

VON WILLEBRAND FACTOR–CLEAVING PROTEASE IN THROMBOTIC THROMBOCYTOPENIC PURPURA AND THE HEMOLYTIC–UREMIC SYNDROME

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ABSTRACT

Background Thrombotic thrombocytopenic purpura and the hemolytic–uremic syndrome are severe microvascular disorders of platelet clumping with similar signs and symptoms. Unusually large multimers of von Willebrand factor, capable of agglutinating circulating platelets under high shear stress, occur in the two conditions. We investigated the prevalence of von Willebrand factor–cleaving protease deficiency in patients with familial and nonfamilial forms of these disorders.

Methods Plasma samples were obtained from 53 patients with thrombotic thrombocytopenic purpura or hemolytic–uremic syndrome. Von Willebrand factor–cleaving protease was assayed in diluted plasma samples with purified normal von Willebrand factor as the substrate. The extent of the degradation of von Willebrand factor was assessed by electrophoresis in sodium dodecyl sulfate–agarose gels and immunoblotting. To determine whether an inhibitor of von Willebrand factor–cleaving protease was present, we measured the protease activity in normal plasma after incubation with plasma from the patients.

Results We examined 30 patients with thrombotic thrombocytopenic purpura and 23 patients with the hemolytic–uremic syndrome. Of 24 patients with nonfamilial thrombotic thrombocytopenic purpura, 20 had severe and 4 had moderate protease deficiency during an acute event. An inhibitor found in 20 of these patients was shown to be IgG in five of five tested plasma samples. Of 13 patients with nonfamilial hemolytic–uremic syndrome, 11 had normal levels of activity of von Willebrand factor–cleaving protease during the acute episode, whereas in 2 patients, the activity was slightly decreased. All 6 patients with familial thrombotic thrombocytopenic purpura lacked von Willebrand factor–cleaving protease activity but had no inhibitor, whereas all 10 patients with familial hemolytic–uremic syndrome had normal protease activity. In vitro proteolytic degradation of von Willebrand factor by the protease was studied in 5 patients with familial and 7 patients with nonfamilial hemolytic–uremic syndrome and was normal in all 12 patients.

Conclusions Nonfamilial thrombotic thrombocytopenic purpura is due to an inhibitor of von Willebrand factor–cleaving protease, whereas the familial form seems to be caused by a constitutional deficiency of the protease. Patients with the hemolytic–uremic syndrome do not have a deficiency of von Willebrand factor–cleaving protease or a defect in von Willebrand factor that leads to its resistance to protease. (N Engl J Med 1998;339:1578-84.)

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THROMBOTIC thrombocytopenic purpura, a disseminated thrombotic microangiopathy, was initially described by Moschowitz in 1924.¹ Three decades later, Gasser et al.² reported a similar disorder in children that they called the hemolytic–uremic syndrome. Today the two conditions are often regarded as variants of a single syndrome called thrombotic thrombocytopenic purpura–hemolytic–uremic syndrome. The syndrome is characterized by thrombocytopenia, hemolytic anemia, fever, renal abnormalities, and neurologic disturbances.^{3,4} The term thrombotic thrombocytopenic purpura is usually preferred for cases in adults in which neurologic dysfunction predominates, whereas cases involving predominantly glomerular damage, which occur mainly in children, are diagnosed as the hemolytic–uremic syndrome. Because some cases of thrombotic thrombocytopenic purpura can involve severe renal abnormalities and extrarenal manifestations can occur in the hemolytic–uremic syndrome, the two disorders are often difficult to distinguish. The terms microangiopathic hemolytic anemia and thrombotic microangiopathy have been used for cases that are associated with infection, bone marrow transplantation, drug therapy, cancer, chemotherapy, and pregnancy. Endothelial injury is generally considered the primary event.

Unusually large forms of von Willebrand factor occur in plasma in patients with chronic relapsing forms of thrombotic thrombocytopenic purpura⁵ and the hemolytic–uremic syndrome.⁶ These extremely large multimers may agglutinate circulating platelets at sites with high levels of intravascular shear stress.⁷ Increased binding of von Willebrand factor to platelets and increased numbers of circulating platelet aggregates have been observed in patients with thrombotic thrombocytopenic purpura.⁸ A specific von Willebrand factor–cleaving protease has recently been isolated from normal human plasma.^{9,10} Severe deficiency of this protease was found in four patients with chronic relapsing thrombotic thrombocytopenic

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nic purpura, two of whom were brothers.¹¹ In another patient with recurrent episodes of thrombotic thrombocytopenic purpura, both a deficiency of protease activity and an autoantibody against von Willebrand factor-cleaving protease were found in plasma.¹² These results suggest that von Willebrand factor-induced agglutination of circulating platelets may be caused by constitutional as well as acquired deficiencies of von Willebrand factor-cleaving protease. Since the prognosis and treatment of patients may be influenced by the severity and nature of the von Willebrand factor-cleaving protease deficiency, we conducted a multicenter retrospective study of the prevalence of this deficiency in patients with acquired and familial thrombotic thrombocytopenic purpura or hemolytic-uremic syndrome.

METHODS

Study Design and Patients

The participating centers (in Bern, Switzerland; Bergamo, Italy; Vienna, Austria; and Frankfurt and Magdeburg, Germany) provided frozen plasma samples from patients who had received a diagnosis of thrombotic thrombocytopenic purpura-hemolytic-uremic syndrome according to clinical and laboratory criteria. The disease was classified as familial if more than one family member was affected and if at least one of the following criteria was met: each affected family member had had multiple acute episodes, the acute episodes had occurred at different times in the case of siblings, and the affected family members were living in different households when the episodes occurred. For each patient, a questionnaire was filled out concerning history; signs of and laboratory data on intravascular hemolysis; the presence of fragmented erythrocytes, fever, neurologic symptoms, and renal dysfunction; the number of acute events; and possible associated risk factors, such as pregnancy, cancer, chemotherapy, bone marrow transplantation, and infection. Furthermore, available information on therapy and its efficacy was collected. The disorders were characterized as relapsing if there were complete remissions between acute bouts and as chronic if complete recovery did not occur between bouts. The diagnosis of the disease as thrombotic thrombocytopenic purpura or the hemolytic-uremic syndrome was made by the investigators at each participating center without knowledge of the results of the von Willebrand factor-cleaving protease assay. The study was approved by the ethics committee of the University of Bern.

Assays

Blood samples were collected from patients before a plasma-exchange session was begun. However, some patients who were examined during an acute episode had received plasma therapy on the days preceding the examination. Blood samples were anticoagulated with either sodium citrate or heparin. Platelet-poor plasma was stored at -20°C until tested. Platelet counts and measurements of hemoglobin, lactate dehydrogenase, total bilirubin, and creatinine were performed according to conventional laboratory methods at each center. Von Willebrand factor antigen was measured by an enzyme-linked immunosorbent assay with a commercial rabbit antiserum against human von Willebrand factor (Nordic, Tilburg, the Netherlands).

The assay of von Willebrand factor-cleaving protease activity was performed as previously described.¹¹ Plasma samples were diluted 1:20 with a solution of 0.15 mol of sodium chloride per liter and 10 mmol of TRIS per liter (pH 7.4) containing 1 mmol of serine protease inhibitor per liter (Pefabloc SC, Boehringer, Mannheim, Germany). The protease was activated by a five-minute incubation at 37°C with 10 mmol of barium chloride per liter.

Immediately after activation, 100 μl of the incubation mixture was added to 50 μl of protease-free von Willebrand factor (with the concentration adjusted to about 3 U of von Willebrand factor antigen per milliliter) that had been purified by gel filtration on Sepharose CL-2B columns (Pharmacia LKB, Uppsala, Sweden) from a cryoprecipitate of normal human plasma. The reaction mixture was dialyzed on the surface of a hydrophilic filter that was 25 mm in diameter (VSWP, Millipore, Bedford, Mass.) for 24 hours at 37°C against 1.5 mol of urea per liter and 5 mmol of TRIS-hydrochloric acid per liter (pH 8.0). The reaction was stopped by the addition of 10 μl of EDTA (0.2 mol per liter, pH 7.4), and the extent of the degradation of von Willebrand factor was assessed by multimer analysis with sodium dodecyl sulfate electrophoresis on 1.4 percent agarose gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes and stained with peroxidase-conjugated rabbit antibodies against human von Willebrand factor (P0226, Dako, Glostrup, Denmark).⁹ A pooled sample of citrated normal human plasma, obtained from 42 healthy male subjects and stored at -70°C , was used to calibrate the protease assay.

Each plasma sample was assessed at least three times for von Willebrand factor-cleaving protease activity, and the results are given as mean percentages of the activity of normal pooled human plasma. Samples with activity levels that were more than 50 percent of the value for normal pooled human plasma were defined as normal. We assayed samples for an inhibitor of von Willebrand factor-cleaving protease by measuring the protease activity in mixtures of plasma from patients and normal plasma at three dilutions (4:1, 1:1, and 1:5) after a 10-minute incubation at 37°C .¹² The amount of protease activity inhibited by 1 ml of the patients' plasma was then estimated; thus, 1 U of inhibitor neutralizes the protease activity in 1 ml of normal human plasma.

Proteolytic digestion of von Willebrand factor from the patients' plasma samples was assayed by multimer analysis after protease activation and dialysis as described above, but without the addition of von Willebrand factor substrate isolated from normal human plasma.

The activity of von Willebrand factor-cleaving protease was also determined in commercially prepared samples of virus-inactivated fresh-frozen plasma provided by the Blood Transfusion Service (Swiss Red Cross, Bern, Switzerland) and Octapharma (Vienna, Austria). In six different batches from the Swiss Red Cross, virus had been inactivated by exposure to light in the presence of methylene blue followed by filtration under sterile conditions with LeukoVir filters (HemaSure, Marlborough, Mass.), whereas six batches from Octapharma had been treated with the solvent-detergent procedure.

Affinity chromatography of IgG was performed on a 5-ml column of protein A-Sepharose (Pharmacia LKB) as described previously.¹² We applied samples of plasma to a column that had been equilibrated with 0.15 mol of sodium chloride per liter and 10 mmol of TRIS per liter (pH 7.4). Plasma proteins were eluted with 0.15 mol of sodium chloride per liter and 10 mmol of TRIS per liter (pH 7.4), followed by 0.1 mol of sodium citrate per liter (pH 4.0). The inhibitor of von Willebrand factor-cleaving protease was assayed in the unbound fraction of the plasma samples as well as in the IgG fraction eluted at pH 4.0 after dialysis against 0.15 mol of sodium chloride per liter and 10 mmol of TRIS per liter (pH 7.4).

RESULTS

The 53 patients with thrombotic microangiopathy who were included in the study were divided into two groups (Table 1): 37 patients without a familial history of the disorder were classified as having nonfamilial thrombotic thrombocytopenic purpura or hemolytic-uremic syndrome, and 16 patients with at least one additional affected family member were classified as having a familial form. The clinical

TABLE 1. CLINICAL CHARACTERISTICS OF THE PATIENTS.*

CHARACTERISTIC	NONFAMILIAL DISEASE		FAMILIAL DISEASE		TOTAL (N=53)
	THROMBOTIC THROMBOCYTOPENIC PURPURA (N=24)	HEMOLYTIC- UREMIC SYNDROME (N=13)	THROMBOTIC THROMBOCYTOPENIC PURPURA (N=6)	HEMOLYTIC- UREMIC SYNDROME (N=10)	
	Sex (M/F)	8/16	4/9	4/2	
Median age at first event (yr)	33.1	44.3	22.7	7.6	28.9
Single acute event (no. of patients)	10	13	0	3	26
Relapsing microangiopathy (no. of patients)	9	0	5	7	21
Chronic microangiopathy (no. of patients)	5	0	1	0	6
Fever (no. of patients)	4†	3‡	ND	ND	ND
Fragmented erythrocytes or hemolysis (no. of patients)	18§	11‡	ND	ND	ND
Neurologic symptoms (no. of patients)	16†	3¶	ND	ND	ND
Renal dysfunction (no. of patients)	5†	11‡	ND	ND	ND
Treatment (no. of patients)					
Plasma exchange	21	10	4	8	43
Corticosteroids	15	6	2	1	24
Vincristine	9	2	0	0	11
Splenectomy	4	1	0	0	5
Death during acute event (no. of patients)	6	2	0	0	8
Dialysis (no. of patients)	0	4	1	7	12
Possible associated risk factor (no. of patients)					
Pregnancy	3	1	2	1	7
Tumor or chemotherapy	0	5	0	0	5
Bone marrow transplantation	1	1	0	0	2
Infection	0	3	1	4	8

*ND denotes not determined.

†Data on 11 patients were available.

‡Data on 12 patients were available.

‡Data on 22 patients were available.

§Data on 20 patients were available.

diagnosis was thrombotic thrombocytopenic purpura in 30 patients and the hemolytic-uremic syndrome in 23 patients. In the group of 24 patients with nonfamilial thrombotic thrombocytopenic purpura, blood samples were obtained from 15 patients during both an acute episode and remission (Patients 1 to 15) and from 9 patients during the acute event only (Patients 16 to 24). Of the 13 patients who were given a diagnosis of nonfamilial hemolytic-uremic syndrome, 8 had plasma samples obtained both during the acute event and during remission (Patients 25 to 32), and 5 had plasma samples obtained during the acute event alone (Patients 33 to 37). Of the 16 patients with familial thrombotic thrombocytopenic purpura or hemolytic-uremic syndrome, plasma was obtained from 5 patients during an acute event and from 11 during remission. Eight patients with nonfamilial conditions died during the acute episode (Table 1). Thirteen siblings of patients with familial hemolytic-uremic syndrome had died; plasma samples from these family members were not available for investigation. In two patients (Patients 37 and 42), the initial clinical diagnosis was thrombotic thrombocytopenic purpura-hemolytic-uremic syndrome: in Patient 37, the kidneys were predomi-

nantly involved, whereas the other patient had a sibling (Patient 43) with recurring thrombotic thrombocytopenic purpura. They were subsequently given diagnoses of nonfamilial hemolytic-uremic syndrome and familial thrombotic thrombocytopenic purpura, respectively.

Laboratory values are presented in Table 2. Among the patients with nonfamilial conditions, most had markedly increased levels of von Willebrand factor antigen during the acute episode, and the levels often remained abnormally high during remission. Of the 24 patients with nonfamilial thrombotic thrombocytopenic purpura, 18 had normal plasma creatinine values during an acute episode, whereas most patients with nonfamilial hemolytic-uremic syndrome had strikingly increased creatinine levels during an acute episode. All 13 patients with nonfamilial hemolytic-uremic syndrome had had only a single acute event, whereas 14 of the 24 patients with nonfamilial thrombotic thrombocytopenic purpura had had more than one acute episode. It must be emphasized that six patients with thrombotic thrombocytopenic purpura and two patients with the hemolytic-uremic syndrome died during the first acute episode, thus precluding a relapse. Six patients from

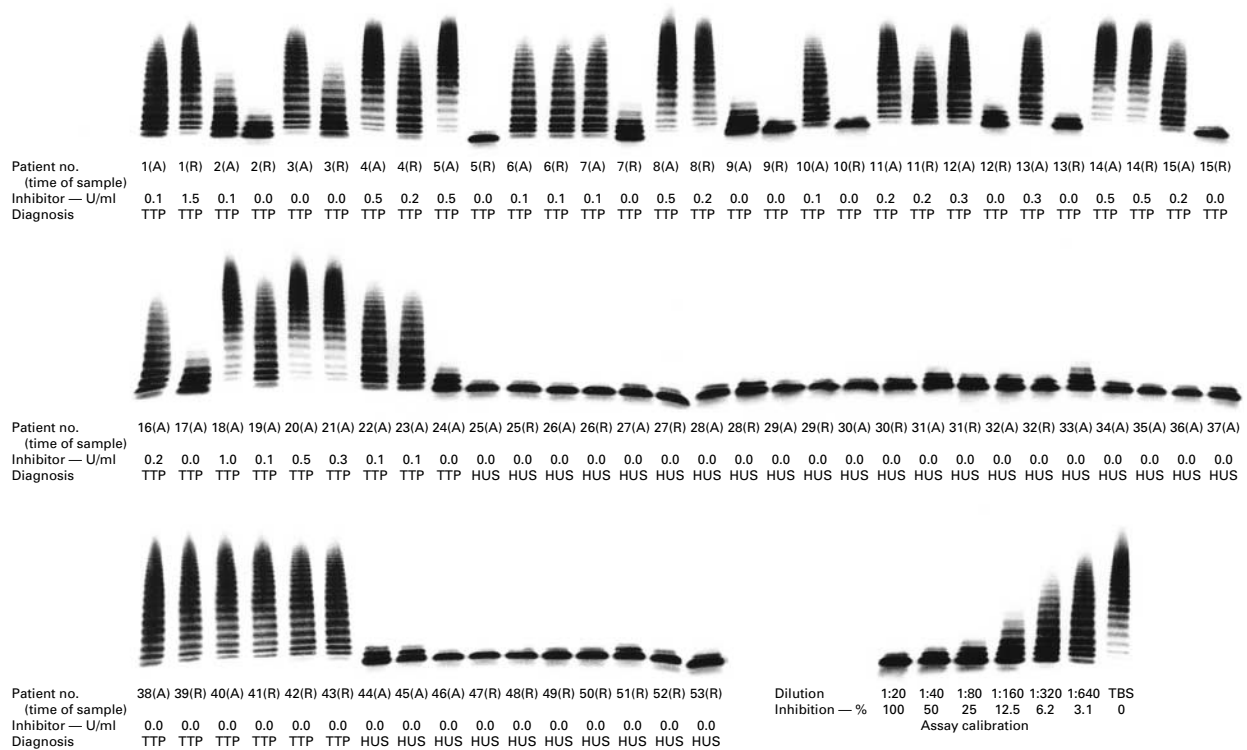


Figure 1. Activity of von Willebrand Factor–Cleaving Protease and the Level of Its Inhibitor in 24 Patients with Nonfamilial Thrombotic Thrombocytopenic Purpura (Patients 1 to 24), 13 Patients with Nonfamilial Hemolytic–Uremic Syndrome (Patients 25 to 37), 6 Patients with Familial Thrombotic Thrombocytopenic Purpura (Patients 38 to 43), and 10 Patients with Familial Hemolytic–Uremic Syndrome (Patients 44 to 53).

The following pairs of patients are siblings: Patients 38 and 39, 40 and 41, 42 and 43, 44 and 45, 47 and 48, 49 and 50, and 52 and 53. The siblings of Patients 46 and 51 had died before the study began. Plasma samples were collected during an acute event (A) and remission (R). The multimeric patterns of purified normal von Willebrand factor substrate after incubation with diluted plasma samples from the patients are shown. The protease assay was calibrated against various dilutions of normal plasma (1:20 to 1:640), and the levels of the protease inhibitor are expressed as the number of inhibitor units per milliliter of plasma. One unit of inhibitor neutralizes the protease activity in 1 ml of normal human plasma. TTP denotes thrombotic thrombocytopenic purpura, HUS hemolytic–uremic syndrome, and TBS 0.15 mol of sodium chloride per liter and 10 mmol of TRIS per liter (pH 7.4).

umn and was eluted from the column at pH 4.0, indicating the presence of IgG.

Eleven of the 13 patients with acute nonfamilial hemolytic–uremic syndrome had normal levels of activity of von Willebrand factor–cleaving protease (defined as activity that was more than 50 percent of the level in normal subjects), and 2 had lower levels of activity (26 to 50 percent of the level in normal subjects). All eight plasma samples collected during remission from patients with nonfamilial hemolytic–uremic syndrome had normal levels of activity of von Willebrand factor–cleaving protease (Fig. 1 and Table 2).

Two patients with acute familial thrombotic thrombocytopenic purpura and four patients with familial thrombotic thrombocytopenic purpura in remission had no activity of von Willebrand factor–cleaving protease. None of these six patients had a detectable protease inhibitor (Fig. 1 and Table 2). Another sib-

ling of Patients 42 and 43 also had complete deficiency of von Willebrand factor–cleaving protease but was not included in the study because he had never had symptoms of thrombotic thrombocytopenic purpura. All 10 patients with familial hemolytic–uremic syndrome had normal protease activity (Fig. 1 and Table 2). No deficiency of von Willebrand factor–cleaving protease was found in 120 normal subjects (data not shown). In plasma samples from five patients with familial hemolytic–uremic syndrome (Patients 45, 46, 50, 51, and 52) and seven patients with nonfamilial hemolytic–uremic syndrome (Patients 27, 28, 29, 30, 31, 32, and 35), von Willebrand factor was completely degraded by von Willebrand factor–cleaving protease (data not shown).

The activity of von Willebrand factor–cleaving protease was determined in 12 batches of commercially prepared samples of fresh-frozen plasma that had been treated with two virus-inactivating proce-

dures. All 12 batches had normal protease activity (data not shown).

DISCUSSION

Thrombotic thrombocytopenic purpura and the hemolytic-uremic syndrome share several clinical features: thrombocytopenia, intravascular hemolysis caused by erythrocyte fragmentation, and injury of the kidneys and the central nervous system. Different triggering factors may be involved, such as bacterial or viral infection, pregnancy, drug therapy, chemotherapy, and bone marrow transplantation. The conditions can occur in several members of the same family. The causes and pathogenesis are unknown. It is generally assumed that endothelial-cell injury is the initial event. Bacterial endotoxins, antibodies and immune complexes, oxidative injury, and certain drugs are possible causes of endothelial damage. Reduced production of prostacyclin, impaired fibrinolysis, and platelet-aggregating agents have been implicated in the development of thrombotic thrombocytopenic purpura-hemolytic-uremic syndrome. Moake et al.^{5,6} and Charba et al.¹³ attributed the enhanced intravascular platelet clumping in these syndromes to the presence of unusually large polymers of von Willebrand factor. Systemic endothelial-cell injury may lead to excessive release from endothelial cells of extremely large polymers of von Willebrand factor that cannot be processed to smaller forms by a specific "depolymerase."^{5,14,15} Normal human plasma or its cryosupernatant was proposed to contain a disulfide-bond reductase capable of degrading unusually large von Willebrand factor multimers to smaller molecular forms.^{16,17} We have recently isolated a specific protease from human plasma that cleaves the subunit of von Willebrand factor between tyrosine at position 842 and methionine at position 843,⁹ the same peptide bond that had been shown to be cleaved *in vivo*.¹⁸ This protease was deficient in four patients with chronic relapsing thrombotic thrombocytopenic purpura and unusually large circulating multimers of von Willebrand factor,¹¹ and no protease inhibitor was detected. The lack of von Willebrand factor-cleaving protease activity in another patient was the result of an autoantibody that inhibited the protease activity.¹² The inhibitor persisted for about one year and disappeared after splenectomy, which was followed by normalization of protease activity, the multimeric pattern of von Willebrand factor, and the platelet count.

In the present retrospective study, we found a high prevalence of von Willebrand factor-cleaving protease deficiency in 24 patients with nonfamilial thrombotic thrombocytopenic purpura: 20 patients had severe protease deficiency (<5 percent of normal activity) and 4 moderate protease deficiency (5 to 25 percent of normal activity) during an acute event. Although blood samples were generally collected

before a plasma-exchange session was begun, some patients who were examined during the acute episode had received plasma therapy on preceding days. Since the biologic half-life of the protease is longer than one day (unpublished data), the protease activity in the four patients with moderate protease deficiency may originate from plasma exchange with fresh-frozen plasma or the inhibitor titer may be affected by this treatment. An inhibitor was found in 20 of 24 patients with acute thrombotic thrombocytopenic purpura and turned out to be IgG in all 5 patients who were tested.

Among the patients with familial thrombotic thrombocytopenic purpura, all six patients (two during an acute episode and four while in remission) were found to have a protease deficiency (<5 percent of normal activity), but none had an inhibitor. We conclude that the deficiency of von Willebrand factor-cleaving protease is highly associated with thrombotic thrombocytopenic purpura. This deficiency may be inherited or acquired as a result of an autoimmune mechanism. A possible association with known triggering conditions has been established in only 4 of 24 patients with nonfamilial thrombotic thrombocytopenic purpura: 3 patients (Patients 5, 6, and 14) had their first episode during pregnancy, and 1 had undergone an allogeneic bone marrow transplantation for acute leukemia. Three patients with nonfamilial thrombotic thrombocytopenic purpura (Patients 9, 12, and 13) entered remission after splenectomy. In all three patients, splenectomy was followed by the disappearance of the inhibitor and normalization of protease activity. Early remission in another patient (Patient 1), despite the persistence of the von Willebrand factor-cleaving protease deficiency due to an autoantibody against the protease, was followed by recurrent episodes of thrombotic thrombocytopenic purpura, and he recovered only after splenectomy and the subsequent disappearance of the inhibitor.¹¹

Plasma exchange or infusion is considered the therapy of choice in patients with thrombotic thrombocytopenic purpura. Since von Willebrand factor-cleaving protease presumably is a hydrophobic high-molecular-weight protein,⁹ the question arises whether the protease activity survives the treatments used to inactivate viruses. We have found normal levels of activity of von Willebrand factor-cleaving protease in commercially prepared samples of fresh-frozen plasma in which viruses had been inactivated either by exposure to light in the presence of methylene blue or by the solvent-detergent procedure. We conclude that the use of these virus-inactivated products is appropriate for plasma exchange in patients with thrombotic thrombocytopenic purpura.

Eleven of 13 patients with nonfamilial acute hemolytic-uremic syndrome had normal levels of activity of von Willebrand factor-cleaving protease, and 2 had

levels of activity that were 26 to 50 percent of normal, whereas all 10 patients with familial hemolytic-uremic syndrome had normal protease activity. Proteolytic degradation of von Willebrand factor by von Willebrand factor-cleaving protease was not impaired in five patients with familial and seven patients with nonfamilial hemolytic-uremic syndrome, indicating that platelet agglutination in these patients is not due to resistance of von Willebrand factor to proteolytic cleavage. Our results thus suggest that neither a structural abnormality of von Willebrand factor leading to its resistance to the specific von Willebrand factor-cleaving protease nor a deficiency of the protease is involved in the pathogenesis of acquired or familial hemolytic-uremic syndrome.

Our results show that patients with nonfamilial thrombotic thrombocytopenic purpura have an acquired deficiency of von Willebrand factor-cleaving protease that is caused by an autoimmune mechanism. Patients with familial thrombotic thrombocytopenic purpura had a complete protease deficiency in the absence of an inhibitor. It is likely that these patients would readily, though only temporarily, recover if they received the protease through plasma-exchange therapy. On the other hand, patients with nonfamilial thrombotic thrombocytopenic purpura and high titers of inhibitor may need large volumes of infused plasma. Treatment with immunosuppressive agents,^{19,20} immunoabsorption with a protein A-Sepharose column,²¹ and treatment with vincristine^{12,22} or splenectomy^{12,23} should be considered as additional therapeutic regimens in these patients. The deficiency of von Willebrand factor-cleaving protease in patients with thrombotic thrombocytopenic purpura and the normal von Willebrand factor-cleaving protease activity in patients with the hemolytic-uremic syndrome provide a means of discriminating between these two syndromes, which are often difficult to distinguish clinically. We conclude that the assay of von Willebrand factor-cleaving protease and its inhibitor may be useful in the differential diagnosis and treatment of patients with thrombotic thrombocytopenic purpura-hemolytic-uremic syndrome.

Supported by grants from the Swiss National Science Foundation (32-47033.96); the Malcolm Hewitt Wiener Foundation, New York; the Central Laboratory, Blood Transfusion Service, Swiss Red Cross, Bern, Switzerland; and Immuno, Vienna, Austria.

We are indebted to Daniel Landau, M.D., Soroka Medical Center, Beer Sheva, Israel, for providing plasma samples from Patients 44 and 45.

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