PATHOGENESIS OF DENGUE VIRAL DISEASES

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Abstract. In recent efficacy trials of a dengue vaccine candidate, about 7-15% of volunteers in the control group manifested at least one of the three dengue hemorrhagic fever (DHF)-defining abnormalities (hemorrhage, thrombocytopenia, and plasma leakage) during symptomatic dengue virus infection. A high risk of developing DHF observed in secondarily infected persons is related to an increased viral burden. Complex interactions between structurally heterogeneous viral particles and pre-existing antibodies specific for the viral envelope glycoproteins, E and prM, are thought to play a role in enhancing virus replication. Concomitant high level of circulating NS1, a virally encoded glycoprotein that is essential for viral RNA replication, may cause plasma leakage through its direct and indirect effects on the vascular endothelial cells. Cross-reaction of anti-NS1 antibodies generated during dengue virus infection with platelets may lead to thrombocytopenia. Dengue serotype cross-reactive T cells rapidly expand during the secondary infection, and their levels in the circulation are associated with the development of DHF. However, the role of T cells in mediating an increase in vascular permeability at the tissue level remains unclear.

Keywords: dengue, dengue hemorrhagic fever, pathogenesis, virus

INTRODUCTION

Diseases caused by dengue virus infection remain a major public health problem. Recent estimates of the global burden of dengue suggest that about 390 million dengue infections occur every year, and 96 million are symptomatic (Bhatt *et al*, 2013). The great majority of dengue infected persons are found in Asia, where outbreaks of dengue hemorrhagic fever (DHF) have been reported since 1954. There are four serotypes of dengue viruses, which are often co-circulating, particularly after the year 2000 (Messina *et al*, 2014). A highly effective vaccine and a licensed specific antiviral agent are not yet available, necessitating a better understanding of dengue virus biology and the pathophysiologic mechanism.

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This review focuses on selected recent findings, and the readers are referred to the following recent reviews for more information (Diamond and Pierson, 2015; Olagnier *et al*, 2016; Souza *et al*, 2016; Yacoub *et al*, 2016; Yam-Puc *et al*, 2016).

The four dengue virus serotypes are classified by *in vitro* cross-neutralization of virus infectivity by employing sera of infected individuals. There are about 30-40% differences in the nucleotide sequence of the viral genome among these serotypes. Infection by a dengue serotype induces prolonged immunity against the infecting serotype, while cross-protective immunity against other serotypes is of limited duration. In the localities where multiple dengue serotypes co-circulate, repeated infections may occur and can result in diverse clinical outcomes.

Following an initial dengue virus infection, the plaque-reduction neutralization test (PRNT) antibody titer determined early after the infection episode provides an indicator of protection against symptomatic re-infection (Katzelnick *et al,* 2016). Among Thai children, the PRNT titers required for protection against local strains that circulated

during 1994-1997 appear to be uneven between persons who had been infected with each of the four dengue virus serotypes, ranging from about 100 for serotype 3-to-300 for serotype 4 and 600 for serotypes 1 and 2 (Clapham *et al,* 2016). The finding that, following an initial infection by serotypes 1 or 2, higher antibody titers are required for the protection against subsequent infection is consistent with comparatively lower efficacies of a tetravalent chimeric yellow fever-dengue vaccine in the prevention of symptomatic dengue caused by these two serotypes in Asian and Latin American children (Capeding *et al,* 2014; Villar *et al,* 2015).

HOW COMMON ARE DHF-DEFINING ABNORMALITIES IN CHILDREN WITH DENGUE?

A range of illnesses that occur during dengue virus infection have been classified originally as undifferentiated fever, dengue fever (DF), DHF of various severities, and expanded dengue syndrome/ isolated organopathy (unusual manifestations) (WHO SEARO, 2011). This classification does not reflect differences in disease severity, particularly between DF and DHF, but represents a syndromic approach in grouping clinical manifestations with distinct underlying pathophysiologic bases.

The pathological abnormalities in DHF include hemorrhage, thrombocytopenia, and plasma leakage. In recent phase III clinical trials of a tetravalent live-attenuated chimeric dengue vaccine candidate in children in Asia and Latin America, a number of volunteers in the non-vaccinated control group experienced symptomatic dengue virus infection during a two-year prospective follow-up period. Among these infected volunteers, up to 15% manifested at least one of the three DHF-defining abnormalities (Capeding *et al*, 2014; Villar *et al*, 2015).

Plasma leakage with clinical signs and marked thrombocytopenia were found at about 4-9% of infected Asian control volunteers. The proportions of children with these abnormalities are consistently lower among infected Latin American volunteers compared with their Asian counterparts (Capeding *et al*, 2014; Villar *et al*, 2015). The disparity may reflect a higher mean age and the greater proportion of children with previous exposure to dengue viruses that manifested as higher baseline PRNT antibody titers among Latin American volunteers. However, other factors may also be involved. These factors include genetic predispositions of the human host, difference in the proportion of circulating dengue virus serotypes, variations in the virus replicative ability, and associated viral burden, as well as virus-host interactions.

DENGUE VIRUS HETEROGENEITY

Recent studies on the intratypic and intertypic diversities of dengue viruses indicate that the four canonical dengue virus serotypes are antigenically more heterogeneous than was previously thought (Katzelnick *et al*, 2015). A series of neutralization test employing large panels of human sera along with temporally and geographically diverse dengue viruses indicated that some dengue virus strains are as different from other strains of the same serotype in their susceptibility to antibody-mediated neutralization as a number of strains of different serotypes (Katzelnick *et al*, 2015). Such diversity is observed despite a clear clustering of members of the same serotype when nucleotide sequence differences were compared. Drastic changes in the susceptibility to antibody-mediated neutralization of virus infectivity, therefore, can occur with non-exceptional levels of sequence variation. This finding may explain why some persons can be infected twice with dengue viruses of the same serotype (Waggoner *et al*, 2016), and why different sequential infections contribute dissimilarly to an altered risk of developing DHF in diverse localities (OhAinle *et al*, 2011).

Structural analyses of dengue virus particles reveal a mixture of immature, partially mature, and mature particles that co-exist in the extracellular compartment, particularly during virus replication in mosquito cells (reviewed in Lok, 2016). An ineffective cleavage of prM by cellular furin enzyme during virus export results in particles with differences in the arrangement of the surface glycoproteins, E and prM. In immature particles and

the 'immature' patch of partially mature particles, three non-covalently linked prM-E heterodimers assume a knob-like protrusion with the receptor-binding domain III of the E protein at its base and the fusion loop at the tip of E domain II hidden by the pr portion of prM. Following cleavage of prM and the release of the pr peptide from extracellular particles, mature particles and the 'mature' patch of partially mature particles display a flat orientation of the head-to-tail E homodimers in which the E domain III is more readily accessible. Association of the E proteins in the homodimeric complex results in formation of E dimer-dependent epitope at the former pr-binding site of E dimer that can be recognized by broadly neutralizing antibodies (Dejnirattisai *et al*, 2015; Rouvinski *et al*, 2015). Also, other epitopes are present in mature particles that are dependent on the quaternary structure formed between adjacent E dimers (Lok, 2016). Particles of different maturation levels are, therefore, variably recognized by antibodies specific to the two surface glycoproteins and differ in their susceptibility to the neutralizing and infection-enhancing potentials of antibodies.

HIGH VIRAL BURDEN IN DHF

Early studies demonstrate higher viremia during the febrile phase in children with DHF than those with DF (Vaughn *et al*, 2000; Libraty *et al*, 2002). Similarly, higher NS1 antigenemia in a period prior to defervescence correlates with subsequent development of DHF (Libraty *et al*, 2002). These findings raise a possibility that virus and/or virus-encoded product(s) are directly involved in the pathogenesis of DHF. Conversely, different components of the immune system may be involved by contributing to an increase in viral burden, or by responding to the high viral burden in such a way that leads to DHF.

Epidemiological studies suggest that secondary infection is a risk factor for DHF (reviewed in Guzman *et al*, 2013). The role of antibody-dependent enhancement of dengue virus infection in increasing viral burden and virus-infected cell mass during secondary infection has been proposed (Screaton *et al*, 2015). Recent studies have found structural heterogeneity of dengue virus particles that affect their inherent ability to infect receptor-expressing host cells as well as 'enhanced' infections of FcγR-expressing leukocytes mediated by IgG antibodies recognizing different viral envelope proteins (reviewed in Flipse *et al*, 2013). In addition to the well-known role of anti-E antibodies in infection enhancement, cross-reactive anti-prM antibodies may contribute to an enhancement of virus infection during natural infection as they are commonly detected in sera of dengue virus-infected persons, are generally non-neutralizing, and are able to enhance infection of FcγR-expressing leukocytes by prM-containing immature and partially mature viral particles (Dejnirattisai *et al*, 2010).

ROLE OF T CELLS IN THE "IMMUNE-MEDIATED" PATHOGENESIS

Adaptive immunological responses to viral antigens and virus-infected cells have been proposed to underlie pathophysiologic derangements observed in DHF. As dengue viruses share common B and T cells epitopes in many viral proteins, cross-reactive T cells that have been primed during the primary dengue virus infection readily expand during the secondary infection. Higher viral burden in DHF cases is likely to result in greater magnitude of activated B and T lymphocytes independent of the type of infection. Indeed, during both primary and secondary dengue virus infections, activated virus-specific T cells are detected in the circulation of DHF cases at higher frequencies than that of their DF counterparts (reviewed in Screaton *et al*, 2015).

Higher proportions of T cells secreting interferon gamma and tumor necrosis factor are found in DHF patients, whereas T cells expressing the degranulation phenotype are more common in DF cases (Duangchinda *et al*, 2010). These results indicate that, in addition to an expected quantitative difference, there is also qualitative difference in dengue virus-specific T cell activity between these two disease entities. However, the current evidence for the temporal association between elevated level of circulating activated T cells and the onset of hemoconcentration is

still lacking (Dung *et al*, 2010). A recent study found that dengue virus-specific T cells migrate to skin during the acute phase of dengue virus infection, but their levels did not correlate the development of DHF or severe dengue (Rivino *et al*, 2015). Whether such activated, virus-specific T cells causally mediate an increase in vascular permeability at the local tissue level in DHF cases remains to be established.

Many cytokines and chemokines are found at different levels in DHF and DF cases, but their direct role in the pathogenesis of DHF is far from clear. More recently, serotonin, known to be involved in platelet aggregation and activation, is found to be decreased significantly in DHF, whereas kynurenine, an immunomodulator, increases significantly in DHF (Cui *et al*, 2016). This is consistent with their proposed roles in causing thrombocytopenia and immunopathology in severe cases of dengue virus infection.

ROLE OF NS1 IN THE 'VIRUS-MEDIATED' PATHOGENESIS

NS1 is a virally encoded nonstructural glycoprotein involved in viral RNA replication. Structural analysis reveals distinct domains with affinity for lipid bilayer, complement components, and homotypic interaction (Akey *et al*, 2014). In dengue virus-infected cells, NS1 dimers localize to viral replication complexes within the cytoplasm and on the cell surface. NS1 is secreted from infected mammalian and mosquito cells into extracellular compartment as homohexameric complexes (Alcala *et al*, 2016) that can bind a number of lipids and serum proteins, including complement components, as well as cell surface molecules, but the role of extracellular NS1 in virus multiplication *in vivo* remains unknown.

In dengue virus-infected persons, variable levels of circulating NS1 are detected initially during the febrile phase, persisting for up to several days, and can be modulated by the serotype of infecting dengue viruses and the sequence of infection (primary *vs* secondary) (Duyen *et al*, 2011). A high level of circulating NS1 early in the illness correlates with subsequent development of DHF during dengue type 2 virus infections (Libraty *et al*, 2002). It is likely that NS1 plays an important role in the pathogenesis of dengue as it is well established that active immunization with NS1 prevents illness in virus-infected mice, and antibodies to NS1 given passively protect mice against lethal virus challenge (reviewed in Muller and Young, 2013; Amorim *et al*, 2014; Akey *et al*, 2015). Partial protection against dengue in recipients of the chimeric yellow fever-dengue virus vaccine observed in Phase III clinical trials is thought to reflect in part a lack of dengue virus NS1 in this chimeric vaccine, which may be unable to induce adequate level of protective immunity against dengue (Screaton *et al*, 2015; Halstead, 2016).

During dengue virus infection, circulating antibodies to NS1 are frequently detected. NS1 shares common epitopes with many cellular proteins, and anti-NS1 antibodies can bind platelets, proteins of the coagulation cascade, and endothelial cell surface (reviewed in Amorim *et al*, 2014). Interference of the function of platelets and coagulation system by anti-NS1 antibodies has been proposed to represent an autoimmune mechanism that leads to thrombocytopenia and bleeding tendency that are observed in dengue cases. Recently, Wang *et al* (2017) reported an increase in the proportion of anti-dengue ENV and anti-NS1 IgG1 antibodies that lack fucosylated glycans in their Fc portion during the early phase of dengue virus infection.

While the proportion of afucosylated anti-dengue ENV IgG1 antibodies was higher in DHF cases as compared with DF cases and their levels correlated with the extent of thrombocytopenia, these anti-ENV antibodies did not bind platelets. Instead, anti-NS1 IgG antibodies cross-reacted with platelets and likely mediated a reduction of circulating platelets upon transfer of IgG from thrombocytopenic patients into FcR-humanized mice. This platelet-lowering effect was dependent on two leukocytes' Fc receptors, FcγRIIA and FcγRIIIA; the latter is known to bind afucosylated IgG1 antibodies more strongly than other IgG molecules (Wang *et al*, 2017). In addition, induction of endothelial cell apoptosis initiated by

direct anti-NS1 antibody binding to endothelial cell surface and/or complement-mediated endothelial cell damage following anti-NS1 antibody binding to surface-bound NS1 molecules may result in an increased vascular permeability. However, the significance of these autoimmune mechanisms needs to be further substantiated by *in vivo* experiments.

Recent studies have revealed a direct stimulatory effect of NS1 on monocytes/ macrophages and vascular endothelial cells (Beatty *et al*, 2015; Modhiran *et al*, 2015). Purified NS1 that is derived from over-expressing insect cells and that is devoid of bacterial lipopolysaccharide interacts directly with Toll-like receptor 4, inducing the secretion of pro-inflammatory cytokines from monocytes/ macrophages. NS1 also disrupts endothelial cell monolayer integrity *in vitro* and triggers an increase in endothelial permeability and vascular leakage in the mouse model (Beatty *et al*, 2015; Modhiran *et al*, 2015). Alteration of vascular permeability and shock in this model can be prevented by vaccination with NS1, the injection of anti-NS1 antibodies, or a Toll-like receptor 4 antagonist (Beatty *et al*, 2015). Moreover, dengue virus NS1 may cause vascular leakage via the induction of autophagy in endothelial cells following the release of macrophage migration inhibitory factor (Chen *et al*, 2016). These results implicate a direct effect of NS1 glycoprotein as a virus-mediated mechanism underlying the pathogenesis of dengue.

CONCLUSION

DHF occurring during secondary dengue virus infection may result from a complex interaction between primarily and secondarily infecting dengue viruses and the host immune system. Low level of cross-reactive antibody from the primary dengue virus infection could enhance virus replication during the secondary infection, leading to a higher viral burden and an increased risk of developing DHF. Rapid expansion of cross-reactive T cells in response to secondarily infecting virus may potentially contribute to an enhanced local production of inflammatory cytokines. Circulating NS1 affects vascular permeability and induces pro-inflammatory cytokines secretion from leukocytes via the interaction with Toll-like receptor 4, and NS1 may contribute directly to plasma leakage in DHF patients. Additionally, cross reactivity of anti-NS1 antibodies with platelets in the presence of infection-associated modification of Fc-linked glycans represents a mechanism that could lead to thrombocytopenia.

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