Vaccine 30 (2012) 4301–4306

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Review

Flaviviruses and flavivirus vaccines

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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.

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

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doi:10.1016/j.vaccine.2011.09.114

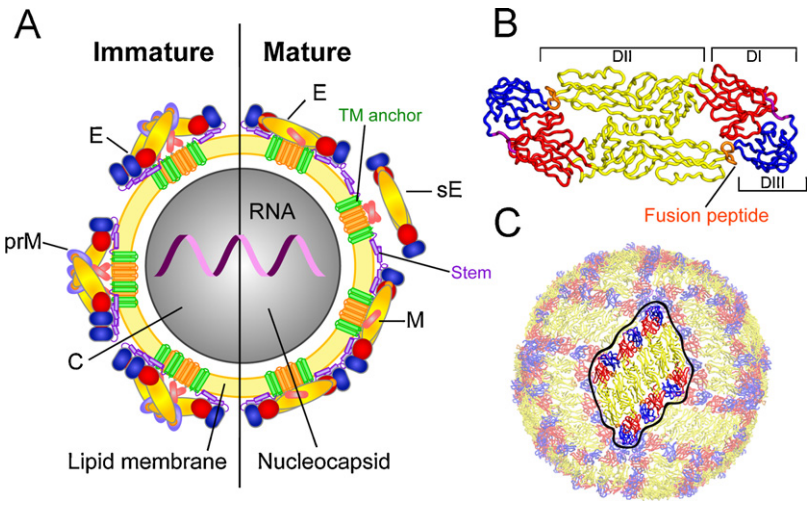

Fig. 1. (A) Schematic model of a flavivirus particle. Left: immature virus, right: mature virus. The spherical capsid contains the viral RNA and multiple copies of the capsid protein C. The surface of immature particles is made up of 60 spikes composed of trimers of heterodimers between the membrane-associated 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 was determined for soluble forms 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 (viperdb 1thd; http://viperdb.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, flaviruses 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