

Impairment of Platelet Aggregation in Hemolytic Uremic Syndrome: Evidence for Platelet "Exhaustion"

By J. S. C. Fong and B. S. Kaplan

Thrombocytopenia, microangiopathic hemolytic anemia, and acute renal failure are the hallmarks of hemolytic-uremic syndrome (HUS). This report presents the results on platelet studies from 10 consecutive HUS patients in childhood. During their acute illness, they all displayed a characteristic pattern of impaired platelet function: no aggregating responses to epinephrine, some to ADP, and moderate to collagen. In addition, platelet contents of beta-thromboglobulin (β TG) were markedly reduced. As these patients improved clinically, their platelet-aggregating responses also normalized despite their uremic state. Incubation of platelets with uremic plasma or guanidinosuccinic acid, a uremic toxin, had minor effects on platelet-aggregating activity. Since low levels of platelet β TG

suggest that these platelets were in an exhausted state, *in vitro* experiments were performed to exhaust normal platelets by incubation at 37°C. A proportional impairment of platelet-aggregating responses and decreasing levels of platelet β TG were noted. Furthermore, the pattern of impairment was similar to that found in the platelet-aggregating activities of HUS patients. Thus, "exhaustion," in addition to azotemia and thrombocytopenia, are factors that contribute to the functional impairment of platelets in these patients. Further studies to reveal mechanisms that lead to platelet exhaustion in HUS are of fundamental importance in the understanding of this illness.

THE HEMOLYTIC UREMIC SYNDROME (HUS) is characterized by the triad of hemolytic anemia, thrombocytopenia, and acute renal failure.¹ Although thrombocytopenia occurs in most of these patients, the role of the platelets in its pathogenesis remains uncertain.^{2,3} The duration of thrombocytopenia usually does not correlate with severity or duration of HUS.⁴⁻⁶ It is unlikely that a thrombopoietic defect leads to thrombocytopenia because of the demonstration of normal megakaryocytes on bone marrow examination.⁷ Increased platelet consumption, as reflected by the frequent demonstration of shortened platelet survival, may account for the thrombocytopenia,⁸⁻¹¹ and platelet survival is inversely correlated with the degree of thrombocytopenia.¹¹ Localized or disseminated intravascular coagulation, microangiopathy, and thrombosis are possible mechanisms that could be responsible for the shortened platelet survival. Studies on adults with HUS suggest that the kidneys may have an important role in platelet survival because, in some patients, nephrectomy has been followed by prompt normalization of platelet counts.¹⁰⁻¹³ In contrast, however, surface counting following infusion of labeled platelets has revealed increased uptake over spleen and liver, and to a much less extent, the kidneys.^{4,11,14} In addition to low levels of circulating platelets, a func-

tional defect with a decrease in thromboplastic and antiheparin activities has also been demonstrated.⁵

Although a clear understanding of the pathogenic role of platelets is lacking, patients have been treated with antiplatelet agents.¹⁵⁻¹⁷ Our previous demonstration of reduced platelet-aggregating activities in three cases^{18,19} has also been found by Pareti et al.²⁰ The purpose of this report is to describe our cumulative studies on platelet functions of 10 patients with HUS. A characteristic pattern of impaired platelet aggregation occurs during the acute phase of the syndrome; platelet exhaustion may account for this functional impairment.

MATERIALS AND METHODS

Patients

Ten consecutive patients with HUS were studied from November 1976 to April 1981. None of the patients had received any agent known to interfere with platelet function during the 10 days prior to these studies. Each patient had a severe hemolytic anemia (lowest hemoglobin concentration ranged from 4.3 to 7.5 g/dl), typical peripheral blood smear with fragmented and burr cells, and acute renal failure (highest serum creatinine concentration ranged from 2.3 to 8.7 mg/dl). Nine patients were thrombocytopenic with platelet counts as low as 5000–101,000/cu mm. One patient, whose hemoglobin concentration was 6.1 g/dl and serum creatinine was 8.1 mg/dl, always had a platelet count exceeding 241,000/cu mm. Six patients were classified as having severe disease;²¹ 5 of these had anuria for 2–12 days. One patient had seizures, hypertension, and oliguria and had a progressive course with gangrene of the colon and cardiac necrosis. He died despite treatment with hemicolecotomy, peritoneal dialysis, infusions of fresh frozen plasma, and plasmapheresis. He was the only patient who died.

Healthy laboratory personnel who did not consume any agent with known antiplatelet functions during the preceding 10 days served as controls. These were designated as normals, and their studies were carried out simultaneously with respective patients.

To study the effect of uremia and thrombocytopenia on platelet aggregation, a group of 6 children of comparable age to that of the

From the Montreal Children's Hospital, Department of Nephrology, Montreal, Quebec, Canada.

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Address reprint requests to Dr. J. S. C. Fong, Department of Nephrology, Montreal Children's Hospital, 2300 Tupper Street, Room E-213, Montreal, Quebec, Canada H3H 1P3.

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HUS group and with chronic renal failure was studied. As a group, their serum BUN averaged 81 ± 32 mg/dl and creatinine was 4.5 ± 2.5 mg/dl. Eight children with normal renal function and normal platelet counts served as controls for this particular study.

Platelet Aggregation Studies

Blood samples were obtained from each patient during the acute and convalescent periods and from controls. Immediately following collection, 9 ml of blood were mixed gently in a polyethylene tube with 1 ml of a 3.8% (w/v) trisodium citrate solution in water. Platelet-poor plasma (PPP) was prepared by centrifugation of the blood at 1000 g for 20 min at 4°C, and platelet-rich plasma (PRP) was prepared by centrifugation of the blood at 300 g for 20 min at room temperature. For more accurate comparison, the platelet counts of the controls' PRP were adjusted with autologous PPP to achieve levels comparable to those of the patients' samples.

A Chrono-log aggregometer (Chrono-log Corp., Havertown, Pa.) coupled to a Fisher Recordall (Fisher Scientific Co., Montreal, Quebec, Canada) was used for the aggregometry study.²² Briefly, 0.45 ml of PRP was added with a stirring bar to a cuvette and placed in the machine. Light transmission was adjusted to 10%; similarly, that for PPP was adjusted to 90%. At zero time, aggregating agents were added in 50- μ l volumes. Platelet aggregation resulted in an increased light transmission and upward deflection of the recording pen.

Collagen and adenosine diphosphate (ADP) were purchased from Sigma Chemical Co., St. Louis, Mo. The collagen suspension was prepared as previously described.²² ADP was dissolved in normal saline to give a final concentration of 1 mM/liter. Epinephrine hydrochloride at 4.5 mM/liter was obtained from Parke, Davis and Co. (Brockville, Ontario, Canada) as an injectable preparation with less than 0.1% (w/v) sodium bisulphite as a preservative.

Since uremic plasma has been shown to suppress platelet function,^{23,24} the abnormal platelet responses noted during the acute illness could be secondary to uremia. To investigate this possibility, PRP from 3 patients in complete recovery were diluted 5-7-fold with homologous acute PPP to obtain similar platelet levels as in the acute illness. These samples were allowed to equilibrate for 15 min at room temperature prior to aggregation studies. Although some uremic toxins might require a longer incubation period to be effective, such experiments were not conducted, as platelets would be exhausted in

direct proportion to the length of the in vitro incubation even in the absence of uremic toxins. For the purpose of comparison and confirmation, aggregation studies were also performed on samples of normal PRP with the addition of guanidosuccinic acid (Sigma Chemical) in a final concentration of 2.5 mg/dl.

Beta-Thromboglobulin Assays

Plasma and platelet β -thromboglobulin (β TG) levels were measured by radioimmunoassay²⁵ (assay kits, Amersham Corp., Oakville, Ontario).

Platelet Exhaustion Study

In view of our demonstration that platelets from patients with HUS in the acute phase were exhausted as defined biochemically, experiments were done to study platelet aggregation activities of normal platelets with exhaustion induced in vitro. PRP samples from 6 controls were shaken in a water bath at 37°C. At 0, 2, and 4 hr of incubation, aliquots were removed for platelet aggregation studies and platelet β TG assays, and the latter were also performed with aliquots incubated for 24 hr.

RESULTS

Platelet Aggregation Studies

Table 1 summarizes the platelet counts and aggregation activities of controls and HUS patients during their acute illness. With the exception of patient 10, all studies were performed with thrombocytopenic PRP samples. Extremely high concentrations of aggregating agents were used. In addition to the apparent impairment of HUS patient platelet aggregation to epinephrine, collagen, and ADP, a characteristic pattern of defective activities was noted: no response to epinephrine, minimal to ADP, and moderate to collagen. In comparison with controls, patient 10 also had impaired platelet aggregation activities. However, the degree of impairment was less than that of the other patients. Aggregation responses of platelets from normal or uremic children were not impaired.

Table 1. Platelet Aggregation Studies of HUS Patients and Controls

Samples	Platelets $\times 10^3/\mu$ l	Aggregation Studies With					
		Epinephrine (450 μ M/liter)*		Collagen (1/100 Dilution)*		ADP (100 μ M/liter)*	
		dT†	Slope‡	dT	Slope	dT	Slope
HUS patients							
No. 1-9	51 ± 28	0	0	36 ± 24	4 ± 1	19 ± 7	3 ± 2
No. 10	375	15	5	15	8	65	17
Normals§							
(n = 6)	55 ± 16	41 ± 7	3 ± 1	70 ± 12	10 ± 4	87 ± 14	13 ± 3
p <	NS	0.001	0.005	0.05	0.02	0.001	0.001
Uremic controls¶							
(n = 6)	50 ± 15	61 ± 24	6 ± 3	77 ± 27	12 ± 4	63 ± 29	12 ± 6
Children controls							
(n = 8)	50 ± 10	54 ± 16	7 ± 2	62 ± 24	16 ± 9	61 ± 15	18 ± 4

*Expressed in final concentrations.

†dT, Maximum change in percent of light transmission as a result of platelet aggregation.

‡Slope, maximum change in percent of light transmission over a 12-sec period during the course of platelet aggregation.

§Platelet counts of normals were adjusted with respective autologous plasma and studied simultaneously with patient samples.

||p, Statistical analyses were performed between normals and HUS patients no. 1-9.

¶Plasma BUN = 81 ± 32 mg/dl; creatinine = 4.5 ± 2.5 mg/dl.

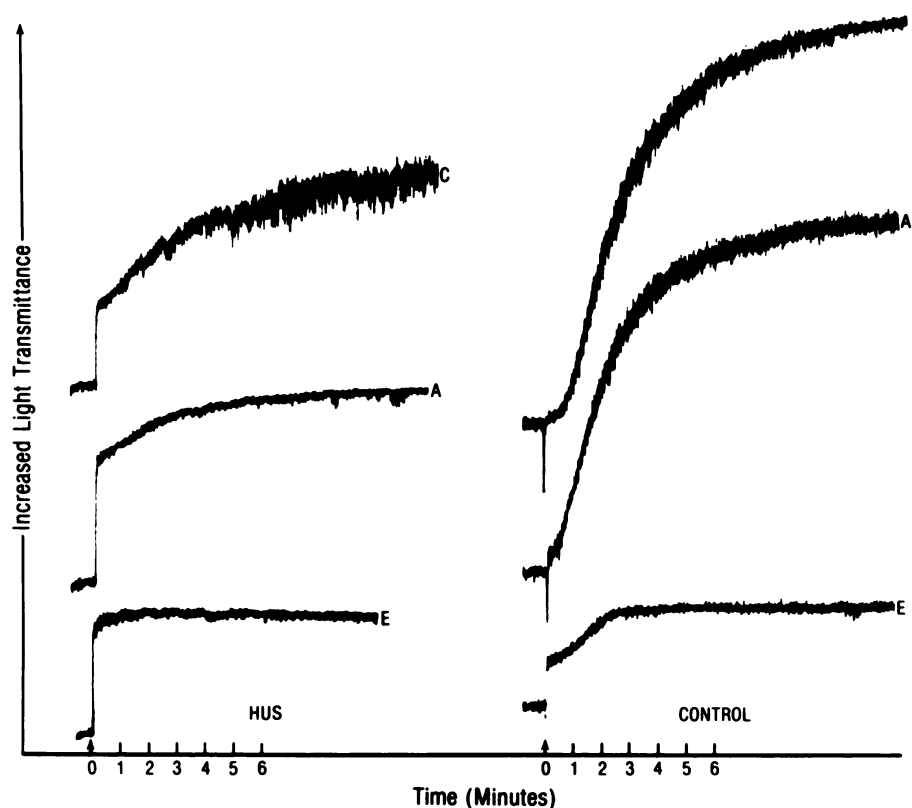


Fig. 1. Tracings of platelet aggregation study of a HUS patient and control. Platelet counts of the platelet-rich plasma used were 55,000/ μ l for the HUS and 50,000/ μ l for the control samples. Aggregating agent of epinephrine (E), ADP (A), or collagen (C) were added at zero time (arrow).

Illustrative tracings of platelet aggregation of a patient with the corresponding control are presented in Fig. 1 to demonstrate the characteristic pattern of impaired platelet aggregation responses in HUS patients. Biphasic responses to epinephrine were not observed at all. Both low platelet counts and high concentration of epinephrine might account for the observed monophasic response.

Results of repeated platelet aggregation studies in HUS patients are presented in Fig. 2, A and B. No attempt was made to adjust the platelet counts of any of the samples. During the acute stage, the platelet counts of the PRP samples of the patients were $51,000 \pm 28,000/\mu$ l. Samples from convalescent patients are presented in two groups according to the platelet counts. In the thrombocytopenic group, the range of the platelet levels was 50,000–100,000/ μ l, while those with a normal platelet count had a range of 195,000–425,000/ μ l. The renal function in both groups were similar: thrombocytopenic patients had BUN concentrations of 38 ± 27 mg/dl and creatinine levels of 2.0 ± 2.3 mg/dl; the other patients had BUN concentrations of 50 ± 23 mg/dl and creatinine levels of 3.1 ± 1.3 mg/dl. Platelet aggregation responses do improve as the patients improve clinically. Furthermore, platelet counts correlate well with the vigor or the aggregating responses, although the patients may still be azotemic.

Results of studies with PRP from patients in remission diluted with autologous uremic PPP and with the addition of guanidinosuccinic acid to normal PRP were similar. In agreement with previous reports on the effect of uremic toxins on platelet aggregation,^{23,24} our studies showed some inhibitory effect of both uremic plasma and guanidinosuccinic acid on platelet aggregation. However, the mild inhibition was reversible and could easily be overcome.

Using high concentrations of aggregating agents (Table 1), there was no inhibitory effect on platelet aggregation by uremic plasma or guanidinosuccinic acid.

β -Thromboglobulin

All 10 HUS patients, irrespective of their plasma platelet levels, had low platelet β TG contents and elevated plasma β TG concentrations. Uremic controls also had elevated plasma levels of β TG, although their platelet contents of this protein were within normal limits (Table 2).

Platelet Exhaustion Study

A total of six in vitro studies were performed with normal PRP samples having platelet counts at $280,000 \pm 120,000/\mu$ l. During the first 2 hr of incubation at 37°C, a rapid loss of endogenous β TG from platelets was noted (Fig. 3). As incubation continued,

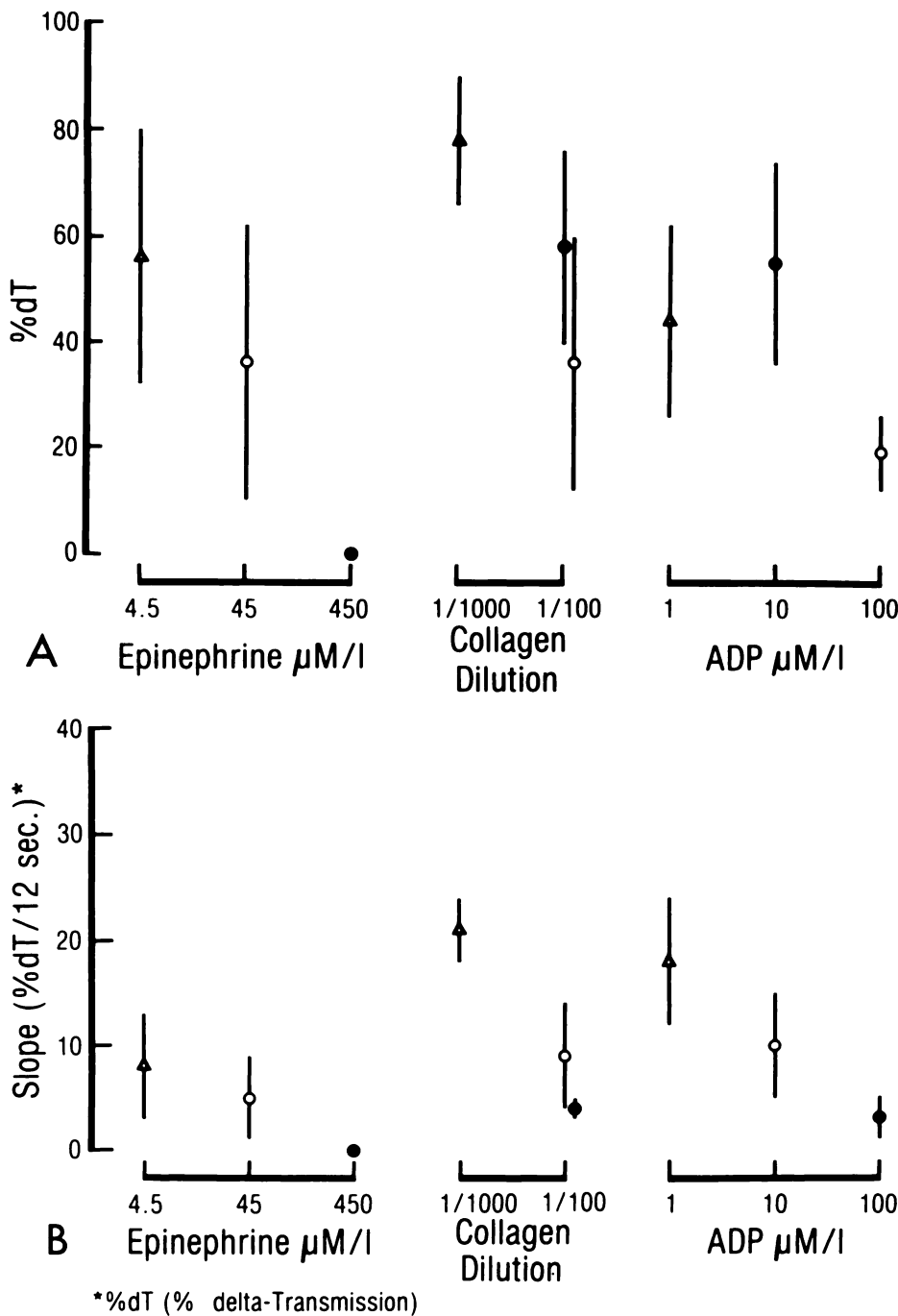


Fig. 2. Platelet aggregation studies of HUS patients when they were acutely ill (●) or in convalescence (○—thrombocytopenic, Δ—normal platelet count). (A) dT represents the maximum change in percent of light transmission as a result of platelet aggregation. (B) Slope represents the maximum change in percent of light transmission over a 12-sec period during the course of platelet aggregation.

the rate of β TG loss gradually decelerated, so that by the end of a 24-hr period, residual platelet β TG was about 40% of preincubation level. In general, platelet aggregation activities declined in proportion to the duration of incubation. Reduction in responses was noted equally for both magnitude and rate of platelet aggregation. Impairment of responses to the three aggregating agents differed in magnitude: most precipitous for epinephrine, moderate for ADP, and mild for collagen. Thus, the pattern of reduced aggregation

activities of exhausted platelets resembled that of platelets from HUS patients.

DISCUSSION

In addition to the thrombocytopenia, patients with HUS also have a functional impairment of the remaining circulating platelets. The reduced platelet-aggregating activities of 9 of these patients displayed a similar and characteristic pattern: no aggregation with

Table 2. Platelet, Beta-Thromboglobulin, and Renal Function in HUS Patients and Controls

Samples	Platelets ($\times 10^3/\mu$)	Platelet BTG (ng/ 10^6 Platelets)	Plasma β TG (ng/ml)	BUN (mg/dl)	Creatinine (mg/dl)
HUS patients					
No. 1-9	75 \pm 43	13 \pm 4	198 \pm 63	83 \pm 14	3.6 \pm 1.6
No. 10	230	6	156	184	8.1
Normals	266 \pm 133	36 \pm 12	83 \pm 20	13 \pm 7	0.7 \pm 0.3
$p < *$	0.01	0.005	0.005	0.001	0.001
Uremic controls ($n = 6$)	267 \pm 139	30 \pm 11	158 \pm 55	81 \pm 32	4.5 \pm 2.5
Children controls ($n = 8$)	218 \pm 101	40 \pm 10	88 \pm 32	15 \pm 4	0.6 \pm 0.2

*Statistical analyses were performed between normals and HUS patients no. 1-9.

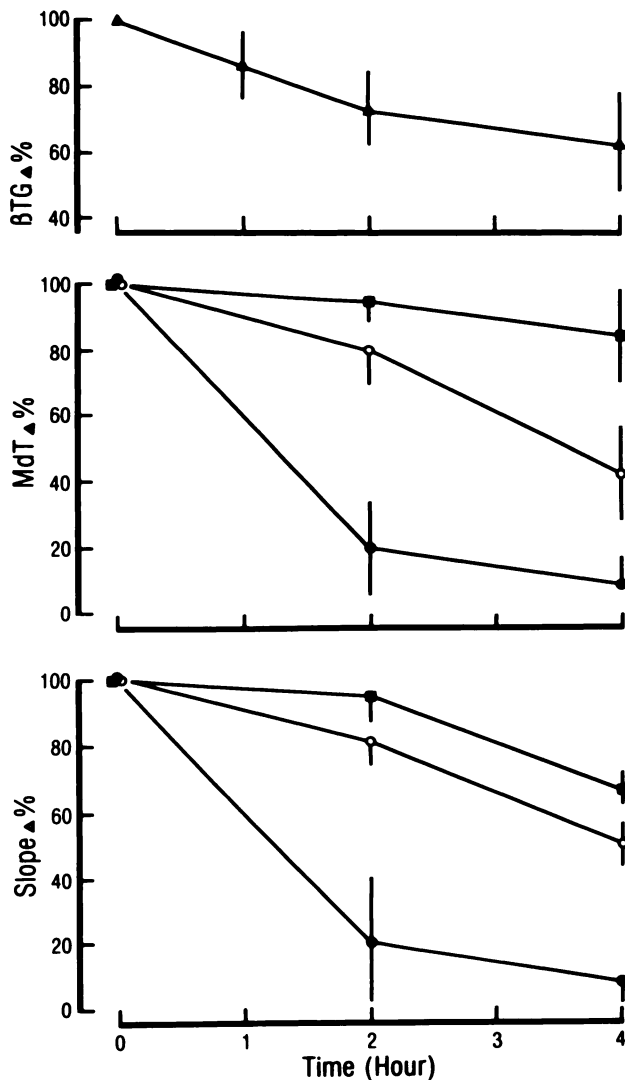


Fig. 3. Platelet beta-thromboglobulin contents and aggregation activities following a 2- or 4-hr period of incubation at 37°C. Results are expressed as percentages of respective samples studied at zero time. MdT represents the maximum change in percent of light transmission as a result of platelet aggregation. Slope represents the maximum change in percent of light transmission over a 12-sec period during the course of platelet aggregation. Aggregating agents used were epinephrine (●), ADP (○), and collagen (■).

epinephrine, some with ADP, and moderate with collagen. Control studies, with platelets from healthy adults, normal or uremic children, showed similar aggregating activities in the presence of high concentrations of aggregating agents (Table 1). As the clinical condition of HUS improved, the platelet aggregation responses also became more vigorous, even though they were still thrombocytopenic and uremic (Fig. 2 A and B). When convalescent patients were studied while still significantly uremic, aggregating responses were directly proportional to the platelet counts. Therefore, platelet number per se did have an influence on the overall reactivity in aggregating responses. Furthermore, uremia and its associated biochemical changes could also have some influence on the platelet aggregation activities during the acute illness. Nevertheless, both uremia and thrombocytopenia did not appear to be responsible for the grossly impaired aggregation activities in HUS patients during the first few days of their illness. Although uremia has been shown to have inhibitory effects on platelet function, this was mild and could easily be overcome using higher concentrations of aggregating agents.^{23,24} Similarly, we could not demonstrate any significant inhibitory effect on platelet aggregation by uremic plasma or guanidinosuccinic acid with high concentrations of aggregating agents. Studies on uremic platelets (Table 1) also supported the notion that uremia itself was not the major responsible factor to account for our observed impaired platelet function in HUS patients. Therefore, biochemical changes as a result of renal failure could not be singularly responsible for the impaired platelet function in HUS.

Aggregation studies with thrombocytopenic samples were technically possible.^{18,20,26} Since maximum sensitivity (maximum "gain" and "offset" settings) was demanded of the platelet aggregometer, normal samples were regularly studied simultaneously with that from patients. These normals were studied as controls needed because of the extreme settings of the aggregometer. Control studies of normal platelets diluted to

comparable levels as those in HUS responded well in the presence of high concentrations of aggregating agents. Comparison between normals and patients revealed such markedly different aggregating activities that it is unlikely that the low counts per se were the cause of the reduced responses, although thrombocytopenia probably did have a minor influence on the aggregating responses. Studies on samples during convalescence showed more vigorous aggregating responses for those samples that were not thrombocytopenic. Patient 10 differed from the other nine patients in that she had a normal platelet count and also demonstrated a lesser degree of platelet impairment.

The concept of "exhausted" circulating platelets has been proposed²⁷ in a report of one patient whose platelets had an undetectable level of intraplatelet serotonin compared to the normal level of 240 ng/10⁹ platelets.²⁸ Another study of two patients confirmed that their platelets had reduced levels of intraplatelet nucleotides and serotonin.²⁰

Platelet exhaustion was demonstrated in all ten of our patients because they had low levels of platelet β TG, a platelet-specific protein that becomes depleted with stimulation of platelets.²⁹ Elevated plasma levels of β TG in chronic renal failure, as reported previously and confirmed by this report (Table 2), were thought to be the result of impaired renal clearance and catabolism of this protein.³⁰ Thus, increased plasma levels of β TG in HUS could result from either platelet release or impaired renal function or both. Although patient 10 had normal platelet counts, her platelets

were exhausted as suggested by a very low level of platelet β TG. Since her platelet aggregation was impaired as compared to controls, platelet exhaustion appeared to be an important factor accounting for the impairment. The dysfunction of her platelet aggregation responses was milder in comparison to that of the other HUS patients.

In vitro experiments with normal PRP showed good correlation between platelet dysfunction (as assessed by aggregation) and the degree of platelet exhaustion (as assessed by β TG). The pattern of impairment of aggregation in response to epinephrine, ADP, and collagen as the platelets became more exhausted also resembled the findings in the patients with HUS. Therefore, in HUS, although thrombocytopenia and azotemia have a minor role, exhaustion had a major role in the impairment. A number of factors therefore seem to contribute to the functional impairment of platelets in HUS: azotemia, thrombocytopenia, and "exhaustion."

Endothelial damage and initiation of coagulation are among the mechanisms that might activate platelets and lead to platelet exhaustion. Treatment in HUS with the intention of normalizing one or both of these events³¹ might indirectly dampen the degree of platelet activation. Whether this would favorably influence the management of these patients is uncertain. Answers are required to these questions in order to provide a better basis for the institution of specific therapy in individual HUS patients whose illnesses need to be more precisely defined and monitored.

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