Platelet Retention in Glass Bead Columns: Adhesion to Glass and Subsequent Platelet-Platelet Interactions

By Jean McPherson and Marjorie B. Zucker

In normal heparinized blood, the retention of platelets in glass bead columns was low in the first 1 or 2 ml, increasing to more than 80% by the 4th or 5th ml. Prior flushing of the columns with platelet-poor plasma or saline lowered retention in all 5 ml. Additional studies were carried out with a two-stage procedure in which a sample of blood (A) was pumped through a column, immediately flushed out with saline or plasma, and followed by a second blood sample (B). When as little as 1 ml of blood A preceded the flushing solution, retention was very high in all 5 ml of the subsequent blood B. This enhancement of retention in B occurred, providing blood A contained platelets (other than thrombasthenic), fibrinogen, and adequate divalent cations. Enhancement did not require von Willebrand factor (vWF) in A, nor was ADP necessary, since enhancement occurred even when heparinized blood as A contained prostaglandin E1 (PGE1) or creatine phosphokinase with creatine phosphate (CPK-CP). However, the presence of PGE1 or CPK-CP in the plasma used to flush the columns prevented the enhancement of retention in the first milliliter of B. Retention in the first milliliter of B (following normal blood as A and saline or normal plasma for flushing) was high when B was afibrinogenemic, moderately high when B contained PGE1 or CPK-CP, and low in thrombasthenic, EDTA, or vWF-deficient blood. Retention declined in subsequent milliliters of PGE1 or CPK-CP blood and remained low in thrombasthenic, vWF-deficient, or EDTA blood. Our findings suggest that (1) the platelets in A adhere to glass; this adhesion requires fibrinogen but not vWF or ADP; (2) the adherent platelets release ADP and become sticky; (3) adhesion of platelets in the first milliliter of B to the sticky platelets from A requires vWF and divalent cations but not ADP; (4) retention is maintained thereafter by repetitive platelet-platelet interactions involving ADP release, alteration of adherent platelets by released ADP, and adhesion of further platelets to these ADP-altered platelets which requires vWF.

Platelet retention in glass bead columns is an in vitro platelet function test reflecting the abnormality in von Willebrand's disease. The mechanism and site of action of the von Willebrand factor in this system is unknown.

ADP has long been implicated in platelet retention. It was originally thought that RBC released ADP which then caused platelets to be retained in the columns. Recent studies from this laboratory demonstrated that ADP and ATP are released from platelets rather than from RBC. The released ADP is essential for normal retention, as enzyme systems that destroy ambient ADP (creatine phosphokinase with creatine phosphate; certain preparations of potato apyrase) markedly inhibit retention. Retention is low when platelets cannot respond to ADP as when divalent cations are chelated, after addition of

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adenosine, and in blood from patients with thrombasthenia. It is also low in blood from patients with storage pool disease. Salzman suggested that two processes are involved in retention: platelet adhesion to glass and the sticking of platelets to other platelets. Cronberg and Holmberg recently obtained somewhat indirect evidence for two processes. Since the results obtained from standard methods of measuring platelet retention involve more than adhesion to glass, the term "platelet adhesiveness test" is misleading.

We have developed a two-stage procedure in an attempt to separate platelet adhesion to glass from subsequent platelet-platelet interactions and to define the roles of ADP, fibrinogen, and von Willebrand factor in platelet retention.

MATERIALS AND METHODS

Platelet retention was measured essentially as previously described. Blood was collected in 50-ml plastic syringes attached either directly to the needle or to a 19-gauge butterfly infusion set (Abbott Laboratories, North Chicago, Ill.). Usually the syringe contained 1/250 volume Panheprin (Abbott), 1000 U/ml. When tested in parallel with blood containing other anticoagulants or reagents, an appropriate volume of saline was added. In other experiments, the syringe contained 1/10 volume of 0.11 M sodium citrate or 1/50 volume of neutral 0.127 M ethylenediaminetetraacetate (EDTA) or ethylenebis (oxyethylenenitrilo)-tetraacetate (EGTA).

Blood was mixed by twirling after collection, before and after transfer to 20-ml syringes, and immediately before testing. The columns contained 2.6 g of glass beads; the beads were washed with distilled water and dried thoroughly before making the columns, and all columns were stored in a desiccator for at least 24 hr before use. In the two-stage experiments, 1 or 5 ml of blood (designated A) was pumped through the column and immediately followed by 5 ml of saline or platelet-poor plasma (PPP) to flush the column. Another 5 ml of blood (designated B) was then passed through the column, and retention was measured on each milliliter emerging from the column. Care was taken to prevent introduction of air when the syringes were changed. Blood and flushing solutions were pumped at 5.7 ml/min. The platelet counts in the first few milliliters of blood were corrected for dilution with residual saline or PPP as assessed by the PCV. Controls were blood passed through a column flushed with saline or PPP only and blood passed through an untreated column.

PPP was prepared at 4°C from heparinized blood centrifuged for 10 min at 2500 g. The plasma was then spun for 15 min at 17,000 g and pumped through a 0.45 μm Millex Tm disposable filter unit (Millipore Corp., Bedford, Mass.); the final platelet count was less than 100/μl. In some experiments the columns were flushed with PPP containing 6 μM prostaglandin E1 (PGE1) (Upjohn Co., Kalamazoo, Mich.), or creatine phosphokinase (CPK) 40 units/ml with 2 mM creatine phosphate (CP), both obtained from Sigma Chemical Co., St. Louis, Mo. Platelet-rich plasma (PRP) was prepared by slow centrifugation (300 g) of heparinized blood in 50 ml polycarbonate tubes and was aspirated into 20-ml syringes prior to testing. For washed platelets, blood was collected into EDTA, and the platelets washed in the cold, resuspended in a mixture of buffers, and warmed before use.

Normal citrated blood was rendered "afibrinogenemic" by gel filtration; it was passed through a Sepharose-2B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) column previously equilibrated with citrated serum from the same donor. A control sample was passed through an identical column equilibrated with citrated plasma from the same donor. Blood in the void volume from each column was used as blood A. Fibrinogen was measured with the tanned red cell hemagglutination inhibition immunoassay.

Disturbed blood, prepared as previously described, was transferred and tested within 20 min of disturbance. Other reagents were added to normal heparinized blood at least 10 min prior to testing to give final concentrations as follows: CPK, 20 units/ml with CP 1 mM; PGE1, 3 μM; ADP, 0.1 μM; antibody to von Willebrand factor (vWF), 1/500. The antibody, produced in goats, was kindly provided by Dr. Harvey Gralnick and Dr. Barry Coller, National Institutes of Health, Bethesda, Md. At a dilution of 1/500 it reduced retention in normal blood to a mean of 82% (4th
and 5th ml) to a mean of 20%. At higher concentrations it inhibited ristocetin-induced platelet aggregation. Antisera were also obtained from Behring Diagnostics (Somerville, N.J.) and dialyzed to remove azide. At 1/200, anti-factor VIII markedly reduced retention in normal blood, whereas anticeruloplasmin and antiprothrombin caused no change. Thus, the effect of anti-factor VIII antibody was specific.

For experiments in which both patient and normal blood were used, the bloods were ABO compatible. M. M. and M. C. (termed TA1 and TA2, respectively) were patients with typical thromboplastinemia who have been previously reported (M. M. by Zucker et al.14 M. C. by Weiss8). Retention was less than 20% in all 5 ml of TA1 and 0% in TA2. M. M. and J. B. have clinically severe von Willebrand's disease (vWD). In M. M. the plasma factor VIII procoagulant activity was 7%, the Ivy bleeding time more than 30 min, and platelet retention 4% in the 4th and 5th ml. This patient was studied through the courtesy of Dr. Margaret Karpatkin, New York University Medical Center, New York, N.Y. J.B. had a plasma factor VIII procoagulant activity of 8%, an Ivy bleeding time of more than 20 min, and 9% platelet retention in the 4th and 5th ml. A patient with the Bernard-Soulier syndrome (T.H.) was described by Weiss et al.15 and was studied through the courtesy of Dr. M. Johnson, Wilmington Medical Center, Wilmington, Del. Blood from the afibrinogenemic patient (C.M.)16 with a plasma fibrinogen level of 13.5 μg/ml was kindly provided by Dr. H. J. Weiss, Roosevelt Hospital, New York, N.Y. and was tested before and after addition of purified fibrinogen to a concentration of 400 μg/ml.

RESULTS

Normal Blood Preceded by PPP or Saline

Retention was lower in columns flushed with normal PPP (Fig. 1A) or saline (Fig. 1B) than in untreated columns. There was a statistically significant difference (p < 0.01) between the values for the 2nd and 5th ml in 12 paired experiments with and without PPP, and between the values for the 1st, 2nd, and 5th ml in 16 paired experiments with and without saline. Retention was iden-
tical whether the preceding PPP contained CPK-CP or PGE₁ or was obtained from blood of a patient with vWF (not illustrated).

**Normal Blood as A and B**

When 1 or 5 ml of normal blood as A was pumped through the column prior to flushing with PPP or saline, retention in the 1st ml of normal blood as B was very high, i.e., retention was enhanced over that seen in columns flushed with PPP or saline without prior passage of blood A (Fig. 1A and B). Since there was no significant difference whether 1 or 5 ml of blood was used as A, the results have been pooled. Retention in the 1st ml of B averaged 79%, when PPP was used to flush the column and 88% when saline was used. Retention remained high in each of the subsequent milliliters of B and did not differ between PPP and saline-flushed columns.

**Test Blood as A, Normal Blood as B**

**Requirement for platelets in A.** Remixed PPP + RBC did not enhance retention in B, whereas PRP caused moderate enhancement, and whole blood or remixed PRP + RBC as A caused marked enhancement (Fig. 2A). When PRP was diluted with PPP to the same platelet count as whole blood (single experiment, not illustrated) its enhancing effect was greatly reduced. Washed platelets enhanced retention as effectively as normal blood (Fig. 2A).

Fig. 2. Retention in B when B was normal heparinized blood and A was test blood. (A) Effect of PPP with RBC (PCV, 45%), PRP (platelet count, 400,000/µl), PRP with RBC (PCV, 44%; platelet count, 271,000/µl), or washed platelets (255,000/µl) as A (representative expts.). (B) A was EDTA blood (seven expts.), EGTA blood (six expts.), or was omitted (Nil) so that B was preceded only by saline. (C) A was citrated blood (three expts.).
Effect of anticoagulants in A. Blood anticoagulated with EDTA considerably enhanced retention in B although to a lesser extent than heparinized blood as A. EGTA blood was usually more effective than EDTA blood (Fig. 2B), and citrated blood (Fig. 2C) was almost as effective as heparinized blood.

Role of ADP. Blood containing PGE₁ (Fig. 3A) or CPK-CP (Fig. 3B) as A enhanced retention in B, although not quite to the extent observed with normal blood as A. When PGE₁ blood was followed by PGE₁-PPP (not illustrated) and when CPK-CP or normal blood was followed by CPK-CP-PPP, retention in B was enhanced to a lesser extent. Disturbed blood and blood pretreated with 0.1 μM ADP caused some enhancement although less than normal blood (Fig. 3C).

Thrombathenic blood as A. Thrombathenic blood caused slight enhancement of B in one experiment and no enhancement in two other experiments. In a fourth experiment, retention in B was actually less after thrombathenic blood as A than in the control PPP-flushed column (Fig. 4A).

Role of fibrinogen and von Willebrand factor. Blood rendered afibrino-
Fig. 4. Retention in B when B was normal heparinized blood and A was test blood. (A) A was thrombasthenic blood (four expts.). (B) A was citrated blood rendered afibrinogenemic in vitro by gel filtration through a serum-equilibrated column (serum GF blood, fibrinogen, 12.4 μg/ml); serum GF blood with added fibrinogen; citrated blood passed through a plasma-equilibrated column (plasma GF blood; fibrinogen, 2500 μg/ml); or normal citrated blood (representative expt.). (C) A was blood from an afibrinogenemic patient (plasma fibrinogen, 13.5 μg/ml) with and without added fibrinogen.

Genomic in vitro (Fig. 4B) and blood from an afibrinogenemic patient (Fig. 4C) failed to enhance retention of normal blood as B. When fibrinogen was added to A, retention in B was markedly enhanced.

Blood from patients with vWD was indistinguishable from normal blood when tested as A. Maximal enhancement was seen whether saline, normal PPP, or vWD PPP was used to flush the columns between the vWD blood as A and the normal blood as B. Blood containing antibody to vWF (four experiments) and blood from a patient with the Bernard-Soulier syndrome (one experiment) were also indistinguishable from normal blood when tested as A.

Normal Blood as A and Test Blood as B

Effect of anticoagulant in B. When EDTA was used as B, there was little enhancement of retention in the 1st ml, and retention was zero in subsequent milliliters. In blood anticoagulated with citrate or EGTA as B, retention was high in the 1st ml but either decreased or failed to increase further in successive milliliters. The variation between experiments was much greater with EGTA blood than with citrated blood (Fig. 5A).

Role of ADP. Retention was high in the 1st ml of CPK-CP blood as B and fell rapidly to very low levels in subsequent milliliters. No difference was noted...
whether normal or CPK-CP blood was used as A or whether PPP or saline was used to flush the column. However, when the column was flushed with PPP containing CPK-CP, there was very little enhancement in the 1st ml of CPK-CP blood as B (Fig. 5B). Similar results were noted with PGE₁: with normal or PGE₁-treated blood as A, retention in PGE₁-treated blood as B was high in the 1st ml and decreased thereafter; when the columns were flushed with PPP containing PGE₁, retention in the initial milliliter was low.

Retention in the 1st ml of disturbed or ADP-treated blood as B was considerably enhanced by normal blood as A and continued to rise in subsequent milliliters (Fig. 5C).

Thrombasthenic blood. When blood from one of the thrombasthenic patients (TA₁) was used as B, retention was consistently enhanced in the 1st ml but fell rapidly in subsequent milliliters. Retention in blood from the other thrombasthenic patient (TA₂) was not enhanced when tested as B (Fig. 6A).

Role of fibrinogen and von Willebrand factor. Retention in blood from the
Retention in blood from patients with vWD was only slightly enhanced by normal blood as A, and this enhancement was poorly maintained (Fig. 6B). Normal blood with added antibody to vWF showed a similar pattern, but retention was maintained at a higher level. Identical results with vWD blood as B were obtained whether saline, normal PPP, or vWD PPP was used to flush the columns between A and B and whether normal or vWD blood was used as A.

**DISCUSSION**

When blood was passed through a column which had been flushed with PPP or saline, platelets were retained, although retention was usually lower than in an untreated dry column. Thus, a blood-air interface was not a requirement for platelet adhesion to glass. The effects of flushing with PPP and saline were similar, indicating that adsorption of plasma proteins to glass beads did not alter subsequent retention in normal blood. Prior passage of normal PPP through the column improved the low retention of afibrinogenemic blood but not that of vWD blood.
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Table 1. Retention in B With Test Blood as A or B

<table>
<thead>
<tr>
<th>Type of Test Blood</th>
<th>Test Blood as A, Retention in 1st ml of Normal Blood as B</th>
<th>Test Blood as B, Retention Following Normal Blood as A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Maximal</td>
<td>Maintained</td>
</tr>
<tr>
<td>vWD</td>
<td>Maximal</td>
<td>Low</td>
</tr>
<tr>
<td>Bernard-Soulier</td>
<td>Maximal</td>
<td>Slow fall</td>
</tr>
<tr>
<td>syndrome</td>
<td>Low</td>
<td>Maximal</td>
</tr>
<tr>
<td>Afibrinogenemia</td>
<td>Low</td>
<td>Maximal</td>
</tr>
<tr>
<td>Thrombasthenia</td>
<td>Low</td>
<td>Virtually zero</td>
</tr>
<tr>
<td>EDTA</td>
<td>Low-moderate</td>
<td>Virtually zero</td>
</tr>
<tr>
<td>EGTA</td>
<td>Moderate-high</td>
<td>Moderate</td>
</tr>
<tr>
<td>Citrate</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>CPK-CP</td>
<td>High</td>
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<tr>
<td>PGE₁</td>
<td>High</td>
<td>Moderate*</td>
</tr>
<tr>
<td>Disturbed</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>ADP, 0.1 µM</td>
<td>High</td>
<td>Moderate-high</td>
</tr>
</tbody>
</table>

*Low if column is flushed with PPP containing CPK-CP or PGE₁, respectively.

In the two-stage procedure used in these studies, blood A was flushed out of the column with PPP or saline, and retention was measured in the subsequent blood B. Results are summarized in Table 1. In contrast to the low retention in the initial few milliliters of blood passed through PPP- or saline-flushed columns, retention was very high in all 5 ml of normal blood as B when the flushing PPP or saline was preceded by normal blood as A. Retention in B was similarly enhanced by remixed PRP and red cells as A, but not by PPP alone or remixed PPP and red cells. PRP with a high platelet count also enhanced retention in B. We conclude that the enhancing effect of A on retention in B is due to the platelets in A. Weiss and co-workers came to a similar conclusion, although in their studies PRP alone failed to enhance retention of subsequent normal blood. In our studies, red cells increased the enhancing effect of PRP but were not essential; they probably acted mechanically to cause more frequent collisions of platelets with the beads or with each other.

Washed platelets were as fully effective as A, supporting the conclusion that it was the platelets in A which caused the high retention in the 1st ml of blood B. George showed marked adhesion of washed platelets to glass, and Packham et al. demonstrated that adhesion was greater when the suspending medium did not contain protein.

Blood deficient in the vWF and blood from a patient with the Bernard-Soulier syndrome as A were as effective as normal blood in enhancing retention in B, even when the columns were flushed with saline or with PPP prepared from donors with vWD. Normal blood anticoagulated with citrate or EGTA, and normal heparinized blood which had been disturbed or which contained CPK-CP, PGE₁, or 0.1 µM ADP were nearly as effective as normal blood when tested as A. EDTA blood gave slight to moderate enhancement, thrombasthenic blood little or no enhancement, and afibrinogenemic blood failed to enhance retention in B unless fibrinogen was added.

The characteristics of blood A necessary for enhancement of B are the same as those noted when platelet adhesion to glass is assessed by direct observation.
Adhesion is reduced in thrombasthenic blood\textsuperscript{24,25} and EDTA blood,\textsuperscript{21,25} but some adhesion may occur\textsuperscript{25}; this is presumably sufficient to cause the slight to moderate enhancement of retention in B seen when these bloods are tested as A. Since blood containing CPK-CP or PGE\textsubscript{1} is as effective as A, adhesion to glass appears not to require ADP.

The marked enhancement of retention in the 1st ml of normal blood B when as little as 1 ml of blood A precedes the PPP or saline contrasts with the low retention seen in the first 1 or 2 ml of blood tested in the standard fashion, and the still lower retention seen when the blood is preceded by 5 ml of PPP or saline. The enhancement suggests that a functional alteration of the adherent platelets from A had occurred during the period of flushing, rendering them sticky. The enhancement of retention in normal, CPK-CP, or PGE\textsubscript{1} blood as B caused by normal, CPK-CP, or PGE\textsubscript{1} blood as A was virtually abolished when the columns were flushed with PPP containing CPK-CP or PGE\textsubscript{1} instead of with PPP alone. When A was omitted, the presence of these ADP-destroying or inhibiting agents in PPP preceding blood B did not further reduce retention. We therefore conclude that these agents may prevent the development of stickiness in the adherent platelets in A, and that this change must depend on the ability of these platelets to release and respond to ADP. The functional alteration may correspond to the marked morphologic changes, including spreading and extensive pseudopod formation, which have been demonstrated in platelets adherent to glass.\textsuperscript{25} The vWF is not required for this alteration since the 1st ml of normal blood as B shows full enhancement when vWD blood is used as A and the column is flushed with vWD PPP or saline.

When no inhibitor is present during flushing, retention is moderately high in the 1st ml of citrated blood, EGTA blood, and blood containing CPK-CP or PGE\textsubscript{1} as B. These reagents all inhibit ADP-induced aggregation; aggregation is less in citrated than in heparinized PRP,\textsuperscript{26} still less in fresh PRP prepared with EGTA, and abolished and markedly inhibited in heparinized PRP by PGE\textsubscript{1} and CPK-CP, respectively, at the concentrations used in this study (unpublished observations). Possibly sufficient responsiveness to ADP remains to account for the high initial retention in the 1st ml of B, but it seems much more likely that, as with platelet adhesion to glass, platelet adhesion to the bed of sticky platelets from A does not itself require ADP.

Although the presence of PGE\textsubscript{1}, CPK-CP, citrate, or EGTA in blood B does not prevent initial platelet-platelet adhesion, it does prevent continuation of the process so that retention is not maintained. This subsequent drop in retention suggests that ADP is essential to sustain retention. Presumably, the adherent platelets in the 1st ml of B must in turn release and be altered by ADP to become sticky. We have summarized elsewhere\textsuperscript{5} our reasons for concluding that this release of ADP does not occur through the classic release reaction as usually observed in citrated platelet-rich plasma. In fact, ADP cannot cause the classic release reaction with a physiologic level of ionized calcium.\textsuperscript{27} The low retention in blood of patients with storage pool disease\textsuperscript{8} suggests, however, that the ADP may come from the “release pool.”

Retention in untreated columns is reduced by prior addition of 0.1 \textmu M ADP,\textsuperscript{4} as well as by disturbance.\textsuperscript{10} Membrane-sequestered ADP is probably
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responsible for this reduction in retention. Disturbed and ADP-treated blood showed similar mild abnormalities as both A and B in the two-stage system. It seems paradoxical that prior exposure to ADP is inhibitory when ADP release is essential for retention. Presumably, inhibition is caused by reduced platelet responsiveness either to subsequent small amounts of ADP, or to vWF as recently demonstrated in aggregation experiments. 

Afibrinogenemic blood as B was indistinguishable from normal blood, with very high retention in the 1st and subsequent ml. Possibly, fibrinogen is not required for platelets to stick to the bed of adherent platelets and for the subsequent ADP-requiring process. More likely, a very low concentration (e.g., 13.6 µg/ml) is sufficient, since ADP-induced aggregation in heparinized PRP was normal in the patient studied. 

With normal blood as A, retention in thrombasthenic and EDTA blood as B was enhanced only slightly, if at all, in the 1st ml and fell to or remained at zero in subsequent milliliters. Retention in blood from patients with vWD was only slightly enhanced in the 1st ml and remained low in subsequent milliliters even when the columns had been flushed with normal PPP. Thus vWF is essential for platelets to adhere to the bed of sticky platelets derived from blood A. Evidence has already been presented that ADP is not involved in this step. The pattern of retention in blood containing antibody to vWF resembles that seen with blood from vWD patients, although the degree of enhancement is greater. Failure of previous normal blood and plasma to improve retention in vWD blood as B indicates that the vWF must be present during the platelet–platelet interactions which follow platelet adhesion to glass.

In conclusion, our findings suggest the following: the initiating event in platelet retention involves platelet adhesion to glass and requires fibrinogen. These glass-adherent platelets release small amounts of ADP and become sticky. In the presence of vWF, platelets adhere to this first layer and, in turn, release ADP and become sticky. Thus, continuing platelet–platelet adhesion (requiring vWF) and alteration of adherent platelets (requiring ADP) achieve platelet retention in glass bead columns. It is not entirely clear, however, why these interrelationships have not been demonstrated in aggregation studies; perhaps they have not been observed because centrifugation of heparinized blood inhibits normal platelet retention and is necessary to prepare PRP for aggregation studies, or because they occur only with specific conditions of flow or are prevented when citrate is used as the anticoagulant.

Tschopp et al. observed that platelets in citrated vWD blood do not adhere normally to rabbit aortic subendothelium, and subsequent studies suggest that ADP is not involved. Possibly, the subendothelium is similar to the surface of a glass-adherent, ADP-altered platelet. The initial event in primary hemostasis is probably adhesion of platelets to vascular subendothelium. Subsequent repetitive ADP-induced platelet alterations and vWF-requiring platelet–platelet adhesion may well be the mechanism for formation of the primary platelet plug.

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