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REVIEW

Current status and perspectives on vaccine development against dengue virus infection

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Dengue virus (DENV) consists of four serotypes in the family *Flaviviridae* and is a causative agent of dengue fever, dengue hemorrhagic fever, and dengue shock syndrome. DENV is transmitted by mosquitoes, *Aedes aegypti* and *A. albopictus*, and is mainly observed in areas where vector mosquitoes live. The number of dengue cases reported by the World Health Organization increased more than 8-fold over the last two decades from 505,430 in 2000 to over 2.4 million in 2010 to 5.2 million in 2019. Although vaccine is the most effective method against DENV, only one commercialized vaccine exists, and it cannot be administered to children under 9 years of age. Currently, many researchers are working to resolve the various problems hindering the development of effective dengue vaccines; understanding of the viral antigen configuration would provide insight into the development of effective vaccines against DENV infection. In this review, the current status and perspectives on effective vaccine development for DENV are examined. In addition, a plausible direction for effective vaccine development against DENV is suggested.

Keywords: antibody-dependent enhancement, antigen, dengue virus, envelope, vaccine

Introduction

Dengue virus (DENV), which is an arthropod-borne single positive-strand RNA virus known to cause dengue fever, dengue hemorrhagic fever, and dengue shock syndrome, is a global public health concern (WHO, 2009). DENV is classified into four antigenically related serotypes, DENV1, DENV2, DENV3, and DENV4. DENV is transmitted by mosquitoes (*Aedes aegypti* and *A. albopictus*) mainly in Southeast Asia, India, and South America, where the vector mosquitoes are prevalent (WHO, 2009). Recently, the area suitable for spread of DENV infection and the population at risk of dengue diseases are expected to increase due to climate changes that promote the replication of host mosquitoes (Messina *et al*., 2019). DENV is estimated to cause more than 390 million infections per year, and more than 96 million cases with clinical symptoms have been reported (Hadinegoro *et al*., 2015). To date, research has been conducted to develop effective vaccines to protect humans from DENV infection. Despite the successful commercialization of a dengue vaccine by Sanofi Pasteur, people are reportedly reluctant to get vaccinated due to the low effectiveness and the side effects of the vaccine (Fatima and Syed, 2018). Consequently, a reliable and effective vaccine against DENV infection currently does not exist. In this review, the current status of vaccine development against DENV infection and the possibility and prospects of dengue vaccine development are summarized.

DENV Antigens (Ags) as Vaccination Materials and Their Characteristics

The role of viral Ags and candidate materials in dengue vaccine development

DENV consists of genes encoding the capsid protein, membrane (M) protein, envelope (E) protein, and non-structural (NS) proteins, including NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Perera and Kuhn, 2008) (Fig. 1A). E protein, which plays an important role in DENV entry into the host cell, and NS1, which is exposed to the host cell surface, are currently major candidate Ags for use in a vaccine to induce effective antibodies (Abs) and inhibit DENV infection. In addition, NS3 and NS5, which have peptide sequences displayed by human major histocompatibility complex class I, are considered major candidate Ags to induce the cellular immune response against DENV infection (Rothman, 2011). The basic functions of the DENV proteins considered the main candidate Ags for dengue vaccine development are further examined in this review.

Similar to other viruses, proteins expressed by DENV are categorized as structural and NS proteins, the former comprising capsid, M, and E proteins. The capsid protein is pre-

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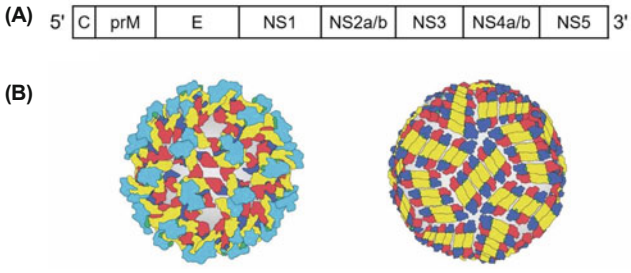

Fig. 1. Genomic profile of dengue virus (DENV) and structures of immature and mature DENV virions. (A) A schematic diagram of the DENV genes. (B) Cryo-electron microscopy image of the structure of the immature DENV1 particle carrying 60 trimeric precursor membrane (prM) E spikes (PDB 4B03) in surface representation (left). Cryo-electron microscopic structure of the mature DENV1 particle with 90 E protein dimers (PDB 4CCT) in surface representation (right). An icosahedral asymmetric unit is indicated by a white triangle, and the icosahedral vertices are marked by white symbols: two-fold, ellipse; three-fold, triangle; and five-fold, pentagon. EDI, E protein domain I; EDII, E protein domain II; EDIII, E protein domain III; FL, fusion loop; S, stem region; TM, transmembrane anchor; pr, precursor peptide; M, membrane protein. (Wilken and Rimmelzwaan, 2020).

sent in the cytoplasm after synthesis and is cleaved by viral proteases (NS2B-NS3). The capsid protein then forms a nucleocapsid and partakes in the initiation of DENV assembly (Byk and Gamarmik, 2016). E and M proteins are major components on the DENV surface and are involved in DENV infection of host cells. E protein is the main protein component of the virus surface and exists as a dimer. E protein is composed of three ectodomains and transmembrane segments, and each E protein domain (ED) has various functions. Of the EDs, domain II of E protein (EDII) has a dimerization interface, two glycosylation sites, and a fusion loop (Cruz-Oliveira *et al.*, 2015). Binding of DENV to its cognate receptor, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), occurs via the interaction between the carbohydrate recognition domain of DC-SIGN and the mannose-rich N-glycan on amino acid residue N67 of E protein (Pokidysheva *et al.*, 2006). Domain III of E protein (EDIII) is important for host cell attachment and virus entry into host cells; DENV interaction with glycosaminoglycans occurs via two lysine residues located on EDIII (Watterson *et al.*, 2012). In addition, mannose receptor and heat-shock protein (Hsp) 90/Hsp70 are host receptors involved in DENV infection, and E protein interacts with these receptors (Cruz-Oliveira *et al.*, 2015). For example, the mannose receptor recognizes residues N67 and N153 of E protein, and Hsp90 reportedly interacts with multiple DENV proteins including E, NS1, NS3, and NS5 (Srisutthisamphan *et al.*, 2018).

M protein, comprising viral particles, is an important protein for virus maturation (Zybert *et al.*, 2008). M protein and viral maturation will be discussed in further detail later in this review. M protein was used as a major target Ag in previous vaccine studies; however, in terms of vaccine development, the results were not promising because the Abs against M protein potentially induce Ab-dependent enhancement (ADE), a significant obstacle in dengue vaccine development.

The monomeric form of NS1 is rapidly dimerized after being expressed in DENV-infected cells and binds to the cell surface, or is secreted in the form of a hexamer (Gutsche *et al.*, 2011). Dimeric NS1 plays a pivotal role in viral genome replication likely via interactions with NS4A and NS4B transmembrane proteins during the early stages of virus infection (Rastogi *et al.*, 2016). Circulating hexameric NS1 in blood modulates the complement system and enhances DENV infection (Gutsche *et al.*, 2011). Cell surface-exposed NS1 during DENV infection can be used as a target for vaccines capable of inducing Ab-dependent cellular cytotoxicity (Chen *et al.*, 2018). However, full-length NS1 is not used for vaccination because the Abs elicited by full-length NS1 cross-react with host proteins, causing pathological manifestations. Consequently, the partial NS1 sequence was used to design a candidate vaccine against DENV infection (Lai *et al.*, 2017). The N-terminal region of NS3 is a trypsin-like serine protease that requires polyprotein processing together with the central hydrophilic domain (~40 amino acids) of NS2B as a co-factor. The C-terminus of the NS3 protein contains a nucleoside triphosphatase, a 5' RNA-triphosphatase, and in the remaining 70% of the protein, a helicase (Norazharuddin and Lai, 2018). NS4 is cleaved into NS4A and NS4B by the protease activity of NS3, and NS4A is involved in the localization of the replication complex in a stable complex within the perinuclear area. NS4B modulates viral replication by interacting with the helicase domain of NS3 to assist its dissociation from the viral RNA. The NS5 protein has an RNA-dependent RNA polymerase domain at its C-terminal end. In addition, the N-terminus of NS5 protein is a capping enzyme site consisting of methyltransferase and guanylyltransferase domains (Zhou *et al.*, 2007).

NS proteins contain target epitopes for $CD4^+$ and $CD8^+$ T cells. In human, $CD4^+$ T cells respond mainly to the capsid, followed by the E, NS3, NS2A/B, and NS5 proteins (Tian *et al.*, 2019). $CD8^+$ T cells respond mainly to the NS3 together with the capsid, NS5, and NS4A/B proteins. Consequently, both structural and non-structural proteins elicit robust DENV-specific T-cell immune responses because T-cell epitopes are distributed in various structural and non-structural proteins of DENV. Although T-cell activity is essential in virus protection, T cells also affect immunopathology during heterologous infection (Mangada and Rothman, 2005). Suboptimal T-cell receptor activation during infection by heterologous serotypes of DENV resulted in increased TNF-α and poor antiviral efficacy, and may have contributed to immunopathology (Rivino, 2018).

Selection of candidate vaccine immunogens based on DENV maturation and breathing

Understanding virus maturation is important in Ab-mediated protection against virus infection because it is closely associated with the binding of neutralizing and infection-enhancing Abs onto the virus, as well as virus infectivity (Shukla *et al.*, 2020). The glycoprotein shell of DENV consists of 180 copies each of E and M proteins in an icosahedral symmetry (Fig. 1B). The two proteins have different conformations in the immature and mature DENV particles and, therefore, confer unique structural features to both the immature and mature forms of DENV particles. In the immature virion, premembrane (prM) and E proteins form 90 heterodimers

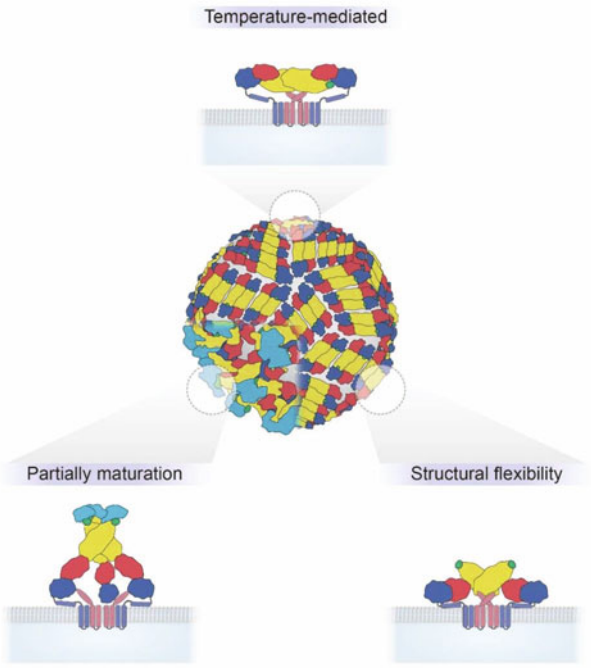

Fig. 2. The changing antigenic landscape of dengue virions. Schematic representations of the diverse viral morphologies arising from (A) inefficient prM protein cleavage (only one type of prM protein-containing particle is shown for simplicity), (B) exposure to temperatures of 34°C and higher in DENV2, and (C) the sampling of multiple E protein conformations at equilibrium. Each of these scenarios individually influences antibody-mediated neutralization of DENV, based on modulating epitope accessibility. The curved double-headed black arrows in (C) indicate viral breathing motions, which in this example, transiently expose the otherwise buried fusion loop. The lipid bilayer and nucleocapsid core are shown in grey and orange, respectively (Wilken and Rimmelzwaan, 2020).

extending as 60 trimeric spikes from the surface of the virus particle. Conversely, E protein in the mature virion consists of 90 homodimers that lie flat against the viral surface forming a smooth protein shell (Kuhn *et al*., 2002).

The prepeptide is cleaved from the prM during maturation, and the M protein remains in the mature virion particle as a transmembrane protein beneath the E protein shell. Notably, the level of uncleaved prM is different depending on the type of cell producing the DENV. The E protein of DENV has a high proportion of prM present in the form of a partially mature virion having both homodimeric and trimeric forms of spikes (Wilken and Rimmelzwaan, 2020) (Fig. 2). Further diversity of DENV stems from the structural flexibility of E protein, termed as 'breathing', which renders the virion structurally dynamic (Shukla *et al*., 2020). This structural flexibility exerts significant effects on epitope exposure. In addition, various factors, such as temperature, pH, and/or host-protein interactions, influence DENV structure. Intensive research on temperature-mediated breathing has been conducted. For example, when DENV infects the host (37°C) from the vector (28°C), varying degrees of structural transitions occur across the various DENVs, and these transitions, such as smooth to rough surface transition, are most prominent in the DENV2 strain (Fibriansah *et al*., 2013). However, DENV1, 3, and 4 serotypes showed no observable structural changes between 37°C and 28°C in cryo-electron microscopy studies (Lim *et al*., 2017). In a recent study, temperature-mediated breathing was correlated with the viral infectivity of DENV2 and DENV1 via intrinsic dynamics and not specific morphologies (Sharma *et al*., 2019). These findings indicate that the DENV particle structure is not a stable but a dynamic state, which should be considered when designing candidate vaccine materials.

Current Dengue Vaccine Development

Currently, various forms of vaccines against DENV infection have been reported and include live-attenuated chimeric-recombinant virus, live-attenuated recombinant virus, live-attenuated virus, inactivated virus, recombinant protein, and mRNA vaccines. The characteristics, efficacy, and perspectives of each form of dengue vaccine are reviewed.

Live-attenuated chimeric-recombinant dengue vaccine

Sanofi-Pasteur's Dengvaxia® is a representative dengue vaccine licensed in 20 countries. The vaccine is based on development of a tetravalent vaccine using Sanofi Pasteur's vaccine against yellow fever virus (YFV-17D) as a backbone to replace the prM and E proteins of DENV1–4 (Fig. 3A). In a phase III clinical trial, children were vaccinated with Dengvaxia® in a three-dose regimen at 0, 6, and 12 months, and the vaccination efficacy was verified by monitoring the symptomatic dengue infection at 25 months after the last vaccination. Dengvaxia® showed efficacies of 65.6% and 44.6% in children older and younger than 9 years, respectively. Vaccine efficiency in terms of hospitalization was observed in 80.75% and 55.9% of vaccinees older and younger than 9 years of age, respectively, indicating that the protective ability decreased in children under 9 years of age (Hadinegoro *et al*., 2015). Although Dengvaxia® is commercially available, it is contraindicated in children under 9 years of age. In addition to the problem of poor protection efficiency in children under 9 years of age, Dengvaxia® differed in its protection among the DENV serotypes, showing a 50.3% protective efficiency against DENV1, 42.3% against DENV2, 74.0% against DENV3, and 77.74% against DENV4 (Villar *et al*., 2015). The vaccine showed lower protection against DENV1 and DENV2, and a type-specific Ab was generated mainly against DENV4; thus, Dengvaxia® appears to be a monovalent vaccine against DENV4 only (Thomas and Yoon, 2019). Therefore, whether Dengvaxia® can be used to protect against DENV infection is questionable, and an effective new vaccine against all four serotypes of DENV is needed.

Live-attenuated recombinant vaccine

TV003/TV005 (United States National Institutes of Health) and TAK-003 (Takeda Inc.) are recombinant attenuated vaccine candidates for DENV currently undergoing clinical phase III trials, and these candidates appear the closest to commercialization since Dengvaxia®. In TV003/TV005, DENV is attenuated via deletion of 30 nucleotides in the 3′-UTR of the gene for the DENV1, DENV3, and DENV4 serotypes; however, only the prM and E protein genes of DENV2 are

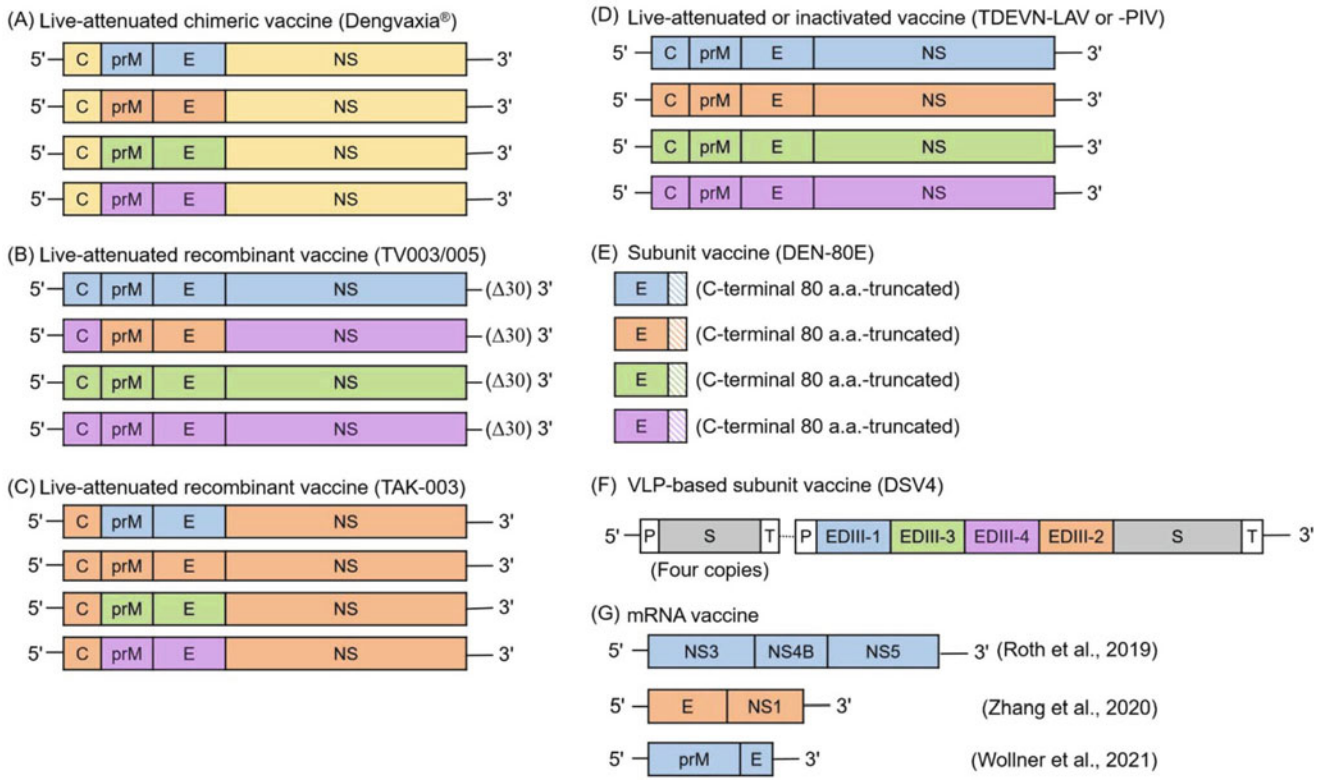

Fig. 3. Schematic diagram of various dengue vaccine constructs. The genetic backbone of each DENV serotype is expressed in different colors: DENV1 (blue), DENV2 (orange), DENV3 (green), and DENV4 (purple). (A) Dengvaxia® uses YFV-17D (yellow) as a backbone to replace the prM and E protein of DENV1–4. (B) TV003/TV005 attenuates DENV by deletion of 30 nucleotides in the 3′-UTR of the gene. The vaccine consists of the complete genes of DENV1, DENV3, and DENV4 serotypes but only DENV2 in the DENV4 backbone. (C) TAK-003 is an attenuated vaccine in which the prM and E proteins of each virus are substituted in the DENV2 backbone. (D) TDENV-LAV and TDENV-PIV consist of the full gene of each DENV serotype. (E) DEN-80E consists of the C-terminal 80-amino-acid-truncated E protein of each DENV serotype. (F) DSV4 consists of four copies of hepatitis B virus surface antigen and one copy of tetravalent EDIII of DENV. S, Surface protein; P, promoter; T, terminator. (G) The target antigens of the DENV mRNA vaccine are diverse: structural proteins (prM and E protein) and non-structural proteins (NS1, NS3, NS4B, and NS5).

used in the DENV4 backbone to prepare a live-attenuated vaccine against DENV2 (Kirkpatrick *et al.*, 2015) (Fig. 3B). The vaccine (TV003/TV005) induced a tetravalent Ab response in most of the subjects, with 94–97%, 94–100%, 100%, and 100% of vaccinated subjects seroconverting to DENV1, DENV2, DENV3, and DENV4, respectively, after the second vaccination (Kirkpatrick *et al.*, 2015). Challenge infection with DENV2Δ30 showed rates of viremia, rash, and neutropenia of 100%, 80%, and 20% in placebo recipients, respectively, whereas TV003/TV005 vaccinees did not show viremia,

Table 1. Summary of dengue vaccine candidates

Vaccine (Sponsor)	Phase	Result summary	References
Dengvaxia® (Sanofi)	A	Clinical trial: DENV1 (50.3%), DENV2 (42.3%), DENV3 (74.0%), and DENV4 (77.7%)	Villar *et al.* (2015)
TV003/005 (Butantan)	III	nAb: DENV1 (94–97%), DENV2 (94–100%), DENV3 (100%), and DENV4 (100%) DENVΔ30 challenge: Placebo (viremia: 100%; rash: 80%; neutropenia: 20%), recipient of TV003 (viremia: 0%; rash: 0%; neutropenia: 0%)	Kirkpatrick *et al.* (2015, 2016)
TAK-003 (Takeda)	III	Clinical trial: DENV1 (69.8%), DENV2 (95.1%), DENV3 (48.9%), and DENV4 (51.0%)	Biswal *et al.* (2020)
TDENV LAV or PIV (WRAIR and GSK)	I/II	Heterologous prime-boost: (sero-conversion rate at 28 days) LAV/PIV: DENV1 (90%), DENV2 (90%), DENV3 (85%), and DENV4 (75%) in the 0–180 dosing group PIV/LAV: all serotypes (100%) in the 0–180 dosing group	Lin *et al.* (2021)
DEN-80E (MSD)	I	Pre-clinical trial: Non-immune (viremia: 5.33, 4.0, 3.33, and 5 days for DENV1, DENV2, DENV3, and DENV4, respectively); low-dose tetravalent (viremia: no detection); high-dose tetravalent (viremia: 2 of 12 animals 1 each in DENV1 and 3)	Govindarajan *et al.* (2015)
DSV4 (ICGEB and Sun Pharma)	-	nAb FNT_{50} titer at 3 months after last injection: 296, 55, 105, and 33, respectively, for DENV1, DENV2, DENV3, and DENV4 in BALB/c mice Passively transferred challenge (DENV2): Anti-DSV4 serum (100% survival); anti-4G2 Ab (all died in 5 days)	Ramasamy *et al.* (2018)

rash, or neutropenia (Kirkpatrick *et al*., 2016). Recently, the TV003/TV005 vaccination was confirmed to induce a serotype-specific neutralizing Ab using the sera from TV003/TV005 recipients (NCT02021968). For example, most subjects (76%) developed serotype-specific neutralizing Abs to three or four DENV serotypes, indicating that immunity is induced by each vaccine component. Importantly, TV003/TV005 can also induce DENV2 serotype-specific neutralizing Abs, which are closely associated with sterilizing immunity in DENV2 infection (Nivarthi *et al*., 2021). Consequently, serotype-specific neutralizing Abs were considered more important than the total level of neutralizing Abs in terms of vaccine efficacy. TV003/TV005 is currently in phase III clinical trials in Brazil, and the end period is projected to be December 2022 according to the clinical trial identifier (NCT02406729).

TAK-003 was made using DENV2 as a basal platform, which was attenuated in primary dog kidney (PDK-53) cells. The prM and E genes of DENV1, DENV3, and DENV4 were used to substitute the corresponding genes of attenuated DENV2 (Fig. 3C). TAK-003, which was produced by combining each monovalent DENV, is one of the most anticipated vaccine candidates against DENV infection after Dengvaxia®. TAK-003 was recently reported in a phase III clinical trial (NCT02747927) targeting children 4–16 years of age, and it showed an overall vaccination efficacy of 80.2% at the primary endpoint of the clinical trial. In the secondary endpoint assessment timeframe, an overall vaccination efficacy of 73.3% was observed. Analysis of the secondary endpoint results showed vaccination efficacies of 76.1% in individuals seropositive at baseline, 66.2% in those seronegative at baseline, 90.4% in those hospitalized for DENV infection, and 85.9% in those with dengue hemorrhagic fever. TAK-003 showed variable vaccination efficacies against each DENV serotype: 69.8% for DENV1, 95.1% for DENV2, 48.9% for DENV3, and 51.0% for DENV4. Children vaccinated with TAK-003 were protected against hospitalization and dengue hemorrhagic fever (Biswal *et al*., 2020). TAK-003 showed strong protection against DENV2 but relatively weak protection against DENV3 and DENV4. Therefore, concerns remain regarding the defense of the TAK-003 vaccine against all DENV serotypes. Recently, type-specific Abs were characterized using samples from human and non-human primates vaccinated with TAK-003, and those against DENV2 were strongly induced. However, type-specific Abs against other DENV serotypes were inefficiently induced, and most of the neutralizing Abs against DENV1, DENV3, and DENV4 were cross-reactive (White *et al*., 2021).

Other live-attenuated and inactivated virus vaccines

The US Walter Reed Army Institute of Research (WRAIR) and GlaxoSmithKline (GSK) are jointly conducting clinical trials of a tetravalent live-attenuated vaccine, TDENV-LAV, and of a tetravalent purified inactivated vaccine, TDENV-PIV (Sun *et al*., 2009; Schmidt *et al*., 2017). TDENV-LAV was subjected to a clinical phase II trial using all DENV serotypes in a tetravalent formulation, which had been attenuated in PDK cells and Rhesus lung cells (Fig. 3D). The results confirmed the safety and immunogenicity of TDENV-LAV. For example, tetravalent formulation 17 of TDENV-LAV was injected subcutaneously into the deltoid on days 0 and 180, and sera were collected 28 days after the secondary immunization. Results showed that tetravalent neutralizing Abs were generated in 63% of subjects (Sun *et al*., 2009). Subjects with a high level of neutralizing Abs against all DENV serotypes were selected and the presence of type-specific and cross-reactive Abs analyzed (Gromowski *et al*., 2018). The results showed that TDENV-LAV induced type-specific Abs against DENV2 and DENV4. In addition, neutralizing Abs against DENV1 and DENV3 were identified as cross-reactive.

WRAIR and GSK also conducted a phase I clinical trial of an inactivated vaccine and a live-attenuated vaccine (Schmidt *et al*., 2017). In the trial, adjuvants such as Alum, AS01E, and AS03B were used to inject the inactivated vaccine intramuscularly on days 0 and 28, and immunogenicity and safety were confirmed. Vaccine-related adverse events were not observed for 12 months after vaccination, and all adjuvanted formulations induced strong neutralizing Abs against all DENV serotypes. Recently, WRAIR and GSK also conducted a phase I clinical trial of a heterologous prime-boost regimen using TDENV-PIV and TDENV-LAV (Lin *et al*., 2021). The experiments were conducted using the TDENV-PIV/LAV or TDENV-LAV/PIV prime-boost protocol, and the immunizations were scheduled on days 0 and 28 or days 0 and 180, respectively. Serious vaccine-related adverse events were not observed in any vaccinee. The results showed that the TDENV-PIV/LAV prime-boost protocol induced stronger tetravalent seroconversion and neutralizing Ab titers compared with the TDENV-LAV/PIV prime-boost protocol. Their research team is conducting a clinical trial to determine the schedule that can induce the most comparable Ab profile between 1 and 6 months and is planning a clinical trial including human challenge studies.

Subunit and virus-like particle vaccines

E protein is involved in DENV binding to host cell receptors, and Abs specific to E protein exert neutralizing effects. Consequently, extensive research has been conducted on the development of subunit recombinant protein vaccine using E protein. Merck and Hawaii Biotech conducted a pre-clinical trial using a C-terminal 80-amino acid-truncated E protein (DEN-80E) (Fig. 3E). Non-human primates were vaccinated with a tetravalent-DEN-80E formulation with ISCOMATRIX™ adjuvant at different concentrations in two immunization regimens (0, 1, and 2 months versus 0, 1, 2, and 6 months). When formulation group 11 (3 μg each of DEN1-80E, DEN2-80E, and DEN4-80 and 6 μg DEN4-80E) was used for vaccination in the regimen conducted at 0, 1, 2, and 6 months, high levels of neutralizing Abs were induced, and viremia was not observed in the DENV1–4 challenge experiment (Govindarajan *et al*., 2015). Next, a phase I clinical trial was conducted using DENV1-80E, however, the results of the experiments have not been reported to date.

Research on dengue vaccine development using a tetravalent virus-like particle formulation with EDIII of DENV is ongoing. Virus-like particles (DSV4) were produced in methylotrophic yeast (*Pichia pastoris*) via fusion with hepatitis B virus surface Ag and tetravalent EDIII of DENV (Fig. 3F). Anti-DSV4 Abs induced neutralizing effects against all DENV serotypes in non-human primates, and ADE was not induced in AG129 mice. Currently, DSV4 is being prepared for

scale-up production and clinical trials (Swaminathan and Khanna, 2019).

mRNA vaccine

An mRNA vaccine for flavivirus has been developed; however, an mRNA vaccine against DENV has not been successfully pursued but has been re-evaluated due to the recent success of the mRNA vaccine against Severe acute respiratory syndrome coronavirus 2. The main target Ag genes for an mRNA vaccine against DENV include NS3, NS4b, and NS5, which can induce $CD8^+$ T cell activation and are devoid of ADE mediated by Abs (Roth *et al.*, 2019). Other research to induce neutralizing Abs using NS1 and 80% of E protein (E80) is in progress (Zhang *et al.*, 2020). The humoral and cell-mediated immune responses in vaccinated BALB/c mice and protective immunity against DENV2 have been confirmed. In addition, the development of an mRNA vaccine using the prM and E proteins of DENV1 is in progress (Wollner and Richner, 2021; Wollner *et al.*, 2021) (Fig. 3G). Constructs with wild-type and mutated fusion loop epitopes were used, and vaccination with the mutated fusion loop epitope reduced the ADE. The mRNA vaccine against DENV is in the early stages compared with other mRNA vaccines; however, the mRNA vaccine platform can be applied to *Flaviviridae* for effective use of NS proteins with $CD8^+$ T cell epitopes. Therefore, development of mRNA vaccines against DENV is expected in the near future.

Obstacles to Dengue Vaccine Development

DENV exhibits ADE, in contrast to the commonly known types of infection associated with other viruses. In ADE, DENV infection is increased by non-protective or less neutralizing pre-existing Abs that bind to DENV (Shukla *et al.*, 2020). Recently, the ADE in DENV was classified as extrinsic and intrinsic ADE. For example, the DENV-induced prM and fusion-loop Abs facilitate immature DENV entry into host cells via the Fcγ receptor and mediate enhanced DENV uptake into host cells, which facilitates a subsequent increase in viral replication (extrinsic ADE). The DENV, which is immune-complexed with these Abs, enters host cells via the Fcγ receptor, resulting in suppression of intracellular cytokine signaling, which promotes a favorable environment for enhanced DENV replication (intrinsic ADE) (Narayan and Tripathi, 2020; Shukla *et al.*, 2020). ADE is an important issue that should be resolved before developing a vaccine against DENV. The other obstacle to dengue vaccine development is antigenic variation based on the DENV genotype. In a recent report, the antigenic variation based on the genotype of DENVs of the same serotype affected the neutralization activity (Martinez *et al.*, 2020). In addition, re-infection with the same DENV serotype was expected to occur after immunization against the DENV serotype, and this re-infection could be due to genetic and antigenic variations. Furthermore, breakthrough infection for specific genotypes was observed in Dengvaxia® recipients, which is likely due to genotypic variation (Martinez *et al.*, 2021). In conclusion, successful vaccine development against DENV infection needs to include a strategy that can avoid ADE and efficiently respond to Ag genotype variations.

Conclusions and Suggestions for Dengue Vaccine Development

Although many difficulties exist in developing successful dengue vaccines, successful vaccine development is closer to being achieved due to the significant amount of recent research efforts. We have several suggestions for achieving a successful dengue vaccine development. First, the Abs produced by the dengue vaccine must induce type-specific Abs against all serotypes (Swaminathan and Khanna, 2019). Both the existing Dengvaxia® and TAK-003 vaccines cannot generate type-specific Abs for all DENV serotypes and induce a skewed Ab response to one serotype (Thomas and Yoon, 2019; White *et al.*, 2021). The TV003/005 induced high levels of type-specific Abs against all DENV serotypes compared with other live-attenuated vaccines (Nivarthi *et al.*, 2021). However, live-attenuated vaccines have prM and fusion loop epitopes, and these Ags have higher immunogenicity compared with neutralization-related Ags; thus, the ADE issue remains (Beltramello *et al.*, 2010; Dejnirattisai *et al.*, 2010). Conversely, several researchers believe that a subunit vaccine using EDIII is a good vaccine platform because prM and fusion loop epitopes are not included. EDIII is an Ag that produces strong neutralizing Abs. However, type-specific neutralizing Abs that recognize EDI and EDII exist, and strong Abs that recognize only EDIII cannot be produced (Cockburn *et al.*, 2012; Fibriansah *et al.*, 2014, 2015; Dejnirattisai *et al.*, 2015). For example, 1F4 Ab, a type-specific neutralizing Ab of DENV1, recognizes EDI and EDI-EDII hinges. Similarly, 5J7, a type-specific neutralizing Ab of DENV3, recognizes the EDI-EDII hinge, EDII, and EDIII quaternary epitopes (Fibriansah *et al.*, 2014, 2015). In addition, the cross-reactive EDE Ab recognizes the E protein dimer and exerts a strong neutralizing effect against all DENV serotypes (Dejnirattisai *et al.*, 2015). We believe that both type-specific Abs and cross-reactive Abs with strong neutralizing ability are important for protection against DENV infection. Therefore, we believe that EDI and EDII are as equally important as EDIII. Recently, a Zika virus vaccine was reportedly developed in which the immunogenicity of the fusion loop epitope was weakened by mutating the fusion loop epitope without affecting the E protein structure (Dai *et al.*, 2021). Based on these mutations, a vaccine immunogen using the entire E protein without concern for the fusion loop epitope can be expected to induce type-specific and cross-reactive neutralizing Abs recognizing EDI and EDII. Second, whereas Ab-based vaccine candidates typically use E protein as an antigen, T-cell activity targets mainly the NS and capsid proteins. Vaccine candidates capable of activating T cells can avoid ADE, a barrier to DENV vaccine development. NS proteins have fairly conserved sequences among DENV serotypes and have potential as targets for vaccines against all DENV serotypes (Tian *et al.*, 2019). Because the live-attenuated vaccine contains the NS protein, it induces strong T-cell activity. Conversely, the E protein-based subunit vaccine does not contain the NS protein and did not stimulate $CD8^+$ T-cell activity compared to the live-attenuated vaccine. This could

be overcome by induction of CD8 T cells by cross-presentation of the Ag using a specific ligand-conjugated subunit vaccine (Kim *et al*., 2021). Collectively, we suggest that a subunit vaccine platform is capable of avoiding ADE as much as possible. In addition, the DENV genotype variation, which is currently considered a significant obstacle, can be managed using the subunit vaccine platform. Compared with the live-attenuated vaccine platform, which is time-consuming to produce vaccine candidates, subunit vaccines may require relatively less time to produce genotype-specific vaccine materials. Consequently, we propose the subunit vaccine platform is a more promising platform compared with the live-attenuated vaccine platform for dengue vaccine development.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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