The Role of Cytokines in Activation of Coagulation and Fibrinolysis in Dengue Shock Syndrome*

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Keywords

Cytokines, coagulation, fibrinolysis, dengue shock syndrome

Summary

In a prospective clinical study of 50 patients with Dengue Shock Syndrome (DSS), we investigated the association of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-1 receptor antagonist (IL-1Ra), and IL-6 with activation markers of coagulation (F1+2 and TATc) and fibrinolysis (t-PA, PAPc, and D-dimer). We found that TNF-α, IL-1β and Il-1Ra, but not IL-6, concentrations were elevated in the circulation during the early stage of infection and at discharge from hospital. TNF-α was significantly associated with D-dimer, an activation marker of fibrinolysis ($p < 0.003$), but not with activation markers of coagulation. IL-1β was significantly associated with t-PA ($p < 0.03$). IL-1Ra was significantly associated with F1+2, TATc ($p < 0.04$ and $p < 0.02$, respectively), whereas IL-6 was significantly associated with both, activation markers of coagulation (F1+2; $p < 0.03$) and fibrinolysis (PAPc; $p = 0.002$). Our data are in line with studies in bacterial sepsis. In severe dengue virus infection the same cytokines are involved in the onset and regulation of hemostasis.

Introduction

The association between infection and activation of coagulation and fibrinolysis has been studied extensively in Gram negative-sepsis and endotoxemia (1). Activation of coagulation, as reflected by increasing plasma levels of prothrombin fragment 1 and fragment 2 (F1+2) and thrombin-antithrombin complexes (TATc), and activation of fibrinolysis, as reflected by increasing plasma levels of tissue plasminogen activator (t-PA) and plasminogen activator inhibitor type 1 (PAI-1), have been detected in sepsis (2-4) and in experimental endotoxemia in humans (5, 6). Cytokines appear to play a crucial role in the initiation of coagulation and fibrinolysis under these circumstances (1). Tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) seem to be the most prominent mediators in this respect (1, 6-10).

Dengue hemorrhagic fever (DHF) is a severe form of the infection caused by any of four serotypes of the dengue virus. It is characterized by fever, thrombocytopenia, bleeding manifestations ranging from mild to severe, and evidence of plasma leakage. According to the 1997 WHO criteria, dengue shock syndrome (DSS) is defined as DHF with signs of circulatory failure, including narrow pulse pressure (20 mm Hg) and hypotension (DHF grade III) or frank shock (DHF grade IV) (11, 12). Plasma leakage and bleeding are the two major pathophysiological changes in DHF/DSS, determining the severity of disease (11-14). Little is known about the pathogenesis of bleeding in DHF/DSS and in an analogy to sepsis it is attractive to speculate that cytokines are key mediators. Preexisting heterotypic dengue antibodies augment dengue virus infection of monocytes or macrophages (15). An increased number of dengue virus-infected monocytes may result in T-lymphocyte activation; the activation of both types of cell may result in the production of elevated levels of the aforementioned cytokines (16).

We designed a prospective clinical study to investigate the association of cytokines with activation markers of coagulation and fibrinolysis in DSS. We investigated those cytokines which are known to play a role in the activation of coagulation and fibrinolysis in bacterial sepsis.

Patients and Methods

Study Setting

The study was executed in Dr. Kariadi Hospital, Semarang, Indonesia, the university hospital of Diponegoro University. The research protocol was reviewed and approved by the institutional Review Board of the Dr. Kariadi Hospital. Written informed consent was obtained from children's parents or legal guardians.

Patients

Between June and November 1996, during an outbreak of dengue in Indonesia, 50 consecutive children with the clinical diagnosis of DSS, and who had been admitted to the Paediatric Intensive Care Unit, were enrolled in the study. The diagnosis of DSS (DHF grade III and grade IV) was based on WHO criteria (1997) (11). The assessment of decreased consciousness was based on the Glasgow coma scale (17). Only children aged ≥ 3 years were included.

Patient Monitoring

Thorough clinical assessments were performed daily during hospitalization, using a medical record form. Laboratory tests to support clinical management included blood cell counts, tests for hemostasis (prothrombin time and activated partial thromboplastin time) as well as biochemical tests for kidney and liver functions and electrolyte status.

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Table 1 Characteristics in 50 patients with dengue shock syndrome

Age in years	6.6±2.8 (3-13)
Days since onset of disease	4.2±1 (2-7)
Male	24 (48)
Female	26 (52)
Clinical diagnosis (WHO criteria)	
DHF III	43 (86)
DHF IV	7 (14)
Positive serology result	50 (100)
Number of death	13 (26)
Bleeding manifestations	
Positive Tourniquet test	23 (46)
Skin bleeding (petechiae, purpura, hematoma)	21 (42)
Epistaxis	10 (20)
Gum bleeding	3 (6)
Hematemesis and / or melena	28 (56)
Hematuria	2 (4)

*) Age and Days since onset of disease are mean ±SD (range); all other characteristics are numbers (%)

Specimen Collection

Blood specimens were collected in vacutaiter tubes (Becton Dickinson, Rutherford, NJ 07417). Specimens for cytokine assays were collected in the acute phase of disease (on day of admission: day 0, on day 1 and 2), and on day 7 or the day of discharge if the patient was hospitalized for less than 7 days. Four ml of blood were collected into sterile tubes containing EDTA in each patient. To this tube 125 μL of aprotinin (Trasylol, Bayer, Leverkusen, Germany; final concentration 625 kallikreine inactivating units/mL) was added through the stopper by a tuberculin needle and syringe. The tube was centrifuged directly at 1250 g for 10 min, and thereafter at 15000 g for 1 min to remove platelets. The plasma was stored in aliquots at –80° C until assayed for cytokines.

Blood samples for analysis of coagulation and fibrinolysis were collected on the same days as the samples for cytokines. Venous blood (9 vol.) for measurement of F1+2, TATc and D-dimers was drawn into vacutaiter tubes containing 0.105 M sodium citrate (1 vol.). For measurement of PAPc, t-PA, blood was collected in siliconized vacutaiter tubes 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 immediately immersed in melting ice and subsequently centrifuged at 4° C for 20 min at $1600 \times g$. Plasma samples were stored at –80° C until assayed.

To collect appropriately timed specimens for serological assays (≥ day 6 after onset of fever) (18-21), 2 ml of blood was collected on day of admission and at discharge. Blood was centrifuged 1000-1500 rpm for 10 min, afterwards, serum was transferred to screw cap Eppendorf tubes and stored at –80° C, until assayed. For the transport from Indonesia to The Netherlands (which lasts longer than 15 h) the samples for cytokines, coagulation, fibrinolysis, and serological assays were kept on dry ice.

Cytokine Assays

TNF-α, IL-β and IL-1Ra were measured in duplicate by nonequilibrium radioimmunoassay (RIA) (22). Recombinant human TNF-α, IL-1β and IL-1Ra were calibrated against standards provided by the National Institute of Biological Standards and Control (Potters Bar, UK), with the sensitivity of the assay with 100 μl sample of 40 pg/ml, 40 pg/ml, and 80 pg/ml, respectively. IL-6 was measured with ELISA obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands, according to manufacturer's instruction; circulating concentrations exceeding 10 pg/ml were considered to be elevated.

Coagulation and Fibrinolysis Assays

F1+2 and TATc levels were determined with the use of commercially available ELISA kits, according to the manufacturer's instructions (Enzygnost F1+2 micro and TAT micro, Dade Behring, Marburg, Germany). For the measurement of D-dimer levels, the TintElize D dimer ELISA from Biopool (Sweden) was used, according to the manufacturer's instructions. t-PA was measured with sandwich ELISA kits using specific monoclonal antibodies as described before (23). PAPc was measured with radioimmuno-assay as described previously (24).

Serological Assays

The diagnosis of dengue infection was confirmed by serological assays. A capture and indirect enzyme-linked immunosorbent assay (ELISA) detected

Table 2 Median circulating plasma concentrations (range) of TNF-α, IL-β, IL-1Ra and IL-6 on day of admission (D0), the following two days (D1, D2) and on day 7 or at discharge if earlier (D7/discharge) in 50 patients with dengue shock syndrome

	D 0	D 1	D 2	D 7 (discharge)	Normal value
TNF-α (pg/ml)	155	140	150	135	90
	(95-2500)	(85-315)	(95-305)	(90-1100)	(50-170)
IL-1β (pg/ml)	72.5	80	80	70	50
	(<40-320)	(<40-140)	(<40-145)	(<40-155)	(<40-115)
IL-1Ra (pg/ml)	795	555	590	395	139
	(220-2500)	(180-2400)	(225-2050)	(240-2900)	(<80-502)
IL-6 (pg/ml)	< 2	2.1	3.5	<2	<10
	(<2-199000)	(<2-1058)	(<2-155)	(<2-48)	

	D 0	D 1	D 2	D 7 (discharge)	Normal value
Markers of coagulation					
F1+2 (nMol/l)	3.2	2.6	2.7	2.2	<1.1
	(1.2-14.2)	(0.9 – 40.8)	(0.8 - 21.4)	(0.8 - 7.6)	
TATc (mg/l)	27.1	15	15.5	6.6	<4.1
	(3.8 - 120)	(1.9 – 120)	(3.3 – 120)	(1.7 - 101.5)	
Markers of fibrinolysis					
t-PA (ng/ml)	52.5	56	41.5	16.5	<10
	(5.5 - 143)	(7.6 – 112)	(9 - 86)	(5.7 - 89)	
PAPc (nMol)	8.6	7.4	10	12	<7
	(2.3-25)	(2.1-14)	(3.3-32)	(3.4-60)	
D-dimer (ng/ml)	241	312	481	265	<39
	(85-2000)	(69-1600)	(53-1778)	(59-1600)	

Table 3 Median plasma concentrations (range) of activation markers of coagulation and fibrinolysis on day of admission (D0), the following two days (D1, D2) and on day 7 or at discharge if earlier (D7/discharge) in 50 patients with dengue shock syndrome

dengue specific IgM and IgG antibodies in serum samples, according to a previously described procedure (25).

Statistical Analysis

Continuous data were described as mean with SD or median with range. Nominal data were described as percentage (%). Multiple linear regression analysis was done to determine the association between cytokines and activation markers of coagulation and fibrinolysis on the day of admission. *P* values of less than 0.05 were considered to indicate statistical significance. All statistical analyses were performed with SPSS for Windows, version 9.0.

Results

Between June and November 1996, 50 children with a clinical diagnosis of DSS, were enrolled in the study. Baseline characteristics of the patients are listed in Table 1. Thirteen patients (26%) died during follow up on the intensive care unit. The clinical diagnosis was confirmed by serological assay in all patients, either by an IgM response or a fourfold rise in IgG titres. Antibody profiles were typical for secondary dengue infection.

Plasma Concentrations of Cytokines in the Course of DSS

The circulating plasma concentrations of TNF-α, IL1β, IL-1 Ra, and IL-6 were assayed on the day of admission (day 0), on day 1 and 2 after admission, and on day 7 after admission or at discharge if this was earlier than day 7. As shown in Table 2, concentrations of TNF-α and IL-1β were mildly elevated and concentrations of IL-1Ra were moderately elevated during the early stage of infection and at discharge. The median plasma concentrations of IL-6 were low during the observation period, although three patients had extremely high plasma concentrations.

Activation of Coagulation and Fibrinolysis

As shown in Table 3, F1+2 and TATc were persistently high from admission to discharge, reflecting activation of coagulation. Also, persistently elevated levels of t-PA, PAPc, and D-dimer were found during the study period, reflecting activation of the fibrinolytic system.

Association of Plasma Concentrations of Cytokines with Activation Markers of Coagulation and Fibrinolysis

As shown in Table 4, TNF-α was significantly associated with D-dimer, an activation marker of fibrinolysis, but not with activation markers of coagulation. IL-1β was significantly associated with activation marker of fibrinolysis t-PA; the regression coefficient (β) for t-PA of IL-1β was 4.05, meaning that for every additional 10 pg IL-1β, t-PA increases 4.05 ng/ml. IL-1Ra was significantly associated with activation markers of coagulation F1+2 as well as TATc. The regression coefficient (β) for F1+2 of IL-1Ra was 0.11, meaning that for every additional 100 pg/ml of IL-1Ra, F1+2 increases 0.11 nMol. IL-6 was significantly associated with F 1+2, an activation marker of coagulation and with PAPc, an activation marker of fibrinolysis. The regression coefficient (β) for F1+2 of IL-6 was 0.05, implying that for every additional 1000 pg/ml of IL-6, F1+2 increases 0.05 nMol. Similarly, β for PAPc of IL-6 was 0.17, and for D-dimer of TNF-α was 13.77. This means that for every additional 1000 pg of IL-6, PAPc increases 0.17 nMol, and for every additional 10 pg of TNF-α, D-dimer increases 13.77 ng/ml.

Discussion

The present study in severe dengue virus infection demonstrates, that cytokines are associated with activation markers of coagulation and fibrinolysis.

Table 4 Results of multiple linear regression analysis showing significant associations of cytokines with activation markers of coagulation and fibinolysis on day of admission in 50 patients with dengue shock syndrome

	Cytokine	*p*-value	β
Markers of coagulation			
F1+2 (nMol)	IL-6 (per 1000 pg)	0.0260	0.05
	IL-1Ra (per 100 pg)	0.0348	0.11
TATc (ng/l)	IL-1Ra (per 100 pg)	0.0108	2.09
Markers of fibrinolysis			
t-PA (ng/ml)	IL-1β (per 10 pg)	0.0265	4.05
PAPc (nMol)	IL-6 (per 1000 pg)	0.0020	0.17
D-dimer (ng/ml)	TNF-α (per 10 pg)	0.0023	13.77

β: regression coefficient

TNF-α was significantly associated with D-dimer, a marker of fibrinolysis, but not with activation markers of coagulation. Studies in bacterial infection have clearly indicated the role of TNF-α in the activation of the fibrinolytic system, but the role of TNF-α in the activation of the coagulation system seems to be less direct. A single intravenous injection of recombinant TNF into human volunteers induced activation of the common pathway of the coagulation cascade, resulting in thrombin formation, as reflected by elevated plasma levels of F1+2 (3), and activation of the fibrinolytic system characterized by increased levels of urokinase-type PA (u-PA) and t-PA, followed by PAI-1 (4). An injection of E. coli endotoxin induced increased levels of TNF-α and IL-6, and resulted in activation of fibrinolysis as reflected by increased levels of t-PA, PAPc, which was subsequently offset by release of PAI-1, and a long-lasting onset of coagulation activation characterized by increase of prothrombin F1+2 and TATc (9). However, further studies have demonstrated that anti-TNF treatment did not affect endotoxin-induced coagulation activation (26-28), but did inhibit the activation of the fibrinolytic system in animal and human models with low-grade endotoxaemia (26, 27, 29). Thus our findings in DHF are in line with these findings in endotoxemia.

We demonstrated that IL-1β was significantly associated with tPA, a marker for activation of fibrinolysis. The role of IL-1β in the activation of coagulation as well as fibrinolysis has been demonstrated by injection of IL-1β, or, indirectly by injection of IL-1Ra (5, 6). Infusion of IL-1 into baboons induced activation of the fibrinolytic system, reflected by increased levels of t-PA, and subsequently the offset of fibrinolysis by PAI-1, followed by the activation of coagulation, characterized by increased levels of TATc (5). Administration of IL-1Ra in baboons with lethal bacteremia, and in patients with sepsis have significantly attenuated the activation of coagulation and fibrinolysis (6).

We also found that IL-1Ra was significantly associated with the activation markers of coagulation F1+2 and TATc, leading to the conclusion that the regulation of coagulation and fibrinolysis by IL-1β and IL-1Ra in this severe viral infection is similar to that in sepsis.

We demonstrated that IL-6 is significantly associated with activation markers of both coagulation and fibrinolysis. The role of this cytokine in the activation of coagulation has been demonstrated by injection of recombinant IL-6, as well as by administration of anti-IL-6 antibody. Infusion of recombinant IL-6 into patients with renal cell carcinoma resulted in increased levels of F1+2 and TATc, while fibrinolysis was not clearly affected (30).

In the present study we found that the median levels of IL-6 were normal, but with extremely high values in 3 patients (5823, 86000 and 199000 pg/ml). Two of these 3 patients died and in these two patients, very high levels of TNF-α (2500 and 2500 pg/ml), IL-1Ra (2500 and 2500 pg/ml), and F1+2 (7.51 and 14.2 nMol/l, normal <1.1 nMol/l) were found. The very high levels of F1+2 reflect the degree of coagulation activation, which may result in a considerable fibrin formation, leading to multiorgan failure and ultimately to death.

We conclude that in dengue shock syndrome the same cytokines are involved in the onset and regulation of coagulation and fibrinolysis as in bacterial infections.

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