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DENGUE VACCINES: NEW DEVELOPMENTS

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SUMMARY

Dengue represents a major, growing public health problem, with an estimated of 2.5 billion people at risk of infection and about 50 million cases. Vector control is the only way of controlling dengue transmission; however, in spite of efforts, an expanded geographic extension of the vector and viruses is observed. Consequently, there is an urgent need for a safe and effective dengue vaccine. Currently, there is no licensed vaccine available, although there are several candidates in different stages of development, a few highly promising. Strategies that have been followed include inactivated viruses, conventional live attenuated vaccines, chimeric live attenuated vaccines, DNA, subunit and vectored vaccines. Advances and challenges of dengue vaccine development are updated here.

INTRODUCTION

A global pandemic of dengue, a mosquito-borne viral disease, started during World War II, intensifying during the 1970s (1-3). Since then, the prevalence of dengue fever (DF), as well as the life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), has increased exponentially, with approximately 2.5 billion people at risk, about 50 million cases and 250,000-500,000 severe cases occurring annually (4). The dengue case fatality rate is estimated at 1-5% and the estimated number of disability-adjusted life years (DALYs) lost to dengue in 2001 was 528 (5). A recent study by the Pediatric Dengue Vaccine Initiative (PDVI) reported figures of 3.61 billion, or half the world's population, at risk for dengue infection, 500 million people infected and 2.1 million severe cases occurring annually (6).

Dengue viruses (DENVs) are transmitted to humans by infected mosquitoes, mainly *Aedes aegypti* and *Aedes albopictus*. Infection by each of the four dengue serotypes can lead to a broad spectrum of outcomes, from asymptomatic and very mild infections to DF and the severe form, DHF/DSS (7).

The disease is reported in Southeast Asia, the Western Pacific, the Caribbean and Latin America, the Middle East and Africa, with more than 70% of the disease burden in the first two regions. The causes of the global emergence and reemergence of dengue, associated with more frequent and larger epidemics, a greater number of severe cases and the co-circulation of several serotypes are not well understood. Unprecedented population growth, unplanned urbanization, deficient water supply, lack of vector control measures, large movement of people towards urban areas and climatic change are some factors that have contributed to the emergence of dengue. The epidemiologic situation in the American region well exemplifies the emergence of this disease (2, 8-11).

In the last 40 years a dramatic spread of DENVs within American countries has been observed, with an increased number of epidemics, shorter intervals among them and increased reports of DHF/DSS cases (10). The total number of reported cases has increased from 1 million in the 1980s to more than 6 million in the current decade. From around 60 DHF/DSS cases reported by 6 countries in the 1980s, these figures have increased to more than 30 countries with more than 165,000 DHF/DSS cases. By September 2010, more than 1,400,000 dengue cases, including 30,820 DHF/DSS cases and 710 fatalities, were reported (http://new.paho.org/hq/index.php?lang=es). Figure 1 shows the increased number of total reported cases in the American region by decade.

Recent reports of dengue in travellers returning from Africa suggest that there was a substantial increase in epidemic dengue activity in this continent during the 1980s (12-14). In the current decade, major DENV outbreaks have been reported in some African countries, suggesting a change in the dengue epidemiology in this region (15, 16).

Currently, vector control is the only way for controlling dengue transmission; however, in spite of efforts, an expanded geographic extension of the vector and viruses, and consequently an increased trend in dengue transmission, the number of countries and regions reporting dengue epidemics, DHF/DSS cases and the cocirculation of several DENVs are observed. In recent years, an increased report of dengue infection in travellers returning from dengue endemic countries, the concurrent coinfection of dengue with other infectious agents and the risk of transmission through transfusion of blood

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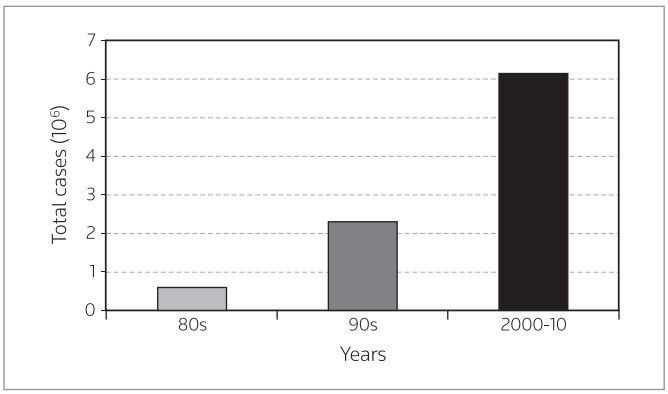

Figure 1. Total reported dengue cases in the American region by decades (period 1980 to February 2010) (http//new.paho.org).

from asymptomatic viremic individuals have been documented (17-22). The report of dengue transmission in Florida, the detection of autochthonous dengue cases in France (23, 24), the rapidly expanding range of *A. albopictus* in Europe and the possibility of dengue transmission in this region, particularly if *A. aegypti* is reintroduced (25), pose a serious concern at the global level.

Because of the increased severity of the global situation, a vaccine against dengue is urgently needed (26, 27). A substantial amount of research has been undertaken over the years to develop a dengue vaccine. Advances and challenges of its development are updated here.

DENGUE VIRUSES

Dengue viruses belong to the family *Flaviviridae*, genus *Flavivirus*. Dengue complex consists of four antigenically closely related serotypes (DENV-1 to -4) (28). They are positive-strand RNA viruses with a genome of about 11 kb encoding three structural proteins (C, capsid protein; prM, membrane precursor of membrane protein; E, envelope protein) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (29).

The viral nucleocapsid consists of the genome surrounded by the capsid protein. The viral envelope consists of a lipid bilayer in which the E and M proteins are embedded (28). In immature virions, prM forms a heterodimer with E protein. It is believed that prM protein serves as a chaperone for proper E folding and assembly. The maturation event of prM to M is necessary to expose the E receptor binding domain, and thus for virus infectivity (30-33).

The E glycoprotein, comprised of 495 amino acid residues, is responsible for viral attachment and entry, elicits protective neutralizing antibodies in the infected host and plays an important role in virus virulence (32). In the mature virion, E exists as homodimers. Each monomer of the dimer has an elongated shape with three domains (I, II and III), the central *N*-terminal domain I, followed by the dimerization domain II and the *C*-terminal Ig-like domain III (34). Domain II contains the hydrophobic fusion peptide (residues 98-110) essential for virus–cell fusion (35-37), while domain III functions as the binding site for cellular receptors (38, 39). Under a low pH environment, E protein undergoes trimerization to expose the fusion peptide.

The NS3 protein is involved in the cleavage of the translated viral polyprotein and plays a role in viral RNA replication through the nucleotide triphosphatase and helicase activities (40, 41). NS5 functions as the viral RNA-dependent RNA polymerase. Also, NS5 has methyltransferase activity (42, 43). NS1 is required for virus replication. This protein has secreted and cell-associated forms, the latter associated with the level of viremia (44). NS1 is an important target for humoral immunity and plays a role in disease pathogenesis, while NS3 is an important target for T-cell epitopes (45, 46).

Nucleotide sequence analysis has allowed classifying DENVs into genotypes, with three to five genotypes depending on the serotype. Studies have suggested that specific viral structures may contribute to increased replication in human cells and to increased transmission by the vector (47). For instance, the replacement of 5'- and 3'-untranslated regions and amino acid E-390 among Asian and American DENV-2 genotypes significantly reduced virus replication in dendritic cells (48). In addition, some studies support that the Asian DENV-2 genotype produces a more efficient infection and dissemination in mosquitoes (49).

Serious DENV disease can result from infection with any of the four serotypes, although some genotypes and specific strains of DENV-2 and DENV-3 have been particularly associated with DHF/DSS epidemics (9, 50-56). Examples of this association include the Cuban DHF/DSS epidemic of 1981, as well as the DHF/DSS epidemics reported in the American region after the 1980s, caused by Asian genotypes of DENV-2 and DENV-3 (52, 55, 57-61). The increased movement of viruses among countries and increased virus transmission have resulted in an increased rate of evolution, with a rise in viruses with greater epidemic potential and a major impact on their virulence for humans (47, 62). Relatively recent, intramolecular recombination has been documented for the four serotypes, although up to now, the epidemiologic and clinical implications of this phenomenon have not been documented (63-67). Also, the possible virus evolution over the course of a dengue epidemic, as well as the potential appearance of neutralizing escape mutants, have been reported (50, 51, 68). In addition, several studies suggest the presence of quasispecies in both humans and mosquitoes (69, 70); however, we still do not know if these populations differ in important phenotypic properties. All these observations suggest that viruses with an expanded range of pathogenic properties could be observed in the future (67).

Besides the urban dengue cycle, transmission cycles involving monkeys and forest-dwelling *Aedes* mosquitoes (*A. luteocephalus, A. furcifer, A. taylori* and *A. niveus,* among others) have been identified in Asia and West Africa (71-74). Sylvatic viral strains from several serotypes have been isolated (47, 75). A documented outbreak of sylvatic DENV-2 in humans was reported in Nigeria during the 1960s (76). In addition, a DENV-2 from a sylvatic lineage was isolated from a DHF case in Malaysia (77). In spite of this evidence, the role of the sylvatic cycle is under discussion, but the suggestion that sylvatic DENV poses a risk for human health and that these viruses may eventually emerge in humans deserves careful study (66, 78).

THE CLINICAL MANIFESTATIONS OF DENGUE

Dengue infection has a wide clinical range that includes very mild illness, mainly in young children. In older children and adults, the classic fever–arthralgia–rash syndrome with fever, myalgia, arthralgia, retro-orbital pain and lymphadenopathy is observed. Minor bleeding, such as petechiae, leukopenia and mild thrombocytopenia, is also common. DHF/DSS is characterized by increased vascular permeability and plasma leakage, with thrombocytopenia and bleeding. An increase in hematocrit, effusions and edema are the expression of plasma leakage. Depending on the severity of plasma leakage, patients can evolve to shock (79, 80). The World Health Organization (WHO) dengue clinical classification has been in use since the 1970s. According to this classification, a DHF/DSS case must fulfill four criteria: fever, bleeding, hemoconcentration and thrombocytopenia of < 100,000/mm^3. Dengue clinical cases represent the peak of the iceberg.

With the globalization of dengue to different tropical areas and age groups, variations in the clinical manifestations of the original description have been reported (81-83). A recent multicountry revision of the 1975 WHO clinical classification was performed and a new one based on the disease severity (dengue and severe dengue) has been proposed (84). The new classification calls attention to the clinical warning signs as a predictive early sign of severity (Table I). At present, some countries have introduced the new classification. In addition, some multicountry studies are ongoing for further validation.

DENGUE PATHOGENESIS

DHF/DSS occurs in children and adults who become infected with a second DENV of different serotype and in infants with a primary DENV infection born from DENV-immune mothers (85-88). Secondary infection has been significantly associated with DHF/DSS, with an estimated 2-4% of cases with a secondary infection developing DHF/DSS. Although secondary infection is considered the main risk factor for DHF/DSS (89-97), other host factors, such as age (higher risk in children), ethnicity (whites have a higher risk of severity compared to black individuals), chronic diseases (bronchial asthma, diabetes, sickle cell anemia) and host genetic factors (polymorphism of HLA, Fc receptors, vitamin D receptor, DC-SIGN), are also a risk (54, 57, 98-106). Some viral sequence of infections, the longer time interval among primary and secondary infections, the serotype and the virus genotype are associated with a higher risk of DHF/DSS (96, 107-111). Table II shows some host risk factors for DHF/DSS.

Earlier studies by Sabin in 1952 demonstrated a short cross-protective immunity among serotypes (around 1-2 months), while other studies suggest that homologous specific immunity is long-lasting (112-116). Neutralizing antibodies can reduce viremia levels, whereas cross-reactive non-neutralizing antibodies may increase viremia (91).

Antibody-dependent enhancement (ADE) was proposed early on to explain DHF/DSS. Complexes containing enhancing immunoglobulin (IgG; from a primary infection) and virus attach to Fc receptor-bearing cells, enhancing the efficiency of virus binding and increasing the number of infected cells (91, 117). The ADE hypothesis postulates that non-neutralizing cross-reactive antibodies from a primary infection could mediate an increased uptake of virus into monocytes via the Fc receptor, leading to an increase in viral replication and immune activation with mediator release (118). Consequently, a higher viral load, plasma leakage and increased severity are expected. Several studies support a higher viral load in secondary DHF/

Table I. *New WHO dengue case classification (84).*

Dengue with or without warning signs		Severe dengue
Probable dengue	Warning signs	Criteria for severe dengue
Live in/travel to dengue endemic countries	a) Abdominal pain or tenderness	*Severe plasma leakage:*
Fever and two of the following criteria:	b) Persistent vomiting	a) Signs of shock
a) Nausea/vomiting	c) Clinical fluid accumulation	b) Fluid accumulation with respiratory distress
b) Rash	d) Mucosal bleed	
c) Aches and pain	e) Lethargy, restlessness	*Severe bleeding as evaluated by clinician*
d) Tourniquet test positive	f) Liver enlargement > 2 cm	*Severe organ involvement*
e) Leukopenia	g) Laboratory increase in hematocrit concurrent with rapid decrease in platelet count	a) Liver: AST or ALT ≥ 1,000
f) Any warning sign		b) CNS: impaired consciousness
		c) Heart and other organs

Table II. *Some identified host risk factors for dengue hemorrhagic fever.*

Risk factors	
Age	Higher risk in children (86, 92, 106)
Nutritional status	Good (92)
Ethnicity	Higher risk in white individuals (57, 98, 100, 101)
Chronic diseases	Bronchial asthma, diabetes, sickle cell anemia (57, 100, 339)
Secondary infection	Sequence of infection, serotype and genotype of the second virus, interval between infections (92, 96, 97, 107, 109)
Host genetics	HLA class I, II, III, FcγR, vitamin D receptor, DC-SIGN1-336, others (99, 104, 105, 340)

DSS cases (119, 120). A detailed analysis of dengue outbreaks in Cuba in 1977 (DENV-1), 1981 (DENV-2), 1997 (DENV-2) and 2001 (DENV-3) supports the role of ADE as an important risk factor for disease severity (95, 97, 121-125). In these epidemics, DHF/DSS cases were significantly associated with secondary infection. A recent study published by Cummings et al. shows that ADE may provide a competitive advantage to any serotype that undergoes enhancement and that this advantage increases with increasing numbers of co-circulating DENVs (126). These results suggest that there is a dynamic fitness landscape for DENV by using enhancement.

E and prM proteins are involved both in neutralization and ADE. E glycoprotein contains the main epitopes recognized by neutralizing antibodies (virus-specific and cross-reactive epitopes) and is considered the primary antigen inducing protective immunity, but also ADE (127-129). On the other hand, infection enhancement and lack of potent neutralization are common properties of anti-prM antibodies, suggesting that prM constitutes another target for infection-enhancing antibodies. Recently, Rodenhuis-Zybert et al. published data demonstrating that antibodies to prM may be involved in ADE (130). These observations were supported by Dejnirattisai et al. (131), who observed that antibodies to prM are highly cross-reactive among the four serotypes, with potent ADE activity and low neutralization capacity (132).

A different but complementary theory of immunopathology involves the reactivation of memory T cells. Serotype-specific and serotype-cross-reactive T cells have been detected in peripheral blood mononuclear cells (PBMCs) of individuals with acute dengue infection (45, 133-136). It has been suggested that the low avidity of serotype cross-reactive T cells raised to the first infecting virus predominates during the secondary infection (original antigenic sin phenomenon), with a low capacity for infected cell clearance (137, 138). A role for T-cell activation has been proposed (134, 139-141). Concentrations of cytokines, mediators and soluble receptors are significantly increased during infection, some associated more with disease severity (142-147). Endothelial permeability and consequently plasma leakage characterize DHF/DSS, although damage to endothelial cells does not appear to be responsible for the increase in permeability. Plasma leakage appears to be the result of transient endothelial permeability caused by one or more soluble mediators released by the endothelium or by immune cells (142, 148-150).

Both mechanisms (ADE and T-cell response) could be involved in the increase in plasma levels of numerous soluble mediators and cytokines, and consequently the increase in plasma leakage; however, DHF/DSS in infants with primary dengue infection cannot be well explained by the T-cell activation (86, 151).

Recently, Sierra et al. reported the results of an ex vivo study. PBMCs from dengue-immune individuals were challenged with homologous and heterologous DENVs. Homologous rechallenge triggered the expression of regulatory cytokines, while heterologous challenge triggered a dominant inflammatory response. The authors suggest that the equilibrium between inflammatory and regulatory patterns of the immune response is important for a balanced and effective control of dengue infection. The uncontrollable inflammatory response (IFN-γ/TNF-α) in the absence of an adequate regulatory control (TGF-β/IL-10) could explain the enhanced risk of DHF/DSS after a heterologous reinfection in individuals exposed to DENV long before (152).

Vascular damage can also be induced by cross-reactive antibodies. It has been documented that an antibody to NS1 protein can cross-react with antigens on the surface of endothelial cells and induce apoptosis, as well as the secretion of proinflammatory cytokines and chemokines involved in the increased vascular permeability (153-156). In addition, anti-NS1 antibodies have also been shown to cross-react with human and mouse platelets and were able to cause transient thrombocytopenia and hemorrhage in mice (157, 158). Cross-reactivity of antibodies against E protein with plasminogen has been associated with bleeding in acute DF infection (159-161).

Complement activation is another important observation during DHF/DSS. Levels of C3a and C5a, complement activation products, correlate with disease severity. It is assumed that complement is activated by various mechanisms, such as the circulating immune complexes observed during DHF/DSS, the possible immune complexes among the secreted NS1 protein and pre-existing cross-reactive antibody, among others (135, 162-164).

In summary, the mechanisms that have been considered to be associated with the pathogenesis of DHF/DSS include non-neutralizing enhancing antibodies in secondary dengue infections with heterologous serotypes, memory T-cell-mediated pathogenesis, antibodies cross-reacting with vascular endothelium, complement activation, cytokine and mediator production, selection of virulent strains, as well as host characteristics (54, 57, 92, 156, 165-167).

DENGUE VACCINES

Objectives and challenges

A dengue vaccine should simultaneously generate long-lasting immunity to the four serotypes. A safe vaccine must address the ADE phenomenon and particularly the immune potentiation of the illness during a second dengue infection. Consequently, the vaccine must be tetravalent. Other challenges for a dengue vaccine include the lack of an animal model to reproduce the main clinical features of DHF/DSS, the incapacity to predict the efficacy of the vaccine candidate, the need for a better understanding of the correlates of protection, the viral interference observed in attenuated live vaccines, the possibility of vaccine sensitization to DHF/DSS in time and the number of doses for an adequate immunization. In addition, phase II and particularly phase III trials, as well as the implementation of vaccination, are also challenges for a dengue vaccine (154, 168-173). A dengue vaccine requires a long follow-up, and also must be tested both in Asia and the Americas. Table III shows some issues and challenges associated with the development of a dengue vaccine.

Correlates of protection

The mechanism of protective dengue immunity is not well understood. Neutralizing antibodies to viruses serve as the most commonly used correlate of protection (174). Although several data support the role of neutralizing antibodies as the main effector of protection against dengue infection, the characteristics of these antibodies, the neutralization mechanism, as well as the neutralizing epitopes, are not well defined (170, 171).

Table III. *Challenges facing vaccine developers.*

a) The vaccine should induce long-lasting immune protection against the four viruses
b) Needs to be tetravalent
c) Antibody-dependent enhancement must be avoided in the short and long term after vaccination
d) Interference with attenuated live vaccines must be avoided
e) No animal model useful for vaccine evaluation or pathogenesis studies are available Mice and monkeys are used for vaccine evaluation but results do not always predict the usefulness of a vaccine candidate
f) Improved laboratory tests need to be developed to determine protective immune correlates
g) Clinical trials need to be performed in dengue endemic areas with different patterns of dengue transmission
h) Logistics of vaccine implementation Cost and cost-effectiveness, vaccination strategy

Antibodies produced during an infection provide lifelong protection to the homologous virus but short-lived protection against the other three serotypes (112, 115). Most neutralizing antibodies recognize E protein, although some recognition has been reported for prM/M proteins (175, 176). Based on epitope mapping data, many type-specific neutralizing antibodies localize at domain III, whereas cross-reactive epitopes localize mostly at domain II of E protein (177-181). Recent studies identified two structurally distinct neutralizing epitopes on domain III of DENV-2: a type-specific and a subcomplex-specific epitope (182). The mechanisms of neutralization include interference in the virus entry pathway, including attachment, internalization and fusion (128).

At present there is interest in developing patterns or "signatures" that correlate with protective immune status, including the cellular immune response. Some data suggest the usefulness of incorporating specific cellular immunity design features into vaccine candidates, particularly to improve antiviral T-cell responses such as cytolysis and IFN-γ secretion with high avidity to variant epitopes to the four serotypes. These responses may improve viral clearance, as well as anamnestic neutralizing antibody response (183, 184). A T-cell response to the four serotypes was studied in volunteers vaccinated with a tetravalent dengue vaccine candidate. In vitro proliferation response, IFN-γ production and cytolytic response were observed in all individuals, but responses to the four viruses were not equal. Proliferation responses were associated with the induction of neutralizing antibodies to the homologous virus (185). These data suggest that a live attenuated tetravalent vaccine can induce CD4 and CD8 T-cell responses to several serotypes, although more research is needed to improve our understanding of dengue pathogenesis and guide the development, testing and evaluation of dengue vaccines (46, 186, 187).

The role of cell-mediated immunity (CMI) for both protection and pathogenesis, as well as the practical aspects of CMI analysis in clinical trials, were analyzed during the scientific consultation on CMI organized by the Initiative for Vaccine Research (IVR) at the WHO (188). Additional studies are needed to better understand the mechanisms of protection and pathogenesis and the role of antibodies, innate immunity and CMI.

Main strategies for dengue vaccine development

Currently, there is no licensed dengue vaccine available, although there are several vaccine candidates in different stages of development, with a few highly promising. Strategies that have been followed include inactivated viruses, conventional live attenuated vaccines, chimeric live attenuated vaccines, DNA vaccines, subunit and vectored vaccines. Each of these strategies has advantages and disadvantages.

Inactivated vaccines

Putnak et al. reported that mice and monkeys immunized with inactivated DENV-2 grown in certified Vero cells for vaccine production were protected from virus challenge; however, the use of inactivated vaccine requires booster doses (189). Very recently, psoralen was used for DENV-1 inactivation. An anti-DENV-1 neutralizing antibody was detected in mice immunized with psoralen-inactivated DENV-1 (190). Also, GlaxoSmithKline entered into a joint venture with Oswaldo Cruz Foundation (Fiocruz) to develop a vaccine (http://www.gsk.com/responsibility/access/technology-transfersn-joint-ventures.htm, http://www.gsk.com/media/pressreleases/2009/2009_pressrelease_10085.htm).

Inactivated whole virus vaccines have two advantages when compared to live vaccines: they cannot revert to a more pathogenic phenotype and do not interfere (no replication competition) with each other in combination. However, the possibility that incomplete protection with the risk of enhancement of the infection requiring booster doses, as well as the low virus replication efficiency in certified cells, represent challenges for this strategy (184, 191-193).

Live attenuated vaccines

Early research on dengue vaccines was based on DENV-1 and -2 propagation in mouse brain. This work was followed by a field efficacy trial in Puerto Rico in 1962, in which partial protection was observed in vaccinated individuals (112, 194-196). During the 1980s, four vaccine candidates were produced in tissue cultures (primary dog kidney [PDK] cells), tested in nonhuman primates and then administered to small numbers of volunteers (194, 197-202). Under- and overattenuation of vaccine candidates were observed, suggesting that biological markers such as temperature sensitivity and plaque morphology were insufficient to predict DENV attenuation for humans (194, 203). After several trials, the safety and immunogenicity of 16 formulations allowed the selection of some tetravalent candidates (204-206). A new study with tetravalent candidates first demonstrated the feasibility of repeated doses and the increasing levels of trivalent or tetravalent neutralizing antibodies (194, 205).

Recently, one of these combinations achieved overall seroconversion of 69%, 100%, 81% and 94%, respectively, to DENV-1, -2, -3 and -4 in all volunteers after two doses (207). This tetravalent formulation was also safe and induced tetravalent neutralizing antibodies in Thai children (208). An important observation of these vaccine candidates developed by the Walter Reed Army Institute of Research (WRAIR) was the low dissemination rates of attenuated viruses in *A. aegypti* and *A. albopictus* (209). The vaccine has been licensed by GlaxoSmithKline and has been evaluated in a phase II clinical trial in Thailand and Puerto Rico. Data confirmed that two doses of vaccine are needed over a duration of at least 6 months to elicit acceptable neutralizing antibody titers.

Another tetravalent vaccine candidate was developed at Mahidol University, Bangkok, Thailand (210). The vaccine viruses were also derived by serial passage of wild-type strains in PDK cells. Initial human trials suggested the DENV vaccine to be safe and immunogenic in adults and well tolerated in children (210-213). The vaccine was licensed by Aventis Pasteur. Although phase II testing was completed in children, with tetravalent seroconversion in all recipients after three doses of vaccine, further development of this candidate was stopped due to reactogenicity and formulation issues with the DENV-3 component (214-216).

Because attenuated vaccines consist of replicating agents, they can induce humoral and cellular immune responses. They may also immunize with only one dose and they may be produced at relatively low cost (212). However, they also have some disadvantages, such as potential reactogenicity in vaccinees, interference among vaccine components, genetic instability leading to reversion to virulence and heat lability that requires maintenance of a cold chain for a hydrated vaccine preparation, as well as the potential risk of transmission through mosquitoes of the vaccine virus (193). There is also the potential risk of enhanced disease following dengue vaccination. In addition, enhanced reactogenicity is possible in subjects with pre-existing anti-*Flavivirus* antibody (184). Finally, the theoretically potential risk of recombination between the vaccine viruses and other wild-type viruses, resulting in recombinants with novel properties, has been suggested; however, according to some reports this is unlikely (217, 218). Most of these risks have not been seen in current clinical trials.

Infectious clone-based vaccines

Infectious, genome-length cDNA clones allowed the efficient, directed genetic engineering of mutations in the viral genome and analysis of phenotypic effects of mutations in the clone-derived viruses (193). This technology has been successfully applied and a number of infectious clones derived from several DENVs, as well as from the yellow fever (YF) 17D virus and other flaviviruses, have been developed (219-222). Among these, ChimeriVax™, the tetravalent dengue vaccine based on the YF 17D vaccine wherein the *prM* and *E* genes are replaced by the corresponding genes of each dengue serotype, is at present the most promising vaccine candidate. Preclinical evaluation suggested that the vaccine candidates were replication-competent and genetically stable, do not become more neurovirulent after several passages in Vero cells and are significantly less neurovirulent in mice and monkeys than YF 17D vaccine.

The protective efficacy of the vaccine was demonstrated in a monkey challenge model (223-226). In addition, the tetravalent vaccine infects mosquitoes poorly, suggesting that it is unlikely that a mosquito feeding on a viremic vaccinee would become infected with the chimeric viruses (227). Furthermore, the substitution of wild-type YF Asibi sequences for 17D vaccine sequences in ChimeriVax™-DEN4 did not enhance *A. aegypti* mosquito infection (228). ChimeriVax™-DEN1-4 replicated at a lower level than YF 17D virus in hepatic cells, suggesting that these viruses may be less hepatotropic than the YF-vaccine (229). A phase I evaluation of the DENV-2 monovalent vaccine showed the vaccine to be safe and immunogenic regardless of pre-existing YF immune status. High levels of neutralizing antibodies against DENV-2 were observed after a single dose of ChimeriVax™-DEN2. In YF-immune individuals, neutralizing antibodies to the other three dengue serotypes were observed. These cross-reactive antibodies lasted up to 1 year (230). The tetravalent vaccine induced Th1 and CD8 responses, with an IFN-γ/TNF-α ratio favoring IFN-γ both in *Flavivirus*-immune and -naive individuals (187).

Reactogenicity, viremia induction and antibody responses of the tetravalent vaccine were investigated in three phase I trials in the U.S., Mexico and the Philippines as a 2- or 3-dose regimen over a 12-month period. Adverse events were mild and viremia was transient and low. All volunteers who received the three doses seroconverted to the four dengue serotypes. Geometric mean values and the percentage of seroconverted participants increased after each vaccination. In addition, *Flavivirus* preimmunity had a positive impact on dengue immunogenicity (231-233).

Currently, phase II clinical trials are being conducted on several continents to assess the safety and immunogenicity of the tetravalent vaccine in children, adolescents and adults. Also, proof of concept in Thai children is under way. These children are being vaccinated with three doses and will be followed to assess safety and efficacy. Results are expected in 2012. This vaccine has been licensed by Aventis Pasteur (now Sanofi Pasteur). Phase III trials in Asia and Latin America are in preparation (231), and based on a press release, a phase III clinical trial has started in Australia.

In another approach, the intertypic chimeric DENVs were developed early. In particular, an infectious clone of DENV-4 814669 was employed as the genetic background to express the structural genes of the other three serotypes (234-237). Preclinical studies in monkeys immunized with a bivalent formulation of DENV-4/DENV-1 and DENV-4/DENV-2 viruses elicited homologous neutralizing antibody titers to both DENV-1 and -2, suggesting that chimeric viruses based on the genetic background of DENV-4 were suitable for dengue vaccine following DENV-4 attenuation (238). Several mutations on the genome of the wild-type DENV-4 814669 have been developed (239, 240). From these, the recombinant DENV-4 mutant bearing a 30-nucleotide deletion (Δ30) introduced into its 3′-untranslated region was found to be attenuated for rhesus monkeys (240), was not transmitted to *A. albopictus* mosquitoes that fed on infected volunteers treated with the vaccine candidate (241), and caused a low level of reactogenicity and a high degree of immunogenicity in volunteers (242-244). A similar nucleotide deletion in the 3′-untranslated region of DENV-1 was also highly attenuated and immunogenic in monkeys (245) and well tolerated and immunogenic in humans (246-248). However, the introduction of the Δ30 into DENV-2 and -3 failed to attenuate the resulting viruses (249, 250).

To solve this situation, chimeric viruses were generated by replacement of M and E structural proteins of the attenuated rDENV-4/Δ30 vaccine candidate with those from DENV-2 and -3 (251), being attenuated in the monkey model. The rDENV-2/4/Δ30 vaccine virus was tested in humans and appeared to be safe, infectious and immunogenic (247). All these studies suggest that the introduction of an attenuating Δ30 into the 3′-untranslated region of DENV-1 and -4 and on the chimerization of DENV-2 and -3 with rDENV-4/Δ30 will be useful for a tetravalent vaccine if low reactogenicity is achieved (252). Phase I evaluation of DENV-3/4/Δ30 has shown the vaccine candidate to be safe but to have an unacceptably low level of infectivity. For that reason, additional approaches for developing attenuated DENV-3 in which the 3′-UTR contains two deletion mutations (Δ30/31) or in which the entire 3′-UTR has been replaced with that derived from DENV-4/Δ30 were prepared. A phase I evaluation of the tetravalent vaccine combinations has been initiated (Whitehead, personal communication) (253).

Chimeric viruses based on the attenuated genetic background of Mahidol DENV-2 PDK53 vaccine virus, and expressing the *prM/E* gene region of heterologous DENV-1, -3 and -4 were prepared (254-256). Much work has been dedicated to investigating the genetic stability of the attenuation loci of the DENV-2 PDK53 virus. These viruses were shown to be immunogenic and protective in the mouse model (256). Inviragen announced that the company has launched phase I trials of its tetravalent live attenuated vaccine, DENVax, at St. Louis University, St. Louis, MO, U.S., and will soon launch a parallel study in Medellin, Colombia (257).

Results reported by Caufour et al. and Galler et al. using a recombinant YF-DENV-2 vaccine candidate support the immunogenicity of this vaccine in a monkey model (258, 259). Finally, Suzuki et al. applied the RepliVAX strategy to dengue vaccine development. Specifically, they designed a chimeric RepliVAX that expresses the *prM/E* genes of DENV-2 in place of the *WNV* gene. Good results in terms of immunogenicity and protection in the mouse model were observed (260). This strategy, based on the development of a single-cycle flavivirus can be used as a safe and effective vaccine as the vaccine virus does not replicate.

Naked DNA vaccine

DNA vaccine technology, in which plasmids expressing appropriate viral antigens can induce an immune response against different viruses, is an alternative for developing effective dengue vaccines. One of the potential advantages of applying these vaccines is the expression of viral proteins in situ after DNA immunization, leading to proper folding of the protein (261). Several approaches have been followed for a DNA dengue vaccine. A candidate DNA vaccine expressing DENV-1 prM and E proteins was able to induce complete to partial protection in immunized monkeys after viral challenge (262-265). Previous studies by the same group have shown that a DENV-2 DNA vaccine containing the same genes induced a neutralizing antibody response and a higher survival rate in mice after virus challenge (266, 267).

More recently, DNA shuffling and screening technologies were employed to produce a chimeric tetravalent DNA vaccine expressing antigens that shared epitopes of the four serotypes. Results obtained in immunized monkeys support the usefulness of DNA shuffling as an alternative tool to create tetravalent chimeric dengue DNA vaccines (268, 269). In a different approach, Konishi et al. developed a dengue tetravalent DNA vaccine consisting of a plasmid expressing *prM* and *E* genes of the four serotypes. This vaccine candidate was able to induce neutralizing antibodies against the four viruses in a mouse model. In addition, they also demonstrated the increased immunogenicity of the vaccine in mice by co-immunization with DENV-2 subviral particles or inactivated Japanese encephalitis vaccine (JE-VAX) (270, 271). These results support that co-immunization with a protein vaccine can increase the efficacy of the dengue tetravalent DNA vaccine.

Considering that domain III of E protein plays a role in dengue immunity, a consensus domain III from all serotypes was cloned and expressed as a DNA vaccine (DNA SynCon™). This vaccine candidate provided tetravalent immunity in the mouse model (272). Similarly, tetravalent immunity was established in nonhuman primates. Previously, Mota el al. observed protective dengue antibodies by the tetravalent DNA immunization of mice with domain III (273). Others groups have selected NS1 as a target in a DNA vaccine as a way to avoid the possible risk of ADE. Wu et al. and, more recently, Costa et al. have evaluated a DNA plasmid encoding the *NS1* gene, suggesting the potential value of an NS1-DNA vaccine against dengue infection (274-276). Very recently, a DENV-2 plasmid containing sequences encoding portions of E and NS1 proteins was constructed. The immunogenicity of this plasmid was evaluated in mice in a prime–boost strategy priming with this plasmid and boosting with recombinant full-length GST-E and GST-NS1 fusion proteins. Antibodies to both E and NS1 proteins were detected by ELISA, with neutralizing antibodies observed in some animals (277).

DNA vaccines overcome the concerns of interference with combined use, since these vaccines are not infectious. DNA vaccines are also suitable for use in endemic areas due to the low cost of production and the long-term duration of the immune response (278, 279). However, while DNA approaches offer unique advantages, no human vaccine against infectious diseases has yet been commercialized. This approach also carries unique risks (184), such as the theoretical risk of DNA integration into the host chromosomal DNA (280, 281).

Recombinant subunit vaccines

Structural and nonstructural dengue proteins have been targets for developing subunit vaccines in different expression systems, such as *Escherichia coli*, yeast and baculovirus (168, 193, 282-284). Recently, domain III of E protein has been identified as a critical region for vaccine development. Choosing only domain III as a vaccine candidate instead of the whole E protein has a low potential for inducing cross-reactive antibodies to heterologous dengue serotypes, which could be implicated in the pathogenesis of severe disease (285). Thus, in the last years, much research has been directed to domain III (286, 287). One of these approaches consists of recombinant fusion proteins of domain B fused to maltose-binding protein of *E. coli* (288, 289). Monovalent and tetravalent vaccines, as well as combinations of DENV-2 DNA and recombinant subunit vaccines, have been evaluated in mice with the production of neutralizing antibodies (285, 289). A different approach was the creation of a DENV-2 and -4 bivalent antigen composed of linked domains of both serotypes. This

candidate was able to raise neutralizing antibodies in immunized mice (290). A consensus dengue domain III was developed by alignment of amino acid sequences from different isolates of the four serotypes. Neutralizing antibodies against the four viruses and anamnestic neutralizing antibody responses following viral challenge were observed in immunized mice, supporting the possibility of a vaccine candidate using this strategy (291).

As domain III has emerged as a promising dengue vaccine candidate, the optimization of protein expression in both *E. coli* and *Pichia pastoris*, as well as the conditions for protein secretion and improving the immunogenicity using different adjuvants, are of importance (292, 293). Probably one of the most complete investigations on this topic was that related to the expression of domain III of the four dengue serotypes fused to p64K of *Neisseria meningitidis* and expressed in *E. coli*. Monovalent recombinant proteins of each serotype induced neutralizing antibodies and protection after viral challenge in mice. DENV-1 and DENV-2 vaccine candidates were immunogenic and induced total or partial protection in monkeys (294-301). Finally, as proof of concept, the immune response induced by the recombinant protein in both mice and monkeys was highly serotype-specific (299).

In another approach, the consensus domain III, deduced from the alignment of the amino acid sequences of the four serotypes, was fused with a lipoprotein from *N. meningitidis*. The resulting lipo-immunogen induced a strong neutralizing antibody response, suggesting the usefulness of lipo-immunogens for novel vaccine development (302).

A recombinant tetravalent vaccine comprising the *N*-terminal 80% of E and prM proteins expressed in a *Drosophila* S2 cell expression system was evaluated in mice and monkeys (303). Low doses of the vaccine induced adequate neutralizing antibodies against the four serotypes and protection against challenge with two tested viruses. The DENV-1 vaccine candidate (licensed by Merck) is currently in phase I clinical trials (304).

Recombinant subunit vaccines offer the advantage of low reactogenicity and low cost; however, similar to other vaccine candidates, the risk of enhanced disease upon exposure to wild-type viruses post-vaccination needs to be evaluated. In addition, the need for adjuvants is a disadvantage (184). Attempts to overcome ADE by using nonstructural proteins such as NS1 and NS3 as subunit dengue vaccines are associated with concerns as to the lack of knowledge about the mode of protection of these proteins (305). Recently, anti-NS1 antibodies have been associated with disease severity (306). Particularly, immune-mediated pathogenesis causing endothelial cell damage, thrombocytopenia and hemorrhage needs to be considered, taking into account the molecular mimicry between platelet/endothelial cells and NS1 (154).

Virus-like particles (VLPs) are inert, empty viral capsids with no DNA or RNA from the virus itself, but with the structure of a virus particle. VLPs based on envelope, membrane or nucleocapsid microbial proteins are able to stimulate mucosal, as well as systemic, immunity (307). A recombinant DENV-2 envelope fused to hepatitis B surface antigen was expressed in *P. pastoris*. This hybrid protein has the potential to aggregate into entities similar to VLPs. Sera from immunized mice contained antibodies directed against both DENV-2 and hepatitis B antigen, supporting that this protein can act as a bivalent immunogen (308). In another approach, VLPs of parvovirus B19 that carry DENV-2 epitopes of domain III were able to elicit a strong humoral immune response in immunized mice (309). Recently, VLPs from the recombinant DENV-2 capsid protein, as well as a chimeric protein comprising domain III and the capsid of the same serotype, were obtained. Evaluation of both vaccine candidates in immunized mice showed significant protection after viral challenge, as well as a strong cellular immune response (310-312).

A new project (still at the murine stage) includes the production of a conjugate dengue vaccine following the strategy used by Spohn et al. for West Nile virus (a conjugate vaccine consisting of recombinantly expressed domain III chemically cross-linked to VLPs derived from the recently discovered bacteriophage AP205) (313).

Expression vector-based vaccines

Early investigations studied replication-deficient modified vaccinia Ankara (MVA)-vectored recombinants expressing E protein of DENV-2 and -4. Partial protection was observed in immunized monkeys, suggesting that this could be a strategy for a tetravalent dengue vaccine (314).

Measles virus vaccine, live, attenuated, is an attractive candidate vaccination vector that could provide safe and effective immunity against measles, as well as against other infectious diseases such as dengue. Measles virus vector has been shown to stably express additional foreign genetic materials (315). Brandler et al. inserted into the measles virus vector a sequence encoding a minimal combined dengue antigen composed of domain III fused to ectodomain of M protein from DENV-1. Neutralizing antibodies to DENV-1 were observed in immunized mice. In addition, the presence of ectoM was critical to the immunogenicity of inserted domain III (316). Recently, a recombinant measles vector expressing domain III of the four serotypes fused with the ectodomain of the membrane protein was evaluated in mice (317). This strategy could provide a recombinant vaccine that might simultaneously protect children from measles and dengue.

Another approach includes the use of replication-defective recombinant adenovirus vectors as vaccines. These vectors have the capacity to express antigens at a high level, being ideal for inducing potent immune responses. Using a complex adenovirus platform, Holman et al. were able to express both prM and E antigens of the four serotypes in symmetrical ends of each vector's genome, resulting in a pair of vectors that each expresses the *prM* and *E* genes of two different serotypes. The vaccine induced both humoral and cellular immune responses to DENV in immunized mice (318, 319). In a similar approach, a chimeric tetravalent antigen of domain III of the four serotypes was expressed using a replication-defective recombinant human adenovirus type 5 (rAdV5) vaccine vector. Humoral and cellular immune responses to the four serotypes were observed in immunized mice (320, 321).

Altogether, these strategies represent an alternative approach to developing a dengue vaccine, although pre-existing vector immunity could negatively influence the efficacy of these approaches (322).

DISCUSSION

An effective dengue vaccine is highly feasible. Viral replication is controlled after a short period of viremia and individuals who

recover from a DENV infection are immune to rechallenge to the homologous virus (133). In addition, dengue vaccines seem to be economically viable. Shepard et al. estimated a price per dose to the public sector of US $0.50 and to the private sector of $10; the average cost of vaccinating 1 child would be $7.58 and the gross cost per 1,000 population of the vaccination program would be $144 (323) in order to be cost-effective.

A huge advance in dengue vaccine development has been observed in the last decade. At present, there are several vaccine candidates at the advanced clinical and preclinical stage of development. Live attenuated dengue viruses, while immunogenic, have been associated with some reactogenicity. In addition, a combination of infectious vaccines may cause interference among the four DENVs in terms of virus propagation in vaccinated hosts. Such interference is an important concern for dengue vaccines, since imbalanced immune responses may cause increased disease severity when the vaccinated host acquires an infection with one of the four DENVs to which induction of immunity is insufficient (271). Ways to diminish or avoid interference have been reported (324). Inactivated DENVs have less immunogenicity and recombinant dengue proteins have failed to protect nonhuman primates from homologous dengue virus challenge (325). However, the use of adjuvants, as well as different strategies of immunization, can revert this situation.

A prime–boost strategy is a prominent approach to enhancing DNA and recombinant subunit vaccines. In addition, it could be applied to a live, attenuated vaccine, allowing a decrease in the number of doses and consequently reactogenicity. Chen et al. immunized monkeys with two doses of a DNA-DENV-1 vaccine and a third dose of a Venezuelan equine encephalitis virus replicon particle. Best results in terms of immunogenicity and viremia protection were observed in prime–boost animals compared to controls (326). Recent reports by Valdes et al. and Simmons et al. support the usefulness of the prime–boost vaccination strategy (327, 328). The former assumed the replicative virus as a model of attenuated virus to prove the concept in monkeys of a prime–boost strategy in combination with a subunit vaccine candidate (recombinant chimeric protein based on domain III from DENV-2, fused to the *C*-terminus of the carrier protein P64k of *N. meningitidis*). The second evaluated this vaccination strategy in rhesus monkeys by priming with tetravalent inactivated virus or tetravalent plasmid DNA vaccines expressing the *prmE* gene region, then boosting 2 months later with a tetravalent, live, attenuated virus. An anamnestic antibody response to the virus challenge was observed in both studies. Taken together, these data indicate ways to elicit robust immunity, as well as suggesting the potential utility of the prime–boost vaccination regimen in the development of dengue vaccines. However, one of the difficulties of this strategy is the implementation of the vaccination schedule using two different vaccines.

A dengue vaccine also implies operational challenges. The vaccine needs to be evaluated against changing patterns of transmission intensity and circulating serotypes, including other flaviviruses. Consequently, the vaccine needs to be evaluated both in Asia and the Americas. In addition, the implementation of the vaccination needs to be carefully considered taking into account the characteristics of the vaccine, as well as the dengue epidemiology (170). It is expected that a dengue vaccine will have a complex vaccination schedule, and will probably have to be given from the second year of life, at a time when few other vaccines are given routinely. Catch-up vaccination of older age groups will probably be necessary (329, 330). Immunization of older children and young adults will require new mechanisms that will need to be created or strengthened to reach these populations, using approaches such as school-based programs, immunization campaigns and clinic-based programs (329).

Given the fact of dengue emergence and re-emergence, several international initiatives are ongoing to bring dengue to worldwide attention. PDVI, based at the International Vaccine Institute in Seoul, Korea, has worked to estimate the disease burden and calculations of the social and economic cost of dengue, the establishment of several phase III vaccine trial sites in endemic areas and the building capacity in endemic countries (331, 332). The guidelines for the evaluation of dengue vaccines in populations exposed to natural infections were prepared and published by the WHO (333, 334). These guidelines emphasize the need for a strong regulatory infrastructure, as well as defined clinical trial and laboratory endpoints, among other aspects of importance. As part of the work developed by IVR/WHO, the analysis of important issues (immunological correlates of protection, CMI in dengue and dengue vaccine development, harmonization of the plaque reduction neutralization test for neutralizing antibody determination, dengue vaccine modeling) related to dengue vaccine have been performed (188, 304, 335-337). On the other hand, the Special Programme for Research and Training in Tropical Diseases (TDR/WHO) includes dengue in its portfolio and considers dengue vaccine development one of the main research priorities for dengue control (4, 27).

Dengue vaccines have been under development since the 1940s, and in recent years development has accelerated dramatically. Today, the dengue vaccine pipeline appears to be sufficiently advanced and diverse (170, 171). It is expected that at least one dengue vaccine may be licensed by 2014 (329). While live attenuated vaccines, and particularly the ChimeriVax™ vaccine, are the most advanced, it is expected that novel subunit protein, DNA and vectored vaccines, as well as novel adjuvants and prime–boost strategies will be evaluated (338). However, a better understanding of dengue immunity and pathogenesis is still required, as well as a better comprehension of dengue epidemiology and disease and economic burden.

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DISCLOSURES

The author states no conflicts of interest.

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