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MECHANISMS OF HEMORRHAGE IN DENGUE WITHOUT CIRCULATORY COLLAPSE

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Abstract. To characterize the molecular basis for the hemostatic defects of dengue infections, a study was conducted in Bangkok, Thailand. Febrile children ($n = 68$) hospitalized with suspected dengue were enrolled before their clinical syndromes were classified as either dengue fever (DF) or dengue hemorrhagic fever (DHF). Hospital course and outcome were recorded; blood was obtained during the febrile illness (S1), after defervescence (S2), and 1 month after onset of disease (S4). Patients were classified as DF ($n = 21$) and DHF grades 1, 2, and 3; (DHF1, $n = 8$; DHF2, $n = 30$; and DHF3, $n = 9$). All had marked thrombocytopenia. Bleeding scores were assigned on the basis of bleeding site. Although there was no correlation between bleeding scores and pleural effusion index (a measure of vascular leakage) or bleeding scores and platelet counts, there was a correlation between pleural effusion index and platelet counts. Bleeding scores did not correlate with hemostatic data. Activated partial thromboplastin time was prolonged, with trends toward decreased fibrinogen and increased levels of prothrombin fragment F1.2 in the acute-phase samples. However, no factor level was dramatically decreased. We conclude that most patients with DF or DHF, even without overt hemorrhage, have consumptive coagulopathy. Nevertheless, hemorrhage in dengue without circulatory collapse is most likely due to activation of platelets rather than coagulopathy, which is well compensated. Our data suggest that vascular alteration may be the principal factor involved in the association of thrombocytopenia and hemorrhage with disease severity.

INTRODUCTION

Dengue virus infections can be subclinical or can manifest as dengue fever (DF) or dengue hemorrhagic fever (DHF). Dengue fever is a syndrome of 3–7 days of fever, headache, myalgia, and rash accompanied by leukopenia and variable degrees of thrombocytopenia. The occurrence of a vascular permeability defect differentiates DHF from DF; this defect results in leakage of plasma into the extravascular compartment. This leakage commences after several days of fever and becomes maximal as fever remits. Coincident with plasma leakage is activation of complement and a sudden drop in circulating platelet count. Hemorrhage in DHF is reported to be more frequent and profound than in DF.[1,2]

Over the past 3 decades, the hemostatic defects that occur in dengue infection have been carefully characterized, chiefly in children with DHF. Many investigations have been conducted to describe the coagulopathy, platelet functional defects and shortened survival, and vasculopathy that occur to varying degrees during severe cases of dengue.[3,4] Nevertheless, we identified pathophysiological issues that might be clarified through the application of new laboratory techniques, particularly in patients without circulatory collapse, who are far more commonly encountered than patients with circulatory collapse. Therefore, we conducted an observational cohort study of children hospitalized with dengue in Bangkok, Thailand. Patients were enrolled for study shortly after the onset of fever, before the course of disease was manifest as DHF or DF. Because hemostasis in dengue is most altered during the period when fever subsides, most patients came under observation before overt hemorrhage occurred. Our focus was on patients with typical cases of dengue seen in hospital, not the most severely ill patients.

Blood was collected from each child (aged 2–15 years) during febrile illness (S1); after defervescence (S2); and 1 month after enrollment (S4). Hemostatic abnormalities were compared by paired analysis of cases during illness and after recovery. These were then correlated with a bleeding score as well as disease severity, graded serologically by World Health Organization (WHO) criteria for DF and grades of DHF, and by quantitation of vascular leakage. The data show that the coagulopathic abnormalities that occur with similar frequency in DF and DHF without circulatory collapse are well compensated; moreover, their severity is unrelated to bleeding score or plasma leakage. On the other hand, thrombocytopenia was significantly correlated with plasma leakage, although individually these 2 parameters were unrelated to bleeding. We hypothesize that during dengue infection, platelets become stimulated and unavailable to promote clot formation. Hence, the principal cause of the hemorrhagic diathesis observed in dengue uncomplicated by circulatory collapse is probably platelet activation and consumption.

MATERIALS AND METHODS

Patients. The Queen Sirikit National Institute of Child Health is located in central Bangkok and provides primary pediatric health care to a large urban population. Hundreds of children are seen daily in its clinics. Those with suspected dengue are admitted for observation and symptomatic treatment. Sixty-eight children, aged 2–15 years, hospitalized between October 1997 and February 1998 with suspected dengue, participated in the study. Study inclusion criteria were documented fever on the ward within 8 hr before enrollment (oral temperatures ≥ 38.5°C), no obvious non-dengue source of infection, and informed consent by parents or legal guardians. Because only patients with recent fever were enrolled, most entered the study before their illness could be classified as DF or DHF. Patients were excluded only if they were unwilling to give consent, were enrolled in another protocol requiring venipuncture in excess of hospital routine, presented with serious chronic disease, or had ingested aspirin or aspirin-containing combination drugs within 10 days of admission.

The research protocol was approved by the Scientific Review Committee at the Walter Reed Army Institute of Research, the Ethical Review Committee, Ministry of Public Health, Thailand, and the Human Subjects Research Review Board, Department of the U.S. Army.

Clinical evaluation. Children were treated for DF or DHF following standard hospital practices. Clinical grading of disease severity was as previously reported[5] according to WHO guidelines. Briefly, children confirmed to have dengue on the basis of viremia, antibody responses, or both, without evidence of plasma leakage, were considered to have DF; those with thrombocytopenia and plasma leakage without shock were diagnosed as DHF grade 1 (DHF1, no spontaneous bleeding) or DHF grade 2 (DHF2, spontaneous bleeding). Patients with signs of shock were diagnosed as DHF grade 3 (DHF3); those with undetectable blood pressure were diagnosed as DHF grade 4 (DHF4). Bleeding sites were recorded for all patients so that a bleeding score could be assigned on the basis of the most serious site of bleeding observed (0 = no bleeding, $n = 22$; 1 = petechiae, $n = 20$; 2 = epistaxis or gingival bleeding, $n = 14$; 3 = gastrointestinal bleeding, i.e., blood in vomit or stool, $n = 12$). These scores were compared with the platelet counts and pleural effusion indexes.

The pleural effusion index was determined for all the patients by a right lateral decubitus chest X-ray taken on the day after defervescence to evaluate pleural effusion volume as a measure of cumulative plasma leakage. This is usually the day of maximum plasma leakage.[6,7] The pleural effusion index has become an important research tool because it is an objective measure of plasma leakage that affords accurate grading of disease severity.[8–10]

Blood specimens. The clinicians who performed the blood tests did so in a blinded fashion. Blood specimens were collected on 3 separate occasions: while the patient was febrile (S1); when the patient defervesced (S2); and a month later, when illness had fully subsided (S4), so each patient could serve as his own control. Originally, we had planned to have 4 blood drawings, with the S3 sample being the one when the patient was discharged from the hospital. However, in 34 of 68 cases, defervescence and discharge coincided and a single blood sample represented the S2 and S3 time points. Hence, the S1, S2, and S4 samples are reported here.

Whole blood was divided into 3 aliquots, as follows. A specimen for platelet counts was placed in 2% ethylenediamine tetraacetic acid. A second portion for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and serology/virus isolation studies was collected into a serum separator tube. A final aliquot for coagulation parameters and platelet membrane studies was collected in 3.8% citrate and kept on wet ice. This last aliquot was processed within an hour of collection. To avoid further activation of platelets, prostacyclin I_2 (50 μg/mL, Sigma, St. Louis, MO) was added, and the citrated blood was centrifuged at 800 × *g* for 5 min at 23°C to generate platelet-rich plasma (PRP). A portion of the PRP was centrifuged at 1,000 × *g* for 10 min; the resulting pellet was gently resuspended in phosphate-buffered saline (pH 7.4) and fixed in 2% paraformaldehyde for measurement of membrane-bound markers such as P-selectin. The fixed samples were preserved in the refrigerator and transported to the Walter Reed Army Institute of Research (WRAIR) in boxes containing ice packs. Any remaining PRP was mixed with the rest of the blood and centrifuged at 1,200 × *g* for 20 min at 4°C. The PPP was collected and frozen in aliquots. The frozen samples were transported to WRAIR on dry ice.

Specimen evaluation. Complete blood counts, including a differential white cell count and platelet counts, were performed at the Queen Sirikit National Institute of Child Health. Serology/virus isolation and the determination of liver enzymes AST and ALT were performed at the Department of Virology, The Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. Immunoglobulin M and G antibodies to dengue virus and Japanese encephalitis virus were measured in all specimens by antibody capture enzyme immunoassay.[11] Determination of primary infection (first flavivirus immune response) or secondary (anamnestic flavivirus immune response) dengue virus infection was performed as described previously.[12] Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to identify the infecting dengue virus serotype in the S1 specimen.[13]

Frozen plasma specimens were sent to WRAIR for evaluation of clotting function in the STA Compact, an automated coagulation analyzer (American Bioproducts, Parsippany, NJ). Prothrombin time (PT; measured in seconds), activated partial thromboplastin time (aPTT; measured in seconds), thrombin time (TT; measured in seconds), and levels of fibrinogen (measured in milligrams per deciliter) were measured in the clot detection system of the STA Compact. Assays of the clotting factors (percentage of healthy normals) II, V, VII, VIII, IX, X, XI, and XII were conducted by means of factor-deficient substrates in standardized coagulation tests. Plasminogen (assessed in milligrams per deciliter) and percentages of α2-antiplasmin were also measured in the STA Compact via chromogenic assays. Semiquantitative determination of D-dimer (measured in micrograms per milliliter) was performed by means of visual analysis of the macroscopic agglutination of antibody-coated latex particles. Prothrombin fragment F1.2 (measured in nanomoles) was measured by an enzyme-linked immunosorbent assay (ELISA) kit (Organon Teknika, Durham, NC).

P-selectin, a member of the selectin family of cell surface molecules, is present in a preformed state in the Weibel-Palade bodies of endothelial cells and platelet alpha granules. As a marker of platelet activation, levels of soluble P-selectin (measured in nanograms per milliliter) were measured in thawed PPP by ELISA (R&D Systems, Minneapolis, MN). The ELISA sensitivity enabled detection at levels as low as 10 ng/mL. The ELISA detector antibody was specific for P-selectin, having no cross-reactivity with E- or L-selectins. Membrane-bound P-selectin was assessed in fixed platelets by a Becton Dickinson FACScan (Mansfield, MA). The antibody used in this technique also had no cross-reactivity with E- or L-selectins. *In vitro* studies have shown that the soluble P-selectin ELISA is more sensitive than flow-cytometric analysis of the percentage of P-selectin–positive cells and allows earlier detection of platelet activation.[14]

Statistical analysis. Analysis of paired data was performed by comparing the S1 and S2 samples with S4 from the same patient by 2-tailed Student's *t*-test. Values are reported as the mean ± standard error of the mean. Relationships between bleeding score, pleural effusion index, and

TABLE 1
Patient demographics and disease features

Description	Dengue fever (n = 21)	Dengue hemorrhagic fever (n = 47)
Age (years)	8.3 ± 0.6	8.5 ± 0.5
Sex (M:F)	11:10	25:22
Primary infection (%)	6 (29%)	6 (13%)
Secondary infection (%)	15 (71%)	41 (87%)
Virus serotype		
D1	5 (3)*	10 (8)*
D2	0	4 (3)*
D3	6 (4)*	14 (9)*
Not determined	10	19
Duration of fever (days)	5.1 ± 0.3	5.3 ± 0.2
Positive tourniquet test (%)	17 (81%)	40 (85%)
Hemorrhage (any site) (%)	12 (57%)	34 (87%)†
Bleeding from gums	2 (10%)	3 (8%)†
Epistaxis	5 (24%)	5 (13%)†
Hematemesis	2 (10%)	7 (18%)†
Hematochezia	0	3 (8%)†
Petechiae, ecchymoses	8 (38%)	21 (54%)†
Transfusion	0	1 (2%)

* Patients who bled who were infected with dengue serotypes 1, 2, or 3.
† The dengue hemorrhagic fever (DHF) category includes DHF grade 1 (n = 8), DHF grade 2 (n = 30), and DHF grade 3 (n = 9). The percentages of patients with bleeding are based on a total of 39 patients with DHF grade 2 and DHF grade 3. By definition, patients with DHF grade 1 have no bleeding. Bleeding site data include patients who bled from multiple sites.

other clinical and coagulation parameters were determined by calculating Pearson's correlation coefficient.

RESULTS

Cohort description. Among those patients with confirmed dengue virus infections, there were 36 boys and 32 girls, with an average age of 8.4 ± 0.6 years (Table 1). All cases were confirmed serologically, with 12 primary and 56 secondary infections. There were 21 cases of DF and 47 cases of DHF (DHF1, n = 8; DHF2, n = 30; DHF3, n = 9). There were no DHF4 patients. On the basis of nested RT-PCR studies, dengue 3 virus (n = 20) was the most prevalent serotype. Dengue 1 virus was detected in 15 patients and dengue 2 virus in 4 patients.

Clinical outcomes. Bleeding scores were compared with pleural effusion indexes and the platelet counts. Figure 1A shows that among all patients, bleeding score was independent of platelet count (Pearson's correlation, P = 0.624). Bleeding score also was independent of pleural effusion index (P = 0.156, Figure 1B). However, there was a difference between the average pleural effusion indices in patients with bleeding scores of 2 and 3 (P = 0.02). Although bleeding scores were unrelated to platelet count, pleural effusion index was related (Pearson correlation, $P \leq 0.001$, Figure 1C). The pleural effusion index, normally 0, increased with disease severity, as follows: DF (0); DHF1 (13.3 ± 3.5); DHF2 (16.4 ± 2.3); and DHF3 (37.0 ± 6.4) (Figure 2A). There was a significant increase between DHF1 and DHF3 (P = 0.01). The index was higher among patients with secondary infections compared with those with primary infections (20.9 ± 2.4 versus 2.3 ± 1.3, $P \leq 0.001$).

Levels of ALT and AST in the acute sample (S1) and the defervescent sample (S2) were compared with the control (S4) sample that was taken when the patient had recovered

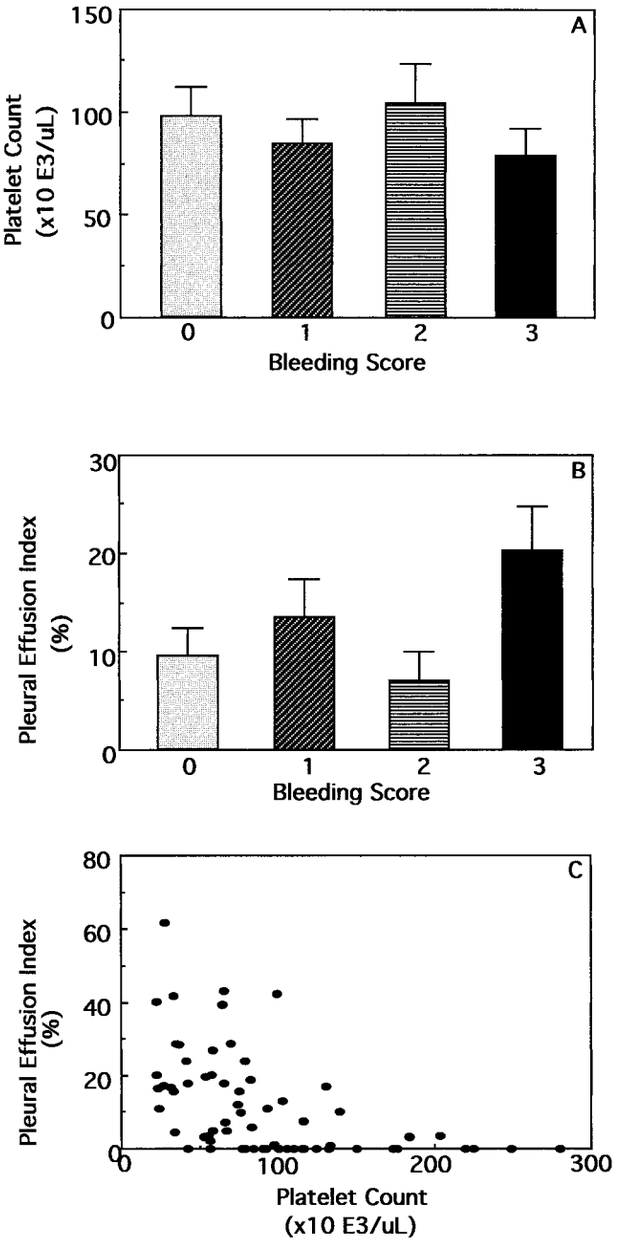
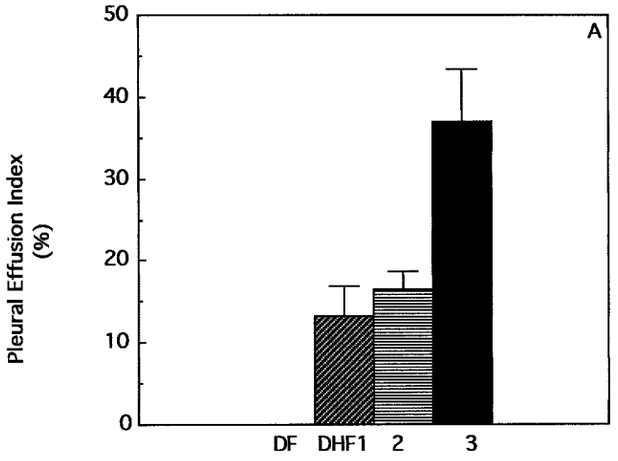


FIGURE 1. Correlation of bleeding scores with the pleural effusion indices and platelet counts in all patients. For each, a score was assigned on the basis of the site of bleeding (0 = no bleeding, 1 = petechiae, 2 = epistaxis or gingival bleeding, 3 = gastrointestinal bleeding—that is, blood in vomit or stool). **Panel A,** platelet counts; **Panel B,** pleural effusion index; **Panel C,** correlation of pleural effusion index versus platelet counts.

completely. All but one patient had elevated AST levels; 36 of 68 had normal ALT levels. However, the mean levels of ALT increased with increasing disease severity in both S1 and S2 samples. For the S1 samples, DF = 37 ± 8 U/mL, DHF1 = 46 ±14, DHF2 = 68 ± 12, and DHF3 = 135 ± 45, as compared with the S4 samples (18 ± 3 U/mL) (Figure 2B). Levels of AST were also elevated in the S1 samples (DF = 86 ± 11 U/mL, DHF1 = 126 ± 38, DHF2 = 164 ± 26, and DHF3 = 283 ± 64) as compared with the S4 sample



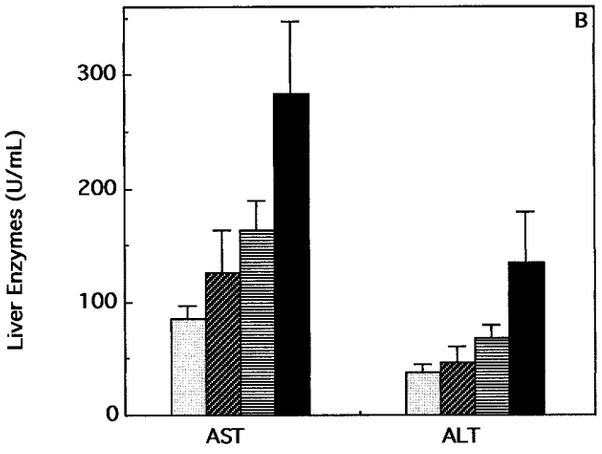


FIGURE 2. Correlation of pleural effusion index and liver enzymes with increasing severity of disease. Patients were categorized according to World Health Organization disease grades: dengue fever (DF □, $n = 21$) and dengue hemorrhagic fever grades 1, 2, and 3 (DHF1 ▨, $n = 8$; DHF2 ▤, $n = 30$; and DHF3 ■, $n = 9$). **Panel A,** pleural effusion index; **Panel B,** liver enzymes.

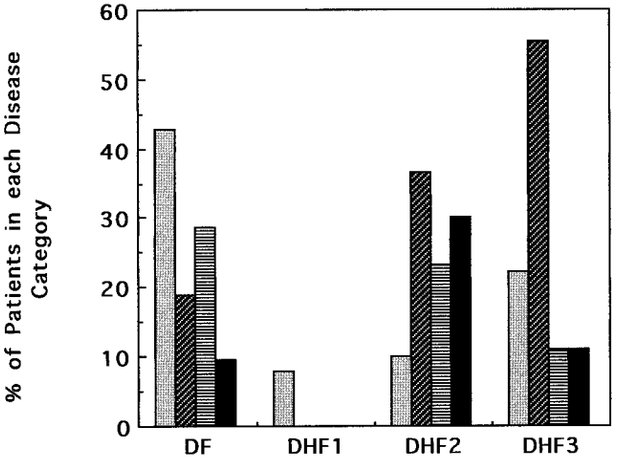


FIGURE 3. Distribution of bleeding scores by World Health Organization disease grade. Bleeding score was the most clinically important bleeding site: none □; petechiae ▨; gingival bleeding or epistaxis ▤; and hematemesis, hematochezia, or both ■.

(31 ± 3 U/mL). These elevated enzyme levels indicated the presence of transient liver injury. Nevertheless, clotting factors were within the normal range, suggesting that hepatic synthesis of proteins was little affected. There was a correlation of pleural effusion index with both AST (Pearson correlation, $P = 0.002$) and ALT (Pearson correlation, $P = 0.002$), although there was no correlation between the assigned bleeding scores and the liver enzymes. Platelet counts were also correlated with both AST (Pearson correlation, $P = 0.002$) and ALT (Pearson correlation, $P = 0.034$).

There was a positive tourniquet test indicating increased vascular permeability in 81% of patients with DF and 85% of patients with DHF (Table 1). However, only 57% of patients with DF had overt hemorrhage compared with 87% of patients with DHF2 and DHF3 (Figure 3). By definition, patients with DHF1 experienced no bleeding. Bleeding sites were similar in patients with DF and with DHF; the most frequent bleeding site was the skin, followed by mucous membranes (nose, gingiva, and gastrointestinal tract). Transfusion was required in only one patient with DHF2, who received whole blood. After excluding patients (22 of 68) with no hemorrhage, 68% had a bleeding score of 1, 2, or 3. There was no certain correlation between bleeding score and disease severity ($P = 0.08$). When the data were analyzed by dengue virus serotype, the percentage of patients who bled was 73% among the patients infected with serotype 1 and 65% among the patients infected with serotype 3. Although the group of patients infected with serotype 2 was small ($n = 4$), 75% of them bled, which was comparable to the other serotypes. Thus, there was no correlation between serotype and frequency of bleeding.

Coagulation data. The acute samples (S1) and the defervescent samples (S2) were compared with the control (S4) samples that were taken when the patient had recovered completely. Because coagulation assay results from S1 and S2 samples were similar, only S1 data are reported.

A significant prolongation in aPTT ($n = 39$) and TT ($n = 36$) was observed in all acute samples (Table 2). However, this prolongation was comparable in all patients whether they had DF or DHF. Levels returned to normal when the patients had fully recovered (S4 samples). There was no significant difference in PT between S1 and S4 samples in any of the patients (data not shown).

Coagulation factors, from the extrinsic (VII), intrinsic (VIII, IX, XI, XII), and common (II, V, X) pathways were measured in 40 patient samples selected to represent the cohort. The factors were measured in the same patients reported in Table 2. No decreases were noted in any one particular factor (data not shown). In this group of 40 patients tested, AST was elevated in all, and ALT was elevated in 53%.

Levels of fibrinogen decreased in the acute S1 samples as compared with the control S4 samples in patients with primary virus infection ($P \leq 0.03$), secondary virus infection ($P \leq 0.001$), DF ($P \leq 0.008$), and DHF ($P \leq 0.001$) (Table

TABLE 2
aPTT and TT in acute (S1) and recovery (S4) samples*

Disease category	aPTT (28.0–40.0 sec) S1 versus S4	TT (15.0–22.0 sec) S1 versus S4
Primary	44.4 ± 1.6 39.4 ± 1.5 $n = 12$, $P = 0.024$	25.3 ± 1.1 19.7 ± 1.3 $n = 12$, $P = 0.001$
Secondary	47.2 ± 1.7 34.8 ± 1.6 $n = 27$, $P = 0.000$	23.3 ± 0.7 19.1 ± 0.5 $n = 24$, $P = 0.000$
DF	46.1 ± 3.0 36.6 ± 1.7 $n = 13$, $P = 0.020$	23.9 ± 0.6 19.7 ± 0.3 $n = 11$, $P = 0.000$
DHF1	48.0 ± 1.7 37.6 ± 2.0 $n = 6$, $P = 0.000$	22.8 ± 0.7 20.7 ± 1.5 $n = 6$, $P = 0.300$
DHF2	45.8 ± 2.2 36.4 ± 1.0 $n = 15$, $P = 0.000$	24.1 ± 1.2 19.0 ± 0.4 $n = 14$, $P = 0.002$
DHF3	46.5 ± 0.9 33.1 ± 1.8 $n = 5$, $P = 0.001$	25.1 ± 2.2 17.6 ± 0.3 $n = 5$, $P = 0.020$

* Values are presented as means ± standard error of the mean. aPTT = activated partial thromboplastin time; DF = dengue fever; DHF = dengue hemorrhagic fever; TT = thrombin time; 1, 2, 3 = grades 1, 2, and 3. Normal ranges for these values are shown in parentheses.

TABLE 3
Fibrinogen and F1.2 levels in acute (S1) and recovery samples (S4)*

Disease category	Fibrinogen (159–400 mg/dL) S1 versus S4	F1.2 (0.19–2.70 nM) S1 versus S4
Primary	252 ± 16 316 ± 24 $n = 11$, $P = 0.040$	3.40 ± 0.52 0.83 ± 0.18 $n = 12$, $P = 0.000$
Secondary	254 ± 13 362 ± 14 $n = 27$, $P = 0.000$	3.72 ± 0.41 1.74 ± 0.26 $n = 33$, $P = 0.000$
DF	280 ± 16 330 ± 20 $n = 12$, $P = 0.065$	2.93 ± 0.46 1.37 ± 0.48 $n = 18$, $P = 0.002$
DHF1	224 ± 19 353 ± 12 $n = 6$, $P = 0.006$	4.30 ± 0.84 1.77 ± 0.27 $n = 6$, $P = 0.02$
DHF2	256 ± 17 365 ± 23 $n = 15$, $P = 0.000$	3.60 ± 0.37 1.77 ± 0.17 $n = 15$, $P = 0.000$
DHF3	221 ± 35 343 ± 43 $n = 5$, $P = 0.031$	3.82 ± 0.85 0.92 ± 0.13 $n = 6$, $P = 0.019$

* Values are presented as mean ± standard error of the mean. DF = dengue fever; DHF = dengue hemorrhagic fever; 1, 2, 3 = grades 1, 2, and 3. Normal ranges for these values are shown in parentheses.

3). Concomitantly, S1 samples showed an increase in levels of the prothrombin fragment F1.2 as compared with the recovery samples (Table 3). Furthermore, fibrinogen levels decreased with increasing disease severity in the acute samples (DHF3 samples as compared with DF) (Table 4). With increasing disease severity, there was a trend toward increased prothrombin fragment F1.2, decreased plasminogen, decreased α2-antiplasmin, and increased D-dimer (Table 4).

The coagulation parameters that showed the most substantial changes with increasing severity of disease were aPTT, fibrinogen, and F1.2. There was, however, no correlation between these parameters and bleeding score. Additionally, neither pleural effusion index nor platelet count correlated with any of these coagulation parameters.

Platelets and disease severity. Platelet counts were decreased in the acute samples (Table 5). All patients had thrombocytopenia, regardless of whether they had DF or DHF, confirming that thrombocytopenia is typical of the disease. With increasing severity of disease, platelet counts were proportionately decreased in the acute samples (Table 5, $P \leq 0.001$). As already reported in the ''clinical outcomes'' section, there was a strong correlation between pleural effusion and platelet counts (Figure 1C), although bleeding scores were unrelated (Figure 1A).

Because P-selectin is mobilized to the surface of platelets and endothelial cells when they are activated by a variety of inflammatory and thrombogenic agents, platelet membrane-attached P-selectin was measured in platelets by flow cytometry. Unfortunately, the lag time for receiving samples from Thailand was rather long (1–2 months after collection and processing). By this time, no surface P-selectin was detectable. Although membrane-bound P-selectin is transient, once shed into the plasma, it can be detected as soluble P-selectin (sP-selectin) by ELISA.[15] However, when sP-selectin was measured in the plasma of the patients by means of ELISA kits, levels were found to be normal in the acute samples of all the patients in the different disease categories. Because endothelial cells could also be a source for the sP-selectin, to estimate the contribution of the sP-selectin shed from the platelets, a ratio of sP-selectin to the actual platelet counts is reported in Table 5. For all disease categories, the ratio was higher than observed in control (S4, recovery) samples. The ratio increased significantly in the S1 samples with increasing disease severity (DF versus DHF3, $P \leq 0.001$), indicating recent platelet degranulation.

DISCUSSION

The purpose of this pilot prospective study was to provide additional information on the pathophysiology of hemostatic defects that occur frequently in dengue. Although descriptive studies of hemostasis have been performed in the past,[2,16,17] to our knowledge, no prospective, integrated examination of hemostasis at the molecular level has been conducted. To accomplish this objective, we conducted a study in typical children hospitalized with dengue. Hemostatic parameters were assessed while patients were febrile (S1), when patients defervesced (S2), and a month later, when the illness had fully subsided (S4), so each patient could serve as his own control. Our results demonstrate that coagulopathy, thrombocytopenia, and frank hemorrhage are common in both DF and DHF. On the other hand, coagulopathy, although substantial enough to result in bleeding if tissue integrity is disrupted (e.g., surgery and menstruation), was well compensated, and in our patients, hemorrhage could not be attributed to it. Instead, platelet activation appeared to be the central event, resulting in thrombocytopenia, possibly aggravated coagulopathy, and hemorrhage. Our data suggest that vascular alteration may be the underlying event that links thrombocytopenia and hemorrhage to disease severity.

Dengue disease severity was assessed primarily by clinical grading. We also used a relatively new measure of cumulative plasma leakage, the pleural effusion index, to reflect clinical grade and extent of endothelial cell alteration.[8–10] Additionally, the hepatocellular enzymes AST and ALT were used as a measure of cellular injury. Our data relating severity of disease with elevated AST levels are in agreement with previous work, suggesting that elevated AST was a strong predictor of dengue infection (particularly DHF) and could be used to make early identifications of high-risk patients.[5]

The patients we studied with mild to moderate dengue disease had clinically important prolongation in aPTT and

TABLE 4
Coagulation parameters in acute (S1) samples

Disease category	Fibrinogen (159–400 mg/dL)	F1.2 (0.19–2.7 nM)	D-dimer, % positive (> 0.25 μg/mL)	Plasminogen (76–136%)	α2-antiplasmin (84–126%)
Primary	252 ± 16 (*n* = 11)	3.4 ± 0.5 (*n* = 12)	0% (*n* = 7)	78 ± 4 (*n* = 12)	107 ± 8 (*n* = 12)
Secondary	254 ± 13 (*n* = 27)	3.7 ± 0.4 (*n* = 33)	42% (*n* = 12)	75 ± 4 (*n* = 27)	94 ± 4 (*n* = 26)
DF	280 ± 16 (*n* = 12)	2.9 ± 0.5 (*n* = 18)	25% (*n* = 4)	84 ± 3 (*n* = 13)	111 ± 6 (*n* = 13)
DHF1	224 ± 19 (*n* = 6)	4.3 ± 0.8 (*n* = 6)	25% (*n* = 4)	73 ± 5 (*n* = 6)	97 ± 10 (*n* = 5)
DHF2	256 ± 17 (*n* = 15)	3.6 ± 0.4 (*n* = 15)	67% (*n* = 6)	74 ± 5 (*n* = 15)	89 ± 5 (*n* = 15)
DHF3	221 ± 35 (*n* = 5)	3.8 ± 0.9 (*n* = 6)	100% (*n* = 1)	62 ± 8 (*n* = 5)	95 ± 15 (*n* = 5)

* Values are presented as mean ± standard error of the mean. DF = dengue fever; DHF = dengue hemorrhagic fever; 1, 2, 3 = grades 1, 2, and 3. Normal ranges for these values are shown in parentheses.

TT, whereas PT was normal. There were no significant alterations in factor levels. These findings could indicate the presence of a coagulation inhibitor such as lupus anticoagulant. Previous studies of patients with severe DHF have found prolonged PT and aPTT with a concomitant decrease in factor VII.[17,18] Some studies also found a mild to moderate decrease in factors V, VII, VIII, IX, and X,[17,19] but all patients in those studies had severe DHF. Moreover, transient liver injury indicated by elevated AST may explain TT prolongation: liver injury may cause dysfunctional fibrinogen. We also observed that levels of fibrinogen, plasminogen, and α2-antiplasmin decreased during acute infection with a concomitant increase in levels of the prothrombin fragment F1.2 and D-dimer. These changes are consistent with activated clot generation and consequent fibrinolysis. This inference is strengthened by the observation that coagulation factors were variably decreased, a finding consistent with a consumptive coagulopathy but inconsistent with dilutional coagulopathy that could occur as a result of the resuscitative fluids infused.

Most earlier studies of the mechanism of bleeding in dengue have examined patients with DHF and identified consumptive coagulopathy as an important cause. Their findings have implied that the contact system was activated to generate thrombi. Because there was no concurrent activation of the fibrinolytic system, disseminated intravascular coagulation was not a major cause of bleeding.[2,4] The association between hemorrhage in severe DHF and coagulopathy was strengthened by the observation of greatest fibrinogen consumption in patients with severe disease and shock.[20] Such observations are less relevant to the more typical hospitalized dengue patients that we studied. Moreover, in contrast to these earlier studies, we observed evidence for activated fibrinolysis (decreased levels of plasminogen and α2-antiplasmin).

We determined bleeding scores for these patients to relate the severity of bleeding to changes in hemostatic and other clinical parameters. Although the bleeding scores were unrelated to platelet counts or pleural effusion index, there was a correlation between pleural effusion index and platelet counts. Neither pleural effusion index nor platelet counts were related to any coagulation parameter. Furthermore, bleeding scores were unrelated to coagulation parameters such as aPTT, fibrinogen, and F1.2. The duration of bleeding was not recorded and therefore was ignored when assigning bleeding scores. Future studies should be designed to address this issue. Nevertheless, we conclude that the absence of a relationship between bleeding and coagulopathy and the presence of one between pleural effusion index (a surrogate for vascular alteration) and thrombocytopenia indicates that endothelial changes could drive hemorrhage caused by platelet activation.

One of the predominant features of dengue is thrombocytopenia. Destruction of platelets appears to occur because of complement activation[21,22] (presumably because platelets bind virus antigens)[23] and also because of peripheral sequestration. Because dengue virus has been shown to suppress marrow production of platelets, both decreased production and increased utilization of platelets may contribute to bleeding early in infection. Bone marrow studies in patients with DHF have shown marked depression of all marrow elements and downregulation of hematopoiesis.[24] As platelet counts reach their nadir, marrow production of platelets resumes. In addition, platelets that escape complement-mediated destruction may nevertheless have diminished function; several previous studies have detected impaired platelet aggregation during the acute phase of DHF.[3,25–27] For

TABLE 5
Platelet activation in acute (S1) samples*

Disease category	Platelet count (250–400 × 10^3/μL)	sP selectin (18–40 ng/mL)	sPsel/Plt Ct (0.13–0.19)
Primary	102 ± 10 (*n* = 12)	26.7 ± 2.4 (*n* = 12)	0.30 ± 0.05
Secondary	92 ± 9 (*n* = 56)	24.2 ± 1.3 (*n* = 30)	0.40 ± 0.05
DF	138 ± 15 (*n* = 20)	27.4 ± 2.0 (*n* = 16)	0.23 ± 0.03
DHF1	70 ± 15 (*n* = 8)	20.5 ± 2.1 (*n* = 6)	0.46 ± 0.12
DHF2	80 ± 8 (*n* = 30)	23.2 ± 1.8 (*n* = 15)	0.41 ± 0.07
DHF3	44 ± 7 (*n* = 8)	27.6 ± 3.7 (*n* = 5)	0.79 ± 0.05

* Values are presented as mean ± standard error of the mean. DF = dengue fever; DHF = dengue hemorrhagic fever; 1, 2, 3 = grades 1, 2, and 3. Normal range for sPsel/Plt Ct ratio is determined from the ratios calculated for control (S4, recovery) samples. Normal ranges for these values are shown in parentheses.

instance, Mitrakul[17] observed shortened platelet survival time and a defect in platelet adenosine diphosphate release in children with DHF and hemorrhage in Bangkok. We hypothesize that during dengue infection, a major fraction of circulating platelets have been activated; in other words, they are either removed from circulation or have lost the ability to promote clot formation. Evidence in support of this hypothesis includes the findings by Srichaikul and others[26] in Bangkok that levels of β-thromboglobulin and platelet factor 4, markers of platelet degranulation, are increased during the acute phase of DHF. Future studies to clarify the role of platelets in the pathophysiology of dengue should focus on assays that measure the secretion of proteins from the α-granules. Such studies would require collection of blood by specific methods that are designed to avoid activation of platelets during collection and processing.

Platelet and endothelial cell activation was assessed through measurement of sP-selectin in the plasma. To assess the contribution of the P-selectin shed from the platelets, a ratio of P-selectin to the actual platelet counts was calculated. The ratio increased in the acute samples with increasing disease severity. If we assume that most sP-selectin originates from activated platelets, and that once activated, platelets are removed from circulation, the excess sP-selectin is consistent with recent platelet degranulation. Increase in F1.2 is indicative of an increase in generation of thrombin, which could also lead to an activation of endothelial cells and platelets.[28] Activation of endothelial cells would produce acute inflammatory changes that could activate platelets and also induce vascular changes.

The prolongation in aPTT and decreased fibrinogen levels would lead to a hypocoagulable condition. However, the normal factor levels and the increase in F1.2 levels suggest a compensatory response to the pathology induced by the viral infection. The hemorrhagic diathesis and the severe thrombocytopenia observed in dengue are most likely due to platelet activation. It is possible that some pathophysiologic mechanisms of dengue infection are similar to those in thrombotic thrombocytopenic purpura, in which endothelial activation is thought to result in platelet activation, causing thrombocytopenia.[29] Additional studies will be necessary to further investigate the similarities between dengue and thrombotic thrombocytopenic purpura.

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REFERENCES

1. Suvatte V, 1981. Haemorrhagic disorders. Part 2: tropical Asia. Luzzatto L. ed. *Clinics in Haematology.* WB Saunders, East Sussex, UK, 933–962.
2. Bhamarapravati N, 1989. Hemostatic defects in dengue hemorrhagic fever. *Rev Infect Dis 11:* S826–S829.
3. Mitrakul C, Poshyachinda M, Futrakul P, Sangkawibha N, Ahandrik S, 1977. Hemostatic and platelet kinetic studies in dengue hemorrhagic fever. *Am J Trop Med Hyg 26:* 975–984.
4. Mitrakul C, 1979. Bleeding diathesis in dengue hemorrhagic fever. *Southeast Asian J Trop Med Public Health 10:* 434–436.
5. Kalayanarooj S, Vaughn DW, Nimmannitya S, Green S, Suntayakorn S, Kunentrasai N, Viramitrachai W, Ratanachu-eke S, Kiatpolpoj S, Innis BL, Rothman AL, Nisalak A, Ennis FA, 1997. Early clinical and laboratory indicators of acute dengue illness. *J Infect Dis 176:* 313–321.
6. Nimmannitya S, Halstead SB, Cohen SN, Margiotta MR, 1969. Dengue and Chikungunya virus infection in man in Thailand 1962–1964. *Am J Trop Med Hyg 18:* 954–971.
7. Innis BL, 1995. Dengue and dengue hemorrhagic fever. Porterfield J, ed. *Kass Handbook of Infectious Diseases: Exotic Viral Infections.* London: Chapman and Hall Medical, 104–146.
8. Setiawan MW, Samsi TK, Wulur H, Sugianto D, Pool TN, 1998. Dengue haemorrhagic fever: ultrasound as an aid to predict the severity of the disease. *Pediatr Radiol 28:* 1–4.
9. Thulkar S, Sharma S, Srivastava DN, Sharma SK, Berry M, Pandey RM, 2000. Sonographic findings in grade III dengue hemorrhagic fever in adults. *J Clin Ultrasound 28:* 34–37.
10. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn S, Endy TP, Raengsakulrach B, Rothman AL, Ennis FA, Nisalak A, 2000. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. *J Infect Dis 181:* 2–9.
11. Innis BL, Nisalak A, Nimmannitya S, Kusalerdchariya S, Chongswasdi V, Suntayakorn S, Puttisri P, Hoke CH Jr, 1989. An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis cocirculate. *Am J Trop Med Hyg 40:* 418–427.
12. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn S, Rothman AL, Ennis FA, Nisalak A, 1997. Dengue in the early febrile phase: viremia and antibody responses. *J Infect Dis 176:* 322–330.
13. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV, 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *J Clin Microbiol 30:* 545–551.
14. Kostelijk EH, Fijnheer R, Nieuwenhuis HK, Gouwerok CWN, de Korte D, 1996. Soluble P-selectin as parameter for platelet activation during storage. *Thromb Haemost 76:* 1086–1089.
15. Michelson AD, Barnard MR, Hechtman HB, Macgregor H, Connoly RJ, Loscalzo J, Valeri CR, 1996. In vivo tracking of platelets: Circulating degranulated platelets rapidly lose surface P-selectin but continue to circulate and function. *Proc Natl Acad Sci U S A 93:* 11877–11882.
16. Isarangkura PB, Pongpanich B, Pintadit P, Phanichyakarn P, Valyasevi A, 1987. Hemostatic derangement in dengue haemorrhagic fever. *Southeast Asian J Trop Med Public Health 18:* 331–339.
17. Mitrakul C, 1987. Bleeding problem in dengue haemorrhagic fever: platelets and coagulation changes. *Southeast Asian J Trop Med Public Health 18:* 407–412.

18. Funahara Y, Sumarmo, Wirawan R, 1983. Features of DIC in dengue hemorrhagic fever. *Bibl Haematol 49:* 201–211.
19. Isarangkura PB, Bintadish P, Pongpanich B, Phanichyakarn P, Valyasevi A, 1986. Hemostatic derangement in dengue hemorrhagic fever in children. *Southeast Asian J Trop Med Public Health 17:* 138–140.
20. Srichaikul T, Nimmannitya S, Artchararit N, Siriasawakul T, Sungpeuk P, 1977. Fibrinogen metabolism and disseminated intravascular coagulation in dengue hemorrhagic fever. *Am J Trop Med Hyg 26:* 525–32.
21. Malasit P, 1987. Complement and dengue haemorrhagic fever/shock syndrome. *Southeast Asian J Trop Med Public Health 18:* 316–320.
22. Malasit P, Mongkolsapaya J, Kalayanarooj S, Nimmannitya S, 1990. Surface associated complement fragments (C3a) on platelets from patients with dengue infection. *Southeast Asian J Trop Med Public Health 21:* 705.
23. Wang S, Patarapotikul J, Innis BL, Anderson R, 1995. Antibody-enhanced binding of dengue-2 virus to human platelets. *Virology 213:* 254–257.
24. La Russa VF, Innis BL, 1995. Mechanisms of dengue virus-induced bone marrow suppression. *Baillieres Clin Haematol 8:* 249–270.
25. Kuberski T, Rosen L, Reed D, Mataika J, 1977. Clinical and laboratory observations on patients with primary and secondary dengue type 1 infections with hemorrhagic manifestations in Fiji. *Am J Trop Med Hyg 26:* 775–783.
26. Srichaikul T, Nimmannitya S, Sripaisarn T, Kamolsilpa M, Pulgate C, 1989. Platelet function during the acute phase of dengue hemorrhagic fever. *Southeast Asian J Trop Med Public Health 20:* 19–25.
27. Zimmerman TS, Muller-Eberhard HJ, 1971. Blood coagulation initiation by a complement-mediated pathway. *J Exp Med 134:* 1601–1607.
28. Kaplan JE, Moon DG, Weston LK, Minnear FL, Del Vicchio PJ, Shepard JM, Fenton JW II, 1989. Platelets adhere to thrombin-treated endothelial cells in vitro. *Am J Physiol 257:* H423–H433.
29. Moake JL, Byrnes JJ, Troll JH, Rudy CK, Hong SL, Weinstein MJ, Colannino MN, 1985. Effects of fresh-frozen plasma and its cryosupernatant fraction on von Willebrand factor multimeric forms in chronic relapsing thrombotic thrombocytopenic purpura. *Blood 65:* 1232–1236.