Dengue Virus Virulence and Transmission Determinants

R. Rico-Hesse

Contents

Abstract The mechanisms of dengue virus (DENV) pathogenesis are little understood because we have no models of disease; only humans develop symptoms (dengue fever, DF, or dengue hemorrhagic fever, DHF) and research has been limited to studies involving patients. DENV is very diverse: there are four antigenic groups (serotypes) and three to five genetic groups (genotypes) within each serotype. Thus, it has been difficult to evaluate the relative virulence or transmissibility of each DENV genotype; both of these factors are important determinants of epidemiology and their measurement is complex because the natural cycle of this disease involves human-mosquito-human transmission. Although epidemiological and evolutionary studies have pointed to viral factors in determining disease outcome, only recently developed models could prove the importance of specific viral genotypes in causing severe epidemics and their potential to spread to other continents. These new models involve infection of primary human cell cultures, "humanized" mice and field-collected mosquitoes; also, new mathematical

R. Rico-Hesse
Southwest Foundation for Biomedical Research, San Antonio, TX 78227, USA
e-mail: rricoh@sfbr.org

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models can estimate the impact of viral replication, human immunity and mosquito transmission on epidemic behavior. DENV evolution does not seem to be rapid and the transmission and dispersal of stable, replication-fit genotypes has been more important in the causation of more severe epidemics. Controversy regarding viral determinants of DENV pathogenesis and epidemiology will continue until virulence and transmissibility can be measured under various conditions.

1 Introduction

Dengue virus (DENV) pathogenesis seems to be determined by numerous, interacting factors: viral virulence, host immunity and immune status, host genetics and possibly others (e.g., preexisting diseases). Because we have no models of severe dengue disease (DHF), all associations of viruses with increased pathogenesis have been indirect and painstakingly slow in being developed. Transmissibility has also been measured indirectly: the successful isolation of viruses from patients and the preponderance of one serotype over another have been documented in numerous countries but this has also introduced biases in our sampling. We do not have available a fully representative set of DENV genomes to study and understand what truly constitutes the natural range of DENV variation; many samples from mosquitoes or less-ill human infections are missing, in addition to those from countries lacking the laboratory and public health infrastructure necessary for detecting and isolating viruses. Virus-mosquito interactions also add a layer of complexity to the determination of which genotype is being transmitted and we are only beginning to measure the effects of this selection. However, many new methods have been applied to the study of DENV genetic variation, replication fitness and their effects on transmissibility and pathogenesis in humans. None of these methods are perfect and we must still regard them as surrogates for measurements of the natural viral determinants of disease. Thus, understanding this complex system will probably require multidisciplinary approaches to solving the mysteries of the interaction of the many factors that contribute to DENV epidemiology. Other, nonviral factors contributing to DENV pathogenicity and transmission are discussed in accompanying chapters.

2 Controversies Over Virulence and Evolution

The first descriptions of DENV virulence differences came from epidemiologic and entomologic studies done in the South Pacific by Rosen and Gubler, in the 1970s (Gubler et al. 1978; Rosen 1977). It was noted that some outbreaks in this region had fewer or no cases of DHF and the transmitted viruses were considered of low virulence; other outbreaks had many cases of DHF, after primary infection and these viruses were therefore more virulent. However, it took the development of RNA nucleotide sequencing techniques and the use of these sequences to generate phylogenetic trees of evolutionary relationships among viruses to discover that

specific variant groups, or genotypes, were more frequently associated with dengue epidemics and severe disease (Chungue et al. 1995; Lanciotti et al. 1997; Lanciotti et al. 1994; Messer et al. 2003; Rico-Hesse 1990; Rico-Hesse et al. 1997). More recently it has been shown that some genotypes associated with DHF have been introduced and become established (endemic) in other continents, sometimes displacing the less-virulent DENV already being transmitted in those regions (causing DF only). "Virulent" genotypes have been described for serotypes 2 and 3 and it remains to be seen if further evolutionary studies will pinpoint similar groups in serotypes 1 and 4 (Rico-Hesse 2003).

In the case of DENV, there is no evidence for rapid evolution and selection as in HIV, influenza or SARS viruses; for DENV, man-made ecologic disruption or increments in the number of mosquitoes or hosts are more important than evolution towards more virulent genotypes. There has yet to be evidence for the circulation of a recombinant DENV and those recombinant genomes described to date have been the product of enzymatic amplification techniques and are thus probably lab artifacts (Aaskov et al. 2007; Holmes and Twiddy 2003; Worobey et al. 1999); no one has isolated and fully characterized a recombinant DENV that is being transmitted in nature, causing disease. Although there is evidence for recombination in other, positive-strand viruses, specific steps in DENV replication might keep this event from occurring, although ample opportunities seem to exist in multiply-infected humans and mosquitoes (Monath et al. 2005). Also, there is no evidence for the epidemic transmission of the sylvatic genotype viruses from West Africa or Malaysia. These older, seemingly less-virulent viruses are transmitted mainly by canopy-dwelling mosquitoes to monkeys and they do not cause outbreaks in the human populations inhabiting those areas. The viruses isolated from dengue patients during outbreaks belong to genotypes imported from other continents (in the case of serotype 2, a genotype originating in the Indian subcontinent was introduced to Africa) (Rico-Hesse 2003). Some researchers, with ample field experience in tropical areas, believe that these zoonotic cycles will eventually disappear, because of the constant reduction of natural forests; thus, these cycles have practically no importance as reservoirs of human dengue (Rodhain 1991).

Although we have not detected increases in replication fitness for any given genotype, some of the evolutionary events leading to more virulent strains seem to have already occurred and by the time these viruses rapidly spread from Southeast Asia to other areas of the world (1940s), they were already virulent or replicatively fit (Gubler 2002). We have yet to measure any specific genetic changes that are fixed in the viral population as virulent genotypes are successfully dispersed to other continents and we do not know whether there have been any changes imposed by selection in their new environments (e.g., for serotype 2, Southeast Asian genotype introduced into the Americas; for serotype 3, Sri Lankan genotype III introduced to the Americas). That is, these viruses had increased fitness at their origin, in that they are directly linked to the appearance of DHF and they are transmitted more efficiently by mosquitoes. However, some researchers believe differences in clinical presentation and severity of epidemics are a function of only immunologic and genetic differences between the human populations in both

continents (Southeast Asia and Americas) (Halstead 2006) or that nonneutralizing antibodies formed during DENV infection play a role in gradually selecting for more pathogenic viruses in humans (Morens and Fauci 2008).

3 Controversies Over Transmission

Of special concern lately has been the effect of global warming on the incidence and spread of dengue disease. Although there are probably no effects on DENV replication in humans, if environmental temperatures rise, many investigators presume there might be an effect on virus transmission by mosquitoes. This is derived from the fact that increases in temperature (along with increases in rainfall) directly affect mosquito development (from larval to adult stages) and their populations can increase dramatically. Also, increases in average temperatures in new climes might make conditions favorable for mosquito breeding and the establishment of new populations. This could surely lead to more chances of exposure to mosquito bites for the human population and thus for infection by mosquito-borne viruses. However, the reason why many mosquito-borne diseases have yet to affect large populations in developed nations seems to be a lack of exposure to mosquito bites; air-conditioning and human behavior have been shown to reduce DENV transmission in the southern United States (Reiter et al. 2003). That is, human activities and their impact on local ecology have generally been more significant in increasing dengue prevalence; thus, dengue disease seems to be influenced more by economic than climatic factors (Gubler et al. 2001; Reiter 2001). Also, research described below has shown that increases in temperature might have more of an impact on selecting for those virulent genotypes that are already being transmitted by mosquitoes. Mosquito survival rates and the time it takes for a virus to infect and be transmitted by the mosquito seem to be more important in this context.

Another subject that has received renewed attention is the possibility of human modification or management of viral virulence by impacting transmission dynamics. This is important for the application of rapid public health measures in the event of the emergence of new strains of parasites, bacteria or viruses (Lipsitch and Moxon 1997). We presume we can influence virulence by the application of changes in human habits or by the use of control factors such as vaccines – and there are numerous precedents of how public health measures have changed the population dynamics of microorganisms (e.g., there are now more cases of vaccine-induced polio or yellow fever in some countries). Efforts to understand the relationship between parasite adaptation to hosts, virulence and transmission have developed into a small industry in evolutionary biology (Bull and Dykhuizen 2003). Although most discussion is still theoretical, the relationships between virulence and transmission have been weighed, presuming that there is an evolutionary trade-off for optimizing either factor within an organism (Ebert and Bull 2003). That is, to increase its chances of transmission to another host, an organism will limit its replication or virulence in so far as to not kill its host. For example, highly virulent,

zoonotic viruses such as Ebola and rabies are less transmissible than measles or common cold viruses, which rarely kill their human host. However, this is overly simplistic when it comes to the evolution of viruses that have multiple strains (multiple infections, with some cross-protection), where virulence involves immunopathology, or where there is another host or an amplifying vector involved in transmission (Day et al. 2007). Thus, the main goal for disease control is to understand how we can reduce virulence in a virus population without making its level of transmission higher. However, this seems a daunting task if we include the effects of evolution by individual versus group selection, bottlenecks and changes in fitness trade-offs. The concern here is whether we will shift DENV evolution and population dynamics by applying incomplete control strategies (e.g., nonsterilizing vaccination or vector transformation).

4 Methodologic Advances and Findings

The controversies mentioned above point to factors we should consider or understand in the control of dengue disease: evolutionary studies of DENV have helped us concentrate on detection (diagnosis and sampling), analysis (genetic and phenotype) and control (transmission dynamics in host and mosquitoes) of those genotypes already shown to be the culprits (Rico-Hesse 2007). The methods described below could help us reach these goals.

4.1 Rapid Sequence Analysis

The determination of viral RNA sequences from different areas of the genome has now become routine and numerous laboratories around the world have this capability; this has added exponentially to the number of DENV samples available for comparison in GenBank. The comparison of these nucleotides and their encoded amino acids 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 data can then be matched with patient viral loads, diagnoses, outbreak characteristics and transmission distribution, to look for specific associations. The RT-PCR technique has allowed for the enzymatic amplification of these sequences from very small amounts of viral RNA from almost any type of tissue but many researchers have now avoided virus isolation and characterization, thus introducing mistakes in some of the banked information (e.g., from amplification, sequencing, or cloning artifacts) and without information on viability or antigenicity. Therefore, it is also important to have access to classic virology techniques, especially if one is to derive information about virus phenotype.

The comparisons of full genome sequences of many DENV have helped pinpoint differences that could be involved in virulence: the comparison of viruses

from two different genotypes associated with DHF or DF only (Southeast Asia and Americas, respectively) showed that there were consistent differences in the 5′-untranslated region (UTR), one envelope protein site (aa390) and the 3′-UTR of the DENV serotype 2 genome (Leitmeyer et al. 1999). Comparisons within the Southeast Asian genotype did not identify any specific nucleotides associated with producing DHF (Mangada and Igarashi 1998; Pandey and Igarashi 2000), so we assume all viruses of this genotype have the potential to produce severe disease. This has also been the case with other DENV, where recent studies have suggested that differences in 5′- and 3′-UTR in the genome can alter levels of replication (Miagostovich et al. 2006; Sirigulpanit et al. 2007; Tajima et al. 2007), which can be extrapolated to viral load in blood or disease presentation (Wang et al. 2006). Another chapter in this volume describes how these influences may occur.

4.2 Infection of Primary Human Cells

The first targets of DENV replication, after mosquito bite, were postulated to be monocytes or macrophages and numerous studies focused on these cell types. However, more recent studies, using newer technologies for cell identification (mainly flow cytometry) have shown that DENV infects human monocytes poorly compared to dendritic cells, including Langerhans cells and monocyte-derived dendritic cells (Marovich et al. 2001; Wu et al. 2000). Primary dendritic cell cultures can be derived from human peripheral blood donations to banks and this is the usual source for studies to compare the replication of low-passage DENV from patients. Because these samples are obtained anonymously, we must be careful to obtain them from blood banks in areas where there is no DENV transmission (i.e., no antigenic priming of cells) and we cannot determine the human genetic background that might lead to differences in virus replication. However, studies reported in 2003 and 2005 (Cologna et al. 2005; Cologna and Rico-Hesse 2003) were able to show consistent differences in replication of DENV of two genotypes of serotype 2, demonstrating that there is an ex vivo correlation to the virulent phenotype derived from evolutionary studies. Although there seem to be innate, probably genetic differences in the yields of virus produced by cells from individual donors, this variation could be accounted for statistically and the correlations with virulence of patient-derived viruses were established (and note that these differences occur in the absence of antibodies). These primary cell cultures were also used to test recombinant viruses, to determine the influence of specific genome regions on virus replication and yields from human cell targets. These studies confirmed that the exchange of three genomic regions (5′ and 3′ UTRs, and E390) could reduce the levels of replication and virus yields of a Southeast Asian virus to those of wild-type, less virulent viruses of the American genotype (Cologna and Rico-Hesse 2003). Other uses for cultured primary human cells include the identification of specific cells that are producing more virus (tropism) and whether virus replication is even required for pathologic effects.

4.3 Infection of Field-Collected Mosquitoes

Another step in determining if a virulent genotype had an increased transmission fitness phenotype was to study differences in replication and dissemination (i.e., the possibility of transmitting virus by bite) in the natural mosquito vector, *Aedes aegypti*. Laboratory-reared colonies of mosquitoes (e.g., Rexville or Rockefeller strains) seem to have lost their selectivity for infection and it is recommended that mosquitoes used in these experiments be from the F4 generation or lower (F0 = field-collected eggs). The virulent, Southeast Asian strains of serotype 2 were shown to infect a larger proportion of mosquitoes than the less virulent, American genotype strains, after feeding mosquitoes on blood containing the same titer of virus (Armstrong and Rico-Hesse 2001); also, a greater proportion of mosquitoes develop disseminated infections with the virulent genotype (Armstrong and Rico-Hesse 2003). If mosquitoes were fed both genotypes simultaneously, they were much more likely (sevenfold) to develop an infection with the virulent strains (Cologna et al. 2005). When the dynamics of virus replication and dissemination were compared for both genotypes, the virulent strains had reached the salivary glands up to 7 days earlier than the less virulent viruses (Anderson and Rico-Hesse 2006). This means that virulent strains may replicate and be transmitted much sooner to human hosts, outcompeting the less virulent viruses and causing many more cases of disease, thus ecologically displacing those that cause less severe dengue. This efficiency of transmission by the vector could explain how certain genotypes have displaced others, shifting the evolution of dengue disease towards more virulence (i.e., more DHF).

4.4 Mouse Models of Disease

During this decade, major advances have been made in the development of new mouse breeds and their transplantation with human stem cells (from umbilical cord blood cells) that may effectively mimic the human immune system or show human signs of disease upon infection. The combination of studies in these mice with those in mice that are defective in interferon production or receptors have led to insights into the mechanisms of dengue pathogenesis (Bente et al. 2005; Kuruvilla et al. 2007; Kyle et al. 2007; Shresta et al. 2006). Thus far, none of these models develop DHF and the production of DENV-specific antibodies has been low or undetectable. However, others have shown that mice engrafted with human hematopoietic cells can be effectively used to study pathogenesis of viruses for which no other models exist (Melkus et al. 2006; Watanabe et al. 2007). It is anticipated that after adaptation of this system to DENV infection by multiple strains, with the acquisition of DENV-specific, functional B and T cells, that the signs of DHF might appear in these “humanized” mice. This would finally allow for the measurement of the many effects of immunopathogenesis, including the protective cross-immunity

created by serial infection and the evaluation of many basic questions, such as dose-dependence of infection, the relevance of mosquito factors to infection (e.g., salivary gland proteins) and the role of other cells as primary targets of infection (e.g., endothelial cells). Most importantly, this model could allow for the immediate testing of antivirals and vaccine candidates, where effective systems for testing products before human use have been lacking.

4.5 Mathematical Models of Transmission

Another promising new field has been the development of mathematical models of DENV transmission, including "evolutionary epidemiology" and virulence management. These models are being used to estimate the effect of changing host immunity or mosquito transmission on the amount of virus circulating and the risks to a hypothetical human population and epidemic "topology." These analyzes have suggested that DENV cross-serotype immunity and mosquito demographics, rather than immune enhancement, are the most important determinants in the dynamics of specific serotype cycles or genotype replacement during epidemics and that the application of incomplete control strategies might actually increase the incidence of severe disease (Adams et al. 2006; Cummings et al. 2005; Nagao and Koelle 2008; Wearing and Rohani 2006). Although these models are complex and still require many basic measurements for their refinement, some of the details are being added as they become available from laboratory or ecological studies (e.g., quantification of cross-protection by various DENV strains and many different measurements of mosquito transmission dynamics, including vector genetics and their effect on competence and capacity, etc.). Other applications of computer modeling have involved measuring the importance of host genetics over parasite contributions to virulence. For virology, the importance of host genetics in disease pathogenesis has been discussed for many years but recent technologies have allowed researchers to weigh the influence of virus virulence over human host genetics, in the case of pandemic influenza A (Gottfredsson et al. 2008; Pitzer et al. 2007). However, these approaches are extremely controversial at this point, as other investigators have reached opposing conclusions when using similar methods and results (Albright et al. 2008).

5 Perspective

Only recently have public health officials in developed countries become concerned about the increased transmission and geographic spread of DENV. In many cases this is due to the increase in cases imported by tourists from less-developed tropical regions. For the United States, there has been a marked increase in imported cases and autochthonous transmission in Texas and Hawaii during this decade (Morens

and Fauci 2008) and for the first time we have been able to document the introduction of a virulent DENV genotype into this country (CDC 2007; Rico-Hesse 2007). This has added a sense of urgency to research using some of the models described here and a hope for additional support for studies of this long-neglected tropical disease.

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