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# Immunopathogenesis of Dengue Virus Infection

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**Key Words**
Dengue virus

**Abstract**
Dengue virus infection causes dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS), whose pathogeneses are not clearly understood. Current hypotheses of antibody-dependent enhancement, virus virulence, and IFN-γ/TNFα-mediated immunopathogenesis are insufficient to explain clinical manifestations of DHF/DSS such as thrombocytopenia and hemoconcentration. Dengue virus infection induces transient immune aberrant activation of CD4/CD8 ratio inversion and cytokine overproduction, and infection of endothelial cells and hepatocytes causes apoptosis and dysfunction of these cells. The coagulation and fibrinolysis systems are also activated after dengue virus infection. We propose a new hypothesis for the immunopathogenesis for dengue virus infection. The aberrant immune responses not only impair the immune response to clear the virus, but also result in overproduction of cytokines that affect monocytes, endothelial cells, and hepatocytes. Platelets are destroyed by crossreactive anti-platelet autoantibodies. Dengue-virus-induced vasculopathy and coagulopathy must be involved in the pathogenesis of hemorrhage, and the unbalance between coagulation and fibrinolysis activation increases the likelihood of severe hemorrhage in DHF/DSS. Hemostasis is maintained unless the dysregulation of coagulation and fibrinolysis persists. The overproduced IL-6 might play a crucial role in the enhanced production of anti-platelet or anti-endothelial cell autoantibodies, elevated levels of tPA, as well as a deficiency in coagulation. Capillary leakage is triggered by the dengue virus itself or by antibodies to its antigens. This immunopathogenesis of DHF/DSS can account for specific characteristics of clinical, pathologic, and epidemiological observations in dengue virus infection.



## Introduction

Dengue fever (DF) is an acute infectious disease caused by the dengue virus, which has four serotypes. It is characterized by biphasic fever, myalgia, headache, pain in various parts of the body, rash, lymphadenopathy, and leukopenia [6, 10, 22]. In most cases, DF is self-limited. However, there is a risk of progressive development into dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). DHF is a severe febrile disease characterized by abnormalities in hemostasis and increased vascular permeability, and severe progression may result in DSS. DSS is a form of hypovolemic shock that is associated clinically with hemoconcentration and which might

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lead to death if appropriate care is not given. Although DF is distinct from DHF/DSS by traditional classification, the various clinical manifestations after dengue virus infection show a continuum from mild to severe reactions, just as in many other viral diseases. The mechanisms involved in the pathogenesis of dengue virus infection, especially the manifestation of DHF/DSS, remain unresolved. An explanation of the pathogenesis of dengue virus infection must account for specific characteristics of clinical, pathologic, and epidemiological observations.

## Current Hypotheses on the Pathogenesis of Dengue Virus Infection

Several hypotheses for the pathogenesis of dengue virus infection have been proposed. Among them, antibody-dependent enhancement (ADE) of infection has long been thought to play a central role [18, 19]. The ADE hypothesis was formulated to explain the finding that severe manifestations of DHF/DSS occur in children experiencing a second dengue virus infection that has a different serotype from the previous one. There are indeed preexisting antibodies to previous dengue virus that cannot neutralize but rather enhance infection in vitro. Sera obtained before infection from children who later developed DHF/DSS were much more likely to demonstrate ADE in vitro than those who had only DF [34]. Newborn babies less than 1 year old who acquire maternal anti-dengue IgG antibody are also susceptible to developing DHF/DSS following primary infection [33]. Epidemiological studies support the association of DHF/DSS with secondary dengue virus infection. However, the association of DHF/DSS with prior immunity to other dengue serotypes by itself explains neither the pathogenetic basis of the association nor the molecular mechanism of DHF/DSS clinical manifestations. It is not known how augmentation of dengue virus infection by enhancing antibodies leads to DHF/DSS. Whether it increases the number of dengue-virus-infected cells or enhances the signal through the Fc receptor remains to be elucidated. As there is no animal model of DHF/DSS, the causal relationship between ADE and DHF/DSS remains unverified [8].

Immunopathogenesis in DHF has been proposed [36, 63]. Serotype crossreactive antibodies from the previous infection bind to virions without neutralization and enhance the entry of virus into monocytes. The number of virus-infected monocytes increases. As a result, the level of T-cell activation is markedly increased, reflecting the increased antigen presentation, the increased frequency of dengue-virus-specific T cells in secondary infection, and the more rapid activation and proliferation of memory T cells. These T cells produce cytokines such as IFN-γ, IL-2, and TNFα, and lyse dengue-virus-infected monocytes. TNFα is also produced by activated monocytes. The complement cascade is activated by a virus-antibody complex as well as by several cytokines to release C3a and C5a that also have direct effects on vascular permeability. The synergistic effects of IFN-γ, TNFα, and activated complement proteins trigger plasma leakage of endothelial cells in secondary dengue virus infection. However, several issues remain unexplained by this theory. Not all DHF/DSS cases are secondary infections. Complement activation may be the result of severe disease, not the cause of DHF/DSS. Most importantly, DHF develops rapidly, usually over a period of hours, and resolves within 1–2 days in patients who receive appropriate fluid resuscitation. No discernible sequelae are usually found. This scenario is not easily reconciled with the known tissue-destructive effects of inflammatory cytokines.

Virus virulence, the capacity of a virus to produce disease in a host, is an alternative hypothesis for the pathogenesis of DHF/DSS. The different manifestations of DF, DHF, and DSS may be caused by variants of dengue virus with different degrees of virulence. The risk of DHF/DSS is higher in secondary infections with dengue virus of serotype 2 compared to the other serotypes [59, 60]. Structural differences have also been found among various isolates of DF and DHF patients [41]. Furthermore, it was reported that high dengue viremia titer was associated with increased disease severity [68–70]. Peak viral titers were 100- to 1,000-fold higher in patients with DSS than those with DF in dengue-infected Thai children. Patients with a secondary antibody response were twice as likely to have DHF, compared with those with a primary antibody response. Apparently, viral load is also a contributing factor in the development of DHF/DSS. Whether viral load is reflective of its virulence or its high growth rate in vivo requires further investigation.

## Clinical and Pathologic Manifestation of Dengue Virus Infection

DF is an acute febrile illness with headache, retro-orbital pain, myalgia, arthralgia, rash, leukopenia, and mild thrombocytopenia. Biphasic fever and rash are the most characteristic features of classic dengue fever. Symptoms resolve after 2–7days. Dengue hemorrhagic fever is an acute vascular permeability syndrome accompanied by

abnormalities in hemostasis. The clinical features include plasma leakage, bleeding tendency, and liver involvement [4, 6, 10, 22]. Capillary leakage develops rapidly over a period of hours, near or at the end of the febrile period when the symptoms of classic DF resolve. Pleural effusion, ascites, and hemoconcentration are indicative of intravascular volume loss. It can quickly progress to shock if patients do not receive intravascular fluid resuscitation. The hemorrhagic manifestations range from a positive tourniquet test to spontaneous bleeding from the nose or the gastrointestinal tract. Hemoconcentration and marked thrombocytopenia are two major characteristic features of DHF/DSS. Liver involvement is common in dengue virus infection with mild elevation of serum transaminases. Three organ systems (hematologic, vascular, and hepatic) are involved in the pathological changes of DHF/DSS. Dysfunction of these systems induced by dengue virus infection, either directly or indirectly, causes the manifestations of DHF/DSS.

## Effects of Dengue Virus Infection on Blood Cells

### *Aberrant Immune Activation during Dengue Virus Infection*

Analysis of blood samples collected during a dengue serotype 3 outbreak from November to December 1998 in southern Taiwan [64] revealed an abnormal immune status in dengue patients. Clinical diagnosis of DF and DHF/DSS was defined according to the criteria of the World Health Organization [73, 74], i.e. all patients with DHF have thrombocytopenia ($<100{,}000/mm^3$) and hemoconcentration (hematocrit >20% of recovery value). In the peripheral blood of an uninfected person, there are more $CD4^+$ than $CD8^+$ T cells. Interestingly, in the peripheral blood of dengue patients, $CD8^+$ T cells outnumber $CD4^+$ T cells, so the ratio of CD4/CD8 T cells declines to <1. This phenomenon was found not only in DHF/DSS patients, but also in DF patients. Among 21 DF and 8 DHF/DSS patients, the CD4/CD8 ratio inversion was found in 10 cases [47]. In 5 of the DHF/DSS patients, the CD4/CD8 ratios ranged from 0.29 to 0.84 (mean 0.51; median 0.48) while in 5 DF patients, the ratios ranged from 0.20 to 0.62 (mean 0.47; median 0.56). The frequency of the CD4/CD8 ratio inversion was higher in DHF/DSS (5/8) than in DF (5/21, $p<0.05$). The kinetic analysis of $CD4^+$ and $CD8^+$ T cell immunophenotypes showed that the CD4/CD8 ratio was reversed during acute infection (days 6–14 after fever onset). The CD4/CD8 ratio gradually bounced back to normal after day 15. Moreover, there were also some $CD4^{dim}$ or $CD8^{dim}$ monocytes, the percentage of which in PBMC was higher or highest on days 6–7, then decreased to levels lower than that of convalescence afterwards. The leukocyte profiles of these 10 dengue patients with the reversed CD4/CD8 ratio showed bandemia, $CD4^{dim}$ or $CD8^{dim}$ monocytosis, and atypical lymphocytosis. The immature neutrophil elevation occurred earliest on days 5–6, while the $CD4^{dim}$ or $CD8^{dim}$ monocytosis manifested itself on days 6–7 after fever onset. Atypical lymphocytosis reached its peak on days 8–10, and then disappeared quickly after day 12. Early activation of mononuclear cells was confirmed by expression of the early activation marker, CD69, on day 4 after fever onset. CD69 was stained on lymphocytes as well as on monocytes, but was expressed more extensively on $CD8^+$ than on $CD4^+$ T cells. Some $CD4^{dim}$ monocytes also expressed the CD69 marker. DHF is a very dynamic illness, so it is important that blood samples be collected at multiple time points from each patient during the course of illness to observe the kinetic changes of the immune response. The CD4/CD8 ratio inversion has only been reported during acute virus infections with the human immunodeficiency virus and Epstein-Barr virus. In dengue virus infection, CD4/CD8 ratio inversion is not observed in all patients, and it is transient with a short window of detection. Nevertheless, the appearance of atypical lymphocytosis and dynamic changes in the CD4/CD8 ratio suggests that aberrant immune activation does occur during dengue virus infection.

### *Cytokine Overproduction during Dengue Virus Infection*

Since mononuclear cells are overactivated during acute dengue infection, it is expected that elevated levels of cytokines can be found in serum. Indeed, there are several reports on serum cytokine levels in dengue patients. High levels of T-cell activation markers such as the soluble IL-2 receptor, soluble CD4, soluble CD8, IL-2, and IFN-γ, as well as monokines, e.g. TNFα, IFN-β, and GM-CSF, were detected in dengue-infected children, and these markers were higher in DHF/DSS patients than in DF patients [37–39, 76]. High serum levels of inhibitory cytokines such as IL-10 or the soluble receptors of sTNFRI and sTNFRII were also found in DHF [15, 16]. In our dengue patients, cytokines such as RANTES, IL-8, and IL-6 increased after dengue virus infection. Serum levels of IL-6 and IL-8 were higher in DHF/DSS than in DF patients [29]. IL-6 has dual roles as both a pro-inflammatory and an anti-inflammatory mediator. Its kinetic analy-

sis showed high variation at different time points and in different individuals, but a transient high elevation of serum IL-6 level occurred either on day 7 or on days 9–11 after fever onset [47]. This suggests that when a host responds to dengue virus infection via generation of inflammatory cytokines, simultaneously, there is also generation of inhibitory cytokines to counteract the inflammation. Cytokines can cause cell activation synergistically or antagonistically; the net outcome will depend on the balance between various cytokine actions.

*Thrombocytopenia and Anti-Platelet Antibodies*

Thrombocytopenia is common in DF and always found in DHF/DSS [4, 22]. The pathogenesis of thrombocytopenia is poorly understood. It was suggested that dengue-virus-induced bone marrow suppression depressed platelet synthesis and resulted in thrombocytopenia [40]. One group found that the dengue-2 virus can bind to human platelets in the presence of virus-specific antibodies, and proposed that the immune-mediated clearance of platelets was involved in the pathogenesis of thrombocytopenia in DHF/DSS [71]. Parvovirus infection is known to be associated with childhood idiopathic thrombocytopenic purpura [21, 77]. Surprisingly, we found IgM but no IgG anti-platelet autoantibodies in dengue patients. The titer of IgM anti-platelet antibodies is higher in DHF/DSS than in DF patients. The presence of these autoantibodies not only induces platelet lysis via complement activation, but also inhibits ADP-induced platelet aggregation [42]. Consistent with human studies, we also found that transient thrombocytopenia caused by dengue virus infection is associated with the generation of anti-platelet antibodies in a murine model of dengue virus infection [24]. The crossreactivity of antibodies directed toward dengue virus proteins, especially NS1, and platelets, suggests the pathogenic role of anti-platelet autoantibodies during dengue virus infection [13]. The production of anti-platelet autoantibodies whose affinities are enhanced in secondary infection not only explains the immune-mediated destruction of platelets, but also raises an important issue of the long-term safety of a dengue vaccine. The molecular mimicry between the dengue virus and endogenous self-proteins should be considered in the presence of autoimmunity during dengue virus infection.

*Immune Deviation Induced by Dengue Virus Infection*

Dengue-infected patients usually are leukopenic for several days during the acute infection, characterized by a decrease in the absolute number of neutrophils and monocytes [32]. A decrease in the proliferative responses of PBMC to mitogen and recall antigen during dengue infection is associated with both quantitative and qualitative defects in the accessory cell population [50]. We also found that the PHA-stimulated T cell responses of dengue patients are impaired during days 6–18 after fever onset during which there is $CD4^{dim}$ or $CD8^{dim}$ monocytosis followed by monocytopenia. The impaired PHA-stimulated T cell response correlates with the deficiency in $CD4^{dim}$ or $CD8^{dim}$ monocytes [47]. Detection of the early activation marker, CD69, on $CD8^+$ T cells, NK cells, and monocytes as well as the appearance of atypical lymphocytosis indicate that lymphocytes are indeed activated by dengue virus infection [14]. Dengue virus can infect Langerhans cells or immature dendritic cells and can replicate more efficiently in these cells than in monocytes or macrophages [75]. The infection of dendritic cells stimulates their maturation and cytokine production of TNFα and IFNα, but not of IL-6 and IL-12 [23]. The levels of IL-12 are higher in DF than DHF patients. No IL-12 could be detected in patients with DHF grades III and IV [55]. This deficiency in IL-12 production might lead to a shift to the Th2-type response and inappropriate CTL generation. This is consistent with observations of the impaired T-cell response associated with both quantitative and qualitative defects in the accessory cell population [50]. NK cells and related cytokines play an important role in defense against virus infection [9]. The selective loss of the $CD16^+CD56^+CD8^+$ NK cell subset was found in HIV infection [49]. Dengue virus infection seems to induce intense immune responses such as early $CD4^{dim}$ or $CD8^{dim}$ monocytosis, transient CD4/CD8 ratio inversion, a high percentage of atypical lymphocytosis, and depressed T-cell proliferation. These immune deviations not only delay virus clearance, but also trigger cytokine overproduction and auto-anti-platelet antibodies that initiate the subsequent pathogenesis of dengue virus infection.

## Effect of Dengue Virus Infection on Endothelial Cells

*Dengue Virus-Induced Vasculopathy*

The most characteristic feature of DHF/DSS and the best indicator of disease severity is plasma leakage. Plasma leakage is caused by a diffuse increase in capillary permeability and manifests as any combination of hemoconcentration, pleural effusion, or ascites. It usually becomes evident on days 3–7 of illness, during which time DF resolves [4, 6, 54]. Plasma leakage occurs systemically,

progressing quickly, but will resolve within 1–2 days in patients who receive appropriate fluid resuscitation. No subsequent tissue or organ dysfunction is observed. Although perivascular edema is obvious, no destruction of vascular endothelial cells is evident. It was previously thought that plasma leakage was due to altered vascular permeability rather than to structural destruction of endothelial cells. The functional alteration in endothelial cells is probably caused by standard effects of cytokine or mediator release in dengue infection. The dengue virus can infect endothelial cells in vitro which leads to the production of cytokines and chemokines such as IL-6, IL-8, and RANTES [29]. Although infection with dengue virus can induce apoptosis of endothelial cells in vitro [2], this direct effect is dependent on the isolate of dengue virus used. In our experience, an isolate such as dengue 2 strain 16681, which has a higher replication efficiency than either dengue 2 strain PL0046 or dengue 3, is more cytopathic at low multiplicity of infection [25]. But PL0046 or dengue 3 at high multiplicity of infection can also induce apoptosis of endothelial cells. Dengue virus-infected endothelial cells are capable of activating complement and inducing the expression of adhesion molecules such as ICAM-1 [27]. The expression of ICAM-1 together with the production of chemokines IL-8 and RANTES increases the adherence of polymorphonuclear cells and mononuclear cells, respectively, and results in increased vasopermeability and thrombomodulin release, a marker of endothelial cell damage. Anti-dengue virus antibodies co-incubated with dengue-virus-infected endothelial cells also cause increased vasopermeability and thrombomodulin release in endothelial cells in the absence of complement. Indeed, we found elevated levels of circulating thrombomodulin in the acute stage of DHF/DSS, indicating that endothelial cell structural damage occurred in vivo [27]. It seems that both direct viral cytopathic effects and immune-mediated damage by leukocyte recruitment and anti-dengue virus antibodies can cause structural injury to infected endothelial cells. This vascular leakage can be induced either directly or indirectly during dengue virus infection. Because endothelium plays a crucial role in maintaining hemostasis, damage of endothelial cells by dengue virus infection may skew the procoagulant/anticoagulant balance of endothelium and increase the bleeding tendency. The sequestration of platelets by activated endothelial cells might also contribute to the development of thrombocytopenia.

*Dengue Virus-Induced Coagulopathy*

Some viral infections can cause hemostatic abnormalities. Hemorrhage is a consequence of either a more pronounced degree of thrombocytopenia and associated platelet dysfunction or disseminated intravascular coagulation. In dengue-virus-induced hemorrhagic manifestations, platelet-vascular abnormalities are more common, but as the severity of illness progresses, massive bleeding with disseminated intravascular coagulation can occur.

Hemostasis is maintained by the balance between coagulation and fibrinolysis. The coagulation system can be activated by both intrinsic and extrinsic pathways to form thrombin, which converts fibrinogen to fibrin. The fibrinolytic system, on the other hand, can break down fibrin into fibrin degradation products. The human fibrinolytic system is comprised of plasminogen, a proenzyme, which can be activated to the active plasmin enzyme by several types of plasminogen activators. The principal endogenous activator of plasminogen is the tissue-type plasminogen activator (tPA) [3]. Plasminogen activator inhibitor (PAI-1), which is produced by platelets, liver, and endothelium, on the other hand, is the major inhibitor of tPA [48]. Generally, coagulation activation triggers a secondary activation of fibrinolysis that is rapidly shut off by the release of large amounts of PAI-1 [67].

During acute dengue virus infection, coagulation parameters such as platelet counts, activated partial thromboplastin time (APTT) as well as fibrinolytic parameters of tPA and PAI-1 are altered. APTT is prolonged while tPA increases. Both coagulation and fibrinolysis are activated, and this activation is much more severe in DHF/DSS than in DF patients [30]. After convalescence, rises in the PAI-1 level and platelet counts are concomitant with the decline in the tPA level, and a return to normal of APTT. The tPA/PAI-1 ratio is higher in DHF/DSS than in DF patients. APTT prolongation and the tPA/PAI-1 ratio increase in the acute stage of dengue virus infection correlate with disease severity, and can be used as early indicators of DHF/DSS [25].

APTT and prothrombin time are indicators of intrinsic and extrinsic pathways of coagulation, respectively. Only APTT, but not prothrombin time, is prolonged in dengue virus infection, suggesting that a defect occurs in the intrinsic pathway of coagulation. This can be caused by either downregulation of the synthesis of specific factors or by increased consumption of specific factors. Because mild hepatitis is found in dengue virus infection, an analysis of the linear correlation and regression between the levels of aspartate aminotransferase (AST)/alanine aminotransferase (ALT) and APTT shows a strong

association between AST/ALT elevation and APTT prolongation in DHF patients. Dysfunction of the damaged liver might be responsible for the decreased synthesis of specific factors in the intrinsic pathway. Increased factor consumption as indicated by high levels of tPA is also associated with APTT prolongation, but in a less significant manner. Therefore, both decreased synthesis and increased consumption of coagulation factors are involved in the prolongation of APTT [25]. The decreased synthesis of coagulation factor XII is discussed below.

The hyperfibrinolysis in the acute stage of DHF/DSS is caused by increased production of tPA. Analysis of the linear correlation and regression shows a significant association between serum IL-6 and tPA in DHF, but not in DF. Dengue virus infection induces endothelial production of tPA as well as IL-6. The de novo synthesis of tPA is blocked by anti-IL-6 antibodies, indicating that tPA production by endothelial cells is IL-6 dependent [28]. Furthermore, antibodies against dengue virus E protein can bind to human plasminogen. It can either inhibit plasmin activity or enhance plasminogen activation [25, 26]. Therefore, both coagulation and fibrinolysis are hyperactivated in the acute stage of dengue virus infection, and are counteracted by increased numbers of platelets and levels of PAI-1 in the convalescent stage. An unbalance between coagulation and fibrinolysis may cause hemorrhage in DHF/DSS [25].

## Effect of Dengue Virus Infection on Liver Cells

Dengue virus is hepatotropic; dengue virus antigen has been detected in hepatocytes, and viral particles were recovered from the liver biopsy specimens of DHF patients [62]. Mouse liver is also a major organ for dengue virus replication [1]. Dengue virus can infect the liver and cause hepatitis. Elevated serum transaminase levels were found in dengue patients, and the degree of AST level elevation correlates with that of hemorrhage [32, 53]. In dengue-viral hepatitis, the level of AST is higher than ALT with a ratio of around 1–1.5, while other types of virally induced hepatitis have more ALT than AST. Using hepatoma cell lines, dengue virus can induce apoptosis as well as RANTES chemokine production via oxidative stress and NF-κB activation [45, 46]. Furthermore, RANTES is preferentially induced by dengue virus but not enterovirus in liver cells [46]. Patients with dengue virus infection have increased RANTES serum levels compared to those with other viral infections. RANTES is a chemokine capable of recruiting lymphocytes and NK cells to sites of inflammation. Whether liver damage caused by dengue virus is a direct effect of virus replication or an indirect effect of RANTES-mediated inflammation needs further investigation. The balance between virus elimination and tissue damage might affect the severity of the disease. Since the liver is known to be the site for synthesis of most coagulation factors, reduced levels of coagulation factors are either the results of increased consumption or impaired synthesis. The latter is the likely consequence of liver injury. IL-6 can downregulate the synthesis of coagulation factor XII, the first factor to initiate the intrinsic pathway of coagulation [12]. APTT prolongation in DHF patients caused by a deficiency in the intrinsic pathway is probably due to impaired synthesis of coagulation factor XII in the liver.

## Animal Model of Dengue Virus Infection

The mouse is not a natural host of dengue virus. However, it is well known that dengue virus can replicate in the brains of suckling mice [51], and there is a strain variation of susceptibility to dengue virus infection [57] when the virus is inoculated intracerebrally. Recently, several groups have reported the growth of dengue virus in either AG129 mice, which lack α/β-interferon and γ-interferon receptor genes, or SCID mice [1, 31, 44]. Dengue-2 caused signs of neurological paralysis and death 2 weeks after injection into K562 or HepG2-engrafted SCID mice.

We have established a murine model for dengue virus infection that resembles the clinical thrombocytopenia manifestation [24]. Transient viremia was found 2 days after intravenous injection of dengue-2 virus. Later, transient thrombocytopenia developed at 10-13 days, and anti-platelet antibodies were also generated. In our mouse model, strain A/J is more susceptible to dengue-2 virus than are BALB/c or B6 mice. This dengue-2-virus-infected mouse system with its thrombocytopenia and anti-platelet antibodies will be a valuable model to study the immunopathogenicity of dengue virus infection. From these murine models of dengue virus infection, we learned that the initial inoculum of dengue virus must be higher than $1 \times 10^8$ PFU/mouse in order to obtain replication of the virus in vivo.

Alternatively, SCID mice with K562 or HepG2 grafts to permit the initial replication of dengue virus were used. Host factors are crucial for virus replication because different mouse strains have variable susceptibilities to dengue virus infection: A/J or AKR strains are more sensitive than BALB/c, B6, or C3H/He strains, probably because

A/J mice have significantly fewer lymphoid, granuloid, monocytoid, and NK cells than do B6 mice [52, 72]. Viremia is low and transient in immunocompetent mice compared with that in SCID or interferon-deficient AG129 mice. Immune responses, especially production of interferon, play a very important role in clearing dengue virus. Moreover, the anti-platelet antibodies were only detected in immunocompetent mice, and are associated with the development of thrombocytopenia. Although clinical symptoms, such as thrombocytopenia, prolonged partial thromboplastin time, and increased hematocrit, were reported in paralyzed dengue-2-infected HepG2-grafted SCID mice; they are not related to the immune responses to dengue virus infection.

Other animals such as the monkey can be infected by dengue virus; however only viremia was observed [20]. No symptoms that mimic clinical manifestation of dengue virus infection have been recorded. Therefore, the murine infection model that manifests viremia and thrombocytopenia is useful; combinations of infection-reinfection with different serotypes of dengue virus can be assayed. Elucidation of the mechanism of DHF/DSS immunopathogenicity, especially the significance of ADE in vivo, can be expected.

*Immunopathogenesis of Dengue Virus Infection*

The characteristic features of DHF/DSS include capillary leakage, thrombocytopenia, and coagulopathy. In our studies, several key observations were found. Dengue virus infection induces transient immune aberrant activation of CD4/CD8 ratio inversion and cytokine overproduction. Anti-NS1 or anti-dengue antibodies can cross-react with platelets and endothelial cells. The binding to platelets causes platelet lysis in the presence of complement, whereas the binding to endothelial cells induces their apoptosis. Dengue virus infection causes the apoptosis and dysfunction of both endothelial cells and hepatocytes. Upregulation of ICAM-1 in endothelial cells and the generation of chemokine increase the adherence of neutrophils and mononuclear cells, which causes more damage to endothelial cells. Dengue virus infection activates both coagulation and fibrinolysis systems. Unbalances between coagulation and fibrinolysis induce hemorrhage in DHF/DSS. Based on these results, we propose a new hypothesis of the immunopathogenesis of dengue virus infection (fig. 1).

Dengue virus infection causes intense immune activation. Aberrant immune responses such as CD4/CD8 ratio inversion not only impair the ability of the immune system to clear the virus, but also cause overproduction of cytokines that can affect monocytes, endothelial cells, and hepatocytes. The rate of viral replication is increased, and a vicious cycle is amplified. Virions expand dramatically. On this basis, both antibody-dependent enhancement and virus virulence theories can be explained. Viral load becomes the common denominator of both theories. Secondary infection by different serotypes of dengue virus produces a higher viral load and a stronger immune deviation than does primary infection. At defervescence of dengue virus infection, endothelial cells are damaged by either direct virus cytopathy or immune-mediated pathology. Plasma leakage is observed clinically because of structural alterations in endothelial cells. Hemoconcentration results from hypovolemic loss. Platelets are destroyed by crossreactive anti-platelet autoantibodies. Titers of anti-platelet or anti-endothelial cell antibodies are higher in DHF/DSS than in DF because of high affinity antibody or immune memory on antibody production.

Dengue-virus-induced vasculopathy and coagulopathy must be involved in the pathogenesis of hemorrhage, and the imbalance between the coagulation and fibrinolysis activation increases the likelihood of severe hemorrhage in DHF/DSS. Hemostasis is maintained unless the dysregulation of coagulation and fibrinolysis persists. Overproduction of IL-6 might play a crucial role in the enhanced production of anti-platelet autoantibodies, elevated levels of tPA, and the deficiency of coagulation factor XII in the intrinsic pathway. Capillary leakage is triggered by the dengue virus itself or antibodies to its antigens; therefore, when the viral load is decreased or the virus is eliminated, the response is quickly terminated. The cytokine storm generated during the early stage of dengue virus infection is antagonized by soluble TNFRI, soluble TNFRII, IL-10, or anti-inflammatory IL-6.

The immunopathogenesis of DHF/DSS is initiated by aberrant immune activation caused by dengue virus. Immune deviation is necessary but not sufficient to trigger the subsequent dysfunction of endothelial cells and coagulation. This immunological basis can reconcile epidemiological data on the association between secondary infection and DHF/DSS. The immune memory or immune enhancement in secondary infection can boost immune deviation, autoantibodies with high affinity, and cytokine overproduction. The ADE hypothesis can be interpreted back to its origin that subneutralizing antibodies enhance the entrance of dengue virus, thereby increasing the virus load. This immunopathogenesis of DHF/DSS can account for specific characteristics of clinical, pathologic, and epidemiological observations in dengue virus infection.

Aberrant activation
•CD69 expression
•CD4/CD8 ratio
•Monocytosis
•Atypical lymphocytosis
•Cytokine over-production
bandemia
monocytosis
Activated PLT
Anti-DV Ab
Auto-antibody
•Anti-PLT Ab (anti-NS1 Ab)
•Anti-EC Ab
CD8
CD4
cytokines
atypical lymphocytosis
Liver damage
•Hepatitis
•Apoptosis
•IL-6
•RANTES
GOT/GPT
oxidative stress
NF-IL-6
apoptosis
RANTES
IL-6
apoptosis
EC
TM
tPA
IL-6
?
Vascular EC damage
•Apoptosis
•Thrombomodulin(TM) release
•IL-6, IL-8, RANTES
•tPA/PAI-1 ratio
•ICAM expression
•Cell-mediated damage (PMN, PBMC)
•Antibody-mediated damage (anti-DV Ab, Anti-EC Ab)
•Complement activation
IL-8
RANTES
Viral Ag
EC Ag
LFA-1
ICAM
Ab
C'

## Biochemical Markers as an Index for DHF/DSS Development

DHF/DSS is a very dynamic illness, and clinical manifestations of dengue virus infection change quickly. Laboratory data vary depending on the time of sample collection during the course of the illness. Thus, it is important to analyze a series of samples that are collected at different times if one is like to find the biochemical markers that predict the development of DHF/DSS. In our previous studies, we had the advantage of being a research team in a medical center that permitted the close monitoring of patients by our researchers. Based on the clinical manifestations of dengue virus infection, several of the following indices can be used as early markers for the development of DHF/DSS. First is the duration from fever onset to defervescence. From the 1998 dengue outbreak in Tainan, Taiwan, we learned that the duration from fever onset to defervescence is longer in DHF/DSS ($11.38 \pm 4.60$ days, $n = 8$) than in DF patients ($5.38 \pm 1.66$ days, $n = 13$, $p < 0.001$). Defervescence was defined as the temperature falling and remaining at $<38\,^{\circ}C$. DF is self-limited, while DHF/DSS shows continuing progression to severe disease. Defervescence in DF represents recovery from disease, but in DHF it reflects increased vasopermeability and heat loss from the skin. Taiwan is not a dengue virus endemic area in which most people are not infected, whereas in Thailand, the majority of infection is secondary infection. The immune memory in patients after dengue infection in Thailand triggers a faster response and shortens disease progression. Therefore, the duration of fever there is around 4–5 days for both DF and DHF/DSS.

Thrombocytopenia is common in dengue fever, and is always found in DHF/DSS. The appearance of IgM antiplatelet antibodies causing the destruction of platelets is a predictor for the development of thrombocytopenia. Hemorrhage is caused by deficiencies in intrinsic-pathway-mediated coagulation or hyperfibrinolysis. The tPA/PAI-1 ratio is 3-fold higher than normal during the first 48 h of hospitalization. Prolonged APTT and the increased tPA/PAI-1 ratio in the acute stage of dengue virus infection are associated with disease severity, and can be used as early indicators of DHF/DSS.

The most characteristic feature of DHF/DSS and disease severity is plasma leakage that results from structural damage of endothelial cells. Therefore, the serum thrombomodulin level can be used as a marker for plasma leakage. Furthermore, IL-6 is an important cytokine for autoantibodies and tPA production, and the serum IL-6 level can also be considered an early marker. These biochemical markers when confirmed in further studies will be very useful clinically because progression from DF to DFH/DSS is not currently predictable. The physician has to be experienced and cautious with dengue patients. With early diagnosis and appropriate supportive care, the mortality rate of dengue virus infection can be greatly decreased.

**Fig. 1.** Immunopathogenesis of dengue virus (DV) infection. Infection by dengue virus causes an intensive immune activation. The aberrant immune responses including CD4/CD8 ratio inversion, monocytosis, atypical lymphocytosis, and depressed T-cell proliferation not only delay virus clearance, but also trigger cytokine overproduction and autoantibodies to platelets (PLT) and endothelial cells that initiate subsequent dysfunctions of these cells. Liver dysfunction in dengue viral hepatitis impairs synthesis of coagulation factors involved in the coagulopathy of APTT prolongation. The infection of endothelial cells (EC) induces apoptosis as well as IL-6, IL-8, RANTES, and tPA production. Structural damage of endothelial cells is caused by either direct virus cytopathy or immune-mediated pathology that results in plasma leakage and hemoconcentration. An unbalance between the coagulation and fibrinolysis activation increases the likelihood of severe hemorrhage in DHF/DSS. The pathogenesis of DHF/DSS is initiated by aberrant immune activation during dengue virus infection. Immune deviation is necessary but not sufficient to trigger the subsequent dysfunction of endothelial cells and coagulation. Hemostasis is maintained unless the dysregulation of coagulation and fibrinolysis persists.

## Antiviral Treatment and Therapeutic Intervention in Dengue Virus Infection

Although multiple cytokines and chemical mediators are released in patients with DHF/DSS, theoretically there should be an early major factor that initiates the amplification cycle of immunopathogenesis. Immunopathogenesis might be mediated by a single or only a few molecules produced during the early phase of immune responses. If the molecular basis of DHF is identified, it is feasible to prevent the disease and treat patients. Viral load is speculated to be the key element inducing the intense immune deviation and triggering the subsequent cascade leading to hemorrhage and plasma leakage.

Ribavirin, an antiviral drug which is a guanosine analogue for RNA viruses, can inhibit dengue virus replication and IL-6 and IL-8 production in endothelial cells [29]. Another clinically used antiviral drug, amantadine, can also inhibit dengue virus [unpubl. observation]. Fur-

thermore, carboxyfullerene (C60), a novel compound with free radical scavenger activity, can inactivate dengue virus in a light-independent manner. It can block viral replication at the attachment and penetration stages, suggesting a direct interaction between C60 and the envelope of the virion [43]. C60 cannot only suppress the replication of enveloped viruses, but it can also regulate immune responses by inhibiting cytokine production or modulating inflammatory cells [65]. The inhibition of dengue-virus-induced disease by C60 can be further evaluated in animal infection models. The search for antiviral drugs and therapeutic agents which intervene in the development of DHF/DSS will become more prevalent when the pathogenesis of dengue virus infection is more clearly understood.

## Vaccine Development

Currently, there is no licensed dengue vaccine available. Several studies on tetravalent attenuated dengue virus vaccines are under clinical trial [5, 7]. Other experimental vaccines such as a chimeric vaccine and a DNA vaccine are also under investigation [17, 35, 58, 66]. Most vaccines inhibit or delay viremia in a monkey infection model following inoculation with dengue virus. The design of safe vaccines with regard to a subunit or whole virus must address the phenomenon of antibody-dependent enhancement, and more generally, the problem of immune potentiation of the disease [11]. Will immunization increase the likelihood of disease severity in future infections when anti-dengue antibodies wane to a subneutralization level 10 or more years after sensitization? To avoid the problem of ADE, non-structural proteins such as NS1 are used instead of the envelope E protein, and it was reported that NS1 immunization can prevent mortality caused by intracerebral injection of dengue virus into mice [56, 78]. However, we willl consider immune-mediated pathology causing endothelial cell damage, thrombocytopenia, and hemorrhage. The immune-mediated pathogenesis is further worsened by immune memory and immune enhancement. The molecular mimicry between platelet/endothelial cells and NS1 of dengue virus further raises the role of autoimmunity in dengue virus infection [13]. Therefore, an animal model with DHF/DSS manifestation is urgently needed. Until the immunopathogenesis of DHF/DSS is clearly understood, we have to be very cautious about the long-term safety of dengue vaccines. Until the epitope that crossreacts with the self-antigen is identified, it is not possible to design a dengue virus vaccine with no side effects.

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