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Review

Domain III peptides from flavivirus envelope protein are useful antigens for serologic diagnosis and targets for immunization

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ABSTRACT

The *Flavivirus* genus of the Flaviviridae family includes 70 enveloped single-stranded-RNA positive-sense viruses transmitted by arthropods. Among these viruses, there are a relevant number of human pathogens including the mosquito-borne dengue virus (DENV), yellow fever virus (YFV), Japanese encephalitis virus (JEV) and West Nile virus (WNV), as well as tick-borne viruses such as tick-borne encephalitis virus (TBEV), Langat virus (LGTV) and Omsk hemorrhagic fever (OHFV). The flavivirus envelope (E) protein is a dominant antigen inducing immunologic responses in infected hosts and eliciting virus-neutralizing antibodies. The domain III (DIII) of E protein contains a panel of important epitopes that are recognized by virus-neutralizing monoclonal antibodies. Peptides of the DIII have been used with promising results as antigens for flavivirus serologic diagnosis and as targets for immunization against these viruses. We review here some important aspects of the molecular structure of the DIII as well as its use as antigens for serologic diagnosis and immunization in animal models.

1. Overview on flavivirus and the significance of the domain III

The Flaviviridae family includes 3 genera: *Flavivirus, Pestivirus* and *Hepacivirus*. *Flavivirus* genera include mostly arboviruses and many of them cause large outbreaks with great impact worldwide. Seventy species of flaviviruses have been described, most of them transmitted to vertebrate hosts by infected mosquitoes or ticks, and 40 of them associated with human disease [1]. Among these flaviviruses, yellow fever virus (YFV), dengue virus (DENV), West Nile virus (WNV), Japanese encephalitis virus (JEV) and tick-borne encephalitis virus (TBEV) are widely distributed in the world resulting in millions of human infections annually, and, probably, a great number of underestimated cases [1].

Flavivirus virions are small, enveloped particles with a diameter of 40–60 nm, and containing a nucleocapsid of approximately 30 nm wrapping one single-stranded linear positive-sense RNA ranging from 9500 to 12,500 nt [1]. The genome contains one open reading frame that encodes a polyprotein which is cleaved by host and viral enzymes, resulting in 10 proteins, three of which are structural and seven which correspond to non-structural proteins. The amino terminus of the genome encodes the structural proteins represented by capsid (C), pre-membrane/membrane (prM/M) and a major envelope glycoprotein (E). Seven non-structural proteins, which are essential for virus replication, are encoded by the remaining portion of the genome [2]. As the major surface protein of flaviviruses, the envelope (E) protein is involved in many events, such as viral attachment, fusion, penetration, hemagglutination, host range and cell tropism. The E protein structure as resolved by X-ray crystallography revealed 3 distinct domains (I to III) (Fig. 1) [3,4]. The domain III (DIII) of the E protein participates in all these events and it is also the major antigenic domain of the whole E protein [5,6].

Based on X-ray crystallographic structural studies, the domain I of E is located in the middle of the protein and contains the N-terminus with glycosylation sites. It is flanked on one side by the elongated dimerization domain II that also contains the fusion peptide at the distal end. The DIII is located on the other side of the E protein (Fig. 1). It is an immunoglobulin-like domain protruding from the otherwise smooth particle surface [4,7]. The DIII is also thought to contain the virus receptor-binding sites [8]. Additionally, the DIII is highly antigenic, consisting primarily of linear epitopes that are recognized by virus-neutralizing monoclonal antibodies [9,10]. Some studies show that this domain is a useful antigen target

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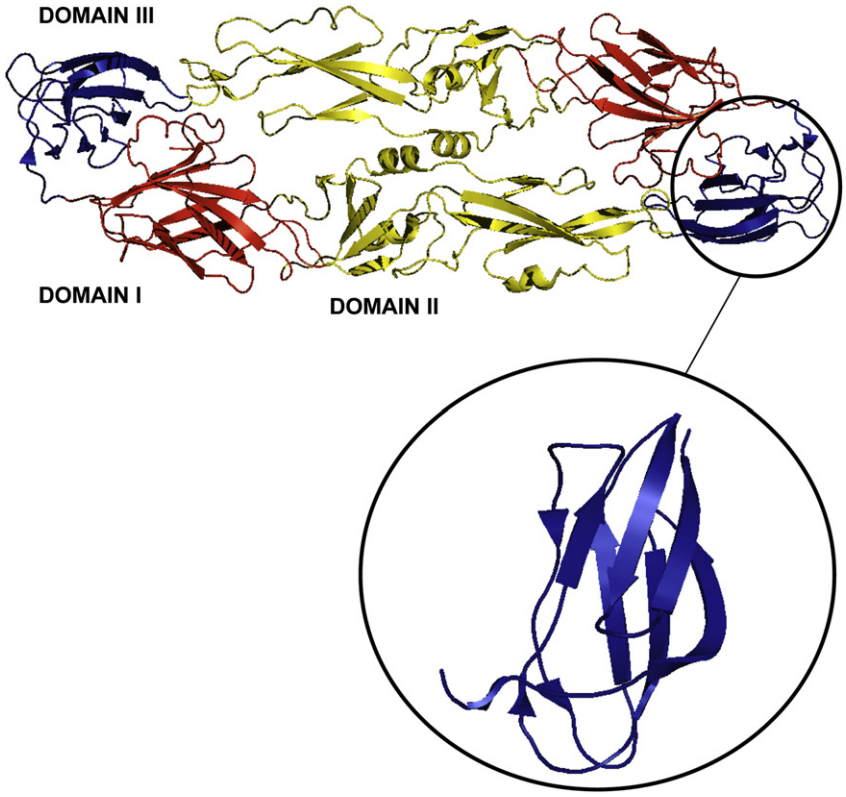

Fig. 1. A stereoscopic drawing of the tertiary structure of the E protein dimer and its domains. The domain I is represented in red, the domain II is represented in yellow and the domain III is represented in blue, with an amplified view. The stereoscopic drawing of the E Protein was constructed based on the structure of DENV-3 (1UZG) using PyMOL program [55]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

for diagnostic assays. It has also been suggested that the DIII could be used as a promising subunit flavivirus vaccine candidate. In this review, we focus on these potential uses of the DIII.

2. Domain III structure

Many flavivirus proteins, including the E protein and the DIII, have been solved by X-ray crystallography and nuclear magnetic resonance (NMR) as shown in Table 1 [4–7,11–23]. The E protein structure from TBEV was first determined by X-ray crystallography showing that it forms a head-to-tail dimer, and each monomer is composed of the three domains, with folding largely based on β-sheets [4]. In the E molecule dimer, the domain I is centrally located in the structure and carries the N-glycosylation sites. The domain II promotes dimerization and bears the fusion loop that inserts into the target host membrane during the pH-dependent virus fusion step. The DIII contains the C-terminal 100 amino acids and forms an Ig-like β-barrel structure composed of 7 anti-parallel β-strands [4]. Further studies on flavivirus E protein DIII were performed with JEV, DENV and WNV showing slight differences in their structures, particularly in areas that constitute virus-neutralizing epitopes [11]. Studies based on monoclonal antibodies showed differences in epitopes involved in neutralization [10,11]. Fig. 2 (A–D) shows the main amino acid residues involved in neutralization of DENV, WNV, JEV and TBEV. Neutralizing epitopes in the DIII identified include residues 306, 331, 333, 337, 360, 373–399, and 387 of JEV [6,24–26]; 384 and 386 of TBEV [27,28]; residues 307, 333–351 and 383–389 of DENV [12,29–31]; and residues 306, 307, 308, 330, 332, 366 and 391 of WNV [13,14]. Mutations in these sites lead to escape from neutralization as previously determined using the monoclonal antibodies mAb E3.3 and mAb E16, both having a high affinity to the DIII peptide of flaviviruses [6,13]. The epitope mapping of JEV by NMR demonstrates that a high affinity binding between mAb E3.3 and residues 302–312, 322–339, 360–372 and 385–392 occurs, since these epitopes are located on the top portion of the β-barrel, the β2–β3,

Table 1
Methods and solved structures of E protein and the Domain III of Flaviviruses.

Structure	Virus	Methodology	Year	Reference
Envelope Protein	TBEV	X-Ray	1995	[4]
Envelope Protein	DENV-2	X-Ray	2003	[7]
Envelope Protein[a]	DENV-2	X-Ray	2004	[15]
Envelope Protein	DENV-2	X-Ray	2004	[5]
Envelope Protein	DENV-3	X-Ray	2005	[12]
Envelope Protein	WNV	NMR	2006	[14]
Envelope Protein	WNV	X-Ray	2006	[13]
Envelope Protein	DENV-1	X-Ray	2009	[16]
Domain III	JEV	NMR	2003	[6]
Domain III	DENV-2	CD IFR	2004	[17]
Domain III	WNV	CD IFR	2004	[17]
Domain III	LGTV	CD IFR	2004	[17]
Domain III	OHFV	CD IFR	2004	[17]
Domain III	WNV	NMR	2004	[11]
Domain III	DENV-3	NMR	2005	[18]
Domain III	OHFV	MNR	2006	[19]
Domain III	LGTV	NMR	2006	[20]
Domain III	DENV-4	NMR	2007	[21]
Domain III	DENV-2	NMR	2008	[22]
Domain III	DENV-1	NMR	2008	[23]

Abbreviations: nuclear magnetic resonance (NMR), circular dicroism (CD), infrared (IFR), tick-borne encephalitis virus (TBEV), dengue virus (DENV), West Nile virus (WNV), Japanese encephalitis virus (JEV), Langat virus (LGTV), Omsk hemorrhagic fever virus (OHFV).

[a] Crystal structure of the E protein interaction with cellular surface.

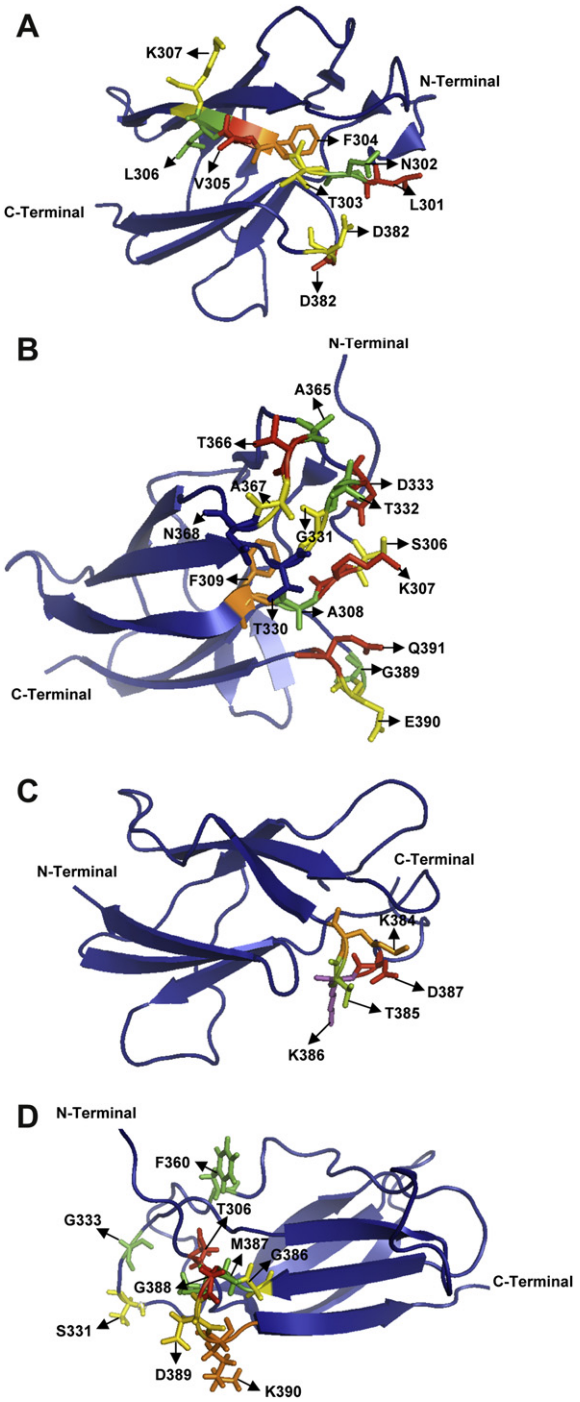

Fig. 2. A stereoscopic drawing of the tertiary structure of domain III of E protein showing amino acids involved in neutralization. A) Residues involved in neutralization of dengue viruses [12,29–31]. B) West Nile virus [13,14]. C) Japanese encephalitis Virus [6,24–26]. D) tick-borne encephalitis virus [27,28], respectively. The domain III drawings were constructed based on the structure of DENV-3 (1UZG), WNV (2P5P), JEV (1PJW), TBEV (1SVB) using PyMOL program [55].

β3–β4 and the β5–β6 loops [6]. The DIII peptides of JEV, DENV-3 and WNV also differ in their amino acid composition as shown in Fig. 3. Alignments of the 100-aa sequences of the DIII among DENV-3, JEV, WNV and TBEV reveal conserved motifs, which contribute to the overall conservation of this region, which can be involved in cross-reactivity of antibodies resulting in neutralization. For example, a unique mAb raised against DENV-2 DIII can cross-react with the all dengue serotypes as well as WNV and promote neutralization in all four DENV serotypes [32]. The DIII peptide forms a β-barrel type structure composed of six anti-parallel β-strands in JEV, ten anti-parallel β-strands in DENV-2, and seven anti-parallel β-strands in WNV [6,11,15]. Those β-strands not present in JEV and WNV-DIII are found near the top of the DENV-2 β-barrel, as β-3 and β-6 strands [11]. The absence of β-3 and β-6 strands in JEV and WNV is due to the lack of dimeric head-to-tail interactions of the DIII with domains I and II since the contact between these two domains may force the loop regions to form more ordered conformations [6,11]. Global folding and secondary structure of JEV, WNV and DENV are similar; however, the protein surfaces presented as antigens or targets for potential therapeutic drugs are significantly different [11]. Amino acids located at the top of the DIII form a pentameric pocket in the virion and the residues near this interface are extremely important. The DIII structures of WNV and JEV present differences in the size, width and amino acid residues involved in the pocket molecular arrangement [11]. The WNV-DIII pocket includes residues Thr332, Ala365, Thr366, and Asn368, while the JEV-DIII pocket includes residues Ser330, Asp331, Pro333, Ser363, Ser364, and Asn366 [11]. Thus, these changes in the structure of the DIII of flavivirus are responsible for different interactions between protein and ligands, such as antibodies, potential drugs and cellular surface proteins [11]. The slight amino acid differences in areas that constitute virus-specific neutralizing epitopes in the DIII may provide the basis for the lack of significant immunological cross-protection between these viruses, even with the cross-reactivity that exists among them [6,11,15].

3. Domain III use as an antigen for serologic diagnosis of flavivirus infections

Serological diagnosis of flavivirus infections is commonly difficult due to the extensive antigenic cross-reactivity among these viruses, especially in regions where two or more of these human pathogenic viruses are endemic. One example is the known antibody cross-reactivity between DENV-3 and Saint Louis Encephalitis virus (SLEV) observed in a study in Brazil [33]. In order to overcome this problem, it has been proposed that recombinant DIII peptides containing virus-specific epitopes could be used for specific serological diagnosis of flavivirus infections [34,35].

Beasley et al. (2004) described the use of a recombinant, bacterially expressed WNV-DIII peptide in an enzyme linked immunosorbent assay (ELISA). In this study, it was possible to clearly differentiate between antibody responses to WNV and those produced by other related flaviviruses, such as SLEV, JEV and Murray Valley encephalitis virus (MVEV). Thus, the WNV-DIII peptide was superior to whole virus antigens in discriminating specific antibody responses to WNV [34]. Another recombinant DIII-based ELISA was reported as able to differentiate tick-borne encephalitis (TBE) serocomplex flaviviruses from mosquito-borne flaviviruses. However, this ELISA could not differentiate mosquito-borne flaviviruses among members of TBE serocomplex, due to high cross-reactivity in antibody responses. On the other hand, the use of DIII recombinant peptides from mosquito-borne flaviviruses in both ELISA and Western blot assays was able to differentiate among viruses in the group [35].

The expression of 20 recombinant polypeptides obtained from the entire genome of DENV-2 was performed in order to establish a diagnostic test and to evaluate serum immune responses. One of the 20 polypeptides, the pD2-3 (E), which was located in the N-terminal portion of the envelope protein, probably corresponding to the DI, was the most reactive when testing sera from dengue patients by Western blot and ELISA [36]. The sensitivity of the ELISA

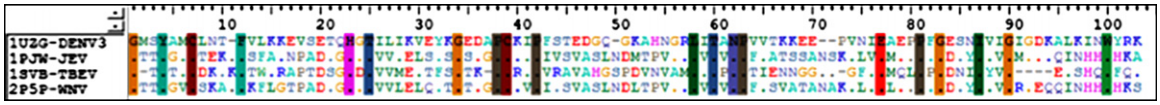

Fig. 3. The amino acid sequences included in the alignment were retrieved from the Protein Data Bank (PDB): DENV-3 (1UZG), JEV (1PJW), TBEV (1SVB) and WNV (2P5P). Alignment was carried out with the CLUSTALW program [56].

using pD2-3 (E) antigen versus mouse brain whole virus antigen was 100% and 79% when testing dengue convalescent and acute phase sera, respectively. The specificity of the test was 100%. Sera from patients infected with different DENV serotypes were also tested showing a clear positive reaction, while positive sera from YFV, rubella and measles patients showed low reactivity. The authors concluded that the use of this recombinant peptide is a useful tool on dengue diagnosis [36]. Thus, not only the DIII, but also other regions of E protein can play useful roles in diagnosis.

In another study whose aim was the development of a diagnostic method for Japanese encephalitis virus, an important arboviral disease in Asia and Polynesia, a recombinant JEV-DIII peptide was obtained after prokaryotic expression and purification using nickel-affinity chromatography. The developed ELISA using JEV-DIII peptide antigen showed 98% sensitivity and 96% specificity when compared to a JE-Dengue Combo ELISA previously in use. The authors concluded that the IgM-ELISA using JEV-DIII peptide antigen is useful for large screening of JEV infections in endemic areas or during outbreaks [37].

Recently, Wahala et al. (2009), have shown that recombinant DIII of DENV is a reliable antigen for ELISA in order to identify the DENV serotype responsible for primary infection [38]. In short, the studies mentioned above show that recombinant DIII peptides are useful and reliable tools for serologic diagnosis of flavivirus infections.

4. Domain III peptide use as a flavivirus vaccine

Since flaviviruses cause a wide variety of diseases, often without available vaccines or antiviral specific drugs, the DIII peptides could be used for therapeutics and as subunit flavivirus vaccines [39–45].

One of the most effective and safe vaccines available for flaviviruses is the yellow fever vaccine using the attenuated virus strain 17D, which was obtained after repeated passages in embryo eggs. The protection mechanism of this vaccine is not completely clear, but it has been shown that the loss of virulence of YFV virus was attributed to mutations in the original virus, mostly in the DIII of the E protein gene [46]. The 17D strain has a DIII that binds more efficiently to glycosaminoglycans (GAGs) of cell surface, reducing the viremia and preventing the viscerotropism of YFV vaccine. This high affinity between virus and GAGs leads to a rapid removal of virus from bloodstream, as a consequence of nonproductive binding of virus to extracellular matrix components, which are rich in GAG, or readsorption of progeny virus to infected cells [46]. In contrast to yellow fever vaccine, other diseases caused by flavivirus do not yet have efficient vaccines available for human use.

The DIII E peptides are potential candidates for flavivirus vaccines, as immunization with these peptides can induce the production of neutralizing antibodies and cellular immune responses as well. For instance, mice immunized with WNV-DIII E soluble peptide showed a predominantly Th1-type cellular immune response, with T lymphocyte proliferation, production of high levels of IFN-γ and IL-2 cytokines as well as higher levels of IgG2a than IgG1 [39]. The antibodies generated by DIII E immunization in mice were able to neutralize 90% of WNV [39] and 50% of DENV-2 in a plaque neutralization assay, at a 1/64 dilution of the antisera [47]. These results also demonstrate that the DIII peptides can induce cross-flavivirus protective effects [48]. Likewise, in vivo protection of WNV infection was observed when the virus was pre-incubated with antisera obtained from mice immunized with a WNV-DIII peptide. Thus, suckling mice inoculated with this incubated solution were protected against WNV infection. The protection was dose-dependent and high concentrations of the antisera resulted in 100% of the animals surviving. The antisera for WNV-DIII peptide were also effective against JEV infection, with 80% of the animals surviving [47]. In the same work, mice challenged with WNV in the absence of WNV-DIII E peptide neutralizing antibodies showed brain tissue damage and virus replication in that tissue, while the viral antigen was not detected in the brain cells of mice in the presence of neutralizing antibodies. Therefore, this work also demonstrates the cross-reactivity of the DIII peptides from different flaviviruses [47]. Another study revealed that mice immunized with JEV-DIII soluble peptides were protected against JEV challenge, with a survival rate of approximately 80% [40,48]. The authors also demonstrated that the use of Freund's adjuvant (FA) and CpG mixed with the DIII peptides was able to enhance the production of neutralizing antibodies by the immunized animals [39,40,43,44]. However, complete FA cannot be accepted as a human vaccine component because it could induce strong hypersensitivity responses and CpG's safety for human use is not well established yet. Alternatively, for use in a human vaccine, the DIII peptides could be linked to cationic liposomes as an adjuvant. Liposomes are known to be safe for human use and when mixed to JEV-DIII and used in mice, induced higher survival rates (80%) after challenge with JEV than when the animals were immunized with JEV-DIII/FA (60%) [48].

Research in flavivirus vaccine development has increased in the last years, with the generation of new tetravalent vaccine candidates for DENV. In this context, it has been shown that recombinant DIII proteins from constructions of consensus sequence of the four DENV serotypes [41], or from in tandem sequences of the DIII of all DENV serotypes, both elicit antibody response in mice against the four serotypes of DENV, as well as T lymphocyte proliferation and IL-4 production [42,43]. Studies were also performed in nonhuman primates in order to test the DIII immunization in an experimental model where antibody responses are qualitatively similar to those of human patients. Thus, monkeys immunized with the DIII peptides of DENV-1 and DENV-2 and challenged with these viruses by subcutaneous inoculation exhibited reduced viremia.

5. Domain III peptide use in therapeutics

The DIII peptides can also represent potential candidates in therapeutics for flavivirus infection. The DIII competes with the virus during entry by preventing viral binding to receptors on the cell surface. Chu et al. (2005) showed that Vero cells pre-treated with a soluble WNV-DIII peptide inhibited more than 60% of the entry of WNV and also 30% of the entry of DENV-2 in the cells. The authors also showed that these virus entry inhibitions were dose-dependent. [47,49].

Another therapeutic approach is the use of monoclonal antibodies to prevent diseases caused by flaviviruses. Sanchez et al. (2005) generated nine different mAbs directed against the E protein of WNV. The majority of the produced mAbs bound to conformation

dependent epitopes in the DIII, while four of the mAbs were able to neutralize WNV and also bound the same region of the DIII with high affinity. In this same study it was observed that some mAbs did not exhibit neutralizing activity and showed cross-reaction with several other flaviviruses, such as Saint Louis encephalitis, Japanese encephalitis, yellow fever and Powassan viruses. Non-neutralizing mAbs bound with different affinities to the DI and DIII. Thus, mAbs obtained with DIII immunization can bind specifically to this domain, and exhibit neutralization, indicating that both affinity and epitope recognition by an antibody are important determinants for virus neutralization [50].

Oliphant et al. (2005) have shown that monoclonal antibodies against WNV-DIII may have therapeutic potential against this virus. Using random mutagenesis and yeast surface display, a panel of monoclonal antibodies was generated. One of these antibodies, E16, neutralized 10 different strains of WNV in vitro and was therapeutically efficient when administered in mice as a single dose 5 days after infection, with a protection greater than 90% against lethal infection and a survival rate of 90%. In this study, E16 was also humanized and retained antigen specificity, avidity and neutralizing activity. A single dose of the humanized version of E16 in post-exposure therapeutic trials in mice protected against WNV-induced mortality [51]. E16 binds to a neutralizing epitope in DIII E of WNV surface and inhibits infection primarily at a step after viral attachment, potentially by blocking envelope glycoprotein conformational changes [52].

Monoclonal antibodies CR4348 and CR4354, isolated from convalescent patients infected with WNV have been reported to bind with high affinity to recombinant DIIIE-WNV and to inhibit virus adsorption in cells. They have also inhibited WNV infection when added after virus adsorption to the cell surface, indicating that at least part of their neutralizing activity was at a post-attachment step of the viral life cycle and they have shown similar neutralizing capacity to humanized E16 [53]. Thus, monoclonal antibodies against WNV-DIII may be a possible treatment option against WNV human infections [51–53]

A recent study showed that the mouse monoclonal antibody 9F12, raised against DENV-2 recombinant DIII E, cross-reacts with the DIII corresponding to other three DENV serotypes and also with WNV, thus preventing the first steps of viral entry. Its virus inhibition activity and cross-reactivity make it a candidate for optimization and humanization into a therapeutic antibody to treat dengue infections [32].

The E3 Fab generated in immunized and challenged monkeys with JEV recognizes DIII E of JEV and presents neutralizing activity against several genotypes of JEV. The mAbs produced from E3 Fab protect mice against lethal challenge with WNV, and use of these mAbs is an alternative to humanized mAbs production for JEV treatment [54]. Thus, based on the results of these studies, monoclonal antibodies against flavivirus DIII may represent important therapeutic approaches for treatment.

6. Concluding remarks

Flavivirus infections are an important public health problem worldwide, especially in North America as a result of the emergence of WNV in 1999. Considering that accurate diagnosis of flavivirus infections and vaccine development is crucial to controlling spread of disease due to these viruses, the use of the DIII peptides as antigens in serologic tests and for immunization could be a very useful tool. However, it is also important to better understand the part that DIII plays in virus attachment as well as on induction of neutralizing antibodies and cellular immune responses against flaviviruses. Another significant aspect of the DIII is that it maintains homology and identity among flaviviruses despite very minor viral species-specific differences, which could explain the lack of antibody cross-protection to infection from most of these viruses [6,11]. Finally, it has been reported that monoclonal antibodies to the DIII peptide can block virus receptor-binding sites, thereby preventing flavivirus infection, as well as neutralize virus (even in mice treated post-exposure), representing promising preventive/therapeutic approaches for human viral infection [50]. In conclusion, the DIII, a key structure in the viral surface E protein, can be used to solve many aspects of flavivirus infection and also to be used for diagnosis and control of diseases by flaviviruses.

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