Dengue Virus Evolution and Virulence Models

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Dengue virus transmission has increased dramatically in the past 2 decades, making this virus one of the most important mosquito-borne human pathogens. The emergence of dengue hemorrhagic fever in most tropical countries has made its control a public health priority, but no vaccines or treatments exist. Little is understood about dengue virus pathogenesis, because no other animals develop symptoms of disease, and research, therefore, has been limited to studies involving patients. Although epidemiologic and evolutionary studies have pointed to host and viral factors in determining disease outcome, only recently developed models could prove the importance of viral genotypes in causing severe epidemics. The influence of host immune status and mosquito vectorial capacity are also being tested in mathematical models to determine virus population dynamics. Therefore, new technologies are allowing us to better understand how specific virus variants cause more disease than others, and these virus variants should be targeted for detection, control, and treatment.

DENGUE VIRUS EPIDEMIOLOGY

Four different antigenic groups or serotypes of dengue virus are transmitted to humans by mosquitoes, mainly Aedes aegypti. These vectors are day-biting mosquitoes that preferentially feed on humans, taking multiple blood meals (from 1 or several human hosts); they breed in containers and are closely associated with human dwellings, thus transmitting virus at higher rates in urban settings. Dengue viruses are currently transmitted in >100 countries, following the distribution of this efficient vector, and persons at risk of infection number in the billions. The World Health Organization has estimated that 50–100 million cases of dengue virus infection occur yearly, with >500,000 cases that require hospitalization and >15,000 deaths [1]. Increasing disease around the world is attributable to many factors but is largely the result of an increase in human populations, increased urbanization, and decreased support for vector control programs, with all of these factors converging in tropical and subtropical areas [2]. Although dengue is not a national notifiable disease in the United States, the Centers for Disease Control and Prevention have confirmed an increasing number of cases of travel-associated dengue in US residents and autochthonous transmission in Texas and Hawaii [3–5]. Nearly as many cases of travel-associated dengue were identified in 2005 (96 cases) as were identified during the preceding 5 years combined (98 cases) [6]. Thus, for clinicians in the United States, it is becoming evident that dengue should be considered in a differential diagnosis when there has been exposure to mosquito bites in any tropical or subtropical area of the world.

DISEASE PATHOGENESIS

Infection by dengue virus can lead to a broad spectrum of outcomes, including an asymptomatic or mild, nonspecific febrile disease, known as classical dengue fever, with 5–7 days of fever, headache, retro-orbital pain, and rash; or dengue hemorrhagic fever (DHF), with multiple bleeding abnormalities, thrombocytopenia, increased vascular permeability, and, possibly, signs of circulatory failure, leading to dengue shock syndrome (DSS). Mortality rates vary from 1% to 10% and are higher in patients as hemorrhagic symptoms worsen, but they are also higher in some countries as a result of lack of clinical experience with management of systemic vascular leakage. Because the same individual may be infected up to 4 times by heterologous virus serotypes, it was noted that those experiencing >1 infection have a much higher propensity of developing hemorrhagic disease. This led to the hypotheses that immune enhancement of disease occurs as a result of antibodies formed against the first infecting virus, which do not neutralize heterologous virus serotypes, and/or as a result of increased...
cytokine release by antigen-presenting cells, endothelial cells, and T cells of the immune system [7, 8]. Also, the genetic background of the human host or other underlying diseases have been hypothesized to increase disease pathogenesis [9, 10]. However, there are numerous documented cases (beginning in the 1970s) of hemorrhagic dengue occurring after primary infection, thus also pointing to differences in viral virulence as a factor in pathogenesis. Recent studies have shown that the first targets of dengue virus infection—dendritic cells and macrophages [11, 12]—have a great influence on the amount of virus replicating and, presumably, circulating in each patient and can thus determine the difference in viral load that seems to correlate with disease outcome [13–15]. The latter 3 articles [13–15] also discuss specific examples of hemorrhagic dengue or DHF occurring after primary infection. Therefore, it seems that both viral genetics and host immune status are major factors in determining the outcome of infection, as has been shown recently for other viruses [16, 17].

**Dengue Virus Evolution**

Although the role of viral genetic variants had been implicated in dengue severity many years ago [18, 19], it was not until the combined application of viral evolution and epidemiologic analyses that dengue virus evolutionary trees (or “phylogenies”) were developed that indicated that specific genotypes within a serotype are associated with disease of greater or lesser severity [20–23]. Thus, for serotypes 2 and 3, the genotype originating in Southeast Asia and the genotype originating in the Indian subcontinent, respectively, have been identified as causing more outbreaks of DHF and/or DSS and more primary infections with severe disease. In certain areas, such as Peru, epidemiologic studies have demonstrated that, even upon secondary infection, patients do not develop DHF and/or DSS when circulation of only less pathogenic genotypes occurs [24]. The more virulent genotypes have also replaced native or autochthonous genotypes in the Americas, leading to more-severe epidemics, with the first appearance of DHF on this continent [25]. The mechanisms leading to increased viral virulence are under investigation, but there is no evidence of increased mutation rates or recombination in creating new variants or genotypes, and the currently identified virulent genotype of serotype 2 (Southeast Asian) has been stably replicating since the 1940s (figure 1); for example, the New Guinea C strain was isolated in 1944, and strain 16681 was isolated in Thailand in 1964. In fact, the Southeast Asian virus has been displacing the less virulent (i.e., not associated with DHF and/or DSS, but still causing dengue fever) American genotype in many countries. In areas of northern Mexico bordering Texas (Reynosa, Mexico, which borders McAllen, Texas, and Matamoros, Mexico, which borders Brownsville, Texas), the last documented circulation (i.e., isolation from a patient or mosquito) of the less virulent, American-origin virus was in 1995, whereas by the end of 2005, the more virulent Southeast Asian virus had displaced it, as in other countries in the Americas (figure 1). The Southeast Asian virus had already been isolated from patients from southern Mexico (Oaxaca State) in 2000. The last reported isolations of the American genotype of serotype 2 were in Peru in 1996, and there is no evidence of the Southeast Asian genotype causing outbreaks in that country. Therefore, it awaits to be seen if dengue transmission in Texas will soon be exclusively of the Southeast Asian genotype of serotype 2 (along with other serotypes, including the virulent genotype of serotype 3, which is found in Mexico [26]) and whether we will see a concomitant increase in DHF cases in the United States.
NEW TECHNOLOGIES

The advent of new laboratory methods has helped to decipher many aspects of dengue virulence in vitro and in vivo; these techniques have also been applied to patient samples, thereby elucidating some of the processes that lead to increased pathology. The availability of RT-PCR in clinical situations has helped to determine the presence of viral RNA in patient samples, such as blood, liver, ascites, or CSF samples. In autopsy specimens, viral RNA can be detected in numerous organs, and viral proteins can be detected in sections using immunohistochemistry (with antidengue monoclonal antibodies) [27]. RT-PCR can also be modified to quantify very small amounts of viral RNA (using quantitative RT-PCR or a TaqMan probe), and this has helped to determine the importance of viral load in dengue pathogenesis [15]. Other modifications of RT-PCR include the use of reaction primers that specifically target either the positive strand of the virion genome or the negative strand that is formed during active viral replication; this helps to determine if specific tissues foster viral amplification that could lead to pathology (e.g., replication in dendritic or endothelial cells). Specific cytokines and chemokines can also be detected in patient blood or tissue samples, either by using RT-PCR to detect cellular mRNA coding for these proteins or by using ELISA to detect the proteins themselves, although the latter method is less sensitive than the former one. Quantitative RT-PCR has also been used to detect viral RNA in mosquito tissues, thereby helping our understanding of how the vector amplifies and transmits different virus genotypes at differing rates [28].

The determination of the viral RNA nucleotide sequence from various areas of the dengue genome has now become more routine, and numerous research and national reference laboratories around the world have contributed to the large collection of viruses that have been compared. The use of RT-PCR on small serum or plasma samples and the sequencing of the amplified nucleotides from the viral envelope gene can help to determine the origin of the infecting virus and, sometimes, its potential to cause more-severe disease (as in the case of serotypes 2 and 3). The comparison of these nucleotides and their derived amino acid sequences can be done with sophisticated computer algorithms that can tell us much about the rates and sites of mutation or evolution in the viral genome. These rates and genome sites can then be matched with patient viral loads, diagnoses, outbreak characteristics (e.g., the presence or absence of DHF), and transmission distribution (e.g., affected population sizes). Other computer algorithms can now be used to estimate the effect of changing host immunity or mosquito transmission on the amount of virus circulating and the risk of exposure to a hypothetical human population [29–31]. These models have suggested that dengue virus cross-serotype immunity and mosquito vector demographics, rather than immune enhancement, are the most important determinants in the dynamics of specific serotype cycles or genotype replacement during epidemics. However, refinement of these models awaits specific data obtained from laboratory and ecological studies, while better animal models of disease pathogenesis are being developed (see New Models of Disease).

Fluorescence-activated cell sorting has also allowed for identification of types of cells and quantities of dengue virus proteins expressed on their surface, thus helping to determine virus tropism and replication rates in cultured primary human cells and patient tissue samples [11, 12, 32]. This technique has been very useful in determining lymphocyte and specific T cell subpopulations in patient blood at different stages of disease and after inoculation with experimental vaccines. It has also helped identify dengue virus receptors on human monocytes and dendritic cells that could potentially be major determinants in the mechanisms of immune enhancement of disease, as well as good antiviral targets [33–36].

NEW MODELS OF DISEASE

Although researchers have been trying to find models of dengue virus virulence and/or pathogenesis (i.e., in vitro or in vivo) for the past 6 decades, little progress has been made because of the fact that even our closest relatives, nonhuman primates, show no signs of disease after virus inoculation [37]. These models are especially important for determining levels of attenuation of potential vaccine preparations and whether specific antiviral drugs will inhibit viral replication and symptoms in humans. Some of the more extensively used nonhuman primate models have necessarily only given information regarding the toxicity of vaccine preparations; there are no good assays for dengue vaccine efficacy, because upon virus inoculation or challenge, there is usually only a transient, low viremia and no other signs or symptoms in monkeys. In fact, because of the possibility of inducing subneutralizing levels of antibodies and immune enhancement of disease in human vaccinees, multivalent vaccine preparations might cause more disease after natural dengue infection [38]. Also, in the context of dengue viral evolution, the use of viruses isolated directly from patient samples for most of these models has been stressed, to derive information that represents reality, rather than laboratory-derived artifacts; virus cultivation or passage in cell culture (or in other organisms, such as mosquitoes, mice, or monkeys) has been shown to select for viruses with altered phenotypes. Therefore, virulence and disease models have necessarily become more complex and include the use of primary human cell cultures, viral infectious clones that have been modified to include specific viral structures [39], whole mosquitoes to study differences in viral replication [40], and stem cell–engrafted severely immunocompromised [41] and IFN-deficient mice [42] to study dengue pathogenesis after inoculation with different viral genetic variants.
Differences in replication in primary human monocyte and dendritic cell cultures, using dengue virus infectious clones of Southeast Asian virus substituted with specific regions of an American genotype virus, have pointed to 3 viral genome regions (3’ untranslated, 5’ untranslated, and amino acid position 390 in the envelope protein) as possible determinants of higher replication and, therefore, viremia in patients [39]. This potential for increased viremia and higher dissemination of Southeast Asian strains in humans and mosquitoes has also been linked to a higher probability of human infection, transmission, and eventual ecologic displacement, as explained in 2 studies in the computer models discussed above will provide a better understanding of the effects of the numerous and complex factors involved in ecologic displacement of one dengue virus by another.

The 2 mouse infection systems mentioned above show higher rates of dengue virus replication and some human-like signs of disease that can be used to measure the effectiveness of drug and vaccine preparations, albeit in a limited fashion. However, the ultimate goal is to develop a model that shows symptoms or markers of hemorrhagic fever, so that the mechanisms of immunopathology can be measured and elucidated, along with the relative contributions of viral genetic determinants. Thus, newly derived mouse strains that can be engrafted with human hematopoietic cells and develop an adaptive immune system show promise in studies of sequential dengue virus infection; it is anticipated that these animals could show signs of DHF and/or DSS.

**CONCLUSIONS AND RECOMMENDATIONS**

Because several dengue virus genotypes have shown the potential to cause more-severe disease by indirect association, it now falls on laboratory researchers to find new models to measure the effects of viral genetic differences in disease. The development of these models has proven to be difficult, but new technologies, especially the use of human stem cells from non-invasive procedures (e.g., cord blood cells from natural childbirth) and better engraftment of mice that develop adaptive humoral (antibodies) and cellular (differentiated B and T cells) immune responses to dengue virus, have great promise in elucidating the role of specific cells and the various host and viral factors leading to severe disease. This field also depends on worldwide monitoring of dengue virus variants, including input from clinicians, epidemiologists, and entomologists, so that specific associations with clinical presentation and ecologic conditions can be made (especially for serotypes 1 and 4, for which little data exist). The renewed transmission of dengue virus in the United States, after large epidemics in the past [43], has added a sense of urgency to research on the distribution of this virus around the world.

**Acknowledgments**

I thank Drs. Jorge Muñoz and Justin Anderson for 2005 vius sample and sequencing.

**Financial support.** National Institutes of Health (AI50123 to R.R.-H.).

**Potential conflicts of interest.** R.R.-H.: no conflicts.

**References**