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Dengue virus markers of virulence and pathogenicity

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Abstract

The increased spread of dengue fever and its more severe form, dengue hemorrhagic fever, have made the study of the mosquito-borne dengue viruses that cause these diseases a public health priority. Little is known about how or why the four different (serotypes 1–4) dengue viruses cause pathology in humans only, and there have been no animal models of disease to date. Therefore, there are no vaccines or antivirals to prevent or treat infection and mortality rates of dengue hemorrhagic fever patients can reach up to 20%. Cases occur mainly in tropical zones within developing countries worldwide, and control measures have been limited to the elimination of the mosquito vectors. Thus, it is imperative that we develop new methods of studying dengue virus pathogenicity. This article presents new approaches that may help us to understand dengue virus virulence and the specific mechanisms that lead to dengue fever and severe disease.

Keywords

dengue virus; epidemiology; evolution; flavivirus; viral pathogenesis

Dengue viruses and the disease they cause were described in early scientific reports dating to the 1940s. However, it took decades of research on patients to derive some information regarding what might predispose an infected individual to become ill with dengue fever (DF; a self-limiting, influenza-like disease with fever, rash and arthralgia), or go on to the more severe dengue hemorrhagic fever (DHF; with massive internal bleeding). Since a person can theoretically be infected up to four different times, each by a different serotype virus, it was discovered in the 1960s that those with secondary infections had a much higher risk of developing DHF. That is, the first infection provides long-lasting immunity to that serotype virus, but subsequent infection by another serotype causes much more severe disease. This has led to the description of antibody-dependent enhancement of the disease and more recent descriptions of cellular immune factors that could also add to the pathology [1,2]. The suspicion that viral factors were also involved in producing more severe epidemics, with more DHF, was first reported in the 1970s and this led to the concept of differences in dengue virus virulence or viral genetic determinants of disease and epidemiology [3,4]. Namely, viral genetic differences could be responsible for different disease presentations in a population and also for characteristics of an epidemic (e.g., number of cases and degree of geographic spread). In addition, more recently, researchers have attempted to link human genetic determinants to higher incidence and/or severity of disease [5–7], while infection of human cells *ex vivo* has

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provided some data to support this [8–10]. Other factors that might lead to more pathology include other underlying diseases or conditions, such as diabetes or pregnancy, but much more research is necessary in order to correlate signs and symptoms of dengue within a more complex disease presentation. However, it seems that all of these factors work in unison to produce a wide gamut of disease presentations characteristic of dengue. Therefore, the study of dengue pathogenesis is complex and, as a first step, it is necessary to have experimental markers of virulence or pathogenesis to measure and evaluate the contributions of all of the previously named factors.

Dengue virus evolution & virulence

Evidence for genetic variability within each of the four dengue virus serotypes first became available in the 1980s, using a technique known as fingerprinting [11,12]. This method used restriction enzymes to cut the entire virus genome into fragments of different lengths according to the nucleotide sequence; however, this method did not pinpoint the actual sequence differences within genes, and virus comparisons were difficult to make. With the advent of nucleotide sequencing techniques in the 1980s, and with enzymatic amplification of RNA genomes in the 1990s, rapid comparisons of specific genes or entire genome sequences became the norm. This led to the comparison of numerous dengue viruses and the derivation of phylogenetic trees of evolutionary relationships between strains within a serotype [13] and across serotypes [14,15]. Thus, the term 'genotype' is now used to describe a genetic variant group within each of the four serotypes; depending on serotype, up to five different genotypes have been outlined within a serotype [13]. Subsequent comparisons of virus genotypes with epidemiologic information led to the linkage of higher rates of transmission and higher incidence of severe disease (DHF) with specific viral groups [16,17]. It is now clear that certain genotypes have higher rates of transmission worldwide, that they are displacing other genotypes on several continents, and that some are associated with DHF while others seem to cause only DF [18,19]. In addition, some authors have speculated as to the nature and origin of the different lineages and as to their rates of evolution or extinction, based on nucleotide sequence comparisons [20,21]; however, these comparisons are biased owing to the lack of uniform sampling of dengue viruses from different times (especially from the past) and areas around the world. It is hoped that some of these studies can be conducted with improved genotypic and phenotypic analyses, to derive data from the viruses themselves and as they evolve in nature [22]. What remains now is to find ways to measure the contribution of specific genotypes to differences in pathogenesis in humans and, ultimately, to evaluate the contribution of viral virulence to the disease process, in contrast to host genetics and immune responses.

In vitro markers of virulence

The most direct measure of virulence is the detection of differences in viral replication. At present, this has taken two forms, either the measure of viral effect on cells (cytopathology) or that of the number of genomes (in the case of dengue, RNA). The visualization of viral particles by electron microscopy is too cumbersome to carry out routinely and does not necessarily reflect infectivity because some particles may not contain full genomes [23]. One of the first methods used to detect and count infectious dengue virus particles was the formation of plaques on cells in culture; these cells were previously derived from hamster or monkey tissues or from mosquito larvae and, thus, are not normal target cells. However, this method was used for decades as the standard to measure amounts of virus and the strength of antibodies to neutralize infectivity of specific viruses (by plaque reduction). Some investigators had noted that viruses isolated from patients directly (or at low passage number) did not plaque well and required further cell or animal adaptation to acquire plaquing ability, thereby selecting for genetic variants that are not representative of the isolated virus [24,25]. It became obvious that only highly passaged laboratory strains of dengue could be measured consistently by this *in vitro*

phenotype, but these strains are scarcely representative of those circulating in nature. The need for measuring numbers of virus particles or infectious units without selecting for mutants became a hindrance to normalizing most experiments, in terms of infectious doses and other replication assays. More recently, quantitative enzymatic amplification of RNA (quantitative real-time PCR or TaqMan®) has become the norm because it allows us to count the number of RNA transcripts in solution as a derivative of the number of viral genomes present. This technique has allowed the comparison of plaquing abilities by the same virus in different cell lines [26] and of various low-passage strains of the same serotype [25]. These comparisons revealed that plaquing abilities could vary by several logarithmic scales; that is, the same virus might be quantified with a 1–2 log difference depending on the cell line used for the assay. It seems that for the customarily used cell lines, the order of plaquing ability is: BSC-1 > BHK-15 > BHK-21 > LLC-MK2; dengue viruses in the C6/36 mosquito cell line form 'foci' instead of plaques. In addition, some viruses will produce ten- to 100-fold more plaques compared with others when an RNA assay is used to normalize input and output; for example, the laboratory strains New Guinea C, PR-159S1 and 16681 produce plaque:RNA ratios of 1:10–100, while low passage strains have ratios of approximately 1:1000 or more. Therefore, the effects of the plaquing phenotype questioned many studies where virus doses were normalized by plaque-forming units and where plaque reduction by antibodies was used to measure genetic relationships, or the ability to induce cross-neutralizing immunity or protection [27–30]. Furthermore, the use of flow cytometry to detect and count the number of infected primary human cells has now been modified to quantify virus and infectivity in cell culture, with results that are more promising than using the plaquing standard [31,32]. However, limits to the interpretation of quantitative RNA assays also exist, owing to the fact that different areas of the genome are amplified and some of these strands are more representative of full-genome RNA copies than others. Until we can actually measure the number of infectious (complete RNA genome) viral particles routinely, we will not be able to directly measure differences in infectivity.

Ex vivo markers of virulence

New advances in flow cytometry or fluorescence-activated cell sorting have helped demonstrate which human cells are targets for dengue virus infection [8,33]. These techniques have shown that dendritic cells, especially those that are normally found in the skin, are infected by dengue viruses at much higher rates than monocytes or macrophages. This phenomenon could help explain some DF disease signs, such as rash, mucosal bleeding and lymphadenopathy, where viral replication causes pathology in the skin and surrounding capillaries and when these infected dendritic cells migrate to antigen-presenting areas. By using recombinant dengue viruses (infectious clones), it has been shown that specific regions or genes of the dengue virus genome can control levels of replication in primary human cells and, therefore, influence viral load in patients [9,34]. These studies showed that several portions of the genome (envelope protein amino acid 390, 5' and 3' untranslated regions [UTRs]) act synergistically to reduce or amplify virus replication in human monocytes and their derived dendritic cells. The availability of recombinant human cell lines expressing major receptors for virus or antibody-virus attachment and entry (DC-SIGN and Fc-γ receptor II, respectively) has also helped compare the infectivity of different viral strains [30,32,35]. However, it is unclear if these receptors alone can determine virus virulence, as there could be many other factors that lead to differences in output and, ultimately, viremia. For example, it has been shown that dendritic cells from anonymous donors differ in their capacity to replicate and secrete infectious dengue virus, reflecting what we assume to be innate immunity (e.g., interferons) [18], with some correlation to DC-SIGN receptors in immature, infection-susceptible cells [10]. Until we can perform these studies in much larger numbers and with human-donor background information (e.g., genetic-linkage analysis), this phenomenon will remain unmeasured and unexplained.

Many investigators have been exploring the mechanisms leading to hemorrhagic signs of disease; it is known that there are drastic changes in hemostasis (coagulation) and a bleeding diathesis (many different signs/symptoms) upon dengue virus infection. However, it is these counteracting forces that lead to plasma leakage in only some patients (with concomitant dengue shock syndrome, if severe). These signs of severe disease could be due to endothelial cell infection and pathology, elimination of platelets, mast cell activation and massive cytokine and chemokine release by immune system cells, among others. Although it is certain that cells of the monocyte/macrophage and dendritic lineages are the first targets of dengue virus replication, the downstream infection of lymphoid, endothelial and hepatic cells has been postulated to add to pathogenesis [36]. However, the ability of the latter to support dengue virus replication *in vivo*, as well as the mechanisms of cell tropism, have yet to be demonstrated. Thus, numerous studies have now concentrated on experimental infection of primary human cells *ex vivo* and the comparison of virus replication and gene activation, as well as protein secretion by these cells. Some of these reports have described the complexities involved in measuring the interactions of primary human cells in culture, where dendritic and T-cell activation and subsequent cytokine secretion vary according to stage of cell maturation and viral infection [37]. Others have specifically addressed the rates of infection in subsets now defined within target cells. For example, plasmacytoid dendritic cells differ from myeloid dendritic cells in terms of infection rates and cytokine secretion; myeloid cells replicate virus at much higher rates while plasmacytoid cells secrete many more cytokines [10]. The role of antibody-dependent enhancement of cell infection has also been measured in dendritic, endothelial and mast cell cultures, with mixed results [38–40]. It seems that the effect of antibodies on infection depends directly on concentration (or titer) and host-immune status (e.g., which sequence of serotype infection). Thus, it is difficult to reconstitute the cell infrastructure and complex interactions that occur within the human circulatory system. For example, it has been very difficult to obtain intact endothelial cell monolayers to measure leakiness after cytokine secretion by dengue virus-infected dendritic or T cells [41–43], and most measurements of cellular apoptosis (a normal end-stage of cells) cannot distinguish between cause and effect of pathology [44,45].

***In vivo* markers of pathogenicity**

As mentioned earlier, one of the main reasons we know little of the mechanisms leading to severe dengue is that there are no animal models of disease that demonstrated all the signs of dengue as those symptoms found in humans. Even our closest relatives, the great apes, do not show any signs of infection other than a low-grade and transitory viremia; albeit, some monkey models have been used to demonstrate that vaccine preparations are not harmful or toxic before going on to human preclinical trials [46]. However, various types of mice have been recently used to test for several markers of pathogenicity. These mice consist mostly of immunodeficient models that demonstrate disease which is not characteristic of dengue (e.g., paralysis and blindness in the AG129 strain of mice), or mice reconstituted with human cell lines or stem cells that show some signs of dengue; wild-type mice do not support dengue virus replication well enough for measurements of pathogenesis [46]. The goal is to develop a system where mice show signs of DF after primary infection and can manifest DHF upon sequential infection with a heterologous (other serotype) virus. This would theoretically require the function of human B and T cells, to produce cross-reactive antibodies and to begin the cytokine and chemokine cascade that seems to lead to dengue immunopathology.

The most promising models of dengue are those of severely immunodeficient mice that have been engrafted with human stem cells (cord blood and/or fetal cells), which reconstitute part of the human immune system [47,48] and show signs of DF on dengue infection [49,50]. These mice, of the NOD/SCID and Rag2 γ ^{-/-} immuno-compromised genetic background, can be transplanted with human CD34⁺ hematopoietic cells that develop into CD45⁺ lymphocytes in

the mice after several weeks. Subcutaneous infection with dengue viruses produces high viremias resulting in fever, rash and thrombocytopenia in some mice, as in human cases of DF. The actual tissues that are infected by these different viruses are yet to be determined, as the quantities of human lymphocytes and target cells, such as monocytes and dendritic cells, are very low in these mice (reported ranges of 1–43% of human lymphocytes in peripheral blood; <5% of monocytes or dendritic cells) [51], and the levels of dengue infection are low even in human patients (one in every 1000 monocytes at acute stage) [52]. Furthermore, the tissues necessary for B- and T-cell development and activation (homeostasis) to produce dengue-specific antibodies are invariably lacking. This can be overcome by transplanting human fetal liver or thymus at the same time as cord blood cells or by inoculating newborn mice that have further immunodeficiencies (NOD/SCID-IL2 γ -null background). Thus, the preparation of these mice is laborious, gives variable results and is still in development for specific application to infectious disease studies [53]. However, new methods of inducing human stem cells to differentiate *ex vivo* and the administration of recombinant human cytokines to mice during and after transplantation have promising results; more CD34⁺ hematopoietic cells can be expanded (up to 68-fold) *in vitro* from a single cord blood sample, engraftment levels have improved substantially (up to 90% of lymphocytes are human) and the injection of human B lymphocyte stimulator cytokine and TNF- α into mice, along with the expanded cells, has led to production of specific antibodies to some antigens (bacterial lipopolysaccharide and toxoid) [54,55]. Thus, these models, using human–mouse chimeras, will most probably continue to improve at a rapid pace, as our understanding of mouse gene knockouts, xenotransplantation and human immune system development increases.

***In silico* predictors of replication**

The advent of reverse genetic techniques has allowed the development and manipulation of infectious clones (full genome) and replicons (partial genome) of dengue viruses, to understand the steps involved in controlling replication [56]. Many studies have now dissected the interactions between different regions of the dengue virus genome that do not code for protein but are necessary for replication. Genome circularization, by interaction between folded RNA strands of the 5' and 3' ends, is now known to modulate RNA translation, replication and encapsidation, by serving as a promoter for viral polymerase RNA synthesis [57,58]. The study of natural variation in these regions of RNA has pinpointed their nucleotide sequence conservation, including some areas and folding patterns that might vary according to virulence or pathogenicity. Full genome-sequencing studies of serotype 2 dengue viruses from patients in the 1990s demonstrated that the 5' and 3' UTRs varied across two genotypes [59] but did not vary according to disease presentation when comparing viruses from the same genotype [24]. That is, viruses that have contrasting virulence and epidemiologic associations have differences in the RNA sequences at the end of the genome that might produce significant differences in replication. The 5' UTRs have been shown to interact with gene-coding regions, such as capsid, and the interaction with the 3' UTR is a dynamic process. However, genetic assays that employ mutagenic approaches can confirm the existence of particular elements in the primary structures. Subsequent studies have confirmed these results, when comparing the 3' UTR among many different viruses – phylogenetic analyses using only the 3' nucleotide sequences correlate with those generated with other genome areas [60]. Viruses belonging to the same genotype but from patients with different disease presentations do not vary significantly in this 3' UTR [61].

Further exploration of these structures revealed that computer-predicted RNA folding patterns varied according to dengue virus serotype and genotype, and some of these folding patterns have been confirmed by enzymatic degradation analysis. However, some structures were also extremely conserved, as a theoretical sign of biological significance. Although the lengths of the 3' UTRs vary (~450 nucleotides long), there are some secondary structures that are

conserved among all dengue viruses; a stem loop at the extreme (3'SL) end of approximately 100 nucleotides and a conserved sequence (CS1) of ten nucleotides that serves as the cyclization sequence to bind with a 5' complementary region that contains the translation initiation codon and an adjacent stem loop, which serves as the viral RNA polymerase promoter [62,63]. Mutagenesis experiments have demonstrated the absolute need for these structures in all dengue viruses, with spontaneous mutations that restore the correct complementary base pairings when small deletions or other changes are made in infectious clones [64,65]. Therefore, specific functions for some of these noncoding regions have been demonstrated, and they could directly influence viral pathogenesis.

Natural variation in the 3' UTR of patient samples was shown to occur at the beginning of this strand (first 150 nucleotides on the 5' side), and when computer algorithms were used to predict secondary structures, results varied according to the software used [24,59,61,66]. There are significant differences in the assumptions made when folding nucleotides *in silico*, where the progressive reordering of base pairings leads to lower levels of energy and prediction methods focusing on a single minimum free-energy structure may not identify functionally relevant structures [67]. Most studies used a very popular program known as M-fold to compute the minimum free-energy structure for a given sequence by systematically sampling structures within a percentage-free energy range, and creating a set of diverse suboptimal structures [68]. Another program, known as RNASTAR, was also used because it can predict pseudoknots (tertiary folding); within RNASTAR, the genetic algorithm simulates an evolution of RNA folding by starting with unfolded structures and increasing the low free-energy fitness criteria in each generation. That is, RNASTAR differs from M-fold in that the optimal structures are generated in a processive manner, folding from the 5' end and changing out structures until reaching the 3' end, mimicking the direction of RNA synthesis [69]. Since the genetic program is a simulation rather than a calculation, its output can be evaluated on how well it converges to a single structure and the reproducibility of the final structure; the user can determine the number of iterations in the simulation. Thus, it is best to compare the structures generated by both programs, and phylogenetic covariation can also be used as criteria for structure selection. The 'best' program is the users' decision that depends on the length of RNA, the available experimental data and computer resources and the intended applications of the structure prediction [67]. To date, there are more than 15 programs described that can perform nucleotide sequence folding into secondary structures, some tertiary structures and some 3D predictions, but no RNA-protein folding predictions have yet been described.

As an example of how 3' UTR secondary structures vary among dengue serotype 2 viruses, a representative from each of the four genotypes is presented here. Figure 1 shows RNASTAR predictions for Southeast Asian, Indian, American and West African genotype viruses; there is genetic covariation in these structures as viruses belonging to the same genotype share many folding pattern features. We had speculated that the differences in these structures might lead to differences in replication, since the more virulent viruses belonging to the Southeast Asian genotype had a common theme of forming tighter folding patterns than the American genotype viruses, thus potentially serving as more attractive binding sites for the viral RNA-polymerase complex [59]. In the examples shown here, we could classify the Southeast Asian and Indian genotype viruses as having more secondary binding (stems and loops), and the possibility of tertiary binding (pseudoknots) than those of the American and West African genotypes (notice how spread out the RNA strands are for the latter two). It is also worth noting that the 3'SL mentioned earlier (150-nucleotide 3'-end stem loop), occurs in all four representatives, but the downstream folding patterns, including the CS1 (ten nucleotide cyclization sequence), are not conserved for all four. Thus, the predicted folding patterns could show us whether the 3' end cyclization with the 5' end and the RNA polymerase promoter are efficiently coupled for genome transcription. However, as previously mentioned, these prediction algorithms are in their infancy and we have no way of determining if these structures actually exist in nature, or

for how long, according to folding dynamics. Whether the predicted structures can serve as visible markers or predictors of pathogenicity remains unanswered, but this awaits confirmation as these genotypes are also proven to vary in virulence and/or epidemiologic associations.

Conclusion

It has been extremely difficult to measure the contribution of viral genetics to dengue disease presentation in humans mainly because there have been no adequate models of disease. New techniques, such as rDNA technology to produce infectious clones of dengue viral RNA with specific modifications, quantitative real-time PCR to measure viral RNA in small quantities, flow cytometry with anti human antibodies to determine the identity of infected cell subsets and xenotransplantation of mice with human immune system cells have all recently contributed to a better understanding of dengue replication. These techniques have also been used to produce and evaluate the potential of vaccine preparations, some of which are currently in human clinical trials [56]. In addition, computer algorithms that predict secondary and tertiary structures of RNA have been tested against chemical analysis of these structures and the resulting folding patterns are becoming more realistic. As we rely on advances in molecular biology to understand the mechanisms of virus replication, the advances in dengue virology should help us confirm the differences in virulence that lead to pathogenicity, in addition to the human immune and genetic factors that make this disease complex. of these structures and the resulting folding patterns are becoming more realistic. As we rely on advances in molecular biology to understand the mechanisms of virus replication, the advances in dengue virology should help us confirm the differences in virulence that lead to pathogenicity, in addition to the human immune and genetic factors that make this disease complex.

Future perspective

Advances in molecular biology have had a great impact on measuring and evaluating the specific steps that control dengue virus replication; some of these techniques have led to the first descriptions of differences in dengue virus virulence. The use of flow cytometry and cell sorting will allow for a better understanding of hematopoiesis and identification of human cell subsets, and these methods should have a great impact on the description of dengue virus cell and tissue tropism, *in vitro* and in patient samples. The 'humanization' of mice is improving rapidly, along with the potential to develop gene knockouts that allow transplantation of the specific tissues or cells identified as targets of dengue replication in humans. Most of the model systems described here can also use recombinant viruses to determine which parts of the viral genome have the most impact on replication and, thus, on viral load in the patient. More of these genome structures can be modeled in terms of secondary- and tertiary-folding patterns, in order to ultimately describe the dynamic and antagonistic processes of dengue genome translation, replication and encapsidation. It is difficult not to imagine progress in the field of structure predictions and the eventual clarification of how these molecules interact with proteins, in order to yield specific phenotypes. Thus, all of these advances should have a major impact on our understanding of dengue virus pathogenesis and should allow for a systematic design of antivirals and vaccines, with a better distinction between causes and effects of pathology and precise identification of targets for control.

Executive summary

Differences in dengue virus virulence have been measured

- Viral replication differences can be measured in cell culture, in primary human cells and in patient blood samples. Natural virus variants show these differences.

- Differences in viremia levels in patients have been correlated with disease presentation.
- Evolutionary analyses have demonstrated that some viral genotypes are responsible for more severe disease and for more geographic spread.
- Other factors, such as human genetic background and immune status, are also important in determining pathogenicity.

New techniques can pinpoint factors involved in virulence

- Quantification of viral RNA is a better determinant of viral dose or input and output compared with the *in vitro* plaquing technique on cell monolayers.
- The use of recombinant viruses allows for the evaluation of virulence-related sequence changes in a uniform genetic background.
- Flow cytometry and cell sorting of human tissues can help identify the cell subsets infected by different dengue viruses.
- Advances in primary human cell culture have helped understand the complex interactions between dengue-infected cells of the skin and circulatory system, the first targets of infection.
- New mouse models of dengue disease have improved and they demonstrate similar signs of disease as those in humans, but they are laborious to prepare.
- Bioinformatic and biochemical analyses have helped identify RNA-noncoding and protein-coding regions that control viral replication.

Impact of understanding viral factors of replication

- The measurement of replication differences in human cells *ex vivo* has demonstrated innate immune factors that might control initial dengue virus replication.
- The comparison of different dengue serotypes and genotypes in new mouse models of disease can help explain mechanisms of pathogenicity, while controlling for some immune effects.
- Recombinant dengue viruses allow for the direct measurement of virus structure changes and can be modeled on natural variants, thus extrapolating to clinical and epidemiologic observations.
- Specific virus genotypes associated with more disease can be targeted for control.
- Antivirals and vaccines can be designed to interfere or accentuate only those viral and immune factors that control pathogenesis rather than contributing to immunopathology.

Future perspective

- The understanding of hematopoiesis and immune system development, using human stem cell technology, will invariably contribute to pinpointing mechanisms of dengue pathogenesis.
- Technological developments aimed at evaluating viral determinants of virulence will help distinguish complex human genetic and immune factors that also contribute to the disease process.
- Antivirals and vaccines can be developed to specifically inhibit stages of viral replication that can ameliorate immunopathogenesis and, thus, severe disease.

- The association of clinical and epidemiologic characteristics with viral genetics will help determine better targets for public health measures to control this disease.

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Figure 1. Predicted folding patterns of the 3' untranslated region of dengue serotype 2 viruses representing each of the four genotypes, shown in a visual order of complexity
(A) Southeast Asian genotype, strain 16681. **(B)** Indian genotype, strain 1349. **(C)** American genotype, strain IQT2913. **(D)** West African genotype, strain ArA2039.