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Pathophysiologic and Prognostic Role of Cytokines in Dengue Hemorrhagic Fever

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Dengue shock syndrome is a severe complication of dengue hemorrhagic fever (DHF), characterized by a massive increase in vascular permeability. Plasma cytokine concentrations were prospectively studied in 443 Vietnamese children with DHF, of whom 6 died. Shock was present in 188 children on admission to hospital, and in 71 children it developed later. Contrary to expectations, certain inflammatory markers (interleukin-6 and soluble intercellular adhesion molecule-1) were lower in the group with shock, and this may reflect the general loss of protein from the circulation due to capillary leakage. Only soluble tumor necrosis factor receptor (TNFR) levels showed a consistent positive relationship with disease severity. In patients with suspected DHF without shock, admission levels of sTNFR-75 in excess of 55 pg/mL predicted the subsequent development of shock, with a relative risk of 5.5 (95% confidence interval, 2.3–13.2). Large-scale release of soluble TNFR may be an early and specific marker of the endothelial changes that cause dengue shock syndrome.

Dengue hemorrhagic fever (DHF) is an important cause of childhood morbidity in many tropical regions. It may progress suddenly to dengue shock syndrome (DSS), a potentially fatal complication that is difficult to predict by clinical examination [1, 2]. The pathophysiologic processes that cause this deterioration are poorly understood. Viral replication is believed to occur primarily in cells of macrophage lineage [3], which are a source of tumor necrosis factor (TNF) and other proinflammatory mediators of septic shock [4]. Data from Malaysia and Tahiti have suggested that the level of TNF production in DHF may be related to clinical outcome [5, 6]. To examine this relationship, we conducted a prospective study in 443 Vietnamese children with DHF of varying severity. As well as measuring TNF and its two soluble receptors (sTNFR-55 and sTNFR-75), we examined four molecules that are produced downstream of TNF in the inflammatory cascade: interleukin (IL)-6 and -8, soluble intercellular adhesion molecule-1 (sICAM-1), and C-reactive protein (CRP).

Methods

Study design. Consecutive cases of suspected DHF were recruited at Dong Nai Paediatric Centre in southern Viet Nam between April and September 1993. The criteria for inclusion in the

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Informed parental consent was obtained before enrollment. The study was approved by the Scientific and Ethical Committees of Dong Nai Pediatric Centre and the Centre for Tropical Diseases, Ho Chi Minh City.

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analysis were as follows: fever or history of fever; hemorrhagic phenomena, including a positive tourniquet test; exclusion of alternative diagnoses, such as malaria, by clinical examination and appropriate laboratory tests; and confirmation of acute dengue virus infection by elevated dengue virus–specific serum IgM titers as measured by capture ELISA or by virus isolation. DSS was defined as a case of DHF with either a narrow pulse pressure (≤20 mm Hg) or unrecordable blood pressure [2]; typically these were accompanied by signs of circulatory insufficiency, such as cold extremities and thready pulse.

Clinical procedures. On admission to the study, patients were examined and results documented on a formal questionnaire, venous blood was taken for platelet count (estimated manually) and differential white cell count in the hospital laboratory, and serum was taken for confirmation of acute dengue virus infection. The sample for cytokine measurement was collected onto EDTA (1 mg/mL) and aprotinin (0.5 TIU/mL) and separated promptly, and plasma was stored at −70°C. Fluid resuscitation was carried out immediately until the child was no longer clinically hypovolemic. All patients were kept under close observation, including hourly measurements of pulse and blood pressure. A second blood sample was collected at discharge.

Confirmation of dengue virus infection. Dengue virus–specific IgM was tested on stored sera by use of a capture ELISA as described elsewhere [7]. If the admission sample failed to confirm acute dengue virus infection, the sample taken on discharge was also tested. Briefly, microtiter plates coated with rabbit anti-human IgM μ chain were used to capture IgM from the patients' sera. The following reagents were then added sequentially, with washing between each step: a mixture of antigens from all four dengue virus serotypes, monoclonal antibody against a dengue virus–reactive epitope, anti-mouse IgG conjugated to horseradish peroxidase, and *o*-phenylenediamine. Absorbance at 492 nm was measured by dual wavelength microplate reader, and a positive reaction was defined as an absorbance of more than three times the mean of negative control samples.

Cytokine assay. All cytokines were measured by sandwich ELISA in 96-well microtiter plates, by use of previously described techniques. Briefly, the TNF [8] and IL-6 [9] assays each used a murine monoclonal antibody for capture (CB6 and 5E1, respectively), followed by a rabbit polyclonal antibody against human TNF, then goat anti-rabbit IgG conjugated with alkaline phosphatase. The assays for IL-8 [10], sTNFR-55 [11], sTNFR-75 [11], and sICAM-1 [12] each used a murine monoclonal antibody for capture (3H7, 1H7, 4C8, and HM2, respectively), followed by a rabbit polyclonal antibody labeled with biotin, then streptavidin conjugated to alkaline phosphatase. The concentration of each mediator was estimated with reference to recombinant standards included on each plate. The detection limits were 5 pg/mL for IL-8, 10 pg/mL for TNF and IL-6, 100 pg/mL for sTNFR-55 and sTNFR-75, and 400 pg/mL for sICAM-1. The CRP assay used murine monoclonal antibody and alkaline phosphatase–conjugated rabbit polyclonal antibody against human CRP (Dako, Carpinteria, CA). Albumin and total protein levels were measured on an Axon automated analyzer by use of bromocresol green dye and the Biuret method, respectively.

Statistical analyses. Distribution of data was assessed by the Shapiro-Wilks test. As most continuous variables were not normally distributed, median values with exact binomial 95% confidence intervals (CIs) were calculated, and differences between groups were evaluated by use of the Kruskal-Wallis test with post hoc significance testing (Bonferroni-Dunn). Nonparametric tests for trend, according to the method of Cusick, were done to examine the effect of disease severity on cytokine levels. Associations be-

Table 1. Admission characteristics of pediatric patients in study of cytokines in dengue hemorrhagic fever.

	No shock (group A) $n = 184$	Pre-shock (group B) $n = 71$	Shock survivors (group C) $n = 182$	Fatal shock (group D) $n = 6$	Kruskal-Wallis test	Trend
Age, years	6 (6.0–7.0)	6 (5.0–8.0)	6 (6.0–7.0)	5 (1.2–7.8)	$P = .4$	$z = -0.47$, $P = .64$
Male sex	101 (55)	35 (49)	97 (53)	3 (50)	$P = .57$	$\chi^2 = 0.08$, $P = .96$
Duration of symptoms, days	4 (4–5)	4 (4–4)	5 (5–5)	5 (4–5)	$P < .0001$	$z = 4.11$, $P < .01$
Axillary temperature, °C	38.2 (38.0–38.5)	39.0 (39.0–39.0)	37.2 (37.0–37.5)	37.5 (37.0–39.9)	$P < .0001$	$z = -6.56$, $P < .01$
Systolic BP, mm Hg	90 (90–96)	90 (90–90)	85 (85–90)	88 (70–120)	$P < .0001$	$z = -5.98$, $P < .01$
Diastolic BP, mm Hg	60 (60–60)	60 (60–60)	70 (65–70)	63 (50–90)	$P < .0001$	$z = 6.97$, $P < .01$
Hepatomegaly	118 (64)	47 (66)	169 (93)	6 (100)	$P < .001$	$\chi^2 = 66.2$, $P < .0001$
Purpura/echymoses	15 (8)	11 (15)	25 (14)	5 (83)	$P < .001$	$\chi^2 = 15.4$, $P < .0001$
Epistaxis	20 (11)	12 (17)	20 (11)	2 (33)	$P = .25$	$\chi^2 = 0.19$, $P = .91$
GI bleeding	13 (7)	10 (14)	38 (21)	3 (50)	$P < .001$	$\chi^2 = 25.8$, $P < .0001$
Other bleeding	5 (3)	3 (4)	16 (9)	0	$P = .02$	$\chi^2 = 7.5$, $P < .01$
Capillary hematocrit, %	40 (39–40)	40 (39–40)	45 (45–46)	45 (27–51)	$P < .0001$	$z = 4.25$, $P < .01$
Manual platelet count × 10^{12}/L	118 (114–120)	116 (110–120)	106 (102–108)	109 (91–157)	$P < .0001$	$z = -6.71$, $P < .01$
White blood cell count × 10^9/L	6000 (6000–6205)	5750 (5160–6040)	6000 (5128–6000)	6950 (5320–7920)	$P = .09$	$z = -1.17$, $P = .24$

NOTE. If units are specified, data are median (exact binomial 95% confidence interval); otherwise, data are no. (%). Group B (pre-shock) refers to children who were not clinically assessed as being in shock at time of this examination but developed shock later in course of illness. Kruskal-Wallis test evaluates overall difference between groups. Trends in relation to increasing severity are analyzed nonparametrically by Cusick's test or χ^2 test for trend. Note that platelet count of <100,000/μL is often used as diagnostic criterion for dengue hemorrhagic fever [2]. In this study, platelets were counted manually in routine hospital laboratory, and true values may have been lower.

tween continuous variables were assessed by use of Spearman's ρ. Nominal data were compared by Fisher's exact tests and χ^2 tests for trend. The software package Stata (Statacorp, College Station, TX) was used for all analyses.

Results

Clinical features on admission. Five hundred forty-eight children were entered into this study with a clinical diagnosis of DHF; of these, 443 were confirmed as having acute dengue virus infection on serologic testing or viral culture and hence fulfilled the entry criteria. On the basis of clinical outcomes, these 443 patients were separated into 4 groups: 184 recovered without developing any symptoms of DSS (group A, non-shock); 71 did not have DSS when admitted to the study but developed this complication later (group B, pre-shock); 182 had DSS on admission but survived (group C, shock survivors); and 6 children were admitted with DSS and died subsequently (group D, fatal shock). The clinical features on admission are shown in table 1. Of the patients in group B, 38 (54%) developed shock either later on the day of admission or on the following day; the remaining 33 patients went into shock between days 2 and 4 after admission. The median time from admission to development of DSS was 1 day (95% CI, 1–2; interquartile range, 1–2; range, 0–4). As expected, the groups differed significantly in respect to blood pressure, temperature, hematocrit, platelet count, hepatomegaly, and bleeding problems. However, among those who were not in shock on admission, the initial examination revealed little difference between those who were about to develop shock and those who were not. That is, the only differences observed between group B and group A were higher temperature and somewhat more purpura or ecchymoses in those who were about to develop shock.

TNF and sTNFR. As shown in table 2, there was a significant positive relationship between disease severity and sTNFR levels, with the effect being most marked for sTNFR-75. TNF levels showed a similar trend, but this was not statistically significant. sTNFR levels tended to fall into three ranges: moderately elevated in children who were not in shock, markedly elevated in children who were in shock or about to develop shock, and massively elevated in fatal shock. Thus, the levels were significantly different between groups A and B (sTNFR-55, $P = .003$; sTNFR-75, $P = .002$) and between groups C and D (sTNFR-55, $P = .0008$; sTNFR-75, $P = .015$) but not between groups B and C ($P = .24$ and $P = .99$, respectively). In children without shock on admission (groups A and B), sTNFR-75 levels of >55 pg/mL gave a relative risk of 3.2 (95% CI, 1.1–9.6) of developing shock subsequently. To evaluate the clinical utility of this as a predictor of shock, we considered all 548 children initially entered into the study with a clinical diagnosis of DHF. Among the 341 who were not in shock on admission, the relative risk for developing shock was 5.5 (2.3–13.2) if the sTNFR-75 level was >55 pg/mL; development of shock was predicted with a sensitivity (95% CI) of 93% (85%–98%), specificity of 34% (29%–40%), positive predictive value of 27% (22%–33%), and negative predictive value of 95% (89%–98%). The value of 55 pg/mL is near the low end of the range of sTNFR-75 in these children, and specificity can be increased at the expense of sensitivity by raising this cutoff. In this series, 20% of patients admitted with suspected DHF but without shock went into shock during the current illness. A rapid sTNFR-75 test with a cutoff of 55 pg/mL (if such a test were developed) would identify 93% of these on admission. These patients could then be more closely monitored.

Overall, there was a weak but significant positive correlation between sTNFR-75 levels and hematocrit (Spearman's $\rho = .13$, $P = .009$). In patients who were not in shock on admission, the same weak association existed ($\rho = .20$, $P = .02$), but there was no difference in mean hematocrit levels between those who went on to develop shock and those who did not, nor was there a difference between those with a raised level of sTNFR-75 on admission (>55 pg/mL) and those without. Assuming that hematocrit is an indirect marker of vascular permeability, the above correlation suggests that the raised sTNFR-75 level in some non-shock patients is accompanied by an as yet clinically undetectable increase in vascular permeability. However, this association is weak, and admission hematocrit is not a clinically useful predictor of shock.

Other factors. None of the other factors showed a consistent positive association with disease severity (table 2). Although IL-8 levels showed a positive trend, this was entirely due to the high levels found in fatal cases, and among survivors there was no difference between the groups. In the case of IL-6 and sICAM-1, we obtained the paradoxic result of a significant negative trend: that is, children in shock tended to have lower levels than did those not in shock. As expected, a similar negative trend was also observed for plasma albumin concentrations. Albumin levels were markedly reduced in children in shock on admission compared with levels in those who were not in shock (group C vs. group A, $P = .0002$) and were lower still in patients with fatal shock. However, among those who were not in shock on admission, the albumin concentration did not distinguish those who were about to develop shock from those who were not ($P = .99$).

The low albumin levels reflect the massive capillary leak that characterizes DSS. Since the inflammatory mediators being analyzed are small- to moderate-sized proteins, their plasma concentrations are likely to have been affected by this capillary leak. In an attempt to correct for this, we calculated the ratio of each plasma cytokine level to that of albumin, to provide an approximation of the level that might have been achieved in the absence of a capillary leak (table 2). When expressed in relation to albumin concentration, the negative trends obtained for ICAM-1 and CRP were reversed, and the corrected value for CRP showed a significant positive correlation with disease severity. The positive association of TNF, sTNFR-55, and

Table 2. Cytokine concentrations in relation to disease severity in study of children with dengue hemorrhagic fever.

	Group				
	Non-shock (n = 184)	Pre-shock (n = 71)	Shock survivors (n = 182)	Fatal shock (n = 6)	Trend
Absolute value					
TNF (pg/mL)	68 (52–84)	88 (57–115)	79 (67–88)	154 (37–524)	
sTNFR-55 (pg/mL)	588 (450–738)	800 (650–1499)	700 (572–900)	6750 (1250–8000)	$z = 2.0$, $P = .04$
sTNFR-75 (ng/mL)	4.4 (3.2–6.0)	8.0 (4.5–10.3)	6.8 (5.5–7.5)	23.3 (3.3–37.5)	$z = 3.6$, $P < .01$
sICAM-1 (ng/mL)	123 (79–175)	110 (90–107)	58 (40–95)	24 (2–530)	$z = -3.4$, $P < .01$
IL-6 (pg/mL)	95 (55–115)	60 (40–100)	30 (22–50)	75 (32–650)	$z = -3.8$, $P < .01$
IL-8 (pg/mL)	0 (0–0)	0 (0–10)	0 (0–0)	200 (20–550)	$z = 2.9$, $P < .01$
CRP (μg/mL)	4.0 (3.0–4.2)	4.0 (3.0–5.6)	3.5 (2.8–4.0)	6.0 (2.0–29.5)	$z = -0.70$, $P = .49$
Albumin (mg/mL)	34 (30–38)	32 (30–38)	21 (18–24)	10 (4–26)	$z = -6.7$, $P < .01$
	n = 95	n = 35	n = 88	n = 6	
Albumin ratio					
TNF ($\times 10^{-9}$)	2.5 (2.1–3.1)	2.8 (1.6–3.8)	4.2 (3.3–5.3)	17.8 (3.7–28.8)	$z = 3.7$, $P < .01$
sTNFR-55 ($\times 10^{-8}$)	2.2 (1.6–2.9)	5.1 (3.3–7.2)	5.4 (4.1–6.3)	44.9 (12.5–200)	$z = 5.0$, $P < .01$
sTNFR-75 ($\times 10^{-7}$)	1.2 (0.9–1.9)	2.8 (1.2–4.4)	3.3 (2.5–3.8)	18.8 (3.3–37.5)	$z = 5.6$, $P < .01$
sICAM-1 ($\times 10^{-6}$)	4.2 (3.1–5.0)	3.9 (2.7–9.0)	4.9 (3.3–6.9)	12.7 (0.2–132)	$z = 1.17$, $P = .24$
IL-6 ($\times 10^{-9}$)	2.6 (1.3–4.0)	2.0 (1.3–4.3)	1.3 (1.0–2.3)	11.4 (3.2–25.0)	$z = -0.46$, $P = .64$
IL-8 ($\times 10^{-9}$)	0 (0–0)	0 (0–0.4)	0 (0–0)	12.6 (2.0–137.5)	$z = 2.2$, $P = .03$
CRP ($\times 10^{-4}$)	1.3 (0.8–1.5)	1.3 (0.6–1.8)	2.1 (1.7–2.8)	15.8 (1.0–43.1)	$z = 2.8$, $P < .01$

NOTE. Data are plasma concentrations (TNF, tumor necrosis factor; sTNFR, soluble TNF receptor; ICAM, intercellular adhesion molecule; IL, interleukin; CRP, C-reactive protein), expressed as medians (exact binomial 95% confidence intervals). Upper panel gives absolute levels. Note that sTNFR-75 and sTNFR-55 are positively associated with disease severity, whereas IL-6 and ICAM-1 show significant inverse relationship. Lower panel gives cytokine concentration divided by albumin concentration. Because of limited sample volume, latter analysis could not be done on every subject. Trends in relation to increasing severity are analyzed nonparametrically by Cusick's test.

sTNFR-75 levels with disease severity was strengthened by correction for albumin level.

Discussion

This analysis reveals a positive association between sTNFR levels and the severity of DHF. Other inflammatory markers studied failed to correlate with severity or, in the case of IL-6 and sICAM-1, showed an unexpected negative correlation. We propose that the latter finding is not due to diminished production of these mediators but is a reflection of the massive increase in vascular permeability in DSS. The extent of this leak can be gauged by the effect on albumin (68 kDa); the median plasma concentration was 36 g/L in patients without shock, 22 g/L in patients with shock, and 10 g/L in patients with fatal shock. Therefore, to gain a better reflection of production rates, we expressed the level of each mediator as a fraction of albumin concentration. This is a considerable simplification, since the leakiness of each molecule is determined by complex factors; based on molecular weight alone, this calculation would tend to overcorrect for the leak of sICAM-1 (95 kDa) and to undercorrect for TNF (active trimer 51 kDa), sTNFR-55 (25 kDa), sTNFR-75 (35 kDa), IL-6 (26 kDa), IL-8 (8 kDa), and CRP (21 kDa). With these caveats, the corrected values suggest that levels of TNF, sTNFR-75, sTNFR-55, IL-8, and CRP all rise significantly with increasing disease severity.

The finding of most interest is that sTNFR levels appear to predict those children with DHF who are about to develop shock. This could theoretically be due to increased production or to reduced clearance. The latter has been noted in renal failure due to septic shock [13], but in our experience, renal impairment is uncommon in nonfatal DSS. We consider a more likely explanation to be increased shedding of sTNFR due to proinflammatory stimulation. It may be relevant that the strongest clinical association was observed for sTNFR-75, since this form of sTNFR appears to be preferentially overexpressed on microvascular endothelium in persons with acute respiratory distress syndrome [14]. Possibly, sTNFR levels in DHF reflect inflammatory events at the endothelial level and might therefore constitute a specific marker of incipient microvascular pathology. In this study, a level of sTNFR-75 >55 pg/mL on admission was a highly sensitive if nonspecific predictor of the development of shock in children with suspected DHF. If similar findings were demonstrated to be important in other geographic locations, it might be worthwhile to develop a rapid test for sTNFR-75 to identify patients at risk of developing DSS; this group could be monitored more closely and may even be candidates for preemptive infusions of colloid.

The above findings are consistent with the view of Halstead [3, 15] that the loss of endothelial integrity in DHF is due to inflammatory factors released by mononuclear phagocytes. TNF is an obvious candidate, since this cytokine can experimentally induce the main pathologic components of DHF, including increased vascular permeability, thrombocytopenia, and hemorrhage [4]. The unresolved question is why capillary leak is such a prominent feature of DHF when a variety of other infectious diseases show large elevations in TNF and other proinflammatory cytokines without profound alterations in capillary permeability. A potential explanation is that the endothelial effects of systemic cytokine release are determined by local events, such as viral replication; alternatively, the cytokines measured here may be markers of endothelial pathology rather than its primary cause.

There are several possible reasons why some infected persons might produce a greater inflammatory response than others. It may be a consequence of virus load and thus of heterotypic antibodies that promote viral replication [1], or it might reflect immunologic variables such as Th1/Th2 balance. Host genetic factors could also be involved. The practical issue is whether DSS might be preventable by suppressing proinflammatory cytokines as soon as DHF is diagnosed, for example with pentoxifylline. Until more is known about the role of host inflammatory mediators in the control of viral replication, such approaches need to be pursued with extreme caution.

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