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# MINIREVIEW

# Recent Advances in Deciphering Viral and Host Determinants of Dengue Virus Replication and Pathogenesis▽

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Dengue virus (DENV) is a member of the *Flavivirus* genus of the *Flaviviridae* family of enveloped, positive-strand RNA viruses. The *Flavivirus* genus includes viruses transmitted by mosquitoes and ticks, as well as zoonotic agents with no known arthropod vector. In addition to DENV, flaviviruses that are significant threats to human health include yellow fever virus, West Nile virus (WNV), Japanese encephalitis virus, and tick-borne encephalitis virus. The dengue viruses are comprised of four distinct serotypes, DENV1 through DENV4, which are transmitted to humans through the bites of two mosquito species: *Aedes aegypti* and *A. albopictus*.

DENV causes a wide range of diseases in humans, from the acute febrile illness dengue fever (DF) to life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). DF is a self-limited though debilitating illness characterized by fever, headache, retro-orbital pain, myalgia, arthralgia, and rash. DHF is marked by increased vascular permeability ("plasma leakage"), thrombocytopenia, and hemorrhagic manifestations; DSS occurs when fluid leakage into the interstitial spaces results in shock, which without appropriate treatment may lead to death. Dengue has spread throughout tropical and subtropical regions worldwide over the past several decades, with an estimated 100 million infections and tens of millions of cases occurring annually. DHF/DSS is one of the leading causes of pediatric hospitalization in Southeast Asia, where the syndrome first emerged 50 years ago, and has become endemic to many Latin American countries over the last 25 years (reviewed in references 64 and 184).

## DENGUE VIRUS REPLICATION

The intracellular life cycles of the flaviviruses are very similar (Fig. 1). Infection with one of the arthropod-borne flaviviruses begins when the vector takes a blood meal and the virus is introduced into the host. The virus binds to and enters a permissive host cell via receptor-mediated endocytosis. Upon internalization and acidification of the endosome, fusion of viral and vesicular membranes allows entry of the nucleocapsid into the cytoplasm and genome uncoating. Translation of the input strand takes place; then the virus switches from translation to synthesis of a negative-strand intermediate, which serves as a template for the production of multiple copies of positive-strand viral RNA (vRNA). Successive rounds of translation produce high levels of viral proteins; the structural protein capsid or core (C), premembrane (prM), and envelope (E) proteins, along with vRNA, are assembled into progeny virions, which are transported through the Golgi compartment and secreted (reviewed in reference 56). While much that is assumed about DENV has been characterized for related flaviviruses such as yellow fever virus, WNV, Japanese encephalitis virus, and tick-borne encephalitis virus, for the purposes of this review we will focus on recent research that has concentrated specifically on the four serotypes of DENV.

## CELLULAR TROPISM, BINDING, AND ENTRY

The replication cycle of DENV begins when the virion infects a permissive host cell. In vitro, DENV has been shown to be capable of infecting numerous human cells, including dendritic cells (DCs), monocytes/macrophages, B cells, T cells, endothelial cells, hepatocytes, and neuronal cells, as well as a number of cell lines used for viral propagation (reviewed in reference 5). Although a general consensus that cells of the mononuclear phagocyte lineage (monocytes, macrophages, and DCs) are the primary targets in vivo emerges from clinical and autopsy studies, some controversy exists as to the primary cell type(s) targeted by DENV in humans (91). In particular, whether hepatocytes, lymphocytes, endothelial cells, and neuronal cells are target cells for DENV replication in vivo has not been conclusively demonstrated. Apart from autopsy studies, further evidence for cellular tropism in vivo has included the detection of DENV in Langerhans cells (skin-resident DCs) after inoculation with an experimental vaccine (197) and in monocytes and, in some instances, B cells in peripheral blood from naturally infected patients (101, 141, 168; A. Durbin, A. Balmaseda, and E. Harris, unpublished data).

The first points in the viral life cycle that determine cellular tropism are the stages of adsorption and entry. It is generally accepted that DENV gains entry to its target cell by receptor-mediated endocytosis (RME). There have been some reports of entry via direct fusion with the plasma membrane (78, 115), although this was shown to result in a nonproductive infection in macrophages (60, 100). Significant effort has been made recently to characterize the cellular receptors of DENV infec-

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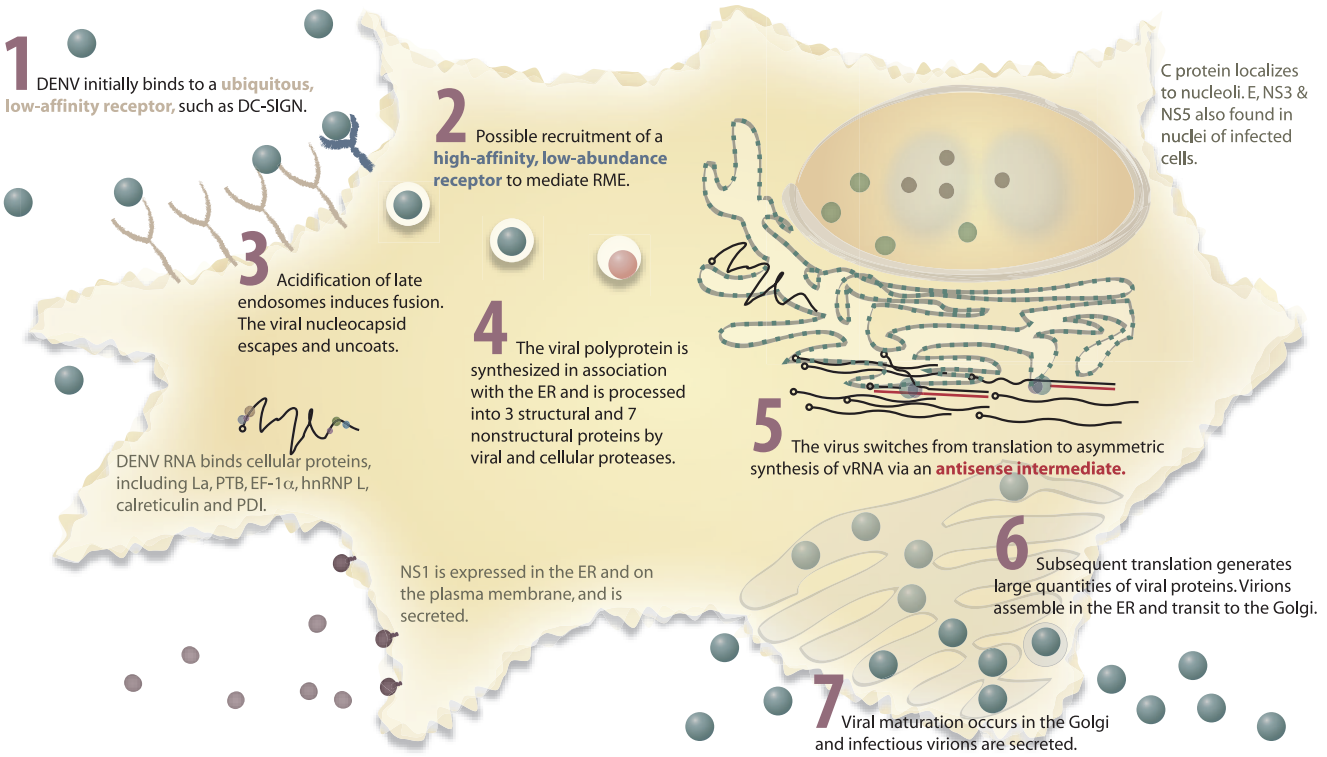


FIG. 1. Intracellular life cycle of dengue virus. DENV binds (step 1) and enters (step 2) cells via an uncharacterized receptor(s) by RME. Endosomal acidification (step 3) results in an irreversible trimerization of the viral E protein, exposing the fusion domain. After being uncoated, the vRNA is translated (step 4) at ER-derived membranes, where it is processed into three structural and seven NS proteins. After the viral replication complex is synthesized, vRNA translation switches off and RNA synthesis (step 5) begins. Subsequently, successive rounds of translation (step 6) are followed by assembly in the ER. The virion is maturated in the Golgi compartment (step 7) and exits via the host secretory pathway. Viral proteins C, E, NS3, and NS5 have been observed in the nuclei of infected cells (57, 96, 177, 186, 190), and the vRNA associates specifically with a number of cellular proteins (41, 58, 187, 198); however, the biological significance is unknown. L-SIGN, liver/lymph node-specific ICAM-3-grabbing nonintegrin; PTB, polypyrimidine tract binding protein; EF-1α, elongation factor 1α; hnRNP L, heterogeneous nuclear ribonucleoprotein L; PDI, protein disulfide isomerase.

tion. A number of different mammalian cell receptors has been proposed, including heparan sulfate (29, 59, 82, 119), heat shock protein 70 (Hsp70) and Hsp90 (187), GRP78/BiP (92), CD14 (30), and 37-kDa/67-kDa high affinity laminin receptor (183), as well as DC-specific intercellular adhesion molecule 3 (ICAM-3)-grabbing nonintegrin (DC-SIGN) (123, 139, 181) and liver/lymph node-specific ICAM-3-grabbing nonintegrin (181).

Among the candidate receptors, the best-characterized interaction is between the virus and DC-SIGN. DC-SIGN can mediate infection of all four serotypes of DENV (181), and the ectopic expression of DC-SIGN confers permissiveness to infection upon normally nonpermissive cell lines (139, 181). DC-SIGN interacts with the virus via carbohydrate moieties on E (123, 139), and the type of glycosylation that occurs in insect cells, specifically the addition of high-mannose glycans to residue N67, is required for DC-SIGN-mediated entry (123, 153). It has been proposed that RME of DENV involves two or more receptors: a ubiquitous, lower-affinity receptor such as DC-SIGN that initially captures the virus at the cell surface, increasing the local concentration, and a less-common, high-affinity receptor that mediates internalization of the virion (Fig. 1). Accordingly, it has been demonstrated that internalization signals in the N terminus of DC-SIGN are expendable for viral uptake (123) and that a site for a possible secondary receptor interaction is left vacant upon virion binding to DC-SIGN (153).

In addition to cell type, recent work has implicated cell cycle status as a potential determinant of cellular tropism in the host. Human hepatocyte-derived HepG2 cells that were stalled in the $G_2$ phase of the cell cycle were shown to be more permissive to infection and to produce higher titers of both DENV2 and DENV3 (151). It was ascertained that uptake of DENV was responsible for the increased infection exhibited in synchronized $G_2$-phase HepG2 cells. In another study, it was demonstrated that cell cycle status was capable of modulating virion assembly in mosquito cells but not in human hepatoma (Huh7) cells (80). Compared with that in asynchronously cycling cells, virus assembly in C6/36 cells stalled in the S phase of the cell cycle was enhanced, corresponding with up to a 30-fold increase in titers of various strains of DENV2. These reports implicate a role for cycling or otherwise activated cells, in addition to nondividing cells, in DENV infections in vivo.

## TRANSLATION OF THE DENGUE VIRUS GENOME

As the replication machinery of positive-strand RNA viruses is not packaged into the viral particle, once inside the cell the

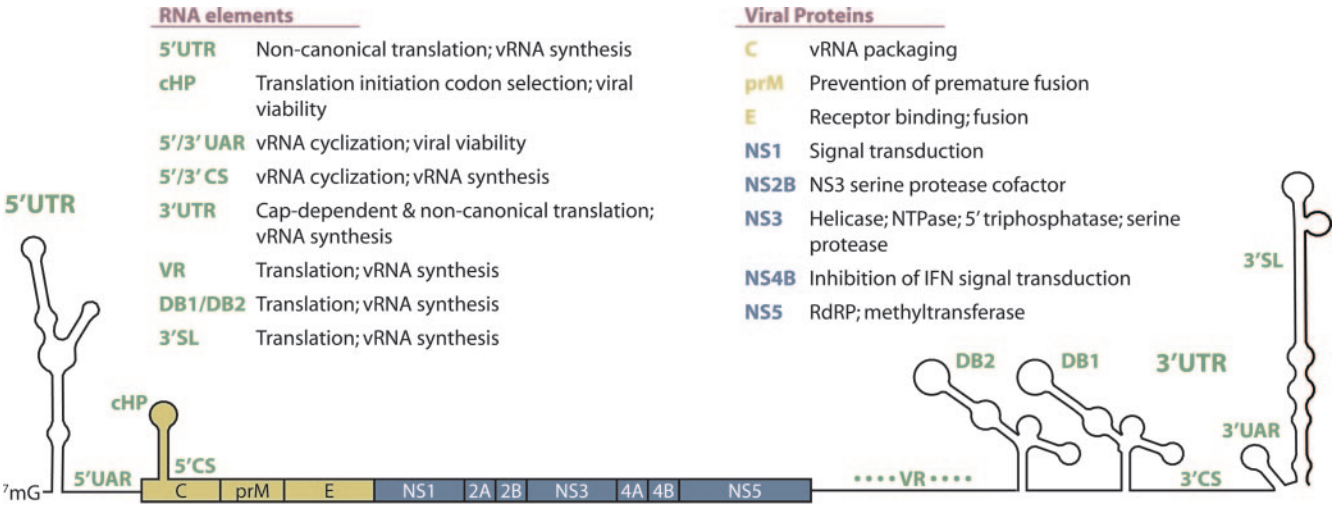


FIG. 2. Structure of the dengue virus genome. DENV vRNA contains a 5′ type 1 cap structure, and the single open reading frame is flanked by 5′ and 3′ UTRs. Conserved RNA secondary structures in the UTRs and in the coding region have been determined to function at various stages of the viral life cycle. Functions for several of the viral proteins have been determined; however, many remain to be elucidated. The secondary structure is based on Southeast Asian strains of DENV2 and is similar for different strains and serotypes. Structures designated cHP, CS, UAR, DB1, DB2 and 3′SL were compiled from a combination of computer predictions, functional analyses, and solution structure probing (3, 34, 71, 132, 148, 154). RdRP, RNA-dependent RNA polymerase; DB, dumbbell.

viral genome must first undergo translation to generate the viral RNA replicase in order to establish a productive infection. Translation and replication of positive-strand viruses occur in association with intracellular membranous structures (reviewed in reference 165). Specifically, DENV is translated in association with endoplasmic reticulum (ER)-derived membranes. The flavivirus genome is structured like a cellular mRNA, containing a 5′ type 1 7-methyl guanosine cap structure, a 5′ untranslated region (UTR), a single open reading frame, and a 3′ UTR; however, unlike nearly all cellular messages, it is not polyadenylated. The lack of a poly(A) tail implies that DENV does not utilize the same mechanism for translation as cellular mRNAs. Moreover, flavivirus translation has been shown to occur under circumstances that inhibit cellular translation, such as during coinfection with poliovirus (167), under conditions of high osmolarity (44, 46, 167), and after sequestration or suppression of critical translation initiation factors such as eIF4E, which leads to an inhibition of canonical cap-dependent translation (47, 48, 108). The ability of flaviviruses to translate under diverse conditions may reflect an adaptation to cellular antiviral responses or to different cell types that contain various levels of essential translation factors.

The efficiency of initial translation may impact the cellular tropism of the virus. It has been determined that different strains of DENV have differing abilities to replicate in various cell types. In one study, DENV2 clinical isolates of different geographic origins and with limited passage in tissue culture ("low passage") were competent for replication in hamster kidney (BHK21) cells, but Nicaraguan strains, unlike Thai strains, did not replicate in human cell lines of myeloid origin (U937), human peripheral blood mononuclear cells, and primary human foreskin fibroblasts (HFF) (42, 46). All isolates bound and entered HFF equivalently, but a Nicaraguan strain was found to be defective at the step of input strand translation (46). In reporter assays, it was determined that point mutations in the 3′ UTR of the Nicaraguan strain conferred the translation defect. This study implicated translation in cellular permissiveness to infection and identified a function of the DENV 3′ UTR in the regulation of translation.

Several groups have begun delineating the roles of the viral UTRs and the cap structure in regulating viral translation. The 3′ UTR of DENV2 was demonstrated to confer similar properties as a poly(A) tail in enhancing translation when coupled with the viral 5′ UTR, a cellular 5′ UTR, or a number of viral internal ribosomal entry sites in reporter assays (31, 85). This enhancement of translation was dependent upon the 5′ cap structure (31, 85) and was shown by sucrose gradient sedimentation analysis to occur at the step of initiation (85). Specifically, the 3′ stem-loop (3′SL), a highly stable and conserved RNA structure at the 3′ end of the 3′ UTRs of flaviviruses (Fig. 2), was partially responsible for the 3′ UTR's ability to enhance translation of reporter RNAs. Additionally, disruption of the top of the 3′SL structure with antisense morpholino oligomers and by mutagenesis decreased translation of DENV2 reporter replicons (86). It has also been reported that deletion of both of the dumbbell-shaped structures (DB1 and DB2) or of the variable region (VR) negatively impacted translation of luciferase reporter constructs (31) and of reporter replicons (2). Conversely, long-range interactions between the 5′ and 3′ cyclization sequence (CS) domains, complementary sequences located in the C-coding region and the 3′ UTR, respectively, were not required for efficient translation of DENV2 reporters (31, 47) and replicons (2, 86). Surprisingly, a complete deletion of the 3′ UTR in a DENV2 replicon system had no significant impact on early translation events (2). However, the role of the 3′ UTR in the regulation of translation in the context of an intact viral genome and life cycle remains to be determined.

Once the DENV RNA has recruited the translation machinery, the transition from translation initiation to elongation

must occur when the small ribosomal subunit has located the initiation codon. The start codon for DENV1-3, located at the beginning of the C-coding region, is in a poor initiation context and therefore is expected to be utilized inefficiently, resulting in the production of N-terminally-truncated C proteins. It has been demonstrated that a predicted RNA hairpin structure downstream of the DENV2 start codon ("cHP" for C-coding-region hairpin) enhances translation from the first AUG in a position-dependent, sequence-independent manner proportional to its stability (34). The presence of the DENV2 cHP element has since been confirmed by solution structure probing of the 5′ end of the DENV2 positive strand (C. Polacek, J. E. Foley and E. Harris, unpublished data). Disruption of the cHP resulted in undetectable virus titers, whereas restoration of base pairing by compensatory mutations rescued viral replication. The degree of defect conferred by the disrupted cHP, beyond what would be expected from the moderate decrease in production of the full-length C protein, implies that this structure plays a role in the viral life cycle beyond start codon selection (34).

## DENGUE VIRUS STRUCTURAL AND NONSTRUCTURAL PROTEINS

The DENV polyprotein is cleaved co- and posttranslationally into three structural (C, prM, and E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) proteins. Polyprotein processing proceeds via a combination of signal peptidases, the viral serine protease, and additional cellular proteases (reviewed in reference 122). The limited size of RNA virus genomes necessitates maximization of the coding capacity of genes; thus, many of the DENV structural and nonstructural proteins serve multiple functions in the viral life cycle. Whereas definitive roles for several of the viral proteins have not yet been established, recent work has implicated a number of the DENV proteins in functions beyond vRNA synthesis and proteolysis, including inhibition of interferon (IFN)-mediated signal transduction, and organization of membranous structures.

The flavivirus particle consists of a nucleocapsid core surrounded by an ER-derived lipid bilayer containing E and prM/M (for a review of flavivirus virion structure, see reference 135). The N terminus of the flavivirus polyprotein contains the viral structural proteins, including the two membrane glycoproteins prM and E. prM is processed to the mature M protein late in secretion in the *trans* Golgi compartment by furin (173). This maturation event is necessary to expose the E receptor binding domain and thus for virus infectivity (reviewed in reference 79). prM is believed to protect E from pH-induced reorganization and premature fusion during secretion (65, 66, 201) and possibly to serve as a chaperone for proper E folding and assembly (79). It was shown recently that regulated efficiency of cleavage of prM/M is important for viral replication; enhanced cleavage only moderately improved DENV infectivity but decreased viral egress, possibly by resulting in retention of the virion or its premature fusion in the Golgi compartment, leading to an overall reduction of viral titers (99).

Although there are numerous candidate cellular receptors that mediate viral entry, there is a consensus that the viral E protein effects viral attachment. Flavivirus E is a membrane glycoprotein comprised of three domains: domain I is a centrally located β barrel, domain II contains a dimerization region and the fusion peptide, and domain III harbors the receptor-binding activity. In a mature virion, E exists as homodimers, with the fusion peptide inaccessible (131). Under low-pH conditions, such as those encountered in late endosomes, E undergoes irreversible trimerization to expose the fusion peptide and mediate endosomal fusion in a manner consistent with the class II fusion proteins of alphaviruses (131; reviewed in reference 135). A flexible hinge region between domains I and II, the kl loop, has been shown to be required for structural changes that precede exposure of the fusion peptide (130, 131). DENV E proteins are differentially glycosylated according to serotype and the cells in which the virus is propagated (93, 109, 123, 139, 153), and glycosylation of E has been implicated in receptor binding (90, 123, 139, 153) and endosomal fusion (67, 109). The DENV E glycoprotein is also a major target of humoral immunity.

Of the viral nonstructural proteins, the most extensively characterized are NS3, its cofactor NS2B, and NS5. NS3 harbors a number of catalytic domains, including a serine protease, which requires the cofactor NS2B (7, 54). NS3 also exhibits the nucleoside triphosphatase and helicase functions required for vRNA synthesis (112), as well as 5′ triphosphatase activity (13, 15), which is the first step in the pathway for $^{7}$mG capping. In addition, DENV2 NS3 has been shown to interact with human nuclear receptor binding protein, which modulates intracellular trafficking between the ER and the Golgi compartment, to impact its cellular distribution and to induce some of the membranous structures seen during flavivirus infection (33). NS3 epitopes are commonly found in the repertoire of DENV-specific cytotoxic T lymphocytes.

The flavivirus NS5 protein serves as the viral RNA-dependent RNA polymerase (144, 180), as well as a methyltransferase (49), another essential enzyme in the capping pathway. NS5 has also been demonstrated to induce transcription and translation of interleukin-8 (IL-8), a neutrophil chemoattractant, via activation of CAAT/enhancer binding protein (128). A role for IL-8 in the pathogenesis of DENV infection will be discussed below.

The NS1 glycoprotein is expressed in three forms: an ER-resident form that colocalizes with the viral replication complex, a membrane-anchored form, and a secreted form (sNS1) (reviewed in reference 122). NS1 is glycosylated at two sites, N130 and N207, and glycosylation of both residues was recently shown to be required for viral replication in mosquito cells and for neurovirulence in mice (36). sNS1 is another dominant target of humoral immunity and may play a significant role in the pathogenesis of disease.

Less well characterized are the small hydrophobic proteins NS2A, NS4A, and NS4B. Their hydrophobic nature, as well as complementation analysis, potentially implicates them in proper localization of viral proteins and vRNA to sites of RNA synthesis and virion assembly (reviewed in references 56 and 122). Recently, it has been demonstrated that NS4B and, to a lesser extent, NS2A and NS4A are capable of blocking IFN-mediated signal transduction (136, 137). While the three proteins in combination are most effective, NS4B alone is a potent inhibitor of beta interferon (IFN-β) and gamma interferon (IFN-γ) signaling.

## VIRAL RNA SYNTHESIS

Positive-strand viruses utilize the same template for both translation and genome replication; however, the two processes cannot occur simultaneously. After input strand translation, the virus switches to production of vRNA, which involves a negative-strand template for further generation of the positive strand. Since the positive strand serves as both viral genome and mRNA, it is produced in excess of the negative strand, although the mechanism by which this asymmetric synthesis occurs has not been described.

For DENV, the ability of the vRNA to cyclize is implicated in the step of viral RNA synthesis. The 5′ and 3′ CS domains have been proposed to mediate circularization (71), and disruption of base pairing between the two regions has been shown to compromise vRNA synthesis (86, 199, 200). The interaction between the 5′ and 3′ CS domains was recently shown for DENV2, using atomic force microscopy (3). Circular and linear RNAs were observed among wild-type sequences, but only linear RNAs were detected when CS base-pairing interactions were ablated. Furthermore, changes in the solution structure of the 5′ end of the DENV2 genome were observed when the 3′ UTR was added in *trans* (C. Polacek et al., unpublished). Additional domains in the 5′ and 3′ UTRs, designated UAR for *u*pstream *A*UG *r*egion, were identified and demonstrated to be required for vRNA cyclization, and complementarity between the 5′ and 3′ UARs was determined to be necessary for viral replication (3). While the specific step of the viral life cycle regulated by the UARs has not been established, given its additive effect on cyclization via the CS domains, the interaction may also modulate viral RNA synthesis.

In addition to their roles in viral translation, conserved structures and sequences in the 3′ UTR have been shown to regulate vRNA synthesis. The DENV 3′SL, in addition to its role in translation, is required for RNA replication (86, 199). Similarly, predicted pseudoknot structures within DB1 and DB2 have been demonstrated to enhance RNA synthesis in cell-free replication (199) and replicon systems (2). Interestingly, the VR of DENV2, just downstream of the stop codon, was shown to enhance replication in mammalian cells but had no effect on replication in insect cells (2). The VR is so named for its lack of conservation in both length and sequence even among isolates within the same DENV serotype. A deletion in the VR region of DENV2 decreased synthesis of replicon RNA in BHK21 cells, and a virus variant with the same deletion exhibited delayed growth kinetics in vitro relative to the wild type. In contrast, a DENV1 variant with a 19-nucleotide deletion in the VR was virtually indistinguishable from the parent virus in replication kinetics, plaque morphology, and titer when propagated in Vero, Huh7, and *A. albopictus* (C6/36) cells (178). Additionally, a number of DF clinical isolates that possess deletions in the VR has been identified (35, 145, 178). Maintenance of this region among DENV strains implies that the VR serves a purpose in the viral life cycle; thus, the role of the VR merits further study.

## DENGUE VIRUS EXISTS AS QUASISPECIES

Viral RNA-dependent RNA polymerases are of notoriously low fidelity (reviewed in reference 45); incorporation of mutations into the progeny RNA strand, coupled with the lack of a second strand for proofreading, results in the generation of a cloud of variant viral RNA species, dubbed quasispecies. Although most often associated with chronic infections with RNA viruses, such as hepatitis C virus, it has become clear that acute RNA virus infections also result in significant intrahost sequence diversity. Work with poliovirus has shown that the degree of sequence diversity of the viral RNA genome must be carefully regulated; too many mutations lead to error catastrophe (38), while too few result in reduced pathogenesis (189). Lately, studies focusing on the C, E, and NS2B genes have indicated that DENV also exhibits substantial sequence diversity in humans (192, 193) and to a lesser extent in mosquitoes (118). An intriguing report recently demonstrated that a defective DENV1 lineage was disseminated and maintained in human populations in Myanmar over at least 2 years, not only providing further evidence of intrahost diversity of viral species but also implying complementation of the defective genome by coinfection of cells with functional viruses (1). Now that it has been established that DENV does exist as quasispecies in vivo, the question naturally arises as to whether the degree of intrahost sequence diversity or particular sequence signatures that are not represented in isolated viruses are associated with viral pathogenesis. Large-scale sequencing efforts of full-length vRNAs from human DENV infections are currently under way to address this issue.

## THE PATHOGENESIS OF DENGUE VIRUS INFECTION

While the past decade has witnessed important advances in DENV biology, including structural determinations of the virion and key viral proteins, dissection of replication and translation mechanisms, and functional characterization of viral proteins, the pathogenesis of DENV remains a challenging puzzle, a complex interplay of viral and host factors. Risk factors for severe disease include age (64, 69), viral serotype (11, 64) and genotype (129, 156), and the genetic background of the host (70, 76), among others. Retrospective and prospective human studies have demonstrated that secondary infection by a heterologous serotype is the single greatest risk factor for DHF/DSS (20, 70, 75, 166, 182), although the ability to cause severe disease in primary infection varies by DENV serotype (11, 143, 188). Here we review recent advances that have contributed to our understanding of how the severity of DENV infection is modulated. For an overview of earlier work, we refer readers to several excellent reviews on the pathogenesis and immunopathogenesis of DENV infection (110, 125, 140, 159, 175).

## DENGUE VIRUS GENOTYPE AND PROTEINS IN PATHOGENESIS

Although it has not been possible to establish a clear correlation between a particular DENV serotype or genotype(s) and the severity of disease outcome, there have been indications that certain DENV2 and DENV3 genotypes are associated with DHF versus DF (129, 156, 194). In general, Asian genotypes appear to be more virulent than those initially found in the Americas and the South Pacific. In the case of DENV2, phylogenetic analyses showed that the native American genotype was associated only with DF, whereas the Asian genotypes

were correlated with DHF cases. Subsequent studies demonstrated that Thai DENV2 strains (Asian genotype) replicated to higher titers than American genotype DENV2 strains in human monocyte-derived macrophages (MDM) and DCs (35, 155). Full-length sequencing of Asian and American genotypes revealed several nucleotide differences, particularly at position 390 in the E protein, and in the 5′ and 3′ UTRs (111). Mutation of N390, found in the Asian genotype, to the American genotype D390 was shown to reduce virus output from both human MDM and DC cultures (35, 155). This reduction was enhanced by replacing the Asian 5′ and 3′ UTRs with those of the American genotype (35). In another example, studies of DENV3 genotypes present before and after the emergence of DHF in Sri Lanka in 1989 showed that a distinct subgroup of DENV3 genotype III appeared at the same time that DHF emerged, in the absence of other changes in transmission patterns (129), implicating the introduction of this DENV3 subtype in the etiology of DHF.

Animal studies have provided further evidence for the correlation between viral sequence and virulence. For example, an experimental chimeric vaccine consisting of DENV1 prM and E genes in a yellow fever 17D vaccine strain backbone was reported to have a single change in E (K204R), resulting in decreased replication in cell culture, decreased virulence in mice, and decreased viremia in monkeys (68). Additionally, it was recently reported that a newly derived DENV2 with a cluster of mutations in a single region of E, including K128E, is associated with increased vascular permeability in mice (171a).

Apart from sequence variation, another correlate of disease severity may be the level of virus or viral proteins in the bloodstreams of DF versus DHF patients, which in itself may result from the more successful replication and/or cell-to-cell spread of certain genotypes. A number of studies have demonstrated a positive correlation between peak levels of viremia and development of DHF for circulating serotypes in Thailand (9, 50, 113, 188), Taiwan (191), and French Polynesia (138). Levels of sNS1 in the bloodstreams of patients also correlated with DENV2 viremia and DHF in two Thai studies (9, 114), which suggests that levels of sNS1 could also be related to disease severity, either as a marker for or as a cause of pathology. Avirutnan et al. (9) reported that higher levels of sN1 were present in the plasma of individuals who proceeded to develop DHF than in that of DF patients during the 3 days prior to onset of DHF, indicating that sNS1 may be useful as a prognostic indicator of DHF. This group additionally demonstrated that complement components in normal human serum were activated by the addition of sNS1 and anti-NS1 antibodies, suggesting a directly pathological effect of sNS1-antibody complexes in inappropriate activation of the host immune response. However, other reports of DENV2 outbreaks in Taiwan (28) and Thailand (176) have not upheld a correlation between viremia and DHF, adding to the complexity of associating levels of circulating virus or viral proteins with the development of severe disease. At the time of onset of DHF/DSS, the virus has typically been cleared from circulation, supporting the conclusion that it is the host response to infection that results in DHF/DSS, including a potentially heightened immune response that persists beyond the stage of peak viremia (reviewed in reference 160).

Other viral proteins have been shown to play a less direct role in pathogenesis by promoting viral evasion of protective host responses, thereby allowing for increased viral replication and spread. DENV proteins NS2A, NS4A, and NS4B have been shown to inhibit signal transduction from IFN receptors; in particular, NS4B was shown to reduce STAT1 phosphorylation in response to IFN-β or IFN-γ treatment (137) and to prevent nuclear translocation of a STAT1-GFP fusion protein in response to IFN-β treatment (136). DENV proteins have also been demonstrated to inhibit IFN-α-mediated signal transduction by decreasing phosphorylation of STAT1 and expression of STAT2 (95). In this study, IFN-γ treatment did not block STAT1 phosphorylation, a difference attributed to the use of a human cell line, K562, versus the simian cell line (Vero) employed previously (95, 137). In human monocyte-derived DCs, it was confirmed that DENV inhibits phosphorylation of STAT1, as well as phosphorylation of STAT3 and of Tyk2, another downstream effector of type I IFN signaling (83). The evolution of DENV proteins that block the signal transduction pathways of IFN-α/β and IFN-γ supports a critical role for IFNs in controlling DENV infection.

## ROLE OF INTERFERONS IN HOST IMMUNITY TO DENGUE VIRUS

During acute DENV infection, innate immune responses very likely play a key role in determining disease outcome, particularly during a primary infection (reviewed in reference 140). IFN-α/β, as well as IFN-γ, has been shown to provide protection from DENV infection in vitro, but only when treatment precedes infection (43, 83). Animal models of DENV infection have also underscored the importance of IFNs in protection against DENV in vivo, in that DENV replicates to much higher levels in mice deficient for both IFN-α/β and IFN-γ receptors, and these mice become uniformly susceptible to DENV-induced disease (94, 170). Both STAT1-dependent and STAT1-independent pathways have been implicated in the IFN-mediated response to DENV infection (171).

In earlier clinical studies, significantly higher levels of circulating IFN-α (105) and IFN-γ (106) were measured in Thai children with DF and DHF/DSS than in healthy controls, and higher levels were observed in DHF/DSS patients than in DF patients. Since then, numerous other studies have also reported higher levels of IFN-α (113) and IFN-γ in DF/DHF patients (for recent reports, see references 23, 27, 28, and 142). The critical role of IFNs in protection against DENV pathogenesis is further supported by ongoing microarray studies. In whole blood of Vietnamese children presenting with DHF, upregulation of multiple IFN-stimulated genes has been observed. However, children with DSS who presented at the same timepoints of their illnesses had substantially lower transcriptional activities among the same genes (C. Simmons, M. Hibberd, and J. Farrar, personal communication). In an interesting parallel, adults in Taiwan with DF and DHF who survived infection exhibited significantly higher levels of circulating IFN-γ than controls, but nonsurvivors did not have significantly higher IFN-γ levels (27). These findings suggest that high levels of IFN-α and IFN-γ in response to DENV infection are part of a protective host response and that lower

levels of IFN-γ in the context of severe disease may reflect an inadequate response.

One of the main producers of IFN-γ are natural killer (NK) cells, which are likely to be important in clearing DENV during acute infections. Researchers in Brazil found that increased percentages of NK cells and of activated NK cells were associated with milder DF, whereas low percentages and lack of activation markers (comparable to healthy controls) were associated with more-severe disease (10). In comparison, an earlier study in Thailand demonstrated decreased absolute counts of NK cells in patients with DHF compared to those with DF or controls, along with decreased absolute counts of all subsets of T cells, although in this case early activation of NK and T-cell subsets was positively correlated with DHF (62). In an interesting link between human and animal studies, NK cells were also demonstrated to be activated early during DENV2 infection of mice (169).

IFN-γ-activated macrophages may play an important role in viral clearance, in part through production of nitric oxide (NO), which has recently been demonstrated to inhibit the replication of DENV in vitro (24). Inducible nitric oxide synthase expression in peripheral blood monocytes of DF patients was found to correlate with the late acute phase of disease and preceded the clearance of DENV from monocytes in these patients (141). Overproduction of NO, however, could also lead to endothelial cell damage, and cross-reactive antibodies against endothelial cells were found to induce cell damage in an NO-dependent manner (121). The fine balance that must be struck, in this case involving an antiviral host defense (NO production) on the one hand and potential damage resulting from NO overproduction on the other, is illustrative of a broader theme in DENV pathogenesis: that disease results from the immune response itself.

## ROLE OF ANTIBODIES IN PROTECTION AND PATHOGENESIS

Infection with one DENV serotype results in immunity to that serotype only; the immune response to the primary serotype is cross-protective against other serotypes only during the first several months after infection (162). It is thought that serotype-specific protection is due to neutralizing antibodies, to DENV-specific memory T cells, or to both (reviewed in reference 160). Both polyclonal serum and monoclonal antibodies against E and prM proteins are capable of neutralizing DENV in vitro and, along with antibodies to NS1, of providing protection in vivo (81, 94, 97, 98, 120, 196). Identification of E residues involved in binding to neutralizing and cross-reactive antibodies, as well as the structural interaction of neutralizing and nonneutralizing antibodies with DENV, has been the subject of much research (reviewed in reference 158). Crill and Chang (37) recently mapped the epitope specificity of cross-reactive monoclonal antibodies that interact with domain II of the E protein. However, most studies have focused on defining neutralizing epitope specificity against domain III with the intent of establishing benchmarks for an effective vaccine response and for developing monoclonal antibodies with therapeutic potential. One group has extensively characterized a neutralizing monoclonal antibody, 4E11, which binds to domain III from DENV1. Mutational analysis of 4E11 demonstrated that residues both proximal and distal to antigen binding sites affect the binding affinity of the antibody (14). Four key residues in domain III of the WNV E protein were identified as the epitope for a dominant neutralizing antibody (E16) that is highly effective against infection. This antibody was found to protect mice from WNV encephalitis, even when administered up to 5 days postinfection, demonstrating a potential therapeutic use for the E16 antibody (147). Analogous epitopes in DENV1-4 are currently being analyzed for neutralization capacity (M. S. Diamond, personal communication). Identification of critical neutralizing epitopes in all four DENV serotypes will allow for the development of tools for evaluating the potential efficacies of tetravalent vaccines and their abilities to generate protective humoral responses.

Fundamental to any discussion of DENV pathogenesis is the association of secondary infection with a heterologous serotype with DHF/DSS, which was first documented in Southeast Asia in the 1960s (20, 75, 166). Since then, numerous other studies in Asia and the Americas have confirmed that secondary infection is an important risk factor for severe disease, although there are cases of DHF associated with primary infection (11, 77, 143), and certainly many secondary infections do not cause severe disease. The antibody-dependent enhancement (ADE) model postulates that some DENV-specific antibodies, either cross-reactive antibodies from a previous DENV infection or subneutralizing levels of serotype-specific antibodies, can interact with DENV without neutralizing the virus and thereby use $Fc_{\gamma}$ receptors on monocytes/macrophages to effect increased viral uptake into these target cells. ADE predicts that nonneutralizing antibodies are, at least in part, responsible for the increased risk of DHF/DSS in secondary infections (reviewed in reference 73). Previous studies had linked ADE in vitro to DHF in DENV2 infections (103), but efforts to reproduce this correlation recently did not yield similar results (107). However, methodological issues may be at the heart of this controversy (19, 61, 72). A computer simulation of ADE predicted that viruses associated with this enhancement phenomenon have increased fitness, particularly in populations with substantial transmission of and immunity to multiple serotypes (39). This is proposed to be to the result of increased transmission of DENV strains that are not susceptible to cross-protective immunity and instead are actually enhanced by the preexisting immune status of the host.

In addition to serotype cross-reactive antibodies, other work has focused on documenting that anti-DENV antibodies cross react with platelets, clotting factors, and endothelial cells in humans and mice (53). In particular, cross-reactive antibodies may contribute to pathogenesis during DENV infection by targeting platelets for destruction (51, 88, 146, 163). Anti-NS1 antibodies have also been demonstrated to bind and induce apoptosis in endothelial cells, as well as to induce secretion of proinflammatory cytokines and chemokines, implicating anti-NS1 antibodies as additional mediators of the increased vascular permeability seen in DHF/DSS (116, 117).

## CELLULAR IMMUNITY AND CYTOKINE PRODUCTION IN DENGUE IMMUNOPATHOLOGY

The immunopathogenesis of DENV probably involves both circulating antibody from previous infections, anamnestic B-

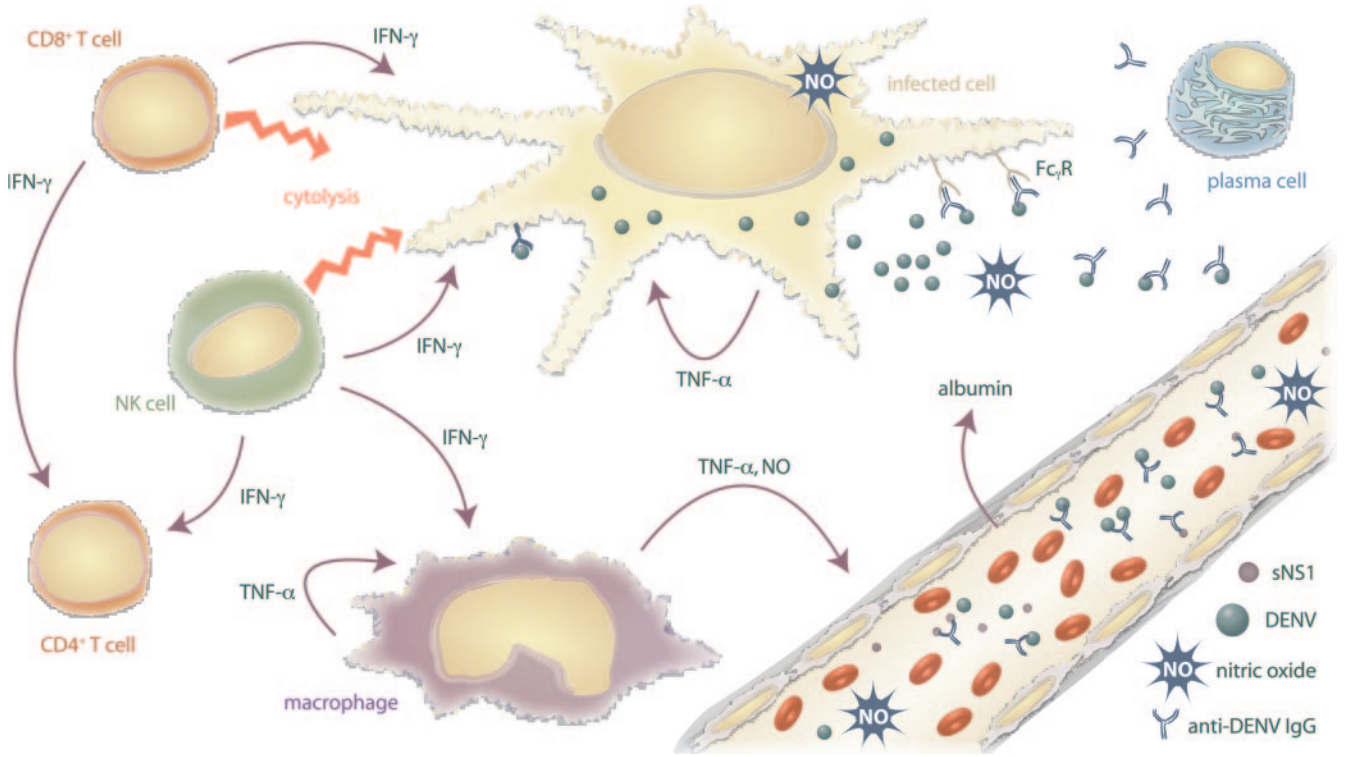


FIG. 3. Pathogenesis of dengue virus infection. DENV initially infects a cell of the dendritic cell/macrophage/monocyte line (reviewed in reference 5) via receptor-mediated endocytosis and/or enhanced uptake via antibody-virus complexes attached to $Fc_\gamma$ receptors (reviewed in references 5 and 73). TNF-α (22, 52) and NO (24, 141) are produced primarily by infected monocytes/macrophages and activate endothelial cells, which can contribute to increased vascular permeability (reviewed in reference 17). Changes in vascular permeability in DENV infections have classically been measured by monitoring levels of albumin in the plasma (195). IFN-γ is produced primarily by NK and $CD8^+$ T cells and activates macrophages as well as $CD4^+$ T cells (17). High levels of DENV and sNS1 circulate in the bloodstream (9, 114), and both have been shown to circulate as immune complexes as well. $Fc_\gamma R$, Fc gamma receptor.

cell and T-cell responses, and the effects of cytokines on both infected and bystander immune cells, hepatocytes, and endothelial cells (Fig. 3). The model of "original antigenic sin" involving memory T-cell responses postulates an inappropriate immune response to a secondary infection due to clonal expansion of cross-reactive memory T cells specific for the previous rather than a current infection, resulting in delayed viral clearance and/or increased cytokine secretion. There is evidence that this may occur during secondary DENV infections (133), and recent work has identified DENV epitopes that, in the context of specific HLA types (genes encoding major histocompatibility complex molecules), may be associated with immune enhancement (134, 172, 202). Other studies have more broadly correlated certain HLA types with disease severity and/or protection from severe disease (102, 174). Defining the link between disease risk and HLA type, race (70, 76), or receptor (164) and cytokine (55) polymorphisms has the potential not only to provide important information regarding the pathogenesis of secondary DENV infection but also to serve as a prognostic tool to identify individuals at increased risk for severe disease.

Whether or not a result of antigenic sin, cytokines are believed to play a direct role in the immunopathogenesis of DENV, due specifically to their proinflammatory effects on vascular endothelial cells. Tumor necrosis factor alpha (TNF-α), the primary cytokine implicated in the pathogenesis of DHF/DSS, acts locally to activate macrophages, but its systemic release may be responsible for the increase in vascular permeability seen in DHF/DSS (6). Serum from patients with acute DENV infection, known to contain increased TNF-α levels, was demonstrated to increase ICAM-1 expression (a marker of activation) in endothelial cells in vitro, and this effect was blocked by antibody to TNF-α (21). DENV proteins may directly induce TNF-α, as NS1 expressed in DCs was reported to interact with STAT-3β and result in increased secretion of TNF-α and another proinflammatory cytokine, IL-6 (32), which is involved in the induction of fever and of increased production of acute-phase proteins from the liver (reviewed in reference 17). TNF-α is released from DENV-infected monocytes (52), and endothelial cell monolayers show enhanced permeability in response to supernatants from DENV-infected macrophages, although the release of TNF-α and virus from the macrophages did not directly correlate with changes in permeability in this study (22). The association of TNF-α with disease severity has been confirmed independently in two mouse models of dengue infection (8, 171a). In both cases, increased TNF-α levels were detected in sick animals and antibodies to TNF-α attenuated the disease phenotype. Given the evidence of increased TNF-α in DF/DHF patients (18, 23, 27, 84) and the combined evidence of in vitro and animal studies, TNF-α is strongly implicated in the pathogenesis of DENV.

A number of cytokines besides the IFNs and TNF-α have been implicated in DHF/DSS, most commonly IL-6, IL-8, and IL-10. IL-10 is an anti-inflammatory cytokine with pleiotropic effects involved in the feedback loop down-regulating inflammatory responses (reviewed in reference 17). IL-10 has been found in some (27, 28, 63, 150), but not all (142), studies to be significantly elevated in DHF versus DF patients. One investigation correlated high levels of IL-6, IL-10, and the proinflammatory macrophage migration inhibitory factor with a fatal outcome in adult DHF patients, compared to those in surviving DHF patients (27). Infection of DCs in vitro by DENV was shown to induce multiple chemokines, including IL-8 (128), a chemokine that attracts neutrophils and may activate monocytes (reviewed in reference 25). IL-8 mRNA levels were increased in peripheral blood mononuclear cells from DF patients in the days just before defervescence and were even higher among those from DHF patients. As mentioned previously, IL-8 expression may be modulated by the viral protein NS5 (128). Infection of endothelial cell monolayers by DENV was shown to result in IL-8 release, cytoskeletal rearrangements, and increased permeability, the latter being partially mitigated by treatment with antibody against IL-8 (179). DENV infection was further shown to induce secretion of tissue plasminogen activator from endothelial cells, and this increase was blocked in the presence of antibodies to IL-6 (89). The combined data describing levels of cytokines in circulation and the response of endothelial cells to direct or indirect infection by DENV supports the role of cytokine-mediated changes in vascular permeability during DENV infections. However, the specific actions of various cytokines and chemokines and their relations to disease severity remain to be fully defined.

Reports concerning levels of various cytokines in DENV infection are abundant; however, TNF-α is clearly implicated in increased vascular permeability, and IFN-γ responses appear to be important in protection and viral clearance. In support of these associations, cytokine levels in $CD4^+$ T cells from individuals receiving a monovalent DENV vaccine showed a higher ratio of TNF-α- to IFN-γ-producing cells when stimulated with antigens from a heterologous serotype than from the homologous serotype (127). These results suggest a model of increased TNF-α production from cross-reactive T cells versus beneficial IFN-γ production from serotype-specific, presumably protective T cells, underscoring the need for development of a tetravalent vaccine that provides robust T-cell immunity against all four serotypes. Further studies on the multifactorial immunopathogenesis of DENV regarding the role of T cells and cytokines may be possible using disease models of DENV infection in mice (4, 16, 26, 149, 171a).

## PROGNOSTIC INDICATORS OF SEVERE DENGUE INFECTION

In the absence of conclusive evidence linking cause and effect, some markers for severe disease may nonetheless prove useful as prognostic indicators. For example, urinary levels of heparan sulfate (195) and plasma levels of pentraxin 3 (126), an acute-phase protein, were both significantly increased in children with DSS, whereas plasma levels of albumin were decreased (195). Additionally, levels of vascular endothelial growth factor were significantly elevated in patients with DHF compared with those in DF patients or control patients (185). Levels of soluble vascular cell adhesion molecule 1, evidence of endothelial cell activation, were found to be significantly elevated in DSS patients compared to those in DF patients, and levels among DSS patients were higher than in DHF patients, although the latter did not reach statistical significance (104). Finally, levels of plasminogen activator inhibitor type 1 at admission were associated with a fatal outcome (124). Once validated, these and other plasma and urine markers for disease severity may serve as useful tools in clinical management of DENV infection and may shed light on the mechanisms of pathogenesis.

## CLINICAL APPLICATIONS AND FUTURE DIRECTIONS

As more is learned about the biological characteristics of DENV infection, one continuing objective is to relate this knowledge to the clinical features of disease. A potential complication in attempting to correlate biological cause with clinical effect involves the current controversy regarding the definition of disease severity. The DHF/DSS classification scheme has indeed been very useful over the years, but many investigators and clinicians report severe presentations of confirmed dengue infection that do not precisely fit the DHF/DSS definitions (10, 12, 77, 152), leading to a growing recognition of the need for an alternative classification scheme(s) (40, 157). Clearly, the definition of severity will impact the study of any parameter of interest or correlate of disease severity, be it a prognostic indicator or a determinant of pathogenesis.

Over the past several years, greater awareness of the public health impact of DENV has led to increased focus on development of tetravalent vaccines (74, 87, 159) and antiviral therapies (reviewed in reference 161). The challenge now is to apply the knowledge gained in understanding viral replication and unraveling the complexity leading to pathogenesis in order to prevent and control dengue and its severe manifestations.

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