

AUGMENTED INFLAMMATORY CYTOKINES IN PRIMARY DENGUE INFECTION PROGRESSING TO SHOCK

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ABSTRACT

Dengue fever (DF) which is caused by four serotypes of dengue virus may in some cases progress into a life threatening situation of dengue haemorrhage fever (DHF) and dengue shock syndrome (DSS). It has been suggested that sequential infection with different dengue virus serotypes predisposes the patient towards DHF/DSS. We report here a primary dengue infection in a 10-year-old boy progressing from DF to DSS while under clinical observation.

The report provides unequivocal evidence for the development of DSS in primary dengue infection caused by virus serotype 4. The close relationship between sequential changes in the levels of tumour necrosis factor (TNF), Interleukin 1 and 6 (IL-1 and IL-6) in the serum, to the clinical progression of the disease from DF to DHF/DSS and then to full recovery implicates a pathogenetic role for the inflammatory cytokines. The child also manifested clinical features consistent with Reye's syndrome and this suggests a common pathogenetic origin for DSS and the Reye-like syndrome induced by dengue virus.

Keywords: cytokines, interleukin 1 (IL-1), interleukin 6 (IL-6), tumour necrosis factor (TNF), dengue shock syndrome.

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INTRODUCTION

Dengue infection is a self-limited febrile disease caused by mosquito-borne dengue virus serotype 1 to 4 which are commonly prevalent in Southeast Asia and Western Pacific countries⁽¹⁾. In some rare cases, the dengue fever (DF) progresses into a life-threatening clinical spectrum of dengue haemorrhage fever (DHF) and dengue shock syndrome (DSS) which are accompanied by thrombocytopenia, extravasation of fluid into interstitial spaces and circulatory collapse⁽²⁾. While the pathology of DHF/DSS are well characterised, the pathogenetic mechanisms for the development of the disease remains uncertain.

Halstead⁽³⁾, from epidemiological and clinical observations had proposed that a second dengue virus infection occurring sequentially in a patient with subneutralising anti-virus antibodies to a different serotype present from an earlier primary infection, enhances the virus replication and predispose the patient towards DHF/DSS. The phenomena of antibody-enhanced dengue virus replication have been experimentally demonstrated in primate leukocytes and in human blood mononuclear cells following the addition of low levels of anti-dengue antibodies to the various models investigated⁽⁴⁻⁶⁾. Apart from the sequential pattern for

development of DHF/DSS, there is unequivocal evidence from clinical observations and laboratory studies of the occurrence of the DHF/DSS due to primary dengue infection^(7,8).

A hallmark of DSS is that the duration of the shock is short and the patient may die within 24 hours of its development if the shock remains untreated. However, recovery from the shock syndrome is often equally rapid with the patient returning to normal state within 2-3 days of onset of shock. Thus, the lack of a diagnostic pathologic findings in the major organs and the rapid recovery without sequelae of survivors, strongly suggest a physiologic dysfunction which is secondary to the action of biologic mediators^(2,9). Recently, we have demonstrated that tumour necrosis factor (TNF), a potent mediator of inflammatory responses, is significantly enhanced in sera of patients with DHF/DSS but not DF⁽¹⁰⁾. We have, therefore, proposed that TNF and other cytokines which act synergistically with it, may have a role in the pathophysiologic changes observed in DHF/DSS. In this report we describe a primary dengue infection in a 10-year-old boy who progressed from DF to DSS while under observation in the hospital. Serial measurements of TNF, Interleukin 1 and 6 (IL-1, IL-6) showed marked enhancement of these cytokines coincident with overt cardiovascular collapse.

CASE REPORT

A 10-year-old Chinese boy (TWN) was admitted for fever of 2 days duration but otherwise in good general condition. Urine were taken for microbiological tests and blood was collected for haematological assessment as well as for various viral studies. The serum was tested for dengue and Japanese encephalitis virus antibodies using the Haemagglutination-Inhibition (HI) Test and a modified IgM-capture ELISA as previously described⁽¹⁰⁻¹²⁾. Briefly, the HI test was conducted using male goose erythrocytes. The patient's serum was extracted with acetone to remove non-specific inhibitors and then with goose erythrocytes to remove non-specific agglutinins. Serial double dilutions of sera, positive and negative controls were reacted against 8 haemagglutination units of dengue antigen from Dengue 1 (Hawaii), Dengue 2 (Trinidad, 1951), Dengue 3 (Hawaii-87) and Dengue (H241). A four-fold rise in titre of acute and convalescent sera was considered positive.

The assay for dengue specific IgM antibody was performed on ELISA plates coated with rabbit anti-human IgM to which

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were added the patient's serum diluted: 1:100, and appropriate controls. Incubation was for 1 hour at 37°C to trap on the solid phase the IgM from the sera. The captured IgM was detected with addition and washes in-between of 50 haemagglutinating units of dengue virus, followed by anti-dengue monoclonal antibody, goat anti-mouse IgG conjugated to horseradish peroxidase and substrate o-phenylenediamine. The colour intensity was read at 490 nm in an ELISA reader (MCC 340 Mark II, Flow Laboratories, UK).

Other serological tests carried out were the immunofluorescent antibody test for detection of the Hantaan virus and the ELISA assay for the detection of types A (HAV-IgM) and B Hepatitis virus (HBsAg) infection.

The sandwich enzyme immunoassay (ELISA) was used to determine the TNF and IL-6 levels, and a radioimmunoassay technique was used to assay the IL-1 levels in the sera following the instructions of the manufacturer (Genzyme, USA) as described earlier⁽¹⁰⁾. Briefly, in the ELISA assay, the TNF or IL-6 in the patient's sera was trapped by anti-TNF and anti-IL-6 monoclonal antibody respectively, which were bound to the wells of microtitre plates. Then an enzyme conjugated to a monoclonal antibody specific to the trapped antigen was added and with washes in between was followed by substrate. The colour intensity was read in a ELISA plate reader and the concentration of the TNF or IL-6 computed from standard graphs established from each plate. The assay was sensitive to TNF concentration at 10 pg/ml and to IL-6 concentration at 1 pg/ml.

The IL-1 concentration in the patient's serum was assayed by a radioimmunoassay containing radioisotope labelled monoclonal antibody to IL-1 in competitive assays. The assay was sensitive to IL-1 level in the sera to 40 pg/ml.

CASE HISTORY

At admission, TWN had a temperature of 39°C but was in good general condition. His pharynx was injected. The liver was palpable 2 cm below the right costal margin but the spleen was not palpable. He did not have petechiae and the Hesse's test was negative. The haemoglobin (HB) level was 11.2 gm/dL, total white blood cells (TWBC) $4 \times 10^9/L$ (neutrophil = 62%, lymphocytes = 38%), platelets $198 \times 10^9/L$ and erythrocyte sedimentation rate (ESR) was 70 mm/hr. In the next five days, the fever persisted but the child remained clinically well. The liver increased in size to 4 cm below costal margin and the spleen became palpable 2 cm below the costal margin. Results of various laboratory tests were as follows: peripheral blood examination for malarial parasites was negative, blood and urine cultures yielded no microorganisms. Other tests including the Paul Bunnell, Widal Weil Felix, Hepatitis A-IgM and HBsAg, Hantaan virus antibody and Japanese encephalitis virus antibody assays were all negative. The only positive findings were the detection of specific IgM antibody to dengue virus as well as a significant rise of HI antibodies to dengue virus Type 4. Cerebro-spinal tap was performed on day 7. The cerebro-spinal fluid (CSF) was free of leucocytes; protein was 150 ml/L and glucose 2.6 mmol/L; direct smear and culture were negative for bacteria and fungi.

On day 6 the condition of TWN deteriorated suddenly and unexpectedly. He had high fever, myalgia, headache and developed recurrent vomiting, haematemesis and diarrhoea. He became combative, irritable, drowsy and hypotensive (blood pressure 50/30 mmHg, pulse 160/min) with cold clammy extremities. He developed oliguria, with urine output decreasing to 0.13 ml/kg/hr.

Investigations performed at this time showed: HB 12.3 gm/dL, TWBC $3.5 \times 10^9/L$, platelets $87 \times 10^9/L$, ESR 11mm/hr, malarial parasites were negative; serum protein 64 gm/L, serum

albumin 27 gm/L, serum bilirubin 44 $\mu\text{mol/L}$ (conjugated 26 $\mu\text{mol/L}$), aspartate aminotransferase (AST) 244 iu/L, alanine aminotransferase (ALT) 73 iu/L, creatinine phosphokinase (CPK) 36 iu/L, alkaline phosphatase (AP) 181 iu/L, urea 108 mmol/L, creatinine 165 mmol/L, sodium 141 mmol/L, potassium 3.2 mmol/L, chloride 104 mmol/L; and repeat blood and urine cultures were negative.

He was managed with intravenous fluids with continuous dopamine infusion to maintain a central venous pressure of 5-8 mmHg. Plasma infusion was required on 2 occasions to stabilise a rapidly declining blood pressure (50/30 mmHg).

On day 7 the fever settled promptly but his clinical condition remained critical as the blood pressure was difficult to maintain and fluctuated between 75/40 and 60/30 mmHg. He remained oliguric with urine output of 0.18 ml/kg/hr. The thrombocytopenia (platelets: $54 \times 10^9/L$) worsened. Renal and liver functions deteriorated further.

At this time the blood chemistry was as follows: serum bilirubin 44 $\mu\text{mol/L}$, AST 2481 iu/L, ALT 982 iu/L, CPK 314, AP 181 iu/L, urea 32.5 mmol/L and creatinine 525 mmol/L.

On day 9 there was a dramatic clinical improvement which was associated with improvement in the haematological and blood chemistry indices. (Platelets $110 \times 10^9/L$, AST 119 iu/L, ALT 327 iu/L, CPK 153 iu/L, urea 33.6 mmol/L, creatinine 527 mmol/L). He continued to improve over the following one week and was discharged well on day 15. The serial clinical condition is graphically depicted in Fig 1. The haematological and blood chemistry results are summarised in Table I.

Fig 1 – Temperature (°) and systolic blood pressure (BP, ■) in patients with dengue fever progressing to dengue shock syndrome.

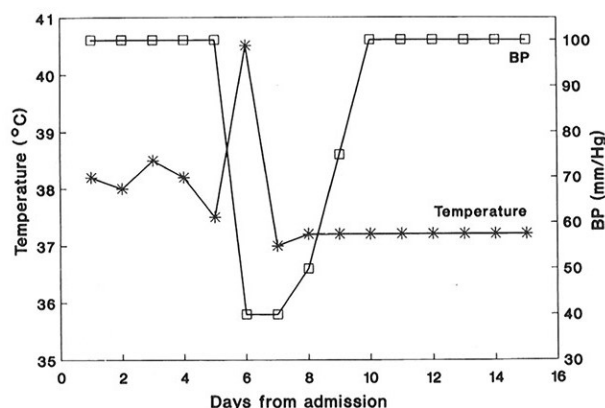


Table I – Haematological and blood chemistry results of patient with dengue infection between day 1 to 15 of illness from the admission date.

Haematological/ Biochemical indices	Day of Illness						
	1	6	7	8	10	12	14
Platelets $\times 10^9/L$	198	87	54	89	110	130	210
Serum albumin, gm/L	30	27	27	26	26	23	30
AST, iu/L	24	224	2481	492	119	67	16
ALT, iu/L	26	73	982	558	327	255	91
CPK, iu/L	–	36	314	404	153	58	9
Urea, mmol/L	–	10.8	22.5	32.5	33.6	33	18.1
Creatinine, mmol/L	–	165	321	525	527	545	214
IgM Anti-dengue virus antibody (ELISA OD units, control 0.167)	6.5	4.1	2.5	2.5	2.9	3.4	4.0

AST = Aspartate aminotransferase, ALT = Alanine aminotransferase, CPK = Creatinine phosphokinase, OD = Optical density

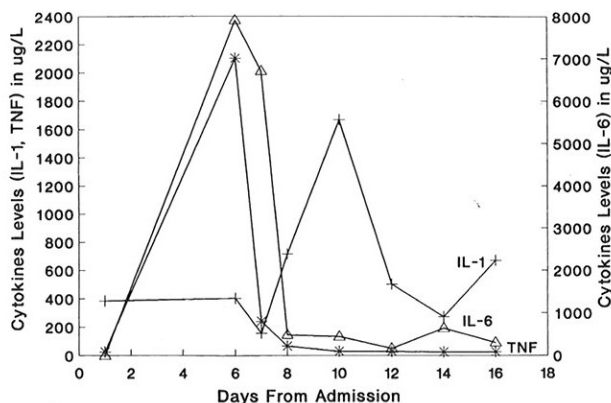
SERUM CYTOKINE LEVELS

Serum was available for day 1, 6, 7, 8, 10, 12, 14 and 16. All the sera were positive for IgM anti-dengue virus antibody. Haemagglutinin-inhibition dengue virus titres were not detectable (<10) at admission for all serotypes but 6 days later only the titre for serotype 4 increased 8 fold (1:40) and other serotypes anti-virus titres remained unchanged. This shows that the infection observed was a dengue serotype 4 in the child.

The ELISA Optical Density (OD) value of the various sera to dengue anti-virus IgM antibody decreased from a high value at admission to low positive values during the period of shock at day 6 and 7 and then the antibody titres again increased (Table I).

Cytokine TNF, IL-1 and IL-6 were assayed in the sera which were obtained in sequence during the period of the infection. Both TNF and IL-6 reached extremely high levels, TNF peaking at 2,100 µg/L and IL-6 at 7,400 µg/L on the day of onset of shock (day 6), but these cytokines were barely detectable at admission and decreased rapidly to low levels from day 7 to day 15. The rise and fall of TNF and IL-6 followed the same pattern. The cytokine IL-1 was enhanced post-shock reaching peak levels of 1,666 µg/L on day 10. (Fig 2). Cytokines were also assayed in the cerebro-spinal fluid on day 7 of admission. The data showed very high levels of IL-6, (1,260 µg/L), and mildly elevated levels of IL-1 (157 µg/L) but TNF (25 µg/L) was barely detectable.

Fig 2 – Change in tumour necrosis factor (TNF, *), Interleukin 1 (IL-1, +) and Interleukin 6 (IL-6, ▲) in patients with dengue fever progressing to dengue shock syndrome.



DISCUSSION

The progression of primary type 4 dengue virus infection from DF to DSS in this patient while under clinical observation in hospital confirms unequivocally the earlier epidemiological observations^(7,8) that DSS can occur without the viral enhancement due to residual heterologous antibody in sequential dengue serotype infections. This shows that both sequential serotype dengue infections and primary infection may induce DSS under appropriate conditions.

At admission the child had high titres of IgM anti-dengue virus antibodies but these levels significantly decreased during the period of the shock which began on day 6 of admission. The observed IgM depletion from serum could be due initially to the formation of immune complexes between IgM and dengue viral antigen and later due to the seepage of IgM into the interstitial spaces or dilution due to administration of intravenous fluids. However, previous studies show that the loss of these proteins could not be attributed to transudation because the reduction in these values exceeded those of transferrin⁽²⁾. These immune complexes may be pivotal in the trigger for the release of

cytokines via mediators in the complement pathway^(13,14). Experimental evidence using the lung model, shows that the intra-alveolar deposition of IgG immune complex results in injury to the lung with damage to the vascular endothelium and alveolar epithelium followed by interstitial edema⁽¹⁴⁾. It appears that complement activation with generation of C5a and stimulation of alveolar macrophages results in production of TNF and platelet activating factor. The TNF on its own can cause lung injury via damage to the vascular endothelial cell and alveolar epithelial cell. Use of specific antibody which neutralised the TNF provided over 70% protection⁽¹⁴⁾. In dengue infection the deposition of virus-IgM or IgG antibody immune complexes in the blood capillaries may initiate a sequence of events resulting in TNF induced injury to the endothelial cells. Vascular endothelial cells have TNF-receptors and are probably one of the most sensitive cell in the body responsive to the action of TNF⁽¹⁵⁾. In vitro studies show that exposure of endothelial cells to TNF or IL-1 results in dramatic increase in adhesive interactions with neutrophils. A correlate of this increased adhesive interaction between endothelial cell and neutrophil is the greatly enhanced susceptibility of the endothelial cell to destruction and damage by neutrophils⁽¹⁴⁾.

The major finding in this report was the demonstration of a close relationship between the sequential changes in the levels of TNF, IL-1 and IL-6 in the serum to the clinical progression of the disease from DF to DSS and then to full recovery. At the time of admission, when the patient was in good condition, serum TNF and IL-1 levels were only mildly elevated and IL-6 was not detected. On day 6 the high activities of TNF and IL-6 coincided with the dramatic onset of signs and symptoms of hypovolumic shock. Over the next 48 hours, the rapid decline in levels of TNF and IL-6, paralleled closely the rapid clinical improvement of the patient. Improvement in the haematological and blood chemistry indices were however observed to occur more slowly and lagged behind the clinical improvement and changes in TNF activities.

Furthermore, the improvement in the constitutional symptoms of nausea, vomiting, malaise and poor appetite appeared to follow more closely the decline in TNF than the blood chemistry indices of liver and renal functions implying a possible direct contributory role of TNF and other cytokines in the origin of these symptoms⁽¹⁵⁾. The clinical manifestation of thrombocytopenia, haemorrhage (petechia and haematemeses) and extravasation of fluid from the vascular compartment progressing to hypovolumic shock evident in this patient are similar to the features of TNF activity observed in the experimental model⁽¹⁶⁻¹⁸⁾ and in clinical situations of meningococcal septicaemia and cerebral malaria^(19,20).

In vivo experimental studies⁽¹⁵⁾ in mice showed that following *E. coli* injection, the initial response consisted of a rapid rise in TNF activity, followed closely by a rise in IL-1 activity and much later by a rise in IL-6 activity. In our present report, TNF and IL-1 activities were detected at low levels on day 1 of illness, rose very sharply on day 6 but these levels decreased rapidly over the ensuing 48 hours. IL-1 activity interestingly increased sharply post-shock when the TNF and IL-6 were low and the child was improving clinically. It is possible that the pattern of cytokine response to bacterial and viral pathogen may differ in these infections.

The interrelationship between TNF, IL-1 and IL-6 in the pathogenesis of DHF/DSS remains to be determined. Nevertheless, it appears likely that TNF plays a dominant role since it is the only cytokine which can, by itself, induce irreversible shock and death⁽²¹⁾. The other cytokines including IL-1 and IL-6 and interferon (IFN)-gamma may act in synergy

to enhance or modulate the severity of the disease.

Kurane et al has demonstrated that significantly higher levels of IL-2 and IFN-gamma exist in acute sera of patients with DF and DHF compared to age matched controls⁽²²⁾. They suggested that the IFN-gamma would facilitate recruitment of dengue virus specific T cytotoxic cells which targeted dengue infected monocytes but the presence of IFN-gamma at similar levels in DF and DHF indicates that the lymphokine alone was not responsible for the pathogenesis in DHF.

The clinical and laboratory features in this patient, namely acute neuropsychiatric symptoms, hepatomegaly, elevated levels of liver transaminases and creatinine phosphokinase and a normal CSF, are consistent with the CDC criteria of Reye's syndrome⁽²³⁾. The augmented levels of cytokines TNF, especially IL-1 and IL-6 in the blood and CSF, could have contributed to the neurological symptoms⁽²⁴⁾. Frei et al has demonstrated that the local production of IL-6 by astrocytes and microglial cells during viral infection of central nervous system appears to serve to stimulate the immune response and also activate tissue repair⁽²⁵⁾. It may be speculated that augmented TNF may have a pathogenetic role in virus associated Reye's syndrome⁽²⁶⁾.

The above observations taken together show that several interlinked factors are associated in the development of DHF/DSS. However, the present report strongly implicates a pathogenetic role for TNF in DSS. It is possible that following dengue virus infection several factors involved in the modulation of the immuno-cytokine responses determine the course of events, whether the DF subsides uneventfully or progresses to DSS. It is also possible that genetic factors as yet unidentified could be involved in determining the response of the individual to the virus infection since only a fraction of the patients with DF progress to DHF/DSS.

TNF antibody given prior to or at the onset of septic shock prevents the deleterious effects of TNF and averts death⁽¹⁵⁾. In the light of these studies, our present findings have significant therapeutic implications⁽¹⁰⁾.

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