



Review

Flaviviruses and flavivirus vaccines

Franz X. Heinz*, Karin Stiasny

Department of Virology, Medical University of Vienna, Kinderspitalgasse 15, 1095 Vienna, Austria

ARTICLE INFO

Article history:

Received 20 June 2011

Received in revised form

16 September 2011

Accepted 23 September 2011

Keywords:

Flaviviruses

Flavivirus vaccines

Molecular antigenic structure

ABSTRACT

Several human-pathogenic flaviviruses (including yellow fever, dengue, Japanese encephalitis, West Nile and tick-borne encephalitis viruses) have a significant public health impact in different parts of the world and the potential of emerging in previously non-endemic regions. For some viruses, the structure of the most important immunogen, the envelope protein E, has been determined to atomic resolution by X-ray crystallography, and the architecture of virus particles has been resolved by cryo-electron microscopy. Through the combination of structural and immunological investigations, we now have a detailed understanding of the mechanisms of virus neutralization and antibody-dependent enhancement (ADE) of infectivity at a molecular level. The latter phenomenon has been proposed to play an important role in the immunopathology of severe forms of dengue virus infections (hemorrhagic dengue fever and dengue shock syndrome) and is therefore of special relevance in the context of dengue vaccines.

Effective human vaccines are in use for the prophylaxis of yellow fever (live attenuated), Japanese encephalitis (live attenuated and inactivated whole virus), and tick-borne encephalitis (inactivated whole virus). Although dengue is the most important flavivirus with respect to global disease incidence, the development and use of vaccines has been hampered so far by the theoretical risk of vaccine-related adverse events such as immune enhancement of infection and the requirement to induce a long-lasting protective immune response against all four dengue serotypes simultaneously. Currently, several kinds of dengue vaccines are in development, but only one of these candidates (a chimeric dengue-yellow fever live attenuated vaccine) has reached the stage of phase 3 clinical trials.

© 2011 Elsevier Ltd. All rights reserved.

1. The worldwide impact of flaviviruses

Flaviviruses comprise more than 70 different viruses, many of which are arthropod-borne and transmitted by either mosquitoes or ticks [1]. Taxonomically, they form a genus in the family *Flaviviridae* which in addition includes the genera *hepacivirus* and *pestivirus* [2]. With respect to disease impact, the most important human pathogenic flaviviruses are yellow fever virus (YFV), dengue virus (DENV), Japanese encephalitis virus (JEV), West Nile virus (WNV) and tick-borne encephalitis virus (TBEV). Several others can also cause severe and even lethal disease in humans but potential exposure to these viruses is apparently limited and the reported case numbers are relatively small. Examples are St. Louis encephalitis virus, Murray valley encephalitis virus, Rocio virus, Kyasanur forest disease/Alkhurma virus, Omsk hemorrhagic fever virus and Powassan virus [1].

Because of their dependence on specific vectors and different natural hosts, flaviviruses have distinct geographical distributions. YFV is endemic in tropical and subtropical regions in Africa and South-America and causes an estimated 200,000 cases with 30,000

deaths annually [3]. Geographically, the endemic regions of DENV overlap with those of YFV in Africa and South-America. However, DEN extends not only to Middle America and southern parts of North America but also to large parts of South-East Asia, where YFV is not found [4]. Infections with DENV are usually mild but extremely frequent, with about 100–200 million infections every year [5,6]. In a small proportion of patients, the disease can exacerbate and lead to dengue hemorrhagic fever (DHF) and/or dengue shock syndrome (DSS). Annually, about 500,000 such cases with more than 20,000 deaths are recorded [7]. The endemic areas of JEV overlap with those of DENV in South-East Asia, but JEV is transmitted by different mosquitoes and has different natural hosts [8,9]. JEV causes severe encephalitis and 25–30% of the 50,000 cases occurring every year are fatal [9]. In contrast to these mosquito-borne viruses, TBEV is not found in the tropics/subtropics but in many parts of Europe as well as Central and Eastern Asia [10]. In these areas, it accounts for one of the most important CNS infections in adults with more than 10,000 cases per year [11]. WNV is an example of the potential of flaviviruses to emerge suddenly in previously unaffected geographical areas. It was known to be endemic in parts of Africa, Europe, Asia, and Australia – causing sporadic cases or small outbreaks of CNS disease – before it first appeared at the East coast of the USA in 1999 and rapidly spread over the North-American continent, to Central-America and finally to

* Corresponding author. Tel.: +43 1 40160x65510; fax: +43 1 40160x965 599.
E-mail address: franz.x.heinz@meduniwien.ac.at (F.X. Heinz).

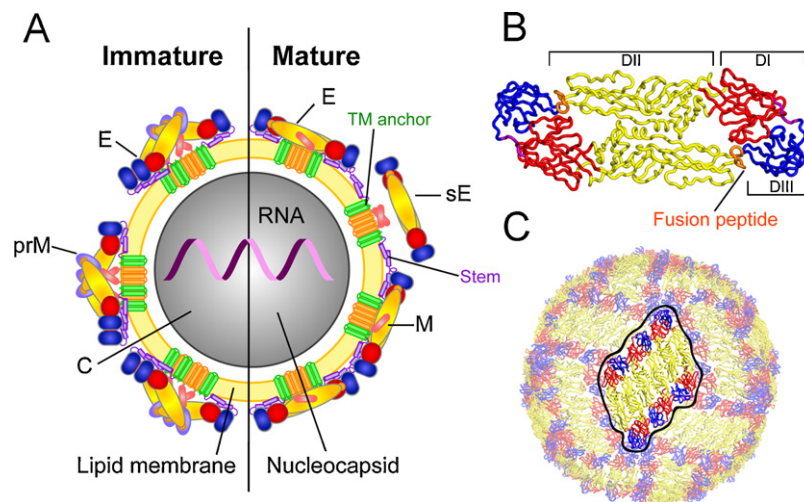


Fig. 1. (A) Schematic model of a flavivirus particle. Left: immature virus, right: mature virus. The spherical capsid contains the viral RNA and multiple copies of the capsid protein C. The surface of immature particles is made up of 60 spikes composed of trimers of heterodimers between the membrane-associated prM and E proteins. The smooth-surfaced mature particles are formed after prM cleavage and contain a tightly packed shell of 90 E homodimers. The structure of E (sE) – schematically lifted off in the panel of mature virions – that lack the so-called stem- and transmembrane (TM) anchor-regions. (B) Ribbon diagram of the crystal structure of the TBEV sE protein (PDB: 1svb). sE is composed of three distinct domains (DI, DII, and DIII). (C) Arrangement of E protein dimers at the surface of mature virions as determined by cryo EM. 30 rafts of three parallel E dimers (one raft is highlighted in the figure) form a specific herringbone-like icosahedral structure. The coordinates were obtained from the VIPERdb Virus Particle Explorer (viperd b 1thd; <http://viperd b.scripps.edu>).

South-America [12]. In the peak year of 2003, 9862 human cases and 264 deaths due to WNV infections were documented in the US [13] and in the light of continued expansion, the need for an effective vaccine appeared to gain high priority [14]. Since then, the annual numbers of cases in the US have declined significantly [15], with a parallel decrease in the interest for commercial vaccine development.

2. Molecular antigenic structure and life cycle

Like all members of the *Flaviviridae* family, flaviviruses are small enveloped positive stranded RNA viruses. Mature viruses have a diameter of 50 nm and contain only three structural proteins, designated C (capsid), E (envelope) and M (membrane) (Fig. 1). Particle assembly takes place in the endoplasmic reticulum and first leads to the formation of immature viruses that contain the precursor of M (prM) (Figs. 1 and 2) [16] which is proteolytically cleaved in the trans-Golgi network during exocytosis by a cellular protease before the virions are released from infected cells (Fig. 2) [17–19]. Immature viruses have spiky projections of 60 trimers of prM-E heterodimers [20], whereas mature virions have a smooth surface made up of a herringbone-like arrangement of 90 E homodimers, organized in rafts of 3 parallel dimers (Fig. 1C) [21,22]. The originally assembled immature virions are non-infectious, and prM cleavage allows E to adopt the conformational state required for its entry functions, i.e. receptor-binding and acidic-pH-induced membrane fusion after uptake by receptor-mediated endocytosis (Fig. 2) [23,24]. Recently, it was shown that fully immature virions can be rendered infectious in the course of antibody-mediated uptake into Fc-receptor-positive cells through the post-entry cleavage of prM in the endosome [25]. The possible contribution of completely immature viruses to the infection process remains to be determined.

Atomic structures of soluble forms of E (lacking the double transmembrane anchor and about 50 additional amino acids in the so-called ‘stem’; Fig. 1A) have been determined for TBEV, DENV, and WNV [26–31]. These structures are very similar, being composed of 3 distinct domains (DI, DII and DIII) in an elongated molecule that forms an antiparallel dimer at the surface of mature virions (Fig. 1B). The tip of DII carries a highly conserved loop (Fig. 1B) that functions as an internal fusion peptide and initiates

endosomal membrane fusion (Fig. 2) after acid pH-induced dissociation of the E dimer [32–34].

Because of its dual role in cell entry – attachment to cellular receptors and membrane fusion – the E protein is the major target of virus neutralizing antibodies that inhibit these functions and thus prevent infection. There is overwhelming evidence that neutralizing antibodies mediate long-term protection from disease and their measurement therefore provides the best correlate of flavivirus immunity [35]. Epitopes involved in neutralization have been mapped to each of the three domains and to sites all over the exposed surface of E, but evidence from work with mouse monoclonal antibodies suggests that those against DIII have a higher neutralizing potency than those to other sites of the molecule [35,36]. Structural and mutational studies revealed epitopes that are (i) confined to single domains [37,38], (ii) located at the junction of domains [38–42], (iii) subunit overlapping (i.e. comprise amino acid residues from both monomers in the dimer) [40,43–45] or (iv) dependent on the specific herringbone-like arrangement of E in the virion [46]. Most interestingly, strongly neutralizing antibodies have been identified that gain access to their partially cryptic epitopes through temperature-dependent conformational movements of E at the virion surface [47], indicating that the particle structure may not be as rigid as previously assumed.

Experiments in animal models have shown that protection from disease can not only be mediated by antibodies to E but also to the non-structural protein NS1, which is produced and secreted from infected cells (Fig. 2). In this case, the mechanism of protection is believed to be dependent on antibodies recognizing NS1 that bind to cell surface-associated NS1 and facilitate phagocytosis and clearance of infected cells through Fc- γ receptors [36]. NS1 has therefore been proposed as a component of new flavivirus vaccines [48,49].

All flaviviruses are antigenically related, as originally shown in hemagglutination-inhibition tests with polyclonal sera [50] but as also revealed in ELISA. Cross-neutralization, however, is confined to more closely related flaviviruses that have been grouped into so-called serocomplexes [51] (Fig. 3). The minimum amino acid sequence identity in the E protein of all flaviviruses is 40–44% and within serocomplexes it is 60–70. Although cross-neutralization and cross-protection are observed within serocomplexes, its extent and duration are strongly dependent on the degree of amino acid

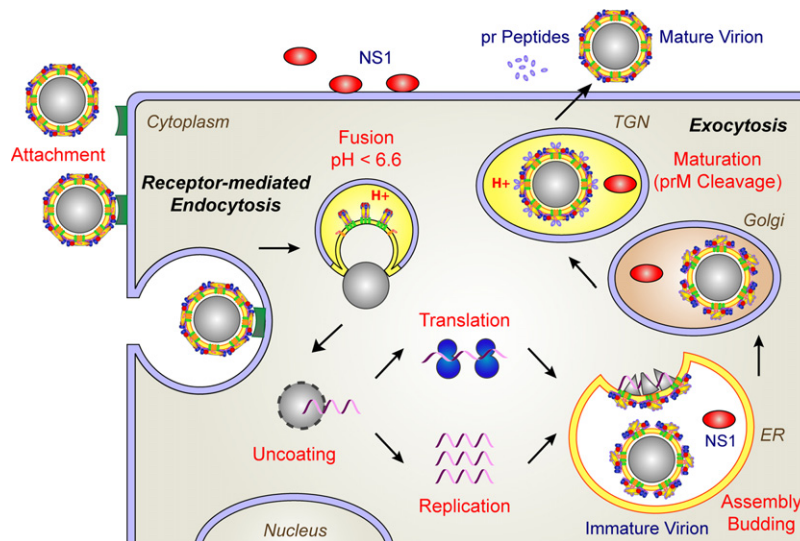


Fig. 2. Schematic of a flavivirus life cycle. Viruses are taken up by receptor-mediated endocytosis and acidic-pH-induced fusion occurs in the endosome. The positive-stranded RNA is translated into the three structural and seven non-structural proteins that are essential for polyprotein processing and RNA replication. Virus assembly takes place in the endoplasmic reticulum (ER) leading to the formation of non-infectious immature particles that are transported through the exocytotic pathway of the cell. The acidic pH in the trans-Golgi network (TGN) causes a rearrangement of the envelope proteins and the proteolytic cleavage of prM into pr and M by the cellular protease furin. pr remains associated with these particles and falls off after the release of infectious virions by exocytosis. NS1 is the only non-structural protein that is released from infected cells.

similarity in E. For instance, infection with any one of the four DENV serotypes induces life-long protection against the same serotype but only for few months against the other serotypes [6].

The epitopes recognized by broadly cross-reactive antibodies have been mapped to the fusion peptide loop at the tip of DII [39,44,45,52] (Fig. 1) which is highly conserved among all flaviviruses. Because of the cryptic nature of this epitope in the context of mature virions, such antibodies usually do not contribute to virus neutralization [52,53]. The accessibility of the fusion loop, however, may be higher in partially immature virions [53,54] that are infectious and released in significant amounts by DENV-infected cells

[55]. FP-specific antibodies may therefore contribute to neutralization of partially immature infectious viruses.

3. Successful flavivirus vaccines

The development of the YFV 17D live-attenuated vaccine was a landmark in the history of viral vaccines, and in 1951 Max Theiler was awarded the Nobel prize in Medicine for his achievements in attenuating the wild-type virus by serial passaging in mouse and chicken tissue [3]. Since its development in 1937, more than 500 million people have been vaccinated and over 98% of vaccinees are believed to be protected for at least 10 years [56]. Despite its great record in protecting from YF, evidence for a significant degree of severe vaccine-associated adverse events has been accumulating in the last ten years. These include YF vaccine-associated viscerotropic disease and YF vaccine-associated neurotropic disease (with a higher incidence in elderly and immunocompromised individuals) at a rate exceeding that of other live virus vaccinations [56,57]. Also, due to a largely unchanged manufacturing process since 1945, the vaccine contains substantial amounts of chicken embryo proteins, and allergic reactions contribute to the adverse events observed with its use [56]. To provide a safer alternative, a purified inactivated vaccine produced in Vero cells has been developed and the results of a recently published phase I clinical trial suggest good immunogenicity and tolerability [57,58].

Effective vaccines are also available for the immunoprophylaxis of Japanese encephalitis, including both inactivated whole virus and live attenuated vaccines. Originally, the substrates for inactivated vaccines were either infected mouse brains or primary hamster kidney cells (China) and vaccine efficacies of 76–95% were reported [9]. Recently, a new inactivated vaccine was developed by Intercell (IXIARO) that is based on the attenuated SA14-14-2 strain grown in Vero cells [59]. Several studies have demonstrated excellent immunogenicity, tolerability as well as non-inferiority to mouse brain-derived inactivated vaccines and this product is now available on the market in many countries, including the US, Europe, Japan, and Australia [60–62]. The attenuated SA-14-14-2 strain was developed in China and is licensed in this country as a live vaccine since 1988 [9]. The field effectiveness is comparable to

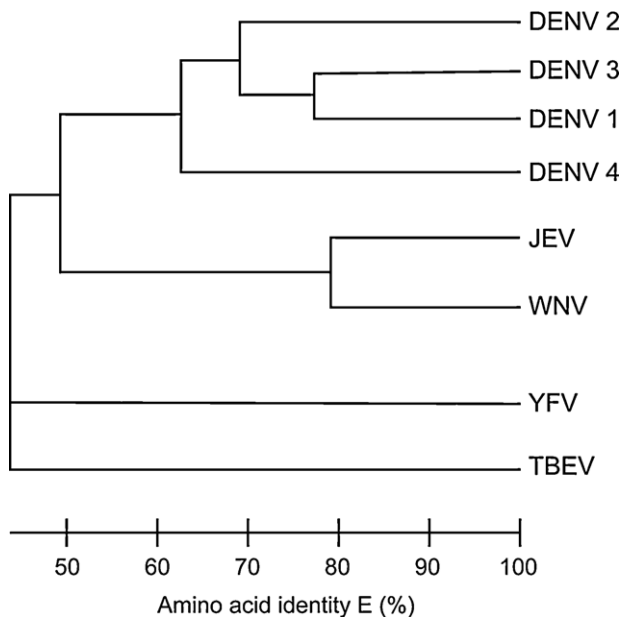


Fig. 3. Dendrogram based on E protein amino acid identity (%) of the most important human pathogenic flaviviruses. DENV, dengue virus; JEV, Japanese encephalitis virus; WNV, West Nile virus; YFV, yellow fever virus; TBEV, tick-borne encephalitis virus.

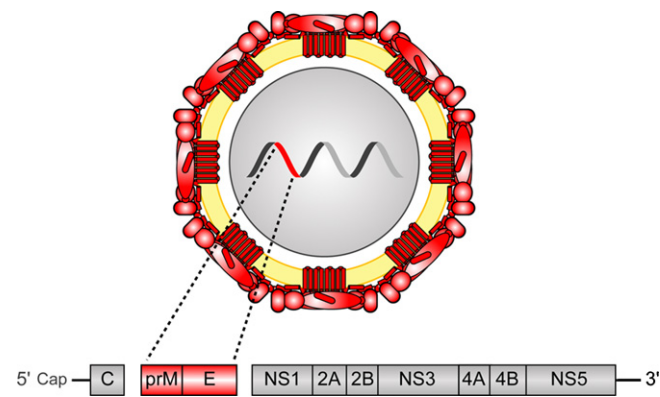


Fig. 4. Schematic representation of the Chimerivax principle. The sequences encoding the prM and E proteins of the vaccine target virus (e.g. JEV or DENV) are engineered into the genome of the yellow fever 17D vaccine strain to replace the authentic prM and E sequences, resulting in the production of chimeric virus particles that are covered by the corresponding heterologous envelope proteins.

that of the inactivated vaccines (88–96%) and more than 300 million doses have been administered since its licensure [9]. A new generation live JE vaccine was developed by Sanofi Pasteur that is based on a chimeric virus containing the prM and E proteins (Fig. 4) of JEV whereas all of the rest is derived from the attenuated YFV 17D strain as a backbone (Chimerivax/IMJOJEV) [63]. Clinical studies revealed excellent immunogenicity without safety concerns and the vaccine is now licensed in Australia [64].

TBE can be effectively prevented by highly purified inactivated whole virus vaccines that are produced in Europe and Russia, using primary chicken embryo cells as a substrate [11,65,66]. Their use in endemic regions differs widely, with the highest vaccination coverages reached in Austria (85%) [67] and the Sverdlovsk district of Russia (81%) [68]. The field effectiveness of both the European and Russian vaccine is very high. It reaches 98% [67,68] when the proper vaccination schedule is applied and has led to a dramatic decline of disease incidence in the vaccinated population.

4. The dengue vaccine problem

The dengue serocomplex of flaviviruses consists of 4 serotypes (Fig. 3), each of which is made up of several genotypes [69]. Consistent with substantial differences in the amino acid sequence of their E proteins, the degree of cross-neutralization and cross-protection between members of different serotypes is limited. After human infection with one serotype, heterologous protection lasts only for few months and epidemiological observations indicate that previous infection with one serotype can predispose to the severe forms of dengue (DHS/DSS) upon re-infection by another serotype [70]. Since endemic regions with co-circulating different serotypes have enormously expanded (establishment of hyperendemic areas) also the incidence of DHF/DSS has increased dramatically in the last decades [5,71]. The precise mechanisms leading to severe forms of dengue are still a matter of investigation, but it is clear that both viral virulence and host factors – including immunopathological mechanisms – can contribute to the exacerbation of the disease [71,72]. Fc receptor-bearing cells such as monocytes, macrophages, and dendritic cells have been shown to be major targets of dengue virus infections in humans [73–75] and increased Fc receptor-mediated uptake of incompletely neutralized virus can lead to the phenomenon of antibody-dependent enhancement of infection (ADE). Cross-reactive non-neutralizing antibodies (such as those present after infection with a heterologous serotype in sequential infections) but also neutralizing antibodies at sub-neutralizing concentrations (e.g. when maternal antibodies drop

Table 1
Tetravalent dengue virus vaccines in clinical trials.

Developer	Vaccine	Clinical trial
Sanofi Pasteur	Chimeric live vaccine (yellow fever 17D back-bone) (Chimerivax)	Phase III
CDC-Inviragen	Chimeric live vaccine (dengue back-bone) (DENVax)	Phase I
NIH	Chimeric and specifically engineered live vaccine strains	Phase I
Walter Reed Army Institute of Research/ GlaxoSmithKline	Conventional live attenuated vaccine	Phase II (on hold)

Modified from Ref. [84].

to sub-neutralizing levels several months after birth) can all contribute to ADE [72,76,77]. In addition, secondary infections have been shown to activate pre-existing cross-reactive T cells that possess higher affinity for the previously encountered but lower affinity for the newly infecting virus [78]. Because of these properties, it has been proposed that the activated T cells are less efficient in viral clearance but through the cytokines they release contribute to the development of severe disease [79]. In current models of dengue immunopathogenesis, the increase in virus load caused by ADE combined with strong anamnestic cross-reactive T cell responses are believed to result in a ‘cytokine storm’ that finally causes capillary leakage and the symptoms of DHF/DSS [78–81].

The risk of inducing an immunological condition in vaccinees that not only does not protect but may even lead to enhanced disease was the major obstacle for the development and use of a dengue vaccine so far. The two most important points of concern are the need to induce an equally protective immunity against all 4 serotypes simultaneously, and the risk of waning immunity associated with the potential of immunological enhancement years after vaccination. An ideal dengue vaccine should therefore induce life-long immunity against all 4 serotypes and have an excellent profile of tolerability, also in children. Despite these hurdles, a number of approaches were pursued for the development of several different types of dengue vaccines [7,82–84]. These include conventionally attenuated live vaccines, genetically engineered chimeric dengue–dengue and dengue–yellow fever live vaccines, inactivated whole virus vaccines, recombinant E protein subunit vaccines, DNA vaccines, and viral vector vaccines expressing either E or only DIII. Ongoing human clinical trials with tetravalent candidate dengue vaccines are listed in Table 1. Currently, the most advanced of these developments is the chimeric dengue–yellow fever live vaccine (Chimerivax; Fig. 4) manufactured by Sanofi Pasteur [85]. It is a tetravalent vaccine that contains chimera of all 4 dengue serotypes, and so far it is the only candidate dengue vaccine that has entered phase III clinical trials [84,85]. Previous studies had indicated that the majority of adverse events observed were mild to moderate and transient in nature [85,86]. The phase III clinical trials in combination with many years of observation will finally reveal whether this vaccine can reduce the burden of severe dengue infections without adverse effects such as enhancement of disease.

5. Concluding remarks

Important new insights into the mechanisms of immune-mediated protection – especially of virus neutralization by antibodies – have been obtained through the elucidation of molecular details of the major flavivirus antigens and their interactions with the immune system [35]. At the same time, however, flaviviruses provide excellent examples of how successful conventional vaccines can be that have been developed in the absence

and/or without the need of such detailed information. This is certified by the effectiveness of the traditional live vaccines against YF and JE as well as the whole inactivated virus vaccines against JE and TBE. Despite these successes, a vaccine against dengue – the most abundant flavivirus infection with the highest disease impact worldwide – is still not available. The application of new technologies and the advancement of a recombinant candidate live vaccine to phase III clinical trials raise hope that an efficient means of immunoprophylaxis against dengue will indeed become available in the foreseeable future.

References

- [1] Gubler D, Kuno G, Markhoff L. Flaviviruses. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, et al., editors. *Fields virology*. 5th ed. Philadelphia: Lippincott Williams & Wilkins; 2007. p. 1153–252.
- [2] Thiel HJ, Collett MS, Gould EA, Heinz FX, Houghton M, Meyers G, et al. *Family Flaviviridae*. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, editors. *Virus taxonomy*. VIIIth report of the international committee on taxonomy of viruses. San Diego: Elsevier, Academic Press; 2005. p. 981–98.
- [3] Monath TP, Cetron MS, Teuwen DE. Yellow fever vaccine. In: Plotkin SA, Orenstein WA, Offit PA, editors. *Vaccines*. Saunders, Elsevier; 2008. p. 959–1055.
- [4] Vasilakis N, Cardoso J, Hanley KA, Holmes EC, Weaver SC. Fever from the forest: prospects for the continued emergence of sylvatic dengue virus and its impact on public health. *Nat Rev Microbiol* 2011;9(7):532–41.
- [5] Gubler DJ. Emerging vector-borne flavivirus diseases: are vaccines the solution? *Expert Rev Vaccines* 2011;10(5):563–5.
- [6] Guzman MG, Halstead SB, Artsob H, Buchy P, Farrar J, Gubler DJ, et al. Dengue: a continuing global threat. *Nat Rev Microbiol* 2010;8(12 Suppl.):S7–16.
- [7] Webster DP, Farrar J, Rowland-Jones S. Progress towards a dengue vaccine. *Lancet Infect Dis* 2009;9(11):678–87.
- [8] Gould EA, Solomon T. Pathogenic flaviviruses. *Lancet* 2008;371(9611):500–9.
- [9] Halstead SB, Thomas SJ. Japanese encephalitis: new options for active immunization. *Clin Infect Dis* 2010;50(8):1155–64.
- [10] Lindquist L, Vapalahti O. Tick-borne encephalitis. *Lancet* 2008;371(9627):1861–71.
- [11] WHO. Vaccines against tick-borne encephalitis: WHO position paper. *Wkly Epidemiol Rec* 2011;86(24):241–56.
- [12] Gubler DJ. The continuing spread of West Nile virus in the western hemisphere. *Clin Infect Dis* 2007;45(8):1039–46.
- [13] Hayes EB, Komar N, Nasci RS, Montgomery SP, O'Leary DR, Campbell GL. Epidemiology and transmission dynamics of West Nile virus disease. *Emerg Infect Dis* 2005;11(8):1167–73.
- [14] Gould LH, Fikrig E. West Nile virus: a growing concern? *J Clin Invest* 2004;113(8):1102–7.
- [15] CDC. <http://www.cdc.gov/ncidod/dvbid/westnile/index.htm>.
- [16] Lindenbach BD, Thiel HJ, Rice CM. *Flaviviridae*: the viruses and their replication. In: Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, et al., editors. *Fields virology*. 5th ed. Philadelphia: Lippincott Williams & Wilkins; 2007. p. 1101–52.
- [17] Yu IM, Zhang W, Holdaway HA, Li L, Kostyuchenko VA, Chipman PR, et al. Structure of the immature dengue virus at low pH primes proteolytic maturation. *Science* 2008;319(5871):1834–7.
- [18] Yu IM, Holdaway HA, Chipman PR, Kuhn RJ, Rossmann MG, Chen J. Association of the pr peptides with dengue virus at acidic pH blocks membrane fusion. *J Virol* 2009;83(23):12101–7.
- [19] Stadler K, Allison SL, Schalich J, Heinz FX. Proteolytic activation of tick-borne encephalitis virus by furin. *J Virol* 1997;71(11):8475–81.
- [20] Zhang Y, Corver J, Chipman PR, Zhang W, Pletnev SV, Sedlak D, et al. Structures of immature flavivirus particles. *EMBO J* 2003;22(11):2604–13.
- [21] Kuhn RJ, Zhang W, Rossmann MG, Pletnev SV, Corver J, Lenches E, et al. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. *Cell* 2002;108(5):717–25.
- [22] Mukhopadhyay S, Kim BS, Chipman PR, Rossmann MG, Kuhn RJ. Structure of West Nile virus. *Science* 2003;302(5643):248.
- [23] Kaufmann B, Rossmann MG. Molecular mechanisms involved in the early steps of flavivirus cell entry. *Microbes Infect* 2011;13(1):1–9.
- [24] Stiasny K, Heinz FX. Flavivirus membrane fusion. *J Gen Virol* 2006;87(Pt 10):2755–66.
- [25] Rodenhuis-Zybert IA, van der Schaar HM, da Silva Voorham JM, van der Ende-Metselaar H, Lei HY, Wilschut J, et al. Immature dengue virus: a veiled pathogen? *PLoS Pathog* 2010;6(1):e1000718.
- [26] Rey FA, Heinz FX, Mandl C, Kunz C, Harrison SC. The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolution. *Nature* 1995;375(6529):291–8.
- [27] Modis Y, Ogata S, Clements D, Harrison SC. A ligand-binding pocket in the dengue virus envelope glycoprotein. *Proc Natl Acad Sci U S A* 2003;100(12):6986–91.
- [28] Modis Y, Ogata S, Clements D, Harrison SC. Variable surface epitopes in the crystal structure of dengue virus type 3 envelope glycoprotein. *J Virol* 2005;79(2):1223–31.
- [29] Kanai R, Kar K, Anthony K, Gould LH, Ledizet M, Fikrig E, et al. Crystal structure of West Nile virus envelope glycoprotein reveals viral surface epitopes. *J Virol* 2006;80(22):11000–8.
- [30] Zhang Y, Zhang W, Ogata S, Clements D, Strauss JH, Baker TS, et al. Conformational changes of the flavivirus E glycoprotein. *Structure* 2004;12(9):1607–18.
- [31] Nybakken GE, Nelson CA, Chen BR, Diamond MS, Fremont DH. Crystal structure of the West Nile virus envelope glycoprotein. *J Virol* 2006;80(23):11467–74.
- [32] Stiasny K, Allison SL, Schalich J, Heinz FX. Membrane interactions of the tick-borne encephalitis virus fusion protein E at low pH. *J Virol* 2002;76(8):3784–90.
- [33] Stiasny K, Kossli C, Lepault J, Rey FA, Heinz FX. Characterization of a structural intermediate of flavivirus membrane fusion. *PLoS Pathog* 2007;3(2):e20.
- [34] Allison SL, Schalich J, Stiasny K, Mandl CW, Heinz FX. Mutational evidence for an internal fusion peptide in flavivirus envelope protein E. *J Virol* 2001;75(9):4268–75.
- [35] Pierson TC, Fremont DH, Kuhn RJ, Diamond MS. Structural insights into the mechanisms of antibody-mediated neutralization of flavivirus infection: implications for vaccine development. *Cell Host Microbe* 2008;4(3):229–38.
- [36] Diamond MS, Pierson TC, Fremont DH. The structural immunology of antibody protection against West Nile virus. *Immunol Rev* 2008;225:212–25.
- [37] Nybakken GE, Oliphant T, Johnson S, Burke S, Diamond MS, Fremont DH. Structural basis of West Nile virus neutralization by a therapeutic antibody. *Nature* 2005;437(7059):764–9.
- [38] Kiermayr S, Stiasny K, Heinz FX. Impact of quaternary organization on the antigenic structure of the tick-borne encephalitis virus envelope glycoprotein E. *J Virol* 2009;83(17):8482–91.
- [39] Oliphant T, Nybakken GE, Engle M, Xu Q, Nelson CA, Sukupolvi-Petty S, et al. Antibody recognition and neutralization determinants on domains I and II of West Nile virus envelope protein. *J Virol* 2006;80(24):12149–59.
- [40] Vogt MR, Moesker B, Goudsmit J, Jongeneelen M, Austin SK, Oliphant T, et al. Human monoclonal antibodies against West Nile virus induced by natural infection neutralize at a postattachment step. *J Virol* 2009;83(13):6494–507.
- [41] Lai CJ, Goncalves AP, Men R, Wernly C, Donau O, Engle RE, et al. Epitope determinants of a chimpanzee dengue virus type 4 (DENV-4)-neutralizing antibody and protection against DENV-4 challenge in mice and rhesus monkeys by passively transferred humanized antibody. *J Virol* 2007;81(23):12766–74.
- [42] Goncalves AP, Chien CH, Tubthong K, Gorshkova I, Roll C, Donau O, et al. Humanized monoclonal antibodies derived from chimpanzee Fabs protect against Japanese encephalitis virus in vitro and in vivo. *J Virol* 2008;82(14):7009–21.
- [43] Daffis S, Kontermann RE, Korimbocus J, Zeller H, Klenk HD, Ter Meulen J. Antibody responses against wild-type yellow fever virus and the 17D vaccine strain: characterization with human monoclonal antibody fragments and neutralization escape variants. *Virology* 2005;337(2):262–72.
- [44] Goncalves AP, Purcell RH, Lai CJ. Epitope determinants of a chimpanzee Fab antibody that efficiently cross-neutralizes dengue type 1 and type 2 viruses map to inside and in close proximity to fusion loop of the dengue type 2 virus envelope glycoprotein. *J Virol* 2004;78(23):12919–28.
- [45] Crill WD, Chang GJ. Localization and characterization of flavivirus envelope glycoprotein cross-reactive epitopes. *J Virol* 2004;78(24):13975–86.
- [46] Kaufmann B, Vogt MR, Goudsmit J, Holdaway HA, Aksyuk AA, Chipman PR, et al. Neutralization of West Nile virus by cross-linking of its surface proteins with Fab fragments of the human monoclonal antibody CR4354. *Proc Natl Acad Sci U S A* 2010;107(44):18950–5.
- [47] Lok SM, Kostyuchenko V, Nybakken GE, Holdaway HA, Battisti AJ, Sukupolvi-Petty S, et al. Binding of a neutralizing antibody to dengue virus alters the arrangement of surface glycoproteins. *Nat Struct Mol Biol* 2008;15(3):312–7.
- [48] Gibson CA, Schlesinger JJ, Barrett AD. Prospects for a virus non-structural protein as a subunit vaccine. *Vaccine* 1988;6(1):7–9.
- [49] Chung KM, Thompson BS, Fremont DH, Diamond MS. Antibody recognition of cell surface-associated NS1 triggers Fc-gamma receptor-mediated phagocytosis and clearance of West Nile virus-infected cells. *J Virol* 2007;81(17):9551–5.
- [50] Clarke DH, Casals J. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *Am J Trop Med Hyg* 1958;7(5):561–73.
- [51] Calisher CH, Karabatsos N, Dalrymple JM, Shope RE, Porterfield JS, Westaway EG, et al. Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. *J Gen Virol* 1989;70(Pt 1):37–43.
- [52] Stiasny K, Kiermayr S, Holzmann H, Heinz FX. Cryptic properties of a cluster of dominant flavivirus cross-reactive antigenic sites. *J Virol* 2006;80(19):9557–68.
- [53] Nelson S, Jost CA, Xu Q, Ess J, Martin JE, Oliphant T, et al. Maturation of West Nile virus modulates sensitivity to antibody-mediated neutralization. *PLoS Pathog* 2008;4(5):e1000060.
- [54] Cherrier MV, Kaufmann B, Nybakken GE, Lok SM, Warren JT, Chen BR, et al. Structural basis for the preferential recognition of immature flaviviruses by a fusion-loop antibody. *EMBO J* 2009;28(20):3269–76.
- [55] Junjhon J, Edwards TJ, Utaipat U, Bowman VD, Holdaway HA, Zhang W, et al. Influence of pr-M cleavage on the heterogeneity of extracellular dengue virus particles. *J Virol* 2010;84(16):8353–8.
- [56] Barrett AD, Teuwen DE. Yellow fever vaccine – how does it work and why do rare cases of serious adverse events take place? *Curr Opin Immunol* 2009;21(3):308–13.
- [57] Monath TP, Lee CK, Julander JG, Brown A, Beasley DW, Watts DM, et al. Inactivated yellow fever 17D vaccine: development and nonclinical safety, immunogenicity and protective activity. *Vaccine* 2010;28(22):3827–40.
- [58] Monath TP, Fowler E, Johnson CT, Balser J, Morin MJ, Sisti M, et al. An inactivated cell-culture vaccine against yellow fever. *N Engl J Med* 2011;364(14):1326–33.

- [59] Tauber E, Kollaritsch H, Korinek M, Rendi-Wagner P, Jilma B, Firbas C, et al. Safety and immunogenicity of a Vero-cell-derived, inactivated Japanese encephalitis vaccine: a non-inferiority, phase III, randomised controlled trial. *Lancet* 2007;370(9602):1847–53.
- [60] Eder S, Dubischar-Kastner K, Firbas C, Jelinek T, Jilma B, Kaltenboeck A, et al. Long term immunity following a booster dose of the inactivated Japanese encephalitis vaccine IXIARO(R), IC51. *Vaccine* 2011;29(14):2607–12.
- [61] Schuller E, Klade CS, Wolff G, Kaltenboeck A, Dewasthaly S, Tauber E. Comparison of a single, high-dose vaccination regimen to the standard regimen for the investigational Japanese encephalitis vaccine, IC51: a randomized, observer-blind, controlled Phase 3 study. *Vaccine* 2009;27(15):2188–93.
- [62] Tauber E, Kollaritsch H, von Sonnenburg F, Lademann M, Jilma B, Firbas C, et al. Randomized, double-blind, placebo-controlled phase 3 trial of the safety and tolerability of IC51, an inactivated Japanese encephalitis vaccine. *J Infect Dis* 2008;198(4):493–9.
- [63] Guy B, Guirakhoo F, Barban V, Higgs S, Monath TP, Lang J. Preclinical and clinical development of YFV 17D-based chimeric vaccines against dengue, West Nile and Japanese encephalitis viruses. *Vaccine* 2010;28(3):632–49.
- [64] Halstead SB, Thomas SJ. New Japanese encephalitis vaccines: alternatives to production in mouse brain. *Expert Rev Vaccines* 2011;10(3):355–64.
- [65] Barrett PN, Plotkin SA, Ehrlich HJ. Tick-borne encephalitis virus vaccines. In: Plotkin SA, Orenstein WA, Offit PA, editors. *Vaccines*. 5th ed. Saunders, Elsevier; 2008. p. 841–56.
- [66] Lehrer AT, Holbrook MR. Tick-borne encephalitis. In: Barrett AD, Stanberry LR, editors. *Vaccine for biodefense and emerging infectious diseases*. Academic Press, Elsevier Inc.; 2009. p. 713–34.
- [67] Heinz FX, Holzmann H, Essl A, Kundi M. Field effectiveness of vaccination against tick-borne encephalitis. *Vaccine* 2007;25(43):7559–67.
- [68] Romanenko VV, Kovalev SY, Esiunina MS, Ankudinova AV. Tactics and strategy of vaccine prevention of tick-borne encephalitis in the Sverdlovsk region. In: TBE: new paradigms in a changing vaccination environment (13th ISW-TBE 2011). Vienna: Austria; 2011.
- [69] Bennett SN. Evolutionary dynamics of dengue virus. In: Hanley KA, Weaver SC, editors. *Frontiers in dengue virus research*. Norfolk, UK: Caister Academic Press; 2010. p. 157–72.
- [70] Guzman MG, Sierra B, Kouri G, Farrar J, Simmons C. Host and virus determinants of susceptibility and dengue disease severity. In: Hanley KA, Weaver SC, editors. *Frontiers in dengue virus research*. Norfolk, UK: Caister Academic Press; 2010. p. 79–101.
- [71] Kyle JL, Harris E. Global spread and persistence of dengue. *Annu Rev Microbiol* 2008;62:71–92.
- [72] Halstead SB. Pathogenesis: risk factors for infection. In: Halstead SB, editor. *Dengue*. London: Imperial College Press; 2008. p. 219–56.
- [73] Halstead SB, Mahalingam S, Marovich MA, Ubol S, Mosser DM. Intrinsic antibody-dependent enhancement of microbial infection in macrophages: disease regulation by immune complexes. *Lancet Infect Dis* 2010;10(10):712–22.
- [74] Jessie K, Fong MY, Devi S, Lam SK, Wong KT. Localization of dengue virus in naturally infected human tissues, by immunohistochemistry and in situ hybridization. *J Infect Dis* 2004;189(8):1411–8.
- [75] Balsitis SJ, Coloma J, Castro G, Alava A, Flores D, McKerrow JH, et al. Tropism of dengue virus in mice and humans defined by viral nonstructural protein 3-specific immunostaining. *Am J Trop Med Hyg* 2009;80(3):416–24.
- [76] Dowd KA, Pierson TC. Antibody-mediated neutralization of flaviviruses: a reductionist view. *Virology* 2011;411(2):306–15.
- [77] Rodenhuis-Zybert IA, Wilschut J, Smit JM. Partial maturation: an immune-evasion strategy of dengue virus? *Trends Microbiol* 2011;19(5):248–54.
- [78] Mongkolsapaya J, Dejnirattisai W, Xu XN, Vasanaawathana S, Tangthawornchaikul N, Chairunsri A, et al. Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. *Nat Med* 2003;9(7):921–7.
- [79] Rothman AL. Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms. *Nat Rev Immunol* 2011;11(8):532–43.
- [80] Mongkolsapaya J, Duangchinda T, Dejnirattisai W, Vasanaawathana S, Avirutnan P, Jairungsri A, et al. T cell responses in dengue hemorrhagic fever: are cross-reactive T cells suboptimal? *J Immunol* 2006;176(6):3821–9.
- [81] Pang T, Cardoso MJ, Guzman MG. Of cascades and perfect storms: the immunopathogenesis of dengue haemorrhagic fever-dengue shock syndrome (DHF/DSS). *Immunol Cell Biol* 2007;85(1):43–5.
- [82] Whitehead SS, Durbin AP. Prospects and challenges for dengue virus vaccine development. In: Hanley KA, Weaver SC, editors. *Frontiers in dengue virus research*. Norfolk, UK: Caister Academic Press; 2010. p. 221–37.
- [83] Chang GJ, Kuno G, Purdy DE, Davis BS. Recent advancement in flavivirus vaccine development. *Expert Rev Vaccines* 2004;3(2):199–220.
- [84] Collier BA, Clements DE. Dengue vaccines: progress and challenges. *Curr Opin Immunol* 2011;23(3):391–8.
- [85] Guy B, Barrere B, Malinowski C, Saville M, Teyssou R, Lang J. From research to phase III: preclinical, industrial and clinical development of the Sanofi Pasteur tetravalent dengue vaccine. *Vaccine* 2011;29(42):7229–41.
- [86] Morrison D, Legg TJ, Billings CW, Forrat R, Yoksan S, Lang J. A novel tetravalent dengue vaccine is well tolerated and immunogenic against all 4 serotypes in flavivirus-naïve adults. *J Infect Dis* 2010;201(3):370–7.