MECHANISMS OF DENGUE VIRUS NEUTRALIZATION BY ANTIBODY

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ABSTRCT

WAHALA MUDIYANSELAGE PADAMASIRI BANDARA WAHALA:
Mechanisms of dengue virus neutralization by antibody
(under the direction of Aravinda de Silva)

Dengue virus (DENV), the causative agent of dengue, is a group of viruses consisting of four different serotypes designated DENV1-4. Each serotype is further divided into different genotypes. Primary DENV infection induces a life-long type-specific immune response against the homologous DENV serotype. It is widely assumed that all the strains of a DENV serotype are equally neutralized by type-specific antibodies irrespective of the genetic variability within the serotype. Studies with mouse monoclonal antibodies (mAb) have demonstrated that serotype-specific neutralization of DENV is mainly mediated by antibody binding to epitopes on domain III of the viral Envelope protein (EDIII).

Although DENV3 has spread worldwide, neutralization mechanisms of DENV3 is poorly studied. Results of my studies with mouse mAbs demonstrate that type-specific neutralization of DENV3 was confined to EDIII lateral ridge as reported for other flaviviruses. I further demonstrated that DENV3 Envelope (E) protein sequences were variable between different genotypes of DENV3. Variable positions were located on or near the known antibody epitopes on E protein, and natural amino acid variations of DENV3 E protein led to complete or partial escape from antibody neutralization. These results suggest that natural intra-serotype variation should be considered when characterizing natural and vaccine induced immunity.

The specificity and functionality of the human antibody response to DENV is poorly characterized.   It is unknown if humans also develop antibodies to EDIII epitopes recognized by mouse mAbs. Using a panel of sera from people exposed to DENV, I demonstrate that people develop low levels of type-specific EDIII reactive antibodies after primary infection and low levels of serotype cross reactive EDIII antibodies after secondary infection. I further demonstrated that these low levels of EDIII reactive antibodies only make a minor contribution to the total neutralization potency of human immune sera. I conclude that the EDIII epitopes identified using mouse reagents, which have been the focus of much recent work, are not the primary target of human antibodies that neutralize DENV. I believe these results will stimulate investigators to study previously neglected regions of the DENV envelope to identify functionally important epitopes engaged by human antibodies.

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AA   amino acid

Ab   antibody

ADE   antibody dependent enhancement

ATCC   American type culture collection

C   capsid protein

CAT   chloramphenicol acetyl transferase

CBS   critical binding site

CEP   conformational epitope prediction

CO_2 carbon dioxide

CMC   Colombo municipal council, Sri lanka

DE-SIGN   dendritic cell-specific intercellular adhesion molecule-3-grabbing non-

            integrin

DEN   dengue

DENV   dengue virus

DF   dengue fever

DHF   dengue hemorrhagic fever

DSS   dengue shock syndrome

E   envelope protein

EDI   envelope protein domain I

EDII	envelope protein domain II
EDIII	envelope protein domain III
EIP	extrinsic incubation period
ELISA	enzyme-linked immunosorbent assay
FC	fragment, crystallizable region of antibody
FMDV	foot and mouth disease virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HRV	human rhinovirus
HSV	herpes simplex virus
IFN	interferon
IgG	immunoglobulin G
IgM	immunoglobulin M
JEV	Japanese encephalitis virus
kDa	kilo-Dalton
M	membrane protein
mAb	monoclonal antibody
MBP	maltose binding protein
nAb	neutralizing antibody
nm	nanometer
nmAb	neutralizing monoclonal antibody
NS	non-structural protein

OD   optical density

PADRE   pan DR helper T cell epitopes

PAN   post attachment neutralization

PCR   polymerase chain reaction

PFU   plaque forming units

prM   pre-membrane protein

PRNT   plaque reduction neutralization test

rEDIII   recombinant EDIII

RTPCR   reverse transcription–PCR

SU   surface protein

TBEV   tick-borne encephalitis virus

TM   trans-membrane

TNF   tumor necrosis factor

UTR   untranslated region

WNV   West Nile virus

CHAPTER 1

Background and Significance

1.1 Introduction:

Antibodies are a main line of defense against dengue and other flaviviruses. However, the antibody response against dengue virus (DENV) can either be pathogenic or protective [1]. Studies conducted with mouse monoclonal antibodies (mAb) have demonstrated that strongly neutralizing antibodies bind to domain III of the envelope protein (EDIII) [2]. Very little is known about how human antibodies neutralize DENV. Moreover, although it is widely assumed that a neutralizing antibody against one DENV strain is effective against all strains within a DENV serotype, there is little experimental evidence to support this assumption. This thesis describes studies to characterize the binding and functionality of human polyclonal and mouse mAbs to DENVs. Using DENV type 3 as a model, studies were conducted to determine if strain variation has an impact on DENV-antibody interactions.

The following sections briefly introduce dengue (DEN) disease, the responsible viruses, the host immune response, general mechanisms of virus neutralization by antibody and, more specifically, current state of knowledge about antibody binding and neutralization of flaviviruses, including dengue. Following this background, I will introduce the main questions to be addressed in this thesis.

1.2 Dengue disease, dengue virus, its transmission and global distribution:

Dengue is a re-emerging mosquito-borne viral disease of the tropics and subtropics caused by a group of viruses designated DENVs. Although, the disease was first described in 1779, DENV was first isolated from infected patients in the mid 19th century [3-5]. DENVs are transmitted by *Aedes* mosquitoes. Two distinct DENV transmission cycles

namely sylvatic cycle and endemic cycle occur. In the sylvatic or enzootic cycle, DENVs are transmitted between non-human primates and arboreal *Aedes* spp. However, in the urban or endemic cycle, DENVs, which are thought to have evolved from sylvatic DENVs over two hundred years ago, are transmitted between humans and *Aedes aegypt*i and *Aedes albopictus* mosquitoes [6]. The human is the primary host in the urban cycle and human infection is essential for the survival of the virus in the urban setting [7]. It is estimated that 50 million dengue infections occur annually in about 100 countries worldwide and approximately 500,000 (1%) require hospitalizations with a death rate of ~ 2.5 % (12,000 of 500,000) [8]. Currently no antiviral drugs or vaccines are available to control the disease.

1.3 Classification and the structure of DENV:

DENV is a member of the genus Flavivirus in the family Flaviviridae. The genus flavivirus is further divided into different groups or sero-complexes which include many pathogenic viruses that cause a variety of diseases ranging from fever to encephalitis and hemorrhagic fevers. Some of the flaviviruses of global heath concern are DENV, Japanese encephalitis virus (JEV), West Nile virus (WNV), yellow fever virus (YFV), Murray Valley encephalitis virus (MVEV), St. Louis encephalitis virus (SLEV), and tick-borne encephalitis virus (TBEV) [9]. The DENV group consists of four closely related, but distinct virus species which have traditionally been called serotypes, namely DENV serotype 1 through DENV serotype 4 [10]. Each DENV serotype can be further divided into different genotypes [11]. For instance, DENV serotype 3 consists of four different genotypes namely DENV3-genotype I through DENV3-genotype IV [12].

DENV is an enveloped virus with a diameter of approximately 50 nm [13]. The

genome of DENV, which is approximately 10.7 kb in size, is a positive sense single stranded

RNA with a single open reading frame [9]. Furthermore, the genome is flanked by

untranslated regions (UTR) at both the 3' and the 5' ends, which are important for initiation

and regulation of protein translation, genome replication and virus assembly (figure 1.1)

[9,14,15]. The genome encodes three structural proteins (capsid, envelope, pre-

membrane/membrane) and 7 non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A,

NS4B, NS5) which are translated as a polyprotein and co- and post-translationally cleaved by

signal peptidases, the viral serine protease, and additional cellular proteases (figure 1.1) [9].

The nucleocapsid, which is made of multiple copies of capsid (C) protein that associates with

the ssRNA genome, is surrounded by an envelope [9]. The envelope of the mature virion,

which arranges into an icosahedral symmetry, is primarily made of two structural proteins;

envelope glycoprotein (E protein) and the membrane (M) protein (figure 1.1 and 1.2) [9]. Of

the 7 non-structural (NS) proteins, NS5 is the RNA-dependent RNA polymerase (RdRp) and

methyltransferase whereas NS3 functions as serine protease, helicase and nucleoside

triphosphatase [9,14]. In addition, NS2B is required as a cofactor for the NS3 serine

protease. The known functions of NS proteins are summarized in the figure1.1, although the

functions of some of the NS proteins have yet to be determined.

1.4 Replication and life cycle of DENV:

The primary targets of the DENV are the cells of mononuclear phagocyte lineage

including monocytes, macrophages, and dendritic cells (DCs). However, B cells, T cells,

endothelial cells, hepatocytes, and neuronal cells, are also permissive to DENV [14].

Although, the cellular receptor, which mediates the entry of DENV, is not known, putative attachment receptors such as dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN or CD209), mannose Receptor (MR), heparan sulfate (HS), heat shock proteins (HSP) 70 and 90, and CD14 linked molecule have been studied to date [9,14,16]. The entry of DENV to permissive cells occurs through receptor-mediated endocytosis [9]. Conformational rearrangement of the E protein, which occurs in the acidic endosome, induces the fusion process, which in turn releases the nucleocapsid into the cytoplasm of the cells. Translation and replication of DENV occurs in association with intracellular membranous structures [9]. The new immature DENV particles, which bud into the lumen of the ER, consist of trimeric spikes of pre-membrane (prM) and E protein heterodimers (figure 1.3)[9]. The prM protein is cleaved by furin and the E protein is rearranged into dimers in an icosahedral symmetry when the immature DENV particles egress through the transgolgi network. Consequently, the mature DENV is released by exocytosis (figure 1.3) [9]. However, cell culture-derived DENV, as well as other flaviviruses, is a mixture of mature and immature virus particles, and some immature virus particles consist of partially mature and immature E protein on the virion envelope [17].

1.5 Dengue: the disease and pathogenesis.

All four serotypes of DENV often cocirculate in DEN endemic areas [8]. Therefore, heterologous, sequential secondary DENV infections are common [18]. The spectrum of disease in both primary and secondary infection is quite diverse ranging from asymptomatic or mild undifferentiated fever, to classic dengue fever (DF), to more severe disease such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [18-20]. In children

most of the DENV infections remain asymptomatic and only 3-5% of infected children develop DHF/DSS [18]. The pathogenesis of DHF/DSS is not well understood mainly due to the lack of suitable animal models [21]. Nevertheless, secondary DENV infection is a major risk factor for developing DHF/DSS [1]. Antibody-dependent enhancement (ADE) model has been proposed to explain the increased risk of DHF/DSS in secondary DENV infection [1,22]. According to the ADE model, cross-reactive, weakly neutralizing or non-neutralizing antibodies from a previous DENV infection interact with a heterologous DENV strain without neutralizing it, thus facilitating the uptake of the virus by Fcγ receptor-bearing cells such as monocytes/macrophages and increasing the viral burden in the host [1]. Although ADE induces caspase-dependent apoptosis and the production of inflammatory cytokines and chemokines that modulate vascular permeability, the molecular mechanism underpinning ADE-driven DHF/DSS is not yet well characterized [23]. In addition, a secondary infection is also believed to induce the expansion of low affinity, cross-reactive memory T cells, which are generated upon the primary DENV infection. These cross-reactive memory T cells, which are incapable of eliminating the heterologous DENV virus, induce over production of pro-inflammatory cytokines such as IFNγ and TNFα that increase vascular permeability leading to DHF/DSS [24].

In addition, some DENV antibodies, that cross-react with endothelial cells, blood clotting factors and platelets, are additional mediators believed to induce DHF/DSS [25,26]. Host genetic factors such as human leukocyte antigen (HLA) type, polymorphism in the genes of TNFα, CD209, Gluose 6 phosphate dehydrogenase (G6PD), FCγ receptor, receptor for vitamin D, as well as age, race and nutrient status of the host are also important factors that can contribute to DHF/DSS [27]. In addition, some DENV strains are more virulent than

the others and may contribute to severe disease [11]. The virulence of such strains has been attributed to the prM, E, NS4b, NS5 proteins and 3'and 5' UTR of the DENV genome [28].

1.6 Protective host immune response against DENV infection:

The innate immune response, especially IFNα/β and IFNγ, likely protects from DENV infection [29]. However, it has been demonstrated that DENV (and other flaviviruses), use a variety of mechanisms to evade the IFN-mediated antiviral response [30,31]. In addition, DENV-specific CD4 and CD8 cells are cytolytic against DENV-infected cells [32,33]. In contrast, cross-reactive T cells are believed to be pathogenic particularly in heterologous secondary DENV infection [32].

Humoral immunity is a key factor in controlling DENV infection in the host. Studies conducted with humans and experimental animals have demonstrated that passively transferred polyclonal sera and monoclonal Abs protect from DENV infection [3,34,35]. In a typical natural DENV infection, viremia peaks around day 4 after the onset of the disease and declines when the initial Immunoglobulin M (IgM) antibody response develops. The IgG response appears 6-7 days after the onset of fever, and although IgM is detectable in patient sera for only a few months post-infection, IgG titers can remain in the patients' sera throughout their lifetime [36]. After a primary infection with one serotype of DENV, a short period of broad cross protection against multiple serotypes is observed. People subsequently develop a serotype-specific, long-term neutralizing antibody response against the serotype responsible for the primary infection only [3,36]. Primary DENV infection also elicits cross reactive, weakly neutralizing and non-neutralizing antibodies. Instead of neutralizing, these antibodies may enhance secondary infection with a heterologous DENV [1].

Many studies have focused on understanding the mechanisms of neutralization of flaviviruses using monoclonal antibodies. Although, most of these studies identified neutralizing mAb binding epitopes and their mechanisms, very little is known about the neutralizing antibody response and the mechanisms of neutralization of virus by human immune polyclonal sera.

1.7 Mechanisms and theories of virus neutralization by antibody

The humoral immune response plays a major role in protecting from flavivirus infection including DENV. Therefore, understanding the mechanism of virus neutralization by antibodies is pivotal toward developing better vaccine candidates and understanding the characteristics of different epitopes of the enhancing Abs that induce ADE. The mechanisms of virus neutralization are usually studied in vitro by using monoclonal antibodies (mAb). Such studies have shown that viral neutralization can be accomplished at different stages of the virus life cycle. In the following section, the known stages of the virus life cycle targeted by neutralizing antibodies are described in detail.

Aggregation and disintegration of virus particles

The simplest mechanism of neutralization occurs when Abs neutralize viruses by cross-linking and aggregating viral particles. The aggregation of viral particles by neutralizing antibodies has been studied in detail using poliovirus with mAb 35-1f4 [37,38]. These studies demonstrated that while the virions in the aggregates were still infectious, aggregation reduced the infectivity of the virus proportionately to the number of virions in an aggregate. When the aggregates were disintegrated, individual virions regained infectivity

[37-39]. However, it was further demonstrated that aggregation only occurred when the antibodies interacted with the viral particle monovalaently, allowing both Fab arms to bind to different virions. However, aggregation was dependent upon a concentration phenomenon, in that aggregation did not occur in the presence of high IgG concentration when epitopes were saturated [38].

Neutralization of some viruses can be obtained by destabilizing the virus particle. Poliovirus and foot and mouth disease virus (FMDV) are disintegrated and become noninfectious after binding to certain antibodies. As mentioned earlier, poliovirus can be reversibly neutralized by the mAb 35-1f4 by aggregating the virus particles under normal physiological conditions. However, under different physiological conditions, the same antibody neutralizes the poliovirus in vitro by dismantling the virus structure. At physiological ionic conditions and 39C, or low ionic conditions and 37C, mAb 35-1f4 changes the structure of poliovirus irreversibly and neutralizes by releasing RNA prematurely from the virion [40,41]. A similar destruction of poliovirus at 39C at physiological ionic strength occurs with polyclonal immune sera as well [41]. Neutralization of FMDV by mAb 4C9 is also mediated by structural changes in the capsid protein, which induces the release of viral RNA. However, unlike poliovirus, structural changes of FMDV happen under normal physiological conditions and temperatures [42]. Although the exact mechanisms of such conformational changes are not known, it is likely that Ab binding mimics the virus-receptor attachment and thus induces the conformational changes leading to premature uncoating of the virus

Block receptor attachment

Attachment of a virus to its cellular receptor or group of receptors is essential for successful infection. Receptor interactions may be cell-type specific for one virus although the same virus may use different receptors on different cell types. Moreover, some viruses use more than one receptor. The primary receptor is used as the attachment factor which brings the virus closer to the co- or ancillary receptor, whose attachment induces the fusion or internalization and fusion of the virus. It has been shown that antibody blocking the receptor attachment of virus is a major mechanism of virus neutralization [43]. In this mechanism of neutralization, antibodies inhibit the receptor - virus interaction by binding to the specific receptor binding domain of the viral protein and thus occupying the interacting sites of the receptors. Neutralization of DENV in Vero cells, and human immunodeficiency virus-type1 (HIV-I) in T cell lines is mediated in this fashion [44,45]. Furthermore, neutralization of HIV-1 by human monoclonal antibody F105 occurs by inhibiting the attachment of the cellular receptor, CD4, to the viral glycoprotein 120 (gp120). The epitope that mAb F105 binds is located on the V3 loop of gp120 and partially overlaps with the CD4 binding site [46,47]. However, receptor attachment can also be blocked by an Ab without directly engaging the receptor binding motif but by occluding the binding area. A classic example for such a mechanism of neutralization occurs in neutralization of human rhinovirus 14 (HRV-14) by mAb 17IA [48]. The attachment domain for the intercellular adhesion molecule-I (ICAM-I) cellular receptor is located in the canyon of the capsid of HRV14 and inaccessible to mAb17IA. The structural and mutational studies conducted by Smith et al have revealed that Fab fragment of mAb 17IA binds to an immunogenic site over the canyon

area [48]. Thus the antibody binds bivalently across the two fold axis of the protein masking the canyon and subsequently blocking receptor attachment [48].

The conformational rearrangement of the viral protein, which exposes the co-receptor binding sites upon binding to the primary receptor, also plays a role in virus neutralization by Abs that target the co-receptor attachment. [49]. Viruses such as HIV-1, herpes simplex virus (HSV), and FMDV use a primary receptor to bind to the cell and co-receptors for completing fusion with cellular membrane. Once the primary receptor, CD4, binds to gp120 of HIV-1, co-receptor binding sites on gp120 are exposed. Antibodies 17b and 48d bind to these co-receptor binding sites, which are termed as CD4-induced gp120 epitopes [49]. These antibodies can neutralize HIV-1 only after the virus binds to its primary CD4 receptor. In addition, some HIV-1- neutralizing antibodies can bind to the gp120-CD4 binding site as well as the gp120-co-receptor binding sites [49].

Neutralization of virus at a post attachment step:

Another important step in the viral life cycle that is often targeted by neutralizing Ab (nAb) is the entry of the virus into the cells. Since this step is preceded by the attachment of the virus to the receptor and/or co-receptor, the step is frequently designated post-attachment neutralization (PAN). PAN is usually differentiated from standard neutralization (STAN) in vitro by investigating the neutralization potential of an antibody after the virus has attached to the cells. PAN has been documented with many viruses including HIV-1, influenza A virus, poliovirus, respiratory syncytial virus, Venezuelan equine encephalitis virus (VEEV), rabies virus, enterovirus 71 , rotavirus , Newcastle disease virus, transmissible gastroenteritis virus, vesicular stomatitis virus, adenovirus, human cytomegalovirus, African swine fever

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virus and various flaviviruses. (cited in [50,51]). One well studied mechanism of PAN is inhibition of the viral-cell membrane fusion. Influenza, HIV-1 and flavivirus fusion proteins have been well studied, and these proteins are present on the native virus particle in a metastable pre-fusion stage [52]. The pre-fusion proteins undergo conformational rearrangements, which are induced by acidic pH (for influenza and flavivirus) or attachment to the primary receptor and co-receptor (for HIV-1), to become fusogenic [52]. Therefore, binding of an antibody to the envelope protein may block the fusion by interfering with attachment of the fusion receptor, by obstructing the juxtaposition of the cellular membrane and viral membrane, or by stabilizing the viral fusion protein in the metastable pre-fusion stage. As mentioned earlier, mAb 17b blocks the binding of co-receptor to gp120 of HIV and consequently blocks the fusion of HIV particles to the T cell membrane. Monoclonal Ab E16 of WNV and human mAb CR6261 of influenza virus, which recognize epitopes on the E protein and the hemagglutinin (HA) protein respectively, block the conformational rearrangement of the protein thus impeding membrane fusion [51,53,54]. Moreover, hepatitis C virus (HCV) is neutralized by mAb as well as human IgG purified from HCV immune sera by blocking viral fusion with the cell membrane [55].

Even though inhibition of receptor binding and fusion are the main attachment and entry steps targeted by many antibodies that neutralize viruses, some antibodies inhibit post-entry events to neutralize viruses. It has been proposed that some Abs that bind to the CD4 receptor inhibit the reverse transcription of HIV-1 thereby preventing viral propagation [56]. Similar mechanisms have been reported with influenza virus neutralization, as well [57,58]. In addition, inhibition of virus budding has also been described as another mechanism used by Abs to neutralize viruses. For instance, the mAb 14C2 has been shown to bind to the M2

protein on the surface of influenza virus-infected cells, restricting the release of the virus [59].

1.8 Models of Virus neutralization.

Irrespective of the replicative stage of the virus life cycle targeted by antibodies, several models have been proposed to describe the possible mechanisms whereby antibodies neutralize viruses. All models proposed to date are based on the results of in vitro neutralization assays conducted using monoclonal antibodies. The rule of thumb for these neutralization assays is that the Abs need to be in molar excess over the antigen (Ag) used and therefore the amount of antigen-bound Abs is negligible compared to the amount of free Abs in the solution throughout the assay [60,61].

The most discussed physical aspects of neutralization are kinetics of binding and molarity of antibody which have been used to describe different models of the neutralization. Studies conducted by Dulbecco et al, showed a linear relationship between the rate of neutralization and the concentration of an antibody, and these authors proposed that binding of a single antibody molecule to one virion particle can successfully neutralize the virus [62]. This model is widely known as "single-hit phenomenon" or the "single-hit model", and it obeys the rule of first order kinetics. However, the single-hit phenomenon has proven to be of the "pseudo first order kinetics" since the Abs were in molar excess in the neutralization reaction, and therefore this model is largely refuted today [60,63].

On the other hand, "multi-hit phenomenon" which describes the requirement of more than one Ab molecule to neutralize a virus particle, is widely accepted today [61,64]. Neutralization of a virus by multi-hit phenomenon is mediated by multiple Ab molecules that

bind and occupy the epitopes of surface proteins (SUs) on the virion particle [64]. The multi-hit model is widely known as the occupancy model of virus neutralization [61]. Furthermore, this model is strengthened by recent work conducted with HIV and WNV [65-68]. The molecularity of neutralization, which is the number of hits required per virion or the stoichiometry, can be calculated if the number of available epitopes on the virion particle for the particular Ab is known. However, the experiments need to be carefully designed in such a way that neutralization and binding of the Ab are measured under the same experimental conditions [60,69]. In addition, the following assumptions are necessary to satisfy the biological requirements of this model: 1) The neutralizing Abs bind to infectious and non-infectious particles equally well, and 2) each virus particle contains the same number of epitopes [60]. The binding of antibody can also be expressed as the relative occupancy which is the percentage of Ab-bound epitopes compared to the total number of available or exposed epitopes on the SUs. It is proposed that a minimal number of free SUs on the virion is required to achieve a successful infection [60,64]. Neutralization starts at a threshold of occupancy whereby antibodies occupy the SUs in such a way as to maintain the number of unoccupied SUs below minimum number of SUs required for the infectivity [60,64]. According to the assumption of the occupancy model, it is obvious that the stoichiometry and the relative occupancy of antibodies on a virion particle depends on the density of SUs, the spacing of the epitopes, mobility of the SUs, and accessibility of the epitope with regard to the properties of the SUs [64]. In addition, affinity and isotype of the antibody are important properties of an antibody, which affect the relative occupancy [69,70].

Nonetheless, the "critical binding site concept" (CBS concept) has also been proposed by Dimmock to explain the mechanism of virus neutralization [71]. According to this

concept, certain binding sites are involved in neutralization and therefore simply coating or occupying many SUs of the virion is not sufficient to neutralize the virus if the critical sites are not occupied by the antibodies. According to the CBS concept, the single-hit model is described such that all the available epitopes are critical sites, and therefore binding of a single Ab molecule to any of these sites can induce structural changes and inhibit viral replication. The CBS concept can also be used to explain the multi-hit phenomenon, as well. Even though antibodies occupy more than one SU, not all are considered as critical sites for neutralization. However, the evidence from many biochemical and structural experiments with different viruses strongly supports the occupancy model [65-68].

1.9 Neutralization of Flaviviruses by antibodies

The envelope (E) and M proteins are flavivirus structural proteins found on the surface of mature flavivius particles. E protein is the major envelope protein (~ 55 kDa) that mediates receptor attachment and membrane fusion [9]. Most neutralizing monoclonal antibodies (nmAb) against flaviviruses described to date recognize epitopes on the E protein [72]. However, some nmAb have been reported to bind to epitopes on the prM/M protein of the flavivirus, although such antibodies are not common [72-74]. In addition, anti-NS1 antibodies are important in protection as well as in disease pathogenicity, even though NS1 protein is not surface exposed [75-80]. Interestingly, NS1 protein has been detected on the surface of infected cells, and anti-NS1 antibodies have been shown to be cytolytic in some cases [76]. Nevertheless, E protein is considered as the major immunodominant protein of the flaviviruses since most neutralizing antibodies studied so far are E protein reactive [72].

<u>Structure of the E protein</u>:

The crystal structures of E protein of TBE, DENV and WNV have been resolved recently, and these structures have advanced our understanding of the involvement of E protein in the life cycle of flaviviruses and how antibodies against this protein lead to viral neutralization [81-84]. In addition, the crystal structure of the post-fusion conformation of E protein has also been resolved [85,86]. Cryo-electron density mapping of mature and immature virion particles and the crystal structure of E protein have enabled researchers to model the E protein superstructure arrangement on the surface of mature and immature virion particles [82,87,88]. One hundred and eighty E protein monomers are arranged into dimers in an antiparallel orientation, whereby the dimers lay flat on the surface of the virion to form a T=3 pseudo icosahedral symmetry in the mature virion (figure 1.2) [13]. Each monomer of E protein is about 495 amino acids (AA) in length, and is anchored to the viral lipid membrane by two transmembrane (TM) helices (figure 1.4) [81-84,89]. The ectodomain of the E protein, which lies flat on the surface of the virion, is connected to the TM domain by a stem region that contains two amphipathic helices. The amphipathic helices of the stem region also lie flat on the viral membrane but are partially buried in the outer leaflet of the lipid bilayer (figure 1.4) [89]. The helices in the stem region participate in the conformational change of E protein during the fusion process. The M protein is also comprised of a partially buried amphipathic helix and two trans-membrane helices which are closely associated with TM helices of the E protein. These helices serve to stabilize the E protein in mature virion particles, as well as the E-prM heterodimers in immature virions [89].

The flavivirus E protein, which is categorized as a class II fusion protein, differs from the class I fusion protein of HIV-1 and influenza virus in its structural arrangement [52].

Class I fusion proteins are arranged perpendicular to the virion surface where they are lightly pack in the particle, whereas class II fusion proteins are arranged in parallel as dimers that pack very tightly in the virion [52]. Structural studies identified three different domains in the flavivirus E protein monomer, and these are designated domain I (EDI), domain II (EDII) and domain III(EDIII) (figure 1.4)[81,90]. Twelve highly conserved cysteine residues stabilize the structure of the E protein through six disulfide bonds, and therefore all flavivirus E proteins fold into a similar 3 domain structure [81,90]. All three domains of the E protein consist primarily of β strands except two small α helices in the EDII compared to class I fusion proteins which are mainly made of α helices. EDII is the dimerization domain and therefore E monomers are not contacted through the entire E monomer to make an E dimer [81,90]. Furthermore, EDII contains a hydrophobic fusion peptide at its distal end, which is buried at the dimer interface due to the contact of EDIII from a paired E monomer.[81,91]. EDI lies in the middle of the monomer connecting both EDII and EDIII through flexible hinges. EDIII, which is connected to EDI by a stretch of 15 amino acids, is an immunoglobulin (Ig) like domain and contains a putative receptor binding domain (figure 1.5)[92]. It is important to note that the β barrel of EDIII is orientated perpendicular to the lipid layer, whereas β barrels of EDI and EDII are parallel to the surface (figure 1.4) [81]. This orientation allows the EDIII to protrude out from the surface and therefore be more solvent exposed compared to EDI and EDII. EDIII consists of anti-parallel β strands and loops connecting each of these β strands (figure 1.5) [81]. The highly exposed lateral surface of EDIII is collectively made of 3 loops, with the loop connecting EDIII with EDI being designated as the N-terminal linker region of EDIII (figure 1.5) [81,90].

The flavivirus E proteins are glycosylated at Asn-153 with an additional glycosylation site present at Asn-67 for DENV. Furthermore, glycosylation at Asn-153 masks the fusion peptide in the mature virion (figure 1.4)[81]. Although earlier studies demonstrated a differential selection of glycosylation sites among different serotypes of DENV, recent work from Hacker et al. indicates that both sites are used by all serotypes of DENV [93,94].

Conformational changes of E protein

Flavivirus E proteins undergo major conformational rearrangements from E dimer to E trimer before fusion with cellular membrane in the acidic endosome [95]. EDI and EDII swing outward keeping EDIII and the stem region preserved in the nearly identical pre-fusion orientation. Later, EDIII is rotated ~20^0 and the stem region helices are zipped up, driving the trimeric E protein into to its fusogenic orientation allowing fusion of the viral membrane with the cell membrane [95]. The exogenous addition of recombinant EDIII (rEDIII) blocks the fusion of the E protein indicating the important role of EDIII for the rearrangement [96]. In addition, it has been proposed that protonation of histidine residues at the EDI/III interface may initiate the conformational rearrangement or trimerization process [97,98]}. Therefore, it is quite possible that antibodies that bind to the hinge regions between domains of monomeric E protein might block the structural rearrangement and thereby neutralize the virus at the post attachment step.

The primary target of neutralizing antibodies is the E protein, and neutralization of flavivirus is primarily accomplished by either blocking virus-cellular receptor interaction or blocking fusion of the viral-cellular membranes [72]. The epitopes of known neutralizing Abs are distributed throughout the E protein and located in distinguishable clusters (figure 1.6) [54,72,99-103]. Binding specificities and neutralization capacities of these antibodies are varied, and therefore Abs have been grouped into type, sub-complex, complex, sub-group and group-specific categories [72]. Most strongly neutralizing antibodies are type-specific, and interestingly, many of the strongly neutralizing mAb bind to the lateral ridge of EDIII [72,100,103-105]. On the other hand, most of the EDI and EDII binding antibodies are cross-reactive antibodies that weakly neutralize the virus, compared to EDIII reactive Abs. Therefore, most of EDI/EDII reactive, weakly neutralizing Abs have a potential to induce ADE in vitro [72,99,106]. Even though early studies indicated that EDIII antibodies neutralize virus by blocking receptor attachment, recent studies conducted with WNV clearly showed that EDIII elicits Abs that also block the fusion or post attachment steps [107]. Monoclonal antibody E16, is a strongly neutralizing type-specific Ab against WNV EDIII, which blocks the rearrangement of the E protein in the endosome and consequently blocks the fusion step [107]. These findings lead to generalizations such as: 1) E protein is the major protein which elicits neutralizing Ab (nAb); 2) E reactive antibodies can either be type specific or cross reactive; 3) type-specific Abs strongly neutralize the virus as compared to cross-reactive antibodies; 4) the potent, type-specific neutralizing Abs are EDIII-reactive whereas EDI and EDII Abs are mostly cross reactive and weakly neutralizing [72,103,106].

<u>Occupancy and flavivirus neutralization</u>.

The average number of Ab molecules required for neutralization has been calculated for many viruses [108] and the number required increases with the size of the virion, ranging from as low as 4 mAb molecules for polioviruses and up to 225 molecules required for neutralizing rabies virus [108]. The neutralization of flavivirus is a multi-hit phenomenon, requiring more than one Ab molecule per virion for neutralization [2,68,109]. A molecular docking study of the EDIII reactive, strongly neutralizing anti-WNV mAb, E16 revealed that this Ab did not bind to all of the E protein monomers in the mature virion [110]. Instead, epitopes on 120 E monomers were found to be occupied by the E16 at saturation. Thirty Ab molecules are required for neutralization of WNV, demonstrating that blocking approximately 25% of the available 120 E monomers is sufficient to neutralize the virus [68]. Thus, an antibody that binds to a flavivirus exceeding the threshold value of 30 Ab molecules per virion is capable of neutralizing the virus. On the other hand, it is proposed that if the maximum number of Ab molecules bound to the virus is below the threshold value, such antibodies are unable to neutralize flaviviruses, but may enhance the infection [68]. The epitopes on the E protein are not exposed to an equivalent environment on the 3 different axes (2-fold, 3-fold and 5-fold axes) of the pseudo-icosahedral symmetry of flavivirus envelope protein [13]. Therefore, if the epitope of an antibody is not highly accessible or cryptic, even at the maximum occupancy of the available epitopes, such antibody may not be able to neutralize the virus [68,109]. Furthermore, dynamic movements of the E protein on the virion particles may also be an important factor that determines the accessibility of cryptic epitopes and consequently, the stoichiometry and neutralization potential of antibodies that bind to cryptic epitopes [111].

<u>Type-specific neutralization, known epitopes and their role against DENV infection</u>

Infection of DENV elicits type-specific, life-long immunity against the homologous virus, but not against the heterologous DENVs. Thus, an individual who has recovered from a primary DENV infection against one DENV serotype is still susceptible to sequential secondary infections from other DENV serotypes [3]. After a primary infection of DENV, the antibodies elicited are mainly against the E protein even though anti prM/M Abs are also found [72]. In addition, the presence of Abs against C protein and NS proteins 1, 3 and 5 have also been reported particularly in secondary DENV infection [112]. Although, anti-NS1 antibodies are not neutralizing, such Abs are protective through antibody-dependent cytotoxicity and complement-mediated clearance [113]. It is proposed that the cross-reactive, non-neutralizing or weakly neutralizing Abs increase disease severity through ADE, especially in a secondary DENV infection [1], Furthermore, it has been reported that DENV immune human polyclonal sera are mainly comprised of antibodies that target the fusion loop, which is known to harbor weakly neutralizing mouse mAb epitopes. However, this conclusion was made using western blots with the sera whereby some antibodies that target conformational epitopes on other domains of the E protein may not have been detected [114]. Thus, the mechanism of neutralization and, the epitopes involved in both type-specific neutralizing and enhancing Abs in human immune sera, are poorly understood.

DENV2 and DENV3 are the serotypes that have been isolated most often from severe disease epidemics around the world, although all four serotypes of DENV can cause severe disease [19,115-117]. However, the neutralization mechanisms of DENV have been mainly studied with DENV2, whereas the neutralization of DENV3 is poorly understood. Anti-DENV2 mouse mAbs have been well characterized [72,104,118,119]. Most of the epitopes

recognized by these mouse mAb are located on all three domains of E protein [72]. As already mentioned, the most potent neutralizing mAbs bind to the lateral ridge of EDIII, whereas weakly neutralizing and non-neutralizing antibodies mainly target EDII [72]. However, poorly neutralizing, DENV cross-reactive antibodies have also been mapped onto the AB loop of EDIII of DENV2 [118]. Furthermore, EDIII also harbors the epitopes of DENV sub-complex Abs, which neutralize more than one DENV serotypes but not all 4 serotypes, and this epitope is found on the A sheet of EDIII [118,119]. In addition, a recent study conducted by Rajamanonmani and colleagues reported that mouse mAb 9F12, which binds to residues mainly located on the A sheet and the BC loop of DENV2 EDIII cross-neutralizes all four serotypes of DENV [102]. However, the complete epitopes of these Abs have not been identified, and therefore more structural studies with such Abs are necessary to identify the neutralizing epitope on EDIII in addition to the type-specific lateral ridge epitope. In contrast, relatively few EDI-reactive antibodies against DENV have been isolated and characterized [120,121]. Recently a chimpanzee mAb 5H2, which neutralizes DENV4 type specifically, has been mapped on EDI at residues 174 and 176 [34]. Furthermore, passive transfer of 5H2 protected monkeys from DENV infection. However, neutralization escape mutant viruses with a mutation at AA residue 174 were also isolated from monkeys who received 5H2 [34]. Interestingly, genotype III of DENV4 strains isolated from DHF patients naturally contained the same AA substitution at 174 of the E protein [34]. This finding may suggest that humans also elicit neutralizing Abs which target a similar epitope as that of 5H2, and strain variability within DENV4 may interfere with neutralization potential of such Abs. Therefore, strain variability may play a role in epitope shuffling, and this may induce DHF/DSS as a result of ADE. Furthermore, a chimpanzee mAb known as 1A5,

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which efficiently cross-neutralizes DENV1 and DENV2, binds to an epitope located on the fusion loop of EDII suggesting that antibodies that target the fusion loop may also neutralize virus even though fusion loop Abs are at best only weakly neutralizing [122]. Intriguingly, no EDIII-reactive strongly neutralizing Abs were isolated from these non-human primates suggesting that EDIII-reactive Abs are rare after DENV infection in primates [34,122]. However, the role of EDIII antibodies and the mechanisms of type-specific neutralization after natural DENV infection in humans are not well understood.

1.10 Objectives of the study:

Epidemics of DHF/DSS in Sri Lanka emerged after 1989 with the introduction of a new clade of DENV3 genotype 3 [123,124]. However, evolution of other DENV serotypes with regard to emergence of severe epidemics in Sri Lanka had never been studied. Intriguingly, the numbers of DHF/DSS cases have dramatically increased since 2000 with a massive epidemic reported in 2004. Since the emergence of DHF in Sri Lanka had already been linked to the introduction of a novel strain of DENV3, we hypothesized that the recent DHF epidemic in 2004 was due to the continuation of evolution of DENV3 or other DENV serotypes in Sri Lanka. Therefore, one goal of the current study was to analyze the molecular epidemiology of DENV serotypes over three decades, especially referring to the 2004 DHF/DSS epidemic in Sri Lanka.

Studies conducted with mouse mAb had identified the E protein of flavivirus as the major immunodominant protein that elicited neutralizing Abs [72]. Interestingly, strongly neutralizing type-specific antibodies were shown to be EDIII-reactive. The central tenet of the type-specific neutralization of DENV has been that all the strains that belong to one

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DENV serotype are uniformly neutralized by the same antibodies [72]. However, phylogenetic analyses demonstrated that DENV strains of one serotype display a considerable amount of genetic heterogeneicity which can be used to subdivide that serotype into genotypes [11]. We hypothesize that strain variation within a serotype of DENV can influence the neutralization potential of an Ab. Therefore, one of the major goals of the current research was to determine the effect of intra-serotypic strain variability on neutralization by type-specific strongly neutralizing antibodies using DENV3 as a model.

Although, type-specific strongly neutralizing mouse mAb were shown to bind to the lateral ridge of EDIII of flavivirus, it was unclear if the polyclonal immune response from flavivirus-infected individuals followed the same. There are indirect reports to suggest that the humoral immune responses after a natural flavivirus infection in humans may be different. Analysis of the human B cell repertoire from WNV-infected individuals suggested that the humoral response mainly produces antibodies that target the fusion loop [125], and western blot analysis of DENV human immune sera with recombinant E protein also revealed the same specificity of human sera [114]. Therefore, a third major goal of the study was to determine whether the type-specific neutralization acquired during a natural DENV infection was in fact driven by EDIII-reactive, strongly neutralizing antibodies as indicated by the mouse monoclonal antibody studies.

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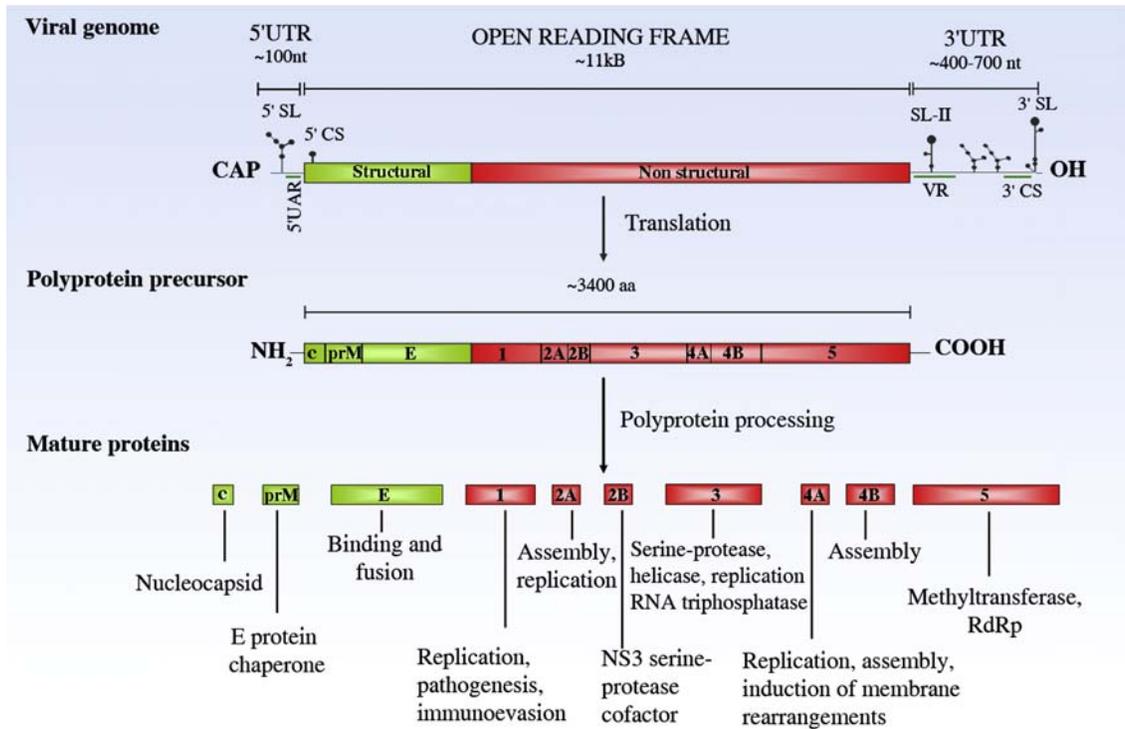
Figure 1.1 Genome structure of dengue virus and functions of mature proteins
Viral genome is translated to a polyprotein precursor and then cleaved by host and viral proteases into 3 structural proteins and 7 non-structural proteins.
(Adapted from Cell Host Microbe. 2009 Apr 23; 5 (4):318-28)
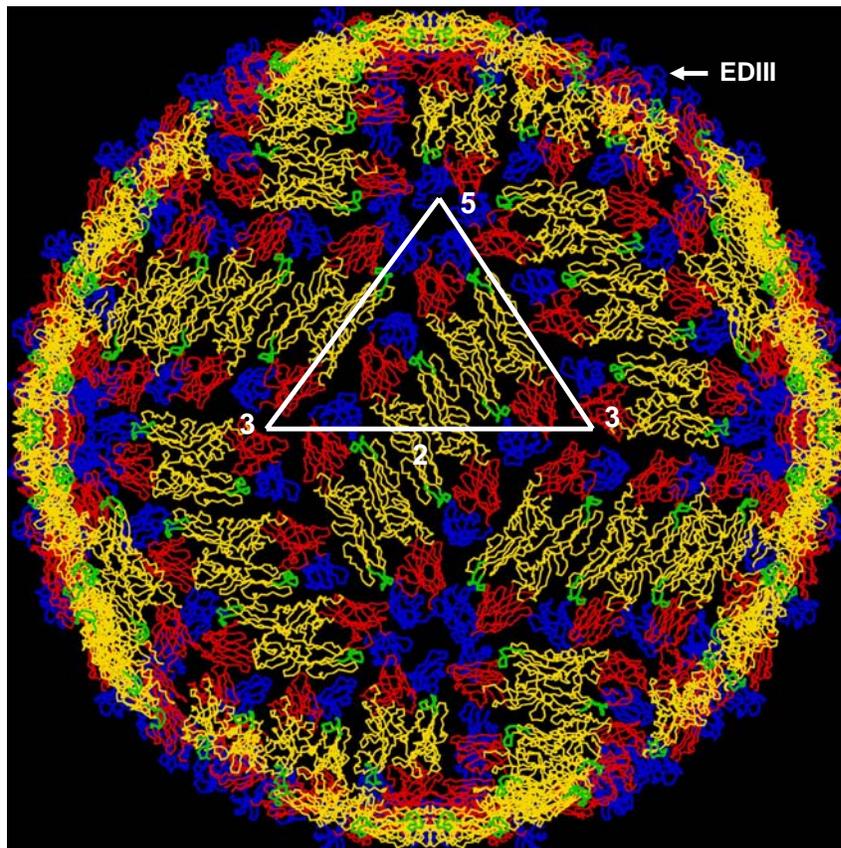
Figure 1.2 Structure of mature DENV particle
E protein dimers are arranged in icosahedral symmetry on the surface of the virion.
The triangle shows an icosahedral unit with 5 fold, 3 fold and 2 fold axes. EDIII
protrudes out from the surface of the virion (arrow).
(Adapted from ftp://ftp.purdue.edu/pub/uns/kuhn.dengue1.jpeg)
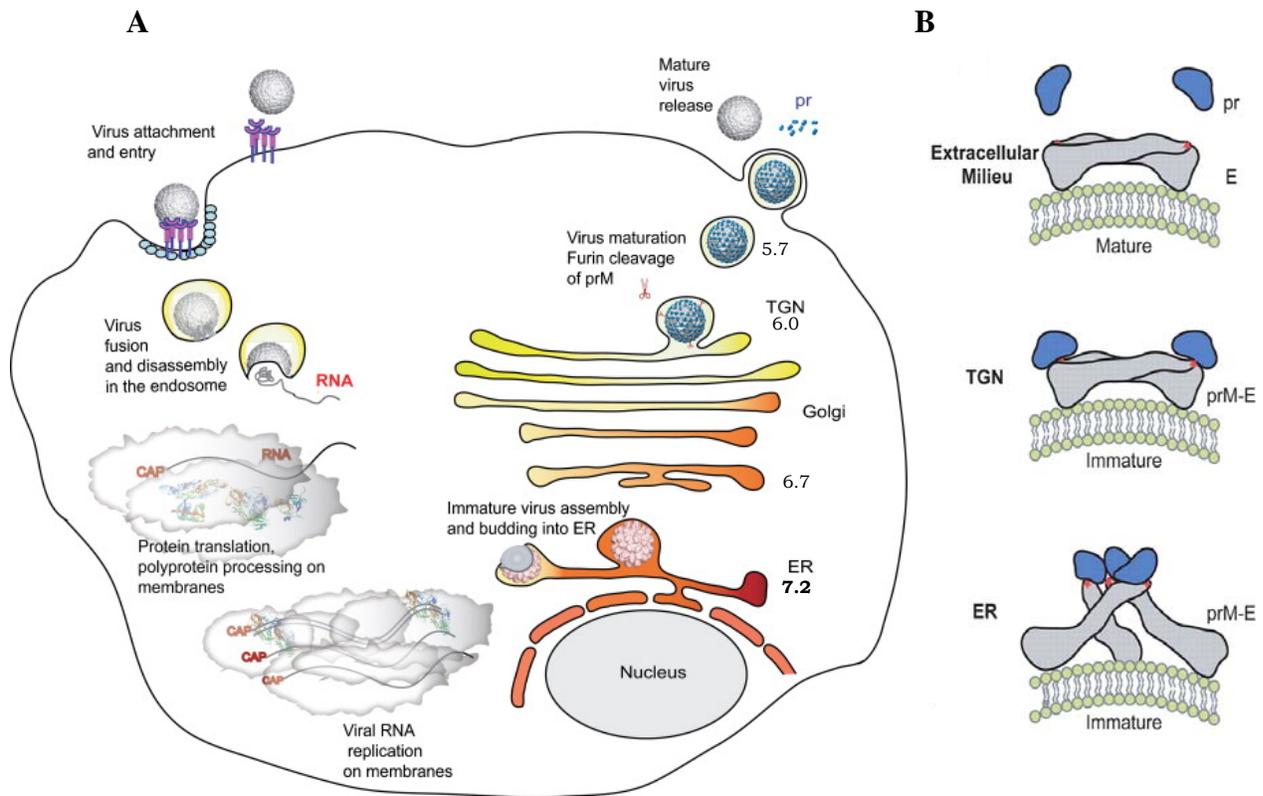
Figure 1.3 Life cycle of dengue virus.
A. Different stages of the life cycle of DENV along with the pH of the different components of the secretory pathway are shown. **B.** Major conformational rearrangements of the E protein in the maturation process of DENV.
(Adapted from Antiviral Research Volume 81, Issue 1, January 2009, Pages 6-15)
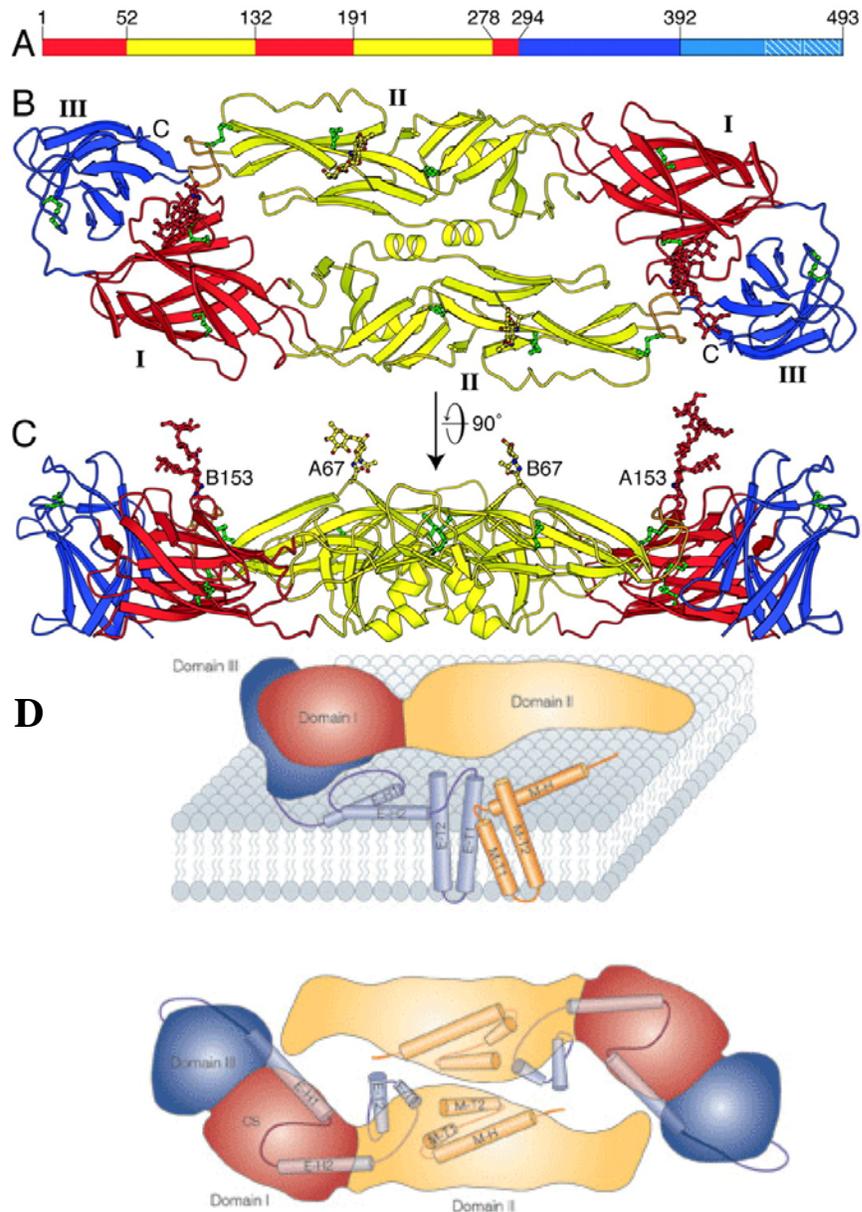
Fig 1.4 Structure of DENV E protein
A. Three domains of E protein. EDI is Red, EDII is Yellow and EDIII is Blue. Amino acids 1-392 form the ectodomain of E protein. Amino acids 392-494 form the stem region and the trans-membrane domain of E protein. **B**. Ectodomain of E protein viewed from the top (2 fold symmetry axis). **C**. Side view of E protein ectodomain (perpendicular to 2 fold axis). Two glycans on residues 67 and 153 of the sub units A and B are labeled. **D**. Ecotodomain of E is connected to the TM domain by the stem region containing two amphipathic helices, which are partially buried in the outer leaflet of lipid bilayer. (*Adapted from J Virol. 2005;79(2):1223-31 and Nature Reviews Microbiology. 2005; 3: 13-22*)
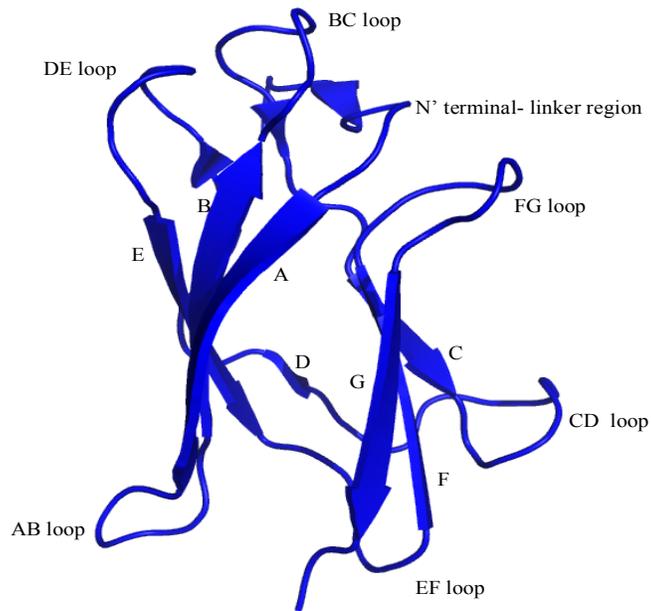
Figure 1.5 Structure of DENV3 EDIII.
EDIII consists of seven β strands linked by connecting loops. The β strands are labeled from A to G. The lateral ridge of EDIII is collectively made of 3 loops (DE, BC, FG loops) with the N' terminal linker region.
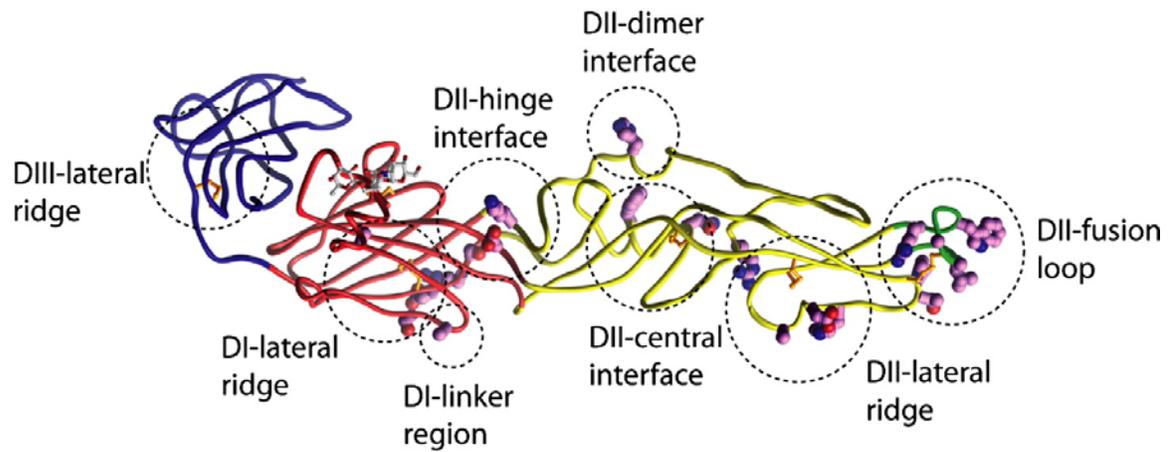
Figure 1.6 Distribution of known neutralizing epitope on E protein
All three domains of E protein contain epitopes recognized by mouse mAb.
Each circle represents a cluster of epitope *(Adapted from Cell Host Microbe.2008;4(3):229-38)*

Chapter 2

Phylogenetic analysis of dengue virus in Sri Lanka from 1981 to 2006 with a special reference to the severe epidemic in 2004.

[Kanakaratne N, **Wahala WMPB**, Messer WB, Tissera HA, Shahani A, Abeysinghe N, et al. **Severe dengue epidemics in Sri Lanka, 2003–2006**. Emerg Infect Dis, 2009;15: 192-199 Collection of serum samples, clinical information, epidemiological data and diagnosis of DENV in serum by PCR were carried out by Karunartahne and colleagues in Sri Lanka. *Virus isolation from PCR positive samples, genotyping of virus isolates, designing of PCR primers for gene sequencing, gene sequencing, phylogenetic analysis of DENV and interpretation of phylogram, were carried out by Wahala WMPB.*]

2.1 Abstract

Recent emergence of dengue hemorrhagic fever in the Indian subcontinent has been well documented in Sri Lanka. We analyzed the phylogenetic relationship of dengue virus (DENV) during a severe epidemic during 2003-2006 in Sri Lanka by comparing the virus isolates over a period of three decades. The four serotypes of DENV have been cocirculating in Sri Lanka and their distribution has not changed over 30 years. During this period, DENV1 and DENV4 showed a genotype switch, which is not observed in the phylogeny of DENV2 and 3. Instead, new clades of DENV3 genotype III viruses have replaced older clades and DENV2 also showed a similar trend. Emergence of new clades of DENV3 in 1989 and 2000 coincided with abrupt increases in the number of reported dengue cases, implicating this serotype in severe epidemics.

2.2 Introduction:

Dengue virus, a member of family Flaviviride, comprises four different viruses designated DENV serotype 1 (DENV1) through DENV serotype 4 (DENV4). Each serotype of DENV is further sub-divided into genotypes depending on nucleic acid sequence similarities [1]. An infection of DENV leads to a wide range of disease symptoms from mild dengue fever (DF) to more severe, life threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [2]. *Aedes aegypti* and *Ae. albopictus* are the main mosquito vectors that transmit DENV [3]. Dengue viruses are now widely spread over the tropics and subtropics causing a major global health problem [4]. Globalization, urbanization, increase of mosquito density in urban settings and especially global travel of dengue viremic people are among the factors that contributed to the global spread of DENV [3-6].

Pathogenesis of DHF/DSS is poorly understood mainly due to the lack of a well defined animal model. However, prospective and retrospective human cohort studies have indicated that secondary infection of DENV is a major cause for DHF/DSS, although the exact mechanism that causes DHF/DSS is not known [7-11,12 ,13,14]. In addition to secondary infection, many virus and host genetic factors are involved in determining disease severity [15-18]. Differences in virulence among different DENV serotypes and genotypes have contributed to severe disease in human [17-19]. Differences of virulence in two genotypes of DENV2 have been well documented [17]. The Asian genotype of DENV2 is more likely to cause DHF compared to the American genotype of DENV2. Furthermore, studies have revealed that differences in amino acid sequence of the envelop (E) gene and the untranslated regions of the genome (UTR) correlated with the differences in replication and virus output between these two genotypes of DENV2, respectively [20-22].

Phylogenetic analysis of DENV strains has been used to understand the evolution of the virus as well as its association with severe disease outbreaks [23]. Whole genome, or partial genome sequences have been used for these analyses with the same outcome [1,24]. Therefore, most of the phylogenetic studies with DENV have been conducted by analyzing a portion of the DENV genome. Such analyses revealed that turnover of new viral lineages or clades is a regular occurrence, and sometimes, clade replacement has been correlated with different disease outcome [23,25-31]

After 1989, recurrent DHF epidemics have been reported in Sri Lanka, an island located in the Indian subcontinent [32]. Although the number of DHF/DSS cases has increased after 1989, the intensity of dengue transmission and the relative abundance of each serotype have remained unchanged [32]. However, DHF has now become a regular occurrence on the island. The magnitude of the epidemics has increased over time, and especially large island-wide epidemics occurred in 2000, 2004 and 2009 (figure 2.1).

The phylogenetic analysis of viruses isolated from 1981 to 1997 clearly grouped DENV3 into two clades designated pre-DHF and post-DHF viruses [19]. Messer et al proposed that the sudden appearance of DHF epidemics in the island was related to the introduction of a new clade of DENV3 [19]. Although both DENV2 and 3 predominated in the period of these studies along with low circulation rates of DENV1 and 4, the phylogenetic relationships among DENV serotypes other than DENV3 are not known [19,32]. Therefore, molecular epidemiology of all serotypes of DENV from 1981 to 2004 was examined in the current study to understand viral evolution over this time and its contribution to one of the largest DENV epidemics that occurred in 2004 in Sri Lanka.

2.3 Materials and Methods:

Sample Collection

Genetech Molecular Diagnostics Institute (GMDI) in Colombo, Sri Lanka, receives diagnostic specimens for dengue testing from clinics and hospitals in Colombo. This study used excess serum samples that remained after diagnostic testing. Only samples collected from patients with 1–4 days of fever were included in the study. All patient-identifying information was removed from specimens before their use in the study. The study was reviewed and approved by the Institutional Review Boards of the University of North Carolina, Chapel Hill, NC, USA, and the University of Sri Lanka, Peradeniya, Sri Lanka.

Reverse Transcription–PCR for Detection and Serotyping of DENVs

Reverse transcription–PCR was performed at GMDI by using the DV1 and DV3 primer set and the ALD 1 and ALD 2 primer set in one reaction [33,34]. The DV primers amplify a 470-bp fragment of the nonstructural protein 3 (NS3) gene of all flaviviruses. The ALD1 and ALD2 primers amplify a 229–240-bp product from the 3´ untranslated region of all DENVs. The DV primers were not as sensitive as the ALD primers for detecting dengue infection. However, the 470-bp fragment amplified by the DV primers was used as the template in a second nested PCR to serotype the virus. All the virus isolates and a subset of PCR positive samples were also serotyped by using D1,D2 and TS1-TS4 primers using semi-nested multiplex PCR at University of North Carolina at Chapel Hill (UNC CH) [35]

Isolation of DENV from acute serum samples:

Different parameters were optimized to increase the isolation rate of DENV from acute serum samples. Briefly, 15 μL from PCR positive serum was mixed with 185 μl of minimal essential medium containing 2% fetal bovine serum and added to C6/36 cells growing in 6-well tissue culture plates. The inoculum was incubated for 1 hour at 28C and 2 ml of media was added. Plates were incubated for 10 days in a CO_2 incubator at 28C. Cells were tested for DENV by staining with monoclonal antibody 4G2, which binds to the envelope (E) protein of all 4 DENV serotypes. Supernatants were harvested from positive wells and frozen as P1 DENV stocks in the presence of 20% FBS.

Sequencing and Phylogenetic Analysis of DENV:

The P1 virus stocks were used as a source of RNA for sequencing and genotyping viruses. Before genotyping, the serotype of each virus isolate was confirmed by PCR using semi-nested PCR with D1, D2, TS1, TS2, TS3 and TS4 primers [35]. Once the serotype of the isolates was confirmed, reverse transcription–PCR (RT-PCR) was performed with different primer pairs to amplify selected regions of the genome of DENV1-4. For DENV-1, a 536-bp segment at the envelope-NS1 junction was amplified by using primers D1F 2034–2055 (5´-CCTTTTGGTGAGAGCTACATCG-3´) and D1R 2570–2551 (5´-ACACACACCCTCCTCCCATG-3´). For DENV-2, we amplified a 519-bp segment at the E-NS1 junction by using primers D2F 2050–2071 (5´-CCATTCGGAGACAGCTACATCA-3´) and D2R 2569–2548 (5´-GAGCCTTCTGGATAGCTGAAGC-3´). A 1,057-bp segment encompassing part of the capsid (C) protein, the premembrane (prM) protein, and part of the E protein of DENV3 was amplified by using primers D3F 132–159 (5´-

TCAATATGCTGAAACGCGTGAGAAACCG-3´) and D3R 1189–1171 (5´-

CTCCTCAGGCAAAACCGCT-3´). A 962-bp segment encompassing part of the C protein,

prM protein, and part of the E protein of DENV4 was amplified by using primers D4F 137–

162 (5´-TCAATATGCTGAAACGCGAGAGAACCG 3-´) and D4R 1099–1074 (5´-

CCACTTCCTTGGCTGTTGTCTTGATC-3´).

PCR products were gel purified using QIAEX II Gel Extraction Kit (Qiagen,

Valencia, CA, USA) according to the manufacturer's instruction and sent to the University of

North Carolina–Chapel Hill Genome Analysis Facility. Overlapping individual nucleic acid

sequences were assembled by using Vector NTI (ContigExpress, Bethesda, MD, USA).

Sequences were aligned by using ClustalX (www.clustal.org), and pylogenetic analyses were

conducted by, PHYLIP (http://evolution.gs.washington.edu/phylip.html), and MEGA4

(www.megasoftware.net) software [36,37]. The evolutionary history of each DENV serotype

was inferred by using the minimum- evolution method [38]. Percentages of replicate trees, in

which the associated taxa are clustered in the bootstrap test (1000 replicates), are shown next

to each branch of the phylogram. All new viral sequences were deposited in GenBank (gene

bank accession number FJ225443 - FJ225471). The virus strains used to create phylogenetic

trees are summarized in table 2.1.

2.4 Results:

Circulating Dengue Serotypes in Sri Lanka:

Of 930 PCR-positive samples collected during 2003–2006, 605 samples were sero-

typed by nested PCR. DENV serotypes 2 (40%) and serotype 3 (46%) were common, and

serotypes 1 (7%) and 4 (7%) were rare. We examined the relative abundance of each

serotype at monthly intervals during October 2003–September 2006 (Figure 2.2). DENV2 and DENV-3 were the dominant serotypes throughout the study period. DENV1 and DENV4 were also regularly isolated but in low numbers. All 4 serotypes were detected in 2004 and 2006. In 2005, DENV activity was low and DENV1 was not identified in samples. These results demonstrate that although all 4 serotypes co-circulate in Sri Lanka, DENV serotypes 2 and 3 are predominantly responsible for clinically apparent cases.

Isolation of DENV from acute serum samples:

Initial attempts to isolate DENV virus from a subset of PCR positive acute serum samples using standard protocols were successful only for some serum samples. We noticed that most of the serum samples contained an unidentified serum components that was toxic to C6/36 cells. After titrating the amount of serum and the duration of incubation with the C6/36 cells at 28C, the optimum conditions for isolation of DENV from acute serum samples were identified and given in detail in the Materials and Methods section. The optimized protocol was successfully used to isolate DENV from acute serum samples with an isolation rate of 82 % (181of 220). Each virus isolate was sero-typed by semi-nested PCR. The 181 isolates consisted of 18 DENV1 strains, 76 DENV2 strains, 64 DENV3 strains, and 23 DENV4 strains. It has been reported by others that adaptation of C6/36 to 33C has increased the replication rate of DENV and consequently increased the isolation rate from acute serum samples [39,40]. Although the recovery rate of the present study was also high, optimizing virus isolation at higher temperature may contribute to a further increase in the rate of isolation.

Phylogeny of DENVs in Sri lanka

The emergence of DHF epidemics in Sri Lanka after 1989 was closely correlated with a replacement of the non-pathogenic clade of DENV3 genotype III by a pathogenic clade of the same genotype. Subsequently, these two clades were designated pre-DHF or genotype IIIA and post-DHF or genotype IIIB viruses [19]. In addition, viruses belonging to the post-DHF clade of DENV3 genotype III have also been introduced to Latin Amaerica leading to severe DHF epidemic in these regions as well [19]. Although DHF emerged in Sri Lanka after 1989, the number of cases and deaths was low until 2000. A rapid increase of DF/DHF cases and the number of deaths have been documented in 2000 and thereafter (figure 2.1). Therefore, phylogenetic analysis of representative virus strains isolated between 1981 and 2004 were conducted to further characterize the DENVs responsible for recent epidemics in Sri Lanka after 2000, and to identify genotypes of DENV that have been circulating in this country over three decades from 1980s. All virus strains and sequences used for this analysis are listed in the table 2.1. Phylogenetic grouping of DENV serotypes and their nomenclature are based on that described by Rico-Hesse [41]. Genotypes are named on the basis of country of origin of the earliest isolates and not necessarily on the current distribution of viruses.

Phylogeny of DENV1 during 1983–2004:

DENV1 has been subdivided into 4 genotypes designated South Pacific, Asia, Thailand, and Africa/America [41]. A 498-nt fragment at the E/NS1 junction (from positions 2056 to 2554) was used to create a phylogenetic tree with virus isolates obtained in 1983, 1984, 1997, 2003, and 2004. Results demonstrate that the DENV-1 genotype circulating in

Sri Lanka has changed over the study period. The two isolates from Sri Lanka obtained in 1983 and 1984 belonged to the South Pacific genotype (Figure 2.3). The phylogenetic tree further indicates that the South Pacific genotype was replaced by the Africa/America DENV1 genotype sometime during 1984–1997. The number of Sri Lankan DENV1 isolates as well as their temporal distribution is limited in the analysis and therefore, clade turnover cannot be identified temporally.

Phylogeny of DENV2 during 1981–2004:

DENV-2 has been subdivided into 4 genotypes designated Malaysian/Indian subcontinent, Southeast Asian, American, and West African (Sylvatic) [41]. The Sri Lankan DENV-2 strains in our collection were isolated in 1981, 1982, 1983, 1984, 1985, 1989, 1990, 1996, 1997, 2003, and 2004. Nucleic acid sequences from a 240-nt fragment at E/NS1 junction (nt positions 2311–2550) were used to generate a phylogenetic tree along with existing sequences in GenBank representing all genotypes of DENV2 strains. All DENV2 isolates from Sri Lanka were closely related and belong to the Indian subcontinent/Malaysia genotype (figure 2.4). Moreover, there is no evidence for the recent introduction of a DENV2 strain from outside the island, because the DENV-2 strains from Sri Lanka are more closely related to one another than to any other DENV-2 strain used in this analysis.

Phylogeny of DENV3 during 1983–2004:

DENV3 has been divided into 4 genotypes designated Southeast Asian/South Pacific (I), Thailand (II), Indian subcontinent (III), and American (IV) [41,42]. Previous studies have demonstrated that all DENV-3 strains from Sri Lanka isolated in the 1980s and 1990s

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were categorized into the Indian subcontinent genotype (III) [19,42]. Within genotype III, DENV3 strains from Sri Lanka form two distinct clades linked to mild (pre DHF or IIIA clade) and severe (post DHF or IIIB clade) disease epidemics on the island [19]. DENV-3 IIIB viruses are likely to have been introduced from East Africa [19]. In 1994, a DENV-3 genotype III from South Asia or East Africa was also introduced into Latin America where it is now well established and responsible for severe disease epidemics [19].

Considering the backdrop of the recent expansion of DENV-3 genotype III viruses, the relationship of DENV-3 genotype III strains from Sri Lanka isolated in 2003 and 2004 to other DENV-3 genotype III viruses currently circulating in Africa, the Americas, and the Indian subcontinent was determined. A 966-nt fragment from a portion of C, all of preM, and a portion of E gene (nt positions 179–1144) was sequenced. A phylogenetic tree was created by using new sequences and existing sequences in GenBank from representative DENV-3 genotype III strains (figure 2.5). The DENV-3 sequences used were from isolates obtained in Sri Lanka in 1983, 1984, 1985, 1989, 1990, 1993, 1994, 1997, 1998, 2003, and 2004. As previously demonstrated, genotype III of DENV3 consists of pre-DHF (IIIA) and post-DHF (IIIB) clades from Sri Lanka, as well as Latin American and East African clades (figure 2.5 and figure by Messer et al [19]). However, DENV3 strains from Sri Lanka isolated in 2003 and 2004 form a new, distinct clade that is closely related but distinct from the DENV3 post-DHF clade (IIIB) viruses that were isolated in the 1990s. Therefore, pre DHF (IIIA) and post-DHF (IIIB) clades were re-named as pre-1989 clade and post-1989 clade respectively. The new 2003–2004 clade, which is designated post-2000 clade, includes an isolate from 1993 indicating that post-2000 clade viruses have emerged from a previously rare virus present in Sri Lanka. In addition, after 1993, the earliest isolate of the post-2000

clade virus was reported in 2000 [43]. The data of the current analysis indicate that post-2000 clade viruses have probably re-appeared in 2000 and replaced the post-1989 viruses.

Phylogeny of DENV4 during 1978–2004:

The phylogeny of DENV4 has not been studied as extensively as the other serotypes. This serotype can be broadly separated into two genotypes designated Southeast Asian (I) and Indonesian (II) [41]. The Southeast Asian genotype strains are primarily from Asia, whereas the Indonesian group has a broad distribution in Asia and the Americas. A 296-nt fragment from positions 787–1083 (prM/E junction) was used to create a phylogenetic tree. A DENV4 isolate obtained in 1978 was grouped into the Southeast Asian genotype or genotype I. Unfortunately, no virus isolates were available to represent the period from 1979 to 1991, and therefore, the distribution of DENV4 genotypes during 1979-1991 is not known. However, as depicted in figure 2.6, two virus isolates obtained in 1992 belonged to the Indonesian genotype indicating the introduction of the Indonesian genotype to Sri Lanka. DENV4 isolates collected during 2003-2004 epidemics again fell into the Southeast Asian genotype, suggesting that the Southeast Asian genotype is the predominant DENV4 in Sri Lanka and the Indonesian genotype probably became extinct after a transient introduction. However, since the no virus isolates were not obtained between 1979-1991 and 1993-2002, temporal distribution of DENV4 genotypes is not conclusive, although the Southeast Asian genotype seems to be the predominant. It is quite possible that, after introduction of the Southeast Asian genotypes in the 1970s, the genotype might have been replaced by the Indonesian genotype in the 1990s and again, the Southeast Asian genotype was re-introduced in 2000 by replacing the Indonesian genotype.

2.5 Discussion:

Introduction of new strains has been considered as one reason for the emergence of severe DF/DHF epidemics in the world [17,19,25,30,44,45]. Phylogenetic analysis has been widely used to determine the distribution of virus strains over time and to study the evolution of DENV [6,46]. Although dengue virus infection is one of the major health concerns in the Indian subcontinent, the disease and its viruses remains poorly studied in the region [47-50]. Furthermore, the epidemiology of dengue has changed over time, and recurrent DF/DHF epidemics are now common in the region [19,51-55]. In Sri Lanka, major DF/DHF epidemics appeared after 1989, and ever since, the frequency as well as the severity of DF/DHF epidemics has increased. In 2000, the number of DF/DHF cases significantly increased compared to the late 1990s, leading to a massive epidemic in 2004 (figure 2.1). Moreover, the trend to severe DF/DHF epidemic has further increased, and in the first 6 months of 2009, 11,000 cases and 160 deaths have been reported (figure 2.1). Therefore, a better understanding of epidemiology of dengue virus in the region is important to develop and implement effective control programs, and to effectively use future dengue vaccines, which are currently in phase 2 clinical trials.

Data from the current study indicates that the relative frequency of DENV serotypes during 2003 - 2006 epidemics was not distinct from the frequency reported earlier in Sri Lanka. DENV2 and DENV3 were the predominant viruses before 1999, with the incidence of DENV2 being marginally higher than DENV3 [32]. In the severe epidemic period in 2004, DENV3 was the predominant serotype followed by DENV2 (figure 2.2). However, according to Kularathne et al, proportion of DENV2 had dramatically increased during 1999-2001 compared to DENV3, although DENV3 dominated over DENV2 in the severe DF/DHF

epidemic in 2004 [56]. This high incidence of DENV2 may be due to sampling bias and the small number of samples studied. However, it is obvious that DENV2 and DENV3 are the most dominant DENV serotypes in Sri Lanka followed by DENV1 and DENV4, respectively, throughout the study period (figure 2.2 and Messer et al [32]).

The present study revealed that a new clade of DENV3 in genotype III (post-2000 clade) has become dominant on the Island after 2000. Among the hypotheses to explain the emergence of new DENV variants are adaptive selections due to immune pressure [26,57], purifying selection [58], stochastic events [59], and a genetic bottleneck in the mosquitoes that leads to selection of a virus variant with a high fitness [46]. However, none of these hypotheses can universally explain the appearance of new DENV variants in different epidemics around the world. In fact, it has been shown that adaptive selection, especially in DENV3, is very rare although few studies claimed that such selection has led to emergence of new DENV2 variant [26,57]. Genetic bottlenecks, especially in the inter-epidemic period, have been proposed to be important for emergence of new variants or lineages of DENV3 [60].

Both pre-1989 and post-1989 clade DENV3 viruses co-circulated in Sri Lanka in 1989 [19]. However, post-1989 clade viruses disseminate efficiently in mosquitoes [61] and probably have gained high fitness [61]. Therefore, genetic bottleneck might have been a factor for post-1989 viruses to replace the pre-1989 clade. However, again in 2000, the post-2000 DENV3 clade has emerged with increased spread and severity of dengue epidemics. Therefore, clade replacements in 1989 and again in 2000 were both linked to stepwise increases in the number of DF/DHF cases reported in Sri Lanka. However, further research

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is needed to understand the underlying mechanisms by which post-2000 clade viruses have replaced the post-1989 clade.

Phylogeny of DENV2 was not drastically changed over the period studied. However, even though all of the strains used in the study grouped into the Indian subcontinent genotype, a clear pattern of temporal distribution of DENV2 within the Indian subcontinent genotype was observed. The pattern of subgrouping of DENV2 within the Indian subcontinent genotype is similar to the grouping of DENV3 into different clades within genotype III, indicating pre-1989, post-1989 and post-2000 groups (figure 2.4 and figure 2.5). However, the bootstrapping value in the phylogenetic tree is not strong enough to designate them into different clades. This may be partly due to the short length of the nucleic acid sequence (240 nucleic acids) used for phylogenetic analysis of DENV2. Therefore, further research is needed to understand the evolution of DENV2 in Sri Lanka

DENV1 and DENV4 showed a genotype shift over the study period, which has not been observed in the phylogeny of DENV2 and 3. However, the limited number of virus isolates, which was not evenly distributed over the study period, hampered the understanding of the evolution of DENV1 and 4 (figure 2.3 and 2.6). However, the phylogenetic analysis demonstrated the South Pacific genotype of DENV1, which dominated in the 1980s, was replaced by the introduction of the African/American genotype which is now established as the predominant DENV1 genotype in Sri Lanka after the 1990s (figure 2.3). Interestingly, the South Asian genotype of DENV4, which was present as the predominant genotype in Sri Lanka throughout the study period, was interrupted by the transient introduction of the Indonesian genotype in the early 1990s.

In summary, our data along with previously published data [19] indicate that DENV2 and DENV3 are the predominant virus strains co-circulating in Sri Lanka, and the introduction of new clades, especially new clades of DENV3, are linked with the severe epidemics in Sri Lanka over the study period. However, apart from the phylogeny of virus, the exact mechanisms involved in the introduction and selection of new clades were not clear from these studies. It is known that the mosquito infection rate is higher during epidemics than in the interepidemic phase [62]. In addition, virus strains involved in silent DENV infection (asymptomatic DENV infection) during the interepidemic phase have been considered as predictive of the next epidemic [62]. However, very few studies have been conducted to study any of these parameters in Sri Lanka [63-66]. Most of the information relating to the 1980s and 1990s has been derived from retrospective studies [32]. Although these studies have revealed the overall distribution of different DENV serotypes, turnover of new genotypes or clades cannot be analyzed by such studies. Therefore, it is important to study asymptomatic DENV infection along with vector studies, especially in the interepidemic period, to understand how new virus strains are circulating in a DENV hyper-endemic country like Sri Lanka. Understanding mechanisms involved in evolution of DENV in Sri Lanka may help to predict future epidemics.

2.6 References

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	Taxon name	Source of sequence	Accession number
DENV1	DV1 West Pacific 1974	Genbank	U88535
	DV1 Aust 1983	Genbank	AB074761
	DV1 Japan 1943	Genbank	AB074760
	DV1 China 1980	Genbank	AF350498
	DV1 Thailand 1964	Genbank	AF180818
	DV1 Thailand 1958	Genbank	D10513
	DV1 Abidjan	Genbank	AF298807
	DV1 Nigeria 1968	Genbank	AF425625
	DV1 Brazil 2001	Genbank	AF513110
	DV1 Brazil 1997	Genbank	AF311956
	DV1 Brazil 1990	Genbank	AF226685
	DV1 Malaysia (Sylvatic) 1972	Genbank	EF457905
	DV1 SL 1983	This study	FJ225443
	DV1 SL 1984	This study	FJ225444
	DV1 SL 1992	This study	FJ225445
	DV1 SL 1997	This study	FJ225446
	DV1 SL 2003a	This study	FJ225447
	DV1 SL 2004a	This study	FJ225448
	DV1 SL 2004b	This study	FJ225449
	DV1 SL 2004c	This study	FJ225450
DV2	DV2 Senegal (sylvatic) 1974	Genbank	M32957
	DV2 Mexico 1992	Genbank	U91888
	DV2 Venezuela 1993	Genbank	U91870
	DV2 Colombia 1992	Genbank	U91876
	DV2 Jamaica 1981	Genbank	M32950
	DV2 Brazil 1991	Genbank	U91867
	DV2 Thailand 1983	Genbank	M32947
	DV2 Thailand 1964	Genbank	M32941
	DV2 Taiwan 1987	Genbank	M32949
	DV2 Philippines 1988	Genbank	M32932
	DV2 Seychelles 1973	Genbank	M32952
	DV2 Bukina Faso 1982	Genbank	M32956
	DV2 Indonesia 1978	Genbank	M32934
	DV2 SL 1981	Genbank	M32938
	DV2 SL 1982	Genbank	M32940
	DV2 SL 1983	This study	FJ225451
	DV2 SL 1984	This study	FJ225452
	DV2 SL 1985	Genbank	M32953
	DV2 SL 1989a	This study	FJ225453
	DV2 SL 1989b	This study	FJ225454
	DV2 SL 1990	This study	FJ225455
	DV2 SL 1996	This study	FJ225456
	DV2 SL 1997	This study	FJ225457
	DV2 SL 2003a	This study	FJ225458
	DV2 SL 2003b	This study	FJ225459
	DV2 SL 2004a	This study	FJ225460
	DV2 SL 2004b	This study	FJ225461

	Table 2.1: Continued		
	Taxon name	**Source sequence**	**Accession number**
DV3	DV3 SL 2003a	This study	FJ225463
	DV3 SL 2004a	This study	FJ225464
	DV3 SL 2004b	This study	FJ225465
	DV3 SL 1983a	Genbank	AF547225
	DV3 SL 1983b	Genbank	AF547227
	DV3 SL 1983c	Genbank	AF547228
	DV3 SL 1983d	Genbank	AF547229
	DV3 Mozambique 1985	Genbank	AF547237
	DV3 SL 1985a	Genbank	AF547241
	DV3 SL 1985b	This study	FJ225462
	DV3 SL 1989a	Genbank	AF547230
	DV3 SL 1989b	Genbank	AF547231
	DV3 SL 1989c	Genbank	AF547232
	DV3 SL 1990	Genbank	AF547233
	DV3 Kenya 1991	Genbank	AF547239
	DV3 Somalia 1993	Genbank	AF547240
	DV3 SL 1993	Genbank	AF547234
	DV3 Panama 1994	Genbank	AF547247
	DV3 Sl 1994	Genbank	AF547235
	DV3 SL 1997	Genbank	AF547242
	DV3 El Salvador 1998	Genbank	AF547259
	DV3 Nicaragua 1998	Genbank	AF547245
	DV3 Puerto Rico 1998	Genbank	AF547258
	DV3 SL 1998	Genbank	AF547243
	D3H-87	Genbank	M93130
DV4	DV4 Dominica 1981	Genbank	AY152360
	DV4 Thailand 1991	Genbank	AY618990
	DV4 Thailand 2001	Genbank	AY618992
	DV4 Thailand 1977	Genbank	AY618991
	DV4 Malaysia (Sylvatic) 1975	Genbank	EF457906
	DV4 China	Genbank	AF289029
	DV4 Indonesia 1976	Genbank	U18429
	DV4 Tahiti 1979	Genbank	U18438
	DV4 Sri Lanka 1978	Genbank	AY550909
	DV4 SL 1992a	This study	FJ225466
	DV4 SL 1992b	This study	FJ225467
	DV4 SL 2003a	This study	FJ225468
	DV4 SL 2003b	This study	FJ225469
	DV4 SL 2004a	This study	FJ225470
	DV4 SL 2004b	This study	FJ225471

Table 2.1. DENV strains and the source of the sequence used in the study
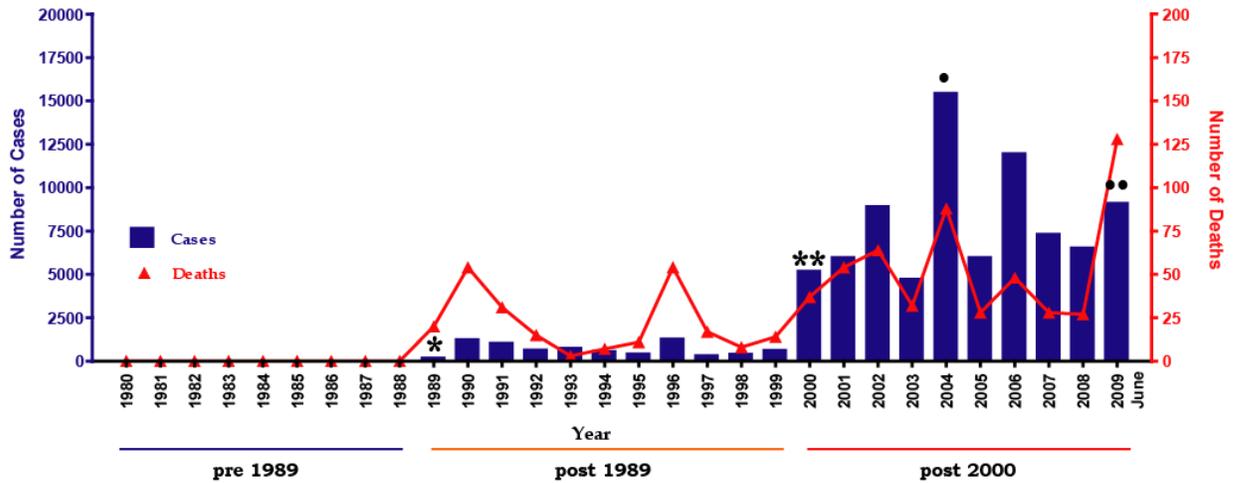
Figure 2.1: **Temporal distribution of number of DF/DHF cases and deaths from 1980 to June 2009.**
Before 1989 (pre 1989 period), no DHF cases and deaths were reported in Sri Lanka. Post 1989 period started with the introduction of DHF along with post 1989 DENV3 viruses to Sri Lanka in 1989 (*). In post 2000 period, starting in 2000 (**), number of DF/DHF cases were dramatically increased with a peak epidemic in 2004 (●) and an ongoing epidemic in 2009 (●●). Information was collected with the courtesy of epidemiology unit, ministry of health, Sri Lanka
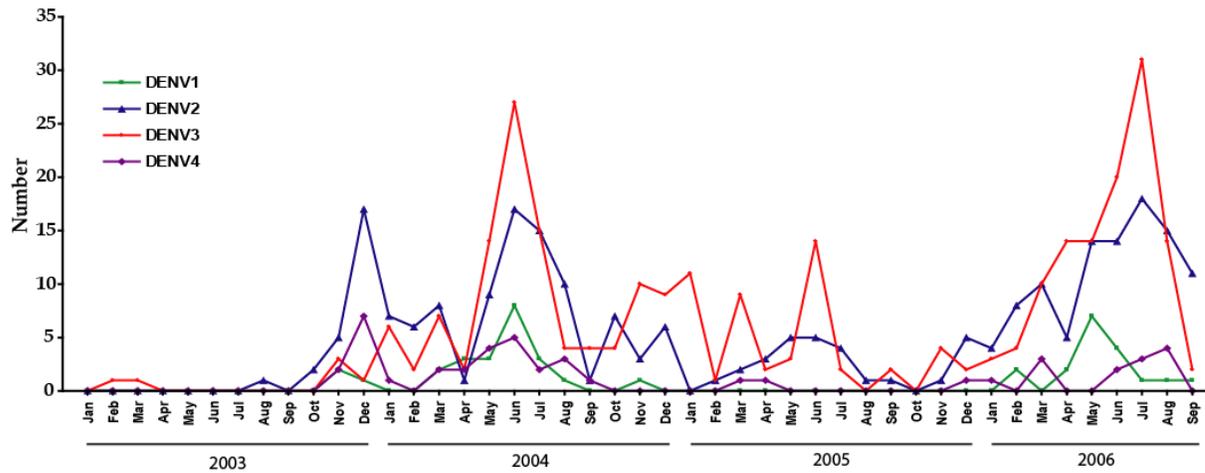
Figure 2.2: Monthly distribution of DENV serotypes identified in patient's sera collected from January 2003 to September 2006: All the PCR positive serum samples were serotyped by semi nested PCR and total number of each DENV serotype was graphed on monthly basis. May to August and November to January are the two seasonal peaks of DENV appearing each year.
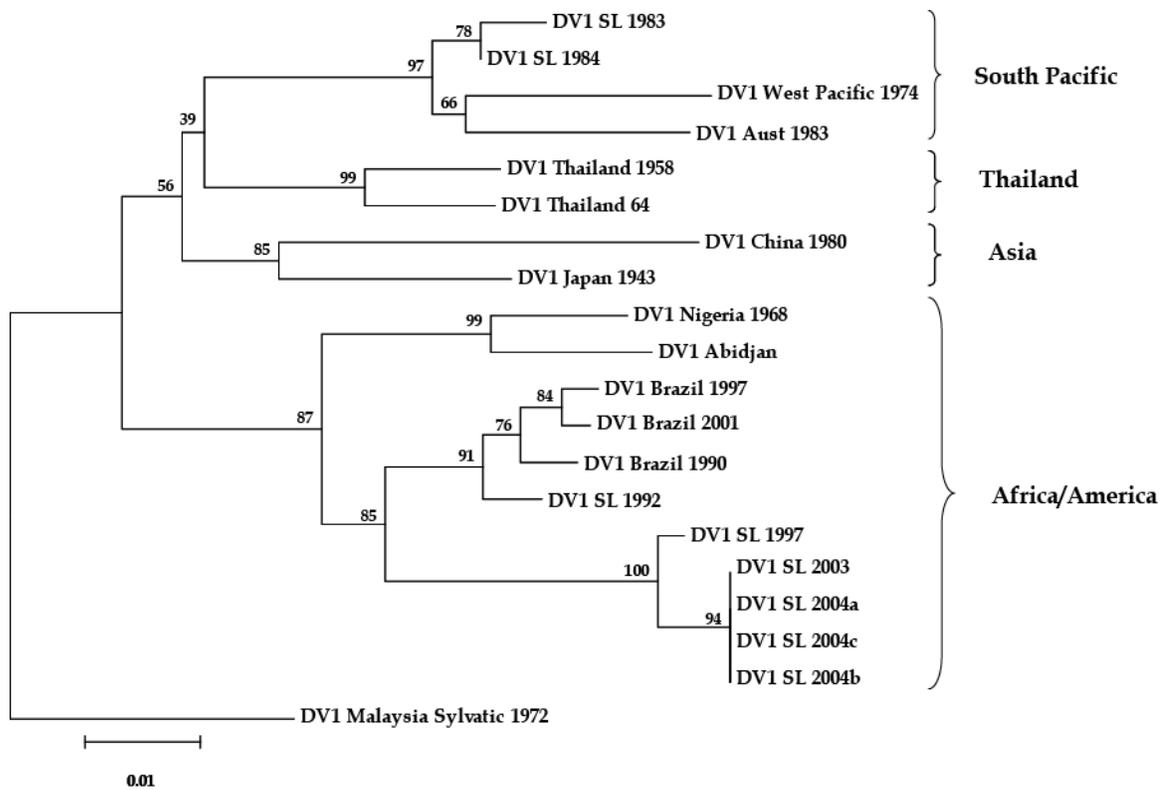
Figure 2.3: Phylogram of dengue serotype 1 viruses (DENV-1) from Sri Lanka (SL), 1983–2004, and other DENV-1 viruses. The tree is based on a 498-bp fragment for positions 2056–2554 coding portions of envelope protein and nonstructural protein 1. The tree was rooted by using a DENV-1 sylvatic strain. Classification and naming of different DENV-1 genotypes is based on the report by Rico-Hesse [41]. Scale bar represents number of base substitutions per site.
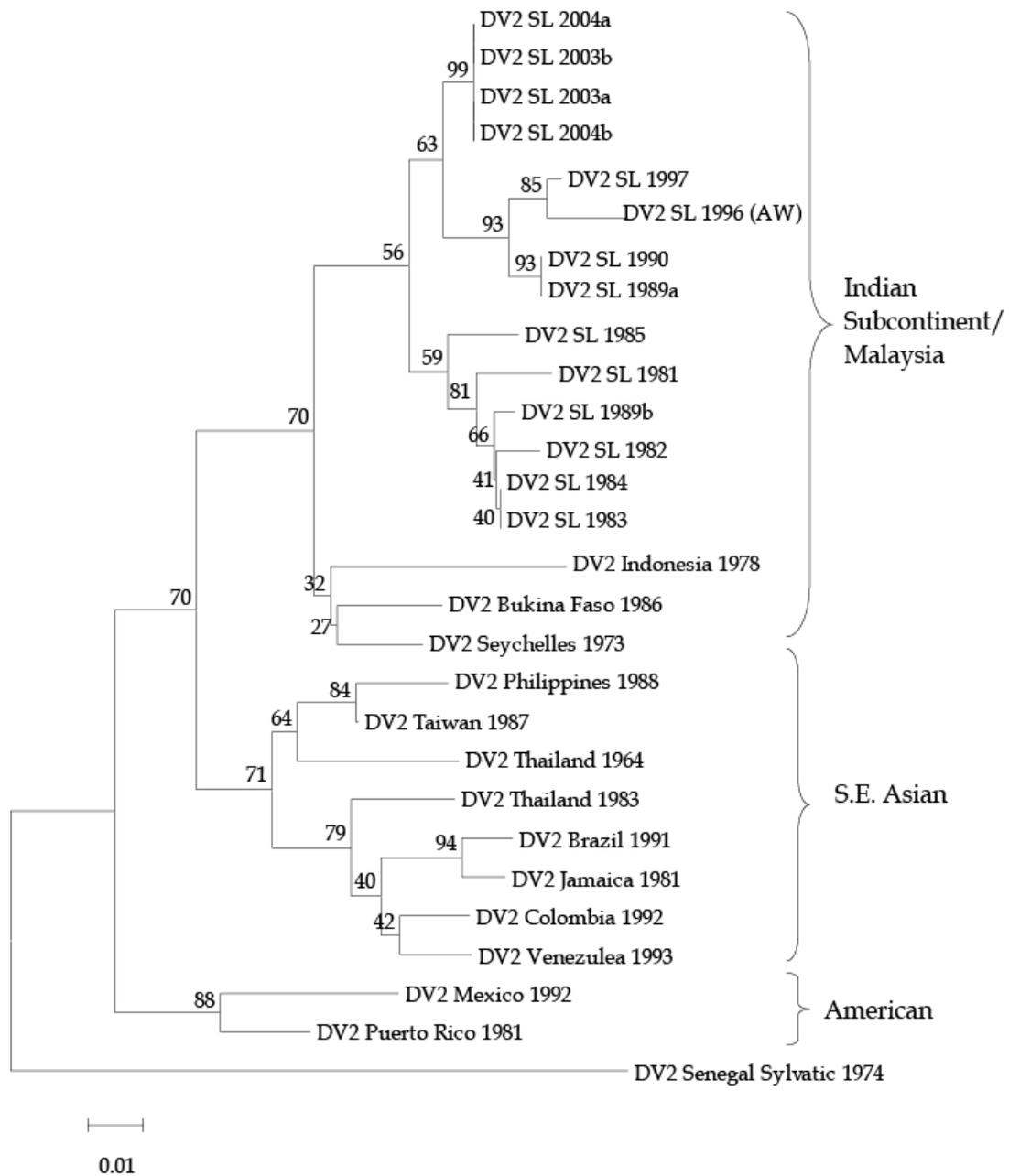
Figure 2.4: Phylogram of dengue serotype 2 viruses (DENV-2) from Sri Lanka (SL), 1981–2004, and other DENV-2 viruses. The tree is based on a 239-bp fragment for positions 2311–2550 coding for amino acids at the envelope protein/nonstructural protein 1 junction. DENV-2 sylvatic strain was used to root the phylogenetic tree. Classification and naming of different DENV-2 genotypes is based on the report by Rico-Hesse [41]. Scale bar represents number of base substitutions per site.
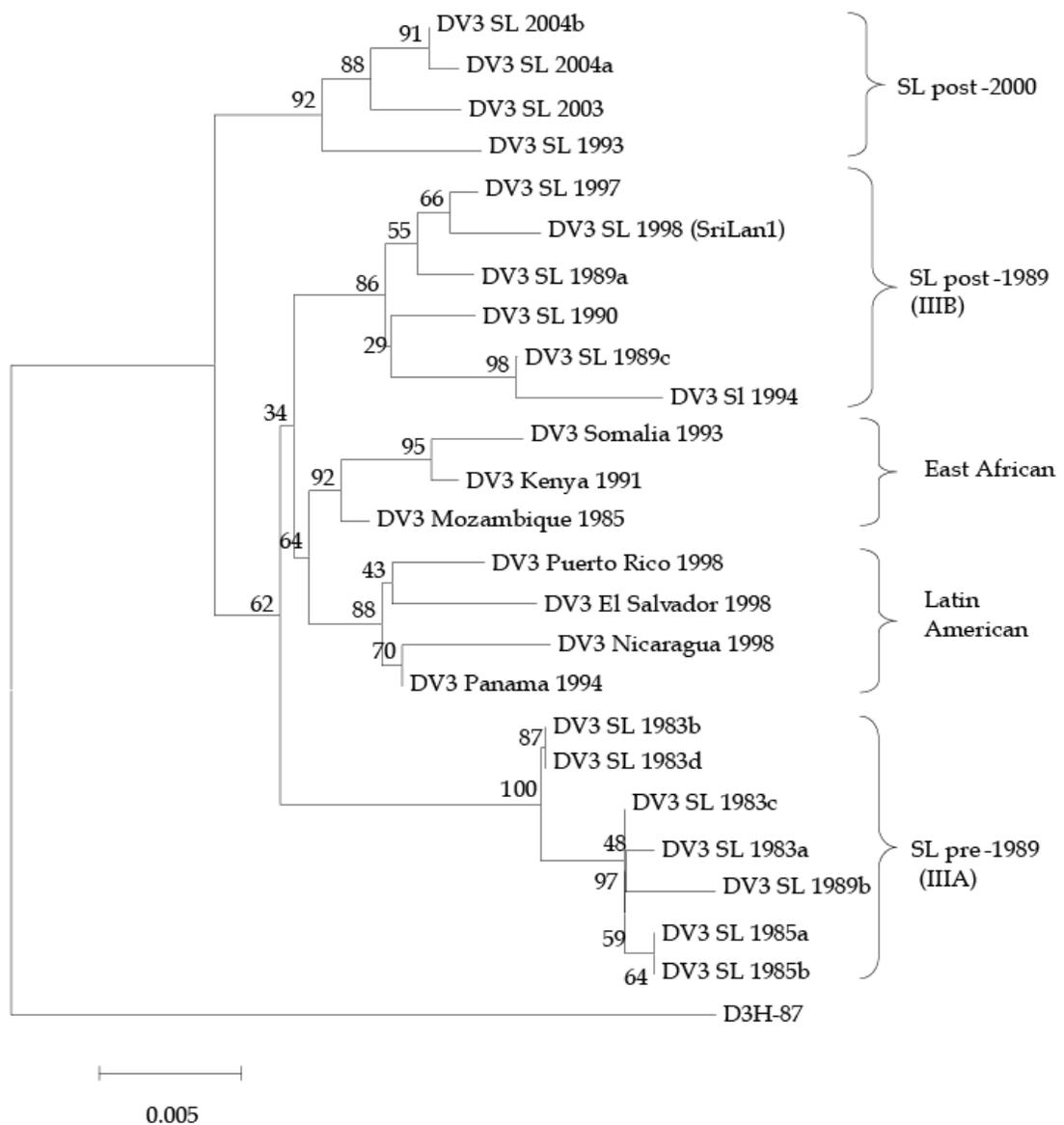
Figure 2.5: Phylogram of dengue serotype 3 (DENV-3) genotype III viruses from Sri Lanka (SL), 1981–2004, and other DENV-3 genotype III viruses. The tree is based on a 966-bp fragment for positions 179–1144 coding for a portion of the capsid protein, all of the premembrane protein, and a portion of the envelope protein. Phylogenetic tree was rooted by using a DENV-3 genotype I virus (H87). Naming of the different groups within DENV-3 genotype III is based on the report by Messer et al. [19]. Scale bar represents number of base substitutions per site.
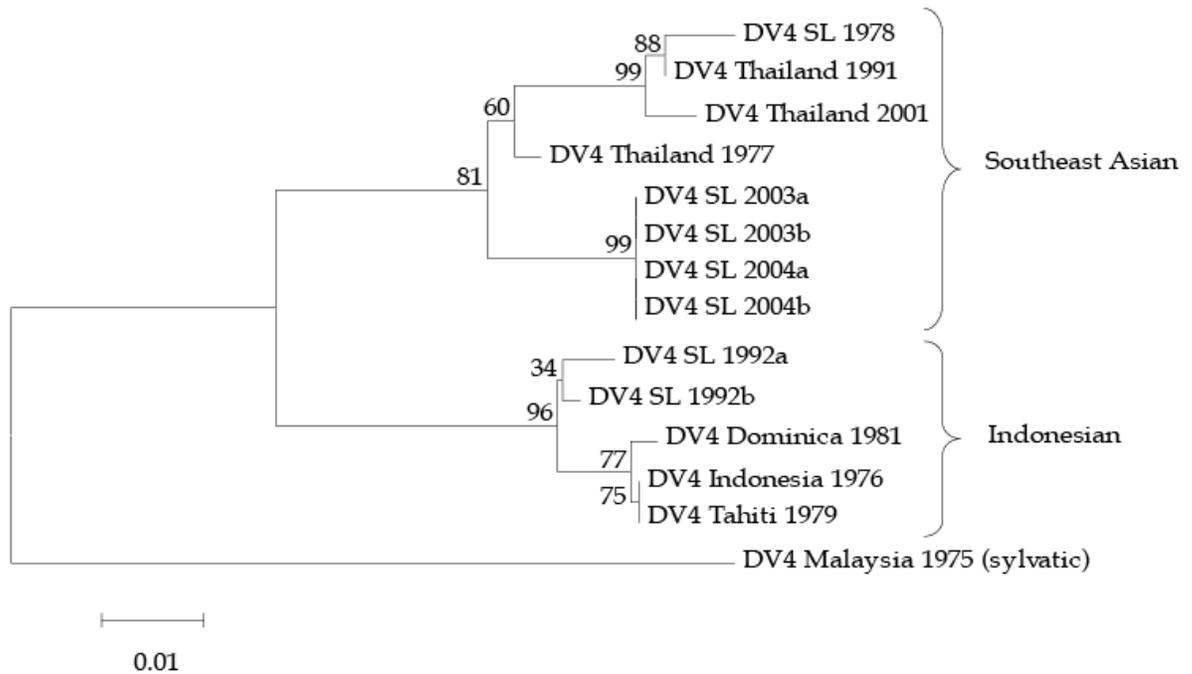
Figure 2.6: Phylogram of dengue serotype 4 viruses (DENV-4) from Sri Lanka (SL), 1978–2004, and other DENV-4 viruses. The tree is based on a 296-bp for positions 787–1083 coding for portions of premembrane and envelope proteins. The tree was rooted by using a sylvatic DENV-4 strain. Classification and naming of different DENV-4 genotypes is based on the report by Rico-Hesse [41]. Scale bar represents number of base substitutions per site.

CHAPTER 3

Development of mouse monoclonal antibody (mAb) against dengue virus serotype 3.

3.1 Abstract :

The mechanism of Dengue virus serotype 3 neutralization is poorly understood although it causes major epidemics in South Asia and Americas. This is mainly due to the dearth of monoclonal antibodies (mAb) against DENV3. We developed a panel of mouse mAb against DENV3 strain CH53489. The panel of mAb includes DENV type-specific, DENV cross-neutralizing and non-neutralizing mAbs. This panel of mAbs is a valuable tool to study the mechanism of DENV3 neutralization and in other DENV studies.

3.2 Introduction:

The neutralization mechanism of flavivirus has been mainly studied with reference to West Nile virus (WNV), especially using the mAb E16 [1,2]. Many other studies conducted with DENV2, Japanese encephalitis virus (JEV) and tick born encephalitis virus (TBEV) have also confirmed that neutralization of flavivirus is mediated bya multi-hit mechanism that requires more than 30 Ab molecules per virion [1,3,4]. However, most of these studies were conducted with EDIII reactive antibodies. Interestingly, a considerable amount of amino acid (AA) heterogenicity of E protein is found among the members DENV3 genotypes, and many of them are located on EDIII (Chapter 4). Such AA heterogenicity may interfere with the neutralization potential of DEV3 type-specific antibodies or polyclonal sera among the members of the DENV3 serotype [5].

Even though monoclonal antibodies (mAb) are valuable tools to investigate the mechanisms of neutralization of flavivirus and other viruses as well , not many DENV3 type-specific mAb are available except the mAb 1H9 [6]. Therefore, a shortage of type-specific mAb against DENV3 has hampered studies of neutralization mechanisms of DENV3, especially to determine whether strain variability affects the neutralization potential of DEN3 type specific antibodies. The aim of the present study was to develop type specific as well as cross reactive neutralizing mouse mAb against all the domains of E protein of DENV3. We were able to isolate mouse mAb that bound to different domains of the E protein and that displayed different potentials of neutralization capacities.

3.3 Materials and Methods:

Purification of DENV.

DENV3 CH53489 reference strain (genotype II) was grown in Vero-81 cells (ATCC CCL-81) at 37C. The virus containing media was harvested 5-7 days after infection and centrifuged to pellet cell debris. The clarified medium was layered on top of a 20% sucrose (wt/vol in PBS) cushion and centrifuged (72,000g for 5 hrs) to pellet the virus. The virus pellet was allowed to dissolve overnight in PBS before layering on a 10%- 40% iodixanol gradient in PBS , then centrifuged at 163,700 x g for 120 min. The virus-containing fractions were harvested. PBS was added to the virus to dilute the iodixanol. The diluted solution was centrifuged (72,000 x g for 5 h) to pellet the virus and remove the iodixanol. The virus pellet was resuspended in PBS and protein content was estimated by spectrometry. The virus was stored at -80°C.

Immunization of mice.

UV-inactivated purified DENV3 antigen was used to immunize mice. Immunization and cloning of hybridomas were conducted in the Immunology Core Faculty at the University of Alabama, at Birmingham. BALB/c mice were immunized with 10 μg of UV-inactivated virus in complete Freund's adjuvant. About 24 days later, mice were boosted with 10 μg of UV inactivated virus in incomplete Freund's adjuvant. Mice were bled at 10-14 days post boost and the sera were tested for anti DENV antibodies. Six weeks after the second boost in incomplete Freund's adjuvant, the mice received an intravenous (IV) injection of 10 μg of virus in saline. Three days after the IV boost, splenocytes from immunized mice were fused using PEG and the P3X63-Ag8.653

75

myeloma line. The fusied cells were plated into 24 well plates in Hypoxanthine

Aminopterin Thymidine (HAT) medium and supernatants were tested for anti-DENV

antibodies, and positive hybridomas were cloned by limiting dilution using peritoneal

macrophage feeders.

Expression and purification of recombinant EDIII (rEDIII).

RNA was extracted from the supernatants of cells infected with DENV3 strain

CH53489 using QIAmp Viral RNA mini Kit (Qiagen). The nucleotide sequences

encoding EDIII of DENV3 (295- 398 AA) were reverse transcribed and PCR amplified.

The PCR products were cloned into the pMAL c2X vector (NEB) to generate

recombinant EDIII (MBP-EDIII) that is fused to maltose binding protein (MBP) at the N

terminus according to the manufacture's instructions. MBP-EDIII from DENV3 was

expressed in *Escherichia coli* DH5α (Invitrogen) and purified using amylose resin

affinity chromatography (NEB).   Purity of the rEDIII protein was confirmed on an SDS-

PAGE gel, and the protein quantity was measured spectrometrically.

**Detection of DENV-reactive antibody in immunized mouse sera and hybridoma
culture supernatants by ELISA.**

Seventy five nanograms (75 ng) of purified DENV3 or 200 ng of MBP-EDIII

were used to coat ELISA plates. ELISA plates were coated using virus or recombinant

protein antigen in carbonate buffer at pH 9.6 for 2 hrs at room temperature. The plates

were washed 3 times in Tris-buffered saline with 0.2% Tween20 (TBST) and incubated

with blocking buffer (Tris-buffered saline with 0.05% Tween20 containing 3% skim milk

and 2% normal goat serum) at 37C for 1 hr. After washing the plates twice with TBST, mouse immune serum or cell culture supernatant diluted in blocking buffer was added to each well and incubated at 37C for 1 hr. Following 3 washes with TBST, alkaline phosphatase-conjugated goat antimouse IgG (Sigma) was added to each well for 1 hour at 37C. After 3 washes with TBST, p-nitrophenyl phosphate substrate (Sigma) was added to each well and the reaction was allowed to develop for 15 minutes before recording optical density at 405nm on a spectrophotometer.

DENV Neutralization assays

DENV neutralizing antibodies in immunized mouse sera or cell culture supernatants were measured using a flow cytometry-based neutralization assay which was performed in 96-well plates with the U937 human monocytic cell line transfected with DC-SIGN as previously described [7]. In brief, mouse immune sera were serially diluted, and cell culture supernatants were used as undiluted or at 1:2 dilutions. Cell culture supernatants or serially diluted mouse sera were incubated with sufficient virus (DENV3- strain CH 53489) to infect 10 to 15% of the cells in the well. The virus/serum or cell culture mixture was incubated for 1hr at 37C and then added to the cells for 1hr at 37C. The cells were washed to remove unbound virus and fresh medium was added before incubating cells for 24hrs at 37C. Cells were fixed, permeabilized and stained with DENV mAb 2H2, which binds to all four serotypes [7]. Cells were analyzed with a FACScan flow cytometer (Becton Dickinson) to identify infected cells. The 50% neutralization titers were determined by nonlinear dose-response regression analysis ((Prism Package, GraphPad Software, Inc., San Diego, CA). Cell culture supernatants

which contained neutralizing mouse mAb were further tested for differential

neutralization potential against all of the genotypes of DENV3. The procedure was same

as mentioned above, except for the use of equal genome copy numbers from each

genotype and incubation times range from 36 hours to 72 hours to get infection of 10-

15% of cells.

3.4 Results:

Mice were immunized with purified, UV-inactivated whole virus as outlined in the

Materials and Methods section. After the initial immunization, each mouse was bled and

tested for the presence of anti-DENV Ab against DENV3 and rEDIII antigens.

Furthermore, before the fusion, sera were tested for the presence of neutralizing Abs, and

the mouse which had the higher 50% neutralization titer against parental DENV3 was

selected for fusion.

We noticed that some of the supernatants collected from the wells after the

fusion consisted of non-specific antibodies with strong affinity to the plastic. Such

supernatants or "plate binders" were not selected, irrespective of their neutralization

abilities. All the wells with non-plate binders were selected and further tested for their

reactivity with purified DENV3 and rEDIII antigens as well as neutralization of the

parental virus strain CH53489. Any supernatant that neutralized parental DENV3 was

further tested for differential neutralization of DENV3 genotypes. Twenty seven mAbs

were selected for further subcloning, purification and characterization such as

neutralization and binding to virus or rEDIII (table 3.1).

One major goal of developing mouse mAb against DENV3 was to isolate EDIII-reactive antibodies. Therefore, the initial attempts were focused on such antibodies, and this resulted in the identification of four EDIII-reactive mAbs. Two of these mAbs were used in experiments described in chapters 4 and 5 of this thesis, and development and characterization of the other mAbs are in progress.

3.5 Discussion:

Mouse mAb against DENV3 were successfully developed. The panel of mAb varied from non- neutralizing antibodies to neutralizing mAb which bind to either virus or rEDIII or both (table 3.1). Amino acid variability of the E protein among the members of DENV3 may be critical in determining the neutralization potential of a mAb. Since the panel of mAbs developed contained rEDIII binding and non-rEDIII binding mAbs, those mAbs will be a useful tool to study the neutralization mechanisms of DENV3, including differential neutralization among the members of DENV3 genotypes. In addition, it has been shown that EDIII-reactive antibodies play a minor role in type-specific neutralization after natural infection in human (chapter 5). Thus, non-EDIII binding neutralizing mAb will be a useful tool to study the epitopes that are targeted by type-specific neutralizing antibodies in human sera using competitive binding assays.

3.6 References:

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Name of the mAb	ELISA reactivity		Neutralization of DENV3 genotype			
	Virus[1]	rEDIII[2]	I	II	III	IV
2B1	+	-	+	-	-	+
2B4	+	+	-	-	-	-
2B5	+	+	-	-	-	-
2D3	+	+	nd[3]	+	nd	nd
2D4	+	-	-	-	-	+
3B1	+	+	+	-	-	+
3B2	+	+	-	-	-	+
3D2	+	+	+	+	-	+
5B5	+	-	+	+	+	-
7A4	+	-	+	+	+	+
7B6	+	+	+	+	-	-
7D4	+	-	+	+	+	-
8A3	+	-	+	+	+	+
8A5	+	+	-	-	-	-
8A6	+	-	+	-	-	-
8C3	+	+	-	-	-	-
9D1	+	-	+	+	+	+
10B6	+	-	+	+	+	-
10C5	+	-	+	+	-	+
12A1	+	+	-	-	-	+
12A6	+	-	-	-	-	+
12B2	+	-	-	+	-	+
12B4	+	+	+	-	-	+
12C1	+	+	-	-	-	+
13A5	+	+	+	-	-	+
13A6	+	+	-	-	-	-
13B1	+	-	nd	+	nd	nd
13B2	+	-	-	-	-	-

1. Strain CH53489 (DENV 3genotype II)
2. from strain CH53489 (DENV 3genotype II)
3. Not determined

Table 3.1 Binding and neutralization properties of mouse mAb
ELISA was conducted with purified virus and rEDIII protein. A representative strain from each genotype of DENV3 was used in neutralization assays.

Chapter 4

Natural Strain Variation and Antibody Neutralization of Dengue Serotype 3 Viruses

[**WMPB Wahala**, Eric F. Donaldson, Ruklanthi de Alwis, Ralph S. Baric and Aravinda M. de Silva**,** submitted for publication.

Design of rEDIII constructs, expression and purification of rEDIII protein, purification of virus, design of experiments, initial sequence analysis to identify the amino acid difference on EDIII lateral ridge, and data analysis were conducted by WMPB Wahala. Identification of informative sites on EDI EDII, and EDIII were done by Eric F. Donaldson and Ralph S. Baric. Ruklanthi de Alwis purified mAbs 8A1 and 14A4.**]**

4.1 Abstract

Dengue viruses (DENVs) are emerging, mosquito-borne flaviviruses which cause

dengue fever and dengue hemorrhagic fever. The DENV complex consists of 4 serotypes

designated DENV1-DENV4. Following natural infection with DENV, individuals develop

serotype-specific, neutralizing antibody responses. Monoclonal antibodies (mAbs) have

been used to map neutralizing epitopes on dengue and other flaviviruses. Most serotype-

specific, neutralizing mAbs bind to the lateral ridge of domain III of E protein (EDIII). It has

been widely assumed that the EDIII lateral ridge epitope is conserved within each DENV

serotype and a good target for vaccines. Using phylogenetic methods, we compared the

amino acid sequence of 175 E proteins representing the different genotypes of DENV3 and

identified a panel of surface-exposed amino acids, including residues in EDIII, that are

highly variant across the four DENV3 genotypes. The variable amino acids include six

residues at the lateral ridge of EDIII. We used a panel of DENV3 mouse mAbs to assess the

functional significance of this naturally occurring amino acid variation. From the panel of

antibodies, we identified three neutralizing mAbs that bound to EDIII of DENV3.

Recombinant proteins and naturally occurring variant viruses were used to map the binding

sites of the three mAbs. The three mAbs bound to overlapping but distinct epitopes on

EDIII. Our empirical studies clearly demonstrate that the antibody binding and

neutralization capacity of two mAbs was strongly influenced by naturally occurring

mutations in DENV3. Our data further demonstrate that the lateral ridge "type specific"

epitopes are not conserved between naturally occurring strains of DENV3. This variability

should be considered when designing and evaluating DENV vaccines, especially those

targeting EDIII.

4.2 Introduction

Dengue viruses (DENVs) are mosquito-borne flaviviruses and the agents of dengue fever and dengue hemorrhagic fever (DHF). According to the World Health Organization, over 2.5 billion people are at risk of contracting dengue, 100 million people develop symptomatic infections and up to 50,000 die from DHF each year. The DENV complex consists of 4 serotypes (DENV1-DENV4). DENVs have antibody epitopes that are unique to each serotype and epitopes that are cross-reactive between serotypes. People who have recovered from primary DENV infections develop long term, protective immune responses against the homologous serotype only. In fact, individuals exposed to a second infection with a different serotype face a greater risk of developing DHF, indicating that pre-existing immunity can exacerbate disease under some conditions [1].

As previously infected individuals do not appear to be re-infected with the same serotype, it is widely assumed that neutralizing antibody epitopes are conserved among strains belonging to the same serotype [2,3]. In fact, the current strategy for developing dengue vaccines is based on the assumption that a neutralizing immune response directed to a single strain will protect against most if not all strains of DENV within the serotype. However, there is considerable genetic diversity within each serotype such that each has been subdivided into genotypes [4]. Despite this diversity, surprisingly few studies have explored how naturally occurring strain variation within each serotype influences DENV neutralization phenotypes. In a study of pediatric dengue cases in Thailand, investigators observed significant differences in the ability of sera to neutralize reference and clinical strains of DENV3 [5]. Guzman and colleagues reported that amino acid sequence differences between DENV3 strains can have strong influences on virus neutralization by

murine and human immune sera [6]. Studies with other flaviviruses have also demonstrated

that neutralization is dependent on the lineages and strains used in the assay [7,8]. Thus, the

current paradigm may not accurately depict the complexity of DENV antigenic relationships,

especially in DENV3.

Antibodies, in particular, have emerged as key effector molecules responsible for

protective and pathogenic immune responses to DENV [1]. The DENV envelope (E) protein

is the major target of neutralizing antibody [9]. E protein mediates attachment to host cells

and low pH fusion of the viral and host cell membranes. The crystal structures of E from

several flaviviruses (Tick borne encephalitis, DENV2, DENV3 and West Nile) have been

solved [10-13]. Individual subunits of E protein consist of three beta-barrel domains

designated domains I (EDI), II (EDII) and III (EDIII) and the native protein is a homodimer

[10,11,13]. Mouse monoclonal antibodies (mAbs) that bind to all three domains of DENV E

have been generated and characterized [9,14,15]. The most potent neutralizing mAbs bind to

an epitope on the lateral ridge of EDIII of flaviviruses [9,16,17]. This epitope, which is not

conserved between dengue serotypes, has been the focus of much recent work because it

might be the target of the natural human immune response that leads to type-specific

neutralization. Investigators are also testing EDIII as a vaccine for inducing antibodies that

neutralize a specific serotype, without inducing serotype cross-reactive antibodies with

potential for disease enhancement [18,19].

In the present study, we have examined the evolution and phylogenetic relatedness of

the E protein sequences from a large number of viruses representing the different genotypes

of DENV3. These data reveal a complex evolutionary pattern that is not characterized by a

sequential progression of changes over time as has been described for influenza and

noroviruses [20,21]. Rather, many surface exposed amino acids were variable between established genotypes of DENV3. Especially noteworthy was the observation that the EDIII lateral ridge, which is a known site targeted by neutralizing mAbs in related flaviviruses, was variable between DENV3 genotypes. We experimentally demonstrate that naturally occurring amino acid differences in DENV3 EDIII lead to differential binding and neutralization by mAbs.

4.3 Materials and Methods

Cells and Viruses:

Aedes albopictus C6/36 cells were maintained at 28C in DMEM supplemented with 10% FBS, penicillin, streptomycin in the presence of 5% CO_2. Human leukemic monocyte lymphoma cell line U937 expressing DC-SIGN (U937 DC-SIGN) were maintained at 37C in RPMI supplemented with 10% FBS, penicillin, streptomycin and 50 mM of beta mercaptoethanol in the presence of 5% CO_2. DENVs were grown in C6/36 cells in DMEM supplemented with 02% FBS, penicillin, streptomycin in the presence of 5% CO_2. Virus strains used in the study were West Pacific 74 (DENV 1), S16803 (DENV2), UNC 3043 (DENV3 -genotype I), CH53489 (DENV3 - genotype II), UNC 3009 (DENV3 - genotype III), PR77- 1342 (DENV3 -genotype IV), and TVP-360 (DENV4).

Monoclonal Antibodies

mAbs 8A1 and 14A4 against DENV3 were provided by Robert Putnak (Walter Reed Army Institute, MD). mAb1H9 was provided by John Aaskov (Queensland University of

Technology, Australia) [22]. mAbs 8A5 and 12C1 were generated for this study by immunizing mice with purified DENV3 strain CH53489.

Expression and purification of recombinant EDIII (rEDIII)

Envelope gene fragments encoding EDIII from DENV1, DENV3, and DENV 4 (AA295-398) and DENV2 (AA297-399) were amplified using Vent polymerase (NEB, Ipswich, MA). Reverse primers used in the study were designed to introduce either a HindIII (for DENV2-4) or PstI (for DENV1) restriction site at the 3' ends of the PCR products. PCR products were digested with HindIII or PstI and cloned into pMAl C2X (NEB, Ipswich, MA) vector using DH5a competent cells to generate fusion proteins of rEDIII and maltose binding protein (MBP). Recombinant EDIII was expressed and purified using an amylose resin (NEB, Ipswich, MA) and following the manufacture's instructions.

Mutagenesis of recombinant EDIII (rEDIII)

Primers were designed using QuikChange® Primer Design Program (www.stratagene.com) and selected amino acids residues on rEDIII were mutated by site directed mutagenesis using Quickchange multi kit (Stratagene, La Jolla, CA) according to manufacturer's instructions. Mutated single stranded pMal c2X plasmids (NEB, Ipswich, MA) were cloned into DH5α cells for expression and purification of mutant rEDIIIs. All mutants were confirmed by sequencing.

Binding ELISA with rEDIII protein

ELISA plates were coated with 200ng of purified EDIII-MBP protein in Carbonate buffer overnight at 4C. An antibody that binds to MBP (NEB, Ipswich, MA) was used to

confirm equal binding of recombinant EDIII proteins to the ELISA plate.   The plates were washed and blocked with 3% normal goat serum (NGS) in Tris-buffered saline with 0.1% Tween 20 (TBST) for an hour at 37C. Serially diluted mAbs in (blocking buffer) were then added to each well and incubated for one hour at 37C. After washing 3 times with TBST, alkaline phosphatase conjugated goat anti-mouse antibody was added to each well and incubated for one hour at 37C. Plates were washed three times with TBST, and color was developed by adding SIGMA FAST p-Nitrophenyl Phosphate tablets. Optical density (OD) was measured at 405 nm using a spectrophotometer.

Neutralization assays

The neutralization protocol as described by Krause, *et. al*., was used with modifications to determine 50% neutralization values for each antibody [23]. In experiments comparing different genotypes of DENV3, the same amount of virus (genome copies) was used to infect cells with each genotype. Different concentrations of mAbs were mixed with virus strains in a 96 well tissue culture plate and maintained at 37C in CO_2 incubator for one hour. After the incubation, U937DC-SIGN cells were added to each well and maintained for an additional 2 hr for adsorption of virus. The cells were then washed with medium, and 200 ul of fresh media were added to each well and incubated for 24-72 hr at 37C under 5% CO_2. After washing 2 times with PBS, the cells were permeabilized and fixed using CytoFix/ Cytoperm kit (BD bioscience). The cells were then stained with Alexa 488 conjugated anti-dengue mAb 2H2, and the percentage of infected cells was measured in a flowcytometer. EC50 values were calculated using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) and nonliner regression analysis.

DENV3 Sequence Analysis

A total of 175 unique DENV3 full-length envelope protein sequences were downloaded from GenBank and these were aligned using ClustalX version 1.83 [24] using the PAM distance matrix and default parameters. A variety of parameters and substitution matrices for the alignment were evaluated using the program TuneClustalv1.0 (http://www.homepage.mac.com/barryghall/Software.html), and the PAM series matrix was determined to be the most appropriate, with default gap opening and extension values. The alignment was refined manually, and 47 sites of variation, defined as any site with a quality score of less than 100, were exported in table format and ordered by genotype and domain. A representative subset of sequences was selected to represent specific DENV3 genotypes, and these contained 32 informative sites. Informative sites were defined as columns of heterogeneity in the alignment where the same amino acid change occurred in at least three independent sequences.

4.4 Results

Variable Amino acids in the DENV3 E protein

As individuals infected with DENV develop a long term, protective immune response to the homologous serotype, it has been assumed that neutralizing antibody epitopes are conserved within each serotype [9,25]. To further evaluate this assumption, we used phylogenetic approaches to compare the evolution of the full length E protein sequence of 175 DENV3 strains. The sequences, which were obtained from Genbank, were shown to contain representatives of each of the 4 recognized genotypes of DENV3 [26]. Informative sites were defined as positions that were variable in 3 or more independent sequences as

identified by alignment. Of the 493 amino acids encoding the DENV3 E protein, 32 sites

encoded informative mutations (Figure 4.1). Individual subunits of E protein consist of three

beta-barrel domains designated domains I (EDI), II (EDII) and III (EDIII) (Figure 4.2A).

Informative sites were present in all domains of the E protein (Figure 4.1). Many of the

informative sites had mutations that were conserved within but not between DENV3

genotypes (Figure 4.1). As the structure of the ectodomain (AA 1 to 392) of DENV3 E

protein has been solved, we were able to determine if the variable sites were either surface

exposed or buried [11]. Eighteen of 28 sites in the ectodomain were surface exposed, while

others were partially or completely buried in the molecule (Figure 4.1).

Relationship of DENV3 E protein informative sites to known antibody epitopes

We compared the locations of known antibody epitopes on the flavivirus E protein

and the positions of informative sites on DENV3 E protein. Mouse monoclonal antibodies

(mAbs) that bind to EDI and II have been mapped to six regions (Figure 4.2A and Table 4.1)

[9,16]. Most of the informative sites were within or adjacent to these antigenic regions

(Figure 4.2A and Table 4.1). However, the antigenic region at the fusion loop was

completely conserved between DENV3 strains (Table 4.1).

Many mouse mAbs that strongly neutralize flaviviruses bind to EDIII. DENV

serotype-specific (type-specific), neutralizing mAbs bind to epitopes on the lateral ridge of

EDIII, which is formed by three loops connecting the D-E, B-C, F-G beta strands on EDIII

and the linker region connecting EDIII and EDI (Figure 4.2B and Table 4.1) [9,16].

Investigators have also defined an epitope on EDIII recognized by mAbs that neutralize more

than one DENV serotype [14,27,28]. This DENV subcomplex epitope overlaps with the

lateral ridge epitope but is centered at positions 305-308 (DENV3 numbering) on the A strand of EDIII (Figure 4.2B and Table 4.1). We compared the positions of known antibody epitopes and DENV3 informative sites on EDIII. The dengue type specific, lateral ridge epitope overlapped extensively with the informative sites on EDIII (Figure 4.2B, Table 4.1). This analysis supports the hypothesis that the EDIII lateral ridge epitope engaged by strongly neutralizing mouse mAbs is not conserved between DENV3 strains.

Mapping of DENV3, EDIII reactive antibodies

To directly address whether natural amino acid variation in DENV3 EDIII results in altered antibody binding and neutralization, we assembled and mapped a panel of DENV3 EDIII reactive mouse mAbs. The sources and general properties of mAbs used in the current study are listed in Table 4.2. All 5 antibodies selected for this study (8A1, 1H9, 14A4, 8A5, and 12C1) bound to EDIII of DENV3 (Figure 4.3). 8A1 and 1H9 were type specific and only bound to DENV3, EDIII. 14A4 was sub-complex specific and bound to DENV3 and DENV1 (Figure 4.3). 3B12 and 12C1 cross reacted with EDIII from all 4 serotypes (Figure 4.3).

To map the binding sites of the mAbs, we expressed and purified 28 EDIII recombinant proteins with defined mutations, primarily in the lateral ridge loops. The binding of each antibody to wild type and mutant proteins was compared by ELISA (Table 4.3). mAb 1H9 is a type specific, neutralizingDENV3, EDIII reactive IgM antibody that has previously been shown to select for escape mutation at position 386 [22]. We observed a greater than 80% loss of binding of 1H9 when amino acids at positions 302,304,308,310 323,

325-330, 357, 358, 361, 380, 382 or 386 were mutated on EDIII (Table 4.3). Most of these mutations are on the lateral ridge of EDIII (Figure 4.4).

mAb 8A1 is a strongly neutralizing, type-specific DENV3, EDIII-reactive IgG antibody. With this antibody, we observed a greater than 80% loss of binding when amino acids at positions 301, 302, 304, 326-328, 330, 361, 380, 382 or 386 were mutated on EDIII (Table 4.3). As in the case of 1H9, most of these positions overlap with the EDIII lateral ridge epitope. However, 1H9 and 8A1 did not bind to identical epitopes because some mutations that influenced 1H9 had marginal to no effect on 8A1.

mAb 14A4 is a neutralizing EDIII reactive IgG antibody that cross-reacts with DENV3 and DENV1 (Figure 4.3). This DENV subcomplex antibody bound poorly to recombinant proteins with mutations at position 308 (A strand), or positions 326 or 328 (B-C loop) (Table 4.3). These mutations are located at a similar position to a DENV EDIII sub-complex epitope recently described in the literature [14,27]. The subcomplex epitope overlaps with the lateral ridge but is centered on the A strand of EDIII.

The DENV complex cross-reactive mAbs (8A5, 12C1) bound to all the mutant proteins indicating these antibodies likely bind to a cross-reactive epitope outside the lateral ridge region (Table 4.3). In Figure 4.4 we display the structure of DENV3 EDIII and the location of mutations that reduced the binding (>80%) of each mAb.

Binding of mAbs to different genotypes of DENV3

Several amino acid positions (301, 302, 329, 380 and 386) on EDIII implicated in binding to mAbs 8A1 and 1H9 (Table 4.3) were also identified as informative sites that were not conserved between DENV3 genotypes (Figure 4.1). All these positions are located in

close proximity to one another on the EDIII lateral ridge. To directly address whether naturally occurring variation at these informative sites leads to altered antibody interactions, we compared the binding of mAbs 1H9, 8A1 and 14A4 to representative EDIII from each of the 4 distinct genotypes of DENV3.   mAbs 8A1 and 1H9 bound to genotypes I, II and III but not to EDIII from genotype IV (Figure 4.5). The DENV subcomplex specific 14A4 antibody bound to EDIII from all 4 genotypes (Figure 4.5). Studies were also performed to compare the binding of these mAbs to purified viruses (Figure 4.6). As predicted from the recombinant EDIII binding experiments, 1H9 bound to DENV3 genotype I, II and III viruses but not to the genotype IV virus (Figure 4.6). Similarly, mAb 8A1 bound to genotypes I, II, and III but not to the genotype IV (Figure 4.6). mAb 14A4 bound to all 4 genotypes. These results indicate that naturally occurring amino acid variation on DENV3 EDIII influence the binding of type-specific antibodies.

To verify that amino acid differences at the EDIII lateral ridge were responsible for mAb binding differences, further studies were conducted with mAb 8A1 and recombinant EDIII proteins. We systematically changed amino acids in the EDIII genotype IV construct to genotype II and defined the minimum number of changes required to restore the 8A1 epitope. In Figure 4.7 we depict the EDIII amino acid differences between the different genotypes. Simply making single amino acids changes at positions 301 or 302 did not restore binding.   Some binding was regained when both 301 and 302 were changed from SG (genotype IV) to LN (genotype II) (Figure 4.7). Full binding was restored when positions 301, 302 and 380 were changed (Figure 4.7) indicating that these were the critical changes that led to the loss of binding of mAb 8A1 to DENV3 genotype IV.   Residues 301, 302 and 380 are surface-exposed neighbors on the lateral ridge of EDIII, with residues 301 and 380

separated by approximately 4.7 Angstroms. Thus, these three residues are likely to be a part of a single epitope bound by 8A1.

Neutralization of DENV3 genotypes by MAbs

Experiments were conducted to compare the ability of EDIII mAbs to neutralize different genotypes of DENV3. mAbs 8A1 and 1H9 failed to neutralize DENV3 genotype IV, which was expected since these antibodies did not bind to this virus (Table 4.4). Surprisingly, even though we did not observe differences in the binding of 8A1 and 1H9 to genotype I, II and III viruses, we did observe differences in the neutralization titer (Table 4.4). For example the neutralization titers for 8A1 arranged nearly 10 fold between genotype I and III viruses (Table 4.4). 1H9 displayed a 60 fold difference in the neutralization titer between genotype I and II viruses (Table 4.4). These results indicate that two mechanisms influence the ability of mAbs to neutralize virus infectivity. In the first, mutations which ablate binding also ablate neutralization. In the second, genetic differences between DENV3 strains that have little effect on apparent affinity can have significant biological effects on neutralization.

4.5 Discussion

A long-held paradigm in flavivirus research has been that DENVs display little if any intra-serotypic antigenic variation, and this has been the basis for the development of current multivalent vaccines and immunotherapeutics [9,16]. The main goal of the current study was to characterize the extent of envelope protein variation within the DENV3 serotype and to determine if this variation influenced antibody binding and neutralization. Sequence and

structural analysis of the E protein indicated that 7% of the amino acids were variable between the four genotypes of DENV3, and most of the non-conserved residues were surface exposed and located at or proximal to known antibody binding sites. Particularly striking was the variation observed on the lateral ridge of domain III, which has previously been identified as the target of antibodies that strongly neutralize flaviviruses [9,16]. Finally, we demonstrated that natural variation in EDIII influences the ability of mAbs to bind and neutralize DENV3.

Studies with other flaviviruses have demonstrated that neutralization is dependent on the lineage and strain of these viruses [7,8]. Recent studies indicate that strain variation influences the neutralization of DENV3 as well. In a study with Thai subjects, Endy and colleagues observed differences in the ability of human immune sera to neutralize different strains of DENV3 [5]. Guzman and colleagues reported that amino acid differences in EDIII were responsible for poor binding and neutralization of some DENV3 strains compared to others [6]. Our results reported here, together with the other studies mentioned above, challenge the long held view that neutralizing antibody epitopes are conserved across DENV strains belonging to the same serotype.

Dengue virus evolution is complex, and, to date, there is little evidence of herd immunity driving the selection of novel strains [29]. Studies have demonstrated that individuals exposed to a secondary infection with a different serotype have a higher serum viremia and face a greater risk of developing DHF. A leading theory to explain the more severe nature of secondary dengue is that serotype cross reactive, non-neutralizing antibodies from the first infection enhance the ability of the virus to infect Fc-receptor bearing host cells, which initiates a cascade of events that lead to DHF [25]. Our results reported here

indicate that the different genotypes of DENV3 encode variable antibody epitopes on the surface of the viral envelope protein. Studies are needed to address how antigenic differences within a serotype might alter the ability of human antibodies to enhance DENV as well. Although speculative, complex pre-exposure histories may select for specific genotypes or strains of DENV that are poorly neutralized and/or preferentially enhanced by pre-existing immunity.

Our results show that EDIII lateral ridge antibodies 8A1 and 1H9 bound to DENV3 genotypes I, II and III but not genotype IV indicating that naturally occurring mutations in EDIII can lead to a total loss of mAb binding. Even though mAbs 8A1 and 1H9 bound to DENV3 genotypes I, II and III with similar apparent affinity measured by ELISA, we observed striking differences in the ability of the mAbs to neutralize these viruses. The neutralization titers were almost 10 fold different between viruses for 8A1 and nearly 60 fold different for 1H9. The small differences in Kd values (fig 4.6) among the genotypes of DENV3 did not correlate with the rank order of neutralization (table 4.4). Our results indicate that apparent affinity of antibody to virus immobilized on ELISA wells is not always predictive of the neutralization titer. There are amino acid differences on the EDIII lateral ridge of genotype I, II and III viruses (Figure 7.7), and these changes may lead to subtle changes in virus antibody interactions that are not detected in our solid phase binding assay. As viral binding and entry are accompanied by overall changes in the conformation of E protein from a dimer to a trimer, even mutations distal to EDIII might influence the ability of antibodies to bind the lateral ridge and neutralize DENV3. Other investigators have also documented that apparent affinity is not always correlated with the neutralization strength of antibodies against viruses, including flaviviruses [30-32]. Further studies are needed to

dissect the mechanism underpinning the ability of EDIII lateral ridge antibodies to neutralize different genotypes of DENV3.

Studies with DENV2 have demonstrated the presence of antibody epitopes on the lateral ridge, A strand and AB loop. The lateral ridge and A strand epitopes are recognized by type-specific and cross-reactive neutralizing mAbs respectively, whereas the AB loop epitope is the target of weakly or non-neutralizing mAbs [14]. Our results demonstrate a similar distribution of epitopes on DENV3. mAbs 1H9 and 8A1 define a type specific epitope on the lateral ridge of DENV3, EDIII. mAb 14A4 recognizes a dengue sub-complex epitope centered on the A strand. Others have also described a dengue complex or sub-complex epitope centered on the A strand of DENV3 [33]. None of the mutations we made in and around the lateral ridge region influenced the binding of mAbs 8A5 and 12C1, which were serotype cross-reactive, non-neutralizing antibodies. We suspect these antibodies bind to a recently described epitope on the A-B loop that is poorly exposed on the virion and the target of other weakly or non-neutralizing antibodies [14]. Thus, the EDIII epitope landscape of DENV3 may well be similar to DENV2, consisting of type-specific and cross-reactive epitopes.

The experiments reported here focused on antibodies that bind to EDIII. Our DENV3 sequence analysis indicates that many antigenic regions in EDI and EDII are also poorly conserved across DENV3 strains. Further studies are needed to identify key epitopes in EDI and EDII and to evaluate their conservation both within and between serotypes. While this work focused on DENV3, our results suggest that intraserotype strain variation might also influence the neutralization of other serotypes. Kochel and colleagues reported that the American genotype of DENV2 was more sensitive than the Asian strain of DENV2 to cross-

neutralization by DENV1 immune sera [34]. Further studies are needed to characterize the

extent of intraserotype variability and the neutralization characteristics of DENV1, 2 and 4,

as well.

In summary, our results indicate that the EDIII lateral ridge epitope targeted by

strongly neutralizing antibodies is not conserved between the different genotypes of DENV3.

Recently we discovered that people exposed to natural DENV infections have low levels of

EDIII reactive antibody and that antibodies that bind to epitopes outside EDIII were most

likely responsible for the neutralizing activity in human immune sera (chapter 5). Thus, not

only is EDIII variable between strains, but natural human infection does not induce high

levels of antibody to this domain. Antibody epitopes in EDI and II are likely to play a more

important role in the neutralizing human antibody response to DENV.

4.6 References

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Domain of DENV3 E protein[1]	Antigenic region[2]	Flavivirus E protein mutations that influence mouse Mab binding[3]	DENV3 E protein informative sites within antigenic region[4]
EDI (AA 1-52, 132-191, 278-294)	Lateral Ridge	166-169, 179, 291	160, 169 171, 172
	EDI-EDII Interface	49-52, 136, 184-187, 268-277	132, 139, 270
EDII (AA 53-131, 192-278)	Central Region	123-128, 210, 215, 232-233	62, 63, 120, 124, 224, 226, 231
	Lateral Ridge	67-72, 75-76, 81-83, 86, 112	68, 81
	Fusion Loop	99-107	
EDIII (AA 295-392)	Lateral Ridge	301-303, 327-330, 381-382	301-303, 329,355, 380, 383
	A Strand	305-308	

[1]The amino acids that form each domain of DENV3 E protein are according to [Modis, 2005 #95].

[2]The antigenic regions are based on the assignments and nomenclature used by [Pierson, 2008 #100]

[3]The flavivirus E protein mutations that alter binding of MAbs are reviewed in [Pierson, 2008 #100; Roehrig, 2003 #103]. The numbering is based on theDENV3 E protein sequence.

[4]DENV3 E protein informative sites were identified as described in Table 1.

Table 4.1 Location of antigenic sites and informative sites on DENV3 E protein

mAb	Isotype	Source
8A1	IgG1	R. Putnak - WRAIR[1]
14A4	IgG1	R. Putnak - WRAIR
1H9	IgM	Serafin. IL and Aaskov JG. Archives of Virology 2001;146 :2469
8A5	IgG1	UNC-[2] this study
12C1	IgG1	UNC- this study

1. Walter Reed Army Institute of Reserach, MD, USA
2. University of North carolina at Chapel Hill, NC, USA

Table 4.2. Source of mouse mAb used in the current study

		Mouse mAb		
Mutation	1H9	8A1	14A4	8A5	12C1
I301A	97	**13**	100	100	99
I301G	52	**3**	97	100	100
N302A	**9**	**4**	95	100	99
N302G	223	**12**	100	98	99
T303A	107	77	100	100	100
T303G	43	42	100	100	98
F304A	**15**	**5**	30	90	100
K308A	**16**	37	**5**	100	96
V310A	**16**	54	83	97	100
E323G	**17**	69	99	100	95
K325A	57	74	38	99	98
K325G	**12**	25	80	90	100
G326A	**10**	**5**	**18**	96	97
E327A	**13**	**4**	72	98	100
D328A	**10**	**4**	**5**	93	100
A329G	**11**	28	74	100	96
P330A	**11**	**6**	29	95	100
T357A	35	76	100	97	98
T357G	**13**	47	100	99	97
K358G	**17**	50	100	95	100
E361G	**6**	**10**	100	98	100
I380A	**18**	**5**	97	98	96
I380G	**4**	**4**	88	96	98
D382G	**5**	**15**	90	98	95
K383A	106	92	100	100	99
K383G	101	97	99	99	100
K386A	**4**	35	97	96	98
K386G	**4**	**15**	96	98	100

[1] Binding to each mutant expressed as a percentage of binding to
   wild type EDIII protein from genotype II
[2] The values in bold indicate mutations that reduced mAb biding
   by >80% compared to the wild type EDIII

Table 4.3. Binding of mouse mAbs to mutant DENV3 EDIII proteins

	50% Neutralization titer[1] (µg/ml ± std error of mean)			
		DENV3 genotype		
mAb	I	II	III	IV
8A1	0.43 ± 0.15	1.3 ± 0.32	4.4 ± 0.67	NN[2]
1H9	0.02 ± 0.002	1.2 ± 0.69	0.37 ± 0.02	NN
14A4	0.64 ± 0.34	1.8 ± 0.54	2.2 ± 0.18	0.25 ± 0.02

[1] 50% neutralization titer determined using U937+DC-SIGN expressing cells
[2] Not neutralized

Table 4.4 . Neutralization of DENV3 genotypes by EDIII mAbs

Figure 4.1: Informative sites in the Envelope Protein of dengue serotype 3. One hundred and seventy five DENV3 envelope protein sequences were aligned and informative sites exported. DENV3 is divided into 4 genotypes (I, II, III and IV) [26]. The envelope protein is divided into four domains indicated by the coloring on the position numbers. Red, domain I; yellow, domain II; blue, domain III; and cyan, transmembrane domain (TM). Residues that are unique to a given genotype are indicated by unique colors. Brown, genotype I; teal, genotype III; green, genotype IV; pink, unique polymorphisms; light yellow, predominant residues shared among multiple genotypes; and gray, variation shared among multiple genotypes.
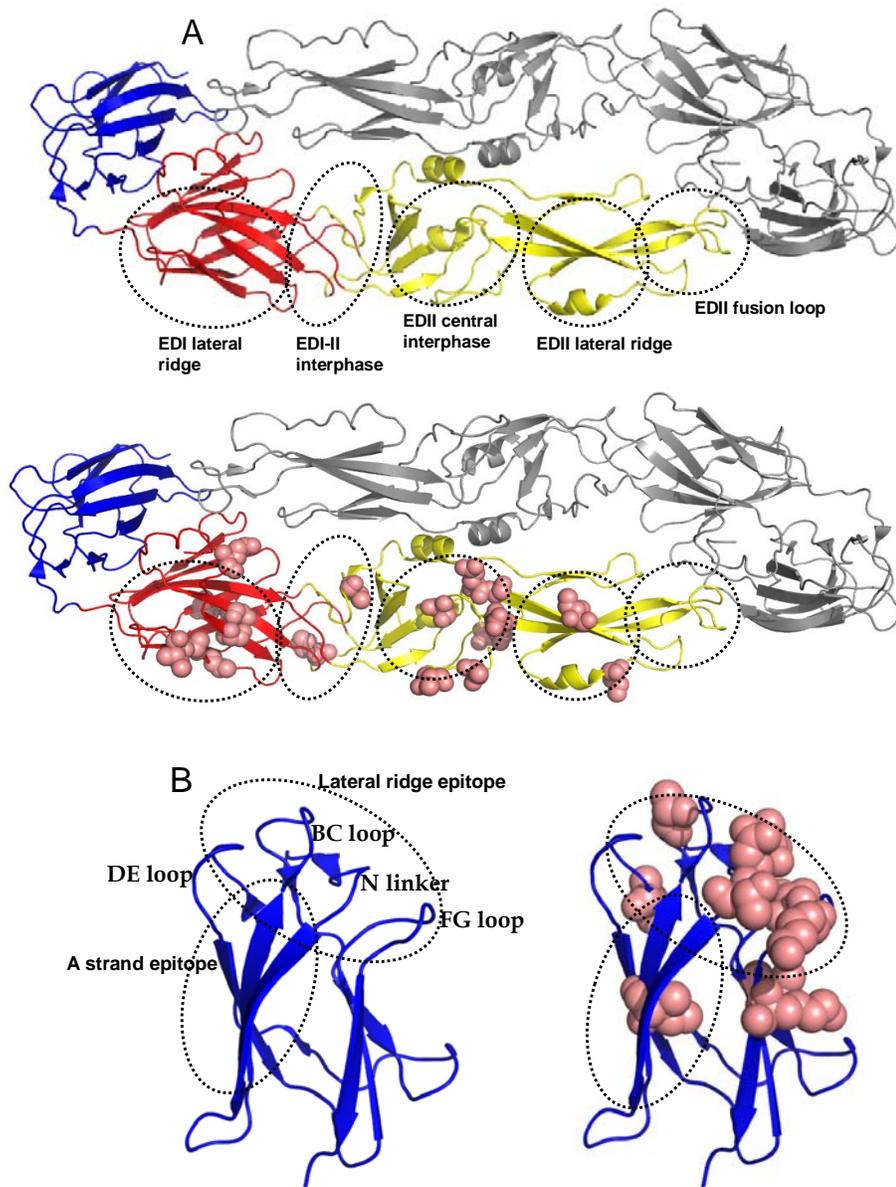
Figure 4.2. Location of mAb epitopes and informative sites on DENV3 E protein. The figure is based on the structure of the ectodomain of DENV3 E protein solved by Modis and colleagues. A. The flavivirus E protein consist of three beta-barrel domains designated domains I (red), II (yellow) and III (blue). The native protein is a homodimer that lies flat on the surface of the virus. The top image depicts the major antigenic sites on domains I and II (see Figure 4.1 for details). The bottom image displays the location of informative sites on domains I and II (pink). B. An enlarged view of domain III displaying antigenic sites and informative sites. The left image displays the lateral ridge and A strand epitopes. The right image displays the domain III informative sites (pink).
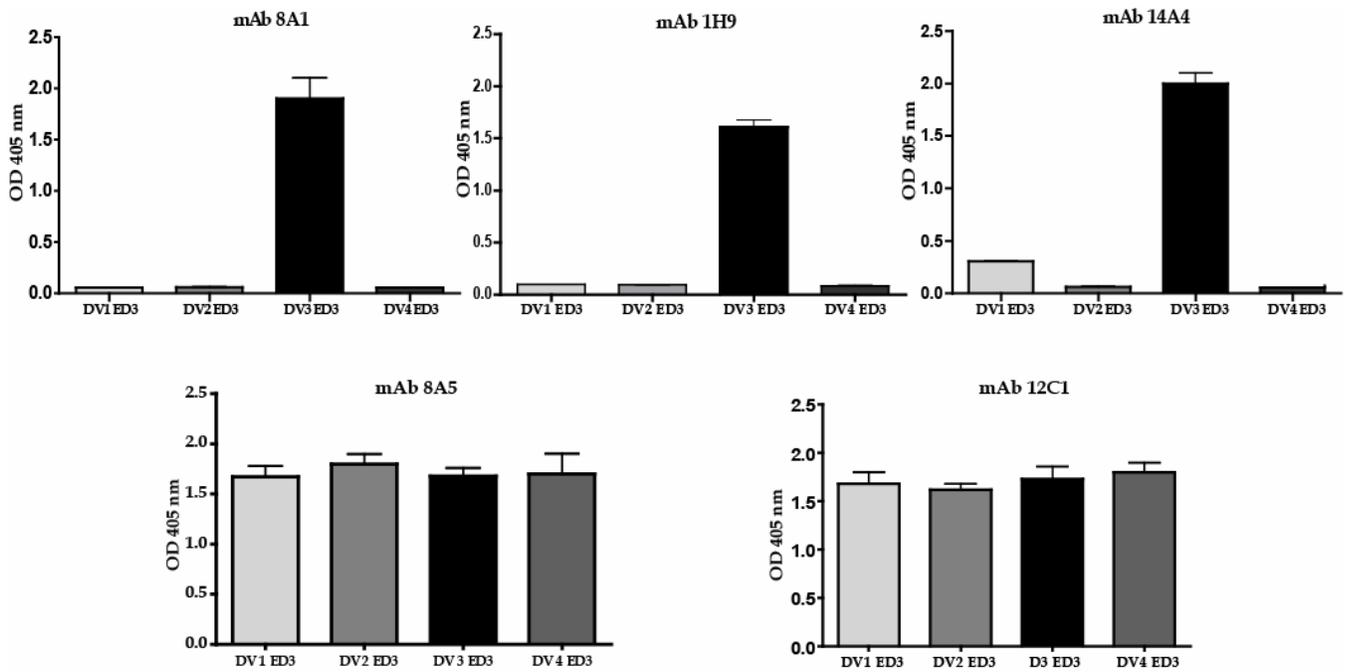
Figure 4.3. Binding of mouse mAbs to recombinant EDIII from the 4 serotypes of DENV. mAb binding was detected by ELISA. mAbs 8A1 and 1H9 bound to EDIII from DENV3 only. m14A4 bound to EDIII from DENV3 and to a lesser extent to EDIIII from DENV1. mAbs 8A5 and 12C1 bound to EDIII from all 4 serotypes.
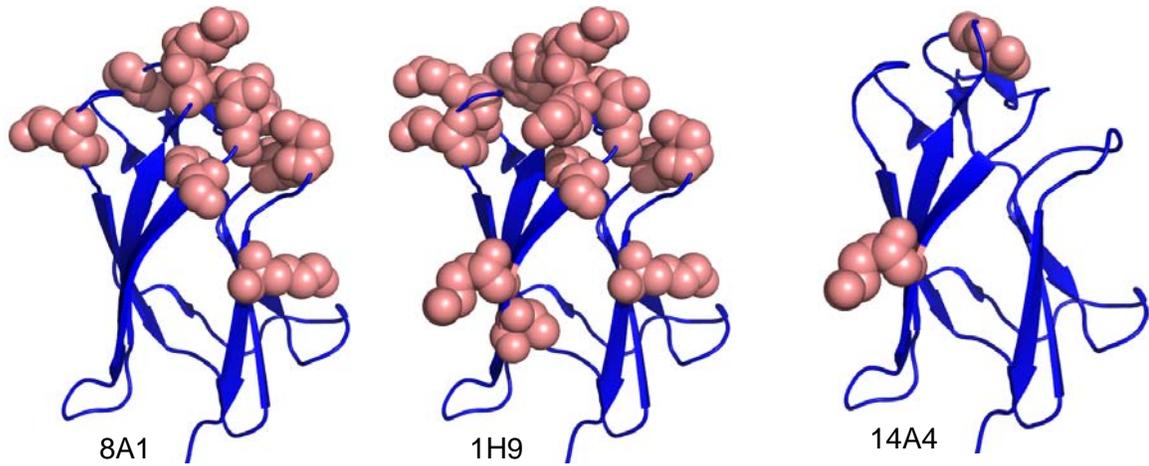
Figure 4.4. Mapping EDIII epitopes for mAbs 8A1, 1H9 and 14A4. The figure depicts the positions of mutations that reduced mAb binding by >80%. Many mutations mainly on the lateral ridge reduced binding of 8A1 and 1H9. In contrast only three mutations inhibited binding of 14A4.
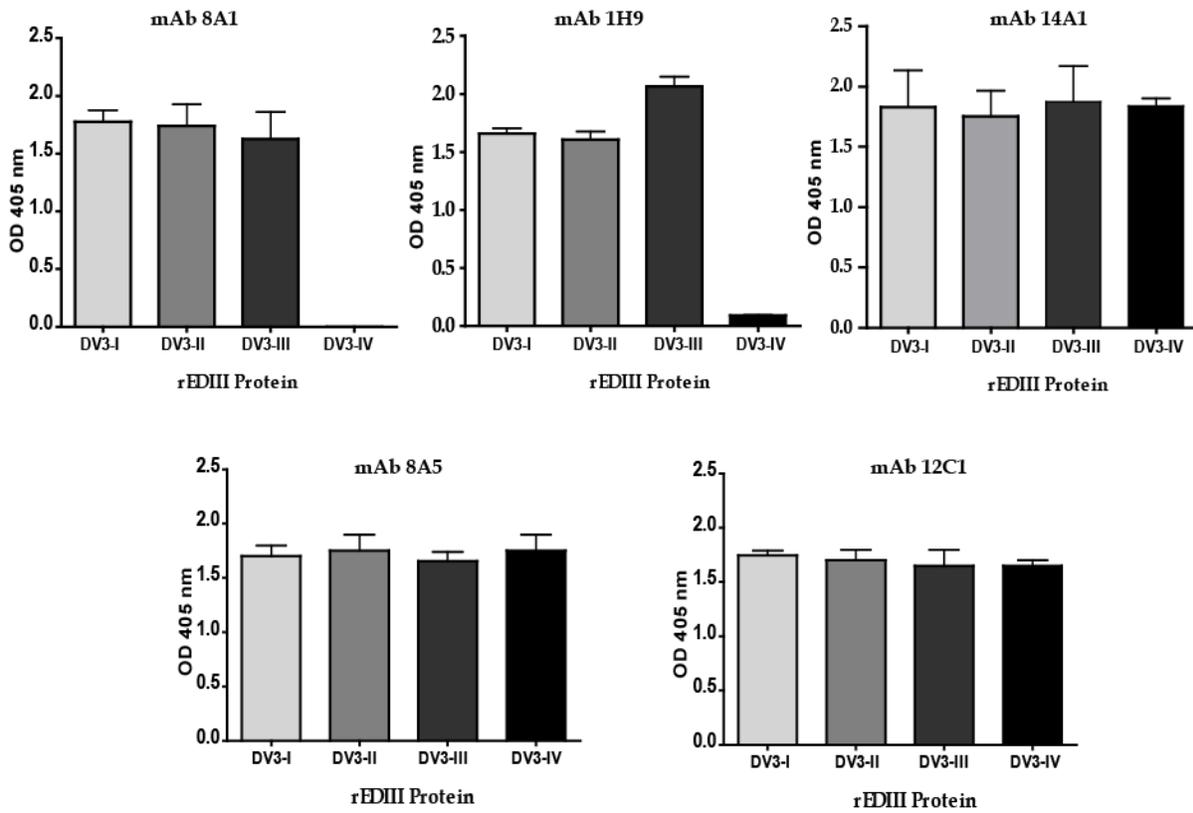
Figure 4.5. Binding of mouse mAbs to recombinant EDIII from the 4 genotypes of DENV3. mAb binding was detected by ELISA. mAbs 8A1 and 1H9 bound to EDIII from DENV3 genotypes I, II and III but not to IV. mAbs 14A4, 8A5 and 12C1 bound to all 4 genotypes.
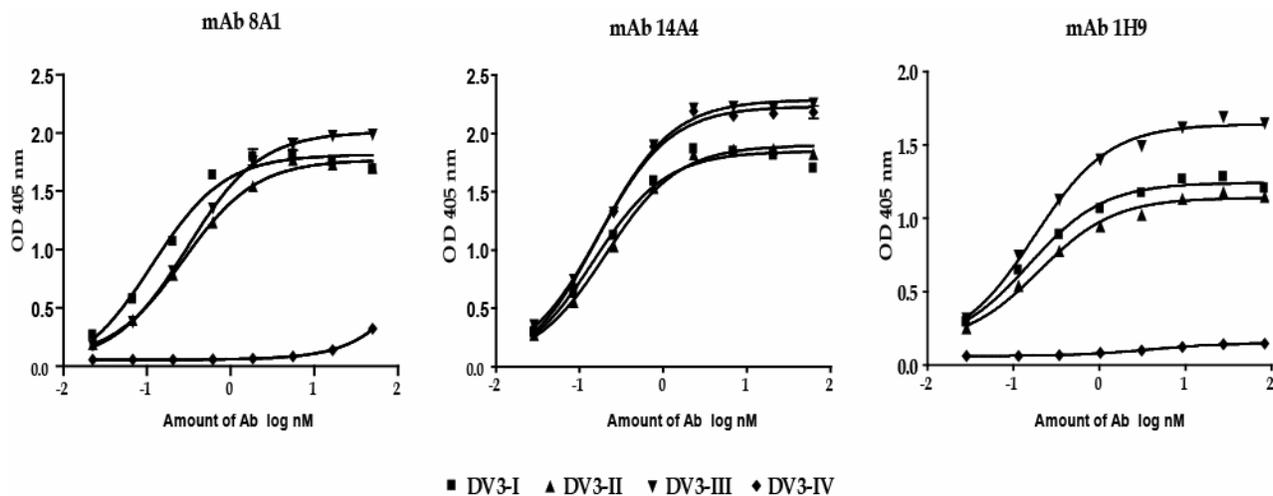
Figure 4.6. Binding of mouse mAbs to DENV3 genotypes. DENV3 genotype I (DV3-I), genotype II (DV3-II), genotype III (DV3-III) and genotype IV (DV3-IV) viruses were purified and used in binding assays with mAb 8A1, 14A4 and 1H9. mAb 14A4 bound to all 4 genotypes with similar apparent affinity. mAbs 8A1 and 1H9 bound to DENV3, genotypes I, II and III with similar apparent affinity, while no binding was detected with genotype IV virus.

Amino acid	DENV3 genotype			
Position	I	II	III	IV
301	L	L	T	S
302	N	N	N	G
303	A	T	T	T
357	T	T	T	I
380	I	I	I	T
383	K	K	N	K
386	K	K	K	R
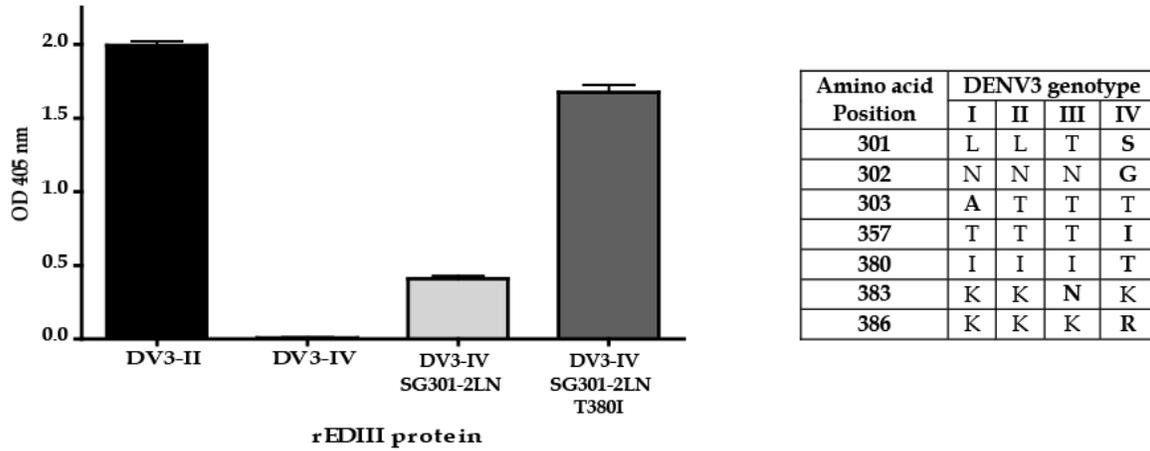
Figure 4.7. Identification of naturally occurring mutations that eliminate binding of mAb 8A1. The table displays the EDIII amino acid differences between the different genotypes of DENV3. mAb 8A1 bound to EDIII from DENV3 genotype II (DV3-II) but not genotype IV (DV3-IV). Binding was partially restored when positions 301 and 302 were changed from the genotype IV to genotype II (DV3-IV SG301-2LN)). Complete binding was restored when positions 301, 302 and 380 were changed (DV3-IV SG301-2LN, T380I.

CHAPTER 5

Dengue virus neutralization by human immune sera: role of envelope protein domain III - reactive antibody

Design of rEDIII constructs, expression and purification of rEDIII proteins, design and

conducting of experiments, data analysis, were done by WMPB Wahala. Kraus AA and

Haymore LB collected blood from human subjects used in the study and conducted initial

characterization of sera to determine the neutralization titer of each serum sample. Accavitti-

Loper MA developed mouse mAb 12C1 and 8A5 in collaboration with WMPB Wahala.]

5.1 Abstract

Dengue viruses (DENV) are the etiological agents of dengue fever (DF) and dengue hemorrhagic fever (DHF). The DENV complex consists of four closely related viruses designated DENV serotypes 1 through-4. Although infection with one serotype induces cross-reactive antibody to all 4 serotypes, the long term protective antibody response is restricted to the serotype responsible for infection. Cross-reactive antibodies appear to enhance infection during a second infection with a different serotype. The goal of the present study was to characterize the binding specificity and functional properties of human DENV immune sera. The study focused on domain III of the viral envelope protein (EDIII), as this region has a well characterized epitope that is recognized by strongly neutralizing serotype-specific mouse monoclonal antibodies (mAbs). Our results demonstrate that EDIII-reactive antibodies are present in primary and secondary DENV immune human sera. Human antibodies bound to a serotype- specific epitope on EDIII after primary infection and a serotype cross- reactive epitope on EDIII after secondary infection. However, EDIII-binding antibodies constituted only a small fraction of the total antibody in immune sera binding to DENV. Studies with complete and EDIII antibody-depleted human immune sera demonstrated that EDIII binding antibodies play a minor role in DENV neutralization. We propose that human antibodies directed to other epitopes on the virus are primarily responsible for DENV neutralization. Our results have implications for understanding protective immunity following natural DENV infection and for evaluating DENV vaccines.

5.2 Introduction

Dengue viruses (DENVs) are emerging, mosquito-borne flaviviruses and the

causative agents of dengue fever (DF) and dengue hemorrhagic fever (DHF). The DENV

complex consists of four serotypes designated DENV 1 through 4. A person infected with

DENV develops antibodies that cross react with all four serotypes [1]. However, the

antibodies only provide long-term protection against the serotype responsible for the original

infection and people can be infected a second time with a different serotype [2,3].

Individuals experiencing secondary DEN infections face a greater risk of developing severe

disease [2,3]. A leading theory to explain the greater risk of severe disease with secondary

DEN infection is that pre-existing cross reactive antibodies bind to the virus and enhance

infection of Fc-receptor bearing cells [4]. Despite the fact that DEN vaccines are entering

large scale clinical testing, we know remarkably little about the relationship between the

binding properties of DEN antibodies in human immune sera and the functional outcome of

these interactions.

The major target of flavivirus neutralizing antibody is the Envelope (E) protein,

although membrane protein (M) and non-structural protein 1 (NS1) antibodies have also been

shown to be protective [1,5,6]. E protein is responsible for viral attachment to host cells and

the low pH fusion of viral and host cell membranes. The crystal structures of E of several

flaviviruses have been solved [7-10]. Individual subunits of E consist of three beta-barrel

domains designated E domains I (EDI), II (EDII) and III (EDIII). Native E is a homodimer

that lies flat on the surface of the viral membrane.

Our current understanding of the interactions between DENV and antibody is largely

based on studies with mouse monoclonal antibodies (mAbs). DENV neutralizing mouse

mAbs have been mapped to all three domains of E. In general, strongly neutralizing mouse mabs are DENV serotype-specific and bind to an epitopes on EDIII that is unique to each serotype [11-16]. A DENV type-specific epitope on EDIII bound by strongly neutralizing mabs has been mapped to 4 loops on the lateral face of EDIII [12,16,17]. Investigators have also mapped flavivirus cross reactive epitopes on EDIII [16,17]. Unlike DENV type-specific mAbs, cross-reactive mAbs that bind to EDIII have moderate to weak neutralizing activity.

Despite the large body of work with mouse mAbs, remarkably little work has been done to characterize the binding properties of human DENV immune sera and to understand the relationship between human antibody binding and neutralization. Convalescent sera from people and horses naturally infected with West Nile virus (WNV), a related flavivirus, had low levels of EDIII-reactive antibody [18,19]. In WNV immune sera, EDIII-binding antibodies were not primarily responsible for neutralization activity [18,19]. People who have recovered from DENV infections also develop EDIII-reactive antibodies [20-24]; however, most human antibody appears to be directed towards a flavivirus-cross reactive epitope close to the fusion loop in EDII of DENV [24,25]. To date, no studies have been done to directly test if EDIII-reactive antibodies are primarily responsible for the neutralizing activity of human DENV immune sera. The goal of this study was to measure the level and specificity of EDIII-reactive antibodies in people who have recovered from primary and secondary DENV infections and to determine the contribution of EDIII-reactive antibodies to DENV neutralization.

5.3 Materials and Methods

Viruses

DENV1 WestPac-74, DENV2 S-16803, DENV3 CH-53489, and DENV4 TVP-360, provided by Dr. Robert Putnak (Walter Reed Army Institute of Research, Silver Spring, MD) were used in this study. . Working virus stocks were obtained by inoculating C6/36 mosquito cells in tissue culture flasks and growing the virus for eight days at 28°C. Supernatants were harvested, clarified at 2500rpm for 5min, supplemented with 15% FBS and stored in aliquots at -80°C. Viral titers were determined by plaque assay on Vero-81 cells as previously described [26].

Immune sera and Antibodies

Convalescent DENV immune sera were obtained from volunteers who had experienced natural DENV infections during previous travel abroad. The protocol for recruiting and collecting blood from people was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill. Sera from 6 DENV-immune subjects were used in the present study. The properties of these sera are listed in Table 1. We also used eight DENV-reactive mouse mAbs that bind to EDIII. mAb 3H5-1 , obtained from Chemicon Co, CA, binds to EDIII of DENV2 only [12]. mAbs 8A1 and 14A4, which bind to EDIII of DENV3, were obtained from Dr. Robert Putnak (Walter Reed Army Institute of Research, Silver Spring, MD). mAb 2Q1899 and 9F16 which bind to EDIII of DENV2 were obtained from United States Biological, Massachusetts. DENV3 EDIII reactive mAb 1H9 was obtained from Dr John G Aaskov (Queensland University of Technology, Australia) [27]. DENV cross reactive EDIII mAb 8A5 and 12C1 were developed in collaboration with

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the South Eastern Regional Center for Excellence in Biodefence monoclonal antibody core facility at the University of Alabama School at Birmingham.

Purification of DENV antigen for ELISA

DENV2 S-16803 and 3 CH53489 reference strains were grown in Vero-81 cells (ATCC CCL-81) at 37°C. The virus containing media was harvested 5-7 days after infection and centrifuged to pellet cell debris. The clarified media was laid on top of a 20% sucrose (wt/vol) cushion and centrifuged (72,000g for 5 hrs) to pellet the virus. The virus pellet was allowed to dissolve overnight in PBS before layering on a 10%- 40% iodixanol gradient and being centrifuged at 163,700 x g for 120 min. The virus-containing fractions were harvested. PBS was added to the virus to dilute the iodixanol. The diluted solution was centrifuged (72,000 x g for 5 h) to pellets the virus and remove the iodixanol. The virus pellet was resuspended in PBS and virus protein content was estimated by spectrometry. The virus was stored at -80°C.

Expression of the ectodomain of E protein (Es) from DENV3

RNA extracted from strain CH53489 of DENV3 was reverse transcribed and PCR amplified to generate a PCR product containing nucleotides coding for the last 15 amino acids of membrane protein and the first 415 amino acids of E protein followed by a 6 histidine tag and a stop codon. This construct was missing the C-terminal amino acids responsible for membrane anchoring of E protein. The PCR product was cloned into pENTR TOPO vector (Invitrogen) and the BaculoDirect baculovirus Expression system (Invitrogen) was used to express recombinant protein according to manufacturer's instructions. Briefly,

the Es gene was recombined with BaculoDirect Liner DNA to generate recombinant baculovirus DNA. pENTR/CAT plamsmid was used to produce recombinant baculovirus expressing the chloramphenicol acetyl transferase (CAT) protein, which was later used as a negative control. Sf9 insect cells were transfected with recombinant baculovirus DNA. P1,P2 and P3 recombinant virus stocks were generated according to the manufacturers instruction and expression of protein was confirmed using western blot with mouse mAb 4G2 (for Es) or anti CAT antibodies. P3 baculovirus stocks were used to express Es and CAT proteins.

Expression and purification of DENV EDIII

RNA was extracted from supernatants of cells infected with DENV2 or 3 using QIAmp Viral RNA mini Kit (Qiagen). The nucleotide sequences encoding for EDIII of DENV2 (297-399 AA) and DENV3 (295- 398 AA) were reverse transcribed and PCR amplified. The PCR products were cloned into pMAL c2X vector (NEB) to generate recombinant EDIII (MBP-EDIII) that is fused to maltose binding protein (MBP) at the N terminus according to the manufacture's instructions. MBP-EDIII from DENV2 and DENV3 were expressed in *Escherichia coli* DH5α (Invitrogen) and purified using amylose resin affinity chromatography (NEB).

Detection of dengue reactive antibody in human immune sera by ELISA

ELISA plates were also coated with 75ng/ per well of purified DENV2 or 3 and the flavivirus cross reactive mAb 4G2 was used to confirm equal binding of each virus to the plate. ELISA plates were coated with 200ng of MBP-EDIII from DENV2 or 3 per well.

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Rabbit anti MBP sera (NEB) was used to confirm equal binding of MBP-EDIII from both serotypes to ELISA plates. ELISA plates were coated using virus or recombinant protein antigen in carbonate buffer at pH 9.6 for 2 hrs at room temperature. The plates were washed 3 times in Tris-buffered saline with 0.2% Tween20 (TBST) and incubated with blocking buffer (Tris-buffered saline with 0.05% Tween20 containing 3% skim milk and 2% normal goat serum) at 37°C for 1 hr. After washing the plates twice with TBST, human immune serum diluted in blocking buffer was added to each well and incubated at 37°C for 1 hr. Following 3 washes with TBST, alkaline phosphatase-conjugated goat antihuman IgG (Fc-specific) (Sigma) was added to each well for 1 hour at 37°C. After 3 washes with TBST, p-nitrophenyl phosphate substrate (Sigma) was added to each well and the reaction was allowed to develop for 15 minutes before recording optical density at 405nm on a spectrophotometer. In ELISAs with mouse mAbs, the protocol was the same except that alkaline phosphatase-conjugated goat anti-mouse IgG was used as a secondary antibody. To compare binding to DENV2 and DENV3 antigens, we normalized the data by using the serum sample # 24 (secondary dengue) that gave the highest OD with each antigen (DENV2, DENV3, DENV2 EDIII and DENV3 EDIII). For each antigen the maximum OD obtained with serum #24 was defined as an OD of 1. In figures 1 and 4 the Y axis is referred to as relative OD to indicate that the data was normalized using serum sample #24.

As the Es antigen bound poorly to ELISA plates, we used an antigen capture method to compare the binding of whole virus and Es. Plates were coated with 200ng of mAb 8A5 in carbonated buffer at pH 9.6. This antibody binds to E protein from all 4 serotypes. The antibody coated plates were washed and incubated with blocking buffer at 37°C for 1 hr. Next, sufficient DENV3 or Es antigen from DENV3 was added to saturate antigen binding to

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the antibody coated plates. CAT protein antigen was used as a negative control. The plates

were washed again before incubating with serial dilutions of dengue immune human sera.

The rest of the assay was performed as described above for the direct antigen coating ELISA.

Depletion of EDIII-reactive antibody in human immune sera

Purified MBP-EDIII was dialyzed against 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1

mM EDTA (column buffer) overnight at 4°C. MBP-EDIII (300 μg) was incubated with the

amylose resin (NEB) in column buffer containing 3% normal human serum (NHS) and

incubated overnight at 4°C. The resin was washed three times with column buffer and 3

more times with PBS to remove unbound MBP-EDIII. The resin was blocked with 5% NHS

in PBS before incubating with 1.5mls of human DENV immune serum diluted at 1:10 in PBS

for 4 hrs at 37°C. The amylose resin was pelleted and the EDIII antibody depleted human

serum was collected. Depletion of EDIII-reactive antibodies was confirmed by ELISA with

MBP-EDIII. In addition, each human immune serum sample was absorbed to an amylose

resin with MBP alone. MBP-absorbed immune sera and MBP-EDIII absorbed NHS were

used as negative controls in subsequent neutralization assays

DENV Neutralization assays

DENV neutralizing antibodies was measured by plaque reduction neutralization test

(PRNT) or a flow cytometry based neutralization assay. The PRNT was performed as

previously described [26]. In brief, Vero-81 cells were seeded into 24 well-plates and grown

until 80% confluent. Serially diluted sera were mixed with 30 plaque forming units (PFU) of

virus and incubated for 1 hr at 37°C. The virus/serum mix was added to the Vero cells and

incubated with a nutrient overlay medium (Opti-MEM® with 1% methylcellulose and 10% FBS) for four days at 37°C. The cells were fixed and stained for viral antigen with monoclonal antibody 4G2 as previously described [26]. The percentage of neutralization was defined as reduction in the number of foci in the test sera compared to the number of foci in the control wells with normal human serum. The 50% neutralization titers were determined by nonlinear dose-response regression analysis (Prism Package, GraphPad Software, Inc., San Diego, CA).

Flow cytometry based neutralization assays were performed in 96-well plates with the U937 human monocytic cell line transfected with DC-SIGN as previously described [26]. In brief, immune sera were serially diluted and incubated with sufficient virus to infect 10 to 15% of the cells in the well. The virus/serum mixture was incubated for 1hr at 37°C and then added to the cells for 1hr at 37°C. The cells were washed to remove unbound virus and fresh media was added before incubating cells for 24hrs at 37°C. Cells were fixed, permeabilized and stained with DENV mAbs 4G2 or 2H2, both of which bind to all four serotypes [26]. Cells were analyzed with a FACScan flow cytometer (Becton Dickinson) to identify infected cells. The 50% neutralization titers were determined by nonlinear dose-response regression analysis ((Prism Package, GraphPad Software, Inc., San Diego, CA).

5.4 Results

Dengue immune human sera were obtained by collecting blood samples from volunteers who might have been infected during foreign travel. Of 35 subjects enrolled in the study, 17 had antibodies that neutralized one or more DENV serotypes. The neutralization patterns of the 17 immune subjects were consistent with past exposures to

DENV1 only (one subject), DENV2 only (four subjects), DENV3 only (four subjects) and secondary DENV infections (eight subjects). These sera were also tested for DENV neutralizing antibody by the Centers for Disease Control (CDC) in Fort Collins, CO and the Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, Maryland. The CDC and NIAID laboratories reached the same conclusions as we did about the past infection history of these subjects (unpublished data from Drs Robert Lanciotti, CDC and Steve Whitehead, NIH). For the current study we selected 6 sera representing 2 subjects each who had recovered from primary DENV2, primary DENV3 and secondary DENV infections. The DENV neutralization titers and the most likely year and place of infection of these subjects are listed in Table 5.1.

DENV Binding Antibodies in Human Immune Sera

Experiments were performed to measure the binding properties of antibodies in the 6 selected immune sera to purified DENV2 and 3. The immune sera were tested at four fold dilutions starting at 1:50. Antibodies in human DENV immune sera cross reacted with both serotypes indicating that the dominant antibodies after primary and secondary infection are serotype cross-reactive (Figure 5.1). End point virus binding titers were calculated for the 6 sera (Table 5.2). As expected, subjects with secondary infections had higher titers than subjects with primary infections (Table 5.2). These results indicate that an ELISA with whole virus as antigen mainly detects serotype cross reactive antibodies and the assay is not predictive of the neutralization properties of the serum sample or past infections history of the subject.

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The DENV particle is made up of envelope (E), membrane (M) and capsid (C) proteins. As E protein is the main target of neutralizing antibody, experiments were done to compare the antibody response to E protein and whole virions. As full length E protein alone is not secreted out of cells, we expressed the soluble ectodomain of E (Es) from DENV3 to be used as an antigen. We used immune serum samples # 03 and 11 from primary DENV3 cases and # 09 and 24 from secondary cases and compared binding to DENV3 and Es from DENV3. Antibodies in human immune sera bound well to both Es and virus particles, but greater binding was observed with virus particles compared to Es (Figure 2). These results demonstrate that although the ectodomain of E is a dominant target of antibody, virions contain epitopes that are absent in recombinant Es.

Purification and Characterization of Recombinant DENV Envelope Protein Domain III (rEDIII)

Studies with mouse mAbs have demonstrated that most DENV serotype-specific antibodies bind to EDIII [11-16]. When using whole virus antigen in an ELISA, the cross-reactive antibodies in human immune sera are likely to dominate and mask signal originating from serotype-specific antibodies. To develop an assay for measuring serotype-specific antibody, recombinant EDIII was expressed as a MBP fusion protein in *E. coli* (Figure 3). Previous studied have demonstrated that EDIII expressed alone or as a MBP fusion protein is folded correctly and displays antibody epitopes present on the virion [28-31]. To confirm that recombinant DENV2 and 3 MBP-EDIII fusion proteins produced in our laboratory were correctly folded, binding assays were performed with eight mouse mAbs that bind to EDIII of DENV2 and/or 3. mAbs 3H5-1, 9F16 and 2Q1899 are antibodies that bind to serotype-

specific epitopes on the lateral ridge of DENV2 [12,16,32]. As predicted, all three antibodies

bound to MBP-EDIII from DENV2 but not DENV3 (Table 3). We used mouse mAbs 8A1,

14A4 and 1H9 which are serotype-specific neutralizing antibodies that bind to EDIII from

DENV3 only [27] (unpublished data, Putnak, Wahala and de Silva). mAb 8A1 and 1H9 bind

to the lateral ridge of EDIII from DENV3, whereas the 14A4 epitope on DENV3 EDIII has

not been mapped yet. mAbs 8A1, 14A4 and 1H9 bound to MBP-EDIII from DENV3 but not

DENV2 (Table 3). We also used two neutralizing mAbs that bind to a serotype cross

reactive epitopes in EDIII and these two antibodies bound to both recombinant proteins

(Table 5.3). Thus, the type specific and cross reactive neutralizing epitopes on EDIII of

DENV are preserved in the recombinant proteins used in the current study.

EDIII-reactive antibodies in human DENV immune sera

After confirming that the recombinant EDIII–MBP fusion proteins expressed

appropriate serotype-specific and cross-reactive epitopes, the antigens were used to detect

EDIII-reactive antibody in our panel of human DENV immune sera. Each immune serum

was tested in four- fold dilutions starting at 1:12.5. At high concentrations of serum,

antibodies in the two subjects with evidence of past primary DENV2 infections bound to

EDIII from DENV2 better than EDIII from DENV3 (Figure 5.4A and B). Similarly,

antibodies in the two subjects with serotype-specific neutralizing antibody to DENV3 bound

to EDIII from DENV3 better than EDIII from DENV2 (Figure 5.4C and D). The two

subjects with evidence of past secondary DENV infections had antibodies that bound equally

well to both antigens (Figure 5.4E and F). These results demonstrate that EDIII-reactive

antibodies that developed after primary infection were specific to the serotype responsible for

infection. The EDIII end-point binding titers also displayed serotype-specificity after primary infection (Table 5.2). In the case of secondary serum samples, the EDIII end point titers were similar for both antigens (Table 5.2).

We compared the amount of antibody in immune sera directed to the whole virus versus EDIII. To measure relative amounts of available EDIII epitopes on viral and recombinant protein antigens used in the binding assays, end-point titers were calculated using mAbs 8A5 and 12C1 (Table 5.3), which bind to a conserved DENV complex epitope on EDIII. The endpoint titers were 5-10 times higher for recombinant EDIII compared to virus (Table 5.2). This result was expected because the DENV complex epitope on EDIII has been mapped to the A-B loop, which is poorly exposed on the intact virus but not on recombinant EDIII [16]. Despite the superior binding of 8A5 and 12C1 to recombinant EDIII, human immune sera bound poorly to recombinant EDIII compared to the virus antigen (Table 5.2). After primary infections the serotype-specific EDIII-reactive antibodies ranged from 0.1 to 8.1% of total virus-reactive antibodies (Table 5.2). Following secondary infections the EDIII-reactive antibodies accounted for 0.2 to 4.0 % of total virus-reactive antibodies. These results indicate that subjects who have recovered from DENV infections have low levels of EDIII-reactive antibody and, in the case of primary infections; antibodies are directed to serotype-specific epitope(s) on EDIII.

Role of EDIII-reactive Antibodies in DENV Neutralization

Experiments were performed to determine the contribution of EDIII-reactive antibodies in human immune sera to DENV neutralization. The immune sera were depleted of EDII- binding antibodies by incubating the serum samples with MBP-EDIII bound to an

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amylose resin. The primary DENV2 and DENV3 immune sera were incubated with MBP-EDIII from DENV2 or DENV3, respectively. The secondary sera were treated with MBP-EDIII from DENV2 (Sample 009) or DENV3 (Sample 024). As depicted in Figure 5.5, incubation with recombinant MBP-EDIII removed most of the EDIII-reactive antibody. The treatment specifically removed EDIII-reactive antibodies as sera treated with MBP alone were indistinguishable from untreated immune sera (Figure 5.5). Interestingly, when the secondary sera were depleted using MBP-EDIII from one serotype, most EDIII reactivity to the second serotype was also lost (data not shown) indicating that in secondary immune sera the antibodies are mainly directed against a cross-reactive epitope on EDIII.

As the recombinant EDIII used in above studies was expressed as a MBP fusion protein, it was conceivable that some human antibody epitopes in EDIII were altered or masked by the fusion partner. To determine if MBP fusion partner altered important epitopes on EDIII, binding assays were performed with purified DENV2 EDIII without MBP (kindly provided by Dr. Michael Diamond, Washington University, St. Louis). Dengue immune sera absorbed with recombinant DENV2 EDIIII-MBP were tested for the presence of antibodies that bound to EDIII without MBP. As depicted in Figure 5.6, immune sera absorbed with DENV2 MBP-EDIII protein failed to bind recombinant DENV2 EDIII without MBP indicating that the MBP fusion partner does not alter or mask the main human antibody epitopes on EDIII.

Next, untreated and EDIII antibody-depleted sera were tested in the flow cytometry-based DENV neutralization assay with U937 cells expressing DC-SIGN. Figure 5.7 depicts neutralization curves for primary DENV2, primary DENV3 and secondary DENV immune sera. Serum samples with or without EDIII-reactive antibodies showed similar neutralization

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patterns (Figure 5.7). The 50% neutralization titers were ~ 10-15% lower for serum samples depleted with EDIII compared to the MBP treated sera (Table 5.4). These results indicate that EDIII-reactive antibodies make a minor contribution to the total neutralizing capacity of human DENV immune sera (Table 5.4).

As it was conceivable that EDIII antibodies might play an important role in DENV neutralization in some cell types but not others, some of the experiments with U937 cells expressing DC-SIGN (Table 5.4) were repeated with Vero cells. We selected EDIII antibody-depleted serum sample # 03 (primary DENV3 immune) and # 09 (secondary DENV immune) and performed neutralization assays with Vero cells. In the case of sample #03, the neutralization titers for DENV3 were similar for untreated and EDIII antibody-depleted serum (50% neutralization titers of 74 and 88 respectively). Similarly, for sample #09, the neutralization titers for DENV2 were similar (50% neutralization titers of 895 and 858 respectively) for untreated and EDIII antibody-depleted sera. These results demonstrate that EDIII reactive antibodies were not required to neutralize DENV infection of U937 cells and Vero cells.

5.5 Discussion

Despite many publications on interactions between DENV and antibody, surprisingly few studies have been published on how the binding properties of human antibodies relate to DENV neutralization. The goal of the current study was to characterize the specificity and functionality of antibodies in DENV immune human sera. Here we have demonstrated that EDIII- reactive antibodies are present in human immune sera. The EDIII antibodies mainly recognized a type-specific epitopes after primary infection and a cross-reactive epitope after

secondary infection. EDIII-binding antibodies were a minor component of the total antibodies in immune sera binding to DENV. Recently Crill and co-workers used DENV2 virus-like particles to measure epitopes specific human antibody responses and they also observed low levels of EDIII-binding antibodies in human sera [24]. Our results demonstrate that EDIII binding antibodies make only a minor contribution to the total neutralizing capacity of human immune sera. Thus, the EDIII neutralizing epitopes that have been the focus of much recent work [12,16,17] were not the target of most neutralizing antibody in primary and secondary DENV immune sera.

Several investigators have reported the presence of EDIII-reactive antibodies following natural infection of people and animals with flaviviruses [18,20-23]. In human WNV-immune sera, EDIII-reactive antibodies were present, although at low levels compared to the total antibody binding to virus [19]. Furthermore, investigators have shown that EDIII-reactive antibodies are specific for the infecting virus, unlike antibodies against the whole virus particle, which are highly cross-reactive [18,20-23]. Our data reported here indicate that DENV cross-reactive antibodies dominate in whole virus binding assays. Our data demonstrate that in subjects who have recovered from primary DENV infection, EDIII-reactive antibodies were mainly directed to an epitope specific for the serotype responsible for infection, whereas in secondary cases EDIII-reactive antibodies bound to a DENV cross-reactive epitope. Recent studies with mouse mAbs have mapped the location of both cross reactive and serotype-specific epitopes on DENV EDIII [12,16,17]. Although it is reasonable to speculate that the cross-reactive and serotype-specific epitopes defined by mouse mAbs are also targets of the human antibody response measured here, further studies are needed to confirm this.

Currently, the primary serological assay to identify the DENV serotype responsible for a primary infection is the neutralization test, which is a laborious and time consuming assay. Our results indicate that a simple ELISA with recombinant EDIII as antigen can be used to identify the DENV serotype responsible for primary infection. Our results also demonstrate that the specificity of EDIII-reactive antibodies is preserved in both early (within first year, data not shown) and late (>4 years after infection) convalescent primary sera. In secondary infections, this assay is unlikely to predict responsible serotypes as the response is directed to cross-reactive epitope(s) on EDIII. Ludolfs and colleagues also reported similar results using recombinant EDIII in an immunoblot assay with human immune sera [23]. Further studies are needed to evaluate the utility of recombinant EDIII as an antigen for identifying DENVs responsible for primary infection. In the case of secondary infections, studies need to address if the EDIII-reactive antibodies simply cross-react with the serotypes responsible for the primary and secondary infections or if they also cross- react with serotypes not responsible for infection.

Studies with mouse mAbs have led to the identification and mapping of a serotype-specific epitope on the lateral ridge of EDIII of several flaviviruses including DENV [1,12,16,33]. An implicit, but untested assumption has been that antibodies directed to this epitope must play a role in serotype-specific neutralization following natural human infection. Our results demonstrate that DENV-specific EDIII-reactive antibodies play a minor role in neutralization observed with human sera. Studies with immune sera from people and horses naturally infected with WNV have also revealed a variable role for EDIII-reactive antibodies in viral neutralization [18,19]. In some cases, EDIII-reactive antibody depletion led to a decrease in WNV neutralization whereas in other cases no significant

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change was observed [18,19]. Our results do not rule out the possibility of inter-domain epitopes involving EDIII making important contributions to neutralization as these epitopes would not be present in the recombinant EDIII proteins used in the current study. We conclude that antibodies directed to inter-domain epitopes, epitopes on EDI or II of E protein and, possibly M protein, are mainly responsible for the neutralizing activity of human immune sera.

One potential concern is that the recombinant EDIII MBP fusion proteins used here may be improperly folded and not display important epitopes present on EDIII in the virus particle. Our studies indicated that the recombinant proteins were correctly folded. Eight EDIII-reactive, neutralizing mouse mAbs bound with appropriate specificity to the recombinant EDIII from DENV2 or 3 used here indicating that the main type-specific and cross-reactive neutralizing epitopes described in the literature were present on our recombinant EDIII proteins [12,16,17,27,32]. Other groups have performed structure studies with EDIII expressed alone or as a MBP-fusion protein and demonstrated that the *E. coli*-expressed protein has a structure similar to EDIII in its native form [28-30]. We also demonstrated here fusing EDIII to MBP did not mask or alter the main human antibody epitopes in EDIII because human sera absorbed with MBP-EDIII failed to bind to EDIII without the MBP fusion portion as well.

In summary, our results indicate that EDIII-reactive antibodies are of minor importance in neutralizing DENV by human DENV immune sera. We propose that the major cross-reactive and serotype-specific neutralizing epitopes targeted by human immune sera are inter-domain epitopes [34] and/or located outside EDIII. Currently live attenuated DENV vaccines are being tested in human clinical trials [35]. It is reasonable to assume that

the protective antibodies induced by these vaccines will be similar to protective antibodies induced after a natural DENV infection. We predict that the quality and quantity of antibodies against EDIII will not determine the efficacy of live attenuated DENV vaccines. As an alternative approach to live attenuated vaccines, several groups have focused on developing recombinant EDIII vaccines [36-38]. Given the low levels of EDIII-reactive antibodies detected here in human immune sera, caution is urged in proceeding with EDIII-based platforms. We currently do not understand why people develop low levels of EDIII-reactive antibody after natural infection. The human immune system may recognize and react to epitopes on EDI and EDII better than to EDIII. However, with appropriate adjuvants and recombinant protein constructs, it might be possible to stimulate an effective immune response directed to relevant epitopes on EDIII as well. Such vaccines are likely to neutralize DENV by a mechanism that is different from neutralization observed after natural infection. The topic of flavivirus-antibody interactions has been dominated by studies to identify and characterize epitopes on EDIII. We hope the results reported here will stimulate more work to characterize epitopes on EDI and EDII and their role in DENV neutralization.

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Sample ID	Likely year and place of infection	Time interval between infection and sample collection	PRNT50 titer[1]				Most probable past infection
			DENV 1	DENV 2	DENV 3	DENV 4	
01[2]	Sri Lanka 1996	9 years	<1:20	1:271	<1:20	1:42	Primary DENV2 infection
13	South Pacific Island 1997	8 years	1:178	>1:1280	1:65	1:140	Primary DENV2 infection
11	El Salvador, 1998	7 years	1:84	1:124	1:1032	1:169	Primary DENV3 infection
03	Thailand 2001	4 years	1:30	1:87	1:338	<1:20	Primary DENV3 infection
09	India or Sri Lanka 2000	5 years	>1:1280	>1:1280	1:290	1:393	Secondary DENV infection
24	Brazil, 1998	7 years	>1:1280	1:640	1:64	1:108	Secondary DENV infection

Table 5.1 Human DENV immune sera used in the study

[1] The plaque reduction 50 % neutralization titer was determined using Vero cells

[2] DENV serotype 2 was isolated from serum sample in 1996

Serum sample or mouse Mab	Most probable past infection	Binding to DENV (end point titer)[1]		Binding to EDIII (end point titer)[1]		Amount of EDIII antibody relative to whole virus antibody (%)[2]	
		DENV 2	DENV 3	DENV2 EDIII	DENV3 EDIII	DENV2 EDIII	DENV3 EDIII
Serum 01	Primary DENV 2	9,864	8,658	67	11	0.7	0.1
Serum 13	Primary DENV 2	11,997	4,509	975	45	8.1	1.0
Serum 03	Primary DENV 3	1,931	4,813	3	50	0.2	1.0
Serum 11	Primary DENV 3	7,119	8,658	0	37	0.0	0.4
Serum 09	Secondary DENV	69,840	74,549	487	164	0.7	0.2
Serum 24	Secondary DENV	53,798	81,411	2,153	2,378	4.0	2.9
Mab 8A5[3]	NA[4]	308	161	1,096	1,778	NA	NA
Mab 12C1[3]	NA	554	195	2,455	2,884	NA	NA

[1] End point titer was calculated from curves displayed in Figures 1 and 3. The end point titer is the reciprocal of the highest dilution that gave a signal greater than 3 standard deviations of the signal for normal human serum.

[2] The amount of EDIII antibody relative to whole virus binding antibody was determined using the following formula: (EDIII end point titer /DENV end point titer) x 100.

[3] To compare accessible EDIII epitopes on viral and recombinant protein antigens from DENV2 and DENV3, end point binding were calculated for 2 mouse Mabs that bind to an epitope on EDIII that is conserved in all 4 serotypes. Both antibodies were used at a starting concentration of 8 µg/ml and tested at 8 four fold dilutions.

[4] Not applicable

Table 5.2 Titer of DENV and EDIII reactive antibody in immune sera

Neutralizing Monoclonal antibody	Binding specificity	Reference	Binding to recombinant MBP-EDIII fusion proteins of	
			DENV2	DENV3
3H5-1	DENV2 Type Specific, EDIII lateral ridge epitope	Gromowski, G D., and Barrett, A.D. 2007 Sukusolvi-petty et al.2007 Henchal, E.A. et al.1985	+[1]	-
9F16	DENV2 Type Specific, EDIII lateral ridge epitope	Gromowski, G D., and Barrett, A.D. 2007 Sukusolvi-petty et al.2007	+	-
2Q1899	DENV2 Type Specific, EDIII lateral ridge epitope	Gromowski, G D., and Barrett, A.D. 2007 Sukusolvi-petty, et al.2007	+	-
1H9	DENV3 Type Specific EDIII lateral ridge epitope	Serafin.IL and Aaskov JG. 2001 and Upublished	-	+
8A1	DENV3 Type Specific, EDIII lateral ridge epitope	Unpublished	-	+
14A4	DENV3 Type Specific, EDIII epitope	Unpublished	-	+
8A5	DENV cross reactive EDIII epitope	Unpublished	+	+
12C1	DENV cross reactive, EDIII epitope	Unpublished	+	+

[1] A positive value was defined as 0.2 OD units above the background signal obtained with the MBP antigen alone.

Table 5.3 Binding of DENV neutralizing monoclonal antibodies to r MBP-EDIII fusion protein

Serum sample	Most probable past infection	Recombinant protein used to absorb immune sera	50% neutralization titer[1]	
			DENV2	DENV3
01	Primary DENV2	None	495	ND
		MBP	495	ND
		DENV2-EDIII	431	ND
13	Primary DENV2	None	375	ND
		MBP	326	ND
		DENV2-EDIII	283	ND
03	Primary DENV3	None	ND	162
		MBP	ND	148
		DENV3-EDIII	ND	141
11	Primary DENV3	None	ND	655
		MBP	ND	625
		DENV3-EDIII	ND	570
09	Secondary DENV	None	1,199	311
		MBP	1,092	326
		DENV2-EDIII	907	358
24	Secondary DENV	None	519	341
		MBP	297	214
		DENV3-EDIII	258	195

[1] The 50% neutralization titers were determined by using a flow cytometry based DENV neutralization assay with U937 cells expressing DC-SIGN.

Table 5.4 DENV neutralization by immune sera depleted of EDIII reactive antibodies
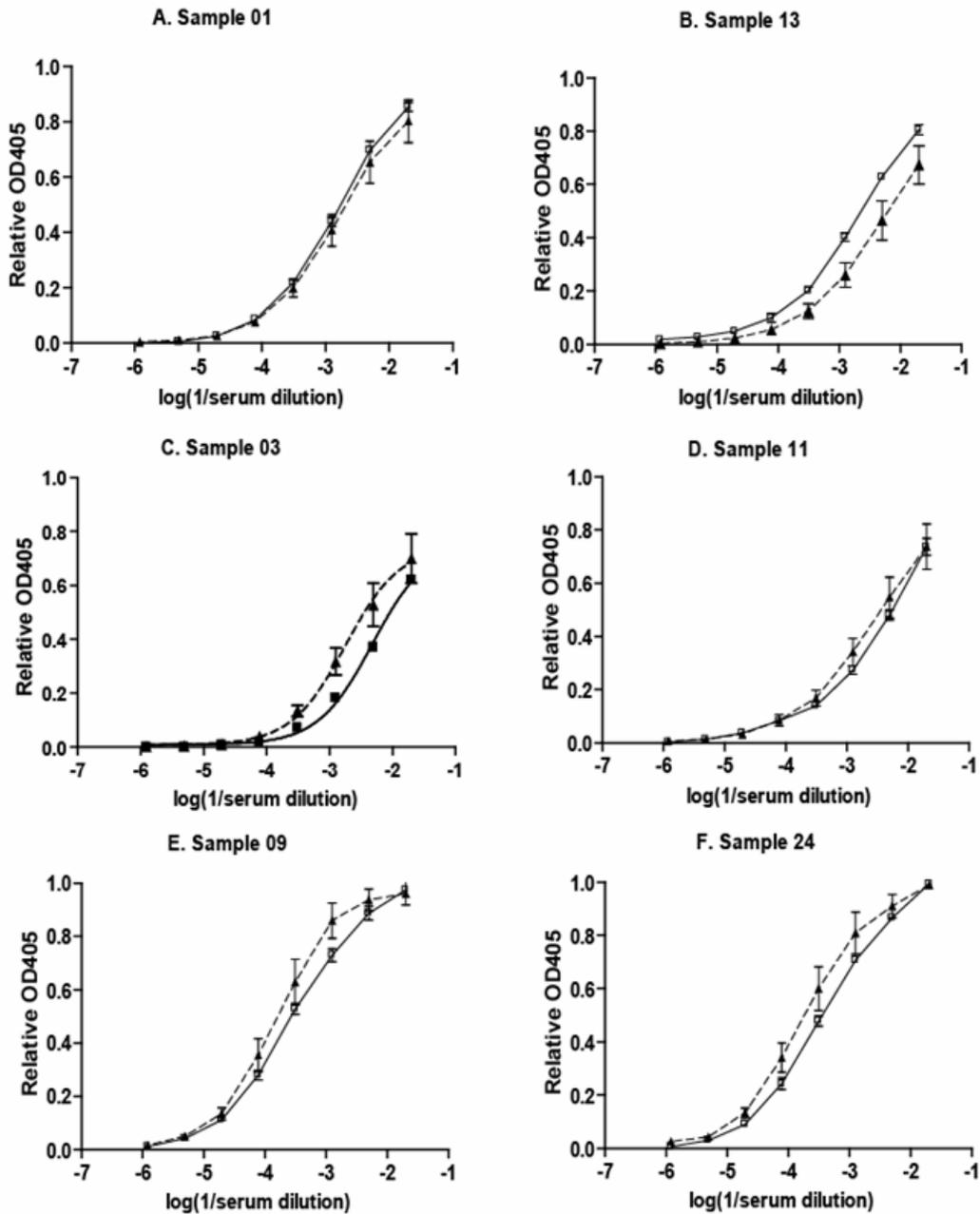
Figure 5.1: Binding of human immune sera to purified DENV2 and 3. The binding of antibodies in convalescent sera from patients who have recovered from primary DENV2 infections (A, B), primary DENV3 infections (C, D) and secondary DENV infections (E, F) to purified DENV2 (solid lines) or DENV3 (dashed line) was analyzed by ELISA. The data points represent mean values and the error bars represent the standard error of the mean. The data shows one of two representative experiments.
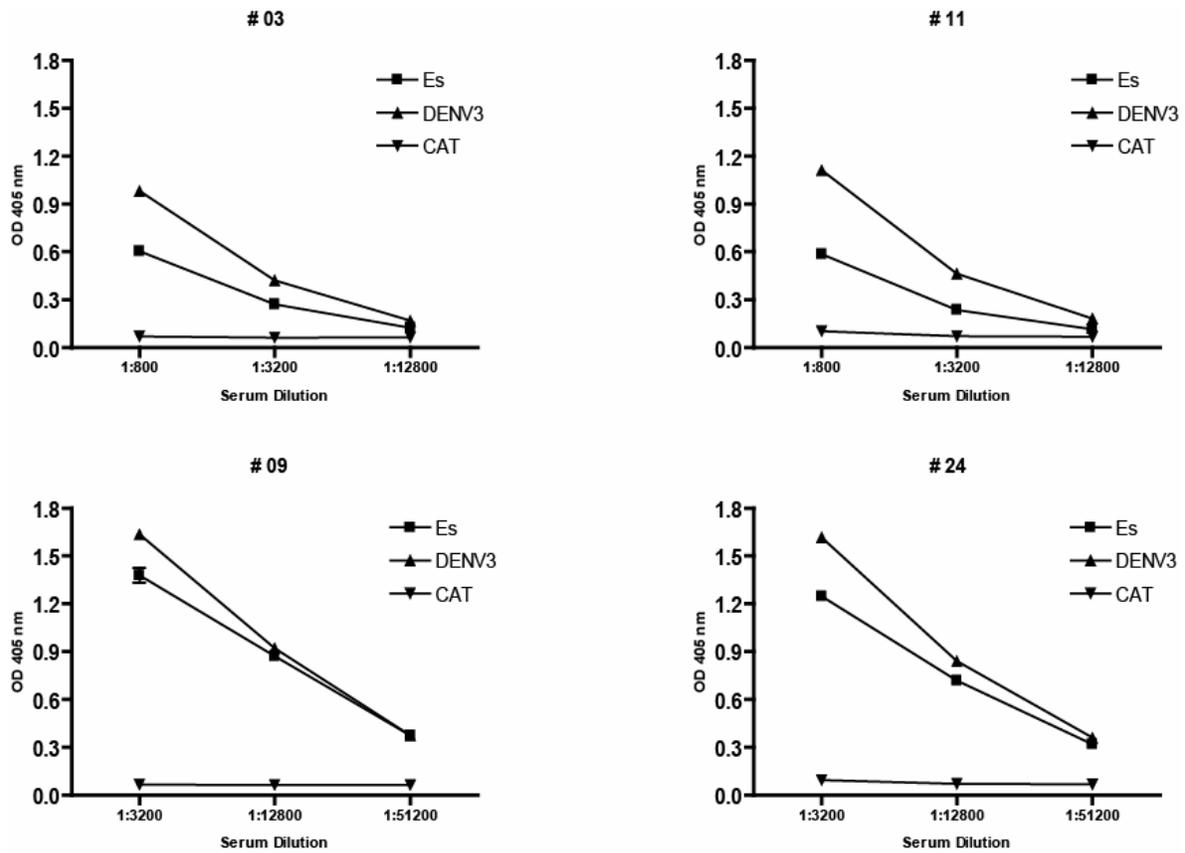
Figure 5.2: Binding of human immune sera to purified DENV3 and the ectodomain of E protein. To compare antibody binding to whole virions and E protein, ELISA plates were coated with purified DENV3 or the ectodomain of E protein (Es) from DENV3. As a negative control, plates were coated with chloramphenicol acetyl transferase (CAT) protein. Sera from patients who have recovered from primary DENV3 infections (03 and 011) and secondary DENV infections (09, and 024) were used to measure virus and E protein specific antibody responses.
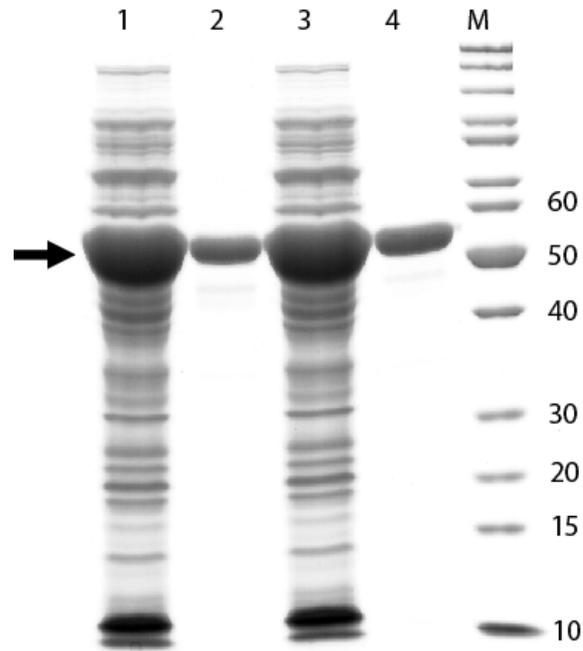
Figure 5.3: Purification and characterization of recombinant MBP-EDIII fusion proteins from DENV2 and 3. The recombinant protein expressed in *E. coli* and purified by amylase affinity chromatography. Lanes 1 and 3 depict the DENV2 and DENV3 MBP-EDIII fusion proteins in *E. coli* lysates. Lanes 2 and 4 depict the purified protein obtained after amylase affinity chromatography. The arrow indicated the band corresponding to the 53kD MBP-EDIII fusion protein.
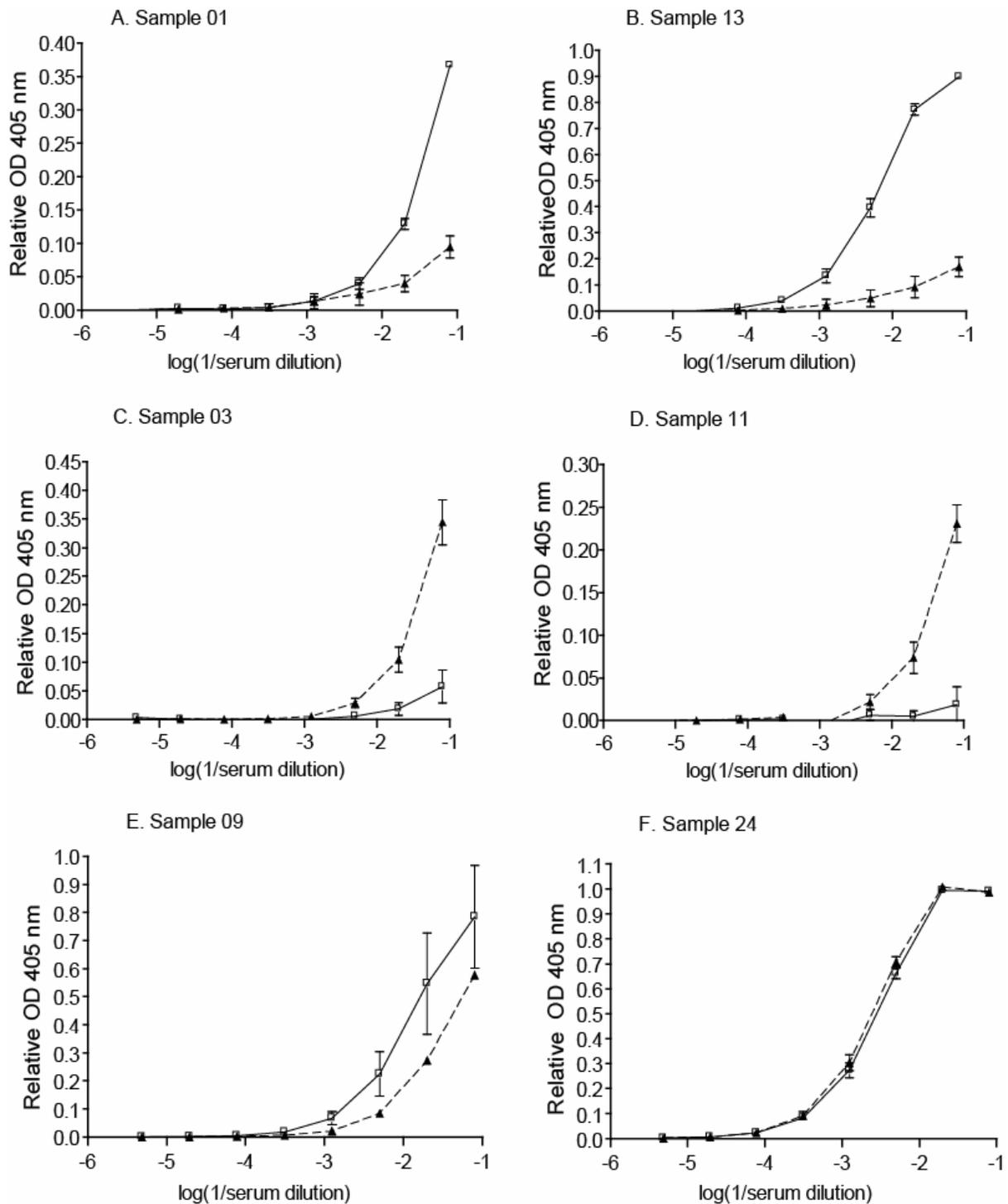
Figure 5.4: Binding of human immune sera to MBP-EDIII from DENV2 or 3.
Convalescent sera from 2 people each who had recovered from primary DENV2 (A, B), primary DENV3 (C, D) and secondary DENV (E, F) infections were tested for binding to recombinant MBP-EDIII from DENV2 (solid lines) or DENV3 (dashed line). The data points represent the mean values and the error bars represent the standard error of the mean. The data are from one of two representative experiments.

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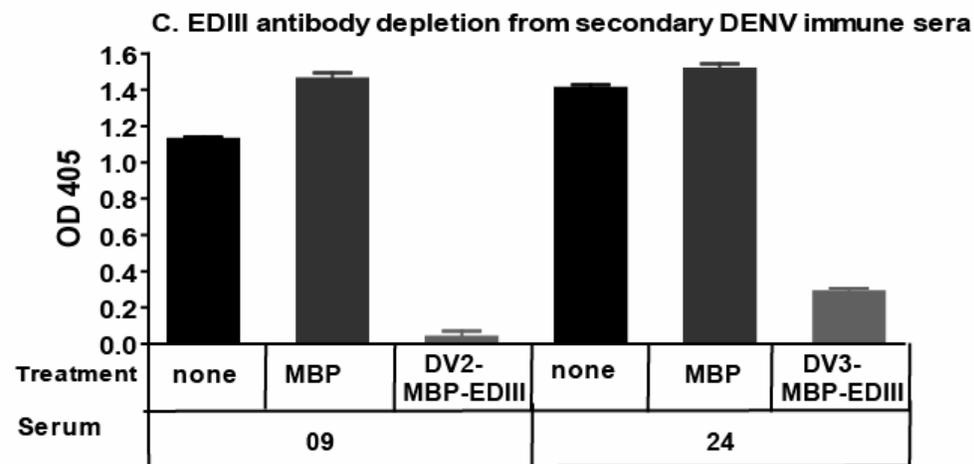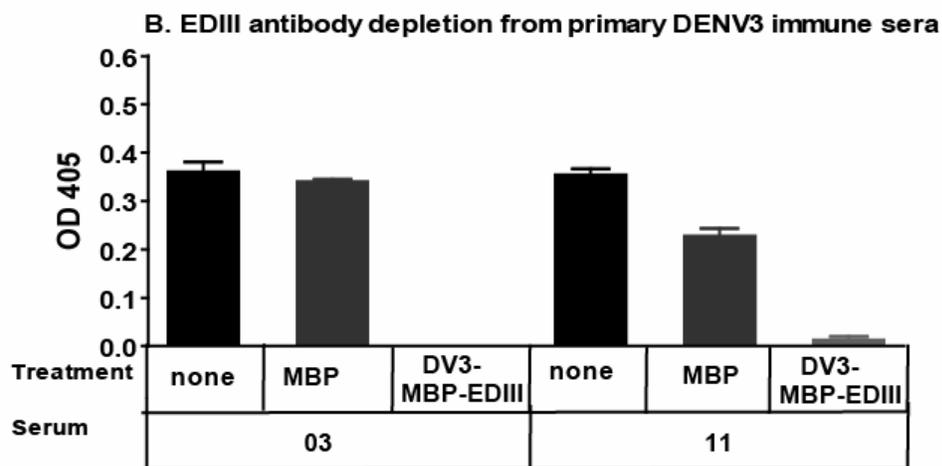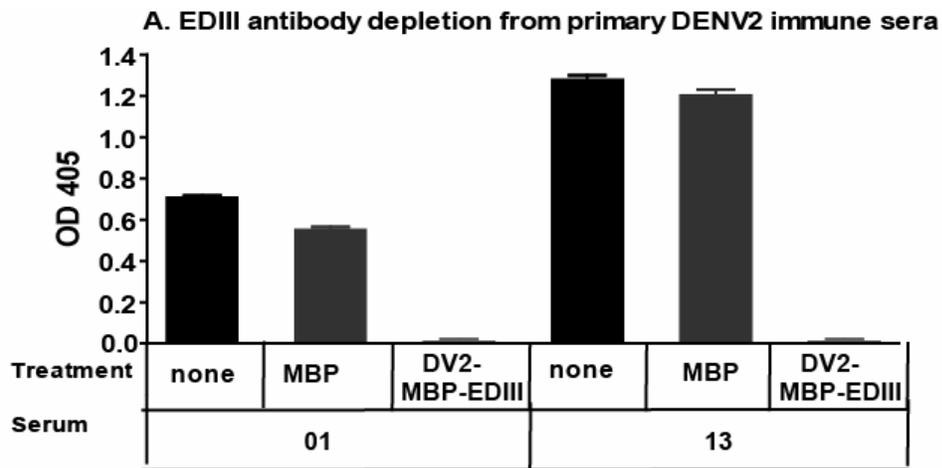
Figure 5.5: **Depletion of EDIII-reactive antibody from human immune sera**. Immune sera were from subjects who had have recovered from primary DENV2 (Panel A), primary DENV3 (Panel B) or secondary DENV (Panel C) infections. The sera were absorbed using MBP alone or recombinant MBP-EDIII-fusion protein from DENV2 (sera # 01, 09 and 13) or DENV3 (sera #03, 11 and 24).
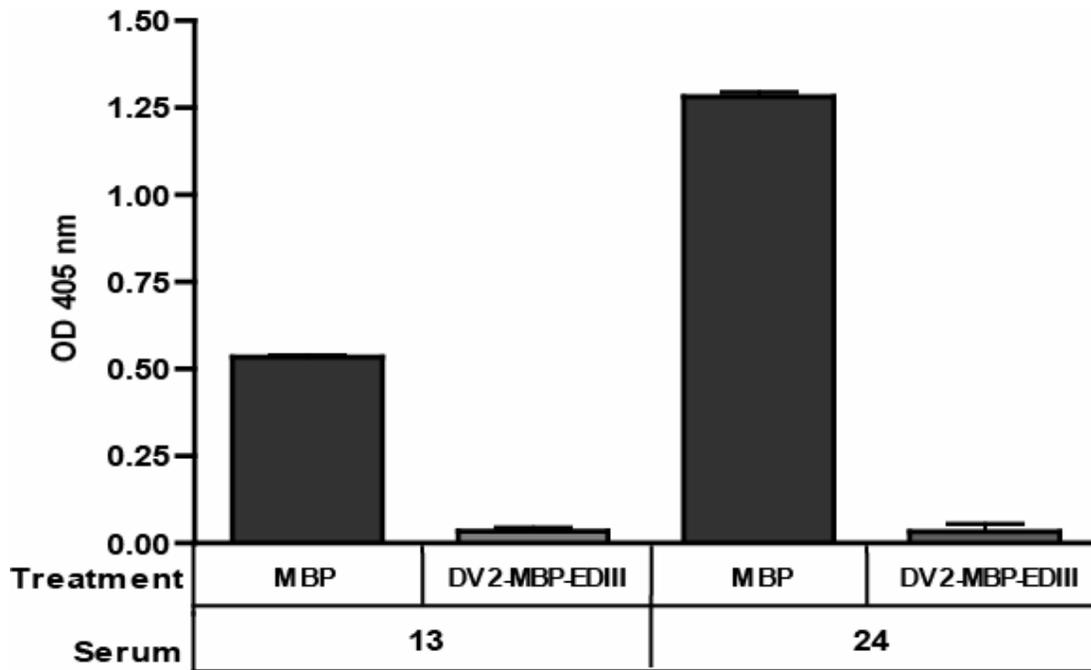
Figure 5.6: Binding of EDIII antibody depleted dengue immune sera to DENV2 EDIII without a MBP fusion partner. Dengue immune sera (# 13, and #24) were absorbed using DENV2 MBP-EDIII or MBP alone. The absorbed sera were tested for binding to DENV2 EDIII expressed without a MBP fusion partner. The MBP-EDIII absorbed sera failed to bind to EDIII alone indicating that EDIII with or without a MBP expressed similar antibody epitopes.

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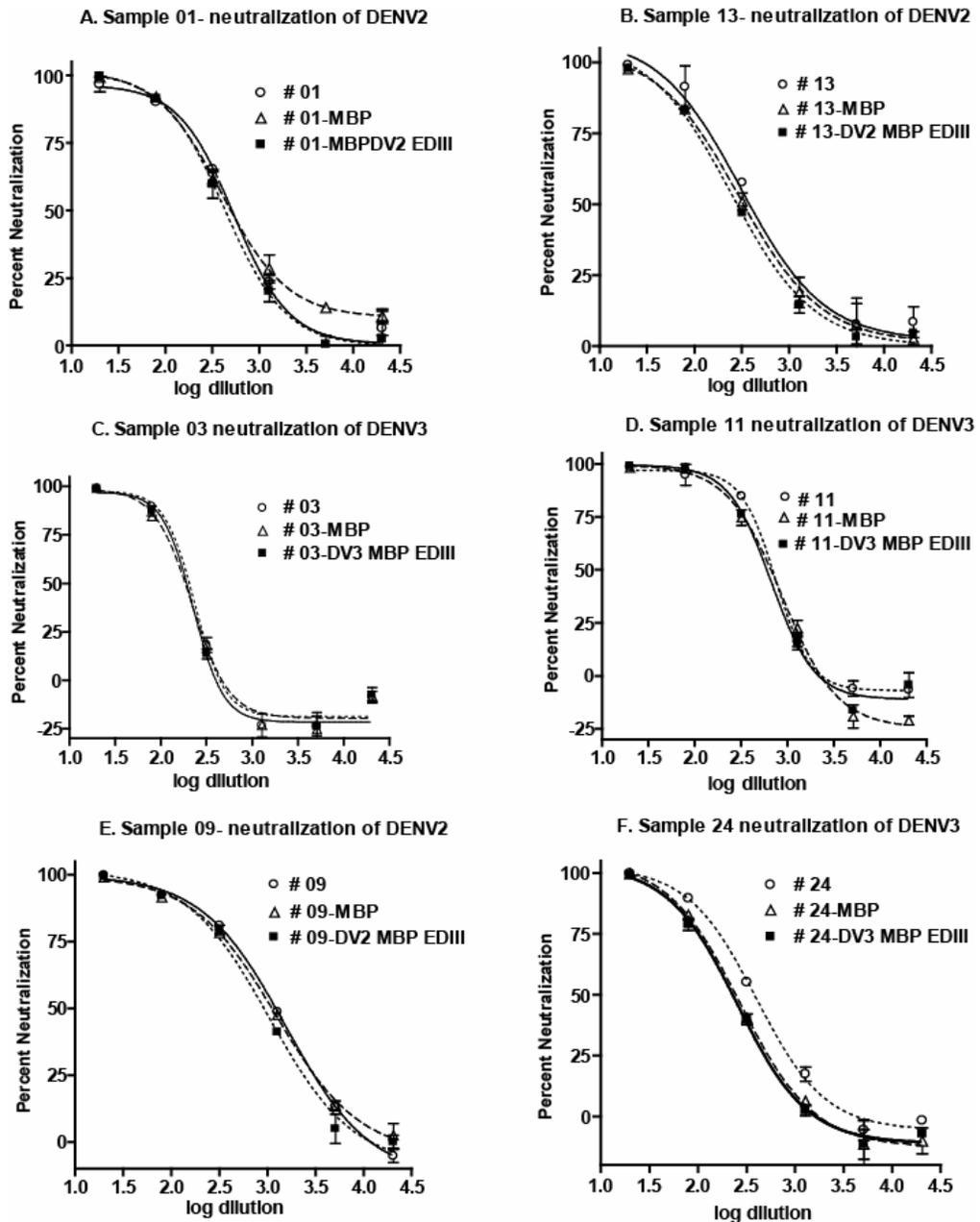
Figure 5.7: **DENV neutralization by human immune sera depleted of EDIII binding antibodies**. Convalescent sera from 2 people each who had have recovered from primary DENV2 (A, B), primary DENV3 (C, D) and secondary DENV (E, F) infections were depleted of EDIII binding antibody and tested for DENV neutralization at different dilutions. Neutralizing antibody was measured using U937 cells expressing DC-SIGN and flow cytometry. Neutralization curves are depicted for untreated (open circles), MBP treated (open triangles) and EDIII antibody depleted (dark squares) sera. The data are from one of two representative experiments.

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CHAPTER 6.

Discussion

The data presented in this thesis can be broadly categorized into two major areas; understanding the evolution of DENV in Sri Lanka, and understanding the mechanism of DENV neutralization by EDIII-reactive antibodies and its relevance to natural DENV infection in people. Therefore, the importance of these findings and the possible future directions are discussed in this chapter under these two categories.

6.1. Clade replacement of DENV3 in Sri Lanka and possible role in pathogenesis.

We studied the evolution and phylogeny of DENVs in Sri Lanka isolated over the past three decades. DENV2 and DENV3 were identified as the predominant DENVs circulating in Sri Lanka during this period. The most interesting change observed was linked to DENV3. It is well established that pre-989 viruses of DENV3 have been replaced by post-1989 viruses when DHF emerged in Sri Lanka in 1989 [1]. However, the present study further revealed that DF/DHF epidemics after 2000 coincide with the emergence of another new clade of DENV3 designated post-2000 clade. Further studies are needed to directly address if the new clades of DENV3 circulating in Sri Lanka are responsible for the recent severe dengue disease epidemics on the Island. We have noticed that DENV2 also followed the same temporal pattern of clade replacement as DENV3. However, our data are not conclusive with respect to DENV2 due to weak bootstrap values and the small number of virus isolates used. Therefore, more comprehensive studies are needed on the phylogeny of DENV2 in Sri Lanka as well.

Analysis of the whole genome of different DENV3 in a recent epidemic in Indonesia indicated that adaptive selection had a minimal role in the microevolution of DENV3 [2]. In this study, the reappearance of an older strain of DENV3 was responsible for the epidemic.

Similarly, the emergence of large epidemics in Sri Lanka after 2000 coincided with the appearance of a new clade of DENV3 that was closely related to a single Sri Lankan isolate from 1993. (chapter 2, figure 2.5) [1]. Starting in 2000, DENV began to spread to new areas in Sri Lanka. This might also have contributed to a steep increase in the number of cases by infecting susceptible people who had not previously been exposed to DENV (Chapter 2-figure 2.1) [3]. Most dengue infections in humans are asymptomatic, and silent infections of DENV during interepidemic periods have been reported [4-6]. Therefore, further research needs to be focused on analyzing the dynamics of silent infections in humans during interepidemic periods and tracing the emergence of new lineages of clades of DENV in Sri Lanka.

Contemporary isolates of DENV3 from Sri Lanka infect and disseminate more efficiently in mosquitoes than viruses circulating before 1989 [7]. This might suggest that new DENV strains on the island are replacing older stains by competitive displacement due to their higher fitness in mosquitoes [7,8]. It is known that the extrinsic incubation period (EIP; time taken to replicate and disseminate virus in mosquitoes after a blood meal) of attenuated DENV is long compared to invasive viruses [9,10]. Therefore, a shorter EIP likely provides a mechanism for the selection and transmission of more efficient virus strains; especially because a mosquitoes requires more than one blood meal for each gonotrophic cycle in its short life span. Furthermore, an increase in ambient temperature has been shown to decrease the EIP in mosquitoes [11]. Apart from that, a subtle change in the daily survival rate of mosquitoes in the environment has been proposed to be sufficient to turn mild epidemics into massive epidemics of Chikungunya virus, which is transmitted by the same Aedes vectors [12]. Therefore, it is important to study these neglected areas of

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vector biology to understand the mechanisms behind the massive epidemics and phylogenetic changes of DENVs in Sri Lanka.

Investigators have argued that some DENV strains are intrinsically different from others by being highly virulent and therefore causing severe DF/DHF epidemics [13-16]. However, this concept is controversial. DENV genomic sequences isolated from DF patients and DHF/DSS patients have been compared, and no detectable, highly reproducible genetic differences have been found among them [17]. However, the pitfall of such analysis is the assumption that a virus strain can only cause DHF and DSS and not DF or vice versa. It is obvious from the epidemiological data collected in DF/DHF epidemics worldwide that only a small percentage of DENV-infected people develop DHF/DSS [18,19]. Furthermore, pathogenesis of DHF/DSS is also governed by the interplay among many host factors [19]. Therefore it is quite possible that some differences among different virus strains can contribute to severe disease along with other host factors. On the other hand, antigenic variation among different DENVs has been proposed as an additional factor contributing to DHF/DSS. For example, sera from people immune to DENV1 were able to neutralize the American but not the Asian genotypes of DENV2, the latter having been shown to cause severe DF/DHF epidemics [20]. This finding was further confirmed in vivo using non-human primates as a model of infection [21].

The only amino acid (AA) difference observed in the viral structural proteins between pre-1989 DENV3 and post-1989 DENV3 was located in the E protein at amino acid position 124 [1]. The amino acid change observed was from Serine to Proline (S124P). In addition, post-2000 serotype 3 viruses contained an additional mutation from Threonine to Alanine at position 219 (T219A) in the E protein (figure 6.1a). Interestingly, AA positions 124 and 219

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are located in the center of EDII in a hinge region (figure 6.1b). AA positions at or near

residue 124 on the E protein of DENV have been identified as part of a mAb binding site by

neutralizing escape mutant viruses [22]. Furthermore, mouse mAb developed against the

New Guinea C strain of DENV2 (NGC) did not neutralize SIN/99 strain of DENV2 [23].

The AA differences found in the E protein between the two DENV2 strains were located at

AA positions 71, 112 and 124 of the E protein. Amino acids at positions 71 and 112 are

proximal to the fusion loop of the E protein whereas the AA at position 124 is located near

the center of EDII [23]. Since the AA mutations found on the E protein of the post-1989 and

the post-2000 DENV3 clades were located near the known epitopes of neutralizing

antibodies, these mutations may alter the binding affinity as well as functional properties of

neutralizing antibodies. Furthermore, the mutations may have some effect in the dynamic

movement of the E protein and thus, may affect the movement of the fusion peptide in the

acidic environment of the endosome. Such impacts on the dynamic movement of the E

protein may also be an important factor in determining the neutralization potential of

antibodies [24]. In fact, Japanese encephalitis virus (JEV) variants with AA mutations at

positions 126 and 219 of the E protein demonstrated neutralization differences with a

neutralizing chimpanzee mAb B3 [25]. The AAs at positions 126 and 219 are located in

similar positions to residues 124 and 219 of the DENV 3 E protein. Furthermore, Cummings

et al proposed that antibody-dependent enhancement (ADE) provides a competitive

advantage over the non-enhanced viruses especially in a hyperendemic situation [26].

Therefore, further experiments are needed to test the possible effects of mutations to residues

124 and 219 on neutralization and ADE by comparing pre-1989, post-1989 and post-2000

DENV3 strains. The DENV3 monoclonal antibody panel developed as a part of this study

will be an important tool for such experiments. Furthermore, it will be very important to determine how convalescent sera collected from DENV infected patients in Sri Lanka before 1989 neutralizes and/or enhances (ADE) during infection with pre- and post-1989 DENV, as well as the post-2000 clade of serotype 3 viruses. In addition, isogenic viruses, which can be made by swapping the E gene from different virus clades and genotypes into a common DENV backbone of the DENV3 infectious clone, will be a useful platform for answering these questions as isogenic viruses can reduce the inherent variability of neutralization assays, and will allow specific targeting of individual domains and epitopes.

The exact role of the mutations at positions 124 and 219 of the E protein of DENV3 has yet to be determined. However, some pre-1989 viruses replicated more efficiently than post-989 viruses in primary dendritic cells as well as in U937 cells expressing DC-SIGN [27]. Interestingly, such differences were not observed in mosquito cells (C6/36) or Vero cells. Importantly, adapting a strain of DENV2 to replicate in mice resulted in a mutation at position 124 of the E protein, suggesting that this position of the E protein may be a determinant of host specificity [28]. According to Prestwood et al., this mutation reduced the binding affinity of E protein to heparan sulfate, a putative cellular receptor for DENV; increased the half-life of the virus in serum; and increased the systemic viral load leading to severe disease in mice [28]. Moreover, mutation at amino acid 122 of E protein of DENV2 has been involved in modulating mid-gut infection and dissemination of the virus in mosquitoes [29]. Therefore it is possible that mutations at positions 124 and 219 of DENV3 E protein may affect the cellular tropism of these viruses. Further studies are needed to test the role of E protein mutations on the phenotypes of Sri Lankan DENV3 strains.

6.2 Neutralization of DENV3 by EDIII reactive antibodies and its role in natural DENV infections in human.

Strain variability and differential neutralization of DENV3:

The data presented in Chapter 4 of this thesis demonstrated that the type-specific neutralization epitopes of DENV3 are also located on the lateral ridge of EDIII as shown for DENV2 and other flaviviruses such as WNV and JEV. In addition, bioinformatics analysis of 175 E protein sequences representing all the genotypes of DENV3 showed that the E protein sequence was not strictly conserved among the different genotypes of DENV3. The variable AA positions of the E protein, referred to as informative sites, were not only restricted to EDIII, but also distributed on EDI and EDII as well. Most of the informative sites were located at or near the known epitopes of E protein-reactive, neutralizing Ab against the flavivirus. Our results further demonstrated that the type-specific lateral ridge epitope on EDIII was not conserved among the genotypes of DENV3 and consequently the binding and the neutralization potential of DENV3 type-specific mAb were affected by natural sequence variation located on the lateral ridge epitope of EDIII.

The long held assumption in the neutralization of DENV is that type-specific antibodies neutralize all the strains of a DENV serotype irrespective of the intra-serotype genetic variability [16,30]. Our results demonstrated that this assumption is not true, at least within the members of DENV3. Due to the fact that EDI- and EDII-reactive Abs cross-react and may enhance virus infection rather than neutralizing it, subunit DENV vaccine candidates based on EDIII are currently being developed [31,32]. However, our results suggest that such vaccines may not elicit EDIII lateral ridge-reactive type-specific antibodies,

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which neutralize all the strains of a serotype with a similar potential. Furthermore, such vaccine formulations must be concerned with the possibility of enhancement of infection. Therefore, EDIII-based vaccines need to be carefully evaluated for their efficacy using all the DENV genotypes within a serotype. On the other hand, 18 informative sites are located on EDI and EDII of the DENV3 E protein as well. The effect of these natural mutations on DENV3 neutralization has not been studied. However, a few studies have already reported differential neutralization of DENV3 by DENV immune sera [33-35]. Therefore, further studies are needed to address these questions in more detail.

Role of EDIII-reactive antibodies after natural infection in people:

Neutralization mechanisms of DENV by human immune sera are poorly studied mainly due to the polyclonal nature of the Abs present in these sera. However, it has been assumed that EDIII-reactive antibodies make up the dominant proportion of antibodies responsible for type-specific neutralization in humans after a natural DENV infection, mainly because EDIII-reactive mouse mAbs have been shown to strongly and type-specifically neutralize DENV. The data presented in chapter 5 of this thesis demonstrated that EDIII-reactive Abs do not make up the dominant proportion of the DENV-reactive antibodies in polyclonal immune sera. Our results further demonstrate that contribution of EDIII-reactive Abs to type specific neutralization in human immune sera is negligible. Furthermore, non-neutralizing type-specific antibodies and non-neutralizing cross-reactive antibodies against EDIII are present at a low percentage compared to total DENV-reactive antibodies present in immune sera after primary and secondary infections, respectively (chapter 5, tables 5.2 and 5.4, figure 5.4). Although, the number of serum samples used in our study was small, the

overall results suggested that type-specific neutralization in human immune sera may be driven primarily by EDI/EDII-reactive antibodies.

The work conducted in this thesis lead to the development of several additional interesting and challenging research questions, which have not been fully addressed by our results. One important research area is to understand why EDIII reactive neutralizing antibodies are not developed after natural DENV infection in humans, and how EDIII based vaccine candidates could be improved to elicit EDIII reactive neutralizing antibodies in humans. Another question is whether non-neutralizing, EDIII-reactive antibodies modulate ADE. Furthermore, identifying the major epitopes on EDI/EDII that are potentially responsible for type specific neutralization after a natural infection in humans is an ongoing and challenging research question, since most of the EDI/II reactive Abs are believed to be weakly neutralizing and/or enhancing.

Although the contribution of EDIII-reactive antibodies in immune human sera to type-specific neutralization is negligible, identifying binding sites (or epitopes) of the EDIII-reactive antibodies will be an essential component of improving EDIII vaccine candidates. Non-neutralizing epitopes on viral proteins can dominate the immune response by out-competing the neutralizing epitope, leading to an attenuated neutralizing Ab response [36,37]. In addition, deletion of non-neutralizing epitope on gp41 of HIV has induced a strong Ab response against a neutralizing epitope [36]. Replacing a key residue or few residues on the epitopes has also been used to reduce the immunogenicity of epitopes [38,39].

A recent study conducted by Webb and colleagues indicated that mice vaccinated with Venezuelan equine encephalitis (VEE) replicon particles which express rEDIII, did not

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elicit neutralizing Abs although EDIII reactive non-neutralizing Abs were detected [40].

Furthermore, it is known that the immunogenicity of rEDIII depends on adjuvant used in the

immunization protocol, and some adjuvants did not sufficiently induce the EDIII reactive

neutralizing Abs. Considering the published literature and the data presented in this thesis,

we hypothesize that weak immunogenicity of EDIII lateral ridge epitopes in humans may be

partly due to suppression from highly immunogenic non-neutralizing epitopes on other

regions of E protein. Epitopes on EDI, EDII and even epitopes on EDIII outside the lateral

ridge may suppress the immunogenicity of the EDIII lateral ridge.

Some exploratory studies were performed to identify the epitops on EDIII bound by

human antibody. Studies conducted with the array of mutant EDIII proteins, which have

been used in chapter 4 of this thesis, and immune sera from people exposed to secondary

DENV infections demonstrated that more than 50% of the binding was lost when AA

positions at 304, 308, (located on A sheet) 326, 328 and 330 (located on BC loop) were

mutated on DENV3 EDIII (figure 6.2). These preliminary data suggest that secondary

DENV immune sera bind to an epitope that is similar to an epitope centered on the A sheet of

EDIII, which is recognized by serotype cross-reactive mouse mAbs. It is also known that

AB loop of DENV EDIII is highly conserved. Mutating highly conserved Histidine and

Threonine residue in the AB loop of EDIII abolished the biding of non-neutralizing mouse

mAb [41]. Therefore, we speculate that non-neutralizing cross-reactive EDIII antibodies,

especially in secondary human sera, may bind to the AB loop as well. However, further

studies are necessary to identify these epitopes.

Immunogenicity of an antigen can be improved by optimizing helper T cell (HTL)

function by using an universal synthetic peptide or pathogen-derived, broadly cross-reactive

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HTL epitopes [42-44]. Such binding sites have been used to improve the immunogenicity of neutralizing epitopes on viral proteins [37,45]. For instance, synthetic Pan DR helper T cell epitopes (PADRE), universal synthetic T helper cell epitope, has been placed next to the epitope of the recombinant viral protein of porcine reproductive and respiratory syndrome virus (PRRSV) to induce the immunogenicity [37,42].

Thus, different approaches such as abolishing non-neutralizing epitopes and introducing T helper cell epitopes have been used to improve the immunogenicity of neutralizing epitopes on proteins from many different pathogens including viruses. Improving immunogenicity to the lateral ridge epitope on EDIII may be accomplished using any of these approaches or combining them. As anticipated, if the non-neutralizing epitopes are identified, those epitope can be mutated to dampen the immunogenicity against them and will probably increase the immunogenicity of the lateral ridge epitope which elicits strongly neutralizing Abs. On the other hand, universal T helper epitopes such as the PADRE epitope may also be a candidate to increase the immunogenicity of the lateral ridge epitope. PADRE has also been placed between non-neutralizing and neutralizing epitopes of recombinant viral proteins to induce immunogenicity of the neutralizing epitope [37]. Therefore, the PADRE sequence may be placed proximal to residues at AA position at 300, which is located at the center of these lateral ridge epitope on the N-linker region of the EDIII, and this will likely exert on a minimal effect on the structure of EDIII. [46,47]. Thus, we anticipate that disrupting non-neutralizing epitopes by introducing mutation in those, and inserting PADRE or similar T helper cell epitopes close to the center of the lateral ridge epitope, may increase the immunogenicity of the lateral ridge epitope and induce a strongly neutralizing Ab response in humans.

It has been well accepted that EDIII-reactive antibodies are strongly neutralizing antibodies and EDI/EDII antibodies are broadly cross-neutralizing and enhancing antibodies [22]. However, such conclusions are made based on the data obtained from studies with mouse mAbs, assuming that natural human infection with DENV elicits a similar immune response to the mouse. In contrast, the present study suggests that this assumption is not correct, and the contribution of EDIII reactive antibodies in human immune sera in the face of neutralization of DENV is negligible. Therefore, it is important to study ADE with regard to non-neutralizing EDIII-reactive antibodies in immune sera as the other possible role of EDIII reactive antibodies. Such studies can be pursued either in an in vitro cell culture model or an in vivo mouse model by comparing EDIII-reactive antibodies depleted sera to un-depleted sera [48].

According to a recent study, human polyclonal sera collected from DENV infected people mainly consist of cross reactive antibodies against highly conserved fusion peptide [49]. Furthermore, EDIII-reactive antibodies in WNV-infected individuals are also very rare and the strongly neutralizing human monoclonal antibody recovered from such an individual binds to EDII and not to EDIII [50-52]. These findings along with the data presented in the present study clearly indicate that individuals who have recovered from natural flavivirus infection mainly developed antibodies against the EDI/EDII portion of the E protein and not to EDIII. Therefore, it is important to map the antibody epitopes responsible for type-specific neutralization after natural human infection.

Developing human monoclonal antibodies from DENV-infected people is one way to characterize the antibody response against DENV in humans. Although human mAbs against DENV will identify interesting epitopes, these mAbs may not represent the total

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antibody repertoire against DENV in humans. Thus, human mAb studies should be done in parallel with studies to characterize the polyclonal human response. However, such analysis is challenging due to the nature of polyclonal serum. Nevertheless, our preliminary ELISA data with mutant EDIII protein and polyclonal sera have indicated that mutational analysis with polyclonal sera can be used to map epitopes of Abs in human sera. Apart from our preliminary data, recent work conducted by Li et al indicated that neutralization specificities can be analyzed and mapped using polyclonal sera [53].

Although many studies focused on EDIII, neutralizing mouse mAbs have also been mapped on EDI/EDII of the flavivirus E protein (chapter 4, table 4.1). The flaviviruses share a similar E protein structure and most of the informative sites on EDI/II of DENV3, which are described in chapter 4 of this thesis, are also located on or near the known epitopes of the mouse mAbs generated against the flaviviruses (chapter 4 figure 4.2). Therefore, these informative sites can also be considered as putative human polyclonal antibody binding sites. This assumption is further corroborated by a recent work conducted by Vogt et al [51]. A strongly neutralizing human mAb against WNV was mapped to EDI/II hinge region which had already been mapped by mouse mAb [22,51]. In addition, conformational epitopes on the E protein dimer can be predicted by using a computer algorithm such as CEP (Conformational Epitope Prediction server; Bioinformatics Centre, University of Pune, Pune 411 007. India). A program such as CEP can also be used to predict an inter-monomeric epitope, which is located between the two adjacent subunits of the E dimer. Inter-monomeric epitopes have been reported for flaviviruses and influenza virus [54,55]. Combining the information from informative sites, mouse and recent Chimpanzee mAb epitopes, and epitopes predicted using CEP; amino acids on EDI/II can be predicted and selected as

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putative antibody binding sites. Such AA can be mutated individually or in groups using the

recombinant DENV3 infectious clone to generate mutant viruses which would subsequently

be used in neutralization assays with human polyclonal sera. In addition it might be possible

to make chimeric viruses comprising EDI/EDII from one serotype and EDIII from another

serotype of DENV [56]. Using recombinant E protein in these approaches may not be

successful since strongly neutralizing EDI/EDII-reactive human monoclonal antibodies

against flavivirus only recognized the whole virus particles and not the recombinant E

protein [51,52]. Depleting antibodies in primary human polyclonal sera with a heterologous

purified whole DENV can also be used to remove cross-reactive antibodies, and such

depleted sera will be a very useful tool in identifying serotype specific EDI/EDII antibodies

and their epitopes, as well.

<u>rEDIII as a diagnostic tool to detect DENV infection and to discriminate primary infection
from secondary infection:</u>

The data presented in chapter 5 indicate that primary immune sera recognize only one

rEDIII protein representing the infected DENV serotype, whereas secondary immune sera

cross-react with rEDIII from all DENV serotypes. Therefore, our data suggest that rEDIII

can also be used as a diagnostic tool. Although, EDIII has already been described as a choice

of antigen for detecting flaviviruses, our study further suggests its use as an ideal antigen to

detect the infected serotype and to discriminate primary infection from secondary DENV

infection. However, further studies are needed to confirm our finding using a larger panel of

sera. Furthermore, in our experiments we observed low level cross reactivity in primary

DENV immune sera among the EDIII from different DENV serotypes, although this cross

reactivity did not hamper type-specific detection. The AB loop of EDIII, which is highly

conserved among DENVs, contains non-neutralizing cross-reactive Ab binding epitopes [41].

Cross-reactive binding can be abolished by mutating Histidine and Threonine residues in the

AB loop, and these EDIII mutant proteins will be a more specific antigen for the diagnostic

purposes.

Neutralization assay is the only available tool to determine the DENV serotype

responsible for the past primary infection from convalescent serum samples. Unfortunately,

neutralization assays are expensive, time consuming and require technical excellence in

tissue culture. Therefore, the neutralization assay is not a feasible technique in larger scale

seroprevalence studies especially in developing countries where dengue is hyperendemic.

However, rEDIII ELISA may be used to identify the infecting serotype if it is a primary

DENV infection. Furthermore, the rEDIII ELISA has shown a wide range cross reactivity

between EDIII from all of the DENV3 serotypes in secondary infection. On the other hand,

neutralization assays also show a wide range of cross neutralization between DENV

serotypes and thus the serotype responsible for secondary infection cannot be identified.

Therefore, an ELISA, based on an improved EDIII protein, will be a better and more cost-

effective tool to diagnose, to discriminate secondary infection from primary infection, and

finally to identify the infecting serotype of DENV in a simple, single assay format.

In summary, the present study demonstrated that severe epidemics of DF/DHF in Sri

Lanka reported after 2000 were due to the emergence of a new clade of DENV3 and possibly

DENV2. Furthermore, our study emphasizes the need for more elaborate studies in

microevolution of DENV in Sri Lanka, especially focusing on neglected research areas. The

major breakthrough of the present study is to confirm that antigenic properties among the genotypes of DENV3 serotypes are not conserved, and that the EDIII reactive antibodies are not the dominant type-specific neutralizing antibodies after a natural infection in humans. I believe that the data presented here in the current study have contributed to a better understanding of humoral immune response after natural DENV infection and will stimulate novel research on natural and vaccine-induced immunity to DENVs.

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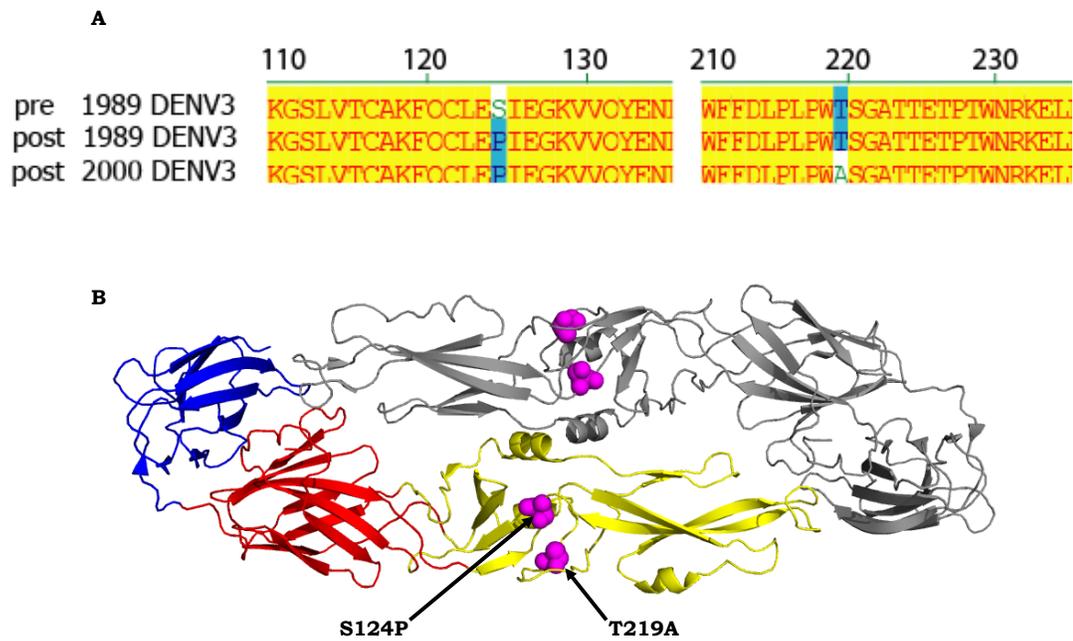
Figure 6.1. Amino acid difference of E protein among pre-1989, post-1989 and post-2000 DENV3 clinical isolates. A. Sequence alignment of representative isolate from each DENV3 clade. Positions 124 and 219 are not conserved. B. Location of amino acid mutation on the E protein dimer. EDI, EDII and EDIII are color coded in red, yellow and blue respectively. Amino acid 124 and 219 are located EDII.
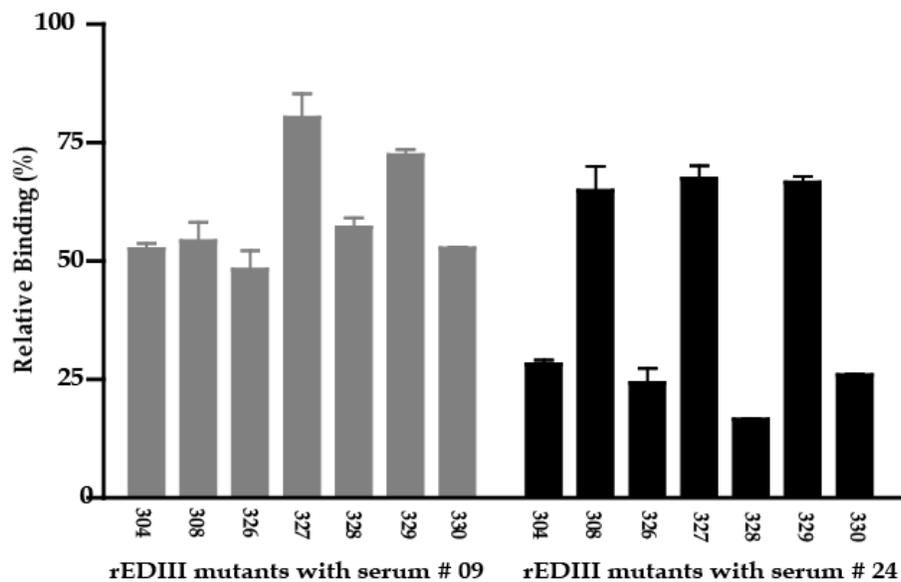
Figure 6.2. Relative binding of secondary DENV immune sera (serum # 09 and # 24) to mutant rEDIII. Amino acid at each position on rEDIII from DENV3 genotype II was replaced by Alanine residue. The binding of immune sera to each mutant rEDIII was graphed as a relative percentage of binding compared to parental rEDIII of genotype II.