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REVIEW

Advances in the development of vaccines for dengue fever

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Abstract: Dengue fever is caused by the mosquito-borne dengue virus (DENV) serotypes 1–4, and is the most common arboviral infection of humans in subtropical and tropical regions of the world. There are currently no prophylaxis or treatment options in the form of vaccines or antivirals, leaving vector control the only method of prevention. A particular challenge with DENV is that a successful vaccine has to be effective against all four serotypes without predisposing for antibody-mediated enhanced disease. In this review, we discuss the current lead vaccine candidates in clinical trials, as well as some second-generation vaccine candidates undergoing preclinical evaluation. In addition, we discuss DENV epidemiology, clinical disease and strategies used for *Flavivirus* antivirals in the past, the development of new DENV therapeutics, and their potential usefulness for prophylaxis and treatment.

Keywords: tetravalent dengue vaccine, live attenuated vaccine, purified inactivated vaccine, DNA vaccine, antibody-dependent enhancement, antivirals

Introduction

Dengue is the most rapidly emerging mosquito-borne viral infection of humans, with an estimated 2.5 billion people at risk globally, more than 70% of whom reside in Southeast Asia, the Pacific, and the Americas. It has been estimated that the dengue viruses (DENVs) cause 50–100 million infections and up to 50,000 deaths each year. In some epidemics, the mortality rate may reach 5%.[1] DENVs are members of the family *Flaviviridae* and are comprised of four antigenically distinct serotypes – DENV types 1, 2, 3, and 4 – which exhibit 65%–70% sequence homology (Figure 1).[2] Dengue virus is transmitted to humans primarily by *Aedes aegypti*. Most infections result in uncomplicated dengue fever (DF), a mild to moderate febrile illness; however, a small but significant number of individuals go on to develop dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which are severe and potentially life-threatening diseases.

While the earliest reported dengue epidemics of the modern era occurred in 1779 and 1780 (in Asia, Africa, and North America), the first recorded cases of a disease likely to have been dengue fever occurred in China during the Jin dynasty (AD 265–420).[3] The term "breakbone fever" was first used by Benjamin Rush in 1789 to describe dengue fever because of the frequent manifestation of symptoms of severe myalgia, arthralgia, and rigors.[4] From 1780 to 1940, infrequent but often large epidemics were reported. It was during and after the Second World War when a global pandemic of dengue transmission began, which prompted the pioneering work on dengue disease and transmission by Albert Sabin et al, along with the development of

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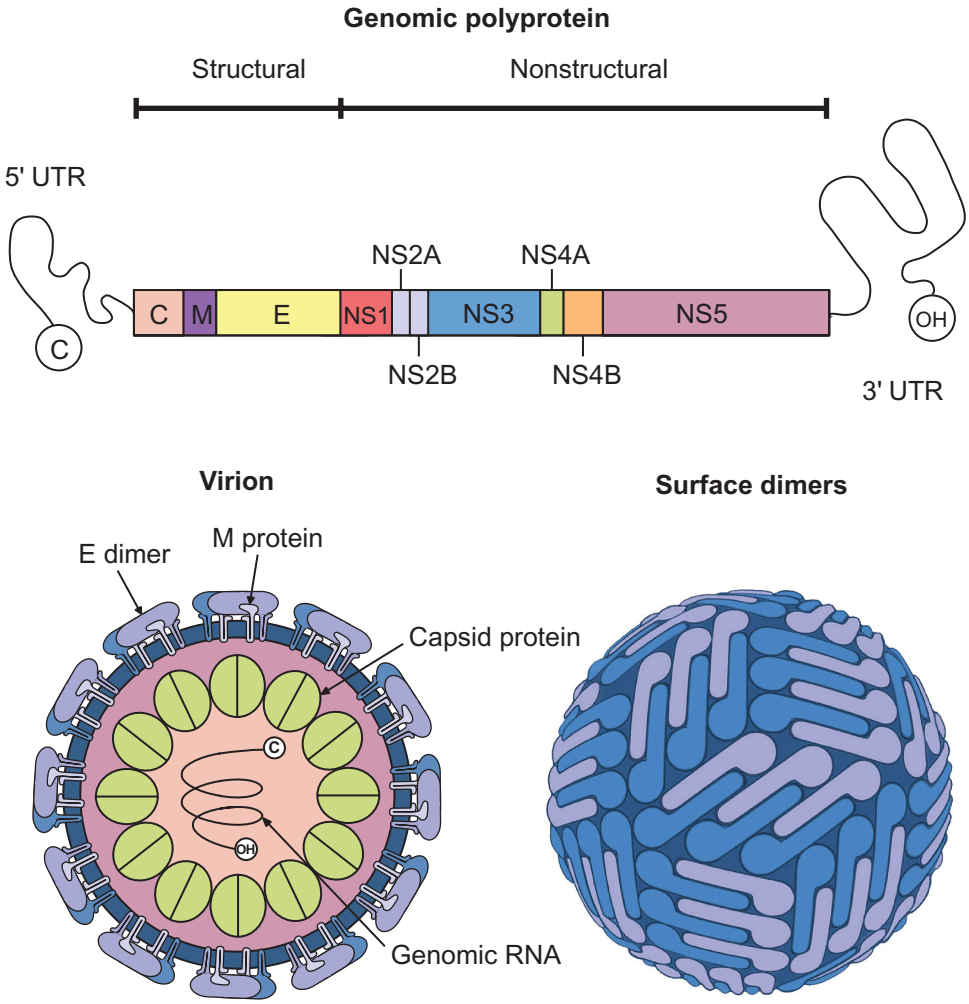

Figure 1 The dengue virus genome and virus particle. Dengue virus is a single-stranded, positive-sense RNA genome of about 11 kb in length that is capped at the 5′ end and serves as the genome and the viral messenger RNA.

Notes: The open reading frame encodes the structural proteins capsid (C), membrane (M), envelope (E), and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The virion attaches to host receptors through the E protein and is endocytosed into vesicles in the host cell. The virus membrane fuses with the vesicle membrane and viral RNA is released into the cytoplasm. The positive sense RNA is translated into a polyprotein, which is co-translationally processed and then cleaved into structural and nonstructural proteins by host and viral proteases. Replication and virus assembly takes place on the surface of the endoplasmic reticulum (ER). The immature virus buds in the ER and is then transported to the Golgi apparatus. While moving through the trans–Golgi network, rearrangements of the M and E proteins result in conformational changes yielding mature infectious virions. The E glycoproteins are aligned as dimers on the virion surface in an icosahedral-like symmetry and are the major target of protective antibodies.

Abbreviation: UTR, untranslated region.

the first successful experimental dengue vaccine.[3,5,6] With the co-circulation of multiple dengue virus serotypes, DHF, a newly described disease, emerged.[3] By the mid-1970s, the epidemic form of DHF had spread throughout Southeast Asia, becoming the leading cause of death among children. DHF has now spread to more than 60 countries, and dengue fever is now endemic in more than 100 countries in the Caribbean, South Pacific, and Africa.[7]

Although all four DENV serotypes are capable of causing DF, the more severe DHF and DSS are more often associated with a second or subsequent heterologous dengue infection. Following convalescence from primary infection, there appears to be lifelong immunity to the infecting serotype and often some degree of cross-protective immunity against other serotypes as well; however, this heterologous immunity is short-lived. Secondary infection associated with the presence of preexisting heterologous dengue antibodies appears to increase the risk for DHF and DSS. This is hypothesized by Halstead et al to be due to antibody-dependent enhancement (ADE) of infection by preexisting "enhancing" antibodies that are acquired actively (eg, by previous infection) or passively (eg, by maternal transplacental transfer) and which form nonneutralized immune complexes capable of infecting phagocytes, macrophages, and other Fc receptor-bearing cells.[8–11] This increased infection rate is also associated with increased levels of cytokines and other proinflammatory

immune mediators, which likely contribute to the hemostatic abnormalities and vascular permeability that characterize DHF/DSS.

Although ADE of DENV infection has been demonstrated under certain experimental conditions in vitro using dengue-immune sera or monoclonal antibodies, laboratory-propagated viruses, and Fc receptor-bearing target cells, there appears to be no absolute requirement for ADE in potentiating severe disease, since not all cases of DHF and DSS occur after secondary dengue infections.[10,12] In the search for alternative explanations for the cause of severe dengue disease, studies have been carried out to look at the role viral genetic factors might play in virulence and whether certain DENV serotypes, genotypes, or strains might be inherently more virulent than others. In this regard, DENV-1 and DENV-3 primary infections were found to be associated with more severe disease than DENV-2 and DENV-4 primary infections.[13] Another study showed that DENV-2 and DENV-3 secondary infections were twice as likely to result in DHF compared to secondary infection with DENV-4.[14,15] However, these findings are based on studies of limited sample size in Southeast Asia, and therefore may not be applicable to all populations. Although DHF and DSS are more prevalent in children and young adults, in recent years severe dengue disease has been reported in older adults as well, for reasons that are not fully understood. The severe forms of dengue have become an increasing cause of morbidity and mortality in many endemic countries. Analysis of serotype-specific associations of dengue disease may help in understanding the factors influencing disease severity. Even though ADE is a widely accepted hypothesis, questions remain about its direct role in severe disease.

Our current knowledge of the humoral immune response to DENV strongly suggests that antibodies mediate protection from disease, but they may also play a role in pathogenesis, as described above. Roles for T cells in dengue pathogenesis as well as immunity have been suggested previously.[16] Studies have demonstrated the presence of dengue virus–specific memory $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ lymphocytes after natural dengue infections.[3] Both serotype-specific and serotype cross-reactive $CD4^+$ T cells are present after infection with a single serotype, whereas the $CD8^+$ T-cell cytotoxic activity is virus-specific. It has been suggested that the vascular permeability observed in DHF and DSS is due to the rapid increase in cytokines and chemical mediators such as tumor necrosis factor (TNF), interleukin (IL)-1, IL-2, IL-6, and interferon-gamma (IFN-γ). A high number of virus-infected monocytes and macrophages may result in increased T cell activation, which in turn results in increased levels of cytokines and chemical mediators leading to vascular permeability, plasma leakage, and shock. Further research is needed to elucidate the role of T cell-mediated immunity in DENV infections.

Available evidence suggests that other factors such as virus strain differences and host immune factors may play a role in the pathogenesis of severe dengue disease.[17] Only selected strains of DENV demonstrated ADE when tested in vitro, and the rate of virus infectivity and replication was found to vary with virus strain. In addition, only certain strains of virus are associated with major epidemics and severe disease.[17] Nevertheless, Zhang et al who performed extensive phylogenetic and sequence analyses of temporally and geographically linked Thai DENV isolates, representing both predominant and nonpredominant phenotypes, and associated with disease ranging from uncomplicated DF to DHF and DSS, failed to find a correlation between specific viral genetic determinants and disease severity.[18]

Despite many years of intensive effort by several research groups, there is still no licensed dengue vaccine.[6,19–22] Since preexisting heterotypic dengue antibody may be a risk factor for severe disease, a safe and effective vaccine will most likely require a tetravalent formulation capable of inducing solid immunity to all four virus serotypes, either simultaneously or near simultaneously. A major challenge in dengue vaccine development is the lack of a good correlate of protection. Although neutralizing antibody levels measured in vitro have been the "gold standard" for dengue, it has recently been reported that people who receive a single injection of the successful live-attenuated yellow fever (YF-17D) vaccine respond with cytotoxic T lymphocytes and exhibit a mixed Th1-Th2 profile.[23] Early gene signatures that predict immune responses suggest possible protection correlates with $CD8^+$ T-cell responses.[24] Further research is needed to identify additional markers of immunity to dengue. Another significant handicap to dengue vaccine development is the lack of an animal disease model. Rhesus macaques have traditionally been used to study immunogenicity and viral replication after experimental infection or vaccination. Although they represent natural sylvatic hosts, they do not manifest disease or severe illness as seen in humans. Nevertheless, nonhuman primates as infection models have been essential for testing the efficacy of potential vaccine candidates before transitioning them to human clinical trials.

Well-designed preclinical and clinical studies are still needed to answer a number of important questions, such as: what are the critical immune correlates of protection; what

is the basis for the immunological interference frequently observed after vaccination with tetravalent dengue vaccine formulations and how can it be eliminated; and finally, what is the duration of protection after vaccination and the effect of preexisting immunity to other *Flaviviruses*? Further important clinical issues that must be addressed are the long-term safety of dengue vaccines and their safety for infants. Children in developing countries arguably suffer the greatest impact from dengue, but travelers to endemic areas and military personnel would also benefit greatly from a vaccine. Therefore, the ideal dengue vaccine must be safe and effective for use in both children and adults with varying histories of past dengue and *Flavivirus* exposure, as well as suitably priced so as to ensure availability to poor children in dengue-endemic areas.

Epidemiology

Aedes aegypti and *A. albopictus* are important vectors for the transmission of DENV among humans. All four serotypes of DENV are maintained through two distinct transmission cycles: the human cycle and the sylvatic cycle.[25] The human cycle involves the two principal vectors *A. aegypti* and *A. albopictus*, both of which are widely distributed in urban and semiurban regions of the tropics and subtropical countries. In the last decade, there has been intensified disease transmission due to ineffective vector control. Figure 2 illustrates the geographic regions in which dengue infection is now endemic. Retrospective studies and use of serological surveys in mammals have demonstrated the presence of epizootics among *Erythrocebus patas* monkeys in Senegal. Similarly, enzootic dengue among nonhuman primates has been reported in Malaysia; however, no studies have demonstrated the presence of a sylvatic transmission cycle in the Americas.[25] Currently, there is no evidence to suggest a significant impact of the sylvatic transmission cycle on human epidemics. The most important transmission cycle leading to periodic urban epidemics is the mosquito–human–mosquito cycle.[3] The rapid expansion and geographic distribution of the dengue viruses, as well as the increasing frequency of dengue epidemics, are thought to be related to multiple environmental and manufactured events. Unplanned urbanization in many developing countries has resulted in lack of infrastructure for appropriate water drainage and waste disposal, thus making the environment conducive for vector multiplication.[3] Globalization and increased international travel also facilitate the introduction of dengue viruses to new regions.[3]

The global distribution of DENV infections is marked by rapid continuous expansion in the tropical regions of Asia, the Indian subcontinent, Central and South America, and Africa. In the Indian subcontinent, the number of recurrent outbreaks in all the countries significantly increased after the year 2000, and the largest number of cases and deaths have occurred in India, Pakistan, Bangladesh, and Sri Lanka. While all serotypes were detected to circulate in these countries, most of the DF and DHF cases in the last 5 years were associated with DENV-2 and DENV-3. The rising incidence of DF and DHF cases in these countries

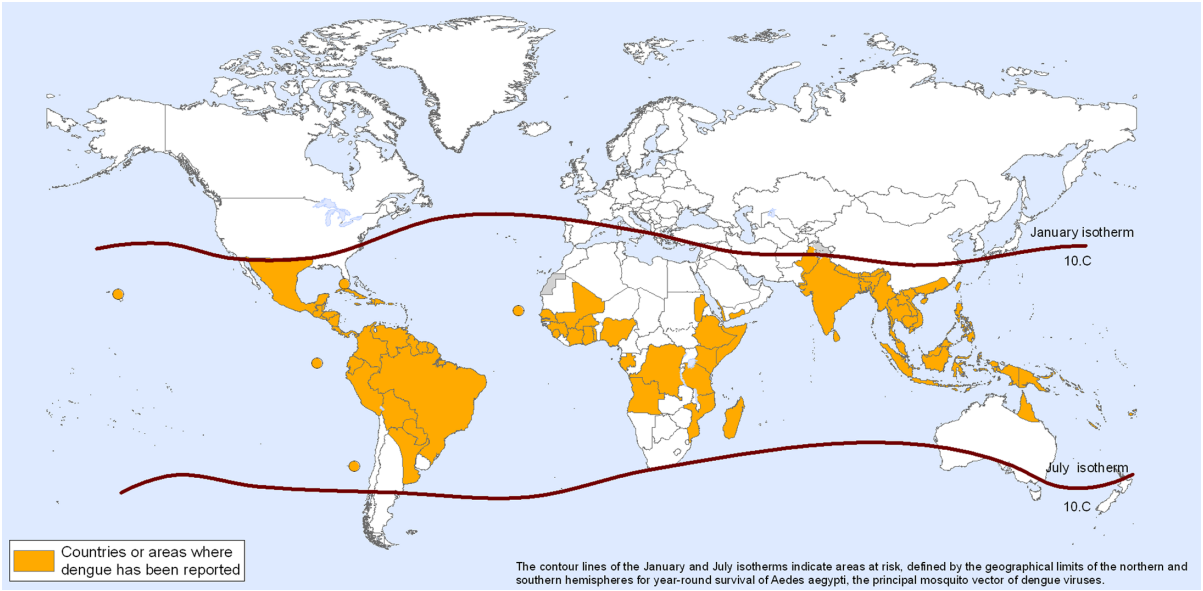

Figure 2 Geographic distribution of dengue virus endemic areas in 2011 with permission WHO 2012, http://who.int/ith/en/, Accessed 17 Feb 2012.

was attributed to rapid population growth and such factors related to climate as prolonged monsoon and flooding.[26] Phylogenetic analysis of DENV-3 strains implicated in the 2004–2006 dengue epidemic in Sri Lanka demonstrated that a new clade (DENV-3, genotype IIIB) was associated with the most recent outbreak, a shift from the clade (DENV-3, genotype IIIA) circulating from 1988–1989.[27] During this epidemic, there was a shift in the average age of patients afflicted with DHF from 15 to 27 years old. These data suggest that contributing factors to the onset of outbreaks include the presence of an immunologically naïve population and the introduction of a new strain against which existing antibodies are not protective. The number of severe dengue cases from Southeast Asia and the Western Pacific regions continues to increase, although the case fatality rates have notably declined to less than 1% since 2007, likely resulting from improved case management.[28] Myanmar, Indonesia, Thailand, Timor-Leste, and Sri Lanka continue to experience recurrent dengue epidemics in Southeast Asia, while Cambodia, Malaysia, Vietnam, and the Philippines have the highest number of cases and deaths in the Western Pacific region. The largest proportion of dengue-related deaths in the world has occurred in the Asia–Pacific region.[28]

The pattern of dengue disease in Central and South America is characterized by rising incidence, expansion of geographic distribution, and an increase in the incidence of severe forms of dengue, ie, DHF and DSS. The annual number of DF cases reported to the Pan American Health Organization increased at least fourfold from approximately 1 million in the 1980s to 4.7 million in the last decade, with cyclic epidemics occurring since 2000.[29] The highest number of reported DF cases was observed in Brazil, with over 3 million cases from 2000–2007, and the highest incidence was in young adults.[30] However, the highest incidence of DHF was seen in Venezuela, where infants were most commonly affected. DENV-1 and DENV-2 were the most prevalent serotypes in the 1990s, but the pattern has changed recently, so that since their introduction into the region in 2000, DENV-3 and DENV-2 are now the most common circulating serotypes.[30]

The incidence of dengue illness in the Eastern Mediterranean region is believed to be on the rise as well. Outbreaks of dengue virus infection in Saudi Arabia and imported cases of DF from Yemen have been reported in the last decade.[31–33] Local transmission and circulation of DENV-1, DENV-2, and DENV-3 have been documented in this region. Knowledge on the incidence of dengue virus infections in Africa is incomplete, mainly due to lack of surveillance data. Dengue activity in Africa, mostly due to DF caused by DENV-1 and DENV-2, has not been associated with significant mortality, and thus, dengue has not been given the same priority as malaria, human immune deficiency virus (HIV), or acquired immune deficiency syndrome (AIDS). Most recently, DENV-3 infection was reported in persons who travelled from Cote d'Ivoire in 2008.[29]

Clinical manifestation and diagnosis

In order to standardize reporting and improve the case management of dengue cases in clinical practice and surveillance, especially in resource-limited settings in which laboratory diagnostic capabilities may be lacking, the World Health Organization (WHO) developed diagnostic criteria for the various clinical syndromes associated with DENV infections. The dengue case definitions in the 1997 guidelines led to the misclassification of numerous severe dengue cases, so the WHO revised the dengue classifications by adding available laboratory values to clinical information when assigning levels of severity.[29,34]

After an incubation period of approximately 2–10 days, a person with primary DENV infection typically presents with DF that begins with the acute onset of high fever (40°C or higher), accompanied by headache, retroorbital pain, generalized myalgias, arthralgias, malaise, anorexia, and flushed skin. DF symptoms may be clinically indistinguishable from acute febrile illness due to other infectious diseases such as influenza, acute mononucleosis, leptospirosis, or HIV seroconverting illness.[28] While the course of primary DF is typically benign and self-limited, a significant number of patients may experience severe, incapacitating symptoms. The fever may last for 2–7 days or longer, after which defervescence occurs and is followed by the gradual resolution of symptoms without treatment. The early phase of DHF or DSS has clinical features similar to those of DF. However, the period following defervescence, called the critical phase, is typically preceded by warning signs such as restlessness, new or persistent abdominal pain, nausea, and vomiting, followed by thrombocytopenia (with platelet count less than 100,000) and a 10%–15% rise in hematocrit. The critical phase may advance to a serious state marked by a massive decrease in the patient's intravascular volume, resulting from sudden vascular permeability generated by cytokines released when T cells attack dengue-infected cells.[35] The affected patient develops a narrowed pulse pressure and hypotension, and without appropriate supportive therapy may progress to shock leading to multiorgan dysfunction, disseminated intravascular coagulation, life-threatening hemorrhage, and death.

Symptoms among adults with DHF were observed to differ from those manifested in children. In DHF, adults developed petechiae, melena, headache, retroorbital pain, nausea, vomiting, joint pain, and arthralgias, whereas epistaxis, oliguria, and liver enlargement occurred more commonly among children.[36] Right upper quadrant abdominal tenderness and liver enlargement were most frequently found in patients with DHF, and these physical findings correlate well with disease severity.

The underlying mechanisms of coagulopathy and vascular permeability in DHF/DSS remain unclear, but are believed to arise from a combination of the following factors: (a) increased virus replication; (b) increased death of cells from infection or from killing of infected cells by cytotoxic immune cells or antibody-dependent cell cytotoxicity; (c) complement activation; (d) activation of subsets of memory T cells that fail to specifically and effectively target virus-infected cells; and (e) increased release of inflammatory mediators and cytokines by infected cells or by immune cells.[37] Supportive therapy consisting of timely volume replacement is the mainstay of treatment for DHF/DSS, and no other effective therapy is currently available.[29]

Development of dengue vaccines

This section of the review will be devoted to a discussion of the current lead dengue vaccine candidates and other candidates that have advanced into clinical trials (for recent reviews, see Hombach,[38] Whitehead et al,[7] Webster et al,[39] Raviprakash et al,[40] Danko et al,[41] Murrell et al,[15] Guy et al,[42] Coller and Clements,[43] and Gubler[44]), followed by a few of the more interesting second-generation vaccine candidates currently undergoing preclinical evaluation (for recent reviews, see Durbin and Whitehead[45] and Schmitz et al[46]), and some of the critical issues faced by today's dengue vaccine developers (for recent reviews, see Edelman,[47] Thomas,[48] and Thomas and Endy[49]). (See Table 1 for a summary of dengue vaccines currently advanced to clinical testing).

The only dengue vaccine candidate currently advanced to Phase 3 clinical trials is the Sanofi-Pasteur (Lyon, France) tetravalent vaccine. The construction of this vaccine takes advantage of the property of *Flaviviruses* to retain their infectivity when the premembrane-envelope (prM-E) structural gene region of one *Flavivirus* is molecularly recombined, using infectious cDNA clone technology, into the nonstructural gene region or "backbone" of a second *Flavivirus*.[50–54] Thus, a chimeric yellow fever (YF) 17D-dengue virus (CYD) vaccine was produced from the YF-17D vaccine and wild-type dengue virus isolates from Thailand and Indonesia (dengue 1 PUO-359/TVP-1140; dengue 2 PUO-218; dengue 3 PaH881/88, and dengue 4 1228 [TVP-980]), with the four individual CYD vaccine viruses combined into a single tetravalent formulation, containing 5 $\log_{10}$ median cell culture infective doses (CCID50) of each serotype. This vaccine has now been safely administered to over 6000 people.[42] In preclinical testing, the chimeric vaccine viruses were determined to be genetically stable with only a few minor, presumably host cell–adaptive, mutations acquired upon propagation in the production Vero cell substrate, which do not involve positions responsible for YF attenuation, and phenotypically stable with respect to plaque sizes and neurotropism in mice and monkeys.[55,56] Initial Phase 1 clinical trials demonstrated the safety and immunogenicity of the CYD vaccine, as both monovalent and tetravalent formulations, in *Flavivirus*-naïve and *Flavivirus*-immunized adults.[57,58] That the vaccine replicates to some degree in humans is evidenced by low levels of viremia (primarily due to CYD-4)

Table 1 Leading dengue vaccine candidates currently advanced to clinical testing

Vaccine strategy	Developer(s)	Current status
Live attenuated yellow fever 17D/DENV chimeric vaccine	Sanofi-Pasteur	Phase 3 trials with a tetravalent formulation in DENV endemic countries
PDK cell-passaged, live attenuated vaccine	WRAIR/GSK	Phase 2 trials with a tetravalent formulation in endemic countries
Live attenuated DENV Delta-30 mutation and intertypic DENV chimeric vaccines	NIH/Johns Hopkins	Phase 1/2 trials with monovalent formulations completed; tetravalent phase 1 initiated
Dengue prM-E DNA vaccine	NMRC	Phase 1 with monovalent vaccine completed
Recombinant 80% E subunit antigen vaccine	Hawaii Biotech/Merck	Phase 1 with monovalent vaccine initiated
Purified inactivated vaccine (PIV)	WRAIR	Phase 1 with monovalent vaccine initiated
Live attenuated chimeric DENV vaccine	CDC	Phase 1 with monovalent vaccine initiated

Abbreviations: DENV, dengue virus; PDK, primary dog kidney cells; WRAIR, Walter Reed Army Institute of Research; GSK, GlaxoSmithKline Biologicals; NIH, National Institutes of Health; prM-E, premembrane-envelope; NMRC, Naval Medical Research Center; CDC, Centers of Disease Control and Prevention.

in vaccinees primarily after the first dose.[42] With the current tetravalent formulation of 5 $\log_{10}$ CCID50 units per serotype, seroconversion to at least three serotypes occurs in a high percentage of vaccine recipients following three doses, at 0, 6 (or 3.5), and 12 months, although compressed dosing schedules as well as priming with Japanese encephalitis (JE) and YF-17D vaccines are being studied.[42,59] Results of clinical trials with a tetravalent CYD vaccine (TDV) in dengue-endemic areas have recently been reported.[60,61] Clinical trials in countries where dengue and other *Flaviviruses* cocirculate are critical for collecting important vaccine safety and efficacy data, particularly given the increased risk for DHF/DSS in dengue-primed individuals. One such trial was a multicenter, randomized, controlled, observer-blinded study in Mexico City in children age-stratified from 2 to 17 and in adults.[60] Three injections of TDV were administered at 0, 3.5, and 12 months; alternatively, subjects were primed with YF-17D vaccine and then boosted with TDV at 3.5 and 12 months. There were no reported adverse effects other than mild to moderate injection site pain, headache, myalgia, and malaise in 14%–40% of subjects in each group, which did not increase with successive injections. Seroconversion against each dengue virus serotype measured by virus plaque reduction neutralization test (PRNT50) was 77%–92% overall and 95% in 2- to 11-year-olds, specifically, for the TDV-TDV-TDV regimen, and 85%–94% overall for the YF-TDV-TDV regimen. Another randomized, controlled trial was done in children, adolescents, and adults in the Philippines.[61] The subjects in this study were age-stratified with groups 2–5, 6–11, 12–17, and 18–45 years of age, similar to the Mexico City trial, and randomized to receive either three injections of TDV at 0, 3.5, and 12 months, or a licensed typhoid vaccine (TyVi) followed by two doses of TDV at 3.5 and 12 months. Other than some reported transient injection site pain, headache, malaise, myalgia, fever, and asthenia, there were no significant adverse events, and vaccine reactogenicity did not increase with successive doses. Low levels of vaccine viremia were also found, again consistent with limited vaccine virus replication. Dengue-neutralizing antibody responses in children against each serotype after three doses of TDV ranged from 83%–100%, and 100% of adults had neutralizing antibodies to all four serotypes after three doses. Interestingly, similar immune responses were reported for groups that received the TyVi-TDV-TDV regimen with the two TDV inoculations spaced more than 8 months apart, which suggests that a wider interval between doses might eliminate the need for a third inoculation to achieve high seroconversion rates.

Researchers from the National Institute of Allergy and Infectious Diseases and Johns-Hopkins are also developing a live attenuated dengue vaccine candidate that has advanced to clinical trials.[7,45] This vaccine was constructed using reverse genetics to introduce a 30-nucleotide-long deletion mutation (delta-30 mutation) into the 3′-untranslated region (3′UTR) of the dengue viral RNA.[62] The resulting delta-30 mutants exhibit several preclinical markers of attenuation, including reduced viral replication in immune-deficient mice transplanted with human liver carcinoma cells, reduced viremia in rhesus monkeys and lower infectivity for mosquitoes.[63–65] For some virus serotypes, DENV-2 and DENV-3 in particular, an effort was made to improve the attenuation phenotype over that conferred by the delta-30 mutation alone by substitution of the viral prM-E gene region of dengue 4/delta-30 virus with the dengue 2 and dengue 3 prM-E gene regions to produce intertypic dengue chimeras, namely, dengue 2prME/4/delta-30 and dengue 3prME/4/delta-30, respectively.[63] This is analogous to the strategy used for making the CYD vaccine described above. Phase 1 clinical trials of monovalent dengue 1/delta-30 and dengue 4/delta-30 viruses administered by a single subcutaneous inoculation of 10^3–10^5 PFU demonstrated that they are safe and immunogenic, with the only reported adverse effects being relatively minor ones commonly associated with nearly all immunogenic live-attenuated dengue viruses, such as faint rash, transient leucopenia (in a reported 7%–40% of vaccinees in one study), and occasional elevations in the liver enzyme alanine aminotransferase (ALT), mainly at higher vaccine doses and not associated with liver enlargement, abdominal pain or nausea.[45,66–68] A Phase 1 clinical trial of the dengue 2prME/4/delta-30 chimera also shows this vaccine to be safe and immunogenic after a single dose of 10^3 PFU.[68] In another clinical trial, dengue 1/delta-30 virus was tested as both a one- and a two-dose vaccine at 10^3 PFU per dose, with the second dose administered 4 or 6 months after the first.[45] The results show that the seroconversion rate by PRNT after the first dose ranged from 84%–100% (aggregate 93%) and that the second dose, regardless of timing after the first dose, failed to boost antibody responses; therefore, for this vaccine, a second dose does not appear to be necessary for inducing immunity. The search for a satisfactorily attenuated dengue 3 vaccine candidate led to the construction of additional dengue 3 viruses modified to improve their attenuation profiles: a 3′UTR double deletion mutant (dengue 3/delta-30/31–7164); a dengue 3 virus with only the dengue 4 3′UTR/delta-30 mutation (dengue 3–3′/4/delta-30), and a dengue 3/4 chimera (dengue 3prME/4/delta-30). In preclinical studies,

all of these viruses exhibited the expected attenuation markers and in clinical trials all were safe; however, dengue 3prME/4/delta-30 was poorly immunogenic even at a higher dose.[45] Further clinical evaluation of dengue 3–3′/4/delta-30 and dengue 3/delta-30/31-7164 viruses confirmed their safety, and demonstrated their immunogenicity in 80% and 95% of volunteers, respectively. The two dengue 3 virus vaccines described immediately above, along with dengue 1/delta-30, dengue 2prME/4/delta-30 chimera, and dengue 4/delta-30, are among the lead monovalent vaccine candidates being considered for incorporation into tetravalent formulations for testing in upcoming clinical trials.[45] In total, vaccines with the delta-30 mutation along with other selected mutations aimed at improving vaccine attenuation or virus yield in the production cell substrate have been safely administered to almost 500 volunteers to date.[45] The vaccines tested thus far appear to have elicited few reported subjective symptoms other than the occasional headache and remarkably little vaccine-related fever. Thus far, the results look very promising, although it remains to be seen whether a single dose of the tetravalent vaccine formulations will be sufficient for inducing high tetravalent seroconversion rates or whether serotype interference will occur to prevent this.

The third dengue vaccine candidate for which human clinical data are currently available is a DNA vaccine that is being developed by a research team at the Naval Medical Research Center.[41] DNA vaccine technology has now been around for almost 20 years, and while initially promising, in preclinical and limited clinical testing it has been difficult to achieve high levels of immunogenicity using this approach. A monovalent dengue 1 vaccine containing the viral prM and E genes expressed under the control of the human cytomegalovirus promoter/enhancer in plasmid vector VR1012 (D1ME^{100}) is the first dengue DNA vaccine to be advanced to a Phase 1 clinical trial.[69,70] Prior to testing in humans the D1ME^{100} vaccine was evaluated in both *Aotus* and rhesus monkeys, where it proved immunogenic and provided 80%–95% protection against viremia after dengue 1 virus challenge.[71,72] A Phase 1 clinical trial with a dose escalation study design was performed in *Flavivirus*-naïve adults randomized to receive 1 mg (low dose) or 5 mg (high dose) of DNA per dose, three immunizations, administered IM at 0, 1, and 5 months by Biojector.[70] There were no reported severe adverse events in this study, and the most commonly reported side effects were mild injection-site pain or tenderness, local swelling, muscle ache, and fatigue. Over 40% of subjects in the high-dose group but none in the low-dose group developed dengue 1 neutralizing antibodies by PRNT after the second injection. All subjects in the high-dose group and almost 80% in the low-dose group developed antibodies measured by IgM and IgG enzyme-linked immunosorbent assay. One subject in the high-dose group exhibited strong IgM and IgG antibody responses after the first injection, which suggests that this individual may have been primed by previous exposure to an unknown flavivirus that was not picked up by screening. In addition to antidengue antibodies T-cell IFN-γ was detected in 50% and 83% of subjects in the low- and high-dose groups, respectively. Based on the results of this Phase 1study, the D1ME^{100} vaccine was determined to have a favorable reactogenicity and safety profile. Studies are currently under way to evaluate dengue DNA vaccines formulated with the lipid-based adjuvant Vaxfectin® (Vical Incorporated, San Diego, CA) in an effort to improve the immunogenicity profile. In a recent study in rhesus monkeys, a Vaxfectin-adjuvanted, tetravalent dengue DNA vaccine was found to give significantly improved antibody responses and better protection against a dengue 2 challenge than DNA alone, which the authors suggest supports the further evaluation of a Vaxfectin-adjuvanted dengue DNA vaccine in an upcoming Phase 1 clinical trial.[73] There are also some recent reports from other groups who are exploring this technology in preclinical studies in mice, successfully inducing immune responses to dengue antigens, including dengue 4 prM-E, dengue 1 prM-E-nonstructural (NS)1, and dengue 2 NS3 antigen.[74–76]

In the mid-1990s, the first highly purified, formalin-inactivated virus (PIV) vaccine candidate for dengue 2 adjuvanted with aluminum hydroxide (alum) was developed at the Walter Reed Army Institute of Research (WRAIR), and successfully tested in mice and rhesus macaques with good virus-neutralizing antibody titers and protection after two doses.[77,78] This was followed by the development of a second-generation JE PIV vaccine using the same technology.[79,80] The new JE vaccine (Ixiaro®; Novartis Vaccines, Cambridge, MA) is now licensed for use in several countries, including the US, as a result of successful multicenter, multination, Phase 3 clinical trials conducted by Intercell.[81,82] Interestingly, licensure of the JE vaccine did not require field efficacy trials because vaccine-induced neutralizing antibody at a titer of 1:10 or greater is an accepted surrogate of protection from Japanese Encephalitis Virus (JEV).[83] Research on the dengue PIV vaccine has recently expanded, and studies carried out in rhesus macaques demonstrated that, when formulated with newer adjuvants, the vaccine is capable of inducing even higher neutralizing antibody titers than when formulated with alum. Furthermore, it is possible to use tetravalent PIV to prime for

a booster inoculation of tetravalent live-attenuated vaccine 1 month later, which results in tetravalent seroconversion with high virus-neutralizing antibody titers, no vaccine viremia, and solid protection against challenge with all four dengue serotypes.[84,85] A monovalent dengue 1 PIV vaccine formulated with alum is now entering a Phase 1 clinical trial at WRAIR in adult flavivirus-naïve volunteers.

Other candidate dengue vaccines that are well advanced preclinically and just now entering or set to enter clinical trials deserve mention. Among these is a C-terminally truncated, recombinant subunit dengue E glycoprotein antigen (r80E) vaccine produced in *Drosophila* cells, which was originally developed at Hawaii Biotech (Aiea, HI).[86] The r80E antigens have been shown to possess a native-like crystal structure.[87] Tetravalent r80E preclinical vaccine formulations have been demonstrated to induce balanced neutralizing antibody responses in mice and monkeys, with no evidence for immune interference among serotypes, and a clinical grade formulation of the r80E vaccine is now being evaluated in a Phase 1 trial in adults.[86] A live-attenuated, genetically engineered, chimeric dengue vaccine called DENVax, developed at the Centers for Disease Control and Prevention (Fort Collins, CO), contains the dengue structural genes of all four serotypes in a dengue 2 virus primary dog kidney (PDK)-53 backbone.[88] Dengue 2 PDK-53 is one of the PDK cell passaged dengue vaccine seed viruses originally developed by Halstead et al and extensively tested in clinical trials by the group at Mahidol University, Bangkok, where it was found to be highly attenuated and immunogenic in both children and adults. DENVax exhibits attenuated neurovirulence and good immunogenicity in mice, and is immunogenic for cynomolgus monkeys with high seroconversion rates after two doses of the tetravalent vaccine, although the neutralizing antibody titers against dengue 4 virus are reported to be lower than for the other serotypes.[88,89] DENVax is currently being evaluated in Phase 1 clinical trials in adults in the US and in Colombia, South America.[88]

Some other novel third-generation dengue vaccine approaches also deserve mention. Recombinant subunit E domain III (ED3) antigen is an interesting vaccine candidate because it appears to contain mainly virus type-specific epitopes, some of which induce potent neutralizing antibodies in mice, although only partial protection was observed in rhesus monkeys (Simmons, unpublished data), and the epitopes may not be immunodominant in the human antibody response to natural dengue infection.[90–94] There is also a recent report of ED3 from all four serotypes expressed from a recombinant pediatric measles vaccine vector able to generate tetravalent memory neutralizing responses in mice.[95] A dengue 1 vaccine candidate inactivated with psoralen/UV, which is a nucleic acid–specific pyrimidine cross-linking agent, was recently reported to be immunogenic and protective in *Aotus* monkeys,[96] suggesting that psoralen may be an alternative, potentially gentler, method for dengue virus inactivation. Finally, dengue 2 viruses genetically altered to contain deletion mutations in the gene region encoding the E protein transmembrane domains (which allow these viruses to replicate well in insect cells but less well in mammalian cells) were recently reported to be genetically and phenotypically stable and highly immunogenic in mice, suggesting that this may be a general method for developing host range-restricted vaccines for enveloped viruses.[97]

Antiviral therapeutics

Mosquito control efforts are currently the only method by which to prevent disease caused by dengue viruses. In the absence of a vaccine to prevent dengue virus infections, antivirals may be useful in some situations. By reducing initial acute viremia, therapeutic agents may be able to reduce disease transmission as well as progression to the severe forms of the disease DHF/DSS. As short-term prophylaxis, antivirals could be used to reduce the potential for transmission. Antivirals against *Flaviviruses* have principally been directed at the inhibition of viral replication, targeting nonstructural proteins, RNA polymerases, and proteases essential for virus replication. Potential inhibitory targets include the NS3/NS2B protease, the NS3 helicase nucleoside triphosphatase (NTPase)/RNA 5′ triphosphatase (RTPase), and the NS5 methyl transferase/RNA-dependent RNA polymerase. An alternative approach to prevent dengue disease is to inhibit entry into cells. Directing inhibitors to the viral envelope protein avoids the difficulty of crossing the plasma cell membrane and internal membranes. The *Flavivirus* E protein mediates binding of the virus to cell surface receptors such as dendritic cells (DC)-Sign, L- Sign, laminin, mannose and glucose-regulated protein 78. Targeting host cellular functions required for viral replication is an additional strategy to inhibiting *Flavivirus* infection. α-glucosidases I and II are enzymes in the endoplasmic reticulum essential for N-linked glycan processing and are essential for proper folding of viral glycoproteins. It has been shown that imino sugars that are glucose mimics act as competitive inhibitors of α-glucosidases and can inhibit enzymatic activity.[98–102]

Since both hepatitis C virus (HCV) and DENV belong to the same viral family, *Flaviviridae*, it is hoped that advances in the HCV drug development may prove useful for DENV

antiviral drug development. Viral protease inhibitors have shown promise as antivirals for HIV, with nine protease inhibitors currently in clinical use, and a number of protease inhibitors of HCV and human rhinovirus are in clinical trials.[103–106] A number of compounds inhibiting HCV helicase have been evaluated, but the majority of inhibitors indicated low potency and/or toxicity.[107–112] Antiviral nucleoside/nucleotide inhibitors are prodrugs, which are pharmacologically inactive compounds that become active by enzymatic transformation. A potent nucleoside analog must first be recognized and phosphorylated by the host nucleoside/nucleotide kinases before incorporation into the viral genome, where it acts as a chain terminator.[113] The nucleoside inhibitor 2′ C methyl deaza-adenosine was reported to be an anti-*Flaviviral* agent for both HCV and DENV.[114] Another report showed that the adenosine analog NITD008 inhibits DENV in vitro and in vivo; however, adverse effects were observed in rats and dogs after a 2-week regimen.[115] Similarly, the adenosine nucleoside prodrug NITD203 inhibited various *Flaviviruses*, including DENV, yellow fever virus, West Nile virus, and HCV, but safety levels were found to be unsatisfactory in a 2-week in vivo toxicity study.[116] Two peptides, 1OAN1 and DN57opt, were recently identified by computational screening to block virus cell binding, and peptide entry inhibitors 1OAN1 and DN59 were subsequently shown to inhibit ADE in vitro.[117,118] Tetracycline and doxorubicin antibiotic derivatives have also been shown to interfere with DENV-2 and YFV-17D virus entry in vitro, but they also proved to be cytotoxic and cytostatic.[119,120] The ability of dengue viruses to utilize multiple cellular receptors provides a considerable challenge to the development of inhibitors of receptor-mediated viral entry.

Small interfering RNAs have been used for gene-specific therapeutics to degrade homologous target mRNAs. RNA interference (RNAi) has been used effectively to inhibit virus replication in vitro for respiratory syncytial virus, hepatitis viruses, influenza virus, polio virus, and HIV.[121,122] A recent study used RNAi to silence the CD-14 monocyte receptor and clathrin-mediated endocytosis to prevent DENV-2 entry and replication in human monocytes.[123] A strategy of combining α-glucosinase inhibitor CM-10-18 and ribavirin, a broad-spectrum antiviral nucleoside analog, resulted in a modest reduction of DENV infection in mice when used alone, but in combination achieved enhanced and statistically significant reduction of DENV-2 viremia.[124]

There have also been a number of reports on the DENV antiviral activity of phytochemicals such as flavonoids, which are low-molecular-weight phenolic compounds found in different kinds of plants.[125–128] Recently, in vitro treatment of infected cells with quecertin resulted in 75% reduction of intracellular replication of DENV-2, and pinostrobin was shown to inhibit DENV-2 NS2B/NS3 protease in an in vitro study.[125,129] Another study reported inhibitory activity of several flavonoid-derived compounds against DENV-2 in HepG2 cells, with a range of potency strengths of 72%–100%.[130] Methanolic extracts of *Andrographis paniculata* and *Momordica charantia* showed 75% and 50% antiviral inhibitory effect, respectively, against DENV-1 replication in Vero cells.[131]

There are currently no antiviral drugs to treat dengue disease, and none of the identified antiviral compounds so far have progressed into clinical trials. This is largely due to the problem of bioavailabilty of small peptides, sensitivity to proteolytic cleavage, low efficacy, and toxicity.[107–112] The potential weakness for some small-molecule inhibitors is the problem of resistance and escape mutations. In the case of HCV, virus heterogeneity poses a problem because inhibitors effective against one genotype may not be effective against other genotypes.[132] One approach would be to develop dengue antivirals that interact with conserved residues, which may delay the emergence of drug-resistant dengue viruses and serve as the basis for developing broad-spectrum antivirals. It is important to consider that most single antiviral compounds may reduce viral replication, but do not eliminate it. Therefore, incomplete downregulation may necessitate the use of a different class of inhibitors in double or triple combination therapy approaches with four or more genetic barriers to resistance. However, downregulation may be sufficient to provide clinically appropriate improvement. The greatest impact of an antiviral is at an early stage of DENV infection. With the use of rapid diagnostics, a drug administered at an early stage would potentially prevent severe disease. During endemic outbreaks, rapid diagnostics could detect infected yet asymptomatic people and allow for prophylactic treatment, leading to prevention of full-blown disease and a reduction in the infected vector population. Dengue antivirals might also have a place in travel medicine and to help bridge the gap for some vaccines that require multiple immunizations over an extended period before they confer protection.

Conclusion

After over 60 years of almost continuous, dedicated effort to develop a vaccine for dengue, the finish line finally appears to be within reach. Nevertheless, there are still critical issues faced by today's vaccine developers.[47–49] First there is the

ever-present risk of enhancement of a subsequent dengue infection by enhancing antibodies or memory T cells, which might result in worsened disease.[133,134] To mitigate this risk, it is assumed that a tetravalent dengue vaccine should be capable of inducing balanced Th1/Th2-type responses with tetravalent or at least trivalent virus-neutralizing antibodies, specific T cells that are efficient at clearing virus-infected cells, perhaps accompanied by a protective IFN-γ response but not other potentially deleterious cytokines, and finally, the vaccine should elicit adequate T- and B-cell memory to protect against reexposure in areas where dengue is endemic.[135] With tetravalent live-attenuated dengue vaccines, the propensity for serotype interference in particular, sometimes necessitating the use of multiple, widely spaced doses, is a concern to vaccine developers, because the initially imperfect protection may pose risks not only for children in endemic areas but also for travelers to those areas.[136] Conversely, with nonreplicating vaccines, the risk is that immunity will wane too rapidly, necessitating frequent reboosting. Clearly, the task to develop and implement a vaccine to protect against dengue is a huge one, with much hard work remaining. While a single dengue vaccine may not provide all solutions to all problems and different vaccines might eventually have to be tailored to suit specific requirements, what is not in doubt is the huge public health and economic impact of dengue infection and therefore the vast benefits a successful vaccine will bring.[137–140]

Antivirals may be a viable option for the prevention and treatment of dengue disease, not meant to replace, but to complement, vaccination. The development of severe disease (DHF/DSS) has been associated with higher levels of viremia.[141] Diagnostic tests to detect viral RNA (reverse transcription polymerase chain reaction) or viral NS1 (enzyme-linked immunosorbent assay) have been developed and are readily available to rapidly detect recent DENV infections.[142–144] When available, antiviral drugs could be given before or shortly after exposure and before illness occurs to prevent symptomatic disease and reduce transmission. Potential targets for DENV antivirals have been identified, and the knowledge gained from the successful drugs against HCV could be applied to DENV drug design. Viral proteases and polymerases have shown to be the most successful targets for inhibitors of HIV and HCV; however, virus entry and assembly are attractive targets as well. Successful inhibitors must be able to suppress viral replication in vivo by ≥tenfold and avoid drug resistance; combination therapy using different classes of inhibitors will improve efficacy.[113]

Disclosure

The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of the Army, Department of Defense, or the US Government. The authors report no conflicts of interest in this work. This work was prepared as part of our official duties. Title 17 USC article 105 provides that "Copyright protection under this title is not available for any work of the United States Government." Title 17 USC article 101 defines a US Government work as a work prepared by a military service member or employee of the US Government as part of that person's official duties.

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