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Activation of coagulation factor XI, without detectable contact activation in dengue haemorrhagic fever

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Summary. A prospective cohort study was performed in 50 patients with dengue haemorrhagic fever (DHF) to determine the potential role of the contact activation system and factor XI activation (intrinsic pathway) in the coagulation disorders in DHF. To establish whether TAFI (thrombin-activatable fibrinolysis inhibitor) was involved in the severity of the coagulation disorders, the TAFI antigen and activity levels were also determined. Markers of contact activation (kallikrein-C1-inhibitor complexes), the intrinsic pathway of coagulation (factor XIa–C1-inhibitor complexes) and TAFI were measured and correlated to thrombin generation markers (thrombin–anti-thrombin complexes (TAT), prothrombin fragment 1+2 (F1+2)) and a marker for fibrinolysis [plasmin–α2–anti-plasmin complexes (PAP)]. Activation of the intrinsic pathway of coagulation was clearly demonstrated by elevated levels of factor XIa–C1-inhibitor complexes, without evidence of contact activation, reflected by undetectable kallikrein–C1-inhibitor complexes. Both TAFI antigen and activity levels were decreased in all patients, which may contribute to the severity of bleeding complications in DHF because of the impaired capacity of the coagulation system to protect the fibrin clot from fibrinolysis. These findings in a human viral infection model are in accordance with earlier findings in bacterial sepsis.

Keywords: factor XI, contact activation, coagulation, TAFI, dengue haemorrhagic fever.

The viral haemorrhagic fevers (VHF), i.e. dengue haemorrhagic fever (DHF), are hallmarked by bleeding complications that may be life threatening. Dengue fever (DF) is the most prevalent VHF worldwide, with over half of the world's population living in areas at risk of infection (Perez-Rigau *et al*, 1998). Annually, millions of people are infected and an estimated 250 000–500 000 patients develop severe DHF, which is associated with a mortality rate of 1–10%.

The pathogenesis of bleeding in DHF is poorly understood. Thrombocytopenia may enhance the risk, but the primary cause of bleeding is unknown. Limited data suggest that activation of coagulation and fibrinolysis play a role in the pathogenesis of DHF (Srichaikul *et al*, 1977; Bhamarapravati, 1989). An imbalance in the regulation of coagulation and fibrinolysis, as in the disseminated intravascular coagulation syndrome (DIC), in conjunction with the characteristic thrombocytopenia may contribute to the bleeding tendency in DHF and other VHFs (Van der Poll *et al*, 1990; Van Gorp *et al*, 1997).

The main route for activation of the coagulation cascade in sepsis and also probably in DHF is the tissue factor (TF) pathway (extrinsic pathway) (Van Gorp *et al*, 1999). The factor XII pathway, or contact system, does not seem to play an essential role in the activation of coagulation in experimental sepsis in baboons (Pixley *et al*, 1993). Blocking of the contact activation system by administration of monoclonal antibodies against factor XII did not affect activation of coagulation, whereas it prevented lethal hypotension. Rather, the contact system contributes to the activation and regulation of the fibrinolytic system in sepsis (Jansen *et al*, 1996). After thrombin formation via the TF pathway, factor XI may become involved in the coagulation process via positive feedback activation by thrombin (Naito

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& Fujikawa, 1991; Broze, 1992). Factor XIa generates additional thrombin via activation of factors IX and X (Von dem Borne *et al*, 1995; Ten Cate *et al*, 1996).

High concentrations of thrombin are needed for the activation of TAFI (thrombin-activatable fibrinolysis inhibitor) (Von dem Borne *et al*, 1995, 1997). Activated TAFI, also known as carboxypeptidase B, carboxypeptidase U and carboxypeptidase R, downregulates fibrinolysis by removing C-terminal lysine residues that are essential for binding and activation of plasminogen (Bajzar *et al*, 1995; Wang *et al*, 1998). Activation of TAFI by the coagulation system results in the protection of the fibrin clot from fibrinolysis. TAFI was found to be responsible for the factor XI-dependent down-regulation of the fibrinolytic system (Von dem Borne *et al*, 1997; Minnema *et al*, 1998a). In fact, any disturbance in the intrinsic pathway resulted in an increased clot lysis because of reduced thrombin formation (Broze & Higuchi, 1996) These regulatory roles of both factor XI and TAFI may explain the bleeding tendency that occasionally accompanies deficiency states of these proteins (Minnema *et al*, 1999).

In this study, we investigated the possible role of the contact activation pathway and activation of the intrinsic pathway in relation to thrombin generation and TAFI. We hypothesized that an inadequate factor XI/thrombin/TAFI feedback loop, resulting in an imbalance between coagulation and fibrinolysis, may contribute to the bleeding tendency in DHF.

PATIENTS AND METHODS

Study setting. The study was carried out in Semarang, Central Java, Indonesia, in the Dr Kariadi University Hospital of the University of Diponegoro in Semarang. Dengue is endemic in Java, with annual outbreaks.

Patients and monitoring. Consecutive patients (children) who were admitted to the paediatric intensive care unit of the University Hospital of Diponegoro, Semarang, Indonesia with a clinical diagnosis of severe DHF (grade III–IV) according to WHO criteria (World Health Organization, 1997) were included. The study protocol received approval from the Institutional Review Board of the hospital and informed consent was obtained from the children's parents or guardians prior to inclusion in the study.

Activation markers of the intrinsic pathway of coagulation, i.e. factor XIa–C1-inhibitor complexes (activation marker of XI), Kallikrein–C1-inhibitor complexes (activation marker of the contact pathway) and TAFI were studied in samples obtained on the day of admission in the hospital and correlated to markers of thrombin generation (thrombin–anti-thrombin complexes (TAT) and prothrombin fragment 1+2 (F1+2)) and plasmin formation (plasmin–α2–anti-plasmin complexes (PAP).

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, according to a previously described procedure (Groen *et al*, 1999). Blood cultures were obtained in all patients to exclude bacterial infections.

Blood collection. Blood samples for analysis were collected on the day of admission. Venous blood was drawn into siliconized vacutainer tubes (Becton Dickinson, Oxford, UK) containing Polybrene (Janssen Chimica, Beerse, Belgium) and EDTA (0·05%, w/v and 10 mmol/l, respectively, final concentrations) to prevent *in vitro* complex formation.

All blood samples were immediately immersed in melting ice and subsequently centrifuged at 4°C for 20 min at 1600***g***. Plasma samples were stored at −70°C until assayed. Routine laboratory tests (haematology and chemistry) were carried out in Indonesia. Research assays were carried out on samples transported to The Netherlands on dry ice.

Coagulation assays. TAT and F1+*2* levels were determined using commercially available ELISA kits, according to the manufacturer's instructions (Enzygnost TAT micro and F1+2 micro, Dade Behring, Marburg, Germany). PAP complexes were measured with a radioimmunoassay, as described previously (Levi *et al*, 1992).

Upon activation in plasma, factor XI forms complexes with several protease inhibitors, the dominant inhibitor being C1-inhibitor (Wuillemin *et al*, 1995). Hence, to assess activation of factor XI in the patients, levels of factor XIa–C1-inhibitor complexes were measured according to a method described previously (Wuillemin *et al*, 1995). In the plasma of normal volunteers, levels of these complexes are below the detection limit (10 pmol/l) of this assay.

Complexes between kallikrein and C1-inhibitor were measured using ELISA, modified from radioimmunoassays (Minnema *et al*, 1998b). Normal values were < 350 pmol/l for kallikrein–C1-inhibitor complexes.

TAFI antigen and activity levels were measured according to a previously described procedure (Mosnier, 1998). A pool of plasma from 150 healthy adult hospital workers was used as a reference for the TAFI assays.

Statistical analysis. The plasma levels of the analytes measured were presented as median values with their corresponding interquartile range (IQR). The Mann–Whitney *U*-test was used to compare the respective plasma levels of patients who died during the study (non-survivors) with those without a lethal clinical course (survivors). Two-tailed *P*-values $< 0{\cdot}05$ were considered to indicate statistical significance. Spearman rank correlation coefficients were calculated. Analyses were performed using statistical software (SPSS 9·0).

RESULTS

Patients

Between July 1996 and October 1996, a total of 50 consecutive children with a clinical diagnosis of DHF were enrolled in the study. Thirteen patients died during follow-up on the intensive care unit (26%) as a result of DIC and multi-organ failure related complications. The base-line characteristics of the subgroups, i.e. survivors and non-survivors, were similar (Table I).

The clinical diagnosis of DHF was confirmed by serological assay in all patients, either by an IgM response or a

Table I. Baseline characteristics of the patients.

	Survivors ($n = 37$)	Non-survivors ($n = 13$)
Age (years)	6·8 ± 2·8	6·0 ± 2·8
Female sex (%)	46	69
Admission day	4·3 ± 0·9	4·1 ± 1·4
Clinical diagnosis		
DHF III	34	9
DHF IV	3	4
Thrombocytes ($\times 10^9$/l)	63·9 ± 35·7	48·7 ± 12·9
White blood cell count ($\times 10^9$/l)	7·5 ± 4·3	10·2 ± 5·1
CRP (mg/l)	22·6 ± 33·5	13·6 ± 18·8

Values are means ± SD. Clinical diagnosis according to WHO criteria (WHO, 1997).

fourfold rise in IgG titres. Antibody profiles were typical for secondary DF infection. All patients were of Javanese origin, excluding racial differences. In all patients, blood cultures revealed no bacterial growth.

Coagulation and fibrinolysis

Thrombin generation was increased in all patients, as reflected by a marked elevation of plasma levels of TAT and F1+2. We also performed a sub-analysis in the survivors ($n = 37$) and the non-survivor group ($n = 13$), which demonstrated that thrombin generation was significantly more pronounced in the non-survivor group (Table II). Likewise, activation of the fibrinolytic system, reflected by a rise in PAP complexes, occurred in nearly all patients, but was most pronounced in the survivor group (Table II).

Activation of the intrinsic pathway of coagulation and the contact system

The intrinsic pathway of coagulation was activated, as demonstrated by an increase in factor XIa–C1-inhibitor complexes. Median values of factor XI-activation complexes were elevated. In the sub-analyses between survivors ($n = 37$) and non-survivors ($n = 13$) no significant differences were demonstrated (Table III).

Although the intrinsic pathway was clearly activated we could not demonstrate contact activation in our patients. Kallikrein–C1-inhibitor complexes, as a marker for contact activation, were only slightly elevated but remained within the normal range. The subgroup analyses between survivors and non-survivors also revealed no significant differences (Table III).

TAFI (thrombin-activatable fibrinolysis inhibitor)

TAFI antigen and activity levels were markedly decreased in all patients (Table III). In the subgroup analyses between survivors and non-survivors we demonstrated that this decrease was significantly more pronounced in the non-survivor group (Table III). Spearman rank correlation coefficients between TAFI levels and the markers of thrombin generation were calculated. We found a negative correlation between TAFI antigen and activity levels and TAT and F1+2 levels (correlation coefficients between −0·2 and −0·6).

DISCUSSION

In the present study, we demonstrated that the intrinsic pathway of coagulation, i.e. factor XI pathway, is involved in the pathogenesis of coagulation disorders in DHF. Factor XI activation was demonstrated and, at the same time, thrombin generation was present, reflected by elevated levels of TAT and F1+2. In contrast, we did not find evidence for contact activation because kallikrein–C1-inhibitor remained in the normal range. This observation was consistent with the observations and results from studies in bacterial infection, but it was the first time that this mechanism had been demonstrated in a viral infection.

The results from earlier studies in sepsis recently resulted in a new comprehensive model for coagulation. In this model, it is thought that the TF pathway is the main route of activation of the coagulation cascade. In 1991, two publications demonstrated the capacity of thrombin to activate factor XI, providing an alternative route for activation of factor XI (Gailani & Broze, 1991; Naito &

Table II. Markers of coagulation and fibrinolysis.

	All patients ($n = 50$)	Survivors ($n = 37$)	Non-survivors ($n = 13$)	*P*-value*
TAT	27·2	21·0	50·9	0·004
N < 4·1 mg/l	(13·3–65·7)	(10·9–52·0)	(28·5–117·5)	
F1+2	3·2	2·8	4·9	0·008
N < 1·1 nmol/l	(2·0–5·0)	(1·9–4·0)	(2·7–7·2)	
PAP	8·6	10·0	6·4	0·03
N < 7nmol/l	(6·3–12·0)	(7·1–13·0)	(4·1–8·5)	

*P-value for comparison between survivors and non-survivors (Mann–Whitney U-test).

Values of markers of coagulation and fibrinolysis at day of admittance: all patients ($n = 50$); survivors ($n = 37$); and non-survivors ($n = 13$). Median values with 25% and 75% interquartile ranges (IQR). N is reference value.

Table III. Activation markers of the intrinsic pathway of coagulation and TAFI.

	All patients ($n = 50$)	Survivors ($n = 37$)	Non-survivors ($n = 13$)	*P-value
Factor XIa–C1-inhibitor complexes	13·5	15·0	15·0	0·7
N < 10 pmol/l	(0–31·8)	(12·0–20·5)	(0–48·0)	
Kallikrein–C1-inhibitor complexes	13·2	9·5	24·4	0·3
N < 350 pmol/l	(5·9–47·8)	(5·8–38·8)	(7·6–69·6)	
TAFI activity	31·8	34·0	20·1	0·004
N 100%	(21·0–39·9)	(25·7–41·1)	(12·7–31·8)	
TAFI antigen	46·0	48·7	33·5	0·006
N 100%	(33·4–61·8)	(39·4–68·4)	(24·7–45·8)	

*P-value for comparison between survivors and non-survivors (Mann–Whitney U-test).Values of factor XIa–C1-inhibitor complexes and kallikrein–C1-inhibitor complexes and of thrombin-activatable fibrinolysis inhibitor (TAFI) antigen and activity for all patients ($n = 50$) and for the subgroups: survivors ($n = 37$); non-survivors ($n = 13$). Median values with 25% and 75% interquartile ranges (IQR). N is reference value.

Fujikawa, 1991). A new comprehensive model for coagulation was presented based on the following concept: coagulation is initiated by the extrinsic, TF pathway, leading to the generation of thrombin and the formation of fibrin. After the formation of small amounts of factor Xa, the extrinsic pathway is rapidly inhibited by the tissue factor pathway-inhibitor (TFPI) (Broze, 1992, 1995). Continued thrombin formation now depends on the intrinsic pathway, activated by positive feedback (amplification) pathways, consisting of thrombin-induced activation of factors V, VIII and XI (Davie *et al*, 1991; Broze & Gailani, 1993). In this model, the contact system does not play a role in physiological blood coagulation. The absence of detectable contact activation and presence of detectable factor XI activation, in conjunction with enhanced thrombin generation, suggests that coagulation activation in DHF follows this revised model. The coagulation system not only results in the formation of the fibrin clot but also protects the fibrin clot by activation of TAFI. Activated TAFI protects the fibrin clot by removing c-terminal lysine residues that are involved in binding and activation of plasminogen (Bajzar *et al*, 1995; Wang *et al*, 1998).

In the present study, TAFI levels, both antigen and activity, were markedly decreased in all patients. Several mechanisms may be involved, such as decreased synthesis and/or increased clearance. However, the most probable explanation may be that excessive thrombin generation results in consumption of TAFI, because *in vitro* experiments have shown that both the antigen and activity levels are affected upon incubation of plasma with thrombin (Meijers *et al*, 2000). This is in contrast to the situation in a hyperfibrinolytic state, such as in acute promyelocytic leukaemia, in which only TAFI activity was decreased (Meijers *et al*, 2000). The underlying mechanism is that plasmin converts TAFI into a form that can no longer be activated by thrombin, but can still be detected by the antigen assay. However, the antigen assay does not detect activated TAFI (Meijers *et al*, 2000). Therefore in the present study, it is very probable that the decrease of both TAFI activity and antigen levels reflects a manifestation of excessive thrombin generation, resulting in a disturbed factor XI–thrombin–TAFI feedback loop. This phenomenon was also demonstrated by the negative correlation between TAFI antigen and activity levels and markers of thrombin generation.

The low TAFI levels found in our patients may also contribute to the impaired capacity of the coagulation system to stabilize clot formation and, thereby, lead to an imbalance between coagulation and fibrinolysis, resulting in haemorrhage. Yet, no data are available about TAFI levels in other infectious diseases and especially infections characterized by a bleeding tendency.

Several issues from the present study merit further comment. First, we have not determined TAFI levels in a control population with the same ethnic background. Recent publications have reported that TAFI antigen levels may vary in populations with different ethnic backgrounds (Chetaille *et al*, 2000). Nevertheless in that study, the lowest values measured in a black African population were above 80% (mean value), whereas antigen levels in our study group varied between 33·5% and 48·7% (median) (Table - III). In a group of healthy Caucasian children ($n = 11$) in the same age group (mean age 7·5 years), we measured mean TAFI antigen levels of 82%. Taking these results together, the TAFI levels measured in our study are extremely low. Second, we only investigated patients with the severest form of DHF. Hence, our findings are only applicable to patients with this severity of disease. Further studies are required to establish the role of the intrinsic pathway of coagulation and TAFI in patients with milder DF. Finally, it was rather surprising that all the enrolled patients, who were clinically classified as having DHF on the basis of widely accepted clinical criteria, had the disease objectively confirmed by serological assays. 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. Taking these restrictions into account, we believe that the possibility to study such a clinical cohort is rather unique.

In conclusion, we have demonstrated that factor XI, i.e. the intrinsic pathway of coagulation, is involved in the activation of coagulation in DHF, whereas there is no

contact activation. This means that in viral infection (DHF) a similar route of activation occurs to that which has recently been postulated and demonstrated in sepsis. The low TAFI levels may be an attributable factor, leading to the imbalance in coagulation, resulting in bleeding complications. These observations may indicate new molecular interactions for therapeutic intervention.

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