

## Investigation of the Role of von Willebrand Factor in Thrombotic Thrombocytopenic Purpura

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Von Willebrand factor (vWF) has been implicated to function as a cofactor in platelet aggregation induced by thrombotic thrombocytopenic purpura (TTP) plasma. To investigate further this role of vWF, we have used rabbit monospecific anti-FVIII/vWF antibodies and a monoclonal antibody to platelet glycoprotein Ib (GP Ib) that blocks the ristocetin-induced platelet aggregation. The monoclonal anti-platelet GP Ib antibody inhibited the platelet aggregation induced by ristocetin in the presence of normal plasma, but not that by any of the five TTP plasma samples.

VON WILLEBRAND factor (vWF) in humans is known to cause the platelets to adhere to the subendothelium<sup>1</sup> and to aggregate in the presence of ristocetin.<sup>2</sup> Recently, it has been reported that large vWF multimers may be responsible for the development of platelet microthrombi in chronic relapsing thrombotic thrombocytopenic purpura (TTP)<sup>3</sup> and may function as a cofactor in the platelet aggregation induced by TTP plasma.<sup>4</sup> To investigate further the latter role of vWF, we have used rabbit monospecific anti-FVIII/vWF antibodies and a murine monoclonal antibody to platelet glycoprotein Ib (GP Ib) that blocks ristocetin-induced platelet aggregation. We found that vWF is unlikely to play a major role in the TTP plasma-induced platelet aggregation.

### MATERIALS AND METHODS

**Preparation of plasma and platelets.** Human plasma and platelets were prepared as described previously.<sup>5</sup> The washed platelets were suspended in the Tris-saline buffer, pH 7.4, containing 0.133 mol/L NaCl, 0.015 mol/L Tris-Cl, 0.005 mol/L KCl, and 0.001 mol/L MgCl<sub>2</sub>, and adjusted to  $750 \times 10^9$  per liter for the platelet aggregation studies. The TTP plasma samples were obtained from five patients with classic manifestation of TTP during active disease. All five patients initially responded to either plasma infusion or exchange plasmapheresis. Four of these patients recovered completely; the other succumbed to the disease after recurrent relapses. All five TTP plasma samples were shown to cause the aggregation of washed platelets, which was inhibited by preincubation with normal plasma, but not with hirudin or heparin in the presence of antithrombin III, using the method described previously.<sup>5</sup>

**Study of the effect of monoclonal anti-platelet GP Ib antibody on the TTP plasma-induced platelet aggregation.** A 1.5- $\mu$ L of buffer or monoclonal anti-platelet GP Ib antibody solutions (which was kindly supplied by Dr Barry Collier, SUNY, Stony Brook, NY, and designated 6D1) containing 0.67  $\mu$ g protein was mixed with 0.1 mL of washed platelet suspension ( $750 \times 10^9$  per liter) and incubated at 37°C for ten minutes. To this, 0.3 mL of normal plasma, 0.074 mL of Tris-saline buffer, and 25  $\mu$ L of ristocetin (24 mg/mL) were sequentially added. It was demonstrated that the anti-platelet GP Ib antibody completely inhibited the ristocetin-induced platelet aggregation in the presence of normal plasma. To determine the effect of this monoclonal antibody on TTP plasma-induced platelet aggregation, a mixture of 0.1 mL platelet suspension and 1.5  $\mu$ L of buffer with or without similar amounts of platelet GP Ib antibody was incubated at 37°C for ten minutes. After this, 0.4 mL of TTP plasma undiluted or diluted with Tris-saline buffer, pH 7.4, was added. The platelet aggregation reflected by the change in optical density was recorded.

The TTP plasma samples from five patients were incubated with the monospecific antibodies to FVIII/vWF. In all of the samples, the FVIII/vWF:Ag was drastically reduced; however, there was almost no effect on the platelet-aggregating activity. Therefore, it is concluded that vWF is unlikely to play a major role in platelet aggregation induced by majority of TTP plasmas and that the site of platelet GP Ib, to which vWF binds in the presence of ristocetin, is not involved in TTP plasma-induced aggregation.

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**Purification of the FVIII/vWF complex.** The FVIII/vWF complex was purified as described previously.<sup>5</sup>

**Purification of anti-FVIII/vWF antibodies.** The rabbit monospecific anti-FVIII/vWF antiserum was produced and collected as described previously.<sup>6</sup> A 7.5-mL sample of the antiserum was brought to 50% ammonium sulfate saturation and stirred at 4 °C for one hour. The precipitate was recovered by centrifugation at 12,000 g for 30 minutes and dissolved in 3.0 mL of 0.05 mol/L sodium phosphate buffer, pH 7.0, containing 0.02% NaN<sub>3</sub>. The solution was dialyzed overnight against the same buffer at 4 °C. The dialyzed sample was spun at 12,000 g for 30 minutes and the clear supernatant was poured onto a 1 × 15-cm protein A-Sepharose 4B column, which was pre-equilibrated with 0.05 mol/L sodium phosphate buffer, pH 7.0. The column was washed with the same buffer at a flow rate of 8.0 mL/h until the absorbency at 280 nm became 0. The bound rabbit IgG was eluted with 0.1 mol/L glycine-HCl buffer, pH 3.0. The IgG fraction was dialyzed against 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.9% NaCl, and stored at -70 °C. Two milliliters of FVIII/vWF complex containing 500  $\mu$ g of protein was mixed with 0.5 mL of protein A-purified rabbit anti-FVIII/vWF IgG fraction containing 2.1 mg protein and incubated at 37 °C for one hour and at 4 °C overnight. The mixture was centrifuged at 27,000 g for 30 minutes. The precipitate containing antigen-antibody complex was washed twice with phosphate-buffered saline (PBS), pH 7.4, centrifuged at 27,000 g for 30 minutes, dissolved in 0.5 mL of 0.05 mol/L glycine-HCl, pH 2.0, and then loaded onto a 1.5 × 16-cm BioGel 1.5-m (200 to 400 mesh) (Bio-Rad Laboratories, Richmond, Calif) column, which was equilibrated with PBS. The column was eluted with PBS, pH 7.4, at 5.6 mL/h. The protein peak containing monomeric IgG was pooled and dialyzed against Tris-saline buffer, pH 7.4, and spun at 48,000 g for one hour to remove any aggregates.

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*Study of the effect of monospecific anti-FVIII/vWF IgG on the TTP plasma-induced platelet aggregation.* A 0.4-mL sample of TTP plasma in a plastic tube was mixed with varying amounts of anti-FVIII/vWF IgG to give a final concentration of 0, 20, or 100  $\mu\text{g}$  of IgG per milliliter. The tubes were incubated for two hours at 37 °C and one hour at 4 °C. The mixture was centrifuged at 48,000 g for one hour at 4 °C to remove antigen-antibody complexes. The supernatant was used for the determination of platelet-aggregating activity<sup>5</sup> and FVIII:Ag<sup>6</sup> as described previously.

## RESULTS

*Effect of monoclonal anti-platelet GP Ib antibody on the TTP plasma-induced platelet aggregation.* After incubation of anti-platelet GP Ib antibody at the concentration of 6.6  $\mu\text{g}/\text{mL}$  at 37 °C for 30 minutes, the platelet aggregation induced by ristocetin (at the final concentration of 1.2 mg/mL) in the presence of normal plasma was completely inhibited. In contrast, at the same concentration of anti-platelet GP Ib antibody, the platelet aggregation induced by any of the five TTP plasma samples was not inhibited (Fig 1).

*Effect of anti-FVIII/vWF IgG on the TTP plasma-induced platelet aggregation.* As shown in Table 1, the FVIII/vWF:Ag was significantly depleted from all of the TTP plasma samples after incubation with rabbit anti-FVIII/vWF IgG; however, there was very little change in the platelet-aggregating activity.

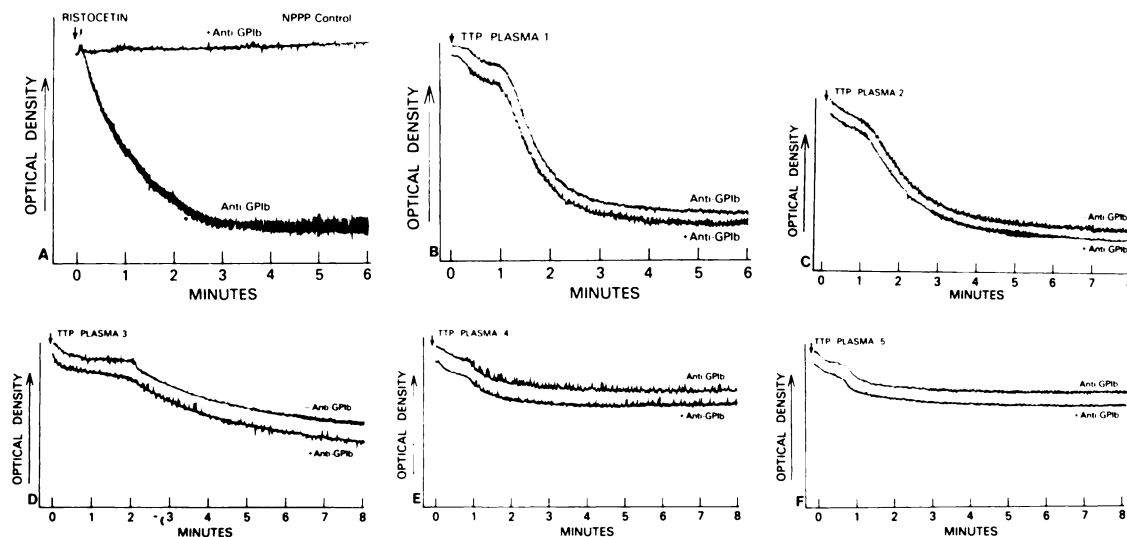
## DISCUSSION

TTP is a syndrome manifested by microangiopathic hemolytic anemia, thrombocytopenia, changing neurologic signs, renal abnormalities, and fever.<sup>7</sup> The syndrome is caused by diverse etiologies.<sup>8-10</sup> The development of TTP is likely caused by the unbalanced interaction between environmental inciting agents and intrinsic host factors. Our laboratory has

discovered that plasmas from some TTP patients induced the aggregation of autologous as well as homologous platelets.<sup>5</sup> The factors responsible for the platelet aggregation are heterogeneous.<sup>9,10</sup> Other than primary intravascular platelet aggregation or agglutination, endothelial cell injury induced by antiendothelial cell antibody,<sup>11</sup> circulating immune complexes,<sup>12</sup> or other agents has also been proposed as the cause of platelet thrombi in the microvessels in TTP. The platelet aggregation or agglutination could be influenced by host factors, such as defective immune response, to produce specific inhibitory immunoglobulin,<sup>9</sup> prostacyclin synthesis,<sup>13</sup> or stabilization,<sup>14</sup> decreased fibrinolysis,<sup>15</sup> hyperactive platelets, and other plasma proteins.

Recently, it has been shown that unusually large vWF multimers are increased in amounts in patients with chronic relapsing TTP during remission.<sup>3</sup> These multimers disappear during relapse of chronic TTP<sup>3</sup> and during active disease of acute TTP. Kelton et al<sup>4</sup> reported that large vWF multimers prepared from normal cryoprecipitate enhanced the agglutination of platelets. These workers suggested that large vWF multimers are possibly consumed in platelet aggregation and patients may be more susceptible to TTP because of congenital or acquired abnormality in processing unusually large multimers of vWF. However, patients with type IIB or pseudo-von Willebrand's disease do not have clinical manifestations of TTP, even if vWF is consumed as a result of platelet aggregation.<sup>16</sup> Furthermore, most of the patients with TTP experience improved conditions by plasma infusion despite the presence of vWF multimers in the normal plasma,<sup>17</sup> from which Kelton et al<sup>4</sup> prepared the cryoprecipitate and large vWF multimers for the *in vitro* platelet agglutination test. These clinical observations raised some doubts that vWF multimers are important in the development of TTP.

Here we demonstrated that the platelet aggregability of



**Fig 1.** Effect of monoclonal anti-platelet GP Ib antibody on the TTP plasma-induced platelet aggregation. Buffer or monoclonal anti-platelet GP Ib antibody (1.5  $\mu\text{L}$ ) containing 0.67  $\mu\text{g}$  protein were admixed with 0.1 mL of washed platelet suspension ( $750 \times 10^9$  per liter) and incubated for ten minutes. To this, (A) 0.3 mL of normal plasma, 0.074 mL of Tris-saline buffer, and 0.025 mL of ristocetin (24 mg/mL) were sequentially added; (B through F) Four-tenths milliliter of each TTP plasma, undiluted or diluted with Tris-saline buffer, pH 7.4, was added. The platelet aggregation reflecting the change of optical density was recorded.

Table 1. Effects of Anti-FVIII/vWF IgG on the TTP Plasma-Induced Platelet Aggregation

Anti-FVIII/vWF IgG	Plasma 1		Plasma 2		Plasma 3		Plasma 4		Plasma 5	
	VIII:Ag U/mL	Platelet Aggregation (%)	VIII:Ag U/mL	Platelet Aggregation (%)	VIII:Ag U/mL	Platelet Aggregation (%)	VIII:Ag U/mL	Platelet Aggregation (%)	VIII:Ag U/mL	Platelet Aggregation (%)
0 µg/mL	3.40	100	0.56	100	1.72	100	3.60	100	0.63	100
20 µg/mL	3.00	100	<0.08	100	1.12	100	2.90	100	<0.08	100
100 µg/mL	<0.08	86	—	—	<0.08	100	0.54	78	<0.08	90

Platelet aggregation induced by TTP plasma without anti-FVIII/vWF IgG is regarded as 100%.

the majority of TTP plasmas was not affected after marked reduction of vWF, and the monoclonal anti-platelet GP Ib antibody that specifically blocks ristocetin-induced platelet aggregation did not inhibit the platelet-aggregating activity of all TTP plasma samples tested. The difference between our results and those of Kelton et al may be due to different platelet-aggregating or -agglutinating factor present in the TTP plasmas or other unknown factors involved in the tests. From our studies, vWF appears unlikely to play a major role

in aggregation by the majority of TTP plasmas, and the site on platelet GP Ib, to which vWF binds in the presence of ristocetin, is not involved in TTP plasma-induced aggregation.

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#### REFERENCES

- Weiss HJ, Tschopp TB, Baumgartner HR, Sussmann II, Johnson MM, Egan JJ: Decreased adhesion of giant (Bernard-Soulier) platelets to subendothelium. Further implications on the role of the von Willebrand factor in hemostasis. *Am J Med* 57:920, 1974
- Howard MA, Firkin BG: Ristocetin—A new tool in the investigation of platelet aggregation. *Thromb Diath Haemorrh* 26:362, 1971
- Moake JJ, Rudy CK, Troll JH, Weinstein MJ, Colannino NM, Azocar J, Seder RH, Hong SL, Deykin D: Unusually large plasma factor VIII: von Willebrand factor multimers in chronic relapsing thrombotic thrombocytopenic purpura. *N Engl J Med* 307:1432, 1982
- Kelton JG, Moore J, Santos A, Sheridan D: Detection of a platelet-aggregating factor in thrombotic thrombocytopenic purpura. *Ann Intern Med* 101:589, 1984
- Lian ECY, Harkness DR, Byrnes JJ, Wallach H, Nunez R: Presence of a platelet aggregating factor in the plasma of patients with thrombotic thrombocytopenic purpura (TTP) and its inhibition by normal plasma. *Blood* 53:333, 1979.
- Lian ECY, Deykin D: In vivo dissociation of factor VIII (AHF) activity and factor VIII-related antigen in von Willebrand's disease. *Am J Hematol* 1:71, 1976
- Moschcowitz E: Hyaline thrombi of the arterioles and capillaries: A hitherto undescribed disease. *Proc NY Pathol Soc* 24:21, 1924
- Bukowski RM: Thrombotic thrombocytopenic purpura: A review. *Hemost Thromb* 6:287, 1982
- Lian ECY, Mui PTK, Siddiqui FA, Chiu AY, Chiu LL: Inhibition of platelet aggregating activity in thrombotic thrombocytopenic purpura plasma by normal adult immunoglobulin G. *J Clin Invest* 73:548, 1984
- Siddiqui FA, Lian ECY: Novel platelet agglutinating protein from a thrombotic thrombocytopenic purpura plasma. *J Clin Invest* (in press)
- Wall RT, Harker LA: The endothelium and thrombosis. *Ann Rev Med* 31:361, 1980
- Morrison J, McMillan R: Elevated platelet-associated IgG in thrombotic thrombocytopenic purpura. *JAMA* 235:1944, 1977
- Machin SJ: Thrombotic thrombocytopenic purpura. *Br J Hematol* 56:191, 1984
- Chen YC, Hall ER, McLeod B, Wu KK: Accelerated prostacyclin degradation in thrombotic thrombocytopenic purpura. *Lancet* 2:267, 1981
- Kwaan HC: The pathogenesis of thrombotic thrombocytopenic purpura. *Semin Thromb Hemast* 5:184, 1979
- Zimmerman TS, Ruggeri ZM: von Willebrand's disease. *Prog Hemost Thromb* 6:203, 1982
- Byrnes JJ, Lian ECY: Recent therapeutic advances in thrombotic thrombocytopenic purpura. *Semin Thromb Hemost* 5:199, 1979