Dengue vaccine – current progress and challenges

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Dengue is a flaviviral disease that is currently a public health problem of global proportions. Vaccines for other flaviviral diseases have been successfully made. But a dengue vaccine has been elusive despite decades of effort. Several factors such as the existence of four antigenically distinct viruses that cause disease, the immune enhancement phenomenon underlying disease pathogenesis and the lack of a good animal model of the disease have collectively contributed to making the task of developing a dengue vaccine a formidable one. Global awareness of dengue in recent years has kindled renewed interest in developing dengue vaccines.

Keywords: Antibody-dependent enhancement, ChimeriVax vaccine, dengue fever, live attenuated vaccine, viral interference.

Introduction

THE earliest efforts to develop a vaccine for dengue were initiated in the early 1940s (ref. 1). Today, at the turn of the first decade of the 21st century, a dengue vaccine has yet to be licensed for human use. Dengue, perceived as an obscure disease of tropical countries, has been neglected for long. This perception has been undergoing a radical reversal in recent years[2]. This is reflected in the important advances made in our understanding of the virus and the disease[3], and vaccine development efforts are proceeding with a new sense of purpose and urgency[3–7]. This has resulted in a keen appreciation of the factors that make the development of dengue vaccines a complex and challenging task. This article will provide a brief overview of dengue disease, the rationale that drives and the hurdles that confront vaccine development efforts, the various approaches to dengue vaccines, current status of clinical trials and conclude with the challenges that must be addressed successfully before a dengue vaccine can become available for human use.

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Dengue disease and need for a vaccine

Dengue is a mosquito-borne viral disease, which has become a major global public health problem with a dramatic expansion in recent decades[3]. The disease is caused by any one of four closely related, yet antigenically distinct, dengue viruses (DENV-1, DENV-2, DENV-3 and DENV-4) of the family *Flaviviridae*, which also includes other members like yellow fever (YF), tick-borne encephalitis (TBE), Japanese encephalitis (JE) and West Nile (WN) viruses. All *Flaviviridae* members possess a single-stranded, (+) sense RNA genome of relatively simple organization, consisting of a single open reading frame (ORF) flanked by 5′ and 3′ non-translated regions (NTRs) as shown in Figure 1. The single ORF directs the synthesis of a long polyprotein that is processed by the combined action of viral and host proteases into 10 viral proteins that consist of three structural proteins (capsid C, membrane M (which is synthesized as the larger precursor prM) and envelope E) and seven non-structural (NS) proteins[8].

DENV infection produces a spectrum of clinical symptoms ranging from inapparent or mild febrile illness, or dengue fever (DF), to severe and fatal dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). DENV inoculated into the skin by a mosquito bite replicates initially in the local dendritic cells[9], followed by systemic infection and release into the bloodstream. DF presents with a sudden onset of fever that coincides with the appearance of DENV in the blood. At the peak of the febrile phase, virus levels in the blood can be as high as 10^6 infectious units/ml. Fever is accompanied by headache, retro-orbital pain, severe general bodyache and skin

Figure 1. A schematic representation of the genomic organization of DENVs and other flaviviruses. The ~11 kb long flaviviral genomic RNA contains a single pen reading frame (ORF), flanked by ~100 base 5′ and ~450 base 3′ non-translated regions (NTRs), shown by the short black lines at either end. The 10 viral proteins encoded by the single ORF are shown by the red and blue boxes, with the alphabets indicating the structural proteins and numbers indicating the NS proteins. The genes encoding the structural proteins prM and E which are used in many recombinant vaccine approaches are shown in blue while the rest are in red.

rashes. In most cases a full recovery from DF occurs. In DHF and DSS, apart from very high fever, the patients manifest 10–100 fold higher viraemia[10], thrombocytopenia, haemorrhagic manifestations and evidence of vascular leakage into interstitial spaces[3,7,11]. In the absence of hospitalization DHF/DSS is associated with high case fatality rates[12,13].

Currently, dengue is prevalent in more than a hundred, mostly economically resource-poor countries in the tropical and sub-tropical regions of the globe, which provide an ideal habitat for the mosquito vectors that spread the disease. In many of these countries, multiple DENV serotypes co-circulate. Outbreaks occur with increasing regularity[11]. The disease which was mostly prevalent in the cities has started spreading to rural areas as well. According to the World Health Organization (WHO), more than 2.5 billion people live in dengue-endemic areas with about 50 million DENV infections and 500,000 DHF/DSS cases occurring each year[13]. Recent estimates of the annual incidence of DENV infections by an international consortium known as the Paediatric Dengue Vaccine Initiative (PDVI) suggest that it may, in fact, be several times higher than WHO estimates[14]. It is now almost universally recognized that this situation prevails due to massive unplanned urbanization, overpopulation, increasing global travel and the inability to eradicate the mosquito vectors. Consequently, there is global consensus that a dengue vaccine is urgently needed[15,16]. The PDVI which emerged subsequently has been charged with the responsibility of accelerating the development of a dengue vaccine.

Rationale underlying dengue vaccine design

The design of the dengue vaccine stems from our current, albeit incomplete[3,17], understanding of the pathogenesis of DHF/DSS. Infection with any one DENV serotype provides lifelong homotypic immunity with only transient cross-protection against the remaining three serotypes[18]. DENV infections tend to be severe in those who have pre-existing antibodies to a different serotype, acquired either through maternal transfer or a primary infection[19–21]. It is believed that non-neutralizing IgG antibodies resulting from prior infection complexed to heterotypic DENVs during a secondary infection are taken up more efficiently by Fc receptor-bearing monocytes and macrophages in a phenomenon termed 'antibody dependent enhancement' (ADE)[11,21]. This, in turn, can lead to increased viral replication and increased viral load. It is beginning to be realized that ADE may not rely solely on Fc receptors to mediate enhanced viral uptake. DENV proteins, particularly prM and NS1, elicit antibodies that cross-react with host endothelial antigens[22,23]. These antibodies complexed to DENV can potentially target the latter for entry into host cells through cross-recognition of host cell antigens. It has been shown recently that anti-NS1 antibody can bind to and damage endothelial cells[24]. Recent studies of the T-cell repertoire after secondary DENV infection suggest that expansion of pre-existing memory T-cell populations (with greater avidity for the primary, but not the secondary serotype), may be responsible for sub-optimal viral clearance[25]. Whether through ADE or sub-optimal viral clearance, the increased virus load triggers a vigorous cytokine storm that damages the endothelium with concomitant capillary leakage and severe disease[3,11,17]. The immunopathologic mechanism underlying DHF/DSS, taken together with the co-circulation of multiple DENV serotypes in the endemic areas, dictates that a dengue vaccine must be tetravalent, capable of affording durable protection against infection by all four serotypes simultaneously. This is easier said than done.

Hurdles in dengue vaccine development

Vaccines against diseases caused by related flaviviruses such as YF, JE and TBE viruses have been made and are licensed in many countries such as US, China, Japan and Europe[11,26]. The expectation that making dengue vaccines using similar approaches would be quickly accomplished has been belied. Why has a dengue vaccine been elusive all these years? Several factors have contributed jointly to making the task of developing a dengue vaccine a complex and challenging task[4,11,27]. First, in contrast to YF, JE or TBE vaccines that are monovalent, targeting single flaviviruses, a dengue vaccine has to be tetravalent. Thus, there is the need to develop not one, but four vaccines that can collectively target all four DENV serotypes. Second, the immune response elicited by such a tetravalent vaccine must be balanced and durable. If solid immunity to all four is not achieved, there is a possibility that the vaccine recipient could be sensitized to severe disease through the immune enhancement phenomena mentioned above. Third, how protective immunity is induced is not well understood. By analogy with the other flavivirus vaccines mentioned above, it is assumed but not proven that neutralizing antibodies mediate protection against DENV infection *in vivo*. Evaluating vaccine induced immunity is a challenging task as it is necessary to demonstrate that each of the four immunogens in the tetravalent vaccine induces immunity. This task is bound to be confounded by the co-circulation of non-DENV flaviviruses in dengue-endemic areas. Finally, one of the biggest hurdles has been the lack of an appropriate animal model system to evaluate experimental dengue vaccines. Mice and monkeys are used, though they do not manifest any dengue-like illness, to carry out pre-clinical assessment and examine if human trials are warranted. Clinical testing and long-term evaluation are the only means of assessing safety, immunogenicity and reactogenicity of experimental vaccines.

Approaches to develop dengue vaccines

Several different strategies, schematized in Figure 2, are being undertaken for the development of tetravalent dengue vaccines[3,4,6,7]. Of these, vaccine candidates based on live viruses, attenuated either empirically or rationally, are in advanced stages of development. The tetravalent live attenuated dengue vaccine candidates being currently evaluated in clinical trials are physical mixtures containing attenuated DENV strains corresponding to the four serotypes (monovalent vaccine strains). The perception of potential difficulties with the live attenuated approach and an increased global awareness of dengue in recent years have led to the exploration of alternative strategies, based on recombinant DENV antigens expressed using different heterologous hosts and genetic vaccination, using plasmids and viral vectors. Again, most of these alternate strategies, with a few recent exceptions, envision the creation of monovalent vaccines, which could be incorporated together in physical mixtures, to produce tetravalent vaccines. Each of these approaches has its unique advantages and drawbacks. For example, the live attenuated vaccines which can provide a robust and durable immune response carry the inherent risk of reversion to virulence. The non-replicating vaccines, on the other hand, which eliminate the safety risk may not be as good at inducing a potent, long-lasting immune response. However, these issues may be addressed through the use of suitable adjuvants and booster immunizations.

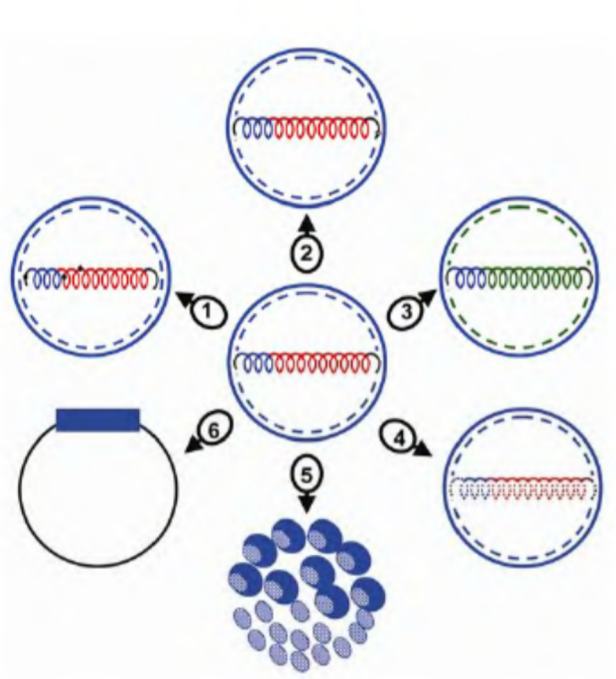

Figure 2. Current strategies for creating experimental dengue vaccines. The native DENV is represented schematically in the centre. The outer envelope made of the M (the mature form of prM) and E proteins is shown by the blue circle. The dashed circle represents the viral C protein covering the genomic RNA which is shown by the linear coil. The structural and non-structural genes are shown in blue and red respectively. The different ways of creating vaccines are shown by the numbers: (1) Passaging the DENV in a semi-permissive host in tissue culture results in attenuation (attenuating mutations are shown by the black dots on the RNA genome). (2) Using infectious clone technology, specific attenuating mutations can be engineered into the genomic RNA. The introduction of the Δ30 mutation (shown by the circle in the 3′ NTR) is depicted. (3) Infectious clone technology can also be used to graft the *prM + E* genes (blue) into the YF17D backbone (green). The resulting chimeric virus which carries this hybrid genome contains YF17D-encoded C (dashed green line), but DENV-encoded M and E. Instead of YF17D, attenuated DENV backbones (for example, DENV-2 PDK-53, rDEN4Δ30), may also be used to make intertypic chimeras. (4) DENV virions can be chemically treated, for example with formalin, to produce inactivated virus vaccine. The RNA genome is shown with a dotted line to indicate inactivation. (5) *E* gene or its carboxy-terminal region encoding EDIII can be expressed in heterologous systems for use as subunit vaccines; recombinant E and EDIII antigens are shown by the solid and hatched blue spheres respectively. (6) The *prM + E* genes or the EDIII genes (shown by the solid blue bar) may be inserted into plasmid vectors (shown by the black circle) for use as DNA vaccines.

Live attenuated dengue vaccines

Propagation of viruses serially in an unnatural cell substrate can lead to the selection of mutants better adapted to replication *in vitro* than in the natural living host. This strategy has been utilized to empirically attenuate DENVs. The development of attenuated DENVs stems from the discovery that they could be propagated in primary dog kidney (PDK) cells with accompanying changes in some of their properties[28]. In general, with increasing passage number, plaques become progressively smaller, and the viruses tend to show increased temperature sensitivity, decreased cytopathicity and mouse neurovirulence[29]. Using these properties as biologic markers, DENVs can be assessed at each passage for the degree of attenuation attained. However, as attenuating mutations have the potential to occur anywhere on the viral genome, the immune response induced by such attenuated viruses may not necessarily be to wild-type viral proteins. Additionally, it must be noted that attenuating point mutations are more prone to reversion than deletions. This is clearly an empirical and time-consuming process to obtain potential vaccine strains, showing an acceptable balance of attenuation and immunogenicity, corresponding to each of the four DENV serotypes. This has been done by two groups independently, one at Mahidol University in Thailand[30], and the other at the Walter Reed Army Institute for Research (WRAIR) in the USA[31]. Both groups have spent nearly three decades to date on this. However, neither group has biologically cloned its attenuated strains[7]. Further, attenuating mutations have been identified for only the Mahidol DENV-2 vaccine candidate known as PDK-53 (ref. 32). This vaccine virus, in which the mutations have been mapped to the 5′ NTR, NS1 and NS3, is currently being used to develop intertypic chimeric vaccine strains (see below).

In contrast to the conventional and empirical approach described above, reverse genetics has been employed more recently to introduce defined attenuating mutations into the DENV genomic RNA. This is also referred to as infectious clone technology. Attenuation is achieved by by site-directed mutagenesis of a plasmid-borne full-length cDNA clone of the DENV genome, followed by its *in vitro* transcription to 'infectious' RNA, from which the recombinant DENV carrying the attenuating mutations can be recovered by transfecting it into permissive cells. It was shown that substituting discrete nucleotides in the terminal part of the 3′ NTR of DENV-1 (ref. 33) and DENV-2 (ref. 34) with corresponding nucleotides from WN virus genome resulted in attenuation. The insertion of this mutation, referred to as 'mutF' into the genomes of the remaining two DENV serotypes has not been described. However, a 30 nucleotide deletion (Δ30) in the 3′ NTR of DENV-4, originally identified as one of a panel of 3′ NTR mutants[35], has emerged as a promising mutation from the perspective of a live attenuated vaccine. This deletion removes nucleotides 10,478–10,507 of the DENV-4 genomic RNA, with concomitant loss of a stem loop structure known as TL2 implicated in replication. The Δ30 mutation has been shown to provide an acceptable balance between attenuation and immunogenicity both in monkeys[35] and humans[36]. As the attenuating mutation is confined to the 3′ NTR, the Δ30 vaccine viruses should be able to elicit immune responses to the wild-type viral-encoded proteins. Another advantage is that deletion mutations are more stable compared to point mutations, minimizing the likelihood of reversion. Live attenuated vaccine strains, corresponding to all four DENV serotypes, carrying the Δ30 mutation have been constructed using reverse genetics at the US NIH[36–39]. Interestingly, introduction of the Δ30 mutation into the homologous 3′ NTR of DENV-1 (ref. 37), but not DENV-2 (ref. 40) and DENV-3 (ref. 39) resulted in acceptable attenuation. While the reason for this is not clear, an alternate approach has been used successfully to develop attenuated Δ30 vaccine viruses corresponding to DENV-2 and DENV-3. This approach termed antigenic chimerization, takes advantage of the fact that serotype-specificity is determined by the structural proteins, predominantly so by the E protein. Additionally, it is common to include the prM protein, as it is believed to assist in the maintenance of the structural/antigenic integrity of the E protein. Using this approach, the prM and E protein-coding region of the rDEN4Δ30 attenuated vaccine vector was replaced with the corresponding region taken from either DENV-2 or DENV-3, to create the intertypic chimeric viruses, rDEN2/4Δ30 (ref. 38) and rDEN3/4Δ30 (ref. 39) respectively. Antigenic chimerization is also being used by researchers at the CDC in the USA to create intertypic chimeric vaccine viruses based on the use of DENV-2 PDK53 vaccine virus mentioned above, as the backbone to carry the *prM* and *E* genes of DENV-1, -3 and -4 (ref. 41). A variation of this approach utilizes the empirically attenuated yellow fever vaccine virus (YF17D) as the vaccine carrier to host the *prM* and *E* genes of the DENVs. YF17D-derived chimeric viruses, known as ChimeriVax viruses, encoding *prM* and *E* genes of DENV-1, DENV-2, DENV-3 and DENV-4 have been developed by Acambis/Sanofi Pasteur[42–44]. It must be noted that in contrast to the DENV/DENV intertypic chimeras which carry all DENV genes, the ChimeriVax viruses carry only the DENV structural genes encoding *prM* and *E* with C and all NS protein genes being provided by YF17D vector (Figure 2). While antigenic chimerization *per se* can contribute to attenuation of the ChimeriVax viruses, the contribution of the attenuating mutations of the YF17D backbone is not quite clear. Attenuating mutations, mapped to the structural genes of YF17D, are lost during ChimeriVax construction.

Alternate dengue vaccines

In contrast to the live, replicating vaccines described above, a host of non-replicating vaccine strategies are also being explored for the development of dengue vaccines. These approaches focus on inactivated viruses, recombinant DENV antigens and the use of non-replicating vectors designed to deliver and express DENV antigens *in vivo*.

Inactivated vaccines, by definition, possess the advantage of conferring immunity without the risk of infection. Purified virus can be inactivated by different methods and used as vaccines. As the purified inactivated virus (PIV) is non-infectious, it has to be formulated in a good adjuvant to elicit a potent immune response. However, the low growth titers attained by DENVs in tissue culture and concerns that inactivation can adversely affect their antigenicity have contributed to a lack of keen interest in developing inactivated dengue vaccines[26]. Using DENV adapted to grow to high titers in certified Vero cells, one group has shown that DENV-2 can be inactivated with low concentrations of formalin with retention of immunogenicity[45].

In contrast to whole virus-based strategies described so far, several initiatives that focus on a subunit approach have emerged recently. Subunit vaccines are non-infectious like the PIVs, but eliminate their disadvantage of yield limitation. These are based on DENV antigens, either produced as recombinant proteins in heterologous systems[46–59] or encoded by plasmids[60–63] or viral vectors[64–69]. In this context, the preferred DENV antigen is the E protein, for a variety of reasons. It is a large, multifunctional protein involved in several critical aspects of DENV biology such as receptor recognition, membrane fusion and virion morphogenesis[8]. The E protein has been implicated in the generation of neutralizing antibodies and the induction of protective immunity[11]. It is responsible for eliciting the

first and longest lasting antibody response to DENV infection[70] as it contains multiple serotype-specific, conformation-dependent neutralizing epitopes[11,71–73]. Recombinant E protein and its derivatives have been expressed and purified using different heterologous systems including *E. coli*[48,50,53–55,58], yeast[51,52,59] and baculovirus[47] expression systems. The results have not been encouraging. Whereas full-length E protein made in *E. coli* and baculovirus tends to be insoluble, in yeast, expression yields are low. The most promising subunit vaccine candidate so far, has been produced by expressing carboxy-terminally truncated versions of the E molecule, using the *Drosophila* expression system[46,74]. Genetic vaccination is another strategy that has been developed for many infectious diseases. It has also been applied to dengue by different groups. Many of the genetic vaccine approaches use the DENV E-encoding sequences inserted into a plasmid[61,62] or viral vector[64,68]. By analogy with the chimeric vaccines described above, many genetic vaccines include prM along with E. This has the added advantage of generating non-infectious virus like particles *in vivo*.

In recent years we have witnessed the emergence of interest in the development of subunit dengue vaccines based on a discrete ~100 amino acid residue sub-domain of the E protein known as envelope domain III (EDIII). This has been fuelled by the realization that several attributes of the larger E protein, critical from a vaccine perspective, are actually associated with EDIII. Thus, experimental EDIII vaccines based on recombinant antigens[48,53–55,58,59], plasmids[60,63] and virus-vectors[65–67,69] have been developed. Interestingly, some groups have developed tetravalent vaccines by incorporating EDIIIs of DENV serotypes 1–4 into a single construct[58,59,63,69].

Current progress

As mentioned already, almost all approaches, with a few recent exceptions, are based on developing single serotype-specific vaccines, also referred to as monovalent vaccines. The four monovalent vaccines are then physically mixed into a single formulation to obtain a tetravalent dengue vaccine. Thus, the making of a dengue vaccine entails the development of five, four monovalent plus a single tetravalent vaccine candidates. In addition to extensive pre-clinical testing in mice and monkeys, each of the four monovalent vaccine candidates needs to be tested in human trials for safety, immunogenicity and efficacy, before being mixed into a suitable tetravalent formulation, which in turn has to undergo extensive clinical development. This is one of the factors that have made this a long drawn process. The live attenuated vaccine candidates are currently the most advanced candidates, having reached phase II clinical testing, as tetravalent formulations (Table 1). Some of experimental vaccines are in early phase I stage, while the majority of the vaccines mentioned under the alternate category are still in pre-clinical phase of development. An overview of the current status of the different dengue vaccines in clinical trials is provided below[75].

Tetravalent formulations of the Mahidol vaccine have consistently been plagued with heightened reactogenicity of the DENV-3 component as evidenced by systemic symptoms in trial volunteers[76–79]. The failure to achieve acceptable levels of DENV-3 attenuation[80] has led to the Mahidol vaccine not being actively developed any further. The WRAIR attenuated DENV vaccine strains also manifested similar problems of balancing attenuation and immunogenicity in phase I trials of 16 different tetravalent formulations. The DENV-1 component was significantly under-attenuated and the DENV-4 component was slightly over-attenuated[81,82]. In an effort to address this, these two components were replaced and dosages modified[83]. A pilot open-label, safety and immunogenicity trial of this modified WRAIR candidate tetravalent vaccine (formulation 17), administered in two doses, six months apart, to a small number of 6–7-year-old Thai children was completed in 2004. The results of this trial which were published recently show that the vaccine was well tolerated with no serious adverse events. One child developed mild transient fever and DENV-4 vaccine viraemia one week after receiving the boost. In the remaining children, neutralizing antibodies to all four DENVs were observed when assayed one month after the second dose. The geometric mean titers specific to DENV-1, -2, -3 and -4 in these children were 55, 475, 350 and 171 respectively[83]. Results of a phase I/II trial completed in mid-2006, designed to look at the effect of giving an additional boost to Thai children who had received two doses of the WRAIR tetravalent vaccine in an earlier study, are not yet available. A phase I/II randomized, controlled, double-blinded trial to evaluate the WRAIR vaccine in 51 flavivirus-naïve 12–15-month-old Thai infants is scheduled to be completed at the end of this year. Multiple formulations of the WRAIR tetravalent vaccine have undergone phase II testing in adults in USA. Formulation 17 which was tested in one of these phase II studies was reported to induce tetravalent seroconversion in 63% of the volunteers[84]. Additional phase II trials are underway in Puerto Rico and Thailand (Table 1).

A monovalent chimeric vaccine created by inserting DENV-2 *prM* and *E* genes into the YF17D backbone, referred to a ChimeriVax-DEN2, was first tested in humans as a prelude to testing the tetravalent formulations. This phase I study revealed that the monovalent ChimeriVax-DEN2 vaccine is safe and immunogenic. Further, this trial also showed that prior immunity to YF facilitates the induction of high levels of long-lasting cross-neutralizing antibody response to all four DENVs. Many of the vaccine recipients manifested transient viraemia[85]. A randomized, double-blind, placebo-controlled phase I study of the safety and immunogenicity of the

Table 1. Dengue vaccines in clinical trials[a]

Vaccine	Targets	Developer	Phase	Trial sites
LAV[b]	All four DENV serotypes	WRAIR/GSK	I/II	Thailand, Puerto Rico, USA
CYD[b]	All four DENV serotypes	Sanofi-Aventis	II	Thailand, Singapore, Vietnam, Peru, Australia, Mexico, USA
Δ30	Single serotypes[c]	NIAID	I	USA
prM/E DNA	DENV-1	WRAIR	I	USA
E (ecto)[d] protein	DENV-1	Hawaii Biotech	I	USA

[a]Data based on a search of the ClinicalTrials.gov website with the search term 'dengue', http://www.clinicaltrials/gov/ct/search?term=dengue
[b]LAV and CYD are being tested as tetravalent vaccines (targeting all four DENV serotypes simultaneously).
[c]Several different Δ30 candidates are being tested as monovalent vaccines (targeting each of the four DENV serotypes separately).
[d]Recombinant DENV-1 E protein ectodomain (N-terminal 80%) formulated in alum.

Chimeric Yellow fever Dengue (CYD) tetravalent vaccine administered in two doses, six months apart, has been carried out[86]. About 45% of the volunteers manifested viraemia, with maximum titers of about 140 PFU/ml, and ~32% seroconverted ($PRNT_{50} \geq 1:10$) to all four DENVs, after one dose. Immunogenicity data from the second dose have not been reported. The interim analysis has revealed the existence of inherent differences in the replication traits of each of the four component viruses in the tetravalent CYD vaccine formulation and the need to readjust the infective dose of the vaccine components in the tetravalent CYD formulation. It was also observed in this study that tetravalent seroconversion was ~69% in recipients with prior YF immunity. To evaluate the role of prior exposure to flaviviruses further, a phase II trial of the CYD tetravalent vaccine was carried out in Australian adults, who had been previously immunized with either an investigational dengue vaccine or YF vaccine. The results from this study which was completed in early 2008 are not available yet. Several phase II trials of the investigational tetravalent CYD vaccine, in flavivirus-naïve and -immune children and adults, are currently underway in different countries and are scheduled for completion starting from the end of 2009 to mid-2015 (Table 1). One of these studies which has been recently initiated in Thailand, aims to recruit ~4000 children in the age range of 4–11 years to assess the efficacy of the tetravalent CYD vaccine candidate administered in three doses.

Unlike the empirically live attenuated and ChimeriVax vaccines above, almost all of the Δ30 vaccine variants are currently in phase I trials. These are being tested as monovalent vaccines. Some trials have been completed and many are still ongoing to evaluate different doses and immunization schedules. Results from some of the completed trials are available. The first vaccine to be tested in this series that paved the way to sustained work on the Δ30 vaccines was rDEN4Δ30. A first trial of this vaccine in 20 human volunteers showed that it was well tolerated, mildly reactogenic, produced low levels of transient viraemia and elicited high titers of neutralizing antibodies[36]. A phase II study reported more recently showed that rDEN4Δ30 administered at a low dose of just 10 PFU was adequately immunogenic, inducing neutralizing antibodies that could broadly neutralize several DEN-4 genotypes[87]. Similarly, rDEN1Δ30 and rDEN2/4Δ30 vaccine viruses, were safe and immunogenic in healthy adult volunteers, inducing a robust and durable immune responses to DENV-1 (ref. 88) and DENV-2 (ref. 89) respectively. Phase I trials of DENV-3 Δ30 vaccine candidates are either currently underway or planned. A tetravalent formulation containing rDEN1Δ30, rDEN2/4Δ30, rDEN3/4Δ30 and rDEN4Δ30 which has been reported to show encouraging results in Rhesus monkeys[90] is yet to be tested in humans.

Regarding the alternate vaccines, so far one phase I study of a plasmid vaccine encoding the *prM* and *E* genes of DENV-1 has been completed in human volunteers for safety and immunogenicity[75]. No results have been posted from this study yet. A phase I study of a carboxy-terminally truncated DENV-1 E protein expressed using the *Drosophila* cell system has just been initiated recently[75]. All other alternate vaccine candidates being developed are in pre-clinical stages. Many of these have been tested in mice and/or monkeys and have been shown to elicit virus neutralizing antibodies. In most instances, with the few exceptions mentioned above[58,59,61,63,69], these are being developed as monovalent vaccines.

Challenges

Dengue vaccine development efforts are proceeding at a hitherto unprecedented pace. However, there are several challenges that need to be addressed before dengue vaccine becomes a reality. The major pre-licensure issues are highlighted below.

The issue of viral interference poses safety concerns. Interference, first seen with the Mahidol vaccine, is a major issue confronting developers of live attenuated

vaccine candidates. When four monovalent live attenuated DENV vaccine strains were mixed together into a single tetravalent formulation, immune responses were predominantly directed to one serotype with the exclusion of the others[76,77,79]. This was correlated to increased replication of one particular component in the tetravalent formulation. Whereas this could be partially addressed by empirical adjustments of the tetravalent formulation, it could not be eliminated altogether. The induction of unequal levels of neutralizing antibodies against the four DENV serotypes has also been seen with the WRAIR vaccine[81,84]. It was felt that interference would not occur if all monovalent vaccine viruses in the tetravalent formulation replicated with similar efficiency. However, the CYD vaccine strains, all of which are based on the YF17D backbone, mixed into tetravalent formulations do manifest evidence of interference[43,44,91]. Interference has been observed with CDC's PDK-53 based tetravalent formulations as well[86]. These data underscore the lack of our understanding of the potential for replication interference among the components of a live attenuated tetravalent vaccine.

Recent pre-clinical data show that further dose adjustments and multiple inoculations of the tetravalent ChimeriVax vaccine can partially ameliorate this problem[91]. Similarly, encouraging results have been obtained using tetravalent Δ30 vaccine formulations in monkeys[90]. But, will these optimized formulations remain 'optimal' in the human recipient is a question that can be addressed only through clinical trials. The fact that humans are the only true models of dengue disease has been a major challenge in vaccine development. Experiments in humans are unethical unless justified by adequate pre-clinical data. Unfortunately, neither the mouse nor the monkey models used in pre-clinical development studies manifests dengue. Clearly, these models have severe limitations and many candidates that show promise in preclinical stages tend to fail in humans. Consequently, clinical trials are the only means of obtaining information of relevance to dengue vaccine development.

Evaluating candidate vaccines in population-based phase III efficacy trials in 'at risk' populations is going to be a major challenge[92]. Because of changes in dengue transmission intensity patterns, circulating DENVs and other co-circulating flaviviruses in different regions, efficacy trials will need to be carried out in multiple trial sites with highly characterized flavivirus epidemiology. Trial sites need to be developed prior to initiating such efficacy trials. PDVI has initiated long-term surveillance of several cohorts to gather data on the incidence and prevalence of dengue in many geographical sites[14]. Once efficacy trials are underway, it will be necessary to evaluate if the experimental tetravalent vaccine confers protection to all four DENV serotypes. Currently, there are no methods to distinguish homotypic and heterotypic antibodies in a tetravalent response. As mostly one DENV tends to predominate in a given geographical region, trials will need to be done at multiple sites to show efficacy against all four DENV serotypes[5]. This would be a complicated task as DENVs co-circulate with other flaviviruses. In addition, the phase III efficacy trials must also include long-term safety and follow up to evaluate the risk of vaccine-induced sensitization to dengue disease.

A major bottleneck in dengue vaccine development is the lack of established correlates indicative of protective immunity against the disease. It is necessary to identify such correlates which can be used as clinical end-points in vaccine trials. This will help in evaluating the vaccine candidates and accelerate the vaccine development process. There is a general consensus that neutralizing antibodies represent a primary correlate of protection against DENV infections. This stems from several lines of data, including animal studies, vaccine trials and population cohort studies[93]. For example, infants in dengue endemic areas are protected against DENV infection during the initial months of their lives when they still have maternally acquired anti-DENV neutralizing antibodies[94]. However, there is no information on the antibody titers required to confer protection against DENV infection. Neutralizing antibody titers are measured using an *in vitro* plaque reduction neutralization test (PRNT). The biological relevance of this test needs to be established. Currently, a $PRNT_{50}$ titre of $\geq 1:10$ is taken as evidence of seroconversion[6] and is assumed to reflect activation of immunologic memory akin to that associated with recovery from infection. It has been suggested that post-challenge, rather than pre-challenge, titer of neutralizing antibodies may be critical for protection[4]. While a role for cell-mediated immunity in the clinical outcome of the disease is recognized, it is considered unlikely to provide a single correlate of protection. WHO has recommended that comprehensive immunological data should be generated from future vaccine trials to define correlates of protection against dengue[92].

Concluding remarks

The live attenuated dengue vaccines are front runners in advanced clinical development and there is cautious optimism that one of these may eventually be licensed for human use. Current indications are that these vaccines will need to be administered in 2 or 3 doses to achieve tetravalent seroconversion. Using such a vaccine, travellers from non-endemic regions can expect to be fully immunized before visiting dengue-endemic regions. However, in dengue-endemic areas, partially seroconverted recipients are likely to face the possibility of infection before completion of the immunization schedule. Another concern in the context of the resource-poor nations in tropical and sub-tropical regions that bear a major burden of

dengue disease is cost. A dengue vaccine for use in these countries has to be not only safe and efficacious, but also inexpensive. From this perspective, there is a need for the development of potent non-replicating subunit vaccines that will not only eliminate safety issues but also be affordable. The alternate non-replicating vaccines that are in early stages of development will benefit immensely from the ongoing efforts aimed at better understanding the pathogenesis and the development of newer models of the disease.

1. Sabin, A. B. and Schlesinger, R. W., Production of immunity to dengue virus modified by propagating in mice. *Science*, 1945, **101**, 640–642.
2. Farrar, J. *et al.*, Towards a global dengue research agenda. *Trend. Med. Int. Health*, 2007, **12**, 695–699.
3. Swaminathan, S. and Khanna, N., Dengue: recent advances in biology and current status of translational research. *Curr. Mol. Med.*, 2009, **9**, 152–173.
4. Hombach, J., Vaccines against dengue: a review of current candidate vaccines at advanced development stages. *Pan. Am. J. Publ. Health*, 2007, **21**, 254–260.
5. Normile, D., Hunt for dengue vaccine heats up as the disease burden grows. *Science*, 2007, **317**, 1494–1495.
6. Guy, B. and Almond, J. W., Towards a dengue vaccine: progress to date and remaining challenges. *Comp. Immunol. Microbiol. Infect. Dis.*, 2008, **31**, 239–252.
7. Whitehead, S. S., Blaney, J. E., Durbin, A. P. and Murphy, B. R., Prospects for a dengue virus vaccine. *Nat. Rev. Microbiol.*, 2007, **5**, 518–528.
8. Lindenbach, B. D., Thiel, H. J. and Rice, C. M., *Flaviviridae*: the viruses and their replication. In *Fields of Virology* (eds Knipe, D. M. and Howley, P. M.), Wolters Kluwer and Lippincott Williams and Wilkins, Philadelphia, 2007, 5th edn, pp. 1101–1152.
9. Wu, S. J. L. *et al.*, Human skin Langerhan cells are targets of dengue virus infection. *Natl. Med.*, 2000, **6**, 816–820.
10. Vaughn, D. W. *et al.*, Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. *J. Infect. Dis.*, 2000, **181**, 2–9.
11. Gubler, D. J., Kuno, G. and Markoff, L., Flaviviruses. In *Fields of Virology* (eds Knipe, D. M. and Howley, P. M.), Wolters Kluwer and Lippincott Williams and Wilkins, Philadelphia, 2007, 5th edn, pp. 1153–1252.
12. Edelman, R., Dengue and dengue vaccines. *J. Infect. Dis.*, 2005, **191**, 650–653.
13. WHO Factsheet N 117, Dengue and dengue haemorrhagic fever, March 2009; http://www.who.int/mediacentre/factsheets/fs117/en/
14. Pediatric Dengue Vaccine Initiative, Global burden of dengue, http://www.pdvi.org/about_dengue/GBD.asp
15. DeRoeck, D., Deen, J. and Clemens, J. D., Policymakers' views on dengue fever/dengue haemorrhagic fever and the need for dengue vaccines in four southeast Asian countries. *Vaccine*, 2003, **22**, 121–129.
16. Almond, J. *et al.*, Accelerating the development and introduction of a dengue vaccine for poor children, 5–8 December 2001, Ho Chi Minh City, Vietnam. *Vaccine*, 2002, **20**, 3043–3046.
17. Rothman, A. L., Dengue: defining protective versus pathologic immunity. *J. Clin. Invest.*, 2004, **113**, 946–951.
18. Innis, B. L., Antibody responses to dengue virus infection. In *Dengue and Dengue Hemorrhagic Fever* (eds Gubler, D. J. and Kuno, G.), CAB International, Wallingford, 1997, pp. 221–243.
19. Burke, D. S., Nisalak, A., Johnson, D. E. and Scott, R. M., A prospective study of dengue infections in Bangkok. *Am. J. Trop. Med. Hyg.*, 1988, **38**, 172–180.
20. Guzman, M. G., Kouri, G. P., Bravo, J., Soler, M., Vazquez, S. and Morier, L., Dengue hemorrhagic fever in Cuba, 1981: a retrospective seroepidemiologic study. *Am. J. Trop. Med. Hyg.*, 1990, **42**, 179–184.
21. Halstead, S. B., Neutralization and antibody dependent enhancement of dengue viruses. *Adv. Virus Res.*, 2003, **60**, 421–467.
22. Huang, K. J. *et al.*, The dual-specific binding of dengue virus and target cells for the antibody-dependent enhancement of dengue virus infection. *J. Immunol.*, 2006, **176**, 2825–2832.
23. Falconar, A. K. I., Antibody responses are generated to immunodominant ELK/KLE-type motifs on the nonstructural-1 glycoprotein during live dengue virus infections in mice and humans: implications for diagnosis, pathogenesis, and vaccine design. *Clin. Vac. Immunol.*, 2007, **14**, 493–504.
24. Lin, C. F. *et al.*, Liver injury caused by antibodies against dengue virus nonstructural protein 1 in a murine model. *Lab. Invest.*, 2008, **88**, 1079–1089.
25. Mongkolsapaya, J. *et al.*, Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. *Nat. Med.*, 2003, **9**, 921–927
26. Eckels, K. H. and Putnak, R., Formalin-inactivated whole virus and recombinant subunit flavivirus vaccines. *Adv. Virus Res.*, 2003, **61**, 395–418.
27. Barrett, A. D. T. and Hombach, J., Opportunities in the development of dengue vaccines. In Report of the Scientific Working Group on Dengue (WHO document TDR/SWG/08), 2006, pp. 61–65.
28. Innis, B. L. and Eckels, K. H., Progress in the development of a live-attenuated, tetravalent dengue virus vaccine by the United States Army Medical Research and Material Command. *Am. J. Trop. Med. Hyg.*, 2003, **69**, 1–4.
29. Hasltead, S. B. and Marchette, N. J., Biologic properties of dengue viruses following serial passage in primary dog kidney cells: studies at the University of Hawaii. *Am. J. Trop. Med. Hyg.*, 2003, **69**, 5–11.
30. Bhamarapravati, N. and Sutee, Y., Live attenuated tetravalent dengue vaccine. *Vaccine*, 2000, **18**, 44–47.
31. Eckels, K. H., Dubois, D. R., Putnak, R., Vaughn, D. W., Innis, B. L., Henchal, E. A. and Hoke Jr, C. H., Modification of dengue virus strains by passage in primary dog kidney cells: preparation of candidate vaccines and immunization of monkeys. *Am. J. Trop. Med. Hyg.*, 2003, **69**, 12–16.
32. Butrapet, S., Huang, C. Y.-H., Pierro, D. J., Bhamarapravati, N., Gubler, D. J. and Kinney, R. M., Attenuation markers of a candidate dengue type 2 vaccine virus, strain 16681 (PDK-53), are defined by mutations in the 5′ noncoding region and non-structural proteins 1 and 3. *J. Virol.*, 2000, **74**, 3011–3019.
33. Markoff, L., Pang, X., Houng, H.-S., Falgout, B., Olsen, R., Jones, E. and Polo, S. Derivation and characterization of a dengue type 1 host range-restricted mutant virus that is attenuated and highly immunogenic in monkeys. *J. Virol.*, 2002, **76**, 3318–3328.
34. Zeng, L., Falgout, B. and Markoff, L., Identification of specific nucleotide sequences within the conserved 3′-SL in the dengue type 2 virus genome required for replication. *J. Virol.*, 1998, **72**, 7510–7522.
35. Men, R., Bray, M., Clark, D., Chanock, R. and Lai, C. J., Dengue type 4 virus mutants containing deletions in the 3′ noncoding region of the RNA genome: analysis of growth restriction in cell culture and altered viremia pattern and immunogenocity in rhesus monkeys. *J. Virol.*, 1996, **70**, 3930–3937.
36. Durbin, A. P. *et al.*, Attenuation and immunogenicity in humans of a live dengue virus type-4 vaccine candidate with a 30-nucleotide deletion in its 3′-untranslated region. *Am. J. Trop. Med. Hyg.*, 2001, **65**, 405–413.
37. Whitehead, S. S., Falgout, B., Hanley, K. A., Blaney Jr, J. E., Markoff, L. and Murphy, B. R., A live, attenuated dengue virus type 1 vaccine candidate with a 30-nucleotide deletion in the 3′ untranslated region is highly attenuated and immunogenic in monkeys. *J. Virol.*, 2003, **77**, 1653–1657.

38. Whitehead, S. S., Hanley, K. A., Blaney Jr, J. E., Gilomre, L. E., Elkins, W. R. and Murphy, B. R., Substitution of the structural genes of dengue virus type 4 with those of type 2 results in chimeric vaccine candidates which are attenuated for mosquitoes, mice, and rhesus monkeys. *Vaccine*, 2003, **21**, 4307–4316.
39. Blaney Jr, J. E., Hanson, C. T., Firestone, C. Y., Hanley, K. A., Murphy, B. R. and Whitehead, S. S., Genetically modified, live attenuated dengue virus type 3 vaccine candidates. *Am. J. Trop. Med. Hyg.*, 2004, **71**, 811–821.
40. Blaney Jr, J. E., Hanson, C. T., Hanley, K. A., Murphy, B. R. and Whitehead, S. S., Vaccine candidates derived from a novel infectious cDNA clone of an American genotype dengue virus type 2. *BMC Infect. Dis.*, 2004, **4**, 39.
41. Huang, C. Y.-H. *et al.*, Chimeric dengue type 2 (vaccine strain PDK-53)/dengue type 1 virus as a potential candidate dengue type 1 virus vaccine. *J. Virol.*, 2000, **74**, 3020–3028.
42. Guirakhoo, F. *et al.*, Recombinant chimeric yellow fever-dengue type 2 virus is immunogenic and protective in nonhuman primates. *J. Virol.*, 2000, **74**, 5477–5485.
43. Guirakhoo, F. *et al.*, Construction, safety, and immunogenicity in nonhuman primates of a chimeric yellow fever-dengue virus tetravalent vaccine. *J. Virol.*, 2001, **75**, 7290–7304.
44. Guirakhoo, F. *et al.*, Viraemia and immunogenicity in nonhuman primates of a tetravalent yellow fever-dengue chimeric vaccine: genetic reconstructions, dose adjustment, and antibody responses against wild-type dengue virus isolates. *Virology*, 2002, **298**, 146–159.
45. Putnak, R. *et al.*, Development of a purified, inactivated, dengue-2 virus vaccine prototype in Vero cells: immunogenicity and protection in mice and rhesus monkeys. *J. Infect. Dis.*, 1996, **174**, 1176–1184.
46. Ivy, J., Nakano, E. and Clements, D., Methods of preparing carboxy-terminally truncated recombinant flavivirus envelope glycoproteins employing *Drosophila melanogaster* expression systems, 2000, US Patent 6136561.
47. Kelly, E. P., Greene, J. J., King, A. D. and Innis, B. L., Purified dengue 2 virus envelope glycoprotein aggregates produced by baculovirus are immunogenic in mice. *Vaccine*, 2000, **18**, 2549–2559.
48. Simmons, M., Murphy, G. S. and Hayes, C. G., Short report: antibody responses of mice immunized with a tetravalent dengue recombinant protein subunit vaccine. *Am. J. Trop. Med. Hyg.*, 2001, **65**, 159–161.
49. Hermida, L. *et al.*, A recombinant envelope protein from dengue virus purified by IMAC is bioequivalent with its immune-affinity chromatography purified counterpart. *J. Biotechnol.*, 2002, **94**, 213–216.
50. Chiu, M.-W. and Yang, Y.-L., Blocking the dengue virus 2 infections on BHK-21 cells with purified recombinant dengue virus 2 E protein expressed in *Escherichia coli*. *Biochem. Biophys. Res. Commun.*, 2003, **309**, 672–678.
51. Muné, M. *et al.*, Carboxy-terminally truncated dengue 4 virus envelope glycoprotein expressed in *Pichia pastoris* induced neutralizing antibodies and resistance to dengue 4 virus challenge in mice. *Arch. Virol.*, 2003, **148**, 2267–2273.
52. Wei, H. Y., Jiang, L. F., Xue, Y. H., Fang, D. Y. and Guo, H. Y., Secreted expression of dengue virus type 2 full-length envelope glycoprotein in *Pichia pastoris*. *J. Virol. Methods*, 2003, **109**, 17–23.
53. Hermida, L. *et al.*, A fragment of the envelope protein from dengue-1 virus, fused in two different sites of the meningococcal P64k protein carrier, induces a functional immune response in mice. *Biotechnol. Appl. Biochem.*, 2004, **39**, 107–114.
54. Hermida, L. *et al.*, A dengue-2 envelope fragment inserted within the structure of the P64k meningococcal protein carrier enables a functional immune response against the virus in mice. *J. Virol. Methods*, 2004, **115**, 41–49.
55. Hermida, L. *et al.*, A recombinant fusion protein containing the domain III of the dengue-2 envelope protein is immunogenic and protective in nonhuman primates. *Vaccine*, 2006, **24**, 3165–3171.
56. Khanam, S., Etemad, B., Khanna, N. and Swaminathan, S., Induction of neutralizing antibodies specific to dengue virus serotypes 2 and 4 by a bivalent antigen composed of linked envelope domains III of these two serotypes. *Am. J. Trop. Med. Hyg.*, 2006, **74**, 266–277.
57. Zulueta, A. *et al.*, Amino acid changes in the recombinant dengue 3 envelope domain III determine its antigenicity and immunogenicity in mice. *Virus Res.*, 2006, **121**, 65–73.
58. Chen, S., Yu, M., Jiang, T., Deng, Y., Qin, C. and Qin, E., Induction of tetravalent protective immunity against four dengue serotypes by the tandem domain III of the envelope protein. *DNA Cell. Biol.*, 2007, **26**, 361–367.
59. Etemad, B., Batra, G., Raut, R., Dahiya, S., Khanam, S., Swaminathan, S. and Khanna, N., An envelope domain III-based chimeric antigen produced in *Pichia pastoris* elicits neutralizing antibodies against all four dengue virus serotypes. *Am. J. Trop. Med. Hyg.*, 2008, **79**, 353–363.
60. Mota, J., Acosta, M., Argotte, R., Figueroa, R., Méndez, A. and Ramos, C., Induction of protective antibodies against dengue virus by tetravalent DNA immunization of mice with domain III of the envelope protein. *Vaccine*, 2005, **23**, 3469–3476.
61. Apt, D. *et al.*, Tetravalent neutralizing antibody response against four dengue serotypes by a single chimeric dengue envelope antigen. *Vaccine*, 2006, **24**, 335–344.
62. Konishi, E., Kosugi, S. and Imoto, J. I., Dengue tetravalent DNA vaccine inducing neutralizing antibody and anamnestic responses to four serotypes in mice. *Vaccine*, 2006, **24**, 2200–2207.
63. Ramanathan, M. P., Kuo, Y. C., Selling, B. H., Li, Q., Sardesai, N. Y., Kim, J. J. and Weiner, D. B., Development of a novel DNA SynCon tetravalent dengue vaccine that elicits immune responses against four serotypes. *Vaccine*, 2009, **27**, 6444–6453.
64. Men, R. *et al.*, Immunization of rhesus monkeys with a recombinant of modified vaccinia virus Ankara expressing a truncated envelope glycoprotein of dengue type 2 virus induced resistance to dengue type 2 virus challenge. *Vaccine*, 2000, **18**, 3113–3122.
65. Khanam, S., Khanna, N. and Swaminathan, S., Induction of antibodies and T cell responses by dengue virus type 2 envelope domain III encoded by plasmid and adenoviral vectors. *Vaccine*, 2006, **24**, 6513–6525.
66. Brandler, S. *et al.*, Pediatric measles vaccine expressing a dengue antigen induces durable serotype-specific neutralizing antibodies to dengue virus. *PLoS Negl. Trop. Dis.*, 2007, **1**, e96.
67. Khanam, S., Rajendra, P., Khanna, N. and Swaminathan, S., An adenovirus prime/plasmid boost strategy for induction of equipotent immune responses to two dengue virus serotypes. *BMC Biotechnol.*, 2007, **7**, 10.
68. Raviprakash, K. *et al.*, A tetravalent dengue vaccine based on a complex adenovirus vector provides significant protection in Rhesus monkeys against all four serotypes of dengue virus. *J. Virol.*, 2008, **82**, 6927–6934.
69. Khanam, S., Pilankatta, R., Khanna, N. and Swaminathan, S., An adenovirus type 5 (AdV5) vector encoding an envelope domain III-based tetravalent antigen elicits immune responses against all four dengue viruses in the presence of prior AdV5 immunity. *Vaccine*, 2009, **27**, 6011–6021.
70. Churdboonchart, V., Bhamarapravati, N., Peampramprecha, S. and Sirinavin, S., Antibodies against dengue viral proteins in primary and secondary dengue hemorrhagic fever. *Am. J. Trop. Med. Hyg.*, 1991, **44**, 481–493.
71. Mégret, F. *et al.*, Use of recombinant fusion proteins and monoclonal antibodies to define linear and discontinuous antigenic sites on the dengue envelope glycoprotein. *Virology*, 1992, **187**, 480–491.

72. Roehrig, J. T., Johnson, A. J., Hunt, A. R., Bolin, R. A. and Chu, M. C., Antibodies to dengue 2 virus E-glycoprotein synthetic peptides identify antigenic conformation. *Virology*, 1990, **177**, 668–675.
73. Roehrig, J. T., Bolin, R. A. and Kelly, R. G., Monoclonal antibody mapping of the envelope glycoprotein of the dengue 2 virus, Jamaica. *Virology*, 1998, **246**, 317–328.
74. WHO Steering Committee Report on 'Dengue and other flavivirus vaccines', VAB/VIR/2003.03, 2–3 April 2003; www.who.int/entity/vaccine_research/diseases/dengue/en/dengue2003minutes_sc.pdf
75. Clinical Trials website of the US NIH, http://clinicaltrials.gov/ct2/search
76. Kanesa-thasan, N. *et al.*, Safety and immunogenicity of attenuated dengue virus vaccines (Aventis Pasteur) in human volunteers. *Vaccine*, 2001, **19**, 3179–3188.
77. Sabchareon, A. *et al.*, Safety and immunogenicity of tetravalent live-attenuated dengue vaccines in Thai adult volunteers: role of serotype concentration, ratio, and multiple doses. *Am. J. Trop. Med. Hyg.*, 2002, **66**, 264–272.
78. Sabchareon, A. *et al.*, Safety and immunogenicity of a three dose regimen of two tetravalent live-attenuated dengue vaccines in five- to twelve-year-old Thai children. *Pediatr. Infect. Disease J.*, 2004, **23**, 99–109.
79. Kitchener, S., Nissen, M., Nasveld, P., Forrat, R., Yoksan, S., Lang, J. and Saluzzo, J. F., Immunogenicity and safety of two live-attenuated tetravalent dengue vaccine formulations in healthy Australian adults. *Vaccine*, 2006, **24**, 1238–1241.
80. Sanchez, V. *et al.*, Innate and adaptive cellular immunity in flavivirus-naïve human recipients of a live-attenuated dengue serotype 3 vaccine produced in Vero cells (VDV3). *Vaccine*, 2006, **24**, 4914–4926.
81. Sun, W. *et al.*, Vaccination of human volunteers with monovalent and tetravalent live-attenuated dengue vaccine candidates. *Am. J. Trop. Med. Hyg.*, 2003, **69**, 24–31.
82. Edelman, R. *et al.*, Phase I trial of 16 formulations of a tetravalent live-attenuated dengue vaccine. *Am. J. Trop. Med. Hyg.*, 2003, **69**, 48–60.
83. Simasathien, S. *et al.*, Safety and immunogenicity of tetravalent live-attenuated dengue vaccine in flavivirus naive children. *Am. J. Trop. Med. Hyg.*, 2008, **78**, 426–433.
84. Sun, W. *et al.*, Phase 2 clinical trial of three formulations of tetravalent live-attenuated dengue vaccine in flavivirus-näive adults. *Hum. Vaccines*, 2009, **5**, 33–40.
85. Guirakhoo, F. *et al.*, Live attenuated chimeric yellow fever dengue type 2 (ChimeriVax^{TM}-DEN2) vaccine: phase I clinical trial for safety and immunogenicity: effect of yellow fever prior immunity in induction of cross neutralizing antibody responses to all 4 dengue serotypes. *Hum. Vaccines*, 2006, **2**, 60–67.
86. World Health Organization, Report of the meeting of the WHO Task Force on clinical trials of dengue vaccine, WHO document WHO/IVB/06.10, 2006.
87. Durbin, A. P. *et al.*, rDEN4Δ30, a live attenuated dengue virus type 4 vaccine candidate, is safe, immunogenic, and highly infectious in healthy adult volunteers. *J. Infect. Dis.*, 2005, **191**, 710–718.
88. Durbin, A. P. *et al.*, The live attenuated dengue serotype 1 vaccine rDEN1Δ30 is safe and highly immunogenic in healthy adult volunteers. *Hum. Vaccines*, 2006, **2**, 167–173.
89. Durbin, A. P. *et al.*, rDEN2/4Δ30(ME), a live attenuated chimeric dengue serotype 2 vaccine is safe and highly immunogenic in healthy dengue-naïve adults. *Hum. Vaccines*, 2006, **2**, 255–260.
90. Blaney Jr, J. E., Matro, J. M., Murphy, B. R. and Whitehead, S. S., Recombinant, live-attenuated tetravalent dengue virus vaccine formulations induce a balanced, broad, and protective neutralizing antibody response against each of the four serotypes in rhesus monkeys. *J. Virol.*, 2005, **79**, 5516–5528.
91. Guy, B. *et al.*, Evaluation of interference between dengue vaccine serotypes in a monkey model. *Am. J. Trop. Med. Hyg.*, 2009, **80**, 302–311.
92. Edelman, R. and Hombach, J., Guidelines for the clinical evaluation of dengue vaccines in endemic areas. Summary of a World Health Organization technical consultation. *Vaccine*, 2008, **26**, 4113–4119.
93. Hombach, J., Cardosa, M. J., Sabchareon, A., Vaughn, D. W. and Barrett, A. D. T., Scientific consultation on immunological correlates of protection induced by dengue vaccines. Report from a meeting held at the World Health Organization, 17–18 November 2005. *Vaccine*, 2007, **25**, 4130–4139.
94. Pengsaa, K. *et al.*, Dengue virus infections in the first 2 years of life and the kinetics of transplacentally transferred dengue neutralizing antibodies in Thai children. *J. Infect. Dis.*, 2006, **194**, 1570–1576.

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