The Presence and Release of $\alpha_2$-Antiplasmin From Human Platelets

By Edward F. Plow and Desire Collen

An antigen immunochemically indistinguishable from plasma $\alpha_2$-antiplasmin, the primary plasmin inhibitor, was detected in human platelets. By radioimmunoassay, 33-114 ng $\alpha_2$-antiplasmin antigen was quantitated in the detergent-soluble extract of $10^9$ washed human platelets from 10 normal donors with a mean level of 62 ± 24 ng/10^9 platelets. Plasma $\alpha_2$-antiplasmin, either in the platelet suspending medium or on the surface of the platelets, could account for less than 8% of the antigen present in the platelet extracts. When stimulated with thrombin, the platelets released $\alpha_2$-antiplasmin antigen without cell lysis, and >85% of the $\alpha_2$-antiplasmin antigen was released at a high thrombin dose. At a lower dose of thrombin, $\alpha_2$-antiplasmin and platelet factor 4 were partially released without concomitant secretion of serotonin. No $\alpha_2$-antiplasmin antigen was detected in extracts of red blood cells, polymorphonuclear leukocytes, and adherent and nonadherent mononuclear cells. Thus, the platelet is the only peripheral blood cell containing significant amounts of $\alpha_2$-antiplasmin.

PLASMIN is the primary enzyme of the plasma fibrinolytic system, and inhibitors with antiplasmin activity regulate proteolysis by this enzyme. It is now established that $\alpha_2$-antiplasmin is the primary plasma inhibitor or plasmin. This inhibitor is a glycoprotein of mol wt 70,000 and is found in plasma at a concentration of approximately 1 μM. Other protease inhibitors such as $\alpha_1$-macroglobulin, $\alpha_1$-antitrypsin, and antithrombin III may also inhibit plasmin in purified systems, but their antiplasmin activity in vivo is apparently restricted to pathophysiologic circumstances that result in a depletion of the $\alpha_2$-antiplasmin. While these statements are generally accepted as describing the status of plasma inhibitors of plasmin, antiplasmin activity has also been detected in a variety of cells. Numerous investigations have reported the association of antiplasmin activity with platelets that appears to constitute from 1% to 10% of plasma antiplasmin activity. Nakabayashi et al. and Ganguly and coworkers have characterized antiplasmins from platelets of mol wt ≤40,000; Moore et al. have reported an antiplasmin of mol wt 70,000 in the supernatant of thrombin-aggregated platelets; and Joist et al. have demonstrated release of an $\alpha_2$-globulin of mol wt 30,000 from platelets following thrombin or collagen stimulation. Additionally, $\alpha_2$-macroglobulin and $\alpha_2$-antitrypsin have also been identified as platelet constituents. If $\alpha_2$-antiplasmin is a platelet constituent, it could play a significant role in the regulation of fibrinolysis via its capacity to rapidly and specifically inhibit plasmin generated within the hemostatic plug. Therefore, in the present study, we have assessed the presence of the primary plasmin inhibitor, $\alpha_2$-antiplasmin, in platelets as well as in other peripheral blood cells.

MATERIALS AND METHODS

$\alpha_2$-Antiplasmin Isolation and Quantitation

$\alpha_2$-Antiplasmin was purified from plasminogen-depleted plasma by sequential affinity chromatography on plasminogen-Sepharose, ion exchange chromatography on DEAE-Sephadex A-50, and molecular exclusion chromatography on Ultragel ACA-44. The isolated protein yielded a single band on 7% polyacrylamide gels in the presence of sodium dodecylsulfate of mol wt 70,000. The inhibitor was radiolabeled to a specific activity of 1–3 μCi/μg with carrier-free Na[125]I by a modified chloramine-T procedure. Antiserum to $\alpha_2$-antiplasmin was produced in rabbits as previously described. The specificity of the antiserum was indicated by immunoelectrophoretic analyses in which it formed a single precipitin line with normal human plasma and the purified inhibitor and did not react with $\alpha_2$-antiplasmin-depleted plasma. The radioimmunoassay utilized for quantitation of $\alpha_2$-antiplasmin was of the double antibody type employing goat antiserum to rabbit immunoglobulin to achieve precipitation. Triton-X100 (J. T. Baker Chem. Co., Phillipsburg, N.J.) at concentrations of <0.1% in test samples had no effect on this assay system.

Cell Separation

All cells were isolated from fresh human blood drawn into either acid-citrate-dextrose or heparin anticoagulant. Platelets were isolated from the plasma of individual donors by differential centrifugation followed by gel filtration on Sepharose 2B-Cl2. The cells contained a total bed volume of 40 ml Sepharose resin and was equilibrated with a modified Tyrode’s buffer containing 2% bovine serum albumin and 1 mM magnesium at pH 7.2. The isolated platelets contained no detectable aggregates upon microscopic examination and exhibited typical aggregation patterns in response to thrombin, collagen, and adenosine diphosphate (ADP) plus fibrinogen. In studies of the release reaction, platelets were labeled with 2-35S-serotonin binoxalate (32 μCi/mole from New England Nuclear, Boston, Mass.) or 51Cr (7 mCi/mmol from Amersham, Arlington Heights, Ill.) in platelet-rich plasma and then isolated as indicated above. The cells (10^7) were suspended in 1 ml of the tyrode’s albumin buffer and stimulated with thrombin (kindly provided by Dr. David A. Arkin, Department of Molecular Immunology, Research Institute of Scripps Clinic, La Jolla, Calif) with stimulation times ranging from 15 to 30 sec.
provided by Dr. John Fenton, New York State Department of Health, Albany, N.Y.) for 10 min at 37°C. After centrifugation, the radioactivity in the supernatant was measured, and the percent serotonin release was calculated relative to the counts solubilized by addition of 1% Triton-X100 to unstimulated platelets. Platelet factor 4 was quantitated with a double antibody radioimmunoassay, and the percent of the antigen released by thrombin was measured as described for serotonin release. Platelet recovery was assessed by counting the 3HCr associated with the platelet pellets in the presence or absence of thrombin.

Other cell types were isolated from 60 to 100 ml of heparinized blood. The blood was diluted with an equal volume of RPMI-1640 (Gibco, Irvine, Calif.), and 10-ml aliquots were layered onto 3 ml Ficoll-Hypaque and centrifuged at 2260 g for 6 min at 20°C. The mononuclear cells were removed from the interface, washed twice with RPMI-1640, and residual RBC were lysed with NH4Cl.24 Subsequently, the cells were washed three additional times with medium and then fractionated on the basis of adherence. To separate adherent from nonadherent cells, the mononuclear cells were resuspended in complete medium (RPMI-1640 supplemented with 100 U/ml penicillin, 50 μg/ml streptomycin, 2 mM glucose, and 10% v/v fetal calf serum), and 20-ml aliquots were placed in T-75 flasks and incubated at 37°C in a humidified 5% CO2 in air for 18 hr. Nonadherent cells were removed by decanting and were washed three additional times in PBS. The adherent cells were removed by a 5-10 min incubation in a 0.1% trypsin-0.25 mM EDTA solution, washed once with complete medium, and then three times with PBS. Purity of the mononuclear cells was assessed by differential staining with Giemsa or neutral red stain. Typically, less than 1% of the adherent and nonadherent cells stained with the Giemsa reagent, indicating minimal PMN contamination. On four separations, 76.5 ± 11.4% of the adherent cells stained with 0.007% neutral red in contrast to 3.1 ± 3.2% of the nonadherent cells.

RBC and PMN were recovered in the pellet from the Ficoll-Hypaque gradients. The cells were resuspended in platelet-poor plasma at one-third the initial volume, and two parts of a 3% dextran (mol wt 250,000) solution added.25 After incubation at 22°C for 20 min, the unsettled cells (mostly PMN) were recovered by centrifugation, the residual RBC were lysed with NH4Cl, and the PMN washed three times in PBS. The settled RBC were also washed three times in PBS prior to lysis. Upon counting of 400 cells within the PMN and RBC preparations, no cross-contamination was detected. All isolated cells were lysed by suspension of 0.5% Triton-X100 to 30-60 min at 22°C. Complete lysis was established by microscopic examination, and cell debris was removed by centrifugation at 3000 g for 30 min.

**Binding of Antibody to α2-Antiplasmin to Platelets**

Antibody to α2-antiplasmin was immunochemically purified from rabbit antiserum by affinity chromatography on α2-antiplasmin coupled to Sepharose 2B. Bound antibody was eluted with 0.1 M glycine-HCl, pH 2.8, and dialyzed immediately. Rabbit IgG, purified by ammonium sulfate precipitation and DEAE-cellulose chromatography was used as a control, and both were radiolabeled with 125I by chloramine-T oxidation.21 After radiolabeling, ≥70% of the radioactivity of the antibody preparation was bound to α2-antiplasmin-Sepharose, whereas ≤8% of the control IgG was bound. To assess the binding of these ligands to platelets, 0.15 ml of platelets at 10^9/ml in modified Tyrode's buffer was incubated with 300 ng of the labeled immunoglobulins (percentifuged to remove aggregates) for 30 min at 22°C. Duplicate 0.05-ml aliquots were then removed and centrifuged for 2.5 min through 0.3 ml of 20% sucrose in a Beckman Microfuge B. This system effectively separated platelet-bound from unbound ligand with ≥95% platelet recovery in the tip of the centrifuge tube.26 The tips were amputated and the ng IgG bound was determined from the specific activities of the ligands. Under the conditions utilized, equivalent concentrations of the nonlabeled ligands failed to induce serotonin release from the platelets. In addition, a threefold increase in platelet number resulted in a 2.8-fold increase in the binding of the labeled antibody indicating that the antibody concentration was not limiting.

**RESULTS**

The presence and quality of α2-antiplasmin with human platelets and other peripheral blood cells was determined employing a competitive inhibition radioimmunoassay. Purified plasma α2-antiplasmin was utilized as a standard for quantitation of the α2-antiplasmin-related antigen within the detergent-solubilized extracts of the cells, and a representative standard curve is illustrated in Fig. 1. The inhibition profile of the α2-antiplasmin standard was characterized by a 50% relative inhibition point at 58 ng/ml, and the limit of sensitivity of the assay was approximately 7 ng/ml. In 10 separate assays performed during the course of this study, the mean sensitivity level of the assay was 8.5 ± 3.2 ng/ml. The inhibition profile produced by normal pooled plasma (10 individual donors) was parallel in its linear portion to the curve produced by the purified inhibitor (see Fig. 1), and the concentration of α2-antiplasmin in plasma was estimated to be 67 μg/ml. This concentration is consis-
The antigen was detected in all 10 extracts, and the range of α2-antiplasmin antigen was 33–114 ng/10⁹ platelets. The mean level of α2-antiplasmin antigen detected per 10⁹ platelets was 62 ± 24 ng.

Two approaches were used to measure the contribution of contaminating plasma α2-antiplasmin to the level of antigen present within the platelet extracts. First, the isolated platelets recovered from the gel filtration column were pelleted by centrifugation and the α2-antiplasmin remaining in the supernatant was quantitated. No α2-antiplasmin was detected in the supernatant, indicating that less than 5 ng/10⁹ platelets of plasma α2-antiplasmin remained in the platelet suspension. Second, ¹²⁵I-α2-antiplasmin was added to the platelet-rich plasma and the recovery of radioactivity in the platelet lysates was measured. Based on this analysis, the plasma α2-antiplasmin accounted for only 5 ng/10⁹ platelets. Neither of these approaches would exclude the presence of tightly bound and nonexchangeable plasma α2-antiplasmin on the platelet surface. To evaluate this possibility, the interaction of immunochemically purified and radiolabeled rabbit antibody to α2-antiplasmin with washed human platelets was assessed. Ligands were incubated with the platelets for 30 min at 22°C, and the platelet-associated immunoglobulin was measured following separation of the cells from unbound ligand by centrifugation through 20% sucrose. Of the antibody to α2-antiplasmin, 7.1 ng were bound per 10⁹ platelets compared to 1.8 ng/10⁹ platelets of the nonimmune IgG. Assuming that one antibody molecule bound one α2-antiplasmin molecule, 3.1 ng α2-antiplasmin resided on the surface of 10⁹ platelets and was accessible to the antibody. Thus, all approaches indicated a minimal contribution of plasma α2-antiplasmin to the level of the platelet-associated antigen.

To assess potential underestimation of the α2-antiplasmin due to its lability in the platelet extracts, isolated plasma α2-antiplasmin was added back to the platelets prior to addition of detergent. After a 30-min extraction with Triton-X100, 2 aliquots of soluble extract were taken. One aliquot was assayed immediately for α2-antiplasmin antigen by radioimmunoassay, and the second aliquot was stored at 4°C for 18 hr prior to analysis. The recovery of α2-antiplasmin in both samples was ≥98%, indicating the stability of antigen in the platelet extract.

Table 1. α2-Antiplasmin Antigen in the Detergent-Soluble Extract of Washed Human Platelets*  

<table>
<thead>
<tr>
<th>Donor</th>
<th>α2-Antiplasmin (ng/10⁹ Platelets)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>33</td>
</tr>
<tr>
<td>2</td>
<td>61</td>
</tr>
<tr>
<td>3</td>
<td>65</td>
</tr>
<tr>
<td>4</td>
<td>71</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
</tr>
<tr>
<td>6</td>
<td>114</td>
</tr>
<tr>
<td>7</td>
<td>85</td>
</tr>
<tr>
<td>8</td>
<td>43</td>
</tr>
<tr>
<td>9</td>
<td>44</td>
</tr>
<tr>
<td>10</td>
<td>65</td>
</tr>
</tbody>
</table>

Mean ± SD: 62 ± 24

*Platelets were lysed with 0.5% Triton-X100 for 30 min at 22°C.
Release of \( \alpha_2 \)-Antiplasmin From Platelets

The release of \( \alpha_2 \)-antiplasmin-related antigen from platelets following thrombin stimulation was assessed. Gel-filtered platelets were resuspended in Tyrode's buffer containing 2% bovine serum albumin and 1 mM magnesium and stimulated with thrombin for 10 min at 37\( ^\circ \)C. After incubation, the platelets were centrifuged, and \( \alpha_2 \)-antiplasmin and platelet factor 4 were measured in the supernatant. In parallel experiments, platelets were labeled with \(^{14}\)C-serotonin and its release was measured under identical conditions. As summarized in Table 2, at 0.01 U/ml of thrombin, 25% of the \( \alpha_2 \)-antiplasmin, and a similar percent of the platelet factor 4 were secreted. In contrast, only 1.2% of the serotonin was released at this thrombin dose. At the higher dose of thrombin, a high percentage of all three constituents was detected in the supernatant, and the release of \( \alpha_2 \)-antiplasmin was 87.5%. At both thrombin concentrations, \(^{51}\)Cr recovery in the platelet pellets was \( \geq \)95%, indicating that release of \( \alpha_2 \)-antiplasmin and the other components occurred without cell lysis.

\( \alpha_2 \)-Antiplasmin in Other Peripheral Blood Cells

The major classes of peripheral blood cells were isolated to a reasonable degree of purity based on differential staining criteria, and the \( \alpha_2 \)-antiplasmin-related antigen in the detergent extracts of the cells was quantitated by radioimmunoassay. With the exception of platelets, no \( \alpha_2 \)-antiplasmin was detected within the extracts of the cells (Table 3). The number of cells available for extraction and the sensitivity of the assay established the maximum level of \( \alpha_2 \)-antiplasmin potentially present within each cell type. When expressed in terms of the number of cells present per milliliter of blood, other cell types contained a maximum of 6–28-fold less \( \alpha_2 \)-antiplasmin than platelets.

**Table 2. Release of \( \alpha_2 \)-Antiplasmin, Platelet Factor 4, and \(^{14}\)C-Serotonin From Thrombin-Stimulated Platelets**

<table>
<thead>
<tr>
<th>Thrombin (U/ml)</th>
<th>( \alpha_2 )-Antiplasmin</th>
<th>Platelet Factor 4</th>
<th>(^{14})C-Serotonin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.1</td>
<td>3.2</td>
<td>0.7</td>
</tr>
<tr>
<td>0.01</td>
<td>25.0</td>
<td>27.8</td>
<td>1.2</td>
</tr>
<tr>
<td>1.0</td>
<td>87.5</td>
<td>92.6</td>
<td>93.4</td>
</tr>
</tbody>
</table>

Platelets (10^9/ml) were stimulated for 10 min at 37\( ^\circ \), and the \(^{14}\)C-serotonin, \( \alpha_2 \)-antiplasmin antigen, and platelet factor 4 present in the supernatant after centrifugation at 11,750 rpm in a Beckman microfuge for 2.5 min was quantitated. Percent release was calculated relative to constituents solubilized by addition of 1.0% Triton-X100 to unstimulated platelets. Serotonin release was measured in the presence of 2 \( \mu \)M imipramine. Results are the mean of three separate experiments with duplicate determinations in each experiment.

**Table 3. Quantitation of \( \alpha_2 \)-Antiplasmin Antigen in Isolated Human Peripheral Blood Cells**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>( \alpha_2 )-Antiplasmin/ml Blood (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td>&lt;3.8</td>
</tr>
<tr>
<td>PBM</td>
<td>&lt;5.0</td>
</tr>
<tr>
<td>Adherent mononuclear cells</td>
<td>&lt;5.0</td>
</tr>
<tr>
<td>Nonadherent mononuclear cells</td>
<td>&lt;1.1</td>
</tr>
<tr>
<td>Platelets</td>
<td>31</td>
</tr>
</tbody>
</table>

*With the sensitivity of the radioimmunoassay at 5 ng of \( \alpha_2 \)-antiplasmin/ml, no antigen was detected in a 1-mI Triton extract of 10^10 RBC, 5 \times 10^9 PMN, 5 \times 10^9 adherent mononuclear cells, and 6 \times 10^6 nonadherent mononuclear cells. To calculate \( \alpha_2 \)-antiplasmin/ml blood, normal peripheral blood levels of cells were taken at: RBC, 7.5 \times 10^9; PMN, 5 \times 10^9; adherent mononuclear cells, 0.5 \times 10^9; nonadherent mononuclear cells, 1.3 \times 10^9; platelets, 5 \times 10^9.

**DISCUSSION**

The detergent-soluble extracts of washed human platelets competitively inhibited a radioimmunoassay specific for \( \alpha_2 \)-antiplasmin. The platelet extract produced complete competitive inhibition at high concentrations and yielded an inhibition slope parallel to that of isolated \( \alpha_2 \)-antiplasmin and plasma. These immunochemical characteristics indicate that the antigen within platelets shared all determinants expressed by plasma \( \alpha_2 \)-antiplasmin, and the antiseraum reacted with these determinants with similar affinity. Thus, the \( \alpha_2 \)-antiplasmin antigen in platelets is immunochemically indistinguishable from plasma \( \alpha_2 \)-antiplasmin. Since a precise immunological identity between a molecule of mol wt 70,000 (plasma \( \alpha_2 \)-antiplasmin) and a molecule(s) of mol wt \( \leq \)40,000 is unlikely, the platelet \( \alpha_2 \)-antiplasmin antigen detected in this study is probably not related to the low molecular weight antiplasmins described by Waks-bayashi et al., Ganguly et al., Mui et al., and Joist et al. The platelet antiplasmin of mol wt 70,000 described by Moore et al. might be identical to \( \alpha_2 \)-antiplasmin, although its lack of urokinase inhibitory activity is in contrast to the weak inhibitory activity of plasma \( \alpha_2 \)-antiplasmin.

Platelets from all 10 individual donors contained \( \alpha_2 \)-antiplasmin, and the mean level was 62 ± 24 ng/10^9 platelets. By two independent approaches, it was estimated that contaminating plasma \( \alpha_2 \)-antiplasmin could account for only 5 ng/10^9 platelets so that plasma carryover could contribute only 8% of the level of \( \alpha_2 \)-antiplasmin detected in platelets. The binding of radiolabeled antibody to \( \alpha_2 \)-antiplasmin to the platelet preparations also suggested that only a small percent of the platelet-associated antigen was available on the platelet surface. Furthermore, \( \alpha_2 \)-antiplasmin added...
to platelets was quantitatively recovered in the detergent extract excluding an underestimation of the level of platelet α₂-antiplasmin due to denaturation or degradation during extraction.

When platelets were treated with thrombin, α₂-antiplasmin antigen was released from the cells, and approximately 85% of the platelet α₂-antiplasmin was released within 10 min at 37°C at a high thrombin dose without cell lysis. This suggests most of the α₂-antiplasmin resides within platelet storage granules. At 0.01 U thrombin/ml, approximately 25% of the α₂-antiplasmin and platelet factor 4 were released without concomitant release of serotonin. Kaplan et al. have shown that α-granule constituents, such as platelet factor 4 and fibrinogen, may be released at lower concentrations of thrombin that serotonin from dense granules. Thus, our results are consistent with an α-granule localization of platelet α₂-antiplasmin although subcellular fractionation will be required to verify this localization.

Compared to the plasma level of 70 μg/ml, with 5 x 10⁶ platelets/ml blood containing 31 ng of α₂-antiplasmin, platelet α₂-antiplasmin constitutes only 0.05% of the blood level on a volume basis. Platelets from 1 ml of plasma yield approximately 0.33 mg protein, so that platelet α₂-antiplasmin constitutes 1/10,000 of platelet proteins. In plasma with 70 mg of protein/ml, plasma α₂-antiplasmin constitutes 1/1,000 of the protein. Thus, there is no relative concentration of α₂-antiplasmin in platelets on a protein basis. Similar relationships have been described for platelet α₂-macroglobulin and α₁-antitrypsin as contrasted to other proteins such as factor VIII and fibrinogen, which are concentrated in the platelets relative to plasma. The low level of α₂-antiplasmin in platelets, however, does not necessarily preclude its physiologic importance. Since platelets are concentrated within the fibrin clot, local concentrations could approach significant levels, and the release of α₂-antiplasmin from stimulated platelets enhances its physiologic potential.

**ACKNOWLEDGMENT**

We gratefully acknowledge the excellent technical assistance of Erich Blase and Doreen Freaney and the secretarial assistance of Mary Gortmaker and Sandy Thompson.

**REFERENCES**

24. Curtiss AK, Edgington TS: Differential sensitivity of lymphocyte subpopulations to suppression by low density lipoprotein


The presence and release of alpha 2-antiplasmin from human platelets

EF Plow and D Collen