Studies Investigating Platelet Aggregation and Release Initiated by Sera From Patients With Thrombotic Thrombocytopenic Purpura

By John G. Kelton, Jane C. Moore, and William G. Murphy

Many patients with thrombotic thrombocytopenic purpura (TTP) have a platelet aggregating factor in their serum that may be pathologically linked with the disease process. To help characterize the type of platelet aggregation and platelet release induced by the sera from seven TTP patients, we measured the ability of a variety of inhibitors of platelet function as well as the ability of monoclonal antibodies (MoAbs) against platelet glycoproteins to inhibit TTP sera-induced platelet aggregation and release. These results were compared with the ability of the same inhibitors to block platelet aggregation induced by ristocetin, collagen, ADP, thrombin, and lgG-immune complexes. Monoclonal antibody directed against platelet glycoprotein lb totally inhibited ristocetin-induced aggregation and release but had no effect on aggregation and release induced by the TTP sera or by any of the other platelet agonists. However, the MoAb against glycoproteins lb/Illa inhibited aggregation and release caused by TTP sera as well as by collagen, thrombin, and ADP but had no effect on aggregation and release induced by ristocetin. The aggregating activity could be abolished by heparin but not by the serine protease inhibitor PMSF (1 mmol/L). And although monomeric human lgG and purified Fc fragments of lgG inhibited lgG-immune complex-induced aggregation and release, they had no effect on TTP sera-induced aggregation and release nor on aggregation and release induced by any of the other agonists. Consistent with these in vitro studies showing no effect of lgG were the in vivo observations that intravenous (IV) lgG was without effect when administered to three patients with TTP. This study indicates that although a von Willebrand factor (vWF)-rich preparation of cryoprecipitate enhances the in vitro platelet aggregation and release caused by sera from the seven TTP patients we studied, the pathway of aggregation and release is not via platelet glycoprotein lb. Also the aggregating factor of TTP sera is not neutralized in vitro or in vivo by lgG.

© 1987 by Grune & Stratton, Inc.

MATERIALS AND METHODS

Patients and Controls

Sera from seven unselected patients with TTP were studied during the acute episodes before treatment. All patients had thrombocytopenia and schistocytic hemolytic anemia. In five of the patients, the pattern of vWF was analyzed by crossed immunoelectrophoresis before treatment was initiated. In all of these patients the large multimers of vWF were reduced or absent. Five of the patients had neurologic signs. One patient died, and postmortem examination confirmed the diagnosis as TTP.

Whole blood was collected into glass Vacutainer tubes (Becton Dickinson, Mississauga, Canada) containing no anticoagulant. The blood was centrifuged (2100 g for 20 minutes); the serum was transferred to plastic tubes and stored in aliquots at –70°C. Before testing, the serum was thawed at 37°C and then incubated for an additional 30 minutes at 37°C to inactivate thrombin, and the pH was adjusted to 7.4.

Control sera were collected from healthy laboratory volunteers receiving no medications. This study was performed in accordance with the guidelines developed by the University-approved Ethics Review Committee.

Aggregating Agents

Platelet aggregation and release was induced using bovine Achilles tendon collagen (final concentration, 55.1 µg/mL; Sigma Chemical Co, St. Louis), thrombin (final concentration, 2 U/mL; Fibrinex,
Ortho Diagnostics, Westwood, MA), adenosine diphosphate (ADP; final concentration, 10 μmol/L; Sigma), ristocetin (final concentration, 1.5 mg/mL; H. Lundbeck & Co, Copenhagen), and IgG immune complexes.

IgG immune complexes were prepared from human IgG. The human IgG was isolated from normal AB sera twice precipitated with 50% ammonium sulphate followed by a diethylaminoethyl (DEAE) Sephadex column (Pharmacia, Dorval, Quebec). The IgG was stored at ~70 °C at a final concentration of 10 mg/mL. IgG immune complexes were made by heating 0.5-mL aliquots (63 °C for 20 minutes). Before using, the largest complexes were removed by centrifugation at 10,000 g for five minutes. Purified Fc fragments were prepared from monomeric IgG using papain (100:1, wt/wt). Undegraded IgG was separated from Fc by gel chromatography, and the completeness of separation was verified by analytic polyacrylamide gel electrophoresis (5% SDS-PAGE). The concentration of Fc was raised to 20 mg/mL by ultrafiltration and the sample frozen at −70 °C before use.

Inhibitors of Platelet Aggregation/Release

The effects of aspirin (3.3 mmol/L), EDTA (1.6 mmol/L) and IgG (5 mg/mL) were tested for their ability to inhibit platelet aggregation and release.

Murine MoAbs 6D1 against human glycoprotein Ib, (IgG, final concentration 10.5 μg/mL) and 7E3 against IIb/IIIa (IgG, final concentration 15 μg/mL) were also used. These MoAbs were a gift from Dr B. Coller of the State University of New York at Stony Brook. A control MoAb lacking platelet glycoprotein activity was also tested at a final concentration of 15 μg/mL.

The effect of two protease inhibitors, heparin (0.5 U/mL), and phenylmethylsulfonylfluoride (PMSF) (1 mmol/L; Sigma) were tested for their ability to inhibit TTP-induced platelet release.

Performance of Platelet Aggregation and Release Studies

Preparation of test platelets. Twenty milliliters of whole blood was collected from different healthy donors into acid citrate dextrose, pH 4.5 (6:1, vol/vol). The platelet-rich plasma (PRP) was obtained by centrifugation (160 g for 20 minutes). The PRP was incubated with 14C serotonin (Amersham, Oakville, Ontario) for 30 minutes at 37 °C at a concentration of 0.5 μCi 14C serotonin per mL PRP. Approximately 50% of the 14C serotonin was taken up by the washed platelets giving a final specific activity of 1.84 × 10−4 μCi of 14C serotonin per 109 platelets. The platelets were pelleted by centrifugation (1800 g for nine minutes) and resuspended in 10 mL of calcium and albumin-free Tyrode’s solution, pH 6.2, containing 50 μL of apyrase. The apyrase (50 μg/mL) was prepared using standard techniques and inhibited aggregation induced by 10 μmol/L ADP.

The platelets were washed once by centrifugation (I 200 g for seven minutes) and resuspended to a final count of 600,000/μL in albumin-free Tyrode’s solution, pH 7.4. Because the reactivity of the platelet preparations occasionally differed from donor to donor, platelets were collected from three ABO compatible donors, pooled and processed as described above.

In a second series of experiments the platelets were resuspended with human fibrogen (Kabi, Sweden) at a final concentration of 2 mg/mL. In these studies the assay was performed at 37 °C and the effects of inhibitors of aggregation and release investigated as described subsequently. In these experiments the cryoprecipitate preparation was not added.

Preparation of solution containing large multimers of vWF. Two units of cryoprecipitate prepared from healthy donors were pooled and diluted with 10 mL of 0.9% sodium chloride. The cryoprecipitate was defibrinated using 0.15 units ancred (Arvin, Connaught Labs Ltd, Willowdale, Ontario) per millimeter of dilute cryoprecipitate. The mixture was incubated for ten minutes at 37 °C, centrifuged (2100 g for 20 minutes), and heat-inactivated for 30 minutes at 56 °C. The solution was stored at −70 °C before using. Immediately before using the solution was thawed at 37 °C and then subjected to centrifugation (12,000 g × 10 minutes) to remove large aggregates. We have previously demonstrated that this preparation contains large multimers of vWF.

Platelet release studies. Twenty microliters of test serum (pH 7.4) was mixed with 60 μL of 14C-serotonin-labeled platelets. The platelet mixture was incubated at room temperature in microtiter wells containing a magnetic stir bar. Following a ten-minute incubation, 3 μL of the preparation containing the large multimers of vWF was added (final concentration of 0.05 units). The suspension was stirred for an additional ten minutes. Both incubations were done on a magnetic stir plate set at a slow speed. At the end of the final incubation period, 100 μL of 0.5% EDTA in saline was added to terminate the release reaction. The platelets were then observed for evidence of visible aggregates and scored as positive (aggregates) or negative (no aggregates). The microtiter plates were centrifuged for five minutes at 1500 g and 50 μL of the supernatant fluid was removed. This aliquot was added to 10 mL of scintillation fluid and the samples were counted in a liquid scintillation counter. The percent release was calculated as follows:

\[
\text{Percent Release} = \frac{\text{Release (test sample)} - \text{Background}}{\text{Total Platelet Radioactivity} - \text{Background}} \times 100
\]

The background was defined as the supernatant fluid radioactivity from platelets handled identically to the test platelets except that buffer was substituted for serum. Total radioactivity was the radioactivity of the test platelet sample. A test result was defined as positive if there was greater than 20% release. All samples were tested in duplicate.

Inhibition of platelet release and aggregation. The experiments were performed as follows: 10 μL of the potential inhibitor (EDTA, aspirin, heparin, PMSF, IgG, Fc fragments, anti-GP Ib, anti-GP IIb/IIIa, or buffer) was added to 60 μL of the platelet suspension followed by a ten-minute incubation at 22 °C. The TTP serum (20 μL) or agglutinating agent (20 μL of ADP, collagen, ristocetin, thrombin, or IgG immune complexes) was then added to the mixture, and it was stirred for ten minutes at 22 °C. The fibrinogen-depleted cryoprecipitate was added and mixed for an additional ten minutes at 22 °C. One hundred μL of 0.5% EDTA in saline was added to terminate the reaction. The microtiter plates were centrifuged, and the radioactivity of the supernatant was measured.

To investigate whether IgG prepared from normal pooled sera or control sera could inhibit TTP sera-induced platelet release, the following experiments were performed: The amount of platelet release initiated by four different TTP sera or one normal control serum was measured using the technique described previously. Control IgG at a final concentration of 1.2 mg/mL or control serum was incubated with each TTP serum for 20 minutes at 22 °C. The 14C-serotonin labeled platelets (60 μL) were added, and, after an additional 20-minute incubation at 22 °C, the amount of release was measured. In these experiments the four TTP sera that were used were selected because their strong reactivity did not require the addition of the preparation rich in vWF to induce the release reaction.

The same experiment was repeated using the same TTP and control sera except that the IgG or control serum was preincubated with the platelets (22 °C for 20 minutes) before addition of the TTP sera.
RESULTS

Case Report 1

A 45-year-old woman was admitted with diagnosis of TTP and was treated with plasma exchange, aspirin, and dipyridamole. After an initial partial response the illness relapsed. The patient was treated with 23 different plasma exchanges but did not respond. She also did not respond to 4 mg of vincristine. Intravenous (IV) IgG (Gamimmune, Cutter Biologicals, Berkeley, CA) was administered over two days at a dose of 2 g/kg. There was no effect on either the platelet count or on the serum LDH level. The patient died two weeks later of intracerebral thrombosis complicated by hemorrhage. Postmortem examination confirmed the diagnosis of TTP.

Case Report 2

A 54-year-old woman was admitted with TTP. She had had an episode of TTP five years previously that had responded to plasma infusion and plasmapheresis and had been well for the subsequent five years. On this admission she was treated with both plasma infusion and plasmapheresis, and her platelet count was maintained above 150,000/μL by weekly 3-L plasma exchanges. Because of difficulties in maintaining vascular access, she was treated with IV IgG (IVEEGAM; Immuno, Vienna, Austria) at a dose of 2 g/kg. She did not respond, and her platelet count fell to 50,000/μL over the next week. She was again treated by a plasma exchange, and her platelet count promptly rose. She is currently maintained by weekly plasma exchanges.

Case Report 3

A 5-year-old girl with congenital chronic relapsing TTP has been treated over the past five years with intermittent (usually three per year) plasma infusions when her platelet count falls below 50,000 per μL. This treatment results in a prompt rise in platelet count and hemoglobin level that persists for weeks to months. She responds to plasma, stored plasma, and cryoprecipitate infusions. On one occasion she was treated with IV IgG at a dose of 3 g/kg (Sandoglobulin; Sandoz Laboratories, Dorval, Quebec) but did not respond to the treatment. Subsequent treatment with plasma infusions promptly raised the platelet count and hemoglobin level. Serum from this child was not used in the in vitro studies described in this report.

Table 1. Results of Platelet Aggregation and Release Studies

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Buffer</th>
<th>Biological Inhibitors of Platelet Aggregation</th>
<th>Chemical Inhibitors of Platelet Aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Monoclonal Antibodies</td>
<td>Heparin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>lb</td>
<td>lIb/IIIa</td>
</tr>
<tr>
<td>TTP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#1</td>
<td>+ (73)</td>
<td>+ (100)</td>
<td>− (0)</td>
</tr>
<tr>
<td>#2</td>
<td>+ (55)</td>
<td>+ (75)</td>
<td>− (18)</td>
</tr>
<tr>
<td>#3</td>
<td>+ (100)</td>
<td>+ (100)</td>
<td>− (18)</td>
</tr>
<tr>
<td>#4</td>
<td>+ (57)</td>
<td>+ (82)</td>
<td>− (2)</td>
</tr>
<tr>
<td>#5</td>
<td>+ (95)</td>
<td>+ (88)</td>
<td>− (4)</td>
</tr>
<tr>
<td>#6</td>
<td>+ (90)</td>
<td>+ (86)</td>
<td>− (0)</td>
</tr>
<tr>
<td>#7</td>
<td>+ (74)</td>
<td>+ (70)</td>
<td>− (0)</td>
</tr>
<tr>
<td>ADP</td>
<td>+ (79)</td>
<td>+ (89)</td>
<td>− (17)</td>
</tr>
<tr>
<td>Ristocetin</td>
<td>+ (100)</td>
<td>− (15)</td>
<td>+ (100)</td>
</tr>
<tr>
<td>Collagen</td>
<td>+ (65)</td>
<td>+ (100)</td>
<td>− (22)</td>
</tr>
<tr>
<td>Thrombin</td>
<td>+ (66)</td>
<td>+ (77)</td>
<td>− (16)</td>
</tr>
<tr>
<td>IgG Aggregates</td>
<td>+ (90)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

The results of various inhibitors (shown along the top) on visible platelet aggregation (indicated by a + sign) and the percent 14C serotonin release from platelets (noted within the brackets). The percent release was the mean of three or more experiments. The concentration of the aggregating agents and antagonists is given in the "Methods" section. ND indicates not done.
neither control IgG nor serum affected the platelet release induced by the TTP sera. Similarly, preincubation of the test platelets with control IgG or serum did not block the effect of the TTP sera. The amount of platelet release initiated by the four different TTP sera with or without control IgG or serum ranged from 70% to 98%.

In the experiments in which fibrinogen was used at a physiologic concentration (2 mg/mL), the preparation rich in vWF was not used. Only three of the seven TTP serum samples caused release, and the amount of release was lower and ranged from 26% to 45% of maximum. However, the pattern of inhibition of release was identical to that noted in Table 1. In particular, the MoAb against platelet glycoprotein Ib was without effect, but the reaction was inhibited by the anti-IIb/IIIa antibody.

The TTP-induced platelet release could be entirely inhibited by heparin (0.5 U/mL). However, the aggregation was not caused by thrombin as shown by the lack of effect of PMSF. PMSF at a final concentration of 1 mM/L had no effect upon the release reaction of seven different TTP sera. The release induced by these sera ranged from 64% to 100%.

**DISCUSSION**

The pathogenesis and optimal treatment of thrombotic thrombocytopenic purpura (TTP) remain uncertain. A number of hypotheses concerning the pathogenesis of TTP have been proposed: most are based on clinical observations, or on the results of in vitro studies, or on the response to certain therapeutic interventions. For example, untreated TTP has a mortality that may be as high as 90%. Treatment with antiplatelet agents reduces the risk of death, suggesting that in vivo platelet aggregation contributes to the morbidity and mortality of TTP. Supporting this hypothesis are postmortem reports of patients with TTP who have disseminated platelet thromboemboli in their small vessels. Also consistent are reports of platelet aggregating factors in the plasma of some patients with TTP. It is possible that by characterizing and identifying the aggregating factor, a more effective therapeutic strategy for dealing with this disorder might be developed.

In vitro and in vivo studies also support an active or passive role for vWF in the pathogenesis of the disorder. Moake and associates demonstrated abnormal patterns of vWF in patients with chronic relapsing TTP, an observation confirmed by ourselves. Consistent with the hypothesis that vWF participates in the disease process are two other observations. First, we have shown that the in vitro reactivity of a platelet aggregating factor in the serum of some patients with TTP can be enhanced by the addition of a preparation rich in large multimers of vWF. And second, pathologic studies have demonstrated the presence of vWF in occluded arteries from patients with TTP. Because vWF-induced platelet agglutination involves the platelet glycoprotein Ib, one might hypothesize that blocking this interaction might inhibit TTP sera-induced platelet aggregation and release.

To investigate this issue more directly, we studied the effect of a variety of inhibitors of platelet aggregation on the aggregation and release induced by TTP sera. The results of these aggregation and release studies were compared with results obtained using other platelet agonists. We found that the in vitro platelet aggregation and release initiated by TTP sera is mediated via platelet glycoproteins IIb/IIIa and, as another group of investigators recently reported, glycoprotein Ib is not involved. Hence our results argue strongly against the reaction being identical to ristocetin-induced agglutination. The MoAb against glycoprotein Ib totally inhibited ristocetin-induced agglutination and release but had no effect on TTP, collagen, ADP, or thrombin-induced aggregation and release. In contrast, the MoAb against glycoprotein IIb/IIIa had no effect upon ristocetin-induced agglutination and release but inhibited the TTP sera-induced aggregation and release as well as the aggregation and release caused by the other agonists. Certain characteristics of TTP sera-induced platelet aggregation are similar to thrombin-induced platelet aggregation. In particular, it can be inhibited by heparin (Table 1). The observation raises the question of whether the TTP aggregating factor could be an artifact, such as thrombin, generated during blood collection. Two observations argue strongly against this possibility. First, as we previously demonstrated, the thrombin-specific inhibitor DAPA was without effect. Second, PMSF, a serine protease inhibitor, also was without effect.

The observation that TTP sera-induced platelet release occurs through glycoprotein IIb/IIIa and not IIb should not be interpreted as indicating that vWF is not involved in the disease pathogenesis. It has been shown that vWF can also interact with glycoprotein IIb/IIIa, and modifications of the molecule dramatically increase its reactivity. Yet the mechanism of aggregation or characteristics of the inhibitory effect of normal plasma that result in remission in TTP remain uncertain. A role for IgG in either initiating the illness or controlling it has been proposed by one group. Elevated levels of platelet-associated IgG have been described in patients with TTP by several groups of workers, including ourselves; others have described circulating immune complexes in these patients. Together these observations might indicate that IgG immune complexes mediate the platelet aggregation. Indeed, IgG immune complexes are potent inducers of platelet aggregation, and aggregation induced by these complexes can be inhibited by IgG or the Fc portion of the molecule. However, our current in vitro and in vivo studies suggest that it is unlikely that IgG plays a causal or protective role in TTP. In vitro platelet aggregation initiated by IgG aggregates did not react similarly to the TTP sera-induced platelet aggregation in that the former could be blocked by either the addition of monomeric IgG or Fc. In contrast, both intact IgG and purified Fc were without effect upon TTP-induced platelet aggregation and release. These results are different from those obtained by another group of investigators studying TTP sera-induced platelet aggregation. They reported that IgG either from a control population or from a patient following recovery from TTP could neutralize the platelet aggregating factor. The reason for the difference in results between groups is uncertain and could be due to differences in experimental techniques. However, our in vitro data, failing to document a role for IgG in neutralizing TTP sera-induced platelet aggregation, is
further supported by the observation that high doses of IV IgG did not produce a remission in three patients with TTP. Although other investigators have reported an apparent response of TTP patients to IV IgG, such observations should be interpreted with caution. It is possible that the remission of the disease was spontaneous and only coincidentally linked to the IV IgG. It should be noted that two of the patients described in this report who failed therapy with IV IgG subsequently responded to more conventional therapy.

In summary, our studies demonstrate that TTP sera-

ACKNOWLEDGMENT

This study was supported by a grant from the Heart and Stroke Foundation of Ontario. Dr. J. Kelton is a Research Associate of the Heart and Stroke Foundation of Ontario.

REFERENCES

Studies investigating platelet aggregation and release initiated by sera from patients with thrombotic thrombocytopenic purpura

JG Kelton, JC Moore and WG Murphy