, 1.9 mmol/L, and 348 KIU/mL, respectively. PC

On each day to produce a total of six pairs of concentrates. On day 7 of 7 cm2/mL or of CPDA-1 (Fenwal half-unit collection sets, No. 4R6004, Deerfield, IL) through a 17-gauge catheter. Immediately following PGE-1 (delivered in 35 μL of absolute ethanol), theophylline, and aprotenin. The final concentrations of inhibitors in the whole blood were: 300 nmol/L, 1.9 mmol/L, and 348 KIU/mL, respectively. PC were prepared by established centrifugation technique15 and transferred to PL-732 storage bags, which were clamped to give an S/V of 7 cm2/mL or 4 cm2/mL for the control unit (as indicated), and 4 cm2/mL for the unit supplemented with inhibitors for reasons discussed elsewhere.16 All PC were stored at 22°C on a slow (2 Hz), tumbler agitator (Helmer Labs, Fenwal). Platelet-poor plasma (PPP) removed during concentrate preparation was also stored at 22°C, but on a flat-bed, to-and-fro agitator.

Experimental design. On day 1 of the experiment, pairs of inhibited and control PC were prepared from each of two donors. On days 3 and 6, this process was repeated with two more donors on each day to produce a total of six pairs of concentrates. On day 7 all 12 PC were sampled and analyzed as described below; at this point the concentrates had been stored 1, 4, or 6 days. In the first experiment the concentrates were sampled again on day 10 and analyzed; at this point the concentrates had been stored 4, 7, or 9 days. There was no significant difference in initial platelet counts (Coulter Counter S Plus IV) between the inhibited concentrates and the matched controls (paired t-test, 1.28

Preparation of

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Quantitation of MP in PC. Figure 1 shows a typical contour plot of fluorescence versus forward angle light scatter in a supernatant plasma sample from a stored PC. In
PLATELET VESICULATION DURING STORAGE

Fig 1. Contour plot showing log green fluorescence versus forward angle light scatter of particles in the supernatant plasma of a stored PC during analysis on the BD FACS-440. (A) Supernatant plasma spiked with 0.5-μm fluorescent latex microspheres, which appear in quadrants I and II. The unstained MP appear in quadrants III and IV. The vertical cursor was set to bisect the singlet bead peak to provide an arbitrary but reproducible discriminator between smaller and larger MP. (B) The same sample without beads but treated with 100 μmol/L DiOC, which fluoresces brightly when taken up into membranes.

In the supernatant plasma of a stored PC during storage, platelets vesiculate and release membrane-bound particles. Filtration of the supernatant plasma through a 0.2-μm pore filter before analysis reduced the MP count by 96%.

Inhibition of MP generation during storage. In the first analysis of paired PC prepared with or without activation inhibitors, the units were at 1, 4, or 6 days of storage. The control concentrates were stored at S/V = 7 cm²/mL, which represents typical conditions of a 60-mL vol in a 300-mL bag. Total MP counts (quadrant III + IV) in samples centrifuged for 30,000g-minute were 41% lower (P = .025) in the inhibited concentrates relative to the matched controls (Fig 2A). The second analysis performed 3 days later, with the concentrates at 4, 7, or 9 days of storage, showed 42% less MP (P = .003) with the inhibitors versus controls (Fig 2B).

This same study was repeated in another set of six donors with only a single analysis when the concentrates were at days 1, 4, and 6 of storage. No significant (P = .622) difference in day 1 platelet counts was noted between the control and inhibited PC. Again we found that the inhibitors had no effect on MP counts in quadrant III (P = .1), but the counts in quadrant IV were 40% lower in the inhibited concentrates (P = .03).

When the experimental design was altered so that the control concentrates were stored at S/V = 4 cm²/mL, as were the inhibitor-treated concentrates, the difference in MP counts and PF3 levels between inhibited and control units was reduced but still significant (Table 1). These

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Figure 2. Comparison of MP counts (quadrants III + IV) between the six pairs of PC with and without platelet activation inhibitors (PGE-1 + theophylline + aprotinin). At the first analysis (A), the concentrates were at days 1, 4, or 6 of the storage period. At the second analysis (B), the concentrates were at days 4, 7, or 9. The MP counts in the inhibited concentrates were significantly lower in both analyses ($P < .02$).

Figure 3. Effect of platelet activation inhibitors (PGE-1 + theophylline + aprotinin) versus controls on MP counts segregated into smaller (quadrant III) versus larger (quadrant IV) size distributions as per cursor settings described in Fig 1. The difference between pairs of concentrates (PC) were not significant in quadrant III ($P = .7$), but were significant in quadrant IV ($P < .001$).
Table 1. Comparison of Paired PC Prepared With (Inhibs) or without (Ctrls) PGE-1 + Theophylline + Aprotinin and Stored at S/V = 4 cm²/mL

<table>
<thead>
<tr>
<th>Quadrant III MP</th>
<th>Quadrant IV MP</th>
<th>Total MP</th>
<th>PF3 Activity</th>
<th>LDH Released</th>
</tr>
</thead>
<tbody>
<tr>
<td>(30,000g-min supernates)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrls</td>
<td>5.3 ± 2.8</td>
<td>20.2 ± 11.1</td>
<td>25.5 ± 10.5</td>
<td>0.34 ± 0.17</td>
</tr>
<tr>
<td>Inhibs</td>
<td>6.5 ± 2.5</td>
<td>12.4 ± 7.0</td>
<td>18.8 ± 7.5</td>
<td>0.16 ± 0.09</td>
</tr>
<tr>
<td>(+23%)</td>
<td>(−39%)</td>
<td>(−26%)</td>
<td>(−53%)</td>
<td>(−46%)</td>
</tr>
<tr>
<td>(7,500g-min supernates)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrls</td>
<td>7.0 ± 2.8</td>
<td>58.7 ± 21.2</td>
<td>64.4 ± 23.1</td>
<td>0.71 ± 0.26</td>
</tr>
<tr>
<td>Inhibs</td>
<td>7.6 ± 1.8</td>
<td>43.0 ± 14.2</td>
<td>50.6 ± 15.5</td>
<td>0.29 ± 0.15</td>
</tr>
<tr>
<td>(+9%)</td>
<td>(−27%)</td>
<td>(−21%)</td>
<td>(−59%)</td>
<td></td>
</tr>
<tr>
<td>Inhibs v Ctrls</td>
<td>*P = .01</td>
<td>*P = .02</td>
<td>*P = .007</td>
<td>*P = .02</td>
</tr>
</tbody>
</table>

Pairs of donor-matched PC at day 1 (n = 2), day 4 (n = 2), or day 6 (n = 2) were analyzed together after a 2-minute or 30-second centrifugation at 15,000g.

MP counts in units of 10⁶/mL (± 1 SD).

PF3 activity in units defined by frozen-thawed platelets = 1 U/mL supernatant PF3.

LDH released expressed as a percent of total platelet content.

Differences in means shown in parentheses as a percentage of the controls.

Abbreviation: ND, not determined.

levels and MP count (r = .597, P < .01), specifically in the larger MP distribution (quadrant IV; r = .748, P < .01) rather than the smaller (quadrant III; r = .04, P > .10). In the samples centrifuged 7,500g-minute, the correlation between PF3 activity and MP count decreased to r = .486 (P > .10). There was very little measurable PF3 in the stored PPP bags, and we did not find a significant difference in PF3 levels in the inhibitor-containing PPP versus controls (P = .5).

In the repeat of this study with the control concentrates stored at S/V = 7 cm²/mL, we demonstrated a similar reduction of 89% in PF3 levels in the inhibitor-treated concentrates (P = .001). Again no difference in PF3 levels was found in the PPP bags between inhibited and controls (P = .5).

Extracellular LDH assays indicated that less LDH release had occurred in the inhibitor-treated concentrates versus the paired controls over all time points (Fig 6). There was an average difference of 61% ± 11% between pairs (P < .001) in the experiment with control concentrates at S/V = 7 cm²/mL, and an average difference of 46% ± 6% when the controls were stored at S/V = 4 cm²/mL (P = .02).
A significant correlation was found between released LDH levels and total MP count \((r = .522, P < .01)\), suggesting that platelet vesiculation may involve a transient or slow leakage of cytoplasmic contents.

**Surface antigens on MP.** Both single and dual label ("two-color") experiments were performed to examine the presence of platelet-specific or other cellular antigens on the supernatant MP. DiOC\(_6\) was added after the final antibody incubation to examine the distribution of antigen and DiOC\(_6\) positivity simultaneously. With each of these antibodies, the negative and positive populations appeared to be distinct and well-separated on the single parameter fluorescence histograms. Less than 5% of the MP in any sample tested were positive with the pan-leukocyte (HLE) or erythrocyte (anti-glycophorin) antibodies. The 10E5 antibody to platelet GPIIbIIIa stained an average of 73% ± 13% \((n = 24)\) of the DiOC\(_6\)-positive MP in samples from inhibited or control PC. There was no demonstrable change in the distribution of 10E5 positivity of MP over storage time. A polyclonal antiserum raised in rabbits against whole human platelets appeared to stain an average of 62% ± 18% \((n = 18)\) of DiOC\(_6\)-positive MP. The AN-51 and SZ-2 antibodies to platelet GPIb stained an average of 46% ± 24% \((n = 18)\) and 43% ± 20% \((n = 18)\) of the MP, respectively. Less than 5% of the MP that stained with any of these antibodies were negative for DiOC\(_6\). Example fluorescence patterns are shown in Fig 7. The small amount of very bright PE-positive material apparent with each of the antibodies, including the non-immune control, was later found to be aggregates of the PE-labeled second antibody.

In the comparison of different experimental designs, there appeared to be a nonsignificant increase (1.2-fold to 1.4-fold) in antibody positivity (AN-51, 10E5, SZ-2, and the polyclonal) in the control samples when centrifuged 7,500g-minute versus 30,000g-minute. A larger increase (1.7-fold to 2.5-fold) was noted with the shorter centrifugation in samples from inhibitor-treated concentrates that was statistically significant \((P < .01)\). Interpretation of this finding is not straightforward. For either supernate preparation, there was no significant difference \((P > .2)\) in marker positivity between samples from control concentrates and those treated with inhibitors. Thus, it seemed that the inhibitors decreased the number of platelet-derived MP found, but did not change the phenotypic profile.

**DISCUSSION**

The flow cytometer is particularly useful in analyzing fluorescent-marker distributions among subpopulations of
cells or particles. In addition, data from measurements of scattered light can yield information on particle size and apparent granularity. The light scatter distribution of platelets is quite broad, reflecting the great heterogeneity of platelet size (2 to 20 FL) and density (1.08 to 1.04 g/cm³), and it has been reported that the distribution broadens during storage of platelet concentrates. Thus, it is difficult to identify clearly where the distribution of intact platelets ends and smaller particles, such as released membranous vesicles, begins. On the assumption that vesiculation of platelets results in particles of various size, but decidedly lower density than the intact cell, we elected to use centrifugation to sediment intact platelets before analysis of remaining MP. However, this approach has the disadvantage of possible loss of MP through co-sedimentation with the intact platelets, leading to underestimation of MP concentration. Our findings were lower than might be expected in that the MP counts were less than 10⁶/mL on average in PC containing greater than 10⁷ platelets/mL. A light centrifugation (7,500g-minute) resulted in more supernatant MP than the usual protocol (30,000g-minute), but there may have also been more platelets remaining as indicated by the Coulter Counter. The distribution of particles greater than 0.5-μm diameter may have included platelet-sized material, or even a few less dense intact platelets, in both types of preparations. We assume that the majority of light scattering events analyzed in these experiments represented vesicles of platelet membrane or the vacuolated ghost left after vesiculation, such as those forms observed by Sims et al. We assume also that there are many that were not observable within our analytical window.

Transmission electron micrographs of MP released during collagen stimulation of human platelets have demonstrated in other studies at least two different populations: one with an apparent diameter of 0.4 to 0.6 μm, and the other in the size range of 0.08 to 0.2 μm. With the light scatter settings used in this study, the flow cytometer could detect unlabeled latex spheres with a nominal diameter of 0.26 μm (data not shown) before encountering significant electronic noise. Further reduction in thresholds or increase in amplifier gain settings caused an immediate overflow of count accumulation that we interpreted as “background” light scatter in the plasma, or in the fluidics of the cytometer, probably originating from lipoprotein structures, macromolecular complexes, or even microbes. With these restrictions, the smaller population of MP (0.08 to 0.2 μm) possibly present in the PC was most likely lost in the background. Our attempts to analyze MP in highly diluted samples at the most sensitive light scatter thresholds produced event counts of 10⁶/mL or higher, but 60% to 80% of the particles were negative for DiOC, uptake and less than 10% were positive for the monoclonal platelet markers. Interestingly, the polyclonal antiserum marked as much as 50% of the material, but the specificity of this finding remains open to question.

Despite these limitations, our data show a significant effect of platelet activation inhibitors and reduced S/V ratio on MP generation in PC stored for transfusion. The predominant population of MP analyzed was larger than 0.5 μm in apparent diameter by forward angle light scatter, and it was this population that was most affected by the inhibitors. Also, this larger population of MP was highly correlated with levels of PF3 activity in the supernatant plasma and showed the most positivity with the antibody probes to human platelet surface antigens. The population of smaller MP (less than 0.5-μm diameter) did not correlate statistically with PF3 activity and was not reduced in the inhibitor-treated PC. This population comprised approximately 16% of the total MP count in control PC and 29% in the inhibitor-treated concentrates, reflecting the specific effect of the inhibitors on generation of larger MP during storage. In stored PPP bags, the smaller MP comprised approximately 35% of the total MP count with no demonstrable shift due to inhibitors. Both large and small MP took up DiOC, but the few negative MP observed at our standard settings were small (in quadrant III).

Staining with MoAbs and a polyclonal antiserum demonstrated that the majority of the observed MP were of platelet origin instead of fragments of erythrocytes or leukocytes. The DiOC hydrophobic dye showed that the particles under study were membranous, rather than aggregates of platelet GP or lipoproteins in the surrounding plasma. Expression of PF3 activity by the MP also indicated the presence of a membrane surface. The relatively greater percentage of positivity seen with the 10E5 antibody to GPIIbIIIa versus the SZ-2 or AN-51 antibody to GPIb suggests a different distribution of surface GP on MP relative to fresh platelets. Previous studies have demonstrated the appearance of a surface GPIb-negative population of platelets during storage, although others have shown that optimizing the agitation method results in no demonstrable loss of GPIb during storage for 5 days. Platelet activation by thrombin has been shown to alter the binding of markers of GPIb. We have shown elsewhere that inhibitors of platelet activation retard the loss of platelet GPIb in extended storage of PC. Perhaps vesiculation of platelets during storage contributes to the loss of surface GPIb, but lack of GPIb on the majority of MP suggests that proteolysis or internalization of GPIb may occur during or after vesiculation.

The release of LDH from platelets and the appearance of supernatant PF3 activity were both correlated with MP count in the PC. It has been known for quite some time that activation of platelets by chemical stimulation results in PF3 expression, but generally very little LDH is released in the short-term incubations usually used. However, extended incubation over several days, as in our storage experiments, with the many potential agonists present, may provide the opportunity for a slow leakage of LDH to accumulate to significant levels. We did not observe a large or consistent decrease in platelet count in the concentrates during storage (≤10% decrease), indicating that complete cell lysis was not the cause of high levels of extracellular.
LDH seen in the controls versus the inhibitor-treated units. We suggest instead that the active process of vesiculation of platelets in vitro results in the generation of membranous MP and a platelet “ghost” with compromised membrane integrity. This hypothesis would appear to be consistent with the morphologic observations of Sims et al. We have not found direct evidence of terminal complement activation in stored PC, but the inner wall of the plastic container may provide a site for occult complement fixation that could interact with platelets during storage. The observed benefit of a reduced bag S/V ratio may be at least partially related to a reduction in platelet collision rate with active complement components on the bag wall. Our finding that a cocktail of platelet activation inhibitors greatly enhanced the reduction in MP count (and PF3 activity and LDH release) is consistent with this hypothesis, but may indicate that also soluble-phase activation of platelets contributes to vesiculation during storage. The specific role of aprotinin in the cocktail of inhibitors has yet to be determined, but it does appear to augment the other components.

We conclude that platelets vesiculate during storage for transfusion largely by an activation-dependent process. It has long been known that stored erythrocytes release membranous vesicles into the supernatant fluid as their adenosine triphosphate (ATP) reserves are depleted. Perhaps the depletion of ATP in platelets after a metabolic burst associated with activation leads to platelet vesiculation in much the same manner. Directly or indirectly, the presence of MP provides more evidence that platelet activation occurs during the storage of PC and may contribute to the resulting platelet storage lesion. It is now apparent that inhibition of MP generation is part of the previously noted beneficial effects of platelet activation inhibitors on storage of platelets.

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Vesiculation of platelets during in vitro aging

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