Abnormal VIII: von Willebrand Factor Patterns in the Plasma of Patients With the Hemolytic-Uremic Syndrome

By Joel L. Moake, John J. Byrnes, Joseph H. Troll, Christine K. Rudy, Mark J. Weinstein, Noreen M. Colannino, and Suchen L. Hong

Plasma VIII:von Willebrand factor antigen (VIII:vWF) levels were elevated approximately two- to eightfold in seven glomerular capillaries and renal arterioles of HUS. In all seven patients, there was an alteration in plasma VIII:vWF patterns during these acute HUS episodes, so that the largest VIII:vWF forms were relatively decreased. Plasma VIII:vWF multimer patterns returned to normal, or nearly to normal, as platelet counts returned to preexisting levels, even in the patients whose recovery of renal function was incomplete and whose plasma VIII:vWF antigen level remained above normal. The sister of one of the HUS patients had a similar clinical prodrome (gastroenteritis) that was not followed by thrombocytopenia or renal failure and was not accompanied by an elevated level or abnormal forms of plasma VIII:vWF. These results suggest that an alteration in VIII:vWF metabolism, distribution, or interaction with platelets is associated with acute HUS episodes. In contrast to patients with chronic relapsing thrombotic thrombocytopenic purpura, none of the HUS patients (either during or after the acute HUS episodes) had a defect in the conversion of unusually large VIII:vWF multimers derived from endothelial cells to the VIII:vWF forms found in normal plasma.

The pathogenesis of HUS has remained a mystery since the initial description by Gasser et al in 1955. The clinical triad of thrombocytopenia, acute renal failure, and microangiopathic hemolytic anemia occurs in both children and adults and has been associated with a variety of infectious agents, immunologic abnormalities, pregnancy, and the use of oral contraceptives. Clumped platelets, fibrin, and subendothelial abnormalities have been described in the glomerular capillaries and renal arterioles of HUS patients.

Clinical and histopathologic similarities have led to the supposition that HUS is a variant of thrombotic thrombocytopenic purpura (TTP). In contrast to systemic platelet agglutination and organ ischemia in TTP, however, abnormalities in HUS are confined predominantly to renal vessels. Recent studies indicate that unusually large VIII:vWF multimers synthesized and secreted by endothelial cells are important in the pathogenesis of chronic relapsing TTP. In this pathogenesis of TTP, the unusually large VIII:vWF multimers are not converted to the VIII:vWF forms normally in circulation. The unusually large VIII:vWF multimers persist between relapses and are associated with a propensity for recurring episodes of systemic intravascular platelet agglutination whenever there is inflammation or tissue necrosis. Studies reported below suggest that VIII:vWF abnormalities are also found in the plasma of patients with HUS, but only during the acute episodes. The type of VIII:vWF abnormality is distinct from that observed in the plasma of patients with chronic relapsing TTP.

Materials and Methods

The preparation of patient and normal pooled platelet-poor plasma from citrated peripheral venous blood samples and the radioimmunoassays of plasma β-thromboglobulin (β-TG, released from platelet α-granules) and fibrinopeptide A (cleaved from fibrinogen by thrombin) have been described. Analyses of β-TG and fibrinopeptide A levels were obtained sequentially on plasma samples from one of our patients (patient 3).

VIII:vWF antigen levels in patient and normal pooled platelet-poor plasma were quantified by Laurell electroimmunoassay, using 0.5% SeaKem HGT Agarose on GelBond backing (Marine Colloids Division, FMC Corp, Rockland, Me) and rabbit anti-human VIII:vWF (Calbiochem-Behring Corp, La Jolla, Calif). VIII:vWF antigen was also quantified by solid-phase immunoradiometric assay (IRMA), using rabbit anti-human VIII:vWF (Dako Antibodies, Accurate Chemical and Scientific Corp, Westbury, NY) and rabbit anti-human VIII:vWF antiserum. Normal pooled platelet-poor plasma contains 100 U/dL (100%) VIII:vWF antigen.

Two-dimensional immunoelectrophoresis was done by a modification of the method of Gralnick et al, using 0.5% SeaKem HGT Agarose on GelBond backing for both dimensions. The buffer was 0.048 mol/L sodium barbital, 0.08 mol/L sodium acetate, pH 8.6. Patient and normal pooled platelet-poor plasma samples were adjusted to the same VIII:vWF antigen levels with barbital-acetate running buffer before electrophoresis. Patient and normal plasma samples were electrophoresed in the first dimension from two different origin wells cut into the same 0.5% agarose gel slab for 2½ to 2½ hours at 100 V. The first-dimensional gel lanes were then cut out and electrophoresed in the second dimension into 0.5% agarose containing rabbit anti-human VIII:vWF antibodies (Calbiochem-
Under the experimental conditions described, the relative chamber. concurrently for the same time periods in the same electrophoresis destained in 25% methanol/7% acetic acid.

Electrophoresis of patient and control normal plasma were superimposed after exact plasma samples in the second dimension was always performed reproducibly. Dried Coomassie Blue-stained individual gels of patient and normal plasma were superimposed after exact matching of the origin sample wells and photographed. Plasma VIII:vWF multimers were separated by sodium dodecyl sulfate (SDS) agarose gel electrophoresis, overlaid with rabbit 125I-anti-human VIII:vWF IgG, and analyzed by autoradiography using 1% agarose and a continuous buffer system in a modification of the method of Ruggeri and Zimmerman.5 Agarose (1%) gel slabs, 1.5 x 80 x 180 mm, were formed in the running buffer, 0.04 mol/L Tris-HCl/0.02 mol/L sodium acetate/2 mmol/L ethylenediamine tetraacetic acid (EDTA)/0.1% SDS, pH 7.4. The VIII:vWF antigen levels in patient and normal platelet-poor plasma samples, previously quantified by electroimmunoassay and IRMA, were adjusted to 6 U/dL by dilution of plasma in 0.02 mol/L Tris-HCl/2 mmol/L EDTA/8 mol/L urea/2% SDS, pH 8.0. The samples were incubated at 60 °C for 15 minutes, and 20 μL were applied to the gel wells. Following electrophoresis for one hour at 50 V and four hours at 100 V, protein was fixed in the gels by 10% acetic acid/10% isopropanol. The gels were washed for one hour in two changes of deionized water, soaked for 3 minutes in 0.036 mol/L sodium borate/0.15 mol/L NaCl containing 1 mg/mL bovine serum albumin (Cohn fraction V, fatty acid free, Sigma Chemical Co, St Louis), pH 8.0, and incubated for 18 hours at 23 °C with rabbit 125I-anti-human VIII:vWF IgG (105 cpm/mL) in borate/NaCl/albumin buffer. They were then washed for four hours in two changes of 0.5 mol/L and 0.15 mol/L NaCl, for 48 hours in two to three changes of 0.15 mol/L NaCl, and then in 2% glycerol/10% acetic acid for one to two hours. The gels were dried in an oven at 56 °C for six hours and placed with XAR-Omat film (Kodak, Rochester, NY) and Chronex Lightning Plus intensifying screen (E.I. du Pont de Nemours and Co, Inc, Photo Products Dept, Wilmington, Del) for 24 to 48 hours at -70 °C. Visual conclusions about the relative distribution of VIII:vWF forms in the gel lanes were confirmed by scanning densitometry (Quick Scan R and D, Helena Laboratories, Beaumont, Tex). Densitograms were digitized onto paper punch tape and loaded into a Hewlett Packard Model 9845A (Corvallis, Ore) desk-top computer. Background baselines were subtracted from the individual scans, and these were then aligned, superimposed, and compared.

RESULTS

Three adults and four children with HUS were studied (Table I). In six of the seven patients, the prodromal clinical condition was an acute gastrointestinal disorder characterized by abdominal pain, vomiting, diarrhea (sometimes bloody), and fever. All patients developed acute severe thrombocytopenia, microangiopathic hemolytic anemia, and oliguric renal failure without initial laboratory evidence for disseminated intravascular coagulation. Only patient 2 had neurologic signs or symptoms (transient generalized seizures in association with rapidly evolving acidosis, electrolyte abnormalities, and uremia). Each patient was treated with fresh-frozen plasma infusion and dialysis. All patients' platelet counts returned to normal (or above). Return to renal function was complete in four patients and incomplete in three. None of the latter patients has required maintenance hemodialysis.

Clinical summaries of the seven patients are presented in Table 1, and additional information is presented below.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Clinical Prodrome</th>
<th>Treatment</th>
<th>Recovery of Renal Function</th>
<th>Plasma vWF Antigen†</th>
<th>Plasma vWF Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Sec. Age)</td>
<td></td>
<td>Plasma</td>
<td>Platelets</td>
<td>Pheresis</td>
<td>Dialysis*</td>
</tr>
<tr>
<td>1 (F, 26 yr)</td>
<td>Pregnancy and delivery</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2 (M, 5 yr)</td>
<td>Gastroenteritis</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>3 (M, 61 yr)</td>
<td>Gastroenteritis</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>4 (F, 60 yr)</td>
<td>Gastroenteritis</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5 (F, 5 yr)</td>
<td>Gastroenteritis</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>6 (F, 3 yr)</td>
<td>Gastroenteritis</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>7 (M, 3 yr)</td>
<td>Gastroenteritis</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

*Peritoneal dialysis or hemodialysis.
†Initial platelet-poor samples were obtained on day 1 of the HUS episode and before any therapy, except in patients 3 and 4. In patient 3, the initial sample was obtained on day 2 of the HUS episode, following 500 mL of fresh-frozen plasma infusion on day 1. In patient 4, the initial sample was obtained on day 1 of the HUS episode, several hours after transfusion of fresh blood, fresh-frozen plasma, and platelets.
Patient 1

In the plasma of patient 1, pretreatment VIII:vWF antigen levels were very high (518%) on the first day of her HUS episode (June 10, 1982), in association with severe thrombocytopenia. Mobility of the VIII:vWF antigen peak in patient 1 plasma was shifted toward the anode (Fig 1). She had exchange transfusions with fresh-frozen plasma (4 L) on June 11–13, and hemodialysis until June 16. By June 18, her platelets were 595,000/μL, VIII:vWF antigen levels had decreased to 274%, and her plasma VIII:vWF pattern was normal (Fig 1).

Patient 2

In patient 2, initial pretreatment plasma VIII:vWF antigen levels (April 12, 1982) were 184% and platelets were 15,000/μL (Fig 2). The plasma VIII:vWF antigen peak was shifted toward the anode. He had peritoneal dialysis for 14 days, and fresh-frozen plasma (200 to 1,300 mL) was infused on April 13–17. His VIII:vWF pattern became nearly normal as his platelet count returned to above normal levels (Fig 2).

Patient 3

Patient 3 developed HUS with platelet counts of 20,000 to 33,000/μL. He was transfused with red cells, transferred to our hospital, and 500 mL of fresh-frozen plasma was infused (day 1). Platelets increased over the next 24 hours to 102,000/μL (day 2). The first plasma samples were obtained on day 2 for VIII:vWF studies (Fig 3). The VIII:vWF antigen levels were extraordinarily elevated (911%) on day 2 and decreased about threefold as patient 3 recovered during the subsequent 15 days (with hemodialysis and no additional plasma infusion).

During this period, fibrinopeptide A levels in peripheral venous plasma were normal, indicating that systemic thrombin generation in vivo was not increased. Platelet α-granule release in vivo also was not excessive. β-TG values in peripheral venous plasma samples (80 to 158 ng/mL) during the HUS episode in patient 3 were insignificantly different from those in uremic patients without HUS (12 plasma samples; range of β-TG, 74 to 142 ng/mL). (β-TG is normally removed from plasma by glomerular filtration.) These latter results imply that the elevated VIII:vWF antigen levels in the plasma of patient 3 was derived from endothelial cells rather than from the α-granules of platelets (where it is stored after synthesis by megakaryocytes).

In the initial (predialysis) plasma sample obtained on day 2, the VIII:vWF antigen peak was shifted toward the anode. His plasma VIII:vWF forms were normal immediately after hemodialysis on day 2 (Fig 3), and the VIII:vWF antigen peak was again shifted toward the anode in plasma samples obtained on days 5 and 6 (44 and 20 hours after hemodialysis, respectively). VIII:vWF patterns returned progressively toward normal in patient plasma samples obtained after day 7, as platelet counts increased to 788,000/μL on day 11. By day 17 (11 days after the last hemodialysis), platelet counts had stabilized at about 550,000/μL, and the VIII:vWF pattern in his plasma was normal (Fig 3).
Patient 4

Patient 4 developed HUS with a platelet count of 17,000/μL. She was transfused with fresh whole blood, fresh-frozen plasma and platelets, and transferred to our hospital. Her platelets decreased rapidly from 92,000/μL (the posttransfusion count) to 33,000/μL, and the initial sample was obtained for VIII:vWF analysis. This day-1 sample contained 324% VIII:vWF antigen, and the VIII:vWF antigen peak was shifted toward the anode (Fig 4). Plasma exchange transfusions were performed during the next two days, and then the patient began hemodialysis. Her platelets increased to levels >100,000/μL by day 5, and remained >150,000/μL after day 7. Renal biopsy on day 18 (platelets, 171,000/μL) showed the presence of vascular lesions characteristic of HUS during the recovery phase, and plasma VIII:vWF forms had returned to normal (Fig 4; VIII:vWF antigen level, 374%).

Patients 5, 6, and 7

Initial vWF antigen levels for patients 5, 6, and 7 were obtained from plasma samples before the beginning of therapy (fresh-frozen plasma infusions and dialysis) and are shown in Table 1. Initial platelet counts in these children were 45,000/μL, 13,000/μL, and 9,000/μL, respectively, and creatinine values were 5.2 to 5.9 mg/dL. VIII:vWF antigen peaks in the initial plasma samples obtained from patients 5, 6, and 7 were shifted toward the anode (patient 5 plasma is shown in Fig 5B).

At the onset of HUS in patient 5, her 4-year-old sister, with a clinically similar gastrointestinal prodrome, was also studied. In the sister, the prodrome was not followed by thrombocytopenia or any other manifestation of HUS, VIII:vWF antigen was not elevated in her plasma, and VIII:vWF forms were normal (Fig 5A).
In patient 6, eight plasma samples were analyzed over a period of 32 days. Complete recovery of both platelet count and renal function was associated with a decrease in plasma VIII:vWF antigen level from 298% on day 1 to 113% on day 32 (platelets, 350,000/μL, and serum creatinine, 1 mg/dL), as well as the return of plasma VIII:vWF forms to normal.

No unusually large VIII:vWF multimers derived from endothelial cells and present in remission plasma samples of patients with chronic relapsing TTP were found in the HUS patients either during their acute episodes or after recovery (patient 4 plasma, for example, is shown in Fig 4). There was no indication that proteolysis of VIII:vWF occurred in patient plasma samples. That is, no VIII:vWF fragments of VIII:vWF bands with mobilities different from VIII:vWF multimers to smaller VIII:vWF forms. Neither selective transeptal suppression of the multimerization of VIII:vWF multimers in the plasma of patients during acute HUS episodes was not observed.

Increased plasma VIII:vWF antigen levels have been observed previously in patients with renal failure of various causes, including HUS. In patients with uremia of other etiologies, however, VIII:vWF patterns have been reported to be normal. This conclusion was confirmed in our HUS patients. When their platelet counts returned to normal, so did their plasma VIII:vWF patterns, even though some renal dysfunction persisted in three of the seven individuals. These results indicate that the abnormal plasma VIII:vWF patterns during acute HUS episodes were not associated with the uremic state per se.

Dilution alone of elevated VIII:vWF antigen levels cannot account for the alterations in the relative distribution of VIII:vWF forms in initial acute HUS patient plasma samples. This can be seen in Figs 1, 3, and 4, which display the VIII:vWF patterns of three of the patients whose renal function did not return to normal after the HUS episode (patients 1, 3, and 4). VIII:vWF antigen levels after recovery of platelet counts had decreased considerably in patient 1 (to 274%) and patient 3 (to 326%), but remained considerably elevated above normal (as in other uremic patients without HUS). Nevertheless, VIII:vWF patterns in the recovery plasma samples of patients 1 and 3 after recovery of platelets counts were normal. In patient 4 (Fig 4), the plasma VIII:vWF antigen level after recovery of platelet counts (374%) was slightly higher than the VIII:vWF level in the initial plasma sample obtained at the onset of the HUS episode (324%). Nevertheless, as the platelet count in patient 4 increased to 171,000/μL on day 18, the plasma VIII:vWF multimer pattern returned to normal.

We have also studied (in unpublished experiments) the effects of dilution of VIII:vWF antigen in normal plasma samples and the progressive dilution of plasma samples from individuals who have very high VIII:vWF antigen levels (ie, some diabetic individuals and some chronically uremic patients who have not had HUS). In these samples, dilution does not result in any shift in the VIII:vWF peak toward the anode on two-dimensional immunoelectrophoresis and does not cause an alteration in the relative distribution of plasma VIII:vWF multimers on SDS-agarose electrophoresis and autoradiography.

The altered VIII:vWF patterns in acute HUS plasma samples indicate either a relative decrease in the largest plasma VIII:vWF multimer forms or a relative increase in the smallest VIII:vWF multimers. Hypotheses for a relative increase in the smallest VIII:vWF multimers in the plasma of patients during acute HUS episodes might include selective proteolysis or reduction of the largest plasma VIII:vWF forms, or transient suppression of the multimORIZATION of VIII:vWF subunits within endothelial cells. Alterations in the VIII:vWF patterns during acute HUS episodes were not accompanied by the appearance on SDS-agarose gels of VIII:vWF bands with mobilities not seen in normal plasma. This is evidence against in vivo proteolysis of the largest plasma VIII:vWF multimers to smaller VIII:vWF forms.

It is more likely that the largest plasma VIII:vWF multimers are relatively decreased in acute HUS
samples. Thrombocytopenia and the abnormality in relative distribution of the plasma VIII:vWF multimer forms occurred concurrently during the HUS episodes in our seven patients. The VIII:vWF multimer patterns became normal as the patients recovered. These largest VIII:vWF multimer forms may have been selectively deposited onto the exposed subendothelial surfaces of injured renal vessels, and then may have augmented platelet-subendothelial attachment in a process similar to that described in vitro. Alternatively, selective interactions between the largest plasma VIII:vWF forms and platelets may have occurred during the acute HUS episode, so that these largest plasma VIII:vWF multimer forms were then relatively diminished in peripheral venous plasma samples.

In buffers containing Ca$^{2+}$/Mg$^{2+}$ (as in circulating blood), the attachment of the largest VIII:vWF forms to platelets in vitro in the presence of ristocetin induces a distinct type of agglutination that is unaccompanied by the release of platelet granule contents. In patient 3, who was evaluated during an acute HUS episode and then following recovery, we detected no increased intravascular release of platelet granule contents or thrombin generation in vivo. There was no increase in peripheral venous plasma levels of the platelet a-granule protein, β-TG, during the acute HUS episode in patient 3, when these values were compared with those of other uremic patients who did not have HUS. Fibrinopeptide A levels in his peripheral venous plasma also were not elevated.

During the HUS episode in patient 3, the plasma distribution of VIII:vWF forms was transiently normalized immediately after hemodialysis. Hemodialysis of uremic patients without HUS usually results in no alteration in VIII:vWF multimer patterns, either in peripheral venous plasma samples or in plasma prepared from afferent and efferent blood samples obtained across dialysis membranes (G.W. Schmitt, J.L. Moake, C.K. Rudy, and J.H. Troll, unpublished observations). One possible explanation of the dialysis results in patient 3, based on in vitro experiments with ristocetin and other small cations, is that a dialyzable cofactor of low molecular weight derived from injured renal (or other) cells was involved in inducing the attachment to platelets of the largest plasma VIII:vWF multimer forms.

It is not known if the infusion of fresh-frozen plasma in HUS patients provides substances that bind and remove this putative cofactor, or if the therapeutic effect of plasma infusion in HUS is due to some entirely different effect. All of our seven patients received transfusions with normal plasma. All recovered hematologically, but three of the seven had residual renal dysfunction.

In five of the seven HUS patients studied, plasma samples were obtained before any therapeutic intervention occurred. Two of the patients (patients 3 and 4) had been transfused during the previous 24 hours with fresh-frozen plasma (along with fresh blood and platelets in patient 4). These transfusions might have increased the VIII:vWF antigen levels in initial plasma samples by 20% to 25%, at most. It can be inferred that the initial VIII:vWF antigen levels in patients 3 and 4 were at least 400% and 800%, respectively, even after allowing for the persistence in patient plasma of most of the VIII:vWF antigen transfused in blood products. If the transfusion of plasma containing a normal distribution of VIII:vWF multimers had an effect on the VIII:vWF patterns observed in the initial plasma samples obtained from patients 3 and 4, it would be expected that the effect was to make the VIII:vWF patterns less abnormal than if the patients had not been transfused.

Unusually large VIII:vWF multimer forms derived from endothelial cells were not found in the peripheral venous plasma of any of the seven patients, either during or after their HUS episodes. In contrast to patients with chronic relapsing TTP, there was no evidence that the HUS patients had a defect in the conversion of unusually large VIII:vWF multimers derived from endothelial cells to the VIII:vWF forms normally in circulation.

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