Artificial Surface Effect on Red Blood Cells and Platelets in Laminar Shear Flow

By Taha M. Alkhamis, Richard L. Beissinger, and Juan R. Chediak

Red blood cell (RBC) effects on platelet adhesion to a nonbiologic test surface (tetrafluoroethylene propylene copolymer) and platelet aggregation during laminar shear flow for shear rates to 5,680 s⁻¹ (corresponding to shear stress to 200 dynes/cm²) were investigated. Results on hemoglobin (Hb) and adenosine diphosphate (ADP) release from RBCs, percent decrease of single platelets in the bulk, and percent of test surface covered with platelets were obtained in a cone-and-plate (CP) viscometer for samples of whole blood, suspensions of RBC ghosts in platelet-rich plasma (PRP), and suspensions of RBCs in either PRP or platelet-poor plasma. Results obtained over the shear rate range studied for samples of normal hematocrit indicated that low-stress shearing led to ADP and Hb release from intact RBCs; shear-induced release of ADP from RBCs was about twice that of platelets, and of the total ADP released, the ADP released from RBCs contributed about six times that of the platelets to single platelet reduction in the bulk and about twice that of the platelets to platelet adhesion, i.e., coverage of the test surface with platelets. Results obtained for various hematocrits showed that above a threshold hematocrit of about 25% to 35% the RBCs (suspended in PRP) had a greater contribution to ADP release, platelet adhesion, and platelet aggregation than the platelets themselves. Single platelet reduction for samples of RBC ghosts suspended in PRP correlated with shear rate level and not with shear stress.

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As a result of the increasing use of artificial organs, prosthetic devices, and extracorporeal flow systems, an improved knowledge of hemolysis (sublytic) and its effect on thrombosis (under a range of physiologically encountered shear conditions) is needed concerning the effects of synthetic material on various blood elements, especially red blood cells (RBCs) and platelets. Under normal circumstances, platelets in the circulation do not adhere to the endothelial-lined blood vessel walls and they usually do not aggregate with other platelets. However, for systems involving foreign surfaces, shear-induced RBC and platelet-surface interactions, which result in release of adenosine diphosphate (ADP), may be essential for platelet adhesion and platelet aggregation. The role of RBCs on platelet events has not been extensively investigated but is of growing importance (eg, in intravascular hemolysis, bleeding noted in uremia, thrombo-embolic events associated to polycythemia vera, transient ischemic attacks, and possibly in coronary artery diseases such as myocardial infarction and unstable angina). In all of these conditions a heightened tendency for spontaneous platelet clumping and or aggregation has been shown to occur. This study attempts to further clarify the importance of RBC mediation (through shear-induced ADP release) in platelet-surface and platelet-platelet interaction leading to thrombosis on artificial surfaces. In particular, this investigation attempts to distinguish between the ADP released from RBCs and that from platelets.

Low-stress hemolysis (which for this study refers to sublytic shear stresses to about 150 dynes/cm²), the loss of hemoglobin (Hb) along with other components that include intracellular nucleotides such as ADP from the RBC into the surrounding plasma, implies cell damage because Hb molecules are too large to leak from the RBCs unless their cell membranes are greatly deformed or ruptured. Although the above-mentioned shear stress threshold of 150 dynes/cm² is considered low stress for studies of hemolysis, it is rather high when considering hemostasis or thrombosis. Moreover, such a shear stress can occur in artificial implants. Under these circumstances the much smaller molecules of ADP would be expected to leak from the RBC more easily than that of Hb. Evidence has been accumulating which suggests that RBCs, through their shear-induced ADP release, chemically affect platelet adhesion on nonbiologic surfaces and platelet aggregation in the bulk. ADP is known to be a primary aggregating agent of platelets and may act through binding with ADP-platelet membrane receptors to activate platelets, which then allows the binding of fibrinogen with the exposed fibrinogen receptors glycoprotein IIb/IIIa; platelet aggregation follows. The shear-induced platelet release studies of Moritz et al suggested that ADP was the major if not sole mediator of shear-induced platelet aggregation. Along with the release of ADP from the RBCs, the rotational and translational motion of the RBCs can physically augment platelet transport (by increasing the effective diffusivity of platelets) in the bulk as well as to an artificial surface, which may lead to the formation of a platelet-rich region near the surface, and consequently (as a result of platelet activation by released ADP) will increase platelet adhesive interaction with the surface as well as their aggregation with other platelets at the surface and in the bulk.

This study investigates low-stress, shear-induced RBC damage and its chemical and physical effects on platelets, specifically the potentiation by ADP released from RBCs on platelet adhesion to a nonbiologic test surface (tetrafluoroethylene propylene copolymer) and platelet aggregation during laminar shear flow for shear rates to 5,680 s⁻¹ (corresponding to shear stress to 200 dynes/cm²), and attempts to determine through correlation whether both these events are related. As a consequence the main hypothesis addressed in these studies is to assess if shear-induced
RBC damage through release of components, especially that of ADP, significantly affect (1) the percent decrease of single platelets, ie, single platelet reduction through platelet adhesion to a nonbiologic surface and platelet aggregation, and (2) the percent of test surface covered with adhered platelets, ie, percent surface coverage.

APPARATUS, MATERIALS, AND METHODS

Apparatus

A Weissenberg rheogoniometer model R16/R18 (Sangamo Weston Controls Ltd, Sussex, England) was used in this study with a cone-and-plate (CP) arrangement to generate viscometric flow.\(^\text{14}\) The CP arrangement, as shown in Fig 1A, comprised a 10.0-cm diameter aluminum cone of 0.34 cone angle and a 10.0-cm aluminum flat plate. Blood and suspension sample volumes of 1.38 mL were contained within the conical gap, with the cone made to rotate at various rotational speeds giving shear rates up to 5,680 s\(^{-1}\). The CP geometry is useful at small cone angles, when a good approximation to uniform shear is achieved if rotational speed is slow enough.\(^\text{15}\) In this geometry, the steady-flow hydrodynamics for Newtonian fluids are known exactly and for non-Newtonian fluids approximately. Expressions for shear stress, shear rate, and sample volume (V) are shown in Table 1.\(^\text{16}\) The shear rate is equivalent to the velocity gradient in the CP geometry and the shear stress, a tangential force per unit area produced by the fluid in motion on the wall resisting its motion, is equivalent to the product of the measured viscosity and the imposed shear rate.

Thin films of fluorinated ethylene propylene copolymer (Teflon FEP film, DuPont, Wilmington, DE) were applied to the viscometer platen surfaces by using double-faced adhesive sheets (Sun Process Company, Elk Grove Village, IL)\(^\text{13,17}\) or an adhesive material (Glue Stick, Faber Castell Dist, Lewisburg, TN). The FEP films were used in this study for their relevance to artificial organs. These FEP implants have been used successfully in heart valves, hip joints, knee joints, jaw bones, arteries, bile ducts, wind pipes, and corneas.\(^\text{17}\)

Materials

Solutions

Acid citrate dextrose (ACD), formula A. This anticoagulant/preservative solution was prepared following methods described previously.\(^\text{6,17,18}\) For studies on platelets and platelet function, ACD is known to stabilize the reactivity of platelets.\(^\text{19}\) In this study, experiments involving platelets were begun 15 minutes after collection of blood samples and involved up to a maximum time of 5 hours after collection during which the cells were studied.

Phosphate-buffered saline (PBS). This buffer was used for suspension and washing purposes and was prepared following methods previously described.\(^\text{6,17}\) A hypotonic form of this buffer was also prepared for osmotic hemolysis of RBCs for ghost preparation.

Tyrode albumin solution (TAS). This solution was used as a plasma substitute for resuspension purposes and was prepared as described in a previous study.\(^\text{17}\)

Methods

Blood Collection

Blood was obtained from volunteer donors. They appeared healthy, fasted overnight, and were asked if they had taken any medications within at least 14 days before donation that might affect or inhibit platelet activation (such as aspirin). Subjects on such medications were excluded from this study. Samples of 10 to 20 mL were withdrawn at either the Illinois Institute of Technology Clinic (Chicago) or at the Special Coagulation Laboratory at Michael Reese Hospital (Chicago, IL). Samples were collected in plastic tubes with ACD (pH 5.5) anticoagulant at a ratio of 15 mL of ACD/100 mL of blood, which resulted in a final pH value of 6.9 at which the experiments of this study were conducted. The samples were transferred to our laboratory within 15 minutes. Apyrase

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**Table 1. Symbols and Expressions for the Different Parameters Describing the Physics of the Cone-and-Plate Viscometric Flow System**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shear rate (γ)</td>
<td>γ = ( \frac{d\omega}{d\gamma} ) = ( \frac{\omega}{\nu} )</td>
</tr>
<tr>
<td>Shear stress (τ)</td>
<td>( \tau = \mu \frac{d\omega}{d\gamma} )</td>
</tr>
</tbody>
</table>

Where: \( \omega \) is the angular velocity of the cone, \( \nu \) is the cone angle, \( \frac{d\omega}{d\gamma} \) is the velocity gradient, \( \mu \) is the viscosity, and \( R \) is the radius of the plate.
(Grade V: from potato, Sigma Chemical Co, St. Louis, MO) was added, following the methods of Moinar and Lorand, to some of the whole blood samples (175 μg/mL) so that these samples could act as controls to show the importance of ADP release from blood cells.

**Blood Component Mixtures**

**Platelet-rich plasma (PRP).** Whole blood was centrifuged at 150 × g and room temperature for 10 minutes, then the supernatant was separated and collected with a plastic pipet and stored in a plastic container until used (within 3 hours). The number of platelets in PRP was adjusted with platelet-poor plasma (PPP) of the same individual to obtain a concentration of 210,000 ± 20,000 platelets/μL (SD).

**PPP.** Centrifugation of PRP at 1,000 × g at room temperature for an additional 10 minutes yielded a supernatant collected as PPP.

**Platelet-free plasma (PFP).** Centrifugation of PRP at 14,000 × g for 10 minutes yielded a supernatant that was devoid of platelets.

**RBC Suspensions**

Two types of RBC suspensions were prepared.

**RBC-PFP.** Packed RBCs, the lower portion of the resultant centrifugation of whole blood to obtain PRP above, were obtained and washed twice by mixing and resuspending in an equal volume of PFP followed by centrifugation at 150 × g for 10 minutes and room temperature. The resultant RBCs, after the second centrifugation, were resuspended and adjusted to the required hematocrit with PFP. The platelet count at a hematocrit level of 45% was less than 5,000 platelets/μL.

**RBC-PRP.** A portion of the packed RBCs obtained above were resuspended and adjusted to the required hematocrit with PRP.

**RBC ghost suspensions.** Ghosts were prepared by osmotic lysis of RBCs obtained from local blood bank (LifeSource, Chicago, IL), followed by rescaling after encapsulation of PBS. Following the methods of Diezil and Girotti and Goldsmith and Marlow, RBCs were washed three times with PBS, then suspended in a hypotonic PBS solution of pH 8.0 containing 0.005 mol/L phosphate buffer with 0.005 mol/L NaCl for 24 hours at 4°C. The membranes were collected and resuspended in either phosphate or dextran (of molecular weight 66,300) buffer to reseal the membranes. The resultant ghosts were washed with PBS of pH 6.9 (similar to PRP pH) containing 0.005 mol/L phosphate buffer with 0.145 mol/L NaCl, and resuspended in PRP to the required hematocrit.

**Percent Decrease in Single Platelets (single platelet reduction)**

Small (0.02 mL) samples of blood obtained before and after shearing (for 2 minutes unless otherwise stated) in the CP viscometer were added to 1.98 mL of 1% ammonium oxalate solution. Platelets were counted following the method of Brecher and Cronkite using a phase-contrast microscope. No platelet aggregates were seen in unsheared blood samples, whereas some aggregates were seen in sheared samples but not counted as platelets and, consequently, not quantified. Single platelet reduction was determined by subtracting the counts in the sheared sample from the counts in the unsheared sample.

**Percent of Test Surface Covered With Platelets (percent surface coverage)**

After the shearing experimental runs the FEP films were removed carefully from the CP platens, rinsed thoroughly with PBS to remove nonadhered material, and incubated in 1% glutaraldehyde solution for 1 hour. After these steps the FEP films were air-dried and prepared for microscopic evaluation, as shown in Fig 1B, by cutting along their diameter and at a distance 0.5 cm from the center, both sides perpendicular to the cut along the diameter. The area was then divided into five equal sub-areas and mounted on a microscopic slide. Phase contrast microscopic pictures were taken using a phase contrast microscope (Micromaster, Fisher Scientific, Itasca, IL) and an Olympus camera (OM-1, Olympus Camera Corp, Overbrook Park, NY). Negatives were used as slides and analyzed using either an interactive digital analysis system (MOP, Eberhardt Instrument Company, Downers Grove, IL) or Scriptel graphic tablet (SPD, AD-Tech, Inc, Park Ridge, IL) interfaced to a Zenith computer for data collection to obtain values of adhered platelet size, platelet surface coverage, and platelet surface density.

The negatives are projected by a slide projector to a 45° angle mirror beneath the digitizing pad. The mirror reflects the image onto the tracing paper covering the transparent pad. The particle pictures are traced to allow surface coverage analysis. Using a known area (A,) and tracing the particles enables the determination of the total area occupied by platelets and platelet aggregates (A,) and permits the calculation of the percent surface coverage by platelet material as A/A, × 100.

**Hemoglobin and ADP Concentration Determination**

The plasma Hb concentration was determined using the benzidine method, which is sensitive to 2 mg%. A high pressure (or performance) liquid chromatography (HPLC) system was used in these studies and was coupled with a fluorescence detector to form a sensitive system for separation and quantification of adenine nucleotides. The method of Ramos-Salazar and Bains that uses a coupling procedure to separate and quantify adenine nucleotides in tissues was adapted for these compounds in blood plasma. Separation was achieved using an isocratic, ion-paired, reverse-phase HPLC method. A Perkin-Elmer (Norwalk, CT) C18 column, 33 mm long × 4.6 mm ID and packed with 3-μm particles, was used. The samples were passed through a fluorescence detector (650 S, Perkin-Elmer) set at 400 nm that used an excitation wavelength of 280 nm. This method is known to recover more than 88% of ADP in the sample.

**Phospholipid Determination**

Two procedures were used: (1) a lipid phosphorous method for determination of plasma phospholipids, and (2) a fluorometric method based on binding phospholipid with the fluorescent probe 1-6 diphenyl hexatriene.
Statistical Methods

The blood damage and shear rate data were analyzed using the student's t test. Each data point represents the mean result of three to five donors; error bars include the corresponding 95% confidence limits.

RESULTS

Viscosity Measurements

The viscosity of various sheared samples was measured to characterize the sample and evaluate the respective shear stress associated with the imposed shear rate. The effect of various hematocrits on the viscosity of samples of RBCs suspended in PRP measured at a shear rate of 2860 s\(^{-1}\) is shown in Fig 2. An increase in viscosity is observed as the hematocrit increases, and at a hematocrit value of 45%, the viscosity was found to be about 3.3 cp in this study, which is about that expected of whole blood samples.

Prepared RBC ghosts suspended in PRP were characterized by their viscosity to ensure that their rheologic behavior was similar to that of whole blood. Figure 3 shows the viscosity of ghosts at 45% hematocrit containing dextran or PBS and suspended in PRP compared with that of whole blood. It was intended that the ghosts containing dextran would show viscosity values similar to those of whole blood because of the similarity of molecular weight compared with Hb. The ghosts containing dextran show viscosity to be a function of the imposed shear rate (non-newtonian), with the viscosity values (measured at 25°C) decreasing from 6.5 cp at 720 s\(^{-1}\) to about 3.3 cp at 5680 s\(^{-1}\). However, the viscosity of ghosts containing PBS is approximately constant over the range of shear rates studied (newtonian) and is about 2.3 cp. As shown, the viscosity of the dextran ghosts is uniformly higher than that of whole blood at a given shear rate, which in turn is higher than the viscosity of the ghosts containing PBS.

Hemoglobin and ADP Release

The effect of RBC volume fraction on Hb and ADP release was studied by variation of the volume fraction of RBCs in suspensions of RBCs in PRP. Figure 2 shows the release of Hb from RBCs as a function of their volume fraction at a shear rate of 2,860 s\(^{-1}\). The plasma Hb concentration increases substantially after a threshold of hematocrit of about 35%, suggesting that at the higher volume percent level the RBCs interact to large extent with the nonbiologic test surface and/or each other, resulting in a significant increase in Hb release.

Results on shear-induced release of ADP from samples of whole blood, ghosts containing PBS suspended in PRP, and RBCs suspended in PFP (all at a fixed hematocrit of about 45%) shown in Table 2, indicate that there is a significant increase in ADP release for whole blood samples compared to the other two types of samples. The amount of ADP released from RBCs was obtained from samples of RBC suspensions in PFP, and it was assumed that the amount released solely from platelets was obtained from samples of ghosts suspended in PRP. These results, which were averaged over the shear rate range tested (see Table 2), indicate that the shear-induced release of ADP from RBCs was significant, ie, about 65% of the total ADP released from whole blood comes from RBCs, while the rest, about 35%, comes from platelets.

The effect of cellular volume fraction on shear-induced release of ADP from RBCs in suspensions of RBCs in PRP and from platelets in suspensions of ghosts in PRP at a shear rate of 2860 s\(^{-1}\) is shown in Fig 4. As can be seen there is a sharp increase in ADP release at the threshold of 35% hematocrit associated with RBC suspension samples. The difference between the two curves indicates the RBC contribution as a function of RBC volume fraction. The increase in ADP release with increasing volume percent is similar for both ghosts and RBCs below the threshold level such that RBCs appear not to be contributing (whereas the platelets do) to the ADP levels measured.

Single Platelet Reduction

The percent decrease in single platelets was used as a measure of the effect of shear on platelet disappearance through aggregation in the bulk and adhesion to the surface. Results of shear-induced single platelet reduction are re-

| Table 2. Shear-Induced ADP Concentration (μmol/L) |
|-----------------|-----------------|-----------------|-----------------|
| Shear Rate (1/s) | Whole Blood (WB) | RBC-PFP          | GST-PFP         |
| 0                | 0.2 ± 0.2        | 0.1 ± 0.1        | 0.1 ± 0.1       |
| 720              | 0.7 ± 0.3        | 0.5 ± 0.2        | 0.4 ± 0.1       |
| 1,432            | 0.8 ± 0.5        | 0.7 ± 0.2        | 0.8 ± 0.1       |
| 2,860            | 1.8 ± 0.3        | 1.4 ± 0.3        | 0.6 ± 0.2       |
| 5,680            | 2.3 ± 0.4        | 1.5 ± 0.3        | 1.0 ± 0.1       |
| Shear-averaged   | 1.2 ± 0.3        | 0.8 ± 0.2        | 0.5 ± 0.1       |

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ported in Table 3 for the same three types of blood component mixtures and suspension samples (all at a fixed hematocrit of about 45%) as mentioned above for ADP release: whole blood, whole blood containing apyrase, and ghosts containing PBS in PRP. Because ADP is converted by apyrase through dephosphorylation to compounds inactive with respect to platelets,\(^\text{20}\) the results of whole blood containing apyrase act as a single platelet reduction control to show the importance of ADP released from RBCs and platelets, and thus help quantify the contribution of ADP released from platelets to single platelet reduction. Measurement of ADP in sheared whole blood samples containing apyrase resulted in undetectable amounts of ADP. The contribution of ADP release from platelets to single platelet reduction was obtained by comparing results of the apyrase-containing samples with those of the ghost suspension samples. The single platelet reduction results, as a function of shear rate, as well as shear-averaged values are shown in Table 3 and indicate that the ghosts in PRP account for about 40% of the whole blood results, suggesting a contribution of about 60% for the ADP coming from RBCs. The whole blood control single platelet reduction results are about 30% of the whole blood results, suggesting that the 10% difference between these results and the ghost suspension samples is due to the effect of shear-induced release of ADP from platelets.

The rheologic effects of RBC ghost content on single platelet reduction are reported in Fig 5. Only small differences in single platelet reduction are observed between the two samples as a function of shear rate. Because sample viscosity is quite different (see Fig 3), the shear stresses experienced by the samples referred to in Fig 5 are proportionally different as shown in Table 4. Therefore, single platelet reduction under these circumstances appears to be controlled by shear rate, not shear stress.

The effect of cellular volume fraction on shear-induced single platelet reduction is shown in Fig 6. The results for both RBCs and ghosts suspended in PRP coincide to about a volume fraction of 25% after which the results diverge, where results for RBCs show a markedly higher effect on single platelet reduction (nearly twice at a hematocrit of 45%). The RBC suspension curve rises sharply between 25% and 45%.

Percent Surface Coverage

The percent of test surface covered with platelets was used as a measure of the extent of platelet adhesion as a function of shear rate. Results of percent surface coverage are reported for the same three types of blood component mixtures and suspensions samples (all at a fixed hematocrit of about 45%) as mentioned above: whole blood, whole blood containing apyrase, and ghosts containing PBS suspended in PRP. The platelet adhesion results, as a function of shear rate, as well as shear-averaged values are shown in Table 5 and indicate that ghost suspensions in PRP are about 70% of the whole blood results, suggesting a contribution of about 30% from RBC-derived shear-induced release of ADP. The whole blood with apyrase sample results are about 55% of the whole blood results, indicating that the 15% difference between these results and the ghost suspension samples is due to the effect of shear-induced release of ADP from the platelets themselves.

### Table 3. Single Platelet Reduction (SPR%)

<table>
<thead>
<tr>
<th>Shear Rate (1/s)</th>
<th>Whole Blood (WHB)</th>
<th>WHB-APR</th>
<th>GST-PRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.8 ± 3.3</td>
<td>5.5 ± 1.6</td>
<td>5.5 ± 1.8</td>
</tr>
<tr>
<td>720</td>
<td>29.3 ± 7.3</td>
<td>10.8 ± 2.5</td>
<td>13.3 ± 1.0</td>
</tr>
<tr>
<td>1,432</td>
<td>37.3 ± 9.1</td>
<td>8.5 ± 1.4</td>
<td>13.8 ± 1.2</td>
</tr>
<tr>
<td>2,860</td>
<td>37.8 ± 3.3</td>
<td>14.3 ± 1.6</td>
<td>17.0 ± 1.7</td>
</tr>
<tr>
<td>5,680</td>
<td>52.4 ± 10.9</td>
<td>16.2 ± 1.9</td>
<td>20.9 ± 1.2</td>
</tr>
<tr>
<td>Shear-averaged</td>
<td>± average variation</td>
<td>34.3 ± 6.8</td>
<td>11.1 ± 1.8</td>
</tr>
</tbody>
</table>
The effect of cellular volume fraction on percent surface coverage is reported in Fig 7 for suspensions of RBCs in PRP and ghosts containing PBS in PRP. The results for both suspensions approximately coincide to a volume fraction of about 30% after which they diverge, with the RBCs showing a higher effect on percent surface coverage (about 33% higher at a hematocrit of 45%).

Phospholipid Release

The fluorescence associated with labeling phospholipid molecules in the aqueous phase after low-stress shearing of samples of RBCs suspended in TAS was measured as a function of shear rate. Results do not show any variations in the amount of fluorescence detected as a function of shear rate, which suggests that phospholipid release from RBC membrane did not occur. To further support this interpretation the procedure of separating the supernatant from the packed cells was evaluated to see if the supernatant contained any phospholipids that were suspected to be released from the sheared RBCs. The centrifugal force of $14,000 \times g$ applied during separation may have removed the suspected membrane fragments from the supernatant. An experiment was performed to investigate this possibility. Sheared samples were centrifuged at $150 \times g$ for 5 minutes to spin down only the RBCs. It was reasoned that at only consistency higher $g$-forces would the phospholipid membrane fragments (if any) be spun down as well. The fluorescence results obtained for the supernatant did not differ from those results reported above.

DISCUSSION

The results obtained in this study indicate the essential role of RBCs in platelet adhesion and aggregation under low stress (up to about 150 dynes/cm²), laminar shear flow conditions and are consistent with the findings of others. The RBCs contribute significantly to the total release of ADP; they release an amount of their ADP contents, which is more than enough to trigger platelet aggregation (since the concentration of ADP that can cause irreversible platelet aggregation in an aggregometer is about two $\mu$mol/L). At a shear rate of 5,680 s⁻¹, RBCs have been found to release about 2% of their ADP content, compared with 0.2% of their Hb content. This order of magnitude difference is probably due to the size difference of the two molecules and the nature of RBC membrane damage, which appears not to be due to irreversible rupture, but rather related to reversible membrane deformation due to the imposed shear. The platelets release about 11% of their total ADP content at a shear rate of 5680 s⁻¹. The shear-induced RBC release of ADP, an important platelet agonist that is believed to be the major if not sole mediator of shear-induced platelet aggregation, contributes to both single platelet reduction in the bulk and adhesion of platelets to nonbiologic test surface in the experimental system studied.

The concentration of RBCs in a blood sample is also an important factor in the shear-induced release of ADP from RBCs and platelets and its effect on platelet aggregation and adhesion. Results obtained for various hematocrits (as shown in Figs 4, 6, and 7) indicated that above a threshold hematocrit of about 25% to 35% the RBCs had a significantly greater (chemical) contribution than the RBC ghosts and platelets themselves to ADP release, single platelet reduction in the bulk, and coverage of the test surface with platelets. However, (as shown in Figs 6 and 7) the ghosts (which were depleted of all releasable ADP that could be detected during shear) suspended in PRP also demonstrated a contribution to platelet aggregation and platelet surface coverage that most likely includes the physical effect of RBCs on platelet transport mechanisms, ADP released from the platelets themselves, and agonists other than ADP.

Reimers et al have also noted an increase in the extent of platelet aggregation, ie, single platelet reduction with increasing hematocrit (to 30%) in PRP. A sharp increase in single platelet loss was apparent in their results at a hematocrit of 20%. This trend is similar to that observed in our studies (Fig

Table 4. Shear Stresses for Equivalent Shear Rates Associated With Samples of Ghosts Containing Either Phosphate-Buffered Saline (GST[PBS]-PRP) or Dextran (GST[DEX]-PRP)

<table>
<thead>
<tr>
<th>Shear Rate (1/s)</th>
<th>Shear Stress (dynes/cm²) GST[PBS]-PRP</th>
<th>Shear Stress (dynes/cm²) GST[DEX]-PRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>720</td>
<td>15.8</td>
<td>47.9</td>
</tr>
<tr>
<td>1,432</td>
<td>30.1</td>
<td>62.4</td>
</tr>
<tr>
<td>2,860</td>
<td>58.1</td>
<td>128.1</td>
</tr>
<tr>
<td>5,680</td>
<td>113.0</td>
<td>164.0</td>
</tr>
</tbody>
</table>

Viscosity values used to obtain the shear stress values presented in this table were taken from Fig 3, in which the viscosities are quite different for the two types of suspensions at the same shear rate.
However, their results showed higher single platelet loss values than those reported herein, i.e., 70% single platelet loss at a hematocrit of 30% and shear rate < 2,000 s⁻¹ compared with 40% at a 45% hematocrit and 5,680 s⁻¹ shear rate. This difference may be due to the difference in the surface used (stainless steel in their study compared with Teflon in this study), the duration of the experiments (5 minutes compared with 2), and the chemical fixation of the RBCs using glutaraldehyde (compared with the preparation and use of RBC ghosts in this study).

The results obtained over the shear rate range studied showed for samples (at a fixed hematocrit of about 45%) of whole blood, ghosts containing PBS suspended in PRP, and RBCs suspended in PFP, that low-stress shearing led to ADP release from intact RBCs, with release of ADP from RBCs being about twice that of platelets. ADP released from RBCs contributed about six times that of the platelets to single platelet reduction. The single platelet reduction results for the whole blood samples containing apyrase were about 55% of the whole blood results, and probably were due to agonists other than ADP, especially thrombin, as reported previously. Also, the whole blood-containing apyrase results include the physical effect of RBCs on percent surface coverage through platelet transport mechanisms.

Single platelet reduction for samples of RBC ghosts suspended in PRP under the conditions studied (as shown in Figs 3 and 5, and Table 4) appears to be controlled by the shear rate, i.e., the velocity gradient imposed by the cone and plate viscometer surfaces on the adjacent fluid layers as well as in the bulk fluid containing the platelets, rather than by shear stress (i.e., not by the frictional forces that are produced by a moving wall on the adjacent fluid layers and transmitted to fluid layers in the bulk). These results further suggest that the mode of single platelet reduction for the whole blood samples may be controlled by shear rate as well. Consistent with these results are those obtained in previous studies by Shapiro and Williams,²⁸ which show that low-stress hemolysis correlates with shear rate rather than with shear stress.

Due to the imposed velocity gradient in the viscometer system the RBCs are transported by diffusion at an augmented rate to the viscometer surface.³ This, along with the above-mentioned result, may suggest that the surface interaction with RBCs along with shear rate controls single platelet reduction. A possible mechanism is that RBCs are temporarily traumatized at the viscometer surface as a function of shear rate leading to partial release of their ADP contents, which diffuse back to activate platelets resulting in platelet adhesion to the surface, as measured by percent surface coverage, and in the bulk resulting in platelet aggregation, as measured by single platelet reduction. This shear rate/surface controlled mechanism may be consistent with the results seen for the threshold level of RBC concentration with respect to ADP release, single platelet reduction, and percent surface coverage. This threshold level, which is about 35%, suggests that below this level there are not enough RBCs interacting with each other and the surface to be traumatized. However, above this threshold level the interaction of the RBCs with each other and the surface is adequate to traumatize the RBCs near the surface, with a sharp increase in the results being seen.

RBC-derived phospholipids could not be detected in this experimental system. The RBCs seem not to release phospho-

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**Table 5. Percent Surface Coverage (PAD%)**

<table>
<thead>
<tr>
<th>Shear Rate (1/s)</th>
<th>Whole Blood (WB)</th>
<th>WHB-APR</th>
<th>GST-PRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.0 ± 1.8</td>
<td>2.5 ± 0.2</td>
<td>3.1 ± 1.6</td>
</tr>
<tr>
<td>720</td>
<td>6.1 ± 1.2</td>
<td>3.2 ± 0.7</td>
<td>3.9 ± 1.1</td>
</tr>
<tr>
<td>1,432</td>
<td>6.2 ± 1.5</td>
<td>4.4 ± 0.8</td>
<td>4.4 ± 1.2</td>
</tr>
<tr>
<td>2,860</td>
<td>10.1 ± 3.5</td>
<td>5.7 ± 1.4</td>
<td>7.0 ± 2.0</td>
</tr>
<tr>
<td>5,680</td>
<td>10.4 ± 1.5</td>
<td>5.7 ± 1.1</td>
<td>8.3 ± 1.2</td>
</tr>
</tbody>
</table>

Shear-averaged ± average variation:

<table>
<thead>
<tr>
<th>Shear Rate (1/s)</th>
<th>Whole Blood (WB)</th>
<th>WHB-APR</th>
<th>GST-PRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.4 ± 1.9</td>
<td>4.3 ± 0.8</td>
<td>5.3 ± 1.4</td>
</tr>
</tbody>
</table>

---

**Fig 7. Platelet adhesion on biomaterial for suspensions of either RBCs or ghosts in PRP as a function of cellular volume fraction at a shear rate of 2,860 s⁻¹ (error bars represent SD about the average).**
lipids from their membranes either as fragments of the membrane or in some molecular form. This result may imply that phospholipids, unlike Hb and ADP, were not released as a result of low-stress hemolysis under laminar shear flow conditions. The RBCs were not damaged permanently, ie, no membrane damage was induced by these shear rate levels and the release of the intracellular components such as Hb and ADP was a result of reversible deformation of RBC membrane interacting with the surface under the influence of the corresponding velocity gradients and shear forces. These results do not exclude the possibility of some shear-induced rearrangement (flip-flop) of phospholipid in the red cell membrane. Also, these results do not exclude the possibility of shear-induced loss of phospholipid-derived material from the RBC membrane such as arachidonic acid (AA), since AA in esterified form is one of the major fatty acids making up the RBC membrane. Then (although there is no supporting evidence from this study) thromboxane A₂, a strong agonist of platelet aggregation, could be formed via the endoperoxide (platelet-derived) enzymatic pathway. Current studies are investigating these possibilities.

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Artificial surface effect on red blood cells and platelets in laminar shear flow

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