Solvent/Detergent-Treated Plasma Suppresses Shear-Induced Platelet Aggregation and Prevents Episodes of Thrombotic Thrombocytopenic Purpura


Two children with congenital chronic relapsing thrombotic thrombocytopenic purpura (TTP) have episodes every 3 weeks. These relapses can be prevented by the infusion of normal fresh-frozen plasma (FFP) without concurrent plasmapheresis. We conducted a study to determine whether the exposure of normal plasma to agents that inactivate human immunodeficiency virus and other viruses destroys the component necessary for the effective treatment of this type of TTP that requires only plasma infusion to prevent or reverse relapses. Clinical responsiveness and von Willebrand factor (vWF)-mediated fluid shear stress-induced platelet aggregation were evaluated before and after the infusion of 10 mL/kg FFP or solvent/tri(n-butyl)phosphate/detergent (Triton X-100)-treated plasma (S/D plasma). Platelet aggregation at shear stresses of 90 to 180 dyne/cm² (similar to those in the partially occluded microcirculation) imposed for 30 seconds was excessive using the citrated platelet-rich plasma of both patients, and was associated with the presence of unusually large vWF forms in patient platelet-poor plasma. Infusion with either FFP or S/D plasma at 3-week intervals caused the platelet count to increase to (or above) normal within 1 week (on 12 of 12 occasions); the disappearance or diminution of unusually large vWF forms within 1 hour (on 6 of 10 occasions studied); and the reversal within 1 to 4 hours of excessive shear-induced platelet aggregation (on 8 of 9 occasions studied). We conclude that a component in normal plasma resistant to S/D treatment is responsible for preventing thrombocytopenia and TTP episodes, and for controlling excessive shear-induced aggregation in these patients. Our results suggest that excessive in vivo platelet aggregation in chronic relapsing TTP and excessive in vitro vWF-mediated shear-induced aggregation may be similar phenomena.

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CHRONIC RELAPSING thrombotic thrombocytopenic purpura (TTP) is characterized by microvascular platelet aggregation, thrombocytopenia (with or without ischemic vascular signs), and intravascular hemolysis. The rarest type of TTP, and usually presents in childhood. Two children with congenital chronic relapsing TTP were studied. Our results suggest that excessive shear-induced aggregation in chronic relapsing TTP may be controlled by a component that is not present in normal plasma. The results of these studies are important for understanding the pathophysiology of TTP and for developing new treatments for this disease.
SHEAR-AGGREGATION AND S/D PLASMA IN TTP

Patients and Methods

Patients. The clinical courses of two male Houston patients (BK and BA) aged 5 and 9 years with chronic relapsing TTP have recently been described in detail. Both of these children initially presented in neonatal life with severe hemolysis and thrombocytopenia that required exchange transfusions, and both have had repeated episodes of thrombocytopenia and hemolysis. FFP infusions every 3 weeks have been required to prevent TTP relapses in both children. For this study, these children were evaluated serially over the course of 18 weeks. A third chronic relapsing TTP patient (MR), an 11-year-old male patient from Ponce, Puerto Rico, received two infusions of S/D plasma. Approval was obtained from the Institutional Review Board for these studies. Informed consent was provided according to the Declaration of Helsinki.

FFP and S/D plasma. The normal FFP units used in this study contained all of the vWF multimeric forms presently present in platelet-poor plasma (PPP, FFP in Fig 1). For preparation of S/D plasma, pooled normal FFP (containing all plasma vWF multimers) was thawed rapidly and stirred for 4 hours with 1% (vol/vol) tri-(n-butyl)phosphate and 1% (vol/vol) Triton X-100 at 30°C. Soybean oil (5% vol/vol) was added, mixed gently for 30 minutes, and removed by centrifugation at 10,000g for 20 minutes. The clarified plasma was applied to a column of Waters Prep C18 resin (Waters Division of Millipore, Milford, MA) with a ratio of plasma to column volume of 6 and a contact time of 3 minutes. The column eluate was passed through a 0.2-μm filter and refrozen in 200-mL bags. For technical reasons that are not yet known, during the preparation of S/D plasma from normal FFP, the largest plasma vWF multimeric forms are lost (Fig 1). S/D plasma, therefore, is similar in vWF multimeric pattern to cryoprecipitate-poor plasma (cryosupernatant). S/D-treated plasma contains near-normal levels of all coagulation and fibrinolytic proteins.

Treatment protocol. Each of the two Houston patients received 3 infusions of either FFP or S/D plasma in alternate 3-week treatment cycles for a total of 6 cycles. Blood samples were obtained 1 hour after completion of the infusion, and then at 4 hours, 8 or 12 hours, 24 hours, 1 week, 2 weeks, and 3 weeks. Each infusion (FFP or S/D plasma) was administered over 2 hours at 10 mL/kg. Each S/D plasma infusion was preceded by a 1-mL test dose and observation for 20 minutes, followed by the infusion. Both Houston patients tested negatively for HIV antibodies before the study, and again 1 to 3 months after its completion. One of the two patients who had received premedications with Benadryl and Solucortef previously in vitro mechanisms of platelet aggregation were to be related, then the infusion of normal FFP should result both in the prevention of recurrences in chronic relapsing TTP patients and in a diminution of the augmented shear-induced vWF-mediated aggregation in their PRP. Furthermore, if the substance in normal FFP that is important in the therapy of TTP episodes were to survive solvent/detergent viral activation processes, then the infusion of S/D-treated plasma should produce the same in vivo and in vitro effects as does FFP.

Fig 1. ULvWF multimers in the EDTA-PPP of patient (Ptn) BA (A) and BK (B) 1 week after transfusion with either normal FFP (A) or S/D plasma (B), as demonstrated by SDS-1% agarose gel electrophoresis and autoradiography. NP, normal pooled EDTA-PPP; EC, HUVEC supernatant; FFP, sample of normal FFP containing all plasma-type vWF multimers; S/D, sample of S/D-treated plasma devoid of the largest plasma-type vWF multimers. vWF antigen levels before dilution to 15% with SDS-urea-EDTA-Tris for electrophoresis are Ptn BA, 106%; and patient BK, 181%. Brackets indicate regions of ULvWF forms.

Fig 2. The shear stress-induced, vWF-mediated platelet aggregation in citrated PRP (130 μL) exposed to various shear stresses was compared in adult normal donors (platelet count, 103,000 to 260,000/μL) and two chronic relapsing TTP patients (BA platelet count, 128,000 to 287,000/μL; BK platelet count, 101,000 to 245,000/μL). Shear stresses of 30, 60, 90, 120, and 180 dyn/cm² were applied for 30 seconds at room temperature in a rotational cone and plate viscometer. The values shown are means ± standard errors; controls, n = 23; BA, n = 6; BK, n = 5. Using the unpaired Student's t-test and comparing the shear aggregation of controls to each patient, at shear stresses greater than 60 dyn/cm², P < .01 for both patients.
during FFP infusions continued to receive these agents before S/D plasma infusions. In addition to whole blood platelet and hemoglobin levels, EDTA-PPP samples were analyzed for vWF multimeric patterns. vWF-mediated, shear-induced platelet aggregation responses were studied in citrated PRP samples. The Puerto Rican patient received two infusions of S/D plasma at 4.7 mL/kg. The vWF multimeric patterns were studied before and after the infusions.

**vWF multimeric analysis.** Patient blood samples were collected for vWF multimeric analysis in EDTA (3.7 mmol/L). PPP was prepared by centrifugation of whole blood at room temperature for 15 minutes at 1,900g, and the samples were frozen at −80°C until analysis.

Plasma levels of vWF antigen were quantified by solid-phase immunoradiometric assay and rapid enzyme-linked immunosorbent assay (ELISA). Normal pooled PPP or PPP samples from patients were diluted to a vWF antigen level of 15 U/dL (15%) with VBS (28 mmol/L sodium barbitol, 125 mmol/L NaCl, 0.02% sodium azide, pH 7.4). The diluted sample (50 μL) was mixed with 75 μL of Tris/EDTA (2% sodium dodecyl sulfate [SDS], 8 mmol/L urea, 2 mmol/L EDTA, 20 mmol/L Tris-HCl, pH 7.4) and heated to 60°C before electrophoresis. HUVEC vWF antigen levels ranged from 5% to 15% before they were mixed (without dilution) for electrophoresis with Tris/EDTA in the same proportions used for PPP. The vWF multimers were separated by SDS-1% agarose gel electrophoresis using a continuous buffer system, overlaid with rabbit antihuman vWF IgG, and analyzed by autoradiography. For the separation and display of ULvWF forms, this electrophoresis procedure is superior to those using less porous (higher percentage) agarose.

**Viscometry experiments.** PRP was prepared by centrifugation of whole blood anticoagulated with 0.38% wt/vol sodium citrate at 150g for 15 minutes. The PRP samples (130 μL) were subjected to controlled shear stress levels ranging from 30 to 180 dyne/cm² in a stainless steel cone and plate viscometer (Model 781; Ferranti Electric, Inc, Commack, NY) for 30 seconds at room temperature. Aliquots of 10 μL were dispensed into 10 mL of Coulter Isoton II (Coulter Electronics, Inc, Hialeah, FL) containing 0.5% glutaraldehyde for preshear platelet counts. After shearing, two 10-μL aliquots of each sample were dispensed into 10 mL of the Isoton/glutaraldehyde. Platelet counts and size distributions were determined with an electronic particle counter (Coulter Multisizer; Coulter Electronics). The platelet counts and size distributions were determined with an electronic particle counter (Coulter Multisizer; Coulter Electronics). The results were compared with those obtained for the other samples.

**RESULTS**

Both patients BA and BK had ULvWF multimers present in their PPP samples in "baseline" samples obtained 1 to 2 weeks after FFP infusion. These ULvWF forms were intermediate in size between the ULvWF forms secreted by HUVECs in culture and the largest vWF multimers present in either normal pooled PPP or single units of FFP (Fig 1A and B). The patient ULvWF forms were more prominent in the samples obtained 1 and 2 weeks after either FFP or S/D plasma infusion compared with the samples obtained at 3 weeks.
weeks (at the time of each incipient relapse, and just before the next infusion). The vWF multimeric patterns, including ULvWF forms, were not detectably different in samples obtained from either patient BA or BK 1 week after infusion with S/D plasma compared with 1 week after treatment with FFP plasma.

Fluid shear stress-induced, vWF-mediated platelet aggregation in PRP samples from both patients BA and BK was significantly increased at 90, 120, and 180 dyne/cm² compared with adult normal control PRP samples (Fig 2). Shear aggregation results were unrelated to platelet counts over a broad range in patient and normal donor PRP samples in the range of 100,000 to 300,000/µL (data not shown).

Shear stress-induced aggregation of normal washed platelets at 150 dyne/cm² in the presence of PPP from patients BA or BK was accentuated by about twofold compared with normal washed platelets in normal PPP (Fig 3). These results indicate that the increase in shear aggregation in patient PRP samples was the result of a component in plasma, rather than an abnormality in patient platelets. shear-induced aggregation could also be increased by about twofold to 2.5-fold by adding 1.5% to 3% vWF (final concentration) released from HUVECs (this includes ULvWF forms) to the normal platelets in normal plasma before shear (Fig 3). In vitro mixing at room temperature of normal pooled plasma (1/4 vol) with the plasma of each TTP patient (1/4 vol) and then addition of this mixture to HEPES buffer (1/2 vol) containing normal platelets (300,000/µL final concentration) resulted in suppression of shear-aggregated platelet aggregation at 150 dyne/cm² by 9%, 14%, 21%, and 24% (4 experiments; data not shown).

On 12 of 12 occasions after the infusion of either 10 mL/kg of FFP or 10 mL/kg of S/D-treated plasma into patients BA and BK, elevations in platelet counts occurred in platelet counts occurred during the following days (Fig 4A and B). There were no adverse reactions to S/D plasma. Mean platelet counts for the two patients were 198,000/µL and 113,000/µL before infusions and 311,000/µL and 232,000/µL, respectively, 1 week after infusions. Based on extensive past experience with these children, plasma infusions were administered every 3 weeks to prevent or reverse their periodic TTP episodes. Therefore, platelet counts less than 50,000/µL, hemoglobin concentrations less than 10 g/dL, elevated LDH, reticulocytosis, and extensive schistocytosis did not develop in either patient before the infusions during this portion of the study.

On three subsequent occasions, patients BA and BK developed more severe thrombocytopenia (12,000/µL to 20,000/µL) in association with viral infections. Each received 10 mL/kg of S/D plasma, and platelet counts were greater than 200,000/µL within 7 days.

After infusion of 10 mL/kg of FFP on 2 of 4 occasions, and after infusion of 10 mL/kg of S/D-treated plasma on 3 of 5 occasions, a partial disappearance of ULvWF forms from EDTA-plasma samples obtained from Houston patients BA and BK was detected. ULvWF multimeric forms present in EDTA-PPP samples obtained from the chronic relapsing TTP patient (MR) also disappeared immediately after 4.7 mL/kg S/D plasma infusion on 2 of 2 occasions (Fig 5).

Figure 6 shows that on 5 of 6 occasions after FFP infusion, and on 6 of 6 occasions after S/D-treated plasma infusion into Houston patients BA and BK, shear stress-induced vWF-mediated platelet aggregation was suppressed within 1 to 4 hours. This suppression persisted for periods of time ranging from hours to days and was to about the same level of shear aggregation seen in control normal PRP (performed concurrently) in 31 of 35 experimental runs using BA and BK PRP samples subjected to 30, 60, 90, 120, and 180 dyne/cm². The suppression was below those observed in normal PRP by (13%, 19%, 21%, and 37%) in only 4 of 35 experiments. After completion of the FFP and S/D plasma infusion studies described above, the effects of polymeric ATA on shear stress-induced aggregation in the PRP of patients BK and BA was studied. ATA in a concentration of 100 µg/mL inhibited shear-induced vWF-mediated platelet aggregation in the citrated PRP of patients BK (2 studies) and BA (3 studies) by 52%, 78%, 90%, 100%, and 100% (Fig 7). These studies were performed at times preceding plasma infusion, and included several occasions when the patients had become more severely thrombocytopenic.

The addition of CP/CPK (50 or 67 mmol/L/0.6 or 0.85 U/µL) to the PRP of one of the Houston patients was as effective in inhibiting shear aggregation as in normal PRP samples (81% to 92% using control PRP with platelets [259,000/µL] v 67% to 100% using patient PRP with platelets [163,000/µL]). Along with the results using ATA, these findings indicate that no substance in patient plasma other than vWF and ADP was responsible for the excessive shear-induced platelet aggregation.
Fig 8. The percentage of shear stress-induced, vWF-mediated platelet aggregation from the citrated PRP (130 μL) of two chronic relapsing TTP patients subjected to (A) 90 dyne/cm² (Pt BA) and (B) 180 dyne/cm² (Pt BK) in a cone and plate viscometer for 30 seconds at room temperature. Patient PRP was tested for shear-aggregation responses before and 1 hour, 24 hours, 1 week, and 2 weeks after transfusions with either S/D plasma or FFP.
DISCUSSION

Shear aggregation is mediated most effectively by the binding of ULvWF multimers derived from human endothelial cells to the platelet vWF receptors, GPIb and GPIIb-IIIa, in the presence of ADP.\(^7\)\(^9\)\(^17\) Shear stress-induced vWF-mediated platelet aggregation was accentuated in the PRP of two Houston patients with chronic relapsing TTP and ULvWF forms in their plasma on all 9 occasions studied before FFP or S/D plasma infusion. The addition of ULvWF multimers to suspensions of normal platelets in normal plasma resulted in increased shear stress-induced platelet aggregation, similar to that seen in suspensions of normal platelets in patient plasma. These findings suggest that there may be a direct association between the presence of ULvWF forms in the plasma of chronic relapsing TTP patients and the accentuated platelet aggregation that occurs at regular intervals in areas of their microcirculation.\(^18\)

One possibility for the frequent, regular, periodic relapses in these children is that it requires about 3 weeks for the excessive release of ULvWF multimers from congenitally defective endothelial cells of chronic relapsing TTP patients to produce levels above a threshold necessary\(^6\) to support fluid shear stress-induced platelet aggregation in the microcirculation. However, this remains unproved because specific quantification of ULvWF forms is not yet possible, and because a progressive increase in endothelial cell output of ULvWF may be accompanied by concurrent ULvWF binding to platelets. Because platelets contain vWF (including ULvWF forms) in their α-granules,\(^8\)\(^9\) it has not yet been possible to analyze directly the attachment of exogenous ULvWF to platelets. However, continuous low-level shear-induced attachment of ULvWF multimers to platelet vWF receptors may account for observations in these and other chronic relapsing TTP patients\(^2\) that the ULvWF forms in patient plasma between episodes are not quite so large as the ULvWF multimers released by stimulated human endothelial cells in culture.\(^1\)\(^2\) The relative decrease in the prominence of these ULvWF forms in patient plasma at times of incipient or complete relapse further suggests the possibility of progressive ULvWF-platelet attachment as remission wanes.

Supplementary quantities of a plasma component that processes ULvWF forms to the normal circulating vWF multimeric sizes is present in normal FFP and cryosupernatant.\(^3\)\(^20\)\(^21\) This component may also be present in S/D-treated plasma, because circulating ULvWF multimers disappeared within 1 hour on 6 of 10 treatment occasions in the TTP patients infused with the quantities of either FFP or S/D plasma used in these studies. However, it is not presently known whether it is the effect of a disulfide bond reductase\(^22\) on ULvWF forms or the effect of some other undefined activity\(^23\) in transfused FFP or S/D plasma that is responsible for the suppression of accentuated shear-induced platelet aggregation in the PRP of chronic relapsing TTP patients.

Either the ULvWF processing activity or another component in normal FFP responsible for controlling excessive shear-induced vWF-mediated platelet aggregation, thrombocytopenia, and TTP episodes in chronic relapsing TTP is not lost during the viral inactivation and filtration procedures involved in the preparation of S/D plasma. Whether S/D plasma in association with plasmapheresis is also as effective as FFP or cryosupernatant in the treatment of single episode TTP, or of recurrent TTP with relapses at irregular or infrequent intervals, has not yet been investigated in a carefully designed clinical trial.

Polymeric ATA, which attaches to large vWF multimers and inhibits vWF binding to GPIb, suppressed shear stress-induced platelet aggregation in vitro in both of the Houston patients studied. This suppression occurred even during the most severe TTP relapses studied. It is not yet known if blocking vWF interaction with either platelet GPIb (as by a recombinant vWF fragment\(^24\) or a derivative of polymeric ATA\(^19\)\(^23\) or GPIIb-IIIa (as by the chimeric mouse-human monoclonal antibody 7E3\(^25\)\(^26\) or the cyclic lysine-glycine-
aspartate—containing peptide, Integrelin²) might eventually be useful in vivo in the treatment or prevention of TTP episodes.

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