Cytokine patterns during dengue shock syndrome

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ABSTRACT. Objective. To investigate the patterns of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, interferon-γ (IFN-γ) and interleukin-1 receptor antagonist (IL-1Ra) during the course of dengue shock syndrome.
Design. Prospective clinical study.
Setting. Pediatric Intensive Care Unit, Dr. Kariadi Hospital, the university hospital of Diponegoro University, Semarang, Indonesia.
Patients. Fifty children with dengue shock syndrome.
Measurements. The plasma concentration and the *ex vivo* production, with and without lipopolysaccharide (LPS), of TNF-α, IL-1β and IL-1Ra were measured in duplicate by nonequilibrium radioimmunoassay (RIA); IFN-γ and IL-6 were measured by ELISA.
Results. During the acute phase, the plasma concentrations and the *ex vivo* production without LPS of IL-1Ra were considerably elevated and returned to normal on recovery. However, the *ex vivo* LPS-stimulated production of the proinflammatory cytokines TNF-α and IL-1β were considerably depressed. Also, these concentrations returned towards normal on recovery. In non-survivors, the plasma concentrations of IL-6 and IL-1Ra were significantly higher than in survivors ($p < 0.00001$ and $p = 0.0005$, respectively). In addition, the *ex vivo* production of IL-1Ra in non-survivors was significantly higher than in survivors, both without LPS stimulation ($p = 0.0008$) and with LPS ($p < 0.004$). IL-1Ra was significantly associated with mortality ($p = 0.007$).
Conclusion. Since IL-1Ra was significantly associated with mortality, this measurement may be used as an index of disease severity in dengue shock syndrome.

Keywords: cytokine, patterns, dengue shock syndrome

INTRODUCTION

Dengue virus infection is caused by any of four serotypes of the virus (DEN-1, DEN-2, DEN-3, and DEN-4). The disease may manifest itself as a dengue fever (DF), characterized by sudden onset of fever and a variety of nonspecific signs and symptoms, including headache, joint and muscle pains, retro-orbital pain, rash and bleeding manifestations [1, 2], or as dengue hemorrhagic fever (DHF), the severe form of the disease. Patients with DHF, almost exclusively children, develop thrombocytopenia, hemorrhage and increased capillary permeability resulting in plasma leakage. DHF may develop into dengue shock syndrome (DSS), the most severe form of DHF, with signs of circulatory failure, including narrow pulse pressure (20 mm Hg), hypotension, or frank shock [1]. Plasma leakage and bleeding are the two major pathophysiological changes in DHF/DSS that determine the severity of the disease. The pathogenetic mechanisms responsible for the increased permeability of vascular endothelial cells in DHF have not been elucidated, however, it has been suggested that cytokines and other mediators, which are released during the immune response to dengue virus infection, may form the underlying mechanism [3]. It is hypothesized that in secondary infections with a virus of a different serotype from the one that caused the primary infection, cross-reactive antibodies enhance the infection by and replication of dengue virus in mononuclear phagocytic cells [4, 5]. In addition, these cells produce and

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secrete pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1 β (IL-1β), and IL-6 [6-8], as well as antiinflammatory mediators, the soluble TNF-receptor p55 (sTNFRp55), sTNFRp75 and IL-1 receptor antagonist (sIL-1Ra) [9]. $CD4^+$T lymphocytes are activated and produce a number of cytokines including gamma interferon (IFN-γ), IL-2, IL-4, IL-5 and IL-10 [10-12]. Previous studies have reported a relationship between high levels of TNF-α, IL-6 and disease severity [6-8]. In addition, the role of TNF-α in plasma leakage has been demonstrated indirectly by the correlation of soluble tumor necrosis factor receptors (sTNFRs) with the degree of subsequent pleural effusion [13]; and, the role of cytokines in the initiation of coagulation and fibrinolysis has been studied intensively in Gram negative-sepsis and endotoxaemia [14].

We performed a prospective clinical study in children with DSS to investigate the patterns of TNF-α, IL-1β, IL-6, IFN-γ and IL-1Ra during the course of the disease, and to identify which of these cytokines were related to mortality.

PATIENTS AND METHODS

Study setting

This prospective study was performed at the Dr. Kariadi Hospital, the university hospital of Diponegoro University, Semarang, Indonesia. The research protocol in this study was reviewed and approved by the institutional Review Board of the Dr. Kariadi Hospital/Diponegoro University. Written informed consent was obtained from the 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 admitted to the Paediatric Intensive Care Unit were enrolled in the study. The diagnosis of DSS (DHF grade III and grade IV) was finally based on the 1997 WHO case definition [1], consisting of four criteria for DHF, plus evidence of circulatory failure. The criteria for DHF consisted of: (i) fever, or history of acute fever, lasting 2-7 days (ii) hemorrhagic tendencies including petechiae, ecchymoses, purpura, epistaxis, hematemesis, melena (iii) thrombocytopenia, 100.000 cells per mm^3 or less (iv) evidence of plasma leakage due to increased vascular permeability, manifested by at least one of the following: a rise in the hematocrit equal to or greater than 20%; a drop in the hematocrit following volume-replacement equal to or greater than 20% of baseline; signs of plasma leakage such as pleural effusion, ascites and hypoproteinemia; evidence of circulatory failure manifested by: rapid and weak pulse; narrow pulse pressure (20 mmHg); hypotension; cold, clammy skin and restlessness. Only children > 3 years old were allowed for blood-sampling.

Patient monitoring

Thorough clinical assesment using a medical record form was performed daily from the day of admission to the Intensive Care Unit until discharge from that Unit or death. 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 electrolytes status. Also, chest x-ray and ECG were done routinely.

Specimen collection and whole blood culture

Blood specimens were collected in vacutainer tubes (Becton Dickinson, Rutherford, NJ 07417, USA). Specimens for cytokine assays were collected in the acute phase of the disease (on the 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. Three, 4 ml samples of blood were collected into sterile tubes containing EDTA, from each patient. To each tube 125 µL aprotinin (Trasylol, Bayer, Leverkusen, Germany; final concentration 625 kallikreine inactivating units/mL) were added through the stopper by a tuberculin needle and syringe. One tube was centrifuged directly at 1,250 g for 10 minutes, and thereafter at 15,000 g for one minute to remove platelets. The plasma was stored in aliquots at –80°C until assayed for cytokines. To one of the two remaining tubes, 50 µL LPS (*E.coli* serotype 055:B5; Sigma, St Louis, USA; final concentration 10 µg/mL) were added to stimulate cytokine production. Unstimulated samples contained aprotinin, but no LPS. Both tubes were incubated at 37°C for 24 hours, and thereafter centrifuged; the supernantants were stored at – 80°C, until assayed for cytokines.

To collect appropriately timed specimens for serological assay (equal to or more than day 6 after onset of fever) [15-18], 2 ml of blood were collected on the day of admission and at discharge. Blood was centrifuged at 1000-1500 rpm for 10 minutes, then, serum was transferred to screw cap Eppendorf tubes and stored at – 80°C until assayed. For the transport from Indonesia to The Netherlands, the samples for cytokine and serological assays were kept on dry ice.

Cytokine assays

TNF-α, IL-1β and IL-1Ra were measured in duplicate by nonequilibrium radioimmunoassay (RIA) [19]. 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. IFN-γ and IL6 were measured with ELISAs obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands, according to manufacturer's instruction. Levels of IFN-γ exceeding 40 pg/ml and levels of IL6 exceeding 10 pg/ml were considered to be elevated.

Serology

In all cases, the diagnosis of dengue infection was confirmed by serological assays. We used a capture and indirect enzyme-linked immunosorbent assay (ELISA) detecting dengue specific IgM and IgG antibodies in serum samples, according to a previously described procedure [20].

Statistical analysis

Continuous data were described as mean (SD), and median; whereas nominal data were described as numbers.

Table 1
Characteristics of 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	50 (100%)
Number of deaths	13 (26%)

Age and days since onset of disease are mean ± SD (range)

Two-tailed *t*-tests were done to compare numerical variables between survivors and non-survivors. The assumption of normality of the data was checked before the *t*-test. Mann-Whitney U tests were done when the data were not normally distributed. The cut-off-point of significance was $p = 0.05$ with a 95% confidence interval. Logistical regression analysis was done to calculate the association between cytokines and mortality. All statistical analyses were performed using 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 the table 1. All children were admitted during the acute phase of the disease. Symptoms had started 2-7 days (mean 4.2 ± 1) before admission to the Intensive Care Unit. Thirteen patients (26%) died during follow up. Death in these patients was due to shock and serious bleeding complications, and diffuse intravascular coagulation. 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 during the course of the disease

In figure 1a, b the plasma concentrations of proinflammatory cytokines TNF-α and IL-1β are depicted during the course of the disease. The median values of these cytokines do not change during the course of the disease. However, (figure 1c) the plasma concentrations of the anti-inflammatory cytokine IL-1Ra were considerably elevated during the acute phase compared to recovery. Median plasma concentrations of IFN-γ and IL-6 were not elevated. In two patients who died, greatly elevated TNF-α concentrations (both 2500 pg/ml) and IL-6 (86.000 and 199000 pg/ml) were found.

Ex vivo *production of TNF-α, IL-1β and IL-1Ra*

As shown in table 2, the *ex vivo* production of IL-1Ra after incubation for 24 hours without LPS was higher during the first days of admission than at recovery; the *ex vivo* production of TNF-α and IL-1β did not change during the course of illness. However, when stimulated with LPS, the median *ex vivo* production of both TNF-α and IL-1β was strongly depressed during the acute phase of the disease as compared to the amounts produced at recovery. The latter amounts are in the same order as those in normal individuals in the Netherlands. The IL-Ra production in the presence of LPS was slightly lower at admission than during the subsequent days.

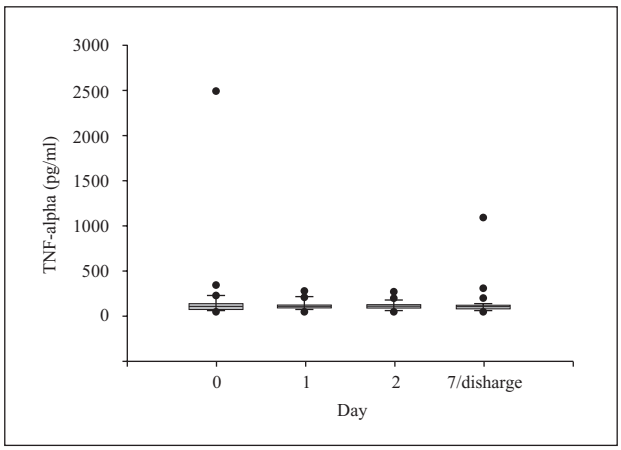

Figure 1a
Median plasma concentrations of tumor necrosis factor-α (pg/ml) with 25 and 75 interquartile ranges, on the day of admission (D0): 155 (120 – 176.2); D1: 140 (120 – 167.5); D2: 150 (125 – 170) and D7 or day of discharge: 135 (115 – 155).

Plasma concentrations of cytokines on the day of admission in survivors and non-survivors

The plasma concentrations of TNF-α, IL-1β and IFN-γ on the day of admission of survivors and non-survivors were not significantly different (mean values ± SD in pg/ml, and *p* value): TNF-α: 154.7 ± 54.0 *versus* 556.6 ± 909.3, $p = 0.254$; IL-1β: 73.2 ± 21.5 *versus* 115.7 ± 91.6, $p = 0.089$; IFN-γ: 22.2 ± 7.8 *versus* 25.0 ± 11.8, $p = 0.376$. However (figure 2), compared to survivors, the mean plasma concentrations IL-1Ra were significantly higher in non-survivors: 802.2 ± 566.4 *versus* 1566.9 ± 675.6, $p = 0.0005$. Also for IL-6, the mean plasma concentrations were higher in non-survivors

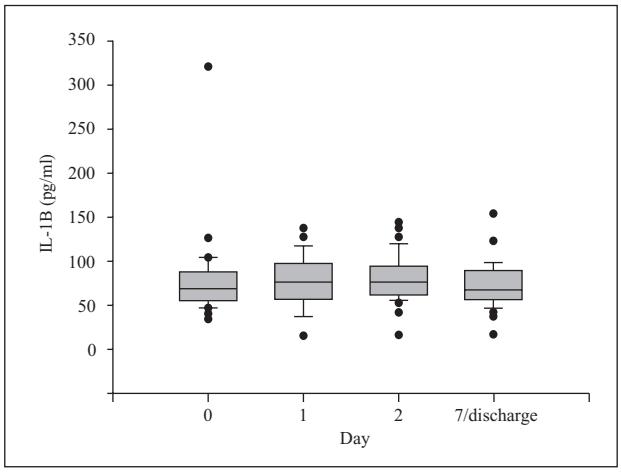

Figure 1b
Median plasma concentrations of interleukin-1β (pg/ml) with 25 and 75 interquartile ranges, on the day of admission (D0): 72.5 (60 – 90); D1: 80 (60 – 101); D2: 80 (65 – 97.5) and D7 or day of discharge: 70 (57.5 – 92.5).

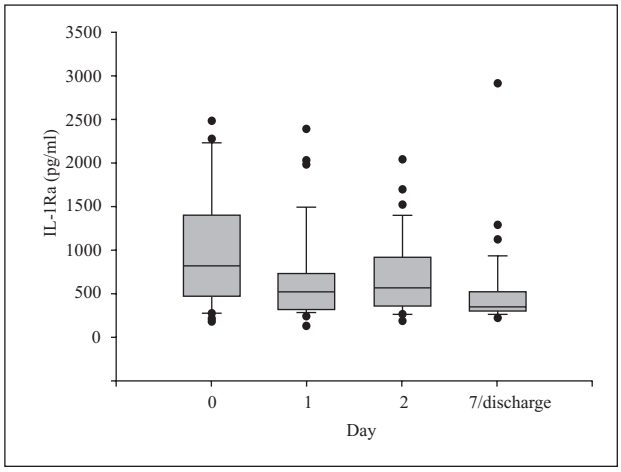

Figure 1c
Median plasma concentrations of interleukin-1 receptor antagonist (pg/ml) with 25 and 75 interquartile ranges, on the day of admission (D0): 795 (505 – 1437.5); D1: 555 (355 – 775); D2: 590 (387.5 – 980) and D7 or day of discharge: 395 (330 –565).

compared to survivors (219.50 ± 58262.0 *versus* 172.1 ± 956.8, $p = < 0.00001$), but the median values of IL-6 in both groups were 9.5 and 1.0 pg/ml, respectively.

Ex vivo *production of TNF-α, IL-1β and IL-1Ra on the day of admission in survivors and non-survivors*

Table 3 shows the patterns of the proinflammatory cytokines TNF-α and IL-1β and of the anti-inflammatory cytokine IL-1Ra. The *ex vivo* production of IL-1Ra was significantly higher in non-survivors than in survivors, both

Table 2
Median *ex vivo* production of cytokines after 24 hr incubation with and without lipopolysaccharide in 50 children with dengue shock syndrome

Cytokine	Day 0	Day 1	Day 2	Day 7/ discharge
	pg/ml	pg/ml	pg/ml	pg/ml
Without LPS				
TNF-α	345	365	330	330
IL-1β	65	80	80	72.5
IL-1Ra	240	2700	3100	1400
With LPS				
TNF-α	520	510	590	3850
IL-1β	660	755	1320	9450
IL-1Ra	6975	8750	9850	10500

LPS: lipopolysaccharide
Day 0 + day of admission

without LPS stimulation ($p = 0.0008$) and with LPS ($p < 0.004$). The LPS-stimulated production of IL-1β was lower in non-survivors than in survivors (516.1 ± 270.0 *versus* 2252.2 ± 4152.9 pg/ml), but this difference did not reach statistical significance.

Association of plasma concentrations of cytokines on the day of admission with mortality

We calculated the association of plasma concentrations of TNF-α, IL-1β, IL-6, IFN-γ and IL-1Ra on the day of admission, with mortality. IL-1Ra was significantly associated with mortality in these 50 children with DSS (Table 4).

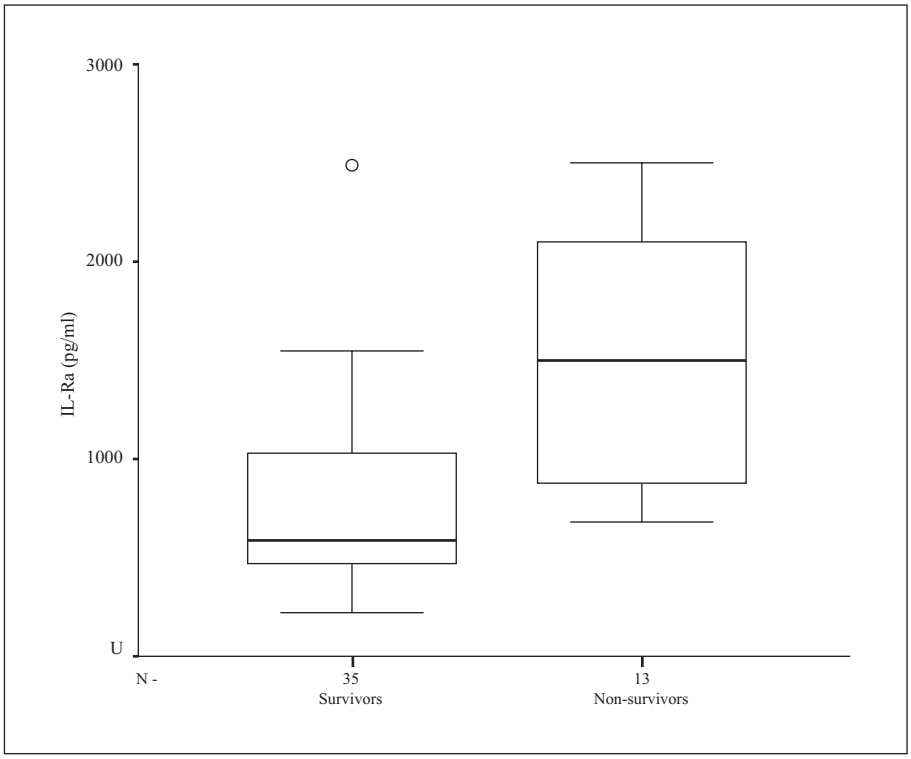

Figure 2
Plasma concentrations of interleukin-1 receptor antagonist on the day of admission (D0) in survivors and non-survivors of 50 patients with dengue shock syndrome. The difference is significant ($p < 0.0005$). Normal value 139 pg/ml (range < 80-502).

Table 3
***Ex vivo* production of cytokines after 24 hr incubation with or without lipopolysaccharide on day of admission in survivors and non-survivors**

Cytokine	Survivors (n = 37)	Non-survivors (n = 13)	*p* value[a]
	pg/ml	pg/ml	
Without LPS[b]			
TNF-α	362.5 ± 219.2	1748.0 ± 3356.1	0.107
IL-1β	76.8 ± 38.9	86.9 ± 49.3	0.584
IL-1Ra	2506.9 ± 2388.9	6823.0 ± 5435.5	0.0008
With LPS			
TNF-α	714.2 ± 791.7	1622.3 ± 2946.7	0.719
IL-1β	2252.2 ± 4152.9	516.1 ± 270.0	0.084
IL-1Ra	7141.4 ± 3436.5	11750.0 ± 5473.9	< 0.004

Values are mean ± SD; [a]Mann-Whitney U-test.

Table 4
Multiple logistic regression analysis of plasma cytokines on day of admission and mortality

Cytokine	β	*p*-value
TNF-α (per 10pg/ml)	– 0.125	0.275
IL-1β (per 10pg/ml)	0.203	0.377
IL-6 (per 1000pg/ml)	0.203	0.734
IFN-γ (pg/ml)	– 0.290	0.887
IL-1Ra (100pg/ml)	0.266	0.007
Constant	2.403	0.953

β: regression coefficient. IL-1Ra is significantly associated with mortality, the predicted value changes by 0.26 each time IL-1Ra increases 100 pg/ml.

DISCUSSION

In 50 children admitted with proven DSS, we found that plasma concentrations of IL-1Ra were considerably elevated and that high concentrations of this antiinflammatory cytokine correlated with mortality.

It is generally assumed that high concentrations of antiinflammatory cytokines in the circulation reflect proinflammatory cytokine production in the tissues. Also, compared to an anti-inflammatory cytokine like IL-1Ra, the proinflammatory cytokine responses are relatively short-lived in serious infections, and elevated concentrations in plasma are only found shortly after onset [21]. Since our patients with DSS were admitted a mean of 4.2 days after onset of disease (table 1), this may explain why we have found only slightly elevated median plasma concentrations of TNF-α and IL-6, although greatly elevated TNF-α concentrations and IL-6 were found in two patients, both of whom died. Other investigators have reported high concentrations of circulating TNF-α in dengue, correlating with disease activity [6, 7]. We could not confirm these data. One reason for this discrepancy may be the timing of blood sampling. In our study, the earliest samples in which cytokines were measured, were collected 4 (median) days (range 2-7) after onset of disease. Therefore, we may have missed possible high concentrations of IL-6 and TNF-α during the first days of infection. From a previous study of mild dengue, we know however that cytokines are not elevated in that situation (unpublished). In addition, the development from mild dengue to DSS often occurs abruptly, making admission urgent. In view of these observations, it is remarkable that the concentrations of cytokines are not found to be more elevated. An additional explanation may be the use of a different TNF-α assay. However, the fact that we also found low median IL-6 concentrations gives support to our TNF-α data, since IL-6 is induced mainly by TNF-α, and is a very sensitive marker for activation of the cytokine network. In comparison with other critical illnesses such as septic shock, it is remarkable that IL-6 concentrations normalize relatively soon in dengue, even when patients are (still) critically ill. These findings on IL-6 are very similar to those found in a study elsewhere in Indonesia, i.e., in Yogyakarta [22].

We did not find evidence for production of the type I cytokine IFN-γ in DSS. Other investigators have observed that IFN-γ levels were increased, decreasing abruptly one day after defervescence [13], coinciding with the disappearance of viraemia [23]. Therefore, as for TNF-α and IL-6, we may have missed the IFN-γ peak due to relatively late sampling.

Our results indicating that IL-RA is a marker of severity are somehow in agreement with those of Pinto *et al.* [9], who found elevated IL-1Ra concentrations in five out of 31 patients with mild dengue.

In the acute phase of the disease, the *ex vivo* production of TNF-α and IL-1β after incubation with LPS was considerably depressed but returned to normal on recovery. Such low production capacity of proinflammatory cytokines has been found in severe bacterial infections such as sepsis [24, 25], typhoid fever [26], and acute meningococcal disease [27], and apparently also occurs in this severe viral infection. The degree of down-regulation of proinflammatory cytokine production has been shown to correlate with disease severity in the bacterial infections mentioned and seems to mirror the magnitude of the initial systemic cytokine response. In our patients, the degree of down-regulation of proinflammatory cytokines did not significantly differ between survivors and non-survivors. The up-regulation of the anti-inflammatory cytokine IL-1Ra, however, was significantly more pronounced in non-survivors.. The down-regulation of cytokine production may be a result of action of the anti-inflammatory cytokine IL-10, which has been shown to be elevated in dengue by other investigators [7, 28, 29]. This cytokine has been implicated in the shift to a type 2 cytokine response in severe dengue [30].

It is remarkable that in all children suspected of suffering from DSS the diagnosis was right. Apparently, in an endemic area during an outbreak, experienced clinicians recognize the rather specific clinical picture of DSS. In adults, the diagnosis is more difficult; this became clear from a prospective study we performed in Semarang, in which 51% of patients with suspected dengue were, in fact, suffering from other diseases. In 20 of the patients without dengue, proof for other infections agents was obtained (influenza A, chikungunya, rubella, hantavirus, Leptospira, *Rickettsia* spp).

We conclude that in hospitalized children with DSS (DHF III and DHF IV), IL-1Ra is a valid prognostic marker, since it is associated with mortality, and because of its relatively long half life, it can be reliably measured. Its presence provides indirect evidence for preceding proinflammatory cytokine activity, such as of IL-1β. The transient proinflammatory cytokine patterns in the blood, with the pronounced antiinflammatory response observed, lend little

support for anti-cytokine strategies in DSS when patients have already been ill for 3-4 days.

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