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Dengue Virus Pathogenesis: an Integrated View

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INTRODUCTION

Dengue virus (DENV) belongs to the family *Flaviviridae*, genus *Flavivirus*, and is transmitted to humans by *Aedes* mosquitoes, mainly *Aedes aegypti*. Based on neutralization assay data, four serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) can be distinguished. DENV infection is a major cause of disease in tropical and subtropical areas, with an estimated 50 million infections occurring each year and more than 2.5 billion people being at risk of infection (75). Infection with any of the DENV serotypes may be asymptomatic in the majority of cases or may result in a wide spectrum of clinical symptoms (87), ranging from a mild flu-like syndrome (known as dengue fever [DF]) to the most severe forms of the disease, which are characterized by coagulopathy, increased vascular fragility, and permeability (dengue hemorrhagic fever [DHF]). The latter may progress to hypovolemic shock (dengue shock syndrome [DSS]). In Asia the risk of developing severe disease is greater in DENV-infected children (≤15 years) than in adults (30, 80, 109, 172). In contrast, in the Americas mainly the adult population is affected, resulting in mild disease (84, 186, 188), although an increasing trend of cases progressing toward DHF/DSS has also been observed in adults there (75, 79, 87, 126, 186). DF is manifested as an incapacitating disease in older children, adolescents, and adults. It is characterized by the rapid onset of fever in combination with severe headache, retro-orbital pain, myalgia, arthralgia, gastrointestinal discomfort, and usually rash. Minor hemorrhagic manifestations may occur in the form of petechiae, epistaxis, and gingival bleeding. Leukopenia is a common finding, whereas thrombocytopenia may occasionally be observed in DF, especially in those with hemorrhagic signs (76, 109). The World Health Organization (WHO) classifies DHF in four grades (I to IV). DHF grades I and II represent relatively mild cases without shock, whereas grade III and IV cases are more severe and accompanied by shock. DHF is characterized by all the symptoms of DF, in combination with hemorrhagic manifestations (positive tourniquet test or spontaneous bleeding), thrombocytopenia, and evidence of increased vascular permeability (increased hemoconcentration or fluid effusion in chest or abdominal cavities). The life-threatening DSS stage occurs at the time of or shortly after defervescence, which is characterized by a rapid, weak pulse (≤20 mm Hg) or hypotension with cold, clammy skin in the early stage of shock (grade III). If patients do not receive prompt and appropriate treatment, a stage of profound shock may set in, in which pulse and blood pressure become undetectable (grade IV), resulting in death within 12 to 36 h after onset of shock (262a). It is important to realize that the WHO case definition was originally proposed as a tool for clinical diagnosis using the results of repeated clinical tests. The WHO classification system poses a problem for everyday clinical practice, because it may be not sufficiently accurate in correctly classifying disease severity and may lack good agreement with clinical practice (213). Consequently, the WHO classification system is currently being reconsidered, and a new classification system is to be expected soon. The stage of prolonged shock may trigger or accelerate the development of disseminated intravascular coagulation (DIC) (228). Data that support or refute the occurrence of DIC in severe dengue are inconclusive, so better studies using prospective cohorts are needed to show the frequency of DIC in DHF/DSS patients and its association with clinical outcome (30, 152, 262). Massive loss of blood is rare in DHF and DSS and if present it is largely restricted to the gastrointestinal tract. This is usually due to prolonged shock resulting in blood being shunted away from

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the gastrointestinal tract, leading to anoxia, cell death, and gastrointestinal bleeding. In contrast, the mild forms of hemorrhages seen early in infection, such as petechiae, result from different mechanisms related to virus infection in combination with the release of vasculogenic cytokines. Understanding the mechanism underlying the development of shock is crucial for the development of novel strategies to improve patient management. It is worth noting that patients classified as having DHF and DSS have no generalized edema; rather, a selective plasma leakage tends to occur in the pleural and abdominal cavities (12, 230, 246, 252, 256), which is detectable by means of radiology or sonography. Ultrasonographic examinations have revealed that plasma leakage occurs before defervescence or changes in hemoconcentration become apparent (12, 230, 252). Attempts to explain the pathogenesis of dengue in all its complexity must consider all the clinical, immunological, pathological, and epidemiological features of DENV infection. The aim of this review is to outline the current views of DHF/DSS pathogenesis and to identify the gaps in our knowledge that represent critical challenges for the future.

THE PATHOGENESIS OF DENV INFECTIONS: CURRENT HYPOTHESES

DENV Tropism

Cell and tissue tropism of DENV may have a major impact on the outcome of DENV infections. The absence of an appropriate animal disease model largely hampers our understanding of the role played by DENV tropism. In vitro data and autopsy studies suggest that three organ systems play an important role in the pathogenesis of DHF/DSS: the immune system, the liver, and endothelial cell (EC) linings of blood vessels. The tropism of DENV for cells of the respective systems, the corresponding pathological effects of DENV infection of these systems, and the relevance of these events for the overall pathogenesis of DENV infection will be described.

Cells of the immune system. During the feeding of mosquitoes on humans, DENV is presumably injected into the bloodstream, with spillover in the epidermis and dermis, resulting in infection of immature Langerhans cells (epidermal dendritic cells [DC]) (136, 263), and keratinocytes (136). Infected cells then migrate from site of infection to lymph nodes, where monocytes and macrophages are recruited, which become targets of infection. Consequently, infection is amplified and virus is disseminated through the lymphatic system. As a result of this primary viremia, several cells of the mononuclear lineage, including blood-derived monocytes (59), myeloid DC (20, 91, 92, 123, 133), and splenic and liver macrophages (18, 54d, 96, 101, 117) are infected. DENV has also been shown to have tropism for circulating mononuclear cells in blood and for cells residing in the spleen, lymph nodes, and bone marrow of infected AG129 mice (124). Leukocytes also have been shown to be infected with DENV in experimentally infected nonhumans primates (156). It should be noted that during secondary infections with heterologous DENV, high concentrations of DENV-specific immunoglobulin G (IgG) will complex newly produced virus that adheres to and is taken up by mononuclear cells. Following infection, mononuclear cells predominantly die by apoptosis (61, 182), while abortively infected or bystander DC are stimulated to produce the bulk of mediators that are involved in inflammatory (22, 47, 91, 133, 145) and hemostatic (48, 60, 97, 120, 236) responses of the host. In this regard, factors that influence the amount of target cells infected, and consequently the levels of viremia, may determine the ratio of different proinflammatory and anti-inflammatory cytokines, chemokines, and other mediators, as well as the way in which the inflammatory response affects the hemostatic system (35, 59). Bone marrow stromal cells have also been shown to be susceptible to infection with DENV (124, 171, 202).

Organ pathology. Although thousands of patients with confirmed dengue have been recognized in Southeast Asia and the Americas in the past 60 years, autopsies have been performed on only a small number of these patients, and whether those cases are representative in reflecting the viral tropism in the acute phase of infection is unclear. Histopathological research is difficult to perform because fatal cases of DHF/DSS are rare and occur mainly in remote parts of the world where appropriate laboratory technology is largely lacking and thus fresh or frozen patient materials are rare. In addition, due to cultural and religious practices, autopsy is not conducted on the majority of fatal cases, and usually families opt for rapid burial or cremation. The interpretation of the pathological findings in fatal cases of DHF/DSS in relation to viral tropism described in the literature is complicated by a skewed age distribution, different times of sample collection, and the range of different techniques used to confirm the presence of virus in affected tissues. DENV cell tropism can be inferred from studies that had used in situ hybridization, immunohistochemistry, or a combination of PCR and virus isolation techniques. A review of the literature describing findings on autopsy samples from a total of 160 fatal cases, mostly children or young adolescents (4 to 18 years old) who died within 36 h of developing shock, revealed, in order of frequency, the presence of DENV in cells in the skin (104), liver (13, 14, 53, 54d, 69, 96, 101, 104, 137, 164, 173, 199, 208), spleen (13, 14, 101, 164, 199, 208), lymph node (13, 14, 101, 104, 173, 199, 208), kidney (14, 78, 101), bone marrow (13, 78, 101, 173), lung (13, 78, 101, 137, 164, 173), thymus (106), and brain (164). The presence of infectious virus in these samples was not always investigated, but in general virus could be isolated only from liver and peripheral blood mononuclear cells. The failure to isolate virus from most organ samples may indicate that those tissues contained primarily degraded virus or virus complexed with antibodies that prevent infection of cells in vitro. In general, the presence of DENV in several organs was not associated with gross or microscopic evidence of severe organ pathology (17), which is in agreement with the pathogenesis of DHF/DSS. Similar organ tropism has been observed in the primate model, with high concentrations of virus isolated from the skin and gastrointestinal tract whereas low concentrations of virus were recovered from the spleen, thymus, and several peripheral lymph nodes (157). DENV has been recovered from the spleen, liver, peripheral lymph nodes, and central nervous system in alpha/beta interferon (IFN-α/β)-deficient mice (13, 267). One notable difference between humans and the mouse model is the tropism of DENV for neuronal cells.

It is interesting to note that a generally used argument in the literature is that when shock sets in, virus is no longer detectable in blood and therefore the host response should play a key

role in pathogenesis (134, 166). Most autopsy data did not specifically compare the presence of viral antigens or nucleic acid in blood and autopsy samples, but the limited evidence suggests that DENV replication may occur in some organs, while viremia is no longer detectable (199). In agreement with these findings it was shown in the rhesus macaque model that DENV could be recovered from some autopsy samples but not from blood.

The liver is commonly involved in DENV infections in humans and mouse models (181, 212), with some reports suggesting an association between elevated liver enzyme levels and spontaneous bleeding tendencies (54e, 124, 261). Cases of dengue-associated hepatitis have been described, which were characterized by moderate midzonal hepatocyte necrosis, microvesicular steatosis, and councilman bodies (63, 78, 124, 181, 254). Although DENV was found in a significant proportion of human hepatocytes and Kupffer cells, little inflammation was seen within the liver, indicating that much of the observed apoptosis and necrosis was virally induced. The higher prevalence of apoptosis over necrosis could explain the limited inflammation seen in the liver, a picture similar to what is observed in the early phase of yellow fever or Rift Valley fever (57, 190, 191). It has been proposed that the severe hepatic damage seen, for instance, in yellow fever, Rift Valley fever, and late Ebola virus infections results in decreased liver function, which could account for the decreased synthesis of coagulation factors and development of coagulopathy (43, 269). Although severe hepatic damage is not common in DENV infections, elevated liver enzymes suggest that the liver is affected, but the role of hepatic damage in coagulopathy and disease severity remains to be established.

EC. EC play an important role in the coagulation response upon severe systemic inflammation. The integrity of the EC bed is physiologically regulated by many factors. The tropism of DENV for EC in vivo remains controversial. Early studies of skin biopsy specimens indicated that the microvasculature located in the dermal papillae is the main site affected, although DENV antigen was not detected in EC but was detected in cells surrounding the microvasculature (21, 203). In contrast, there is evidence for the presence of DENV antigen in the pulmonary vascular endothelium (101). It is important to realize, however, that the mere presence of viral RNA or antigen in EC is no proof for viral replication. In contrast to mononuclear cells, EC do not carry Fc receptors and thus will not take up immune-complexed virus. Therefore, the presence of viral RNA in these cells would more likely be explained by a mechanism of pinocytosis (101). In vitro studies have shown that all DENV serotypes can actively replicate in EC (7, 19, 95), and infection results in functional rather than morphological damage. It is not clear whether EC of different vascular-bed systems have different susceptibilities to DENV infection. In this regard, it has been proposed that the coagulation responses upon severe systemic inflammation by EC in different parts of the vascular-bed system are not the same (200, 201). Similarly, DENV infection patterns in microvascular cells in vitro suggest that EC from different tissues have different activation patterns (184). Although increased peripheral microvascular permeability has been shown to occur in both DHF and DSS patients (16), it is conceivable that EC from the pulmonary and abdominal territories react in a specific way to either infection with or the response to DENV infection (28, 39), resulting in the selective vascular leakage syndrome characteristic of DHF/DSS. Several studies suggest that vascular damage or dysfunction is central in the pathogenesis of DHF/DSS (26, 29, 39, 115, 168). It is interesting to note that selective apoptosis of the microvascular EC in pulmonary and intestinal tissues has been detected in fatal cases of DHF/DSS (137), providing a possible explanation for the profound plasma leakage seen in pleural and peritoneal cavities. In this regard, it is worth mentioning that the major nonstructural protein 1 (NS1) of DENV has been shown to bind preferentially to EC of lung and liver tissues (9). It has been hypothesized that recognition of NS1 by anti-NS1 antibodies could then contribute to the selective pulmonary vascular leakage.

Virus Virulence

According to the virus virulence hypothesis, certain DENV strains are responsible for more severe disease. DENV serotypes can be further classified into different genotypes on the basis of nucleotide variations. Viral genetic differences have been associated with differences in virulence (51, 131, 206, 248). Remarkably, the first outbreak of DHF in the Americas occurred in 1981, which coincided with the introduction of the possibly more virulent DENV-2 Southeast Asian genotype, while the less virulent indigenous DENV-2 genotype was already circulating in the region (118, 195–197). It has also been proposed that intraepidemic evolution of the circulating DENV might be responsible for increased severity of disease. During the 1981 DENV-2 epidemic in Cuba, it was noted that severity of disease manifestations and case-fatality rates were increased toward the end of the epidemic (118, 119), suggesting that the circulating DENV-2 might have become more virulent through passage in hosts during the epidemic. A similar situation was observed in the 1992 DENV epidemic in Townsville, Australia (234), and again in Cuba during the 1997 epidemic (81). Analysis of DENV genomes has shown that DENV indeed evolves during an epidemic (42, 198); however, more data are needed to establish an association between intraepidemic virus evolution and increased disease severity. Epidemiological observations in the Americas and in Singapore suggested that the sequence of infection with particular serotypes and the time interval between primary infection and secondary infection may play an important role in the development of DHF. Epidemics with high incidences of DHF have been linked to primary infection with DENV-1 followed by infection with DENV-2 or DENV-3 (79, 83, 178). Furthermore, these studies indicated that the longer the interval between primary and secondary infections, the higher the risk of developing severe disease. In addition, age has been shown to influence the outcome of disease following a secondary infection with heterologous DENV (80). In Asia, the risk of severe disease is greater in children than in adults, in contrast to the Americas, where the adult population is mainly affected and infection results in milder disease. This difference in disease severity caused by Asian and American genotypes correlated with structural differences in the two strains of DENV (51, 131). It has also been shown that different geographical DENV strains or different serotypes may vary in their ability to infect different cell types or cause severe disease (56, 251). However,

the observation that DHF/DSS is seen primarily in a relative small percentage of secondary DENV infections and to a much lesser extent in primary infections even with allegedly virulent strains suggests that host factors must be crucial determinants of severe disease development. It is important to realize that virulence has traditionally been considered a microbial property, evaluated independently of the host or only in vitro or in often inbred animals. However, an increasing body of evidence incriminates the host immune response in the pathogenesis of many microbial infections (31). Therefore, in studying DENV virulence, both host and viral factors should be considered.

Activation of the Complement System

The complement system is one of the main humoral components of the innate immunity and interacts closely with the hemostatic system to provide the first line of defense against pathogens. These innate immune mechanisms provide the host with the time needed to maximally induce the more slowly developing adaptive immunity. With regard to DENV, investigators noticed that around the time of defervescence, when plasma leakage may become apparent, high levels of the activation products C3a and C5a are measured in the plasma, followed by an accelerated consumption and a marked reduction of the complement components in patients with DSS (50, 174, 214). Therefore, it was hypothesized that complement activation plays an important role in the pathogenesis of dengue. Comparison of global gene expression profiles in peripheral blood mononuclear cells of DF and DHF/DSS patients also suggests the involvement of the complement system in disease severity (249). However, many aspects of complement activation and its role in DENV pathogenesis remain to be investigated.

It has been proposed that NS1 is an important trigger for complement activation (122). Binding of heterotypic antibodies to NS1 expressed on infected cells may result in complement activation (8, 142). In addition, it is believed that NS1 released from infected cells can directly activate complement factors present in the fluid phase (122). Production of the C5b-C9 complex could then trigger cellular reactions and stimulate the production of inflammatory cytokines that are associated with development of DHF/DSS (8). Alternatively, the C5b-C9 complex could independently trigger other local and systemic effects (158), which may be implicated in intravascular coagulation. It is important to realize that coagulation enzymes can also activate the complement system, illustrating the extensive interaction that exists between the complement and the coagulation system.

Several groups have shown that both IgG1 and IgG3 were the predominant subclasses involved in the specific antibody response in human DENV infections (116, 242). Both IgG subclasses can fix and activate the complement system effectively, whereas IgG2 and IgG4 are less effective in this respect (90). Although IgG1, IgG2, and IgG4 are able to activate the classical complement pathway, they require the unlikely event of two IgG molecules binding close to the antigen in order to promote C1q binding (90). IgG3, on the other hand, has the capacity to self-associate into multivalent complexes, thereby increasing functional affinity and the likelihood of C1q binding. The presence of sialic acid in the glycans of IgG subclasses could also affect their complement-fixing properties (100) and possibly their infection-enhancing activity (65, 163, 266). It is important to understand what determines the threshold of activation needed and how activation participates in development of DHF/DSS.

Transient Autoimmunity

Antibodies produced during a DENV infection have been shown to cross-react with some self-antigens, but it is not clear if production of these antibodies is associated with secondary DENV infections. For instance, antibodies recognizing a linear epitope in the E protein have been shown to bind human plasminogen and inhibit plasmin activity (49, 64, 94, 159). The presence of serum antibodies specific to NS1 also has been shown to correlate with disease severity (134, 218). Cross-reaction of anti-NS1 with cells of the liver, EC, and platelets (33, 140, 177, 237) could be at the basis of this observation. Anti-NS1 antibodies cross-reactive with EC could trigger these cells to express nitric oxide (NO) and undergo apoptosis (141). Although NO has been shown to inhibit DENV replication (239), its overproduction could also lead to cell damage (141, 215). It is worth reiterating that morphological damage is not a common observation in lethal cases of DHF/DSS. Anti-NS1 antibodies have also been shown to enhance expression of interleukin-6 (IL-6), IL-8, and intracellular adhesion molecule 1 (ICAM-1) (138). Further studies are needed to see if cross-reactivity of anti-NS1 with EC could lead to the increased permeability that is characteristic of DSS. In addition, anti-NS1 antibodies were also shown to cross-react with human and mouse platelets and were able to cause transient thrombocytopenia and hemorrhage in mice (142, 237), indicating that such cross-reactive antiplatelet antibodies are pathogenic. This observation may have implications for vaccine development, especially for live-attenuated vaccines. Therefore, it is important to understand why the autoimmune phenomenon observed in some DENV-infected patients does not persist. Since the kinetics of anti-NS1 antibodies is difficult to reconcile with the short duration of the sudden hyperpermeabilty event that leads to shock, the autoimmune hypothesis has remained controversial. Although it is likely that the cross-reactive antibodies to self-antigens are of the short-lived IgM isotype (139, 204), vaccination strategies should not result in memory IgG responses to such antigens. Clearly, more efforts should be deployed in identifying the putative self-antigens that are recognized by anti-DENV antibodies and in understanding their role, if any, in dengue pathogenesis.

Host Genetic Factors

Differences in disease severity can be seen at both the individual and population levels. Several epidemiological studies indicated that genetic factors constitute important components in disease susceptibility. Several human HLA class I and II alleles are associated with development of DHF (Table 1). Polymorphism in the tumor necrosis factor alpha (TNF-α), Fcγ receptor, vitamin D receptor, CTLA-4, and transforming growth factor β (TGF-β) genes has been associated with development of DHF/DSS. Certain host factors, such as glucose-6-phosphate dehydrogenase (G6PD) deficiency, may also con-

TABLE 1. Summary of non-HLA and HLA-associated genetic factors involved in the development of DHF/DSS

Genetic factor	Reference(s)
Vitamin D receptor polymorphism	143
FcγRIIa polymorphism	143
G6PD	35
MBL2	1
TGF-β	45
TNF-α308A polymorphism	66
CTLA-4	45
Transporters associated with antigen presentation and human platelet antigen	224, 225
DC-SIGN polymorphism	205
HLA class I alleles A*01, A*0207, A*24, B*07, B*46, B*51	144, 232, 270
HLA class II alleles DQ*1, DR*1, DR*4	125, 187

tribute to increased replication of DENV in monocytes. Deficiency in G6PD, a ubiquitous X-linked enzyme, is the most common enzyme deficiency worldwide, with high prevalence seen in the African population (175). G6PD deficiency causes abnormal cellular redox, thereby affecting production of nitric oxide, superoxide, and hydrogen peroxide. Oxidative stress is known to affect viral proliferation and virulence by increasing viral receptors on target cells or increasing production of viral particles (264). Although, it is possible that G6PD deficiency provides a more suitable milieu for viral replication, it is worth noting that a low incidence of severe disease was reported in populations of African origin in studies conducted in Cuba and Haiti (54b, 54c). Polymorphism in the mannose-binding lectin 2 (MBL2) gene was shown to be associated with thrombocytopenia and an increased risk for developing DHF. MBL is a member of the collectin family and is assumed to play an important role in pattern recognition and innate immune defense. Mutation in the promoter region of MBL results in low serum levels of MBL, resulting in a common immunodeficiency syndrome present in up to 10% of the U.S. population (243). Polymorphism in transporters associated with antigen presentation and human platelet antigen has also been associated with increased risk for developing DHF (224). The risk to develop DHF and DSS following infection with DENV is likely to be determined by a combination of multiple common genetic traits, each with mild to moderate effects, predisposing to a more severe form of disease. It remains to be determined whether single gene defects that confer profound susceptibility to DENV infection exist, as has been identified for a number of common pathogens, such as pneumococci and mycobacteria (185). In this respect, individuals who develop DHF or DSS but are otherwise healthy may serve as a pool to identify polymorphism and single-gene defects predisposing to the development of the most severe forms of DENV infection.

Antibody-Dependent Enhancement

In most acute virus infection models, the presence of antibodies, both neutralizing and nonneutralizing, correlates with control, elimination, and eventually protection. However, a possible detrimental role of virus-specific antibodies has been described for several viruses as measured by in vitro enhancement of infection of cells (72, 73, 93, 98, 189, 235, 238, 240, 255), a phenomenon that is not restricted to viral pathogens only (150). This in vitro phenomenon was also described for DENV infection (86), and epidemiological studies have shown an increased risk of developing DHF/DSS after a secondary DENV infection (74, 111, 207, 245). Halstead and colleagues observed that the incidence of DHF and DSS peaked in two populations of young children. One peak occurred in infants (at the age of 6 to 9 months) who were infected with a DENV serotype different from that which had infected their mothers. The key observation there was that severe disease occurred in infants for whom maternal antibodies had declined to low, subneutralizing levels. The other peak was observed in young children who had experienced an earlier, usually mild or subclinical, infection and were later infected with a different DENV serotype. These observations led to the conclusion that subsequent infection of preimmune individuals with a different DENV serotype could exacerbate rather than mitigate disease, a phenomenon that was claimed to be caused by antibodies and termed antibody-dependent enhancement (ADE) of disease (85). Several subsequent epidemiological studies provided further circumstantial evidence for the role of preimmunity in the pathogenesis of DHF (25, 80, 82, 112). ADE could result in infection of a higher number of target cells, which could lead to the high viral load observed in many studies (132, 221, 245, 251, 258, 259). Despite several clinical studies, evidence for the role of ADE in human disease, such as in DENV infections, remains circumstantial. Although some studies have shown a correlation between enhancing activity of serum, high levels of viremia, and an increased risk for DHF/DSS (38), not all cases of severe disease are associated with ADE or preceded by infection with a heterologous serotype or by high viral loads. In some cases, when DHF/DSS is seen, the presence of viral RNA became undetectable (132). In general, however, a high viral load and the presence of virus on the day of defervescence are important risk factors for the development of severe disease. As stated above, it is not completely clear whether the absence of viremia always correlates with clearance of virus from infected tissues (155, 199).

An alternative or complementary hypothesis is that FcγR-mediated entry suppresses the antiviral immune response. For instance, a study with Ross River virus showed that viral entry via the FcγR pathway could suppress antiviral genes and enhance IL-10 production in murine macrophages, while entry via the normal cellular receptor did not change the antiviral environment (135, 151). Furthermore, it was shown that virus replication was necessary in order to promote IL-10 expression. Unfortunately, the Fc receptor that was involved in ADE was not identified. It was also shown that DENV infection of THP-1 cells via FcR suppressed the transcription and production of IL-12, IFN-γ, TNF-α, and NO but enhanced expression of the anti-inflammatory cytokines IL-6 and IL-10 (36), indicating that ADE of DENV infection also resulted in a milieu that promoted viral replication. These results must be interpreted with caution, however, since the effect of ADE of infection on gene expression may be cell dependent (20). This effect of FcγR-mediated entry on the antiviral state is not unique to viral pathogens. For instance, FcγR-mediated infection of murine macrophages with *Leishmania* amastigotes was required to sustain persistent infection (108, 180, 244). Infection of humans and mice with *Leishmania major* in the pres-

ence of antibodies resulted in development of chronic infection. In this regard, increased levels of IL-10 have been associated with visceral and cutaneous leishmaniases, and IL-10 has been flagged as having an important role in the regulation of the immune response to this parasite, thereby linking ADE to Th2 immune responses. This immunosuppressive effect of FcγR-mediated infection is in agreement with the decrease in the proliferative responses to mitogen and recall antigens that can be measured during acute DENV infection, which is associated with both quantitative and qualitative defects in the antigen-presenting cell (APC) population (148, 161).

The role of preimmunity conferred by vaccination against DENV or other flaviviruses in the pathogenesis of DENV infections has been studied in limited numbers of subjects. Most clinical trials with candidate DENV vaccines were conducted in areas where the disease is not endemic and the chance of acquiring a natural infection is limited. One study conducted in Thailand reported no differences in the incidence of DHF in children who had received a live-attenuated DENV vaccine and unvaccinated controls at 6 to 8 years after vaccination (34). Experimental infection of monkeys with DENV-1 or DENV-4 followed by a secondary infection with DENV-3 a year later did not result in increased viremia or disease (113). The efficacy of DENV candidate vaccines was also not influenced by preimmunity to DENV or other flaviviruses (77, 114). The results from these experimental studies should be interpreted with caution, however, since the interval between primary and secondary infection may have been too short.

Cross-Reactive T-Cell Response

Although memory T cells cross-reactive with a heterologous virus can provide partial protective immunity, they can also cause substantial immunopathology (210). The role of $CD8^+$ T cells during DENV infection is not entirely clear, but they may play a role in clearing infection as well as in immunopathogenesis (3, 166). It is worth noting that a consistent finding in all examples of T-cell-mediated pathology during acute or persistent viral infections is morphological tissue damage as a result of cytolysis or inflammation induced by the high numbers of effector T cells. The efficiency of activated T cells in clearing virus-infected cells is dependent on the avidity of the T-cell receptor (TCR) for the HLA-peptide complex (222), and it is assumed that cross-reactive T cells of low avidity for heterologous virus are not protective (110). Yet, there are only a handful of virus-animal models where heterologous immunity has been shown to cause pathology. These include the combinations of infections with lymphocytic choriomeningitis virus (LCMV) and vaccinia virus (VV), as well as influenza A virus (IAV) and murine cytomegalovirus (MCMV) (209). In one study, peripheral VV infection of LCMV-immune mice resulted in immune-mediated panniculitis (211), whereas respiratory VV challenge of LCMV-immune mice resulted in recruitment of LCMV-specific $CD8^+$ T cells into the lung, causing bronchiolitis obliterans (40). In the IAV-MCMV model it was shown that IAV-immune mice challenged with MCMV developed severe consolidating mononuclear pneumonia as a result of increased viral replication in the lungs (41, 209). One example of cross-reactivity leading to disease in humans has also been described (250, 260). The authors of those studies reported two cases of fulminant hepatitis C virus infection associated with an unusually high frequency of $CD8^+$ T cells. These T cells were shown to recognize a single epitope within the hepatitis C virus NS3 that also cross-reacted with an epitope in the IAV neuraminidase protein. These findings suggest that cross-reactive memory T cells can modify the primary immune response and modulate the immunopathologic response to subsequent infection with other pathogens.

During the acute phase of a secondary infection of humans with heterologous DENV, highly cross-reactive $CD8^+$ T cells with high avidity for the infecting virus are preferentially activated (58, 99). The majority of these cross-reactive T cells produce high concentrations of pro- and anti-inflammatory cytokines such as IFN-γ, TNF-α, and IL-13 but somewhat lower levels of IL-10. These high-avidity cross-reactive $CD8^+$ T cells die through apoptosis, but it is not clear whether cells die as a result of activation-induced cell death or whether apoptosis is selectively induced by cross-reactive epitopes. Other studies have also suggested that epitopes can regulate the level of proinflammatory cytokines produced by T cells (146, 147). Alternatively, low-avidity cross-reactive $CD8^+$ T cells would be preferentially expanded (165, 166). These cross-reactive T cells react differently to the heterologous epitopes than to homologous epitopes by producing high levels of proinflammatory cytokines, but they lose their cytolytic activity. Delayed virus clearance would prolong activation of such cross-reactive $CD8^+$ T cells, which then results in the production of high levels of cytokines such as TNF-α, IL-6, or other soluble factors that affect vascular permeability. The phenomenon where cross-reactive memory T cells for the primary infecting virus are more efficiently activated, due to the increased frequency and higher activation state of memory cells, has been called original antigenic sin (OAS). This phenomenon has also been described for LCMV in mice (110). During a secondary infection with a heterologous serotype, cross-reactive epitopes preferentially reactivate the larger number of memory T cells against the priming virus more effectively than they activate naïve T cells. However, in accordance to what has been described for several other systems, it is possible that during a heterologous DENV infection, only a very small subset of cross-reactive memory T cells will be stimulated to expand because of a narrowing TCR repertoire. This narrowing of the TCR repertoire in combination with the fact that each individual has a unique TCR specificity (private TCR [52, 107]) would result in dominant responses that are unique for individuals. This could explain the variability seen in disease outcome upon secondary infection with heterologous DENV. Much less is known about the $CD4^+$ T-cell response during DENV infection. However, evidence exists that sequential infection with different DENV serotypes may also alter the cytokine response of cross-reactive $CD4^+$ T cells, resulting in production of proinflammatory cytokines (154) that may contribute, together with the $CD8^+$ T-cell response, to a detrimental cytokine release.

Soluble Factors

It is strongly believed by many scientists studying dengue pathogenesis that a high viral load and activation of high num-

bers of nonprotective T cells result in a "storm" of inflammatory cytokines and other mediators, leading to the increased plasma leakage characteristic of DHF/DSS. One of the most daunting challenges in DENV research is the identification of soluble factors that can mediate, either alone or in combination, the functional changes induced in EC that are associated with the increased plasma leakage. Several studies have shown that concentrations of multiple cytokines and other mediators, as well as soluble receptors, are significantly increased during severe dengue infections (reviewed in reference 15). Higher plasma levels of IL-1β, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-13, IL-18, TGF-1β, TNF-α, and IFN-γ have been found in patients with severe DENV infections, in particular in patients with DSS (10, 23, 32, 103, 128, 169, 172, 183, 192, 194, 236). These studies analyzed samples from infants, children, and adults infected with different DENV serotypes. It is reasonable to assume that synergistic interactions between these cytokines will occur. Other mediators and soluble factors found to be increased in severe disease include vascular endothelial growth factor (VEGF), granulocyte-macrophage colony-stimulating factor, monocyte chemoattractant protein 1 (MCP-1), macrophage migration inhibitory factor, thrombopoietin, soluble vascular cell adhesion molecule 1 (VCAM-1), soluble ICAM-1, von Willebrand factor antigen, thrombomodulin, E-selectin, tissue factor (TF), plasminogen activator inhibitor 1 (PAI-1), and tissue plasminogen activator (23, 26, 27, 29, 44, 46, 115, 130, 153, 223, 229). Analyses of several studies reveal conflicting results, as some studies report increased levels of plasma cytokines while others do not. These discrepancies are attributed mainly to the study design and experimental setup, the laboratory tests used to measure cytokines, and the statistical tests applied for the analyses of the results. Furthermore, the time of sampling during infection is difficult to standardize, and this may explain some of the discrepant results. The observation that plasma leakage occurs mainly in the pulmonary and peritoneal cavities justifies the question whether levels of cytokines and mediators in plasma indeed reflect concentrations in different compartments. Infection of humans and animals with seasonal IAV (H1N1 or H3N2) indicated that levels of cytokines and chemokines were much higher locally, at the site of infection, than in plasma or serum (68, 71, 88). In contrast, H5N1 causes fulminant disease in humans, characterized by diffuse alveolar damage and progression to multiorgan dysfunction. In this case disease severity correlates strongly with cytokine levels, both locally and systemically (54a, 129). For instance, hemophagocytic syndrome has been proposed as a cause of the multiorgan dysfunction observed in patients with confirmed H5N1 fatal infections (247, 268). Notably, hemophagocytic syndrome in fulminant virus infections or autoimmune diseases as well as macrophage activation syndrome in hematopoietic cell transplantation has been associated with excessive cytokine production (127, 193, 227). The production and actions of cytokines are thus critically dependent on the context in which they occur. More specifically designed studies are needed to dissect the relationships between levels of several cytokines in the pulmonary (pleural fluid) and peritoneal (ascites) regions compared to plasma or serum in severe DENV infections. It is conceivable that differences in viral replication and damage to selective EC in vivo may account for a differential cytokine profile, resulting in different vascular permeability patterns.

Some evidence to support a role for cytokines comes from animal models of increased vascular permeability and hemorrhage during DENV infections (39, 217). TNF-α, IL-1β, IL-6, and IL-10 levels have been shown to be high in sera of DENV-infected mice (6). In addition, several in vitro experiments have demonstrated high levels of cytokines in culture supernatants of DENV-infected (primary) DC (20), monocytic cells (47, 162), and EC (11). In the presence of anti-NS1 antibodies, EC produce MCP-1, IL-6, and IL-8 in vitro (138). T cells interacting with DENV-infected cells may also produce TNF-α, IFN-γ, IL-4, and/or IL-10 (91).

The biological roles of some cytokines and soluble factors and the implication in the development of hemorrhage have been inferred and are summarized in Table 2. The cytokines TNF-α and IL-10 are of particular interest. For instance, in one study a positive correlation between soluble TNF-α concentrations and thrombocytopenia was found (23). Furthermore, the TNF-α308A allele, which leads to overproduction of the cytokine, is more commonly found in DHF patients (66). These observations, together with experiments showing that TNF-α is capable of increasing EC permeability in vitro (55), suggest its possible role in pathogenesis of DHF. In a mouse model of DENV-induced hemorrhage, high levels of TNF-α in tissues correlated with EC apoptosis and hemorrhage (39). It is worth noting that in most models of immunopathology, pathology is mediated via direct lysis of infected cells by TNF-α, and it has been shown that $CD8^+$ T-cell cytotoxicity can be mediated exclusively by TNF-α. Plasma levels of IL-10 were shown to correlate with platelet decay in DENV-infected patients (10, 132) and may modulate the activation of coagulation. IL-6 is a major mediator of fever and acute-phase reactions and is produced by macrophages and activated EC. Furthermore, IL-6 and IL-8 mediate derangement of coagulation and fibrinolysis, whereas TNF-α and VEGF act synergistically to induce expression of TF on EC (216). TNF-α has a direct effect on production of IL-6 and thus an indirect effect on coagulation and fibrinolysis. IL-2 plays a central role in the regulation of the immune response, as it induces potent proliferation of T cells and to a lesser extent of B cells, stimulates synthesis of IFN-γ and TNF-α, and may damage the integrity of EC. IL-8 has an effect on the expression of adhesion molecules such as ICAM-1 and VCAM-1. MCP-1 causes EC tight-junction openings in vitro (231) and elevates endothelial permeability changes in vivo (265). IL-13 is a pleiotropic type 2 cytokine, and its receptor is expressed on vascular EC. IL-13 downregulates expression of proinflammatory cytokines IL-1, IL-6, IL-8, and IL-12. Furthermore, IL-13 is a potent stimulator of matrix metalloproteinase 9 (MMP-9) and cathepsins and plays important roles in the pathogenesis of emphysema in animal models of respiratory infections. Interestingly, DENV-infected cells have been shown to produce MMP-9, which increases vascular permeability in vitro (145). IL-18 is a type 1 cytokine, produced mainly by monocytes, macrophages, and DC, and has a strong proinflammatory activity. In vitro, IL-18 upregulates expression of adhesion molecules (E- and P-selectins) on EC, which may contribute to a procoagulant state (167). High levels of IL-18 may be associated with neutropenia, thrombocytopenia, and elevated levels of liver enzymes. During DENV infection,

TABLE 2. Summary of soluble factors that are or are likely to be associated with development of DHF/DSS

Soluble factor	Biological function in relation to pathogenesis
Thrombin	Thrombin is thought to act near the site at which it is produced. Thrombin converts circulating fibrinogen to fibrin and triggers platelet activation, which results in platelet aggregation. Thrombin activates EC and increases EC permeability, leading to plasma leakage and edema formation. Thrombin is chemotactic for monocytes and is mitogenic for lymphocytes and mesenchymal cells. Activated platelets release several soluble factors with inflammatory, antimicrobial, and immune modulating activity, such as MMP-9, which enhances EC permeability. Activated platelets also secrete soluble CD40 ligand, which can induce EC to produce reactive oxygen species, adhesion molecules, chemokines, and TF. Thrombin also inhibits IL-12 production by mononuclear cells.
C3a and C5a	C3a activates platelets and enhances their activation and adhesion properties. C5a enhances blood thrombogenicity by upregulating TF and PAI-1 expression on various cell types. C5a stimulates monocytes to produce IL-1, IL-6, IL-8, and TNF-α. Activation of these complement factors is enhanced by thrombin, which cleaves C3 and C5 to C3a/b and C5a/b, respectively. Activated platelets are also involved in C3 cleavage, which induces activation of the classical complement pathway.
C4b	C4b binds to protein S and thereby inhibit the anticoagulant properties of activated protein C-protein S complexes.
IL-1	IL-1β is major mediator of platelet-induced activation of EC, causing enhanced chemokine release and upregulation of VCAM-1. VCAM-1 promotes adhesion of monocytes to the endothelium. IL-1 increases the expression of TF on EC and suppresses the cell surface anticoagulant activity of EC. Depending on its concentration, it may upregulate TNF-α production or downregulate TNF-receptors. IL-1 stimulates the hypothalamus and, as a consequence, the pituitary gland to produce anti-inflammatory mediators such as endorphins, melanocyte-stimulating hormone, and adrenocorticotropic hormone.
IL-6	Together with other proinflammatory cytokines, IL-6 potentiates the coagulation cascade. It can downregulate production of TNF-α and TNF receptors. IL-6, together with IL-1, is a potent inducer of fever.
IL-8	IL-8 is a chemokine that is abundantly produced by monocytes, EC, and hepatocytes. EC damage in the liver may elevate systemic concentrations. Activation of the coagulation system results in increased expression of IL-6 and IL-8 by monocytes, while the APC-PS anticoagulation pathway downregulates production of IL-8 by EC.
IL-10	IL-10 is produced by monocytes and regulatory T helper cells and may cause platelet decay. Thrombin can stimulate IL-10 production by monocytes. The cytokine downregulates the inflammatory response and creates a proviral survival milieu. IL-10 promotes OAS by inhibiting development of effector T cells to new epitopes. IL-10 also inhibits the expression of TF and inhibits fibrinolysis.
TNF-α	TNF-α in a potent activator of EC and enhances capillary permeability. TNF-α upregulates expression of TF on monocytes and EC and downregulates expression of thrombomodulin on EC. It also activates the fibrinolysis system. TNF-α enhances expression of NO and mediates activation-induced death of T cells, and it has therefore been implicated in peripheral T-cell deletion.
TGF-β	TGF-β may act as a proinflammatory or anti-inflammatory cytokine, depending on its concentration. Early in infection, low levels of TGF-β may trigger secretion of IL-1 and TNF-α. However, later in infection, the cytokine inhibits the Th1 response and enhances production of Th2 cytokines such as IL-10. TGF-β increases expression of TF on EC and upregulates expression and release of PAI-1.
NO	NO has a multifaceted role in inflammatory reactions. It enhances vasodilatation and formation of edema. It upregulates TNF-α production in monocytes. At low concentrations it protects cells from apoptosis, while at high concentrations it induces apoptosis. NO downregulates expression of MHC class II and suppresses expansion of Th1 cells. Maintenance of the EC barrier requires a basal level of NO. Both a lack of NO and high NO levels destabilize EC junctions.
VEGF	VEGF is a key driver of vascular permeability. It reduces EC occludins, claudins, and VE-cadherin content, all of which are components of EC junctions. Upon activation, VEGF stimulates expression of ICAM-1, VCAM-1, and E-selectin in EC.

$CD4^+$ T cells have been shown to produce a unique cytokine called the cytotoxic factor, with peak amounts measured in DHF/DSS cases (2, 37). The validity of these observations has to be confirmed by independent experiments.

Clearly, there is a substantial redundancy between cytokines (i.e., the lack of one specific cytokine may be compensated for by another cytokine with overlapping activities), making it difficult to explain DHF/DSS pathogenesis on the basis of a single cytokine. It is more likely that multiple cytokines contribute simultaneously in a complex way to the development of DHF/DSS. Apart from any other considerations, it is reasonable to assume that cytokines and other soluble mediators of the functional, and to a lesser degree the morphological, pathology characteristic of DHF/DSS are also essential for efficient viral clearance. The fact that DSS patients recover extremely rapidly after appropriate fluid therapy suggests that cytokines do not cause tissue destruction like in many immunopathology models but rather cause a reversible EC dysfunction.

THE INTERGRATED VIEW

The mechanisms leading to the severe manifestations of DENV infections are still not completely understood but are likely to be multifactorial (Fig. 1). The genetic background of the host influences the way that the immune response reacts to DENV infection. Upon inoculation of DENV into the dermis, Langerhans cells and keratinocytes will primarily be infected. The virus subsequently spreads via the blood (primary viremia)

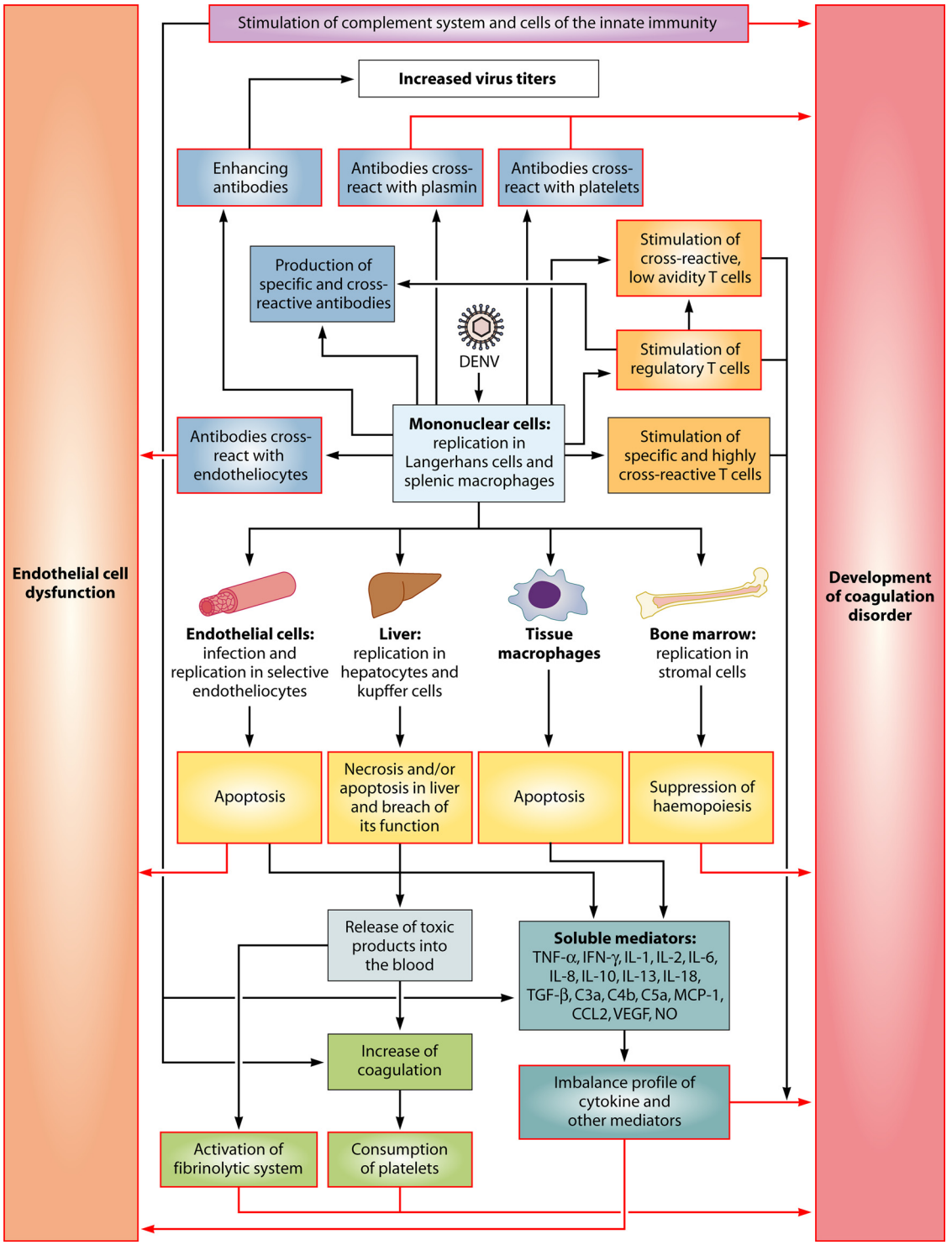

FIG. 1. Proposed model for the pathogenesis of DF, DHF, and DSS, based on an integrated view of the data presented (see section The Integrated View in the text). Black arrows, processes leading to the indicated event; colored boxes with white centers, pathological events. Each event will ultimately affect the EC or the hemostatic system (purple arrows).

and infects tissue macrophages in several organs, especially the macrophages in the spleen. The replication efficiency of DENV in DC, monocytes, and macrophages, as well as its tropism for and replication efficiency in EC, bone marrow stromal cells, and liver cells, collectively determine the viral load measured in blood. This viral load represents an important risk factor for development of severe disease. Essentially, infection of macrophages, hepatocytes, and EC influences the

hemostatic and the immune responses to DENV. Infected cells die predominantly through apoptosis and to a lesser extent through necrosis. Necrosis results in release of toxic products, which activate the coagulation and fibrinolytic systems. Depending on the extent of infection of bone marrow stromal cells and the levels of IL-6, IL-8, IL-10, and IL-18, hemopoiesis is suppressed, resulting in decreased blood thrombogenicity. Platelets interact closely with EC, and a normal number of functioning platelets is necessary to maintain vascular stability. A high viral load in blood and possibly viral tropism for EC, severe thrombocytopenia, and platelet dysfunction may result in increased capillary fragility, clinically manifested as petechiae, easy bruising, and gastrointestinal mucosal bleeding (170), which is characteristic of DHF. At the same time, infection stimulates development of specific antibody and cellular immune responses to DENV. When IgM antibodies that cross-react with EC, platelets, and plasmin are produced, the loop that results in increased vascular permeability and coagulopathy is amplified. In addition, enhancing IgG antibodies bind heterologous virus during secondary infection and enhance infection of APCs, thereby contributing to the increased viral load that is seen during secondary viremia in some patients. Furthermore, a high viral load overstimulates both low- and high-avidity cross-reactive T cells. In the context of certain HLA haplotypes, cross-reactive T cells delay virus clearance, while producing high levels of proinflammatory cytokines and other mediators. Ultimately, these high levels of soluble factors, many of which still remain to be identified, induce changes in EC leading to the coagulopathy and plasma leakage characteristic of DSS.

DISCUSSION

Here we review and discuss the plethora of hypotheses about DHF and DSS pathogenesis presented in the literature. Most of these hypotheses are not mutually exclusive, and together they harbor multiple elements that collectively may explain most of the phenomena observed in the multiple presentations of DENV infection. Table 3 addresses major challenges to the hypotheses described in this review. The pathogenesis of both DHF grades I/II and DSS is complicated and multifactorial, involving both viral and host factors. However, necessary and/or sufficient factors have still not been identified. It may be questioned whether factors that explain the pathogenesis of DHF and DSS in all patients do exist. Genetic predisposition may have a significant effect on disease outcome. Only a few studies have studied host genetics with regard to the severity of DENV infection. Most commonly, clinically significant genetic variations consist of single-nucleotide polymorphisms within genes that affect disease pathways. Many polymorphisms have small and independent effects on disease outcome and often act in concert with other polymorphisms and environmental risk factors (24). This eventually results in complex and variable disease outcomes. Studies using a combination of genomics (transcriptomics, proteomics, and metabolomics), single-nucleotide polymorphism genotyping, and careful phenotypic disease characterization in well-defined cohorts should be adopted to identify individual molecular markers of DHF/DSS.

As discussed in this review, the pathways to DHF involve both viral and host-specific elements. The following points can be summarized.

(i) In contrast to what is generally believed, the involvement of liver and of EC in different organ systems seems to be an important factor in the pathogenesis of dengue. In this respect, it is of paramount importance to understand how EC lining the thoracic and peritoneal cavities are affected. It is worth noting that despite the increased vascular permeability measured in both DHF and DSS patients (16), plasma leakage is highly restricted to the pleural and peritoneal cavities, while no generalized edema is seen. No specific vascular lesions are found in fatal cases of DENV infections. Viruses known to cause hemorrhagic fever, such as viruses belonging to the families *Arenaviridae* (Junin virus and Lassa virus), *Filoviridae* (Ebola virus and Marburg virus), *Bunyaviridae* (Hanta virus and Rift Valley virus), and *Flaviviridae* (yellow fever virus), are also not associated with EC damage (43, 70). In these cases, the pathogenesis of hemorrhagic diathesis is the result of severe liver damage leading to decreased production of coagulation proteins and albumin. Increased vascular permeability as a result

TABLE 3. Challenges for DENV research

Challenge
Developing an animal model of dengue disease
Understanding the role of several DENV strains and serotypes in DENV tropism in vivo
Elucidating the role of intrahost evolution of DENVs in emergence of virulent strains and the role of genetic variants and different serotypes in promoting OAS, ADE, transient autoimmunity, and unbalanced T-cell responses
Elucidating the role of DENV proteins, especially NS1 in pathogenesis
Understanding the interplay between the hemostatic and the complement systems in DENV infection
Elucidating the mechanisms by which innate immunity regulates memory B- and T-cell generation, maintenance, and activation during primary and secondary DENV infections
Elucidating the role of autoimmunity in dengue pathogenesis
Identifying serotype-specific linear and conformational B-cell epitopes and understanding their role in primary and anamnestic B-cell responses
Understanding the role of DENV immune complexes in the signaling network within target cells, especially APCs, and how it influences virus replication and priming of immune responses
Elucidating the role of the T-helper and T-cytotoxic cell repertoire, especially the private repertoire, on evolution of T-cell responses during primary and sequential heterologous DENV infections
Understanding the role of OAS in T-cell-mediated immunity in heterologous DENV infections and elucidating the factors that determine occurrence of OAS for T cells and its role in pathogenesis
Identifying soluble factors that are necessary or sufficient to induce endothelial cell dysfunction and coagulopathy seen in DHF/DSS
Identifying critical components of tight junctions and adherent junctions present in the vascular beds affected during DHF/DSS and elucidating the role that different soluble factors play in expression and functions of the several components of these junctions
Investigating the effect of demographic history of infection on the type of B- and T-cell responses elicited during primary and secondary DENV infections and on pathogenesis
Elucidating the role of host gene polymorphism, such as FcR, cytokines, chemokines, etc., in DHF/DSS pathogenesis
Understanding race- and age-dependent susceptibility to severe DENV infection

of hypercytokinemia and a reduction of plasma osmotic pressure due to severe liver damage contribute to edema formation, as is seen in severe cases of Lassa fever (67). In addition, replication of most of these viruses in the adrenal gland contributes to hypotension and sodium loss, which collectively result in hypovolemic shock. The shock syndrome associated with DSS, however, is unique to DENV infection, and it is of paramount importance to understand how the EC lining the thoracic and peritoneal cavities are affected.

(ii) Hemorrhagic manifestations as seen in DF and DHF grades I/II are usually mild and manifested as petechiae disseminated in the skin, with an increased tendency of bleeding upon bruising. These manifestations are found early after onset of fever and coincide with the window of viremia and degree of thrombocytopenia (121). The pathogenesis of mild hemorrhages seen in DF and DHF grades I/II may be explained by increased capillary fragility as a result of thrombocytopenia or platelet dysfunction, virus infection of EC, and high concentrations of cytokines that disrupt vascular integrity (54, 170, 257). Most mediators that increase vascular permeability affect the organization of the adherens junctions (AJ), a complex network of adhesion proteins that are linked to intracellular cytoskeleton (54), causing retraction of EC and opening of intercellular gaps. Alternatively, the stability of the junctions may be weakened, resulting in vascular fragility. Weakness of the junctions is not reflected by morphological changes, as is exemplified by the fact that internalization of VE-caherin or phosphorylation of AJ proteins reduces junction stability without the opening of intercellular gaps (4, 62). It is noteworthy that the major anatomical sites of bleeding in patients with severe thrombocytopenia are the intercellular gaps in the postcapillary venules, which are rich in AJ (170). Platelets play a role in maintaining the integrity of AJ by constitutively releasing an array of factors such as platelet-activating factor and sphingosine-1-phosphate, which are released as a result of fluid shear stress (5). Therefore, interruption of platelet-EC interaction by severe thrombocytopenia or platelet dysfunction may lead to increased vascular fragility, resulting in hemorrhages or increased tendencies for hemorrhages.

(iii) Several studies have shown that plasma leakage occurs before defervescence or hemoconcentration. As mentioned before, the WHO case definition has mainly a clinical diagnostic purpose and is not an appropriate selection criterion for pathogenesis studies. Pathogenesis studies should be designed to understand the differences between (i) no bleeding tendencies, (ii) increased manifestations of bleeding or tendencies for bleeding, or (iii) plasma leakage as measured by sonography and hemoconcentration. It is crucial to study the pleural fluid in order to understand the pathophysiological cause of plasma leakage and why it is restricted to or more pronounced in thoracic and peritoneal areas.

(iv) Antibody-mediated enhancement of infection either results in a high viral load or represents the link to type 2 cytokine responses.

(v) In some viral systems, cross-reactive $CD8^+$ T-cell responses are involved in the development of pathological changes during secondary infection with a related but heterologous virus. DENV cross-reactive T cells, however, lose their cytolytic activity, while producing high levels of proinflammatory cytokines and other mediators. The role that preexisting immunity to other flaviviruses plays in the development of severe dengue should be investigated.

(vi) Soluble host factors seem to be central in the pathogenesis of both DHF grades I/II and DSS. Although several of these have been associated with severe disease, their concentrations are also elevated in other viral infections without resulting in plasma leakage. High concentrations of cytokines such TNF-α, IL-6, and IL-8 have been implicated in capillary leakage and development of hypovolemic shock in patients with anaphylaxis, meningococcal sepsis, and Jarish Herxheimer reaction (105, 176, 233). Similarly, high concentrations of cytokines have been associated with unfavorable outcomes of filovirus (253), arenavirus (89, 160), and yellow fever virus (241) infections. However, the shock syndrome associated with DSS does not occur in these conditions. Therefore, it may be speculated that soluble factors incriminated in the increased vascular permeability seen in DSS must be qualitatively and quantitatively different from those involved in the conditions described above.

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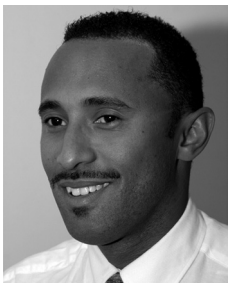

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