Defective ADP-Induced Platelet Factor 3 Activation in Uremia

By HERBERT I. Horowitz, Burton D. Cohen, Pablo Martinez and Mitsu F. Papayonou

Patients with uremia frequently have both prolonged bleeding times,11# suggesting poor platelet plug formation, and impaired prothrombin consumption,1,3,4,6,8-14 ascribed to defective platelet factor 3 activation. Considerable evidence points to the presence of a qualitative platelet defect in uremia, since such abnormalities cannot be attributed to the mild thrombocytopenia occasionally encountered.1,3,11-14 The exact nature of this defect has not been defined.

Adenosine 5'-diphosphate (ADP) plays a central role in the formation of the platelet plug15 and also activates platelet factor 3,15-19 accelerating fibrin formation.16,19 Thus, a defective response to ADP in uremia could account for most of the hemostatic abnormalities observed. In this study we report the presence of an inhibitory factor in uremic plasma which interferes with ADP-induced platelet factor 3 activation. Preliminary studies suggest that this may be a newly described intermediate of urea metabolism, guanidinosuccinic acid.20-22

Material and Methods

Blood for these studies was obtained from patients on the general medical service of our hospital, as well as house staff and laboratory workers. The patient material included 17 with azotemia, 40 with miscellaneous diagnoses not involving the kidneys or liver, and 17 with hepatic disorders. The azotemic group comprised patients with a variety of clinical diagnoses, including both acute and chronic renal failure as well as one patient with presumed prerenal azotemia. Only two of the patients had symptoms of bleeding. The blood urea nitrogen values ranged from 38 to 222 mg. per 100 ml. Siliconized or plastic glassware and syringes were used to process all specimens. Whole blood was anticoagulated with 1/9 volume of 3.8 per cent sodium citrate. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared by differential centrifugation at 4 C.

Russell's viper venom (Stypven) was purchased from Burroughs Welcome Co., Tuckahoe, N. Y., guanidinosuccinic acid (GSA) guanidinoacetoc acid (GAA) and arginine from Mann Research Laboratories, New York, N. Y., and ADP from Sigma Chemical Co., St. Louis Mo. Diguaniidino diphenyl sulfone (DGPS) was kindly provided by Dr. M. B. Zucker. Stock solutions of GSA, GAA, arginine, and ADP were made up in saline buffered to pH 7.35 with one-tenth volume of 0.05 M imidazole. The pH of GSA and GAA in

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imidazole buffer were 4.5 and 5.4, respectively but the pH of the final dilutions of these reagents in plasma was found to be in the range of 7.6 to 7.8. Platelet counts were done by phase microscopy and were adjusted to the desired values by mixing PRP and PPP. By substituting uremic PPP for normal PPP, inhibitory concentrations were achieved.

Activation of PRP with ADP and the effect of inhibitors were evaluated by incubating 0.9 ml. PRP and 0.1 ml. ADP solution with 0.1 ml. of either saline or inhibitor. The components of the incubation mixtures were mixed thoroughly, then incubated at 37 C. for 60 minutes without further agitation.

Platelet factor 3 activity was estimated by the modified Stypven time. Incubated test PRP, 0.05 ml., was added to 0.05 ml. of normal PPP (to provide adequate concentrations of Factors I, II, V, and X). Two-tenths ml. of a mixture of Stypven 1:50,000 and NI/40 calcium chloride was added to the plasma and the clotting time was recorded. Shorter Stypven times indicated greater platelet factor 3 activity.

RESULTS

Evidence that ADP can activate the platelet factor 3 of intact platelets is seen in Figure 1. Normal citrated plasma containing 600,000 platelets per cu. mm. was incubated with graded amounts of ADP. In the absence of ADP some spontaneous platelet factor 3 developed during incubation, but progressively greater activity developed with increasing ADP concentration.

Figure 2 illustrates results of studies undertaken to establish an optimal concentration of platelets and of ADP for a reproducible and sensitive test of this platelet function. Concentrations of 150,000 platelets per cu. mm. and $2 \times 10^{-5}$ M ADP were used as the standardized test for platelet factor 3 activation. Results are shown in Figure 3. Normals and hospitalized patients clustered about 23 seconds, the range of 20 to 25 seconds encompassing 91 of the 95 tests. In contrast, 16 of 17 uremics had distinctly elevated 60-minute Stypven times. Patients with liver disease also had consistently impaired ADP-induced platelet factor 3 activation.

The presence of uremic PPP prevented ADP-induced platelet factor 3 activation in normal PRP, with a rough dose-response relationship. In these experiments a small amount of uremic plasma was added with normal plasma to the platelet button. These results are shown in Table 1. Fully activated platelet...
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Fig. 2.—Relationship of platelet number and ADP concentration to modified Stypven time in normal PRP after 60 minutes of incubation at 37 C. The dots represent individual determinations and the lines connect average values.

Fig. 3.—Sixty-minute modified Stypven times in patients and controls. Incubation mixtures contained 150,000 platelets per cu. mm. and $2 \times 10^{-5}$ M ADP.

factor 3 obtained from frozen and thawed platelets was not inactivated during 60 minutes of incubation in uremic plasma.

The inhibitory effect of uremic plasma could not be attributed to urea for two reasons. First, as seen in Figure 4, no correlation could be found between the 60-minute platelet factor 3 level and blood urea nitrogen. Second, addition of 50 to 1000 mg per 100 ml of urea to normal PRF did not inhibit platelet factor 3 activation with ADP.
Table 1.—Inhibition of ADP-Induced Platelet Factor 3 Activation by Uremic Plasma or Normal Plasma

<table>
<thead>
<tr>
<th>Normal PPP +</th>
<th>Stypven Time (60 minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubated with Normal PPP (sec.)</td>
<td>Incubated with Uremic PPP (sec.)</td>
</tr>
<tr>
<td>Saline + 20% plasma</td>
<td>37</td>
</tr>
<tr>
<td>ADP + 20% plasma</td>
<td>22</td>
</tr>
<tr>
<td>AHP + 10% plasma</td>
<td>22</td>
</tr>
<tr>
<td>AHP + 5% plasma</td>
<td>22</td>
</tr>
</tbody>
</table>

Platelets: 119,000 per cu. mm.
ADP: $2 \times 10^{-5} \text{ M}$

Recently, 1-4 diguanidino diphenyl sulfone (DGPS) has been shown to inhibit ADP-induced platelet aggregation. This inhibitory action can be reversed by addition of a small amount of calcium, which by itself does not cause clotting or platelet aggregation. The effect of DGPS on ADP-induced platelet factor 3 activation is seen in Table 2. Adding DGPS in a final concentration of $5 \times 10^{-4} \text{ M}$ inhibited ADP-induced platelet factor 3 activation. An increment of calcium abolished DGPS inhibition completely. Of further interest was the finding that the previously demonstrated inhibitory effect of 20 per cent uremic plasma could also be overcome by this addition of calcium.

These considerations led us to look for some compound analogous to the inhibitor DGPS which might be present in uremia. Arginine, guanidinoacetic acid (GAA), and guanidinosuccinic acid (GSA) are the only guanidinium compounds known to be present in man. Guanidinosuccinic acid and DGPS with their common guanidinium groups are depicted in Figure 5. At concentrations comparable to those of DGPS, GSA proved to be a potent inhibitor of ADP-induced platelet factor 3 activation. Like DGPS and uremic plasma, the inhibitory effect of GSA could be abolished by adding 2.5 millimoles of calcium.
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Table 2.—Inhibition of ADP-Induced Platelet Factor 3 Activation by Uremic Plasma, DGPS and GSA: Reversal by Calcium

<table>
<thead>
<tr>
<th>Normal PRP +</th>
<th>Saline (sec.)</th>
<th>Plasma (sec.)</th>
<th>GPS (sec.)</th>
<th>GSA (sec.)</th>
<th>GAA (sec.)</th>
<th>Arginine (sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>32</td>
<td>32</td>
<td>33</td>
<td>33</td>
<td>32</td>
<td>30</td>
</tr>
<tr>
<td>ADP</td>
<td>24</td>
<td>33</td>
<td>31</td>
<td>32</td>
<td>25</td>
<td>23</td>
</tr>
<tr>
<td>Calcium</td>
<td>33</td>
<td>33</td>
<td>31</td>
<td>33</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td>ADP + Calcium</td>
<td>24</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
</tbody>
</table>

Platelets: 150,000 per cu. mm.
ADP: 2 x 10^-6 M
CaCl2: 25 x 10^-4 M
GAA: 2 x 10^-4 M
Arginine: 2 x 10^-4 M

Guanidinosuccinic Acid

\[
\text{H} \quad \text{COOH} \\
\text{H-N-C-NH-CH} \\
\text{CH}_2 \\
\text{COOH}
\]

1,4 Diguanidinodiphenylsulfone

Fig. 5.—Guanidinosuccinic acid and DGPS with their common guanidinium groups.

chloride. GAA and arginine were not inhibitory of ADP-induced platelet factor 3 activation (Table 2).

PRP and ADP were incubated with concentrations of GSA ranging from 0.02 mM to 20 mM, with or without addition of 2.5 mM of calcium chloride to the incubation mixture. The findings in a representative experiment are presented in Table 3 and indicate that the antagonistic actions of GSA and calcium are competitive. By increasing the concentration of GSA, the normalizing action of calcium is overcome.

The inhibitory activity is equally demonstrable in uremic serum. It is dialyzable in vitro, and in both of our bleeding patients the inhibitor has been markedly reduced by peritoneal dialysis with improvement in symptoms of bleeding. It is heat stable for 30 minutes at 56 C. but is inactivated at 60 C. after 5 minutes. It is not adsorbed by aluminum hydroxide or barium sulfate. It is absent in the trichloroacetic acid supernate and in the euglobulin precipitate of uremic plasma. When GSA is suspended in normal serum its inhibitory activity is preserved or destroyed under conditions identical to the activity of the uremic inhibitor (Table 4).

Discussion

Several lines of evidence point to a functional platelet disorder in uremia. The clinical features of the hemorrhagic tendency of uremia are those of a
### Table 3.—Reversal of GSA Inhibition of ADP-Induced Platelet Factor 3 Activation by Calcium: Effect of GSA Concentration

<table>
<thead>
<tr>
<th>Normal PRP + ADP +</th>
<th>Incubated without Calcium (sec.)</th>
<th>Incubated with Calcium (sec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>0.02 mM GSA</td>
<td>27</td>
<td>24</td>
</tr>
<tr>
<td>0.2 mM GSA</td>
<td>30</td>
<td>24</td>
</tr>
<tr>
<td>2.0 mM GSA</td>
<td>31</td>
<td>29</td>
</tr>
<tr>
<td>*20.0 mM GSA</td>
<td>33</td>
<td>32</td>
</tr>
</tbody>
</table>

PRP: 152,000 per cu. mm.
ADP $2 \times 10^{-8}$ M
Calcium $2.5 \times 10^{-2}$ M

* This concentration was obtained by dissolving GSA directly in PRP.

### Table 4.—Comparison of Some Properties of the Uremic Inhibitor and of GSA

<table>
<thead>
<tr>
<th>Property</th>
<th>GSA in Serum</th>
<th>Uremic Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialyzable</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Stable, 56 C., 30 min.</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Stable, 60 C., 5 min.</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Adsorbed by Ba SO₄</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Adsorbed by Al (OH)₃</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>In Trichloroacetic Acid Supernate</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>In Enolglobulin Precipitate</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

defect in primary hemostasis. The bleeding time has been noted to be excessive in many uremic patients and is invariably prolonged in those with overt clinical bleeding. The unique platelet contribution to blood coagulation, platelet factor 3 activity, has been found to be abnormal by some but not by others. Abnormal platelet thromboplastic function can best explain the most common coagulation abnormality of uremic patients, poor prothrombin consumption.

The effect of ADP in aggregating uremic platelets has recently been investigated. Castaldi et al. have found a significant abnormality in this parameter, but Salzman and Neri, employing lower concentrations of ADP, failed to find any abnormality in their patient material. They did find abnormal platelet aggregation with low concentrations of thrombin. The platelet-aggregating action of thrombin is believed to be induced by ADP and can be blocked by ADP-inhibitors. These apparently conflicting results are likely due to patient variability and, even more important, to differences in the tests employed.

ADP, in concentrations of $10^{-5}$ M or higher, activates platelet factor 3 in citrated PRP. Hardisty has suggested that this action of ADP may play a role in physiological platelet factor 3 activation. Castaldi et al. found an abnormality of platelet factor 3 activation induced by kaolin in some of their patients.

We have developed a standardized method for evaluating platelet factor 3...
activation using ADP. Previous studies have indicated that this test is specific for the platelet factor 3 activity of citrated PRP. Previous studies have indicated that this test is specific for the platelet factor 3 activity of citrated PRP. A reproducible and reliable test was devised through careful control of the following variables:

Agitation. In the presence of ADP, platelet factor 3 activation is markedly enhanced by agitation. Arbitrarily, agitation was kept to a minimum.

Platelet number. Platelet factor 3 activation varied with the numbers of platelets suspended in plasma. Since uremics are frequently (though mildly) thrombocytopenic, concentrations of 100,000 to 200,000 platelets per cu. mm. were used.

ADP concentration. Figure 2 shows that an optimal ADP concentration for platelet factor 3 activation can be identified for each range of platelet count. A final concentration of $2 \times 10^{-5}$ M ADP gave the most consistent results at the low platelet counts indicated above.

Our data demonstrate that an inhibitor is present in uremic plasma which interferes with ADP-induced platelet factor 3 activation. Such an inhibitor was anticipated by Marcus and Zucker in their recent review and is in accord with observations on cessation of bleeding and improvement in abnormal tests of platelet function in patients after peritoneal or extracorporeal dialysis. Cahanlane et al. have previously described an inhibitory activity of uremic plasma against platelet factor 3. Uremic plasma slowly, over an 18-hour period, inactivated platelet factor 3 extracted from human platelets. This activity differs from the inhibitor described in the present report, which prevents the activation of platelets during 1 hour of incubation.

Hellem et al. have reported on a dialyzable factor present in uremia which interferes with platelet aggregation; they believe this substance to be urea. Salzman and Neri found that urea aggregated platelets itself at 200–400 mg. per 100 ml. while failing to inhibit ADP-induced aggregation. We did not find any evidence of inhibition of ADP-induced platelet factor 3 activation of platelets by urea.

A fortuitous set of circumstances led us to consider that the uremic inhibitor might be GSA. Zucker et al. investigated a series of guanidino compounds as inhibitors of platelet aggregation; the strongest inhibitors were diguanidino compounds such as DGPS, whose inhibitory activity could be overcome by small increments of ionic calcium. One of us has recently found a guanidino derivative, GSA, to be uniquely increased in the urine of uremics. This substance, like DGPS and uremic plasma, can be shown to inhibit ADP-induced PF-3 activation. In each case the inhibitory activity is abolished by additional calcium. Preliminary characterization of the inhibitor assigns it properties comparable to GSA. These studies suggest, but do not prove, an identity.

The mechanism of action of the inhibitor may be comparable to that postulated for DGPS. ADP reversibly complexes with calcium ions, and may act upon platelets as a calcium complex. DGPS competes with calcium for combination with ADP; adding more calcium favors formation of the Ca-ADP complex leading to platelet aggregation. However, the experimentally determined association constant of DGPS with ADP was by itself insufficient to fully account for DGPS inhibition of ADP-induced platelet aggregation.
That the inhibitor may be so readily overcome by added calcium raises serious questions as to its significance in vivo, where ionic calcium is present in much larger amounts than in citrated plasma. The data presented in Table 4 bears on this relationship if we assume that the inhibitor is GSA. For example, the inhibitory activity of 2.0 mM (or 35 mg. per 100 ml.) of GSA on ADP-induced platelet factor 3 activation was not overcome by the addition of 2.5 mM (or 10 mg. per 100 ml.) of calcium. The amount of calcium added was comparable to that originally present in native plasma. However, since the incubation mixture did not clot, it is probable that excess citrate kept the ionic calcium below in vivo levels. Higher GSA levels conceivably would remain inhibitory in the face of higher levels of ionic calcium. It may be that the citrated test system makes it possible to detect the presence of the inhibitor in many uremics who are not clinically bleeding. Patients who are bleeding due to this mechanism would be expected to have high levels of GSA, low levels of ionic calcium, or a combination of the two.

ADP-induced platelet factor 3 activation is also abnormal in some patients with liver disease. Our modification of the Stypven time provides a source of those procoagulants which may be depleted in liver disease so that the Stypven time abnormality cannot be ascribed to a clotting factor deficiency. In contrast to the uremic, plasma from these patients does not inhibit platelet factor 3 activation when added to normal PRP. An intraplatelet defect may be present, as has been suspected previously, but further studies will be needed to define the nature of this abnormality.

**Summary**

A test for platelet thromboplastic function measuring the activation of platelet factor 3 on incubation of ADP with citrated platelet-rich plasma has been standardized in terms of ADP and platelet concentrations, and amount of agitation, to give sensitive and reproducible results. Abnormal results have been found in patients with elevated blood urea nitrogen; the abnormality is due to a plasma inhibitor and is reversible on the addition of small amounts of calcium. Guanidinosuccinic acid, a newly described metabolite found in the urine of patients with azotemia, also inhibits ADP-induced platelet factor 3 activation. The properties of guanidinosuccinic acid and the uremic inhibitor so far studied suggest that they may be identical.

**SUMMARIO IN INTERLINGUA**

Un test pro le function thromboplastic de plachettas—mesurante le activation de factor plachettal 3 per le incubation de ADP con citratate plasma ric in plachettas esseva standardisate relative al concentrationes de ADP e de plachettas e al quantitate de agitation de maniera que sensibile e reproducibile resultatos pote esser obtenite. Resultatos anormal esseva trovate in patientes con elevate valores de nitrogeno de urea del sanguine. Iste anormalitate es causate per un inhibitor plasmatic. Illo pote esser corrigite per le addition de micre quantitates de calcium. Acido guanidinosuccinic, un novemente describite metabolito trovate in le urina de patientes con azotemia, etiam inhibi le activation de factor plachettal 3 per ADP. Le proprietates de acido guanidinosuccinic e del inhibitor uremic—in tanto que illos ha essite studiate usque a iste tempore—suggestiona le possibilitate que illos es identic.
REFERENCES


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