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Impaired Fibrinolysis in the Pathogenesis of Dengue Hemorrhagic Fever

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The mechanisms contributing to bleeding complications in dengue hemorrhagic fever were studied by investigating the pattern of activation of the coagulation and fibrinolytic systems in 50 children with severe dengue hemorrhagic fever. Thirteen patients (26%) died, and activation of coagulation was most pronounced in the deceased group. Fibrinolysis was also activated, but this activation was relatively weak compared with that of coagulation as a result of persistently high plasminogen activator inhibitor levels. Plasminogen activator inhibitor also prevented a switch from the procoagulant to the profibrinolytic state in lethal dengue hemorrhagic fever, which was further enhanced by an acquired protein C deficiency. The present study is the first to demonstrate such a mechanism in a viral infection. This imbalance between coagulation and fibrinolysis may be used as a prognostic marker, but it may also be a target for future therapeutic intervention. ***J. Med. Virol. 67:549–554, 2002.***

KEY WORDS: dengue hemorrhagic fever; coagulation; fibrinolysis; infection; disseminated intravascular coagulation

INTRODUCTION

Dengue fever is the most prevalent viral disease transmitted by arthropod vectors worldwide [Perez-Rigau et al., 1998]. The clinical picture varies from mild, uncomplicated dengue fever to dengue hemorrhagic fever and dengue shock syndrome. Dengue virus infections form a major and growing health care problem worldwide, especially in endemic areas such as South East Asia and Latin America. Each year millions of people are infected, and about 250,000–500,000 patients develop severe dengue hemorrhagic fever, accompanied by a mortality rate of 1–10% [Hayes and Gubler, 1992; Gubler and Trent, 1993; Perez-Rigau et al., 1998].

Limited data suggest that activation of coagulation and fibrinolysis play a role in the pathogenesis of dengue hemorrhagic fever [Srichaikul et al., 1977; Bhamarapravati, 1989; Huang et al., 2001; van Gorp et al., 2001]. However, the pathogenetic mechanism underlying bleeding complications and multiorgan failure has not yet been elucidated. Systemic infections in general may influence hemostasis, leading to thrombohemorrhagic complications and disseminated intravascular coagulation (DIC), hemolytic uremic syndrome, thrombotic thrombocytopenic purpura, or vasculitis. Bleeding or thrombosis, or both, may be dominant symptoms. DIC may contribute to multiorgan failure and is associated with a high mortality [ten Cate et al., 1993; Bauer and Weitz, 1994; Levi and ten Cate, 1999]. However, current insights into the relationship between infectious diseases and the coagulation system are based mainly on data obtained from clinical and experimental studies of bacterial sepsis and little is known about the relevance of this system in severe viral infections [Cosgriff, 1989; van Gorp et al., 1999].

A curative treatment or vaccine for severe dengue virus infections and other viral hemorrhagic pathogens

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is not available, which underlines the need to understand the pathogenesis of these infections. In the present prospective longitudinal study we investigated the balance between the activation patterns of the coagulation and fibrinolytic systems in the course of severe dengue hemorrhagic fever.

MATERIALS AND METHODS

Study Setting

The study was undertaken at the Dr. Kariadi University Hospital of the University of Diponegoro, in Semarang, Central Java, Indonesia, where dengue is endemic and is known to cause yearly outbreaks.

Patients and Monitoring

Consecutive patients (children) admitted to the pediatric intensive care unit, with a clinical diagnosis of severe dengue hemorrhagic fever (grade III–IV) according to criteria of the World Health Organization [WHO, 1997], were included. In brief, the clinical diagnosis of dengue hemorrhagic fever grade III was based on the presence of fever, a hemorrhagic tendency, plasma leakage manifested by pleural effusion, and/or a rise in hematocrit ($\geq 20\%$), thrombocytopenia ($< 100 \times 10^9$/L), and evidence of circulatory failure manifested by a narrowing pulse pressure (≤ 20 mm Hg) or clinical signs of shock with the presence of a cold clammy skin. Those with profound shock were diagnosed as dengue hemorrhagic fever grade IV.

To evaluate coagulation activity, the following tests were carried out: activated partial thromboplastin time, prothrombin time, fibrinogen, prothrombin fragment $1+2$, thrombin–antithrombin complexes, protein C, and protein S. Fibrinolytic activity was evaluated by the measurement of tissue-type plasminogen activator, plasmin–antiplasmin complexes, plasminogen activator inhibitor and D-dimers. All samples were obtained during hospitalization on 3 consecutive days, starting on the day of admission, with a fourth and final sample on day 7 or the day of discharge (survivors) or the day before death (nonsurvivors).

The study protocol received approval from the institutional Review Board of the University Hospital of Diponegoro University in Semarang. Informed consent was obtained from children's parents or guardians before inclusion in the study.

Diagnosis

The presence of dengue was objectively confirmed by serological assays. A capture and indirect enzyme-linked immunosorbent assay (ELISA) detected dengue-specific IgM and IgG antibodies in serum samples [Groen et al., 1999]. Dengue infections were defined as primary when the ratio of dengue-specific IgM to IgG serum antibodies was >1. When the ratio of dengue specific IgM to IgG serum antibodies was <1, the infections were defined as secondary dengue cases [WHO, 1997]. Blood cultures were obtained in all patients, to exclude bacterial infection.

Blood Collection

Venous blood (9 vol) for measurement of the activated partial thromboplastin time, prothrombin time, fibrinogen, prothrombin fragment $1+2$, thrombin–antithrombin complexes, protein C and protein S and D-dimers was drawn into Vacutainer tubes containing 0.105 M sodium citrate (1 vol) (Becton Dickinson, UK). For measurement of plasmin–antiplasmin complexes, tissue-type plasminogen activator and plasminogen activator inhibitor, blood was collected in siliconized Vacutainer tubes (Becton Dickinson) containing Polybrene (Janssen Chimica, Belgium) and EDTA (0.05%, w/v, and 10 mM, respectively, final concentrations) to prevent in vitro complex formation.

All blood samples were immersed immediately in melting ice and subsequently centrifuged at 4°C for 20 min at 1,600*g*. Plasma samples were stored at −70°C until assayed. Routine laboratory tests (hematology and chemistry) were done in Indonesia. Research assays were carried out on samples transported to the Netherlands on dry ice.

Coagulation Assays

Fibrinogen concentrations (Clauss method), activated partial thromboplastin time and prothrombin time were measured by means of a KC 10 (Sigma Aldrich, Germany) mechanical coagulometer, using bovine thrombin (Fibrinomat, bioMérieux, France), actin FS (Dade Behring, Germany), and Thrombomat (bioMérieux, France), respectively. Prothrombin fragment 1+2 and thrombin–antithrombin complexes were determined by ELISA kits available commercially, according to the manufacturer's instructions (Enzygnost F1+2 micro and TAT micro, Dade Behring, Marburg, Germany). Protein C antigen and free and total protein S antigen (with and without precipitation with PEG 25) were assessed with sandwich-type ELISA kits according to the manufacturer's instructions (Stago Boehringer, Ingelheim, Germany; DAKO-ITK Diagnostics).

Fibrinolysis

For the measurement of D-dimer levels, the TintElize D-dimer ELISA from Biopool (Sweden) was used, according to the manufacturer's instructions. Tissue-type plasminogen activator and plasminogen activator inhibitor were measured with sandwich ELISA kits, using specific monoclonal antibodies as described by de Boer et al. [1993]. Plasmin–antiplasmin complexes were measured with a radioimmunoassay (RIA), as described by Levi et al. [1992].

Statistical Analysis

The plasma levels of the analytes measured are presented as median values with their corresponding interquartile range. The Mann-Whitney U-test was used to compare the respective plasma levels of patients who died during the study (nonsurvivors) with those who recovered (survivors). The composition of the

group changed and the number of patients became limited, due to mortality. The level of statistical significance of these data: two-tailed P-values of < 0.05 were considered to indicate statistical significance. Analyses were performed using SPSS 9.0.

RESULTS

Patients

A total of 50 consecutive children with a clinical diagnosis of dengue hemorrhagic fever were enrolled in the study. The baseline characteristics of the subgroups, i.e., survivors and nonsurvivors, were similar (Table I). All patients were of Javanese origin, excluding racial differences.

The serological assays confirmed dengue virus infections in all patients, either by an IgM response or by a fourfold rise in IgG titers. Antibody profiles were typical for secondary dengue virus infection, as only IgM to IgG ratios of < 1 were found.

Thirteen patients (26%) died during follow-up on the intensive care unit with laboratory-proven DIC and complications of shock. In all patients, blood cultures showed no bacterial growth.

Coagulation

Activation of the coagulation system was more pronounced in the deceased group than among survivors (Table II). On admission, nonsurvivors had a more pronounced prolongation of activated partial thromboplastin time and prothrombin time than survivors, suggesting activation of both the intrinsic pathway and the extrinsic or tissue factor pathway. The activation pattern is clearly manifested by an early increase in markers of thrombin generation i.e. prothrombin fragment $1 + 2$, thrombin–antithrombin complexes as well as a diminished fibrinogen level. During follow-up, these activation parameters tended to improve in the survivor group but not in the deceased group (Table III). In accordance with this activation pattern, the coagulation inhibitor proteins C and S were lower among the deceased than among survivors, also reflecting pronounced activation of coagulation in the deceased group.

Fibrinolysis

The fibrinolytic system was activated as reflected by a rise in levels of tissue-type plasminogen activator, plasmin–antiplasmin complexes and D-dimers in both groups on day of admission (Table II). Tissue-type plasminogen activator and D-dimer levels were increased to a similar degree in both groups and plasmin–antiplasmin complexes were found to be lower in the nonsurvivor group compared with the survivor group. Levels of plasminogen activator inhibitor, an inhibitor of fibrinolysis, were initially (Table II) and persistently (Table III) elevated in the deceased group during the course of the disease, whereas there was a slow decrease in plasminogen activator inhibitor levels in the survivor group (Table III).

These data show that the fibrinolytic system, when compared with the degree of activation of the coagulation system, was relatively impaired in the nonsurvivor group, resulting in an ongoing procoagulant state in this group, as reflected by a high thrombin–antithrombin to plasmin–antiplasmin ratio and lower levels of circulating plasmin–antiplasmin complexes. In the survivor group, there was a switch to a profibrinolytic ratio during the course of disease, reflected by a thrombin–antithrombin to plasmin–antiplasmin ratio of < 1 on the final day of follow-up.

TABLE I. Baseline Characteristics of the Patients Classified as Having Dengue Hemorrhagic Fever Grade III and IV*

	Survivors (n = 37)	Nonsurvivors (n = 13)
Age (yr)	6.8 ± 2.8	6.0 ± 2.8
Female sex (%)	46%	69%
Admission day	4.3 ± 0.9	4.1 ± 1.4
Clinical diagnosis of dengue hemorrhgaic fever		
Grade III (n)	34	9
Grade IV (n)	3	4
Thrombocytes ($\times 10^9$/L)	63.9 ± 35.7	48.7 ± 12.9
Hematocrit (highest %)	38 ± 6.4	36 ± 7.4
White blood cell ($\times 10^9$/L)	7.5 ± 4.3	10.2 ± 5.1
C-reactive protein	22.6 ± 33.5	13.6 ± 18.8
Pleural effusion		
Right-sided (n)	12	3
Bilateral (n)	2	6
Lowest blood pressure		
Systolic (mm Hg)	92 ± 11	83 ± 11
Diastolic (mm Hg)	59 ± 24	45 ± 31
Clammy skin (n)	3	6
Cold extremities (n)	35	13

*Values are reported as means with their corresponding standard deviation or numbers (n). Patients were clinically diagnosed as dengue hemorrhagic fever grade III or IV, according the criteria of the World Health Organization [WHO, 1997]. Normal values for hematocrit 33–40%; leukocytes $4–11 \times 10^9$/L; thrombocytes $150–400 \times 10^9$/L.

TABLE II. Markers of Coagulation and Fibrinolysis on Day of Admission in Patients With Dengue Hemorrhagic Fever Grade III and IV[†]

	All patients (n = 50)	Survivors (n = 37)	Nonsurvivors (n = 13)	Normal	*P**
Activated partial thromboplastin time (sec)	52.0 (43.4–64.1)	47.6 (40.1–59.7)	71.1 (52.6–97.4)	24–36	0.001
Prothrombin time (sec)	13.2 (11.9–15.4)	12.6 (11.4–13.9)	16.3 (15.0–25.8)	10.5–13.5	0.001
Prothrombin fragment 1 + 2 (nmol/L)	3.2 (2.0–5.0)	2.8 (1.9–4.0)	4.9 (2.7–7.2)	< 1.1	0.008
Thrombin–antithrombin complexes (mg/L)	27.2 (13.3–65.7)	21.0 (10.9–52.0)	50.9 (28.5–117.5)	< 4.1	0.004
Fibrinogen (g/L)	1.6 (1.3–1.9)	1.7 (1.5–2.1)	1.3 (0.8–1.6)	1.7–4.0	0.005
Protein C (%)	52.5 (36.8–66.3)	56.0 (48.0–68.0)	38.0 (10.0–63.5)	100%	0.04
Protein S activity (%)	51.0 (39.0–64.3)	54 (42.5–66.5)	34.0 (30.5–48.0)	100%	0.002
Protein S free (%)	23.0 (19.0–28.0)	23.0 (20.0–28.0)	18.0 (14.3–24.8)	100%	0.02
Tissue-type plasminogen activator (ng/ml)	52.5 (41.0–61.0)	54.0 (41.0–61.0)	51.0 (25.0–69.0)	< 10	0.93
Plasmin–antiplasmin complexes (nmol)	8.6 (6.3–12.0)	10.0 (7.1–13.0)	6.4 (4.1–8.5)	< 7	0.03
Plasminogen activator inhibitor (ng/ml)	183.0 (86.8–392.5)	130.0 (65.0–280.5)	394.0 (270.0–1573.0)	30–60	0.001
D-Dimer (ng/ml)	241.0 (197.3–761.0)	234.0 (179.5–715.5)	301.0 (205.0–1303.0)	< 39	0.23
Thrombin–antithrombin to plasmin–antiplasmin ratio	2.8 (1.3–6.5)	2.3 (1.1–5.8)	7.8 (3.3–23.7)	—	0.03

[†]Values of markers of coagulation and fibrinolysis on day of admission of all children (n = 50) with dengue hemorrhagic fever grade III and IV. Values of the subgroup survivors (n = 37) and nonsurvivors (n = 13) are also given. The reported values are medians with their 25th and 75th interquartile ranges.
*Denotes the resulted *P*-value after comparison of survivors and nonsurvivors, using the Mann-Whitney U-test.

DISCUSSION

In the present study, it was demonstrated that severe dengue hemorrhagic fever is characterized by activation of the coagulation and fibrinolytic systems, reflected by both a rise in circulating levels of markers of thrombin generation and markers of activation of fibrinolysis. However, during the initial phase of infection these activation processes were not in balance since thrombin–antithrombin to plasmin–antiplasmin ratios were increased (ratio higher than 1), yielding a procoagulant state. In survivors this procoagulant state was counteracted by an activated protein C system, an activated protein S system, and ongoing fibrinolysis, resulting in a net anticoagulant state, as reflected by an inverse thrombin–antithrombin to plasmin–antiplasmin ratio (ratio < 1). In the nonsurvivor group such a change from the procoagulant to the anticoagulant state did not occur. Quite remarkably, plasminogen activator inhibitor levels remained high during the course of the disease in the deceased group, contributing to an ongoing procoagulant state by a relative inhibition of fibrinolysis. The acquired protein C deficiency observed in our patients may contribute to a further enhancement of this procoagulant state and hence to DIC and/or multiorgan failure.

Persistently high circulating plasminogen activator inhibitor levels and increased thrombin–antithrombin to plasmin–antiplasmin ratios were associated with an adverse clinical outcome in our study. This phenomenon has also been observed in patients with systemic bacterial infections [Brandtzaeg et al., 1990; Westendorp et al., 1992; Kornelisse et al., 1996; Mesters et al., 1996]. Studies of experimental bacterial sepsis models demonstrated that specific cytokines mediate the derangement of coagulation and fibrinolysis [ten Cate et al., 1997]. Injection of endotoxin or tumor necrosis factor (TNF) into healthy volunteers resulted in early activation of fibrinolysis, as reflected by an early rise in tissue-type plasminogen activator, which was rapidly shut off by increasing levels of plasminogen activator inhibitor [Suffredini et al., 1989]. Coagulation was also activated early in these models and remained activated for hours [Levi et al., 1997]. Hence, administration of endotoxin or TNF induced a procoagulant state, characterized by an increased thrombin–antithrombin to plasmin–antiplasmin ratio [Suffredini et al., 1989]. A similar procoagulant state has been described in experimental studies of baboons with lethal sepsis [de Boer et al., 1993]. In these experimental models, coagulation and fibrinolysis appeared to be activated independently, which may lead to an imbalance between fibrin formation and breakdown [van der Poll et al., 1990]. Whether such a procoagulant state also occurs in viral infections has not been investigated. This study is the first to demonstrate such a mechanism in patients with a severe viral infection.

Several issues in the present study merit further comment. First, only patients with the severest form of dengue hemorrhagic fever were investigated. Hence, the findings are applicable only to patients with this spectrum of disease. Huang et al. [2001] studied 25 patients with dengue fever and dengue hemorrhagic fever. These investigators also found a deranged fibrinolytic system, as demonstrated by elevated levels of tissue-type plasminogen activator and plasminogen

TABLE III. Markers of Coagulation and Fibrinolysis During Follow-up in Patients With Dengue Hemorrhagic Fever Grade III and IV*

		Day of admission	Day 2	Day 3	Final sample	Normal
Thrombin–antithrombin complexes (mg/L)	Survivors	21.0 (10.9–52.0)	14.0 (6.2–26.6)	13.1 (6.2–59.9)	6.6 (4.0–18.1)	< 4.1
	Nonsurvivors	50.9 (28.5–117.5)	23.8 (14.1–79.8)	39.2 (22.7–51.2)	57.3 (17.5–97.1)	
Plasmin–antiplasmin complexes (nmol)	Survivors	10.0 (7.1–13.0)	7.8 (5.7–12.0)	10.0 (7.4–13.0)	12.0 (9.0–14.0)	< 7
	Nonsurvivors	6.4 (4.1–8.5)	7.0 (4.4–10.6)	7.9 (6.7–23.8)	33.4 (6.8–60.0)	
Plasminogen activator inhibitor (ng/ml)	Survivors	130.0 (65.0–280.5)	145.0 (59.5–597.0)	93.0 (56.0–150.0)	44.0 (23.0–74.0)	30–60
	Nonsurvivors	394.0 (270.0–1573.0)	1235.0 (230.5–3057.0)	469.0 (195.5–835.8)	200.0 (55.0–345.0)	
Thrombin–antithrombin to plasmin–antiplasmin ratio	Survivors	2.3 (1.1–5.8)	1.6 (0.9–3.1)	1.6 (0.5–5.0)	0.6 (0.3–1.7)	—
	Nonsurvivors	7.8 (3.3–23.7)	4.1 (1.6–13.6)	4.2 (1.1–7.7)	2.1 (1.6–2.6)	

*Values of markers of coagulation and fibrinolysis during hospital stay on 3 consecutive days and a fourth and final sample on day 7 or day of discharge (survivors) or day before death (nonsurvivors). All patients were diagnosed with dengue hemorrhagic fever grade III and IV. Number of survivors is 37 and number of nonsurvivors is 13 on day of admission, 8 on day 2, 6 on day 3, and 2 on day 7. Reported values are medians with their 25th and 75th interquartile ranges.

activator inhibitor, although the levels of plasminogen activator inhibitor were 4- to 10-fold higher in our series. Whether this is a result of differences in patient baseline characteristics or the inclusion predominantly of patients with mild dengue hemorrhagic fever remains to be determined. Second, all the enrolled patients who were classified clinically as having dengue hemorrhagic fever on the basis of widely accepted clinical criteria had serologically confirmed dengue virus infections. This occurred despite the inclusion of consecutive patients in order to avoid selection bias and explains the lack of a non-dengue control group in this study.

Allotypic variations in the promoter region of the plasminogen activator inhibitor gene may contribute to inter-individual variations in plasminogen activator inhibitor responses [Eriksson et al., 1995]. In patients with meningococcal disease, these allotypic variations are associated with an enhanced risk of septic shock after infection with Neisseria meningitides [Westendorp et al., 1999]. It is speculated that these allotypic variations of the plasminogen activator inhibitor gene may also be associated with the severity of dengue hemorrhagic fever. In addition, other genetic polymorphisms, such as those involved in the production and release of cytokines, may contribute to the outcome of dengue virus infections [Westendorp et al., 1994; van Dissel et al., 1998]. The role of genetic factors in the risk of developing severe dengue hemorrhagic fever is the subject of present prospective studies.

In conclusion, severe dengue hemorrhagic fever is associated with a relative impaired fibrinolytic response as a result of elevated circulating plasminogen activator inhibitor levels, which may contribute to a high mortality. In addition, high levels of plasminogen activator inhibitor and elevated thrombin–antithrombin to plasmin–antiplasmin ratios may be used as prognostic markers in daily management. The procoagulant state demonstrated may be a target for future intervention among patients with this disease.

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