

Clinical correlates with immunopathogenesis in dengue haemorrhagic fever/dengue shock syndrome

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INTRODUCTION

Studies on the link between pathogenesis and clinical manifestation in dengue virus infection are of interest because they may reveal answers which will affect the outcome of management of dengue virus infection. Close similarities are observed clinically between dengue shock syndrome (DSS) and its bacterial counterpart, sepsis syndrome. The sepsis syndrome can be defined in terms of the systemic response to infection expressed as tachypnoea, tachycardia, fever or hypothermia, and evidence of inadequate organ perfusion or organ dysfunction such as impaired cerebral perfusion.^{1,2} When sepsis is accompanied by hypotension unresponsive to fluid therapy it is often referred to as septic shock.¹ Laboratory investigations reveal hypoxia, acidosis, elevated plasma lactate, renal impairment and hepatic dysfunction. Anaemia, thrombocytopenia and disseminated intravascular coagulation supervene. Oedema, jaundice, worsening acidosis, respiratory and cardiac failure may occur. These features and the complex multisystem derangement they indicate are largely the result of three basic processes affecting the blood vessels.

1. Increased vascular permeability

Alterations in the endothelial lining of the blood vessels are of key importance in the pathophysiology of sepsis.⁴ The vascular endothelial cell is remarkably specialised to maintain vascular homeostasis in the following ways:

- a) Water and small molecules are permitted to pass freely to the tissues while albumin and larger plasma proteins or blood cells are confined to the intravascular space.
- b) Although blood cells pass at high speed through capillaries, damage to the blood cells is prevented by the non-reactive endothelial surface.
- c) Platelets and proteins of the coagulation pathway are protected from activation by the non-thrombogenic and platelet inhibi-

tory properties of the endothelium.

- d) Neutrophils and other inflammatory cells do not normally adhere to the intact endothelium.
- e) Regulatory factors such as prostacyclin, Factor VIII, von Willebrand factor and plasminogen activator are synthesised and secreted by vascular endothelial cells.

These characteristics of the endothelium are partly due to the high concentration of anionic glycosaminoglycans, heparin sulphate and chondroitin sulphate on the luminal surface of the endothelium. These glycosaminoglycans confer a fixed negative charge on the endothelial surface which is important in the permeability properties and in reducing cellular interactions on the endothelium. The glycosaminoglycans have anticoagulant activities and localise molecules such as antithrombin III, lipoprotein lipase and platelet factor 4 on the endothelial surface.

An alteration in the permeability properties of the endothelium and the capillary wall allows albumin and other colloids to leak from the intravascular space. This results in hypovolaemia, diminished venous return and diminished cardiac output. Accumulation of extravascular plasma proteins and fluid in the tissues leads to oedema in the pulmonary and other interstitial areas.

2. Vasoconstriction

Vasoconstriction of arteries and arterioles increases vascular resistance in the systemic and pulmonary circulation, decreasing cardiac output and diminishing perfusion to the kidneys, gastrointestinal tract and other organs. Dilatation of postcapillary venules and some precapillary arterioles results in peripheral pooling of blood, shunting of blood past some vascular beds, and in further reduction in both tissue perfusion and venous return.

3. Microvascular obstruction

Microthrombi composed of platelets, leukocytes and fibrin form within the capillaries of the

pulmonary, renal and other vascular beds. A further reduction in organ perfusion takes place, together with the clinical picture of a consumption coagulopathy.

These three processes produce a pathophysiological cycle which, if maintained, results in septic shock, multiorgan failure and death.

PATHOGENESIS

There are two contrasting theories to the pathogenesis of dengue haemorrhagic fever (DHF). The first is that DHF is related to the virulence of the virus infecting lymphoid cells which are destroyed, releasing mediators which cause the signs and symptoms of DHF and DSS.⁵ The other hypothesis is that DHF is due mainly to the host immune response to dengue infection.

Antibody response to primary dengue infection

Primary dengue virus infection with one of the four antigenically distinct types elicits an immune response directed at elimination of the virus and recovery by the patient.⁶ The serotype specific antibody produces lifelong homotypic immunity. It is accompanied by a brief period of cross protection against the other three serotypes.⁷ If infection later occurs with a homologous dengue virus the host memory response eliminates the virus before disease is produced. Such an immune response is protective to the individual and prevents dengue reinfection.

Antibody response to heterotypic dengue infection

Should later infection occur with a different dengue type, known as a heterologous or heterotypic virus, the antibodies produced will have an enhancing effect on viral replication within the mononuclear phagocyte. Antibody dependent enhancement was first observed in experimentally infected monkeys.^{8,9} Monkeys monotypically immune to dengue virus types 1, 3 or 4, when infected with dengue 2 virus, circulated more virus than did nonimmune animals infected with the same virus strain administered by the same route and at the same dose.

There may be two reasons why antibodies fail to neutralise dengue virus. First, antibodies directed against epitopes on another dengue virus serotype may lack specificity for the binding site of the dengue virus. Second, antibody titres may be too low to prevent binding. In either case, serotype crossreacting non-neutralising antibodies can enhance dengue infection and

contribute to the pathogenesis of DHF/DSS.¹⁰

DSS can also occur during primary infection in infants. Mothers of affected infants have all been shown to have had dengue antibodies from previous infections." The presence of these antibodies, which are placentally transferred, and memory T-cells may be important in the pathogenesis of DHF/DSS.¹²

The dengue antibodies promote viral entry into the monocyte by forming complexes with Fc gamma receptors on the monocyte. In the presence of enhancing antibody, virus engulfed by monocytes replicates within monocytes. Dengue-infected monocytes are destroyed in the process of immune elimination, probably mediated by dengue-specific cytotoxic T lymphocytes or by natural killer (NK) cells.^{13,14} A variety of mediators or lymphokines are released with effects on vascular permeability, complement and coagulation systems, resulting in shock and haemorrhage.

Human T-lymphocyte responses to dengue infection

Two populations of T-lymphocytes proliferate after dengue virus infection. CD4- CD8+ T-cells lyse dengue virus-infected monocytes and recognise the viral envelope glycoprotein E and non-structural (NS3) proteins. Proliferating CD4+ CD8- T-cells produce gamma interferon (IFN- γ) which augments dengue virus infection in the presence of antibody to dengue viruses.^{15,16,17} Lysis of dengue virus-infected monocytes releases lymphokines which may lead to DHF/DSS.¹⁸ Examples of lymphokines which are released in septic shock states include¹⁹:

- Vascular permeability factor
- Complement activating factors, C3a, C5a
- Thromboplastin
- Fibrin-split products
- Kinins
- Histamine and histamine-like products
- Interferon (e.g. IFN- γ)
- Interleukins (e.g. IL-1, IL-2, IL-6)
- Macrophage activation factor
- Monocyte chemotactic factor
- Platelet derived growth factor.

The release by the macrophage of lymphokines such as Tumour Necrosis Factor (TNF), Lymphotoxin, Interleukin-1 (IL-1), Interleukin-6 (IL-6), Interferon and Platelet Activating Factor in bacterial sepsis leads to speculation that similar peptides may be released in DSS. During bacterial infection these lymphokines are important in mediating the inflammatory response.

However, when produced in very large quantities, some have a **synergic** interaction and become responsible for tissue injury and cell death.

IL-1 produces fever by inducing prostaglandin E2 synthesis in the **thermoregulatory** centre. In animals, IL-1 produces the features of septic shock by activating phospholipase to release arachidonic acid from phospholipids in cell walls.²⁰ A IL-1 receptor antagonist has been shown to reduce the lethality of **endotoxin-induced shock** in rabbits.²¹ Human urine contains a protein (molecular mass, 27 kDa) which binds to TNF and protects cells against TNF cytotoxicity.²²

Cytotoxic Factor

In mice, dengue 2 virus induces a calcium-dependent lymphokine called cytotoxic factor (CF) (molecular mass, 43 kDa) with broad **proteinase-like activity**.^{23,24} CF kills macrophages, T-helper cells and megakaryocytes and, when inoculated into mouse or monkey, increases capillary permeability. Pretreatment with rabbit anti-CF antisera or with H1 or H2-receptor antagonist drugs prevents the effects of CF on mouse spleen cells and macrophages. Such a lymphokine may be responsible for thrombocytopenia, reduced megakaryocytes and plasma leakage from increased capillary permeability in DHF/DSS. CF also produces a breakdown in the blood brain barrier leading to cerebral oedema during dengue-2 virus infection.²⁵

Arachidonic acid pathway

Arachidonic acid undergoes metabolism by different pathways leading to a variety of active metabolites²⁶ via:

15-lipoxygenase to the lipoxins.
5-lipoxygenase to hydroxy peroxyeicosatetraenoic acid (HPETE) which is converted to leukotrienes. The leukotrienes are mediators of allergy and inflammation.
cyclooxygenase to the prostaglandins. With thromboxane synthase, PGG₂ forms thromboxane (TXA₂), mainly in platelets. TXA₂ is a powerful vasoconstrictor and platelet aggregator. PGI₂ (prostacyclin, epoprostenol), produced from PGG₂ via prostacyclin synthase in the endothelial and smooth muscle cells in the blood vessels, is a potent vasodilator and inhibitor of platelet aggregation. A balance between PGI₂ and TXA₂ is essential to maintain vascular and

platelet functions.

In DHF/DSS extensive capillary damage occurs and generalised petechiae are present. Increased PGI₂ production has been described in DHF patients during impending shock, compared with normotensive controls.²⁷ This has been described as a mechanism to limit thrombosis in the damaged vessel and contribute to vasodilatation. However, patients with DSS present more commonly with hypovolaemia and vasoconstriction and the exact role of PGI₂ in DHF/DSS, in analogy with the use of prostacyclin in septic shock,^{28,29} remains to be elucidated.

Thrombocytopenia

Thrombocytopenia is a primary cause of haemostatic disorder in dengue infection. In-vitro studies suggest three possible causes of thrombocytopenia in dengue infection.³⁰

First, infection of megakaryocytes by dengue virus leads to maturation arrest.

Second, dengue virus incubated with cultured endothelial cells from human umbilical vein showed an increase in dengue viral titres within endothelial cells. When platelets were added to the cultures, the platelet count decreased whereas the platelet count was unaltered in virus free cell cultures. This suggests that there is an interaction between platelets and dengue antigen on the endothelial cell, causing thrombocytopenia. The mechanism of destruction of the endothelial cell is unknown but leads to exposure of subendothelial collagen which promotes platelet aggregation and thrombocytopenia.

Third, dengue virus also attaches to platelets without an immune mediated reaction. Destruction of that platelet is triggered in the presence of dengue antibodies and by immune complexes, causing thrombocytopenia.

Immune complexes

Circulating immune complexes formed between dengue antigen and antibody may play a central role in the pathogenesis of DHF/DSS. The circulating complexes have been detected using C1q binding assay, RIA and solid phase conglutinin binding assay.

Immune complexes were demonstrated on the surface of platelets, peripheral blood B lymphocytes, skin capillary walls and renal glomeruli. Those formed on platelets could enhance platelet destruction by the RES in the liver and spleen, adding to mechanisms of thrombocytopenia already discussed.^{31,32,33}

Complement

The main action of immune complexes is the activation of complement. The onset of DHF is associated with a fall in C3, C3Proactivator, C4 and C5, with marked depressions occurring in DSS.^{32,33,34} They indicate that complement is activated by classical and alternate pathways in DHF/DSS. Decreased synthesis of complement and increased extravasation of complement were excluded as causes of complement reduction by radioisotope studies of complement metabolism.

Massive complement activation can produce large amounts of C3a and C5a which are potent mediators of vascular permeability, causing decreased plasma volume and hypovolaemic shock. Although plasma contains powerful inactivators of C3a and C5a, it is quite possible that these peptides contribute to the development of shock before they are inactivated. In humans convalescence is associated with a return to normal levels of C3a and C5a.

However, immune complexes do not explain DSS in infants with primary dengue infection. As described earlier, cell mediated immunity may be an important mechanism of shock in this group of patients.

Haemostatic derangement

The pathogenesis of bleeding in DHF is complex. The spectrum of clinical effects includes vasculopathy, thrombocytopenia, platelet dysfunction and blood coagulation defects.³⁵ The positive tourniquet test demonstrates that capillary damage is present in the early days of dengue infection.

In children with DHF, prolongation of PT and APTT was caused by decreases in multiple components of coagulation. Decreases in Factor VII, prothrombin and antithrombin III were not as prominent as decreases in Factor VIII and fibrinogen. This suggests that fibrinogen and Factor VIII were consumed not only by coagulation but also by fibrinolysis. Alpha-2 Antiplasmin activity was low in the acute stage and returned to normal during convalescence. It was also observed that the FDP fraction was high in patients whose platelet count was below 20,000. This supports the view that fibrinolysis occurs in acute DHF.³⁶

Bleeding manifestations have been observed in 45-75% of DHF patients.³⁷ The degree of thrombocytopenia correlates well with the degree of severity. Fibrin degradation products are slightly increased (1.8-50mg/dl) in all grades of dengue infection. In children, infection, hypoxia

and consumptive coagulopathies may induce prothrombin complex deficiency through liver impairment.

Plasma protease inhibitors

The danger which proteolytic enzymes, such as neutrophil elastase, present to the body is perhaps indicated by the existence of at least 8 plasma proteins which function to neutralise proteolytic enzymes:

- alpha-1 antitrypsin
- alpha-2 macroglobulin
- alpha-2 plasma inhibitor
- antichymotrypsin
- antithrombin III
- C1 esterase inhibitor
- heparin co-factor II
- protein C inhibitor.

These proteins form an inactive complex with their target proteolytic enzyme which is then cleared from the circulation by the reticuloendothelial system. Proteolytic enzymes, when infused into animals, are initially neutralised by the protease inhibitors and the corresponding concentrations decrease. As soon as the protease inhibitors are overwhelmed and free proteolytic enzymes appear in the circulation, shock, disseminated intravascular coagulation and complement activation occur. In sepsis, release of neutrophil elastase and other proteolytic enzymes in excess of the neutralising capacity of the plasma protease inhibitors may have similar consequences to that seen in animal models.^{19,4}

The concentration of alpha-1 antitrypsin, the major plasma inhibitor of neutrophil elastase, increases in infection, suggesting an important role for alpha-1 antitrypsin in regulating the inflammatory response. Although alpha-1 antitrypsin is normally present in adequate concentrations to neutralise neutrophil elastase and localise any elastase to the immediate site of infection, massive release of neutrophil elastase may overwhelm the protease inhibitors.

Neutrophil elastase destroys a similar peptide sequence on alpha-1 antitrypsin and on antithrombin III, inactivating these inhibitors. In inflammation, if elastase is released in sufficient amounts to overwhelm alpha-1 antitrypsin and antithrombin III, endothelial damage can be accompanied by uncontrolled coagulation and intravascular thrombosis. This sequence of events has yet to be confirmed in humans, yet it is a potentially important mechanism linking neutrophil activation with disseminated intravascular coagulation. It is a pathophysiological se-

quence which may be interrupted therapeutically by infusion of fresh frozen plasma containing protease inhibitors, or purified protease inhibitors such as alpha-1 antitrypsin and antithrombin III.

Neurovirulence

Of great interest is the question: are the cerebral effects associated with DHF/DSS the result of primary CNS damage by dengue virus or are clinical manifestations such as encephalopathy and cerebral oedema the result of secondary phenomena? It is certainly suggested that the large numbers of patients who have made a full recovery from DSS without neurological sequelae is more common than the converse and this appears to lessen the argument for neurotoxicity.

A histopathological study of neurovirulence was carried out by CNS inoculation of monkeys using a wild strain and an attenuated strain of dengue virus.³⁸ There was no evidence of extensive CNS lesions. There were minimal lesions in 4 of 8 inoculated and 1 of 2 control monkeys. It was concluded that the dengue virus has a low propensity for severe neurovirulence.

In a report of four cases of dengue fever with neurological symptoms, CSF and serum were processed with IgM capture ELISA to detect specific IgM antibodies. CSF and serum IgM titres were positive but the duration and levels of IgM in CSF were shorter and lower than in serum.³⁹ The detected CSF antibodies could arise in two ways:

1. Increased permeability across the blood brain barrier. A lymphokine, CF, produced in dengue-2 virus infection in mice, has been shown to affect the integrity of the blood brain barrier for a short period, resulting in increased cerebral oedema.²⁵ CF has not been isolated in human dengue virus infection.
2. Stimulated by dengue virus infection. Intra-peritoneal inoculation of mice with dengue 2 virus does not result in an increase in brain virus titres.²⁵

Evidence for neurotoxicity caused by direct dengue viral infection of the CNS in humans remains to be conclusively demonstrated.

Envelope glycoprotein

The dengue 4 virus has an envelope glycoprotein, E, (molecular mass, 55 to 60kDa) which contains three specific antigenic sites. Specific neutralising antibodies are directed against E but the low immunogenicity of E has been a major

obstacle to the development of an effective dengue virus vaccine. Recently however, parts of E have been sequentially truncated to delineate E sequences that have shown improved immunogenicity responsible for inducing resistance to dengue virus challenge in mice.⁴⁰

Plasminogen

In computer analysis of the amino acid structure of glycoprotein E, unexpected similarities were revealed in sequence between dengue 4 E and a family of clotting factors including plasminogen, urokinase, Factor X, prothrombin and Tissue Plasminogen Activator.⁴¹ These factors play a role in the generation of fibrin or fibrinolysis. Factor X promotes the conversion of prothrombin to thrombin, which catalyses the proteolysis of fibrinogen to produce fibrin monomers.

Plasminogen is cleaved by tissue plasminogen activator, urokinase or other plasminogen activators to plasmin, the major effector of fibrinolysis. Plasmin also activates C3 and C4. Plasmin activity is modulated by alpha-2 antiplasmin. Dengue virus infection (types 1,2,3,4) in humans elicits glycoprotein E-specific antibodies which cross-react with plasminogen. This cross-reactivity with plasminogen was not seen in patients with Japanese encephalitis. These *in vitro* results have yet to be confirmed in *in-vivo* studies. Testing of sera from dengue patients with haemorrhage will be necessary to demonstrate that cross-reacting antibodies are important in modulating plasminogen and plasmin activity leading to haemorrhage in DHF/DSS.

CONCLUSION

Prevention, management and therapy will continue to be the aims of research so long as dengue virus infections are a major health problem in many parts of the world. The answers to research will contribute to understanding the roles of serotype cross-reactive antibodies and T-lymphocytes and the roles of mediators in the immunopathogenesis of DHF/DSS, and lead to the development of dengue virus vaccines.

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