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Abstract
The dengue virus (DENV) with its four unique serotypes is transmitted by the *Aedes* mosquito vector in tropical countries worldwide. All serotypes can cause illness ranging from asymptomatic, self-limiting flu, Dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). DHF and DSS are characterized by a sudden increase in vascular permeability due to a unique set of cytokines released by DENV-infected monocytes. The method by which DHF/DSS arises is largely accepted to be due to Antibody Dependent Enhancement (ADE) whereby upon secondary infection with a different serotype, the immune system generates non-neutralizing antibody-antigen complexes that permit a greater number of viruses to enter monocytes via Fc-receptors than DENV alone. These DENV-infected monocytes release cytokines resulting in DHF/DSS, the severe and life-threatening forms of the disease. Although the number of people that develop potentially fatal DHF/DSS is relatively small, the effect of all symptomatic DENV infections on Dengue endemic areas is impetus for the large-scale prevention of viral transmission. Given the serotype specific immunity to DENV infections and the possibility of ADE-induced DHF/DSS, prevention of DF is critical. There is currently no Dengue vaccine for the estimated 2.5 billion people at risk of infection; however vaccines under development are being designed to provide protective immunity against all four DENV serotypes to minimize the devastating consequences of ADE-induced DHF/DSS.

Introduction

Dengue is considered the most common and most important mosquito-borne viral disease in the world today. Recently, this disease has become a major international public health concern. There are an estimated 2.5 billion people at risk to contracting the dengue virus, and up to 50 million infections and 22,000 deaths annually, the majority of which are children (WHO 2007). The dengue virus (DENV) is an arbovirus that causes dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) in humans. DENV belongs to the family *Flaviviridae,* a family of viruses that include the viruses that cause yellow fever, St. Louis encephalitis, West Nile fever and Japanese encephalitis. The dengue virus is transmitted by the female *Aedes* mosquito, notably *Aedes aegypti* and *Aedes albopictus (*Figure 1). There are many characteristics that make *Aedes* a very efficient vector for viral transmission including its feeding and living habits. The *Aedes* mosquito is a daytime feeder that prefers human blood and is capable of biting several people during one blood meal. They are also well adapted to urban settings and breed in stagnant waters found in containers, such as discarded tires, cans and other trash. Distribution and incidence of DENV has increased 30-fold in the last 50 years due to increased mosquito distribution, increasing urbanization, poor living conditions with inadequate mosquito control and increased travel (WHO 2007; Guzman and Kouri 2001). The dengue virus is transmitted and prevalent in tropical and subtropical countries worldwide and due to the reproductive characteristics of the mosquito vector itself, DENV epidemics express a strong seasonality that coincides with the rainy season.

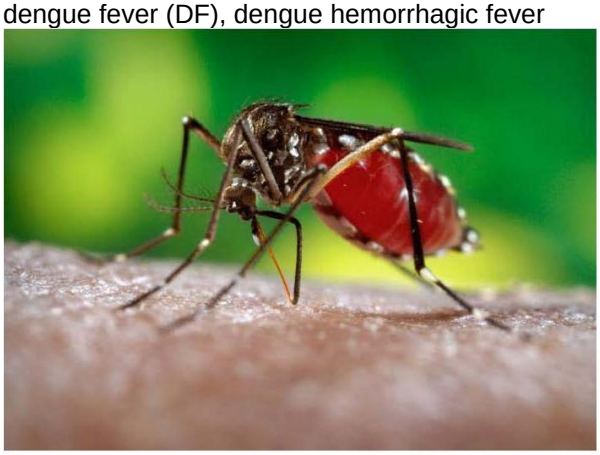

Figure 1. Female Aedes aegypti mosquito in the midst of acquiring a blood meal from her human host. (Photo Credit: James Gathany, 2006. Content Provider(s): CDC/ Prof. Frank Hadley Collins, Dir., Center for Global Health and Infectious Diseases, Univ. of Notre Dame)

Epidemiology

DENV is composed of positive single strand RNA and four serotypes, known as DENV-1, 2, 3, and 4. Each virus serotype elicits specific immunity against the same homologous serotype but does

not provide immunity against the other three heterologous serotypes (Halstead 2007). The 11 kb DENV genome encodes three structural proteins: the core nucleocapsid protein (C), the membrane protein (M), and the envelope glycoprotein (E) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) (Guzman and Kouri 2001). The Viral E protein of the dengue virus contains the sites required for virus binding, penetration into susceptible cells, hemagglutination of erythrocytes, neutralising antibody induction and protective immune response (Gubler and Kuno 1997). The major envelope (E) protein is a large (~500 amino acids in length), cysteine rich, multifunctional protein that is involved in receptor recognition, membrane fusion and virion morphogenesis. This E protein therefore contains multiple, serotype-specific, conformation-dependent, neutralizing epitopes with an Ig-like domain that plays a role in binding to the Fc receptors of host monocytes (Khanam et al. 2006).

The dengue transmission cycle, as shown in Figure 2, begins when an *Aedes* mosquito bites

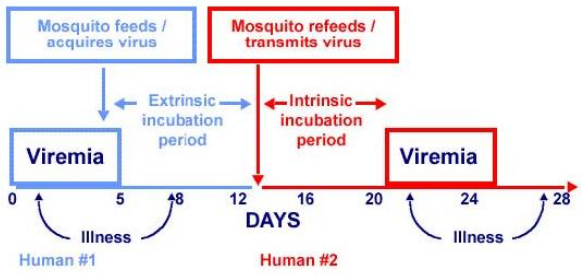

Figure 2. Transmission of Dengue Virus by Aedes aegypti (Source: CDC Division of Vector Borne Infectious Diseases 2007)

a person infected with the dengue virus. A recently infected individual is normally viremic for about five days following infection, during which time the virus can be transmitted to other mosquitoes during a blood meal. Dengue viruses replicate in the *Aedes* female during the extrinsic incubation period of 8-12 days. Once the extrinsic period is complete, the next time that the mosquito feeds she is able to infect another individual with the newly replicated viruses and will continue to infect through bloodfeeding for the rest of her life (CDC 2007). The virus is now able to replicate inside its primary human host during an intrinsic incubation of 4-7 days. The viremia stage begins just before the manifestation of Dengue Fever symptoms which may last 3-10 days if the individual experiences his/her primary encounter with the Dengue Virus.

Pathogenesis

Infection by DENV can result in asymptomatic or flu-like symptoms, classical Dengue Fever, Dengue Hemorrhagic Fever, or Dengue Shock Syndrome. The most common form of DENV manifestation is a form of asymptomatic or undifferentiated fever which results in a fairly prevalent 'silent transmission' of the virus between populations (Burke et al. 1988; Halstead 2007). The clinical characteristics of classical Dengue Fever (DF) include fever, headache, muscle and joint pain, nausea/vomiting, rash and hemorrhagic manifestations (WHO 2007). Dengue fever is also known to induce intense retro-ocular pain, a maculopapular rash and skin eruptions (Rigau-Perez et al. 1998). Although DF can be extremely debilitating during the acute phase, patients are generally able to recover fully from the illness.

During the viremic phase, diagnosis is possible only by clinical signs or isolation of the virus. To this end, reverse transcriptase PCR has permitted development of several protocols which have greatly enabled molecular diagnosis of dengue infection by DENV serotype (Guzman and Kouri 2001). PCR has provided for dengue diagnosis with sera, tissue, mosquito pools, mosquito larvae and inoculated cell cultures.

The World Health Organization has established four necessary criteria for the diagnosis of Dengue Hemorrhagic Fever (DHF). These include an acute fever, hemorrhagic manifestations, low platelet count (100,000/mm^3 or less), and objective evidence of plasma leakage due to an increase in vascular permeability (WHO 2007). DHF hemorrhage may take the form of petechaie, epistaxis, ecchymosis, gingival or gastrointestinal bleeding and hypermenorrhea (Gould and Salomon 2008; Guzman and Kouri 2001). Plasma leakage caused by dengue viral infection is seen as an extravasation through endothelial gaps without inflammation or necrosis of the capillary endothelium (Gubler and Kuno 1997). Plasma leakage is the key difference between DF and DHF and results in DHF patients requiring intravenous fluids. Dengue Shock Syndrome (DSS) is actually a further manifestation of DHF whereby a patient exhibits all of the four criteria for DHF and shows evidence of circulatory failure. Clinical signs of impending shock include a progressively rising haematocrit and decreasing platelet count accompanied with sudden, intense abdominal pain, vomiting, lethargy and a change from fever to hypothermia (Rigau-Perez et al. 1998). Symptoms of circulatory failure include

weak or rapid pulse, hypotension or frank shock due to a systemic loss of plasma (CDC 2007). DHF and DSS patients must be administered intravenous fluids to counteract the increased vascular permeability and plasma leakage. DHF/DSS mortality can range from 12-44% and prognosis often depends on how early shock is identified and treated (Rigau-Pérez et al. 1998).

Immunopathogenesis

The pathophysiology which causes some patients with DF to develop DHF and/or DSS remains controversial although several theories have gained substantial support. It is accepted that individuals who have immunity to one of the DENV serotypes, either through naturally acquired immunity or maternal antibodies, are at higher risk of DHF and/or DSS with a secondary infection to another DENV serotype. In the majority of DHF/DSS cases this phenomenon is explained by the Antibody Dependent Enhancement (ADE) theory. When infected by DENV for the first time, an individual's immune system is able to mount a response to clear the virus, creating homologous antibodies specific to the invading serotype of the virus 5-6 days post infection (Halstead S 2007). Homologous antibodies bind to their pathogen and mark it for ingestion and destruction by phagocytes through a process known as opsonization. Neutralization can be completed through both partial and complete opsonization of the viral antigens with homologous IgG antibodies through inhibition of membrane fusion (van der Schaar et al. 2008). Viral particles are then degraded and disposed of in the cell's lysosomes. Pathogen clearance is further aided through activation of the complement system, a biochemical cascade whose end products amplify immune response and comprise the cytolytic membrane attack complex (MAC). MAC forms a transmembrane channel through a pathogen causing lysis through osmosis. Homologous antibody viral clearance provides lifetime immunity against that one serotype for the individual due to the presence of memory B and T cells. Upon secondary infection with a different serotype, these same DENV IgG antibodies have enough affinity to bind to the heterologous antigen, but are non-neutralizing and therefore unable to clear the virus from the system (Halstead 2007). These non-neutralizing antibody-antigen complexes can enter a greater proportion of monocytes than the virus alone through host-cell Fc Receptor binding via the Fc component of IgG. Antibody-dependent enhancement is the process in which unique serotypes of dengue virus, associated with non-neutralizing antibodies, are able to infect a greater number of mononuclear cells. The virus is then able to replicate unchallenged within these monocytes resulting in significantly increased viral production and corresponding to a massive infection. The DENV envelope (E) protein allows specific targeting of cells of the immune system that possess Fc receptors, such as monocytes, macrophages, dendritic cells, mast cells and hepatocytes, where the virus can reproduce (Chareonsirisuthigul T et al. 2007). The mechanism through which the dengue virus escapes the endocytic degradation pathway is currently unknown. One theory is that the nonneutralizing antibodies dissociate from the Dengue viral E-protein once inside the acidic lumen of the endosome, allowing the virion to infect the cell through membrane fusion (van der Schaar et al. 2008). *In vitro* studies have shown that DENV membrane fusion is triggered by an exposure to low pH which is concordant with the internal endosomal acidity (Heinz et al. 2004). Through ADE infection, the non-neutralizing heterologous antibodies enhance the entry of the virus into the very cells in which it can replicate, resulting in a significantly enhanced viral titer. It is important to note that although ADE has never been shown in vivo, it has been demonstrated in vitro with non-neutralizing but cross reactive antibodies (Gould and Salomon 2008).

After DENV infection, these monocyte cells produce cytokines and vasoactive mediators which correlate with the severity of DHF/DSS symptoms (Chaturvedi et al. 2000). Cytokines are soluble proteins produced by cells involved in adaptive and innate immune systems that act as chemical messengers for regulating immune responses. In Th1 immune responses, CD4+ Th1 cells secrete IL-2, IFN-γ and TNF-β (Tumour Necrosis Factor β) and result in cell-mediated immunity and inflammatory responses, while Th2 cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13 which are responsible for humoral immunity and B cell antibody production . Th2 responses are primarily mediated by the secretion of IL-10 while Th1 is regulated by IFN-γ. In a number of parasitic, viral, fungal and bacterial infections, a Th1 response is linked to recovery, while a Th2 response has been shown to result in severe pathology and exacerbation of the illness (Abbas et al. 2007). Recent studies have shown that a shift from Th1 in patients with DF to a Th2 response correlated with the development of DHF/DSS pathology (Chaturvedi et al. 2007). In dengue ADE infections, IL-10 was dramatically upregulated which in turn suppressed IL-12 and

INF-γ and therefore inhibited the activation of macrophages, dendritic cells and T cells through a Th1 immune response (Chareonsirisuthigul et al. 2007). Th2 responses in DHF patients have also demonstrated elevated levels TGF-β which may act as a pro-inflammatory or an anti-inflammatory agent depending on concentration. TGF-β also inhibits Th1 cytokines, especially IL-12 and enhances Th2 cytokines such as IL-10 (Chaturvedi et al. 2000). Suppression of the intracellular production of antiviral chemokines has also been shown in ADE infections. Antibody-facilitated dengue virus entry into monocytes suppressed the expression of nitric oxide synthase which is responsible for the production of nitric oxide, NO (Chareonsirisuthigul et al. 2007). NO is normally produced by macrophages and plays a key role in immune system clearing of viral infections. NO results in the suppression of viremia by blocking viral genome synthesis and viral proteases (Charnsilpa et al. 2005). By suppressing the production of NO, ADE prevents the immune system from producing one of its most potent viral defense products and allows the virus to continue replicating. ADE infection also results in the excessive production of IL-6 which stimulates B-cells to differentiate into plasma producing cells and produce antibodies (Restrepo et al. 2007).

Cytokines	DF	DHF
IL-1 β	no change	no change
IL-2	+++	+
IL- 4	-	+++
IL-6	+	+++
IL-8	-	+++
IL-10	-	+++
IL-12	+++	-
IL-13	-	+++
IL-18	+	+++
TNF-α	+++	+++
INF-γ	+++	+
TGF-β	-	+++
hCF	+	+++

Table 1: Changes in key cytokines with Dengue Fever (DF) and Dengue Hemorrhagic Fever (DHF). + increase , +++ marked increase, - decrease. Source: Chaturvedi et al. 2000.

This overproduction of IL-6 results in B-cells pumping out non-neutralizing antibodies which could further contribute to the uptake of the replicating virus into macrophages through ADE and thereby compounding the severity of the infection. Current research has identified a unique cytokine produced during severe DENV infections, human cytotoxic factor or hCF. A recent study found that 95% of DHF cases have peak levels of hCF while individuals diagnosed with DF did not demonstrate hCF production (Chareonsirisuthigul et al. 2007). Human CF causes increased capillary permeability and damage to the blood brain barrier leading to cerebral edema and exacerbating DHF/DSS pathogenesis. Human CF also stimulates macrophages to produce free radicals and reactive oxygen species. Besides killing the target cells through apoptosis, these free radicals also stimulate the production of pro-inflammatory cytokines. The ultimate factor switching the immune response from one of the Th1 to the Th2 pathways seems to be hCF produced by CD4+ helper T cells upon secondary infection with a heterologous DENV serotype as levels of hCF seem to peak on the 2nd day of infection in those who develop DHF (Chaturvedi et al. 2000). The acute inflammatory response seen in patients with DHF/DSS is due to the production of pro-inflammatory cytokines IL-1, IL-6 and TNK-K (Chareonsirisuthigul et al. 2007). Abnormally high levels of IL-8 produced by macrophages may be

responsible for the intravascular coagulopathy seen in DHF patients (Chaturvedi et al. 2000).

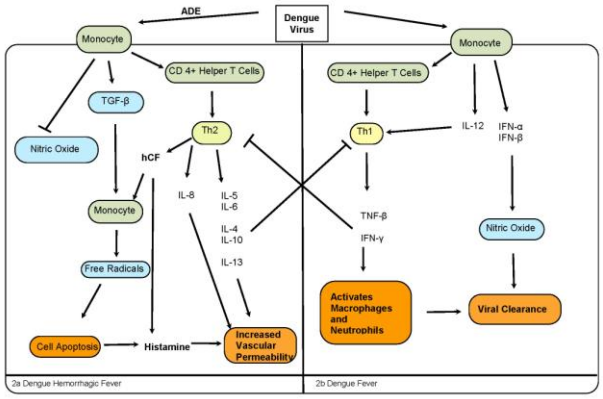

Figure 3. Figure 3 proposes the immune system's respective responses to primary (2b) or Antibody Dependent Enhancement (2a) infections with DV. 2a demonstrates the effect of cytokines and chemokines produced through a Th2 response on the pathogenesis of DHF. 2b demonstrates the effect of cytokines and chemokines produced through a Th1 response on the pathogenesis of DF.

High local levels of IL-8 have been found in patients with severe pleural infusions suffering from DHF/DSS and could therefore be a major cause of plasma leakage (Raghupathy et al. 1998). Vascular permeability is increased due to the combined production of hCF, histamines, free radicals, pro-inflammatory cytokines and products of the complement system. Extensive plasma leakages in body cavities (pleural, pericardial and peritoneal) can result in profound shock and death. The significant differences in cytokine production seen in DF and DHF infections (see Table 1, Figure 3) seem to be responsible for the differences in pathogenicity and severity of these two DENV manifestations.

Alternative theories for DHF/DSS pathogenesis

While most DHF/DSS infections occur in patients with secondary DENV infection or in infants with circulating maternal antibodies, some DHF/DSS cases have been identified in patients with primary DENV infections and in children lacking maternal antibodies. Alternative theories for DHF/DSS pathogenesis include viral virulence factors and molecular mimicry (McBride and Bielefeldt-Ohmann 2000; Rico-Hesse 2007). Some investigators postulate that the virulence of certain strains are sufficient enough to trigger DHF in certain individuals (Rico-Hesse 2007; Wearing and Rohani 2006). Molecular mimicry occurs when the immune system produces antibodies that are cross-reactive to self peptides, resulting in an abnormal autoimmune response and subsequent pathology. This phenomenon can arise when the genetic sequence of an invading antigen is similar enough to that of endogenous protein that the immune system makes cross-reactive self antibodies. While cross-reactive antibodies have been detected in children infected with DENV, no relationship between the presence of molecular mimicry and DHF/DSS pathology has been found in humans (Chungue et al. 1994).

Viral virulence is the theory that the pathogenic severity resulting in clinical DF or DHF/DSS is determined largely by the virulence of a variant strain of DENV (McBride and Bielefeldt-Ohmann 2000; Wearing and Rohani 2006). This could provide an explanation for the higher risk of developing DHF/DSS through secondary infection with DENV-2 as compared to other serotypes (Rico-Hesse et al. 1997; Rico-Hesse et al. 1998). Each DENV serotype exhibits unique genetic variances that have been postulated to correspond with viral virulence. These factors may involve the virus' ability to infect cells, generate more progeny, evade the host cell's defense mechanisms, or the ability to provoke increased production of inflammatory cytokines (McBride and Bielefeldt-Ohmann 2000). While this could explain some of the inconsistencies concerning cases whereby disease severity seems to strongly correlate with DENV serotype, the lack of a suitable model organism for Dengue makes studying virulence difficult and potentially flawed.

Furthermore, it is also possible that as yet unidentified individual host susceptibility factors influence a DHF/DSS that could provide further alternatives to ADE.

Future Treatments and Prevention

Due to the strong correlation between IL-12 levels and Th1 vs. Th2 immune responses, research concerning the use of IL-12 as an antiviral drug is currently underway (Restrepo et al. 2007). There is hope that IL-12 injections could prevent the switch to Th2 immune response and therefore prevent the development of DHF or DSS. The development of a dengue vaccine remains elusive despite years of work. While dengue infection becomes increasingly common, a relatively small proportion of those infected develop severe DHF/DSS. Since there is no way to predict which patients with DF will progress to DHF/DSS, it is necessary that efforts move towards preventing infection altogether or ensuring strong protection against all DENV serotypes. Based on the Antibody Dependent Enhancement theory, neutralizing antibodies against each of the four

serotypes are required to avoid Dengue Hemorrhagic Fever and its manifestations. An ideal vaccine would have to be tetravalent, providing immunity to all four of the serotypes, and cost-effective in order to permit its use in the millions at risk in developing countries. Potential vaccine development is now focused on recombinant strategies, geared towards developing four monovalent vaccines targeting single DENV serotypes and then creating a tetravalent vaccine by physically mixing the four monovalent components into a single formulation (Khanam et al. 2006). Tetravalent vaccine candidates currently in development include mixtures of four different live attenuated viruses, recombinant live attenuated viruses, protein subunit vaccines and DNA vaccines (Normile 2007; Halstead 2002). Another potential future treatment would be the development of a vaccine that targets hCF, the main pathogenic factor of DHF and DSS, and thereby targeting the cytokine instead of the antigen itself (Chaturvedi et al. 2000). While this obviously wouldn't garner broad immunity to all of the DENV serotypes and therefore protection from DF, DHF and DSS, this vaccination approach could prevent the development of life- threatening DENV complications such as increased vascular permeability leading to shock and potential death. Researchers have also targeted the DENV major envelope (E) protein as a potential field for vaccine development. The structural conformation of this E protein is crucial for Dengue viral transmission through entry into vulnerable host cells. On the Ig-like E protein domain three, there is a β-OG hinge region that is essential for the conformational changes that allow the DENV antigen to bind to monocyte Fc receptors and subsequent entry into host cells for viral replication (Laile and Roche 2004). By blocking this β-OG hinge region with a small inhibitory molecule, a vaccine could prevent the dengue virus from performing its conformational changes necessary for host cell entry, thereby severely limiting DENV transmission and preventing viremia. A vaccine targeted towards preventing the entry of DENV into host cells could be useful as both a prophylactic treatment for those traveling to DENV-endemic areas and for those routinely challenged by Dengue.

One of the foremost challenges for DENV research is the lack of a model for dengue disease. While models exist to study viral replication and the development of neutralizing antibodies, there are no models for DHF/DSS pathogenesis (Johnson AJ and Roehrig JT 1999; Rico-Hesse 2007).

Until a dengue vaccine is available for those in DENV endemic regions, vector control is the most important method for reducing dengue transmission and reducing the risk of DHF/DSS. It is therefore crucial that the importance of controlling dengue transmission be recognized and established at all levels of society and reiterated through political support, public health and educational campaigns and active community participation (Gubler and Kuno 1997).

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