DISSECTING THE HUMAN ANTIBODY RESPONSE TO DENGUE VIRUS

Adamberage Ruklanthi de Alwis

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Approved by:
Aravinda M. de Silva, MPH, PhD
Stephen H. Clarke, PhD
Edward J. Collins, PhD
Stanley M. Lemon, MD
Ronald I. Swanstrom, PhD
Dengue fever (DF) and severe dengue (SD) are two forms of an emerging infectious disease that presents a severe public health crisis predominantly in developing countries. Its etiological agent, Dengue virus (DENV), is a mosquito-borne pathogen, which exists as four different serotypes (named DENV1 through 4). Primary natural infections in humans stimulate a highly cross-reactive antibody response, however, protection is observed to be only against the serotype of infection. Extensive work on the mouse antibody response to DENV has mapped strongly neutralizing antibodies to the domain III (EDIII) of the DENV envelope (E) protein. However, recent work showed that after a natural infection in humans, anti-EDIII antibodies contribute very little to protection. Therefore, the human memory antibody response against a natural DENV infection remains poorly understood. Our present studies characterized both circulating polyclonal antibodies from human sera and human monoclonal antibodies from memory B-cells, after late convalescent primary DENV infections. The present body of work shows that after late convalescent natural primary DENV infections, humans produce two uniquely different antibody groups: 1) A cross-reactive, weakly neutralizing group that makes up the dominant proportion of anti-DENV antibodies, and 2) a minor group of strongly neutralizing, type-specific antibodies. Subsequently, we mapped some of these strongly neutralizing type-specific antibodies to a novel complex, quaternary epitope that includes the hinge region between domains I and II.
(EDI-II) of the E protein. Due to the role of the EDI-DII hinge region in DENV fusion and entry, this epitope offers a functional advantage. As hypothesized, we observed that all neutralizing EDI-DII hinge-binding monoclonal antibodies that were isolated blocked DENV infection at a step post-attachment, while the mechanism of neutralization by human polyclonal sera was more variable. In parallel, we found that the weakly neutralizing, cross-reactive group of antibodies was responsible for antibody-mediated enhancement of infection by heterotypic DENV serotypes. Further investigation mapped these enhancing cross-reactive antibodies to the DENV surface glycoproteins prM and E protein. These studies shed some light on the protective and enhancing DENV epitopes targeted by the human immune response, and set the stage for a safer and efficacious human vaccine against DENV.
ACKNOWLEDGEMENTS

“We are like dwarfs sitting on the shoulders of giants. We see more, and things that are more distant, than they did, not because our sight is superior or because we are taller than they, but because they raise us up, and by their great stature add to ours”

Bernard of Chartres, 12th Century

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<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>amino acid</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>ADE</td>
<td>antibody dependent enhancement</td>
</tr>
<tr>
<td>cryo-EM</td>
<td>cryo-electron microscopy</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>CNBr</td>
<td>cyanogen bromide</td>
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<tr>
<td>CS</td>
<td>conserved stem region</td>
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<tr>
<td>DC-SIGN</td>
<td>dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin</td>
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<tr>
<td>DENV</td>
<td>dengue virus</td>
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<tr>
<td>DF</td>
<td>dengue fever</td>
</tr>
<tr>
<td>DHF</td>
<td>dengue hemorrhagic fever</td>
</tr>
<tr>
<td>DSS</td>
<td>dengue shock syndrome</td>
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<tr>
<td>E</td>
<td>envelope protein</td>
</tr>
<tr>
<td>E85</td>
<td>85% of E protein</td>
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<tr>
<td>EBV</td>
<td>Epstein barr virus</td>
</tr>
<tr>
<td>EDI</td>
<td>envelope protein domain I</td>
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<tr>
<td>EDI-II or</td>
<td>envelope protein domains I &amp; II</td>
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<tr>
<td>EDII</td>
<td>envelope protein domain II</td>
</tr>
<tr>
<td>EDIII</td>
<td>envelope protein domain III</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immuno-sorbant assay</td>
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<td>FcR</td>
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FRNT  focus reduction neutralization titer
GC    genome copies
hMAb  human monoclonal antibody
IFN   interferon
IgG   Immunoglobulin G
IL    interleukin
LDC   Langerhans dendritic cells
MAb   monoclonal antibody
mMAb  mouse monoclonal antibody
Neut_{50}  50% Neutralization titer
NS    nonstructural protein
PBMC  peripheral blood mononuclear cells
prM/M pre-membrane/membrane protein
rE    recombinant envelope protein
PCR   polymerase chain reaction
RT-PCR reverse transcription polymerase chain reaction
TBEV  tick-borne encephalitis virus
TGN   trans-Golgi network
UTR   untranslated terminal region
VEEV  Venezuelan equine encephalitis virus
VRP-rE virus replicon particle expressing 80% DENV E protein
WNV   West Nile virus
YFV   yellow fever virus
CHAPTER ONE

Introduction

1.1 Dengue: Epidemiology and clinical disease

Dengue is a re-emerging neglected disease that affects individuals in over 100 countries and is the pre-eminent arthropod-borne viral disease of humans (47, 89). Dengue disease is caused by dengue virus (DENV), which exists as four closely related serotypes (named DENV1 through DENV4). DENV spreads efficiently between humans primarily through the mosquito vectors Aedes aegypti and Aedes albopictus. DENV is estimated to infect over 390 million individuals globally each year (8, 11, 89, 131). The mosquito vectors reside in subtropical and tropical regions; thereby placing 2.5 billion individuals at risk from acquiring a DENV infection. Unfortunately, there are currently no approved therapeutics or vaccines against DENV.

Historically, due to similar geographical circulation and clinical symptoms, infections by DENV and the alphavirus, chikungunya virus, were frequently confused (15). Nevertheless, epidemics of dengue fever (DF)-like illness have been reported as far back as the 10th century (reviewed in (44)). However, it was not until after the 1950s that severe forms of dengue fever, such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), emerged in the
Indian subcontinent and Southeast Asia (51). Since the 1970s, severe forms of dengue have spread from five to over 100 countries (reviewed in (44)). It is estimated that at least 500,000 cases of DHF and DSS cases (with 5% mortality) occur globally each year (131). This continual rise in morbidity, mortality and geographical spread is due to increasing urbanization, globalization, lack of basic infrastructure, and the spread of the mosquito vectors to new territories.

Successful transmission of DENV by the mosquito vector requires circulating titers of around $10^7$-$10^9$ mosquito infectious doses in an infected person (34). When an *Aedes* species mosquito injects DENV into the skin of a new susceptible human, the virus undergoes the first round of replication in the resident skin dendritic cells, called Langerhans dendritic cells (LDCs) (92). The infected LDCs then migrate to the draining lymph node where the virus infects monocytes and macrophages (162). Within the lymphatic and circulatory system, the virus is then disseminated to other organs, such as the liver, spleen and bone marrow (64).

Dengue manifests as a broad spectrum of clinical symptoms in humans. Between 50-95% of DENV infections are asymptomatic or present sub-clinical symptoms. Symptomatic DENV infections are predominantly cases of DF, with classical symptoms of fever, headache, eye pain, myalgia, athralgia, rash, nausea and abdominal pain, following the 3-7 day viral incubation period. Most patients recover after fever subsides, while a small proportion goes on to develop a systemic vascular leak syndrome (i.e. DHF and or DSS), with petechiae, thrombocytopenia and shock (108). Prior classification of DENV-induced disease, DF and DHF/DSS, used to be based on a number scale rated from 1 to 4 (with 4 being most severe and classified as DHF/ DSS) (108). This classification led to delayed diagnosis and untimely treatment of severely ill DENV infected patients (30, 119).
Therefore, the WHO recently revised the guidelines, and now patients are diagnosed as either having dengue or severe dengue (SD) (109). Hence in the present document, we will use SD and DHF/DSS interchangeably.

1.2 DENV genome and life cycle

DENV belongs to the arthropod-borne, single-stranded positive-sense RNA (+ssRNA) virus family called flaviviruses. Other significant human pathogenic flaviviruses include West Nile virus (WNV), yellow fever virus (YFV), Japanese encephalitis virus (JEV) and tick-borne encephalitis virus (TBEV) (45). Important work on these related flaviviruses have contributed significantly to our understanding of the life cycle and structure of DENV (reviewed in (120)). Similarly in the presently described work, we have on numerous occasions delved into published material on related flaviviruses to gain further insight while studying the human antibody response to DENV.

Flaviviruses attach to the surface of the host plasma membrane and enter the host cell through receptor-mediated endocytosis (149). The internalization receptor for DENV is as yet unknown. However, several important attachment factors that aid DENV infection in vitro have been identified; these include C-type lectin (DC-SIGN, dendritic cell-specific ICAM-3 grabbing non-integrin) (142), glycosaminoglycans (Heparin Sulphate, manose receptor in macrophages) (19, 28, 96, 114) and phosphatidylserine receptors (93). As the virion travels through the endosomal pathway, the low pH environment within the endosome triggers large conformational changes on the virion surface, leading to viral fusion with the anionic lipid-containing host membrane of the late endosome (2, 12, 149, 166). Virus envelope-host membrane fusion releases viral RNA into the host cytoplasm, where the virus hijacks the host protein translation machinery and translates the (+)ssRNA DENV genome
into a single polypeptide. As shown in Figure 1.1A, the viral polypeptide (with protein sequence C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5) is subsequently processed by viral and host proteases into three structural (C, capsid; prM, membrane precursor; E, envelope) and seven nonstructural proteins (NS) (reviewed in (120)). During protein synthesis on the rough endoplasmic reticulum (ER), several viral membrane proteins are anchored into the ER membrane and the process of viral assembly begins within the ER lumen (Figure 1.1B). Viral translation initiates formation of convoluted membrane structures, where the viral NS proteins form replication complexes and actively replicate the RNA genome (88, 146). The newly transcribed viral genomic RNA strands are encapsulated within a nucleocapsid, which in turn is packaged within an ER-membrane derived envelope containing prM and E proteins in a trimeric conformation (Figure 1.1B and C) (reviewed in (120)). These assembled viral particles are immature and non-infectious (Figure 1.1C). While the immature virions are transported through the trans-Golgi network (TGN), they undergo glycosylation modifications and processing by the low pH-dependent host protease, furin (Figure 1.1B and C) (133, 164). Mature, infectious virions are subsequently exocytosed from the host cell.

1.3 DENV surface glycoproteins

When a DENV virion is initially assembled, each virion surface is embedded with heterodimers of the two glycoproteins, prM and E. The prM protein primarily functions to prevent premature fusion of the E glycoprotein with host cell membranes, and chaperones the folding and assembly of E protein (86). The prM protein may also play roles in immune evasion, by eliciting weakly neutralizing and infection-enhancing antibodies in the human host (31, 121). Despite the low amino acid sequence identity (~30%) in prM protein within
the flavivirus family, there is high prM protein sequence conservation (~70%) among the four DENV serotypes (31). The prM protein contains approximately 166 amino acids arranged in three domains; 1) the pr peptide (91 amino acids) which folds into a 7 stranded β-sheet, 2) the M portion, which is predicted to consist of an amphipathic helix, and 3) two C-terminal transmembrane segments (M-T1 and M-T2) (Figure 1.2C), where M-T2 contains the signal sequence for the secretion of E protein (78, 91). X-ray crystallography and cryo-electron microscopy (cryo-EM) structures indicate that in the immature virus, the pr peptide resides on the distal end of the E protein and covers the fusion peptide to prevent viral fusion within the TGN membranes (78, 170). During DENV maturation (Figure 1.1C), the prM protein is cleaved by host furin into the soluble pr peptide and the membrane-anchored M protein (~75 amino acids) (2, 164). Structural studies indicate that the M protein is partially hidden below the surface-exposed E proteins in the mature virion (as shown in the cartoon representation in Figure 1.2C), and thereby presumably inaccessible to antibodies and predicted to play an insignificant antigenic role (168).

The flavivirus E glycoprotein (E) (~495 amino acids) is the most surface-exposed protein on the mature, infectious virion particle (Figure 1.2A) (75). E protein plays critical roles in determining cell tropism, attachment, fusion and entry into host cells. The E protein is also the most antigenically significant protein across members of the flaviviral group and the major target of neutralizing antibodies (122). The initial identification of four DENV serotypes was based on the antigenic differences in the E protein. The existence of four distinct DENV serotypes was first identified using immunological assays, and later supported by the 60-70% amino acid sequence similarity of the E proteins among the serotypes (156). As represented in Figure 1.2C, each E protein consists of three structurally different regions;
1) the ectodomain, 2) the two amphipathic helices (E-H1 and E-H2) and conserved stem region (CS), and 3) the two C-terminal transmembrane helices (E-T1 and E-T2) (103, 168). Since the helices and CS region are both hidden by the E ectodomain and membrane associated, very little structural information is known about these regions (168). Several molecular and biochemical studies have shown that these regions are involved in viral assembly (i.e. through prM-E heterodimer stability, and membrane association), low pH rearrangement events and fusion (12, 70, 83, 98, 115, 171). It is also assumed that because these helices are not surface exposed they are inaccessible to antibodies and therefore, irrelevant for studying neutralizing antibody responses.

When the ectodomain region of the E protein (80% of E protein) is expressed without the helices, the resultant recombinant E protein is soluble and secreted (27). The x-ray crystallography structures of several flaviviral E proteins were solved using the soluble E ectodomain, also termed recombinant E protein (rE) (Figure 1.2B) (97, 99, 117). The E ectodomain is folded into three distinct β-barrel domains (named domain I, II and III); the N-terminal domain I (EDI) is an eight-stranded β-barrel, connected on one side with four peptide chains to the dimerization domain (EDII, domain II) and on the other side with one peptide linker to the immunoglobulin (Ig)-like domain (EDIII, domain III). Domain II contains the highly conserved fusion peptide, which is inserted into the host membrane during viral fusion and entry. Domain III of the E protein has the receptor binding sites (25, 62, 77). The DENV E protein has putative surface N-linked glycosylation sites at Asn-67 (EDII) and Asn-153 (EDI), with the latter glycosylation being conserved in most flaviviruses (59, 117). The N-glycan at Asn-67 plays roles in viral attachment (by interacting with DC-SIGN) and viral propagation in mammalian cells, while the sugar at Asn-153 was captured
by x-ray crystallography as stabilizing the E protein dimers by stretching across the dimer interface to cover a region of the fusion peptide (97, 100, 113, 117).

The E protein ectodomain under neutral pH has been solved by x-ray crystallography for both DENV2 and DENV3 (97, 99). Despite a two amino acid deletion in EDI of the DENV3 E protein, the E protein structures of both serotypes were found to share very high structural similarity. One of the few structural differences was observed when the E structures of the two serotypes were super-imposed on each other; it was noted that a 10° difference in rotation at the EDI-II hinge region of the E protein translated into a 13Å and 18Å difference in position of the fusion loop and EDIII, respectively (99). Therefore, it is important to keep in mind that the EDI-DII hinge region is highly flexible and undergoes large conformational changes during viral maturation and the low pH-induced fusion process (Figure 1.3B) (168).

1.4 Virus Structure

Similar to other flaviviruses, the assembled, infectious DENV virion is 500Å in diameter. Each virion consists of the viral genomic RNA-containing nucleocapsid enveloped in a lipid membrane containing the two membrane-embedded glycoproteins, (prM/M and E protein) (75). The DENV genome encodes a single open reading frame of 11 kb in length, with untranslated terminal regions (UTRs) that are critical for regulation of genome replication and translation. Highly asymmetrically charged capsid (C) proteins package this DENV RNA for virion assembly (87). A host ER membrane-derived lipid envelope consisting prM/M and E proteins surrounds the nucleocapsid. Since the inner lipid leaflet and the nucleocapsid are ~30Å apart, it is hypothesized that there is very little connection between them, and that flavivirus envelope formation is primarily dependent on the prM and
E proteins (75, 102, 168, 170). This is further evident by the routine observation of subviral particle formation in virus-infected or prM-E transfected cells in culture, where 30 prM-E heterodimers are sufficient to arrange into particles with an icosahedral symmetry ($T=1$), induce fusion and stimulate protective immunity (2, 23, 35, 72, 103).

**Mature virion**

Recent x-ray crystallography and cryo-EM images have revolutionized our understanding of the DENV virion structure (75, 97-99, 170). The DENV virus is made of 180 heterodimers of prM/M and E proteins, ordered into an icosahedral structure. The surface exposure and arrangement of the glycoproteins on the particle is dependent on the maturity of the virus (Figure 1.1C) and the pH of the surrounding environment (75, 103, 117, 170). The earliest cryo-EM studies of DENV were of the mature, infectious virion (75). In the mature virion, E protein monomers are arranged into head-to-tail dimers, with the fusion loop of one monomer hidden below EDI and EDIII of the adjacent monomer (see Figure 1.2B). Three sets of these dimers are arranged into herringbone-structured rafts, and 30 of these repeated raft structures assemble into the infectious virion (Figure 1.2A). As the E monomers are assembled into the virion particle, the hinge angle between EDI and EDII reduces by about 10-20° (Figure 1.3B), and the EDIII is pushed outwards. It was recently found that the M protein, which is hidden below the surface-exposed E protein, also has a vital role to play in keeping the virion intact (169). The N-terminal 20 amino acid region of M protein contains three pH-sensing histidine residues that at neutral pH latch onto the membrane-facing side of E protein, and forces the E protein to lie flat on the viral membrane and keep the fusion peptide unexposed (169).
Cryo-EM studies of TBEV subviral particles lead to the hypothesis that flaviviral particles are assembled in a T=3 symmetry (35). However, cryo-EM studies of the infectious virion show that the orientation of the 30 rafts in the virion form 5-3-2 axes of symmetry; the EDIII of five E proteins come together to form a 5-fold axis, the EDI of three E proteins form the 3-fold axis and two E proteins come together to form the 2-fold axis (Figure 1.3A) (75). Therefore in the assembled virion, not all the E protein monomers are in the same chemical environment, and this could have significant consequences on attachment, fusion and neutralizing epitopes available for antibody binding.

Immature virion

Surface structure of the immature DENV virus particle is vastly different from that of the mature DENV virus (Figure 1.1C). In the immature prM-E heterodimers, the uncleaved prM extends along the length of the E protein, and the pr peptide β-barrel structure sits on the fusion loop at the distal tip of EDII preventing premature fusion events (78). In the immature virion, these prM-E heterodimers arrange into 60 repeated trimer structures. Unlike the smooth outer surface of the mature virion, these trimers give the immature virus an irregular, spiky surface and an external diameter of 600 Å (i.e. 100 Å greater than the mature virion) (170). The highly flexible hinge region between DI and DII of E protein in the immature virus differs by 30°, when compared to the mature virion, and about 5-15° as compared to the crystal structures of the soluble E protein (75, 97-99, 170). Differences in the EDI-DII hinge angle, E protein epitope exposure and the presence of uncleaved prM could have significant consequences on antibody binding, neutralization or enhancement of mature versus immature virus. Interestingly, recent EM studies have shown that a vast majority of DENV particles exocytosed in cell culture systems are not fully mature (66). In fact, a majority of the cell
culture-derived DENV virions were found to be only partially mature, with almost 90% of the virions containing uncleaved prM (6, 66, 67, 121). The minimum degree of viral maturity required for infectivity is unknown. Recent unpublished data from our lab indicates that the ratio of mature and immature virus may have vast consequences on attachment and infectivity of different cell lines. Furthermore, it is unknown whether natural in vivo infections in humans or mosquitoes also produce DENV virions with a range of maturity.

Major viral rearrangements: maturation & fusion

During the DENV lifecycle, the virion particle undergoes two major conformational changes: 1) during viral maturation in the TGN (reviewed in Perera, R et al, 2008 (111)), and 2) during viral fusion with the host membrane in the late endosomes. Elegant biochemical studies coupled with studies of virus structure have given us some insight into the conformational changes the virus experiences during these events. 1) Viral maturation occurs in two important steps. Spiky immature virus buds from the ER and enters the TGN, where a gradient decrease in the surrounding pH causes pH-sensing histidines in the C-terminal ectodomain region of prM to induce latching on to the membrane-facing side of the E proteins, causing a 30° rotation at the EDI-DII hinge region and forcing the E proteins to lie flat on the membrane surface (Figure 1.3B) (111, 164, 169). This results in the rearrangement of 60 prM-E trimers into 90 E protein dimers (Figure 1.1C). This low pH-induced virion conformation exposes the furin cleavage site, enabling efficient processing of prM into pr peptide and M protein (133, 164). The pr peptide prevents premature fusion by remaining associated with the E protein, until the virion is released from the host cell. Once exocytosed, the neutral pH causes the pr peptide to dissociate from the virion, leaving the virion ready to infect new host cells.
The second major structural rearrangements of the surface glycoproteins occur during viral fusion with the host membrane in the late endosomes (Reviewed in Harrison, SC, 2008 (56)). In the recent years, DENV membrane fusion and the resulting conformational changes in the E, have been studied extensively. Flaviviral fusion is a class II fusion process (117, 134, 135). The structural rearrangements associated with membrane fusion begin when the pH drops in the endosomal compartment, leading to protonation of several histidine residues in both E and M glycoproteins (2, 38). This leads to disruption of the E protein raft structures and dissociation of E protein dimers into monomers. As a result, EDII of E protein is pushed upwards, exposing the fusion loop at the tip of EDII. The E protein monomers rearrange to form the low pH-induced unstable trimer structure. As the virus passes through the endosomal pathway, the viral fusion loop is inserted into the late endosomal membranes enriched with anionic lipids (166). After the insertion of the fusion loop into the outer leaflet of the host endosomal membrane, EDIII folds towards EDII and brings the viral membrane and host membrane together to form the unstable hemifusion intermediate. This is followed by the formation of the fusion pore, and the release of the RNA-containing viral nucleocapsid into the cytosol. X-ray crystallography has been able to capture only the pre-fusion and post-fusion trimer conformations (Figure 1.3)(98, 117). Therefore, while biochemical studies have tried to fill in the gaps of our understanding of this fusion process (79, 130), there is still much work to be done in this area. Furthermore, during the membrane fusion process, the EDI-DII hinge undergoes a large angular change of ~35° (Figure 1.3) (171). Therefore, it is conceivable that antibodies or inhibitors that target this region would potently neutralize DENV by efficiently blocking fusion.
1.5 Human immune response to natural DENV infections

DENV causes an acute self-limited infection, which a majority of individuals clear asymptotically. Studies have shown that the human adaptive immune response is instrumental in the resolution of DENV infection and prevention of re-infection with the same serotype. A human cohort study showed that during secondary infections, cross-reactive CD8+ T cells contributed to severe disease through a process known as “original antigenic sin” (101). However, several recent studies indicate that CD8+ T cells might play a more protective role during secondary infections (163). Therefore, the role of T cells in primary and secondary infections is unclear and requires further examination. Protection against DENV has been directly correlated with the neutralizing antibody response. Furthermore, natural infections with DENV lead to the life-long production of protective antibodies, preventing re-infection with the serotype of infection. Therefore, it is widely accepted that in humans neutralizing antibodies are the primary mediators of protection against DENV.

Primary DENV infections

The human antibody response following a DENV infection is very similar to other acute viral infections. During a primary infection, DENV initially stimulates an IgM response within 4-5 days of fever. DENV-specific IgM reaches a peak titer at about 2 weeks and decays to undetectable levels around 2-3 months after infection (Figure 1.4)(63). Isotype switching occurs and a rapidly rising DENV-specific IgG response is detected within two weeks of illness (Figure 1.4). This life-long IgG (predominantly IgG1 and IgG3 subclasses) response is highly cross-reactive with all four DENV serotypes and even with other species in the flavivirus family (73, 90). Due to the high structural similarity within the E proteins of
the four DENV serotypes, this observed cross-reactivity in the antibody response is hardly surprisingly. Unfortunately, these cross-binding antibodies prevent the use of simple binding assays (such as conventional ELISAs or dipstick assays) to identify the infecting DENV serotype, and require the use of more expensive and technically challenging assays, such as reverse transcription polymerase chain reaction (RT-PCR) and virus neutralization. These cross-reactive antibodies provide a transient cross-protection against the DENV serotypes not seen by the immune system (i.e. heterotypic serotypes) (Figure 1.4). Human DENV-challenge studies in the 1950s, along with epidemiological and more recent computational investigations have calculated this transient individual and herd population cross-immunity as lasting for approximately 6-12 months and 2-3 yrs, respectively (1, 26, 126). Unlike the cross-protective IgG response, the type-specific neutralizing IgG response can be detected even 60 years after infection, and is generally assumed to provide life-long immunity against the serotype of infection (48, 62, 63, 107). Nevertheless, the main basis of current vaccine platforms is to stimulate balanced, life long, neutralizing responses to each of the four DENV serotypes.

*Secondary DENV infections*

Due to the lack of long-lasting cross-serotype protection after primary DENV infections, individuals are susceptible to secondary infections from heterotypic serotypes (14, 128). Pre-existing sub-neutralizing, cross-serotype binding antibodies to heterotypic serotypes lead to a higher probability of severe disease during secondary infections, which is classically thought to be driven by a phenomenon called antibody dependent enhancement (ADE). Secondary infections result in a minor, rapidly diminished IgM response, followed by a rapid increase in IgG antibodies to titers far exceeding those observed during primary
infections. Soon after a secondary infection, both naïve B cells and DENV-specific memory B cells are stimulated, and a rapid elevation in neutralizing antibody titers towards the primary infection serotype is observed; a phenomenon explained by “original antigenic sin” (36, 54, 95). This has strong implications for vaccine strategies, since boosting with heterologous serotypes will preferentially amplify the antibody repertoire towards the serotypes of initial vaccination. A common feature of secondary infections is the eventual development of a broad cross-immunity to include serotypes yet unseen by the immune system. Furthermore, severe dengue in tertiary and quaternary infections are rare, suggesting the presence of long-lasting antibodies targeting cross-protective epitopes after secondary infections (3, 155).

**DENV Antigens targeted by the human antibody response**

Natural DENV infections stimulate the development of specific antibodies towards several structural and non-structural proteins. By western blot binding analysis, primary DENV infections were found to raise antibodies primarily towards E, NS1, NS3 and NS5 proteins, while secondary infections broaden the response to include NS1, prM and C proteins as well (21, 147). It is unclear whether secondary infections truly induce a broader antibody response, or whether the overall higher level of antibodies following a secondary infection enables the detection of anti-NS1, prM and C antibodies. It is unlikely that antibodies directed against the intracellular proteins, NS3 and NS5 proteins, contribute to protection or enhancement of the disease (141). On the other hand, NS1 protein is a soluble viral glycoprotein that is secreted into the blood stream and detectable during active infection (47). DENV NS1 has been implicated in both protective and pathogenic roles (ref). Studies with passive transfer of anti-NS1 antibodies were shown to protect mice from lethal DENV
challenge (60, 129). Furthermore, several vaccine designs expressing DENV NS1 protein (either alone or in combination with E protein) also induced protective responses against DENV (22, 24, 161). Similar protective roles of NS1 protein have been shown in several other flaviviruses (32). However, anti-NS1 antibodies have also been implicated in severe disease by promoting vascular leakage. DENV NS1 was shown to stimulate autoimmune antibodies that not only bind NS1, but also cross-react with platelets and endothelial cells, leading to endothelial cell damage and inflammation (reviewed in (81)).

The DENV E protein is the most surface-exposed protein on the infectious virion, and the principal target of neutralizing antibodies (21, 147). Therefore, E protein has been the subject of intense research for several decades. A significant portion of these circulating anti-E protein antibodies are directed towards the highly conserved fusion loop of E protein, and are cross-reactive (76, 82, 107). Since primary infections only provide long-term protection against the serotype of infection, it is highly unlikely that these cross-reactive, fusion loop-binding antibodies are cross-neutralizing. Results using mouse model systems led to many studies investigating the possibilities of EDIII as being the primary target of neutralizing antibodies made in humans. Unexpectedly, these studies with DENV-immune human sera showed that natural infections produced only a few anti-EDIII antibodies, and this small proportion of anti-EDIII antibodies contributed to a minor proportion (10-15%) of the neutralizing capacity of the polyclonal sera (153, 154). Similarly, mutating a critical lateral ridge epitope on EDIII in WNV did not significantly affect neutralization titers in infected humans or horses (107, 127). This further establishes that flavivirus neutralizing antibodies in humans do not target EDIII.
Memory B-cells and human monoclonal antibodies

For many decades, the DENV antibody field has been restricted to using DENV-immune human polyclonal sera or mouse monoclonal antibodies (MAbs) to map epitopes targeted by the antibody response. Due to the inherent complexity of polyclonal sera, only a limited number of studies have tried to map the DENV epitopes recognized by DENV-immune human sera, and even fewer studies have tried to map the neutralizing epitopes. It is only in the past decade, that the technology to efficiently generate human MAbs from human peripheral memory B-cells has been worked out. Generation of human MAbs from memory B-cells in peripheral blood mononuclear cells (PBMCs) must follow several critical steps; 1) isolation of antigen-specific memory B cells, 2) Epstein-Barr virus (EBV) transformation of the isolated memory B cell, 3) fusion of EBV-transformed B-cell with a fusion partner to create a hybridoma cell line, and 4) purification of the secreted antibody. Memory B-cells specific to an antigen of interest are usually particularly rare, making the isolation of these cells particularly difficult. Until recently, EBV transformation of memory B-cells was inefficient, only transformed CD21⁺ B-cell subsets, and led to transformed B-cells with poor growth, low antibody production and chromosomal instability. Recent studies found that the addition of CpG dramatically increases the efficiency of EBV transformation (7, 57, 144). Furthermore, recent improvements in cell fusion technologies (such as the generation of heterohybridomas) have dramatically improved the number of suitable fusion partners available (165). The above-mentioned improvements in human MAb generation have led to the very recent isolation and characterization of numerous human MAbs specific to DENV virus (6, 29, 31, 132).
1.6 Lessons from DENV-specific mouse MAbs

Until recently, a majority of studies investigating the epitopes of antibody neutralization or enhancement in DENV were conducted by characterizing DENV-specific mouse MAbs raised via viral inoculation or vaccination with recombinant E (rE) protein (reviewed in (33, 155)). Mouse MAbs have been mapped primarily to the most surface-exposed E protein. Few prM and NS1 mouse MAbs have been isolated, but these were found to be only weakly or moderately neutralizing in vitro. Isolated mouse MAbs have been mapped to all domains of E protein; 1) a few were cross-reactive, weakly neutralizing and bound the fusion loop in EDII, 2) a few strongly neutralizing MAbs bound the lateral ridge of EDI, 3) several strongly neutralizing MAbs bound the dimer interface, and 4) most importantly, a large proportion of the strongly neutralizing mouse MAbs targets the EDIII (Figure 1.5) (25, 80, 85, 123, 138, 139).

There are two overlapping epitopes within the EDIII that are recognized by neutralizing mouse MAbs: 1) the 4-looped lateral ridge region of EDIII, and, 2) the A-strand of EDIII (Figure 1.5B). The lateral regions of EDIII have been identified as containing high variability between serotypes, with high conservation within serotypes. Therefore it is understandable that the strongly neutralizing mouse MAbs that have been mapped to the lateral ridge of EDIII are type-specific (42, 138, 139). Many of the mouse MAbs that mapped to the A-strand epitope within EDIII are sub-complex binding (i.e. MAbs binding to two or three serotypes, but not to all four serotypes), and only moderately neutralizing (43, 85, 139). For many years, EDIII was thought to be the epitope of neutralizing antibodies, even within humans. As a result, many vaccine platforms were initially designed with recombinant EDIII antigen and anti-EDIII antibodies were researched for therapeutic applications (9, 48, 61). As
noted above, recent studies indicated that natural infections in humans were found to only rarely elicit EDIII-binding antibodies, and that human neutralizing antibodies targeted an epitope that is yet unmapped.

1.7 Mechanisms of DENV neutralization

Despite differences in the neutralizing epitopes targeted by humans and mice, the use of mouse MAbs in mechanistic studies has vastly expanded our knowledge of the mechanisms of DENV neutralization (reviewed in (33)). The antibody-mediated neutralization of flaviviruses (including DENV) is a “multiple-hit” phenomenon, where neutralization entails a virion to be bound with an antibody stoichiometry that exceeds a required threshold number of bound antibodies for neutralization (Figure 1.6). Work by Pierson and colleagues using WNV and a panel of anti-EDIII mouse MAbs, have calculated the antibody threshold number for flaviviruses as being ~30. This means that for successful antibody neutralization of a flavivirus particle that contains 180 E protein epitopes, there needs to be ≥ 30 antibody molecules bound to that virion (94, 112). It is hypothesized that when antibodies are bound to virions at a stoichiometry less than the threshold number, then there is the risk of ADE (33). It is unclear whether this threshold requirement of at least 30 antibody molecules to neutralize a virion is true for all epitopes. Furthermore, the effect on the threshold number when a polyclonal mix of antibodies is binding to several different epitopes on the same virus (as is the case when DENV enters a DENV-immune human body) is also not known. Nonetheless, exceeding the antibody threshold requirement of neutralization is governed by at least two important factors; 1) antibody affinity, and 2) epitope accessibility (reviewed in (33)).
**Antibody affinity**

Antibody affinity defines the percentage of epitopes on the virion that are occupied by that antibody at a given concentration. Therefore, at any given concentration, antibodies with higher affinity will bind to the virion in greater numbers than low affinity antibodies. Similarly, E protein mutations that reduce antibody affinity, will lower the number of viral epitopes occupied at any given concentration, and reduce the neutralization potential of that antibody. Several studies have observed that decreases in antibody affinity due to intra-serotype, strain variation (i.e. 4-10% within E protein) lead to reduced neutralization of those strains (13, 138, 152). It is unclear whether these intra-serotype variations also affect *in vivo* protection against re-infection from any strain within a particular serotype.

**Epitope accessibility due to complex virion structure**

Due to the complex structural properties of flaviviruses (and more particularly DENV), epitope accessibility is multifaceted. As detailed above, the 180 E proteins are arranged in a dense 5-3-2-fold axes of symmetry, dividing the E proteins on the mature virion into three different chemical environments. Therefore, depending on the epitope recognized by a particular antibody, steric constraints of that epitope would decrease the epitope’s accessibility for binding (20, 68, 69, 85, 106). For example, cryo-EM structures of the neutralizing mouse MAb, E16, in complex with WNV showed that although E16 binds the highly exposed lateral ridge region on EDIII, it can only occupy 120 of the available 180 EDIII epitopes on the mature virion. It was observed that E16 could not occupy the EDIII epitopes at the 5-fold axes of symmetry due to their close proximity to each other (68). Since the threshold number for neutralization is less than the maximum accessible epitopes available, greater than 30 molecules of E16 can bind and neutralize a virion. On the other
hand, in the mature virion the highly conserved fusion peptide is buried beneath EDI and EDIII of the opposing E protein, and inaccessible to binding by anti-fusion loop MAbs, such as E53. Therefore, MAbs similar to E53 need to bind almost all accessible epitopes to exceed the threshold of neutralization, making these MAbs only weakly neutralizing (20, 137).

**DENV structural dynamics**

Despite our earlier detailed description of the mature flavivirus virion, we know that proteins assembled into viral particles are not static structures. In fact, viral surface proteins are thought to be capable of constant movement or “breathing” (65, 160). This dynamic movement was shown to also be true of DENV, and enable the exposure of epitopes that would otherwise have been inaccessible. The mouse MAb, 1A1D-2, is potently neutralizing, DENV-subcomplex binding and more specifically, recognizes an epitope on the A-strand of EDIII (85, 123). Detailed mapping of 1A1D-2 showed that this MAb binds an epitope that is hidden in the mature virion. Experiments at 4°C showed that 1A1D did not bind the virus at low temperatures, while 1A1D binds and efficiently neutralized DENV at 37°C. Cryo-EM studies showed that “breathing” of the DENV virion at 37°C allowed binding of 1A1D-2, which caused E proteins to rotate in a manner that further exposed its epitope (85). Studies with 1A1D-2 and the more recently described DENV1-E11 MAb are evidence of the effects of DENV structural dynamics on the accessibility of epitopes to antibody binding (Figure 1.6) (4, 85).

**Epitope accessibility & DENV maturation**

DENV virus populations released from cell culture are heterogenous in the ratio of cleaved and uncleaved prM, containing a combination of completely immature, completely mature and partially mature-immature virions. Recent studies show that heterogeneity in the
virus population is higher than previously assumed, with up to 90% of the virions containing uncleaved prM (66). Although the minimum requirement of cleaved prM for infectivity is unknown, a majority of partially mature virions are observed to be infectious (66). The arrangement of E proteins during the mature E dimer conformation is vastly different from the immature prM-E trimer conformation (Figure 1.1C). This vast difference in conformation has drastic consequences on epitope accessibility (Figure 1.6), with many instances where a subset of the virus population is resistant to neutralization even at saturating antibody concentrations (105, 112). For example, it was observed that the fusion loop was more exposed in the immature E trimer conformation. Hence, several anti-fusion loop MAbs are better at neutralizing more immature virus, leaving completely mature virions resistant to neutralization from these MAbs (105). Although no detailed cryo-EM mapping of an anti-prM antibody has been conducted yet, it is also easily conceivable that antibodies targeting prM are especially sensitive to DENV maturation.

Complement protein, C1q, & decrease in threshold number

It has been known for years that serum complement components can boost the neutralization of flaviviruses by antibodies (reviewed in (33)). However, it was only within the past few years that the complement protein responsible for this observed increase in neutralization was identified as being C1q. As part of the classical complement pathway, the ~460 kDa C1q multimeric protein activates the pathway by binding the Fc-portion of antibodies. In humans, C1q preferentially binds to IgG3 and IgG1 with higher affinity, than IgG2 and IgG4. Recent work has directly shown that exogenously added C1q can augment the neutralization of flavivirus by antibodies. This increase in neutralization in the presence of C1q is possibly due to the ability of C1q to cross-link antibodies, increasing the avidity
and therefore, decreasing the antibody stoichiometry or threshold number required for neutralization (Figure 1.6) (94). The effects of C1q on antibody neutralization should be considered when testing DENV-specific MAbs and DENV-immune polyclonal sera in \textit{in vitro} neutralization assays.

\textit{Pre-attachment and post-attachments blocking Abs}

DENV-specific MAbs have been documented to neutralize DENV using two main mechanisms. Many MAbs prevent a productive DENV infection by binding regions on the virion surface that are involved in receptor attachment, and are named pre-attachment blocking antibodies. Another group of MAbs (named post-attachment antibodies) allow the attachment of DENV to host cells, but block an essential step in the entry process and prevent viral fusion (25, 40, 58, 106, 123, 136, 143, 151). Post-attachment antibodies bind regions on the virus that are critical for the low pH-induced large conformational change required for fusion. MAbs that neutralize post-attachment bind several different epitopes on the E protein, including EDIII and the fusion loop (40, 123, 136, 143, 151). Both pre-attachment and post-attachment blocking MAbs can be strongly neutralizing (25, 40, 58, 106, 123, 136, 143, 151). Only one study has investigated the dominant mechanism of neutralization in DENV-immune polyclonal human sera (58). This study found that human serum from acutely DENV-infected patients primarily blocked attachment of DENV (58). However, this study used serum samples that were not conducive to investigate long-term protection. Therefore, it is still unclear which mechanism of neutralization is dominant in DENV-immune polyclonal sera.
1.8 Antibody dependent enhancement of DENV: the other edge of the double-edged sword.

One of the most compelling explanations for the higher proportions of severe disease during secondary heterotypic DENV infections (when compared to primary infections) is the phenomenon of ADE (52, 53, 150). As shown in Figure 1.7, ADE of DENV infection is expected to occur when pre-existing sub-neutralizing antibodies in the body (such as from a primary infection) bind to a heterotypic virus during a subsequent infection, and facilitate the entry of the virus through Fc receptor (FcR)-mediated endocytosis into FcR-bearing myeloid cells (such as monocytes and macrophages). Through mechanisms that are largely unclear and possibly furin cleavage-dependent, the antibody-bound virus escapes the phagolysosome and establishes a productive infection within the host cell (Figure 1.7) (reviewed in (52)). It is important to remember that primary infections can also sometimes lead to DHF/ DSS, which means that severe DENV-induced disease is multi-factorial and ADE is only one important mediator of severe disease.

Apart from inducing severe secondary infections, there are several other epidemiological observations that tie the theory of ADE to DENV infections. 1) In DENV endemic regions, infants between the ages of 6 and 12 months are a high-risk group for severe forms of DENV such as DHF or DSS (17, 18, 71). Since the infant immune system is facing DENV for the first time, this occurrence is independent of a cellular adaptive immune response. One of the explanations for a time-dependent severe disease phenotype in infants is the ADE of DENV infection by passively transferred maternal antibodies. The DENV-specific maternal antibodies initially protect the infant for several months, but as the maternal antibody decays over time and reaches sub-neutralizing titers, the infant is at risk of an ADE-
mediated DENV infection for several months (17, 18, 71). 2) Severe disease in both secondary infections and infant cases are observed to reach high serum viremia titers (150). This is not always due to higher virulence of the infecting virus strain (49). Several studies have clearly observed that a DENV infection through the ADE pathway (as opposed to the conventional receptor-mediated entry pathway) leads to significantly higher replication and secretion of infectious virus (41, 55).

ADE of DENV infection is easily reproducible in cell culture using FcR-bearing cells and DENV-specific MAbs or DENV-immune polyclonal sera. Many cell lines, both primary (PBMCs, primary dendritic cells, monocytes and macrophages) and immortalized cells (K562, U937, THP-1), are presently being used to recapitulate the ADE phenomenon in vitro (6, 10, 16, 41, 74, 124, 145, 159). A majority of these immortalized cell lines are only permissive to DENV infection in the presence of DENV-specific antibodies, hence removing the complexity of traditional mechanisms of entry while studying ADE. It was initially postulated that the higher viremia when infected through ADE is not due to more efficient FcR-mediated uptake of virus and higher numbers of infected cells (aka extrinsic ADE) (reviewed in (155)). However, recent in vitro studies show that entry of DENV through ADE is not very efficient, and does not infect more cells than the traditional method of entry (145). Instead the FcR-mediated mechanism of uptake of virus into the host cell suppresses the antiviral responses within that cell, which allows greater viral replication and output (a process termed intrinsic ADE) (145). Despite the discrepancies in the exact cytokine profiles between ADE of DENV across different cell lines, it was generally observed that DENV infection through ADE subverted the host antiviral response by decreasing and increasing secreted levels of type I interferon (IFN) and interleukin (IL)10, respectively (10, 16, 74,
Whether FcR-mediated uptake is more immune suppressive than the traditional method of receptor-mediated entry, is yet unclear. Further studies need to be conducted to define the exact molecular mechanism of how the virus escapes the phagolysosomes, and the proceeding steps that lead to a suppression of the cellular anti-viral response.

The concept of ADE (as opposed to the competing ‘cytokine storm’ explanation) causing severe disease in DENV infected patients has been a topic of much debate for several decades (118). Because DENV is an obligate pathogen of humans (including non-human primates) and the mosquito vector, there is no natural DENV small-animal model, which has hampered in vivo investigations of ADE. Although DENV infection is generally asymptomatic in non-human primates, DENV replicates to low titers in several non-human primate models. DENV infection following passive transfer of DENV-immune sera or DENV-specific MAb into monkeys showed signs of ADE through a 10-100-fold increase in viremia, when compared to virus only treated monkeys (41, 50). However, since non-human primates do not show severe DENV-like symptoms, they are not considered a very good animal model for dengue disease/DHF. One of the most successful animal models for studying ADE of DENV is the type I and II IFN receptor knockout mouse model, AG129 (148). When AG129 mice are passively injected with DENV-specific MAb or polyclonal sera and then challenged with a mouse adapted virus strain, within 5 to 6 days the mice develop a lethal vascular leakage disease that is similar to DHF in humans. During ADE, The infected cells within the AG129 are FcR-bearing cells (such as monocytes and macrophages etc), and sinusoidal endothelial cells in the liver (5, 167). Similar to the DENV-induced disease in humans, the lethal vascular leakage disease in these mice also presents thrombocytopenia and elevated levels of cytokines, such as TNFα, IL-6 and IL-10 (5, 167).
Therefore, although the absence of type I and II IFN-receptors makes the AG129 mouse model imperfect for studying immune responses to DENV, it represents the human DHF disease closely and serves well as a passive antibody transfer model for mapping out the DENV epitopes targeted by enhancing antibodies. Almost all DENV-specific MAb s or polyclonal sera, whether neutralizing or not, when diluted out far enough, will enhance DENV infection. Therefore, it is critical to identify the heterotypic DENV epitopes targeted by antibodies in human sera at physiological concentrations. Determining the targets of enhancing antibodies in human sera will hopefully enable the design of safer, non-DENV-enhancing vaccines.

1.9 Prospective DENV vaccines

The presence of four related serotypes, each capable of inducing cross-reactive antibodies that at sub-neutralizing concentrations can enhance infection of heterotypic virus, makes DENV vaccine development a very challenging task. Vaccine developers have several factors to keep in mind when trying to create a DENV vaccine (reviewed in (104)). First, since most DENV endemic countries have all four DENV serotypes co-circulating, it is imperative that the vaccine elicits a protective response against each of the four serotypes. Second, this protective response needs to be life-long. If antibody levels fall to sub-neutralizing levels, this places the vaccinee at risk of enhanced DENV infection and therefore, more severe disease. Third, as with all vaccines, a DENV vaccine must be safe and produce minimum symptoms in the recipients. Fourth, the immunization protocol must include the entire population, since even adults are at risk from tertiary and quaternary infections. Finally, since the countries most affected by DENV are developing countries with
poor health care, any approved DENV vaccine must be economical and affordable for individuals residing in these countries.

Scientists are using many strategies to develop a DENV vaccine. Nonliving vaccine platforms being tested against DENV are inactivated subunit, subviral particles and whole virus vaccine. Although, nonliving vaccines have the advantage of being safe, these vaccines are not very efficient at stimulating strong adaptive responses, and hence require adjuvants and serial boosts. Furthermore, subunit vaccines currently being developed using E ectodomain protein do not present the immune system with the same repeated E protein raft structure that is displayed on the whole virion. Several studies have pursued the development of subviral particle through the expression of prM and E proteins, where vaccine recipients are either immunized with purified subviral particles (84) or with viral vectors (such as adenovirus, vaccinia, or alphavirus) that express DENV prM and E proteins (37, 116, 157, 158). Although subviral particles have a T=1 symmetry, they also lack some of the complex epitopes formed when E assembles into the virion particle. Whether this repeated E protein raft structure is essential for raising life-long DENV neutralizing antibodies is unclear.

The three DENV vaccines most advanced in clinical trials are live attenuated vaccines. One is a live, attenuated DENV vaccine (LAV) developed by the National Institute of Health. This vaccine contains a DENV4 genetic background with attenuation mutational deletions within the 3’UTR region. The prM and E proteins from the three other serotypes were inserted into this attenuated DENV4 background. This vaccine has just passed phase I clinical trials, and there have also been successful human challenge studies (140). The second LAV, DENVax, was initially developed by the Center for Disease Control (CDC) and later acquired by the private company, Inviragen (110). This vaccine utilizes the weakened
DENV2 strain, PDK-53, into which the prM and E proteins from the other three serotypes are inserted. This vaccine is also in clinical trials at the moment. The third LAV, ChimeriVax, is a chimeric virus between DENV and the highly successful (and presently licensed) yellow fever vaccine strain, YFV 17D (46). The prM and E proteins are expressed in the YFV 17D background, thereby producing 4 chimeric, monovalent viruses. This ChimeriVax vaccine, designated CYD-TDV (licensed by Sanofi Pasteur) has reached phase IIB clinical trials. Unfortunately, these clinical trial data, published late last year, showed that this vaccine had an overall protective efficacy of only ~30% against natural DENV infections, with no protection against DENV2 infections (125). Several hypotheses about genotypic differences between immunized and circulating viruses are being put forward (unpublished data from Sanofi Pasteur). Since we know that the most successful DENV immunizations in humans at present are natural infections, we need to ask another vital question; are the vaccines being developed eliciting similar T cell and B cell responses as natural wild type DENV infections? Unfortunately, with very little known about the DENV epitopes targeted by protective T cells and neutralizing antibodies, the answer to this question is still pending.

1.10 Objectives of this dissertation

At a time when DENV is endemic in over 100 countries with over 390 million new infections globally, we known very little about the neutralizing and enhancing human antibody response after a natural DENV infection. The lack of a suitable animal model led to the generation of MAbs in immune-compromised mouse models (39). For many years, mouse MAb based studies were used to gain insight into the neutralization and enhancement of DENV. Type-specific neutralization was mapped to DIII of the E glycoprotein (25, 80, 85,
However, as described above, more recent studies have clearly shown that EDIII is not the target of human neutralizing antibodies following natural flaviviral infections (107, 127, 153, 154). Therefore, there is a significant knowledge gap in our understanding of the epitopes targeted by the human antibody response after a natural DENV infection. One of the goals of this dissertation is to expand our knowledge of the human antibody response after primary DENV infections. To achieve this goal, we have devised a simple technique to fractionate DENV-specific antibodies in human immune sera into groups, and then characterize their binding and functional properties. Additionally, in collaboration with other groups, we have also generated, isolated and characterized the binding and functional properties of DENV-specific human MAbs from individuals with past natural DENV infections.

A recent study has mapped a potently neutralizing anti-WNV human MAb to a complex viral surface epitope centered on the DI-DII hinge region of the E protein. This epitope is not conserved on the soluble E protein and hence, this potent anti-WNV MAb does not bind soluble E protein (69, 151). Primary DENV-immune human sera are very reactive to soluble DENV E protein from all serotypes. Despite the large cross-reactivity across serotypes, late convalescent sera after primary DENV infections only neutralize the serotype of infection. This indicates that the neutralizing antibodies in human sera are very rare and possibly very difficult to isolate. Fortunately, advances in human B-cell technologies have vastly improved the transformation and fusion of human memory B-cells, so that even rare populations of memory B-cells can be isolated and the secreted MAbs can be characterized (7, 57, 144, 165). Another central goal of this dissertation is to isolate and characterize strongly neutralizing human MAbs, and identify the neutralizing epitopes targeted by human
antibodies. Successful characterization of the neutralizing epitopes will allow us to probe and understand the mechanisms of DENV neutralization by human MAbs, and how they differ from those already described using DENV-specific mouse MAbs.

Almost any DENV-specific MAb (binding E or prM protein) can enhance DENV infection in vitro. Therefore, the importance of characterizing the enhancement properties of MAbs is unclear. What is critical is to define the epitopes of antibodies in DENV-immune human sera that enhance infection of heterotypic serotypes. Hence, the third goal of this dissertation is to characterize the epitopes targeted by human polyclonal antibodies that enhance heterotypic infection. These experiments will be conducted using in vitro ADE assays and the in vivo AG129 mouse model. In summary, the primary goal of this dissertation is to expand our understanding of the neutralizing and enhancing human antibody response to DENV, for purposes of understanding the biology of antibody-mediated protection in DENV infection and for the rational development of successful vaccines.

**Hypothesis:** We hypothesize that humans exposed to primary dengue infections develop two unique antibody sub-populations that 1) neutralize homotypic and 2) enhance heterotypic serotypes of virus. Moreover, we propose that human antibodies neutralize DENV at a post-attachment step by binding to novel conformational epitopes that include the DI-DII hinge of the dengue envelope (E) protein.

**Aim 1.** To characterize the binding and neutralization properties of the human antibody response to natural DENV infections, by studying the DENV-specific memory B-cell and plasma cell response.
**Aim 2.** To map and characterize the neutralizing epitope(s) targeted by the human antibody response after a natural DENV infection, by mapping both DENV-immune human polyclonal sera and strongly neutralizing human monoclonal antibodies. Furthermore, mechanistic studies will be conducted to gain insight into the mechanism of DENV neutralization by human antibodies.

**Aim 3.** To determine the epitopes of enhancing antibodies circulating in humans after a primary DENV infection. In vitro observations will be extended to *in vivo* experiments, using the AG129 ADE mouse model.
**Figure 1.1 DENV genome and life cycle.** A) The DENV RNA is translated into 10 proteins, (i.e. 3 structural and 7 non-structural proteins) which are processed by viral and host proteases. B) The DENV virion binds its putative receptor and enters the host cell through clathrin-mediated endocytosis, undergoes low pH fusion with the host membrane in the late endosome, undergoes replication and translation in the cytosol, assembly in the ER, post-translational modifications and processing of the virion in the TGN, after which infectious virions are exocytosed. During different stages in the DENV life cycle, the virion can exist in one of three important virion structures, i.e. 1) mature virion, 2) immature virion, and 3) the post-fusion conformation. C) The effects on virion structure and prM-E conformation before, during and after prM processing. Figures A) & C) taken and modified from *Perera, R et al, Current opinions in microbiology, 2008*, while figure B was taken and modified from *Mukhopadhyay, S et al, Nature reviews microbiology, 2005* (103, 111).
Figure 1.2 DENV E glycoprotein structure and its arrangement on the mature virion.

A) Six E proteins are arranged into 30 repeated E protein raft structures, which assemble into the infectious DENV virion with 5-3-2 axes of symmetry. B) Each E protein raft is made of three E protein dimers. C) Flaviviral E protein is a transmembrane glycoprotein, made of three distinct domains, two amphipathic helices, conserved stem region and two transmembrane helices. Figure panels A), B) & C) were taken and modified from Mukhopadhyay, S et al, Nature reviews microbiology, 2005 (103).
Figure 1.3 DENV E protein undergoes large conformational changes during virus maturation and viral fusion. A) X-ray crystallographic structure of the post-fusion conformation of DENV2 E protein. B) The EDI-DII hinge angle changes drastically during virus maturation and fusion with host membrane. Figure modified from Mukhopadhyay, S et al, Nature reviews microbiology, 2005 (103).
Figure 1.4 The antibody response in humans following natural primary DENV infections. Protective polyclonal response after a primary DENV infection is only transiently cross-protective, while life-long protection is type-specific and against the serotype of infection.
Figure 1.5 Epitope mapping of DENV-specific mouse MAbs. While neutralizing mouse MAbs against DENV have been mapped to all domains within the E protein (A), the majority of mouse MAbs were mapped to the lateral ridge and A-strand epitopes of EDIII (B). Image taken and modified from Wahala, WMPB et al, PLOS Pathogens, 2010 (152).
Figure 1.6 Structural dynamics, virion maturation and presence of complement affect the antibody threshold of DENV neutralization. Using mouse MAb s, successful neutralization of DENV was predicted to require at least ~30 antibody molecules bound per virion. It is predicted that antibody-virion stoichiometry less than the ~30 threshold will provide conditions for ADE. Image taken from Dowd, KA et al, Virology, 2011 (33).
Figure 1.7 Antibody dependent enhancement of DENV. In the presence of sub-neutralizing concentrations of DENV-specific antibodies (IgG), antibody-coated virus can enter FcR-bearing cells (such as monocytes and macrophages), suppress the host cell antiviral response and release high viral loads. Image taken and modified from Murphy, BR et al, Annu. Rev. Immunol., 2011 (104).
References


CHAPTER TWO

In-depth analysis of the antibody response of individuals exposed to primary dengue virus infection

2.1. Overview

Humans who experience a primary dengue virus (DENV) infection develop antibodies that preferentially neutralize the homologous serotype responsible for infection. Affected individuals also generate cross-reactive antibodies against heterologous DENV serotypes, which are non-neutralizing. Dengue cross-reactive, non-neutralizing antibodies can enhance infection of Fc receptor bearing cells and, potentially, exacerbate disease. The actual binding sites of human antibody on the DENV particle are not well defined. We characterized the specificity and neutralization potency of polyclonal serum antibodies and memory B-cell derived monoclonal antibodies (hMAbs) from 2 individuals exposed to primary DENV infections. Most DENV-specific hMAbs were serotype cross-reactive and weakly neutralizing. Moreover, many hMAbs bound to the viral pre-membrane protein and other sites on the virus that were not preserved when the viral envelope protein was produced as a soluble, recombinant antigen (rE protein). Nonetheless, by modifying the screening procedure to detect rare antibodies that bound to rE, we were able to isolate and map human antibodies that strongly neutralized the homologous serotype of DENV. Our MAbs results

indicate that, in these two individuals exposed to primary DENV infections, a small fraction of the total antibody response was responsible for virus neutralization.

2.2 Introduction

Dengue virus (DENV) complex consists of 4 serotypes. People exposed to primary DENV infections develop robust antibody responses that cross-react with all serotypes (Reviewed in (30)). Despite the extensive cross-reactivity, individuals only develop long term, protective immunity against the homologous serotype responsible for the primary infection (11, 32). Indeed, the risk of progressing to DHF is greater during secondary compared to primary infection (12). A prevailing theory that explains severe dengue during secondary infection is that pre-existing, non-neutralizing dengue specific antibodies enhance DENV entry and replication in Fc-receptor-bearing cells, which leads to a higher viremia and more severe disease (12). Antibodies have been demonstrated to enhance DENV in cell culture (13, 14) and in animal models of dengue pathogenesis (1, 8, 43).

Our current understanding of how antibodies interact with DENV and other flaviviruses is primarily based on studies utilizing mouse monoclonal antibodies (MAbs) (Reviewed in (26)). The DENV envelope (E) protein is the principle target of neutralizing antibodies. Antibody neutralization occurs by blocking critical functions of the E protein, including attachment to host cells and low pH-dependent fusion of the viral and host cell membranes (27). The crystal structures of the E protein of several flaviviruses have been solved (19, 20, 23, 28). Individual subunits of E protein consist of three beta-barrel domains designated domains I (EDI), II (EDII) and III (EDIII), with the native protein forming a head-to-tail homodimer. Mouse MAbs that bind to all three domains of DENV E have been
generated and characterized (6, 9, 10, 17, 18, 31, 36, 37). Although neutralizing mouse MAbs have been mapped to all three domains of E, the most strongly neutralizing MAbs recognize epitopes on the lateral ridge and A strand of EDIII (35).

Following a primary DENV infection, humans develop antibodies that cross-react with all 4 serotypes, but mainly neutralize the homologous serotype responsible for the infection (Reviewed in (32)). Studies with human immune sera and, more recently, human monoclonal antibodies have demonstrated that the dominant antibody response is cross-reactive and weakly neutralizing (2, 5, 7, 16, 34, 40). Multiple viral antigens including E protein, pre-membrane (prM/M) protein and non-structural protein 1 (NS1) are recognized by the human humoral response (2, 5, 7, 16, 34, 40). Nonetheless, few studies have defined the actual epitopes of DENV recognized by type-specific and cross-reactive human antibodies at the structural level and compared this to the epitopes defined using mouse antibodies. The target(s) of dengue type-specific, strongly neutralizing human antibodies remain unknown. The goal of this study was to study two subjects in-depth to define the major antigens and epitopes recognized by antibodies that develop following primary human DENV infection. Defining the human B-cell epitopes on DENV is a key step towards understanding how antibodies can both enhance and inhibit the severity of DENV infections.

2.3 Materials and Methods

Viruses, recombinant proteins and immune sera.

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 the present study (40). Recombinant envelope (rE) proteins from the 4 DENV serotypes were kindly provided by Dr. Beth-Ann Coller (Hawaii Biotech, Inc) (19). The recombinant proteins bind to conformational MAbs and X-ray crystallography studies have demonstrated that these proteins retained a native-like structure (19, 20). Convalescent DENV immune sera were obtained from volunteers who had experienced natural DENV infections during travel abroad. The protocol for recruiting and collecting blood samples from people was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill. Written informed consent was obtained from all subjects before collecting blood.

*Whole DENV and recombinant E antigen ELISAs*

ELISA plates were coated with 50 ng of purified virus or 100 ng of rE in carbonate buffer at pH 9.6 for 2 hrs at room temperature and incubated with blocking buffer (0.05% TBS-T containing 3% skim milk or 3% normal goat serum) at 37°C for 1 hr. Human immune sera or hMAbs serially diluted in blocking buffer were added for 1 hr at 37°C followed by alkaline phosphatase-conjugated goat anti-human IgG (Sigma) for 1 hr at 37°C. Finally, 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 405 nm on a spectrophotometer.

*DENV Neutralization assays*

DENV neutralizing antibodies was measured by a focus reduction neutralization test.
(FRNT) with Vero cells or using a flow cytometry-based neutralization assay with the U937 human monocytic cell line stably transfected with DC-SIGN as previously described (15).

Production of human M Abs (hM Abs) from dengue immune travelers

Peripheral blood samples were obtained from two healthy adult donors who were infected by DENV during foreign travel. The dengue neutralization profiles confirmed previous primary DENV2 (Donor 013) and DENV3 (Donor 033) infections (Table S1). From both donor hM Abs were produced as previously described (3). B-cells producing DENV specific antibody were identified by screening culture supernatants by flow cytometry for antibodies that bound to C6/36 insect cells infected with DENV2 (Donor 013) or DENV3 (Donor 033) (3). A secondary screen to identify antibodies that bound to the rE was performed by ELISA as previously described (3).

Epitope mapping of EDIII binding hM Abs

DENV antibody escape mutant viruses were selected for by infecting Vero cells with DENV2 (strain S-16803) in the presence of hM Ab concentrations estimated to neutralize greater than 99% of infectious virus (i.e. 1.0 µg/ml for DVC 3.7 and 1.5 µg/ml for DVC 10.16). Equivalent GC copy numbers of control and antibody treated viruses were repeatedly passaged in the presence of hM Abs until equivalent DENV genomic copy numbers were observed for MAb treated and control samples (4-6 passages under antibody pressure). Escape mutant viruses were plaque purified and amplified. E genes were amplified by RT-PCR and sequenced to identify mutations linked to antibody escape. Antibody binding sites
were also mapped by using yeast cells expressing a library of EDIII as previously described (25). Mutations were mapped onto the DENV2 EDIII structure using the atomic coordinates of DENV2 EDIII (RCSB accession number 1OAN) and displayed using PyMOL Molecular Graphics System, Version 1.3 (Schrödinger, LLC).

2.4 Results

The objective of the current study was to characterize the primary human antibody response to DENV by comparing immune sera and MAbs derived from two individuals previously exposed to primary DENV infections. Donor 033 had reported a high fever following a visit to India in 2005 and laboratory investigations confirmed a primary DENV3 infection (data not shown). One year later, when serum and peripheral blood mononuclear cells (PBMCs) were isolated, the subject had a neutralizing antibody response that primarily targeted DENV3 (Table S1). Donor 013 developed a fever, clinically diagnosed as dengue, while visiting a Pacific Island in 1996. Eight years later when serum and PBMCs were isolated for the current study, the subject had a neutralizing antibody profile consistent with a past primary DENV2 infection (Table S1).

Initially we characterized the binding properties of serum polyclonal antibodies in both subjects using purified DENVs and recombinant DENV E proteins from the 4 serotypes. We calculated endpoint titers (reciprocal of highest serum dilution that was positive in the assay) to estimate relative levels of antibody against whole virus and rE antigens (Table 1). The quantity of antibody binding to E was only 10 to 35% of the quantity that bound to whole virus suggesting that antibodies bound to sites on the virus that were not present on rE protein (Table 1). Thus, in both subjects the predominant antibodies binding to dengue
virions were serotype cross-reactive and directed to epitopes on the virus and, to a lesser extent, to epitopes on rE protein.

Identification of DENV-reactive memory B cells following primary infection

We have previously reported that dengue reactive memory B cells are common following both primary and secondary DENV infection (2). To further characterize the human B cell response in donor 033, we immortalized memory B cells. PBMCs were isolated and IgG+ memory B cells were immortalized with EBV and CpG as previously described (38). The immortalized B cell culture supernatants were screened for antibodies that bound to C6/36 insect cells infected with DENV3. Thirty five percent of the B cell cultures generated from donor 033 was positive following this initial screen (Table 2). From the dengue positive cultures only 7.5% of the cultures bound to rE protein (Table 2). To further characterize the binding and functional properties of human antibodies, we isolated hMAbs from donor 033.

Isolation and characterization of DENV-specific hMAbs from donor 033

To isolate hMAbs specific for DENV from donor 033, positive B cell cultures were cloned by limiting dilution and 16 clones were isolated and expanded. All the hybridomas produced IgG1 with the single exception DV20.10, which was IgG3 (Table S2). To identify antibodies that bound to structural viral antigens, the hMAbs were tested for binding to purified DENV3 particles in ELISA. Fourteen out of 16 hMAbs bound to DENV3 virus. Of the 14 hMAbs that bound to DENV3 particles, 13 hMAbs cross-reacted with all four dengue serotypes dengue complex reactive and one antibody (hMAb DV51.3) bound to serotypes 1
and 3, but not 2 and 4 (dengue sub complex reactive)(Table S2). Dengue virions were solubilized and subjected to Western blot analysis to identify the viral structural proteins recognized by hMAbs. Ten of the 14 hMAbs bound to prM and only a single hMAb (DV64.3) bound to E protein (Figure 1). Three hMAbs did not bind to viral antigens by Western blot, although they reacted with DENV3 virion. The 16 hMAbs were also tested for binding to rE protein in ELISA. Only hMAb DV64.31, which had also recognized E protein by Western Blot, bound to rE protein from all 4 DENV serotypes (Table S2).

We next tested the ability of the 16 antibodies generated from donor 033 to neutralize DENV. Five hMAbs, including the two that did not bind to the virion, lacked neutralizing activity (Figure 2). The remaining antibodies ranged from weak to moderately neutralizing and had DENV3 50% neutralization titers that ranged from 0.09 to 1 µg/ml (Figure 2A). The neutralizing antibodies had similar 50% neutralization titers against all 4 serotypes, which was consistent with their broad binding specificity (data not shown). In general, prM antibodies had neutralization curves that were shallow and did not reach 100% neutralization, indicating that a fraction of the virus population was resistant to antibody neutralization (Figure 2B). The single E reactive antibody (DV64.31) exhibited a steeper neutralization curve and neutralized 100% of virus at high concentrations (Figure 2C). In summary, the hMAbs generated from donor 033, who had experienced a primary DENV3 infection, were broadly cross-reactive, weakly neutralizing, and mainly directed to epitopes on prM. None of the hMAbs mimicked the functional properties of immune serum from donor 033 that displayed strong type-specific neutralization of DENV3 (Table S1). A complete summary of the functional profiles of all sixteen hMAbs from donor 033 is included as supplementary material (Table S2).
Characterization of Human MAbs generated from donor 013

Next, we characterized the dengue specific memory B cell response in donor 013, who had recovered from a primary DENV2 infection. We have previously reported on some of the properties of B cells and hMAbs generated from this donor (2). Here we expand on these previous results by comparing properties of serum antibodies and hMAbs from this donor and by epitope mapping a subset of hMAbs from this donor. When immortalized B cell culture supernatants from donor 013 were screened for antibodies that bound to C6/36 insect cells infected with DENV2, 28% (567/2016) of the cultures were found positive for DENV-specific B cells following this initial screen (Table 2). From the DENV positive cultures only 2.9% of the cultures bound to rE protein (Table 2). Thus, as in the case of donor 033, although dengue specific memory B cells were frequent, only a small fraction of the positive cultures from donor 013 produced antibodies that bound to rE protein.

Since a relatively unbiased selection scheme for producing hMAbs from donor 033 indicated that most hMAbs were cross-reactive, weakly neutralizing and directed to antigens other than rE, we altered the selection scheme for donor 013 to enrich for hMAbs that recognized rE from DENV2. After the initial screening of memory B cell culture supernatants, the relatively rare rE protein binding cultures were selected for cloning and expansion. Ten rE binding hMAbs were produced (all IgG1). When the antibodies were tested for binding to heterologous serotypes, 5 hMAbs were DENV2 type-specific, 2 hMAbs were dengue subcomplex-specific and 3 hMAbs were dengue complex-specific (Figure 3A). Six of the ten hMAbs bound to EDIII from DENV2 (Figure 3A). The hMAbs from donor 013 displayed variable neutralization properties (Figure 3A-C), with 2 non-neutralizing
hMAbs (DV1.6, and 21.5; 50% neutralization titers > 1 ug/ml), 4 weakly to moderately neutralizing hMAbs (DV14.21, 35.3, 25.5 and 18.21; 50% neutralization titers between 0.1 and 1 ug/ml) and 4 strongly neutralizing hMAbs (DV3.7, 10.16, 13.6 and 23.13; 50% neutralization titers < 0.1 ug/ml). The strongly neutralizing hMAbs, including those that were cross-reactive in binding assays, neutralized DENV2 better than the other serotypes (Figure 3). Thus, by biasing the initial screen for hMAbs that bound to rE protein, we identified hMAbs with neutralization profiles that were more similar to the immune sera of donor 013, which strongly neutralized DENV2.

**Epitope mapping donor 013 hMAbs binding to EDIII**

Several hMAbs from donor 013 bound to EDIII (Figure 3). Two approaches were used to map the binding sites of these hMAbs on EDIII. Two strongly neutralizing hMAbs that bound EDIII, which were type- (DV3.7) or subcomplex- (DV10.16) specific, were mapped by identifying neutralization escape variants after passaging DENV2 in the presence of each antibody (Figure 4A and B). When the escape mutants were sequenced, each had acquired a mutation on EDIII. The virus passaged in the presence of hMAb DV3.7 acquired the single point mutation V382G (Table 3 and Figure 4C). This residue is located on the EDIII lateral ridge, which is a target of previously mapped type-specific strongly neutralizing mouse MAb (9, 37) (Table 3 and Figure 4C). The virus passaged in the presence of hMAb DV10.16 acquired the point mutation E311K (Table 3 and Figure 4C). This amino acid is located on the A strand of EDIII and forms part of a dengue subcomplex epitope recognized by neutralizing mouse MAb (10, 37) (Table 3 and Figure 4C).

As an independent approach to mapping EDIII-reactive hMAbs, we used a yeast surface
display assay that has been previously used to map numerous flavivirus antibodies (25, 36, 37). Table 3 summarizes all mutations that resulted in loss of binding or neutralization of EDIII antibodies generated from donor 013. All the human EDIII-binding MAbs mapped to the lateral ridge or the A strand regions that have previously been described as targets of mouse MAbs (37) (Figure 4C).

2.5 Discussion

Our main objective was to define viral antigens and epitopes recognized by 2 individuals exposed to primary DENV infections. In both these subjects the dengue virion was a major target of the humoral immune response but many of these antibodies did not bind to rE protein. The DENV virion displays 180 E and 180 prM or M proteins that are arrayed with pseudo-icosahedral symmetry. Depending on the maturation state of the virus particle, the 180 E protein molecules are organized as 90 head-to-tail dimers that lie flat on the virion surface or 60 trimers that protrude as spikes from the surface (21). Since the E protein, with ~500 amino acids, is considerably larger than the 166 amino acid prM protein, the majority of surface exposed viral protein consists of the ectodomain of E. Thus, our finding that many antibodies bound to epitopes on the virus that were not preserved on rE was somewhat unexpected. These findings are consistent with the results of another study from our group (2) as well as a study by Dejnirattisai and colleagues who observed that many hMAbs that bound to DENV particles did not bind to rE protein (7). As the rE protein used in the current study lacked ~20% of the protein, which include the membrane proximal regions and the transmembrane domains, it is possible that some antibodies bind to these regions. Moreover, antibodies may recognize E protein epitopes that are only available in the context of the native oligomeric array on the virion. Several hMAbs generated from
individuals infected with West Nile virus bound the virion but not rE protein; these hMAbs recognized epitopes that were created by adjacent E protein molecules on the surface of the virion or formed by hinge regions (39).

When producing MAbs from donor 013, we increased the probability of identifying strongly neutralizing antibodies by biasing the selection scheme for MAbs that specifically recognized rE protein. In contrast to the weak or non-neutralizing hMAbs isolated from donor 033, several hMAbs from donor 013 displayed strong type-specific DENV2 neutralization. Of note, even hMAbs from donor 013 that bound to more than one serotype (dengue subcomplex or complex MAbs) displayed type-specific neutralization of DENV2, which was the serotype that infected donor 013. Thus, by biasing the selection to enrich for rare rE protein binding antibodies, we generated hMAbs from donor 013 that were functionally similar to the polyclonal immune serum from the same donor that neutralized only DENV2. It is unclear how the results with hMAbs relate to the neutralization properties of polyclonal immune serum from these donors. One possibility is that the abundance of hMABs reflects the functional properties of antibodies in immune sera, where a small fraction of DENV-specific antibodies in immune sera are responsible for neutralization. Alternatively, it is conceivable that individual antibodies that are weakly neutralizing become strongly neutralizing due to cooperative effects that only occur in a polyclonal milieu. Further studies are needed to understand how the properties of hMABs from DENV immune donors relate to the properties of circulating serum antibody.

From the neutralizing MAbs from donor 013 that bound to rE, some bound to the lateral ridge and A strand epitopes on EDIII. We also identified several neutralizing hMABs (35.3, 18.21, 13.6, 23.13) that bound to rE but not EDIII and these antibodies most likely
bind to epitopes on EDI or EDII. We are especially interested in mapping the binding sites of these hMABs as recent studies indicate that epitopes other than the lateral ridge and A strand of EDIII can be the targets of strongly neutralizing mouse, horse and primate antibodies (24, 33, 40) (8, 36),

Our results indicating that prM was a dominant target of the primary antibody response are in agreement with other recent studies (2, 7). The prM protein is required for the proper folding and assembly of flavivirus particles in the endoplasmic reticulum (42), while also preventing adventitious fusion of the virus (41) in the acidic environment of the trans-Golgi network. Recent studies have demonstrated that DENV virions produced in cell culture are often partially processed and contain a mixture of unprocessed prM and fully processed M (4). The maturation state and relative amount of prM on a virion can alter the potency of antibodies that bind specific epitopes on E, including the cross-reactive antibodies that bind the fusion loop in DII (22). Additionally, immature, non-infectious particles became infectious in the presence of prM antibody by enhancing the ability of immature dengue virions to infect Fc-receptor bearing cells in vitro (7, 29). Given our findings and those of others (7) establishing that prM antibodies are common following primary and secondary DENV infections, more work needs to be performed to address their contribution to dengue pathogenesis in humans.

In summary, the studies reported here demonstrate an unexpected antibody profile in two individuals following primary dengue infection. In both individuals a majority of the DENV-specific human antibodies were broadly cross-reactive and weakly neutralizing. Many antibodies bound to prM and sites on the virus that were not preserved on rE protein. Only a minor fraction of the total dengue specific antibody response was responsible for
potent neutralization of the homologous virus. Given the difficulty of identifying suitable donors and generating antigen specific hMAbs, we only characterized the antibody response in two subjects here. Further studies with more dengue immune subjects are needed to determine if our findings are broadly applicable to primary dengue exposure.
Figure 2.1 Antigens recognized by hMAbs produced from donor 033. DENV3 virions were purified and the viral proteins were separated by SDS-polyacrylamide gel electrophoresis. Western blots were performed to identify the viral antigens recognized by hMAbs from donor 033. The figure displays Western blot results for selected hMAbs that bound to E protein (64.31) and prM protein (DV38.1, 59.3, 65.5, 4.3, 11.12, and 18.5). 4G2 and 2H2 are control mouse MAbs that bind E and prM proteins, respectively.
Figure 2.2  DENV3 neutralization by donor 033 human MAbs. The 50% neutralization titers against DENV3 were determined for all 16 hMAbs from donor 033 using a flow cytometry based neutralization test that utilizes U937 cells expressing DC-SIGN. (A) Dengue antigen recognized and the 50% neutralization titer for each hMAb. The hMAbs for which a specific antigen has still not been identified are designated with a *?*. (B) Neutralization curves for three representative prM antibodies. Note that prM antibodies have shallow neutralization curves, which plateau when ~60% of the virions are neutralized. (C) Neutralization curve of the only E protein reactive antibody obtained from donor 033. This antibody has a steeper neutralization curve compared to prM antibodies.
Figure 2.3  Binding and neutralization properties of donor 013 hMAbs. Ten hMAbs from donor 013 were tested for binding dengue virus, recombinant E (rE) and EDIII from DENV2. Cross-reactivity was determined by using whole virus antigen from all four serotypes. For each hMAb the 50% neutralization titer was determined using flow cytometry and U937 cell expressing DC-SIGN. (A) Summary of the binding and neutralization data for all hMAbs from donor 013. B, C and D display representative neutralization curves for DENV2 type-specific (B), subcomplex- specific (C) and complex-specific (D) hMAbs.
Figure 2.4 Epitope mapping of anti-DENV2 hMAbs binding to EDIII. To identify antibody binding sites, DENV2 was serially passaged in the presence of neutralizing hMAbs DV3.7 or DV10.16. Viruses growing in the presence of hMAbs were plaque purified and expanded. Neutralization escape was confirmed by growing the parental and the cloned antibody selected viruses in the presence of each hMAb 3.7 (A) or hMAb 10.16 (B). (C) Localization of neutralizing human antibody epitopes on the structure of DENV2 EDIII (strain 16681) using residues identified by neutralization escape selection (blue) or yeast surface display screening (see Table 3) (orange). Ribbon diagram of DENV-2 EDIII was generated from a published X-ray crystallographic structure. The disulfide bond is highlighted in yellow. Human MAbs 3.7 and 25.5 are type-specific antibodies that bind to epitopes centered on the lateral ridge while 10.16 is sub-complex-specific and bind to an epitope centered on the A strand of EDIII.
### Table 2.1 Relative levels of virus and rE protein binding antibody in immune sera

<table>
<thead>
<tr>
<th>Serum</th>
<th>Serotype</th>
<th>End point binding titer</th>
<th>rE antibody relative to whole virus antibody (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Whole virus</td>
<td>rE protein</td>
</tr>
<tr>
<td>Serum 013</td>
<td>DENV1</td>
<td>3,579</td>
<td>358</td>
</tr>
<tr>
<td>(primary DENV2)</td>
<td>DENV2</td>
<td>8,906</td>
<td>1,536</td>
</tr>
<tr>
<td></td>
<td>DENV3</td>
<td>3,579</td>
<td>1,242</td>
</tr>
<tr>
<td></td>
<td>DENV4</td>
<td>3,141</td>
<td>485</td>
</tr>
<tr>
<td>Serum 033</td>
<td>DENV1</td>
<td>13,164</td>
<td>1,338</td>
</tr>
<tr>
<td>(primary DENV3)</td>
<td>DENV2</td>
<td>8,906</td>
<td>2,050</td>
</tr>
<tr>
<td></td>
<td>DENV3</td>
<td>20,768</td>
<td>6,931</td>
</tr>
<tr>
<td></td>
<td>DENV4</td>
<td>12,334</td>
<td>1,815</td>
</tr>
</tbody>
</table>

*a The end point binding titers are based on ELISAs performed with virus or recombinant E (rE) protein antigen and serial dilutions of immune serum. The end point titer was the reciprocal of the highest dilution that produced a signal 2 standard deviations above the signal for normal human sera.

*b The amount of antibody that bound to rE protein relative to the virus was calculated using the following formula: (end point titer for rE antigen/end point titers for whole virus)X 100. When endpoint titers were calculated using a E protein cross reactive mouse MAb (4G2), similar end point titers were obtained for well coated with virus or recombinant E indicating both antigens had a similar number of accessible E molecules.
**Table 2.2 Screen for isolating DENV-specific human MAbs**

<table>
<thead>
<tr>
<th>Donor</th>
<th>No. of positive cultures after primary screen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Proportion of DENV positive cultures binding to rE protein&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. of human MAbs produced&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>033 (primary DENV3)</td>
<td>332/960 (35%)</td>
<td>7.50%</td>
<td>16</td>
<td>This study</td>
</tr>
<tr>
<td>013 (primary DENV2)</td>
<td>567/2016 (28%)</td>
<td>2.90%</td>
<td>10</td>
<td>Betramello et al 2010</td>
</tr>
</tbody>
</table>

<sup>a</sup> Primary screen was conducted by flow cytometry using C6/36 cells infected with the homologous serotype.

<sup>b</sup> Proportion of rE protein reactive cultures was determined by ELISA using recombinant protein from homologous virus.

<sup>c</sup> For donor 013, the selection of cultures for DENV-specific MAbs production was biased to enrich for rE and EDIII reactive MAbs.
<table>
<thead>
<tr>
<th>Human MAb (Donor 013)</th>
<th>Binding specificity</th>
<th>DENV2 neutralization potency</th>
<th>Neutralization escape mutations</th>
<th>DENV2 Yeast EDIII mapping</th>
<th>EDIII Epitope</th>
</tr>
</thead>
<tbody>
<tr>
<td>DV3.7</td>
<td>DENV2 type specific</td>
<td>Strong</td>
<td>V382G</td>
<td>P384A, P384N</td>
<td>K307Q</td>
</tr>
<tr>
<td>DV10.16</td>
<td>DENV subcomplex</td>
<td>Strong</td>
<td>E311K</td>
<td>K305E, K310E</td>
<td>NA</td>
</tr>
<tr>
<td>DV21.5</td>
<td>DENV complex</td>
<td>None</td>
<td>NA</td>
<td>K305E, K310E, K317Y</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA = Not Applicable

*Only EDIII reactive MAbs from donor 013 were mapped.*

*The MAbs were tested for binding to the four DENV serotypes.*

*Neutralization potency is based on 50% neutralization titers in Figure 3.*

*DENV2 escape mutants were obtained with strongly neutralizing MAbs only.*

*EDIII mutations that led to a complete loss of binding and partial loss of binding are indicated separately.*
**Table 2.S1.** Dengue immune human sera used in the present study

<table>
<thead>
<tr>
<th>Donor ID</th>
<th>Location &amp; year of infection</th>
<th>Interval between infection and sample collection</th>
<th>PRNT50 titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Infecting Serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>033&lt;sup&gt;b&lt;/sup&gt;</td>
<td>India 2005</td>
<td>12 months</td>
<td>1:49</td>
<td>1:129</td>
</tr>
<tr>
<td>013</td>
<td>South Pacific Island 1997</td>
<td>8 years</td>
<td>1:178</td>
<td>&gt; 1:1280</td>
</tr>
</tbody>
</table>

<sup>a</sup> The 50 % plaque reduction neutralization titer was determined using Vero cells.

<sup>b</sup> DENV3 was isolated from the subject in 2005.
Table 2.S2. Properties of MAbs from donor 033 (Primary DENV3 infection).

<table>
<thead>
<tr>
<th>MAb ID</th>
<th>IgG Isotype</th>
<th>Whole virus binding ELISA (OD$_{405}$)</th>
<th>Recombinant E protein ELISA (OD$_{405}$)</th>
<th>Recombinant DENV3 EDIII ELISA (OD 405)</th>
<th>Target antigen (DENV3)</th>
<th>Neut$_{50}$ (µg/ml) of DENV in U937 DC-SIGN cell:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DENV1</td>
<td>DENV2</td>
<td>DENV3</td>
<td>DENV4</td>
<td>DENV1</td>
</tr>
<tr>
<td>1.12</td>
<td>γ 1</td>
<td>0.48</td>
<td>0.76</td>
<td>1.13</td>
<td>1.04</td>
<td>0.06</td>
</tr>
<tr>
<td>4.30</td>
<td>γ 1</td>
<td>0.99</td>
<td>1.48</td>
<td>1.86</td>
<td>1.88</td>
<td>0.07</td>
</tr>
<tr>
<td>11.12</td>
<td>γ 1</td>
<td>1.08</td>
<td>1.77</td>
<td>1.78</td>
<td>1.95</td>
<td>0.07</td>
</tr>
<tr>
<td>12.70</td>
<td>γ 1</td>
<td>1.01</td>
<td>1.76</td>
<td>1.71</td>
<td>1.53</td>
<td>0.07</td>
</tr>
<tr>
<td>18.50</td>
<td>γ 1</td>
<td>1.18</td>
<td>1.69</td>
<td>1.72</td>
<td>1.86</td>
<td>0.07</td>
</tr>
<tr>
<td>20.10</td>
<td>γ 3</td>
<td>0.08</td>
<td>0.09</td>
<td>0.08</td>
<td>0.08</td>
<td>0.09</td>
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<tr>
<td>21.20</td>
<td>γ 1</td>
<td>1.17</td>
<td>1.68</td>
<td>1.63</td>
<td>1.63</td>
<td>0.07</td>
</tr>
<tr>
<td>22.70</td>
<td>γ 1</td>
<td>0.74</td>
<td>1.49</td>
<td>1.46</td>
<td>1.55</td>
<td>0.06</td>
</tr>
<tr>
<td>32.40</td>
<td>γ 1</td>
<td>1.01</td>
<td>1.68</td>
<td>1.63</td>
<td>1.59</td>
<td>0.06</td>
</tr>
<tr>
<td>36.50</td>
<td>γ 1</td>
<td>0.85</td>
<td>1.59</td>
<td>1.49</td>
<td>1.60</td>
<td>0.07</td>
</tr>
<tr>
<td>38.10</td>
<td>γ 1</td>
<td>1.09</td>
<td>1.65</td>
<td>1.62</td>
<td>1.62</td>
<td>0.06</td>
</tr>
<tr>
<td>47.70</td>
<td>γ 1</td>
<td>0.07</td>
<td>0.09</td>
<td>0.08</td>
<td>0.08</td>
<td>0.07</td>
</tr>
<tr>
<td>51.30</td>
<td>γ 1</td>
<td>2.16</td>
<td>0.09</td>
<td>0.82</td>
<td>0.09</td>
<td>0.07</td>
</tr>
<tr>
<td>59.3</td>
<td>γ 1</td>
<td>1.27</td>
<td>1.93</td>
<td>1.72</td>
<td>1.59</td>
<td>0.06</td>
</tr>
<tr>
<td>64.31</td>
<td>γ 1</td>
<td>1.33</td>
<td>1.83</td>
<td>2.30</td>
<td>1.85</td>
<td>0.86</td>
</tr>
<tr>
<td>65.50</td>
<td>γ 1</td>
<td>0.78</td>
<td>1.49</td>
<td>1.53</td>
<td>1.48</td>
<td>0.06</td>
</tr>
</tbody>
</table>
References


CHAPTER THREE

Identification of human neutralizing antibodies that bind to complex epitopes on dengue virions

3.1 Overview

Dengue is a mosquito-borne flavivirus that is spreading at an unprecedented rate and has developed into a major health and economic burden in over 50 countries. Even though infected individuals develop potent and long-lasting serotype-specific neutralizing antibodies (Abs), the epitopes engaged by human neutralizing Abs have not been identified. Here, we demonstrate that the dengue virus (DENV)-specific serum Ab response in humans consists of a large fraction of cross-reactive, poorly neutralizing Abs and a small fraction of serotype-specific, potently inhibitory Abs. Although many mouse-generated, strongly neutralizing monoclonal antibodies (MAbs) recognize epitopes that are present on recombinant DENV envelope (E) proteins, unexpectedly, the majority of neutralizing Abs in human immune sera bound to intact virions but not to the ectodomain of purified soluble E proteins. These conclusions with polyclonal Abs were confirmed with newly generated human MAbs derived from DENV-immune individuals. Two of three strongly neutralizing human MAbs bound to E protein epitopes that were preserved on the virion but not on recombinant E (rE) protein. We propose that humans produce Abs that neutralize DENV infection by binding a complex,
quaternary structure epitope that is expressed only when E proteins are assembled on a virus particle. Mapping studies indicate that this epitope has a footprint that spans adjacent E protein dimers and includes residues at the hinge between domains I and II of E protein. These results have significant implications for the DENV Ab and vaccine field.

3.2 Introduction

Dengue viruses (DENVs) are emerging arboviruses and the causative agents of dengue fever and dengue hemorrhagic fever (DHF). The DENV complex consists of four distinct but related viruses, designated as serotypes (1, 19). A person infected with DENV develops an antibody (Ab) response that, to varying degrees, cross-reacts with all four serotypes. Despite the cross-reactivity, Abs that are produced durably only prevent re-infection by the same homologous serotype. Serotype-specific neutralizing Abs can be detected 60 y after a primary infection, suggesting that Abs provide lifelong protection against the homologous serotype (13). People experiencing a secondary DENV infection with a different (heterologous) serotype face a greater risk for developing DHF. Ab-dependent enhancement by cross-reactive, weakly neutralizing Abs is the most widely suggested theory explaining the higher risk for DHF associated with secondary infection (12). The identity of DENV epitopes recognized by human Abs responsible for potent and long-term neutralization remains unknown. This is a significant knowledge gap impeding the current global effort to develop dengue vaccines that induce protective neutralizing Abs and not cross-reactive Abs with potential to enhance disease.

The DENV envelope contains two integral membrane proteins designated envelope (E) and premembrane/membrane (prM/M) proteins. DENV E protein, which binds to cellular
receptors and mediates viral fusion during entry, is thought to be the major target of neutralizing Abs (29). The ectodomain of E proteins has been crystallized, and atomic structures have been determined for several flaviviruses (22-24, 28). Individual subunits of E protein consist of three β-barrel domains designated domains I (EDI), II (EDII), and III (EDIII), with the native protein forming a head-to-tail homodimer on the mature virion. The mature DENV particle consists of 90 dimers that cover the surface of the virion (16). Although several groups have characterized mouse monoclonal antibodies (MAbs) that neutralize DENV infection (12, 29) and mapped them to all three domains on the E protein (29, 35, 36), the strongest neutralizing mouse MAbs were serotype-specific and bound to two overlapping and adjacent epitopes on the lateral ridge and A-strand of EDIII (3, 11, 31, 35, 36, 39).

To understand how human Abs neutralize DENV, investigators have begun to characterize human immune sera and human monoclonal Abs (hMAbs) (6, 7, 33). Humans also produce EDIII-reactive Abs, including strongly neutralizing MAbs that bind to similar epitopes recognized by murine EDIII Abs (2, 6). However, several recent observations indicate that EDIII-specific Abs alone are unlikely to account for the strong type-specific neutralizing Ab responses observed in people following natural infections. DENV-immune humans have low levels of serum EDIII-specific Abs, and these sera retained potently neutralizing activity even after depletion of EDIII-binding Abs (25, 30, 42). Moreover, recombinant DENVs with mutations in EDIII epitopes recognized by neutralizing Abs remained sensitive to neutralization by human DENV-immune sera (40). Collectively, these observations suggest that humans produce neutralizing Abs that bind to epitopes other than those on EDIII. Here, we characterized polyclonal sera and hMAbs generated from DENV-
immune individuals to identify DENV epitopes engaged by potently neutralizing human Abs. We demonstrate that human neutralizing Abs recognize a complex epitope that is preserved on the intact virion but is not present on the soluble E protein.

3.3 Methods

Serum Samples

Human serum samples were collected from individuals who had experienced a DENV infection during travel to an endemic region. Rhesus macaque (Macaca mulatta) sera were taken from animals vaccinated with a VEEV replicon particle (VRP-rE) expressing 80% of DENV3 E protein. More information is provided in SI Methods.

Virus and rE Proteins

The DENV1 (West Pac 74), DENV2 (S-16803), DENV3 (CH-53489 and Thailand 95), and DENV4 (TVP-360) strains were used in the present study. All viruses used in the neutralization assays were grown in C6/36 Aedes albopictus mosquito cells at 28°C and titered on Vero-81 cells as previously described (15). DENV was purified as previously described (42). The rE proteins from each of the four DENV serotypes were purchased from Hawaii Biotech, Inc.

Depletion of DENV-Specific Abs from Human Immune Sera

Purified DENVs were adsorbed onto 4.0 μm Polybead polystyrene microspheres.
following the manufacturer’s instructions (Polysciences, Inc.). Control beads were adsorbed with BSA instead. Human immune sera were depleted of virus-specific Abs by incubating sera with virus-adsorbed beads at 37 °C. Detailed information is given in SI Methods.

Depletion of DENV rE-Specific Abs from Human and Monkey Immune Sera

DENV rE proteins were covalently conjugated to cyanogen bromide (CNBr)-activated beads following the manufacturer’s protocol (Sigma). Control beads were conjugated with the blocking reagent instead of rE protein. DENV rE-specific Abs were depleted by incubating human and rhesus macaque immune sera with rE-conjugated beads at 37 °C. Detailed information is given in SI Methods.

Detection of DENV or rE-Binding Abs by ELISA

ELISAs were conducted as previously described (5). Sera were used at dilutions of 1:40 and 1:25 for the depletion confirmation ELISAs in the virus and rE depletion experiments, respectively. More information is provided in SI Methods.

Detection of rE-Binding Abs by Western Blot

Detailed information is provided in SI Methods.
3.4 Results

Depletion of Homologous DENV-Specific Abs from Immune Sera

Studies were undertaken to characterize Abs in human immune sera responsible for potent and long-term neutralization of the homologous virus serotype. We assembled a panel of eight immune sera from healthy volunteers who had been exposed to primary DENV2 or DENV3 infections \(\sim2–9\) yrs before blood collection (Table S1). Human serum from individuals lacking a past history of DENV infections (confirmed by ELISA and neutralization assays) was used as a negative control.

To define the Ab subpopulation in immune sera responsible for DENV neutralization, we developed a bead-based technique to fractionate DENV-specific Abs in immune sera. Polystyrene beads coated with virions of the homologous serotype were incubated with immune sera at 37 °C to deplete DENV-binding Abs. Untreated and control-depleted serum samples bound to whole virus from each of the four DENV serotypes by ELISA and efficiently neutralized DENV (Fig. 1 A and B). Serum samples depleted using beads coated with the homologous DENV displayed greatly reduced binding and neutralization of DENV (Fig. 1 A and B), indicating that beads coated with the homologous serotype successfully removed most DENV-specific Abs from immune sera.

Depletion of Heterologous DENV-Specific Abs from Immune Sera

Next, we assessed the contribution of DENV cross-reactive Abs in immune sera to virus binding and neutralization. We used polystyrene beads coated with virus of a
heterologous serotype (a serotype that has not infected the DENV-immune subject) to deplete cross-reactive Abs from primary immune sera (Fig. 1 and Table 1). Depletion of primary DENV2-immune sera with DENV3-coated beads led to the removal of all cross-reactive Abs, with the remaining Abs binding to DENV2 in a type-specific manner (Fig. 1C). Reciprocal depletion of primary DENV3-immune sera with DENV2-coated beads removed all binding to DENV2 and DENV4 but not to DENV3 and, to a lesser extent, DENV1 (Fig. 1E). This residual DENV1-binding signal may be attributable to Abs targeting sub-complex epitopes that are preferentially shared between DENV1 and DENV3 (11, 18, 36). Removal of cross-reactive Abs from primary immune sera did not change the capacity of the sera to neutralize the virus responsible for infection (Fig. 1 D and F, Fig. S1, and Table 1). These results demonstrate that the DENV-specific human Ab response consists of both cross-reactive and type-specific Abs. Although the serotype cross-reactive Abs were abundant, in the samples we analyzed, their contribution to neutralization was negligible. Thus, type-specific Abs appear to be primarily responsible for neutralizing the homologous serotype.

Depletion of DENV Recombinant E Protein-Binding Abs from Immune Sera

The organization of DENV E protein dimers on the surface of the infectious virus has been modeled using crystal structures of DENV recombinant E (rE) and cryo-EM reconstructions of the virion (16, 17, 22, 44). Furthermore, neutralizing mouse MAbs have been mapped extensively to the rE protein, and DENV subunit vaccines using the rE protein are currently being developed (3, 4, 10, 11, 35-37, 39). We next assessed whether epitopes targeted by neutralizing Abs in human immune sera were preserved on the rE protein. DENV
rE protein that was covalently coupled to agarose beads was used to deplete Abs in immune sera. Sera were incubated with either control beads or homologous rE-conjugated beads at 37°C. The structure of DENV rE on the beads was confirmed to be conformationally preserved, and rE dimers were confirmed to be intact by successfully depleting mouse MAbs previously mapped to the fusion loop (MAb 4G2), EDIII (MAb 9F16) (36), and E dimer interface (MAbs DV2-10, DV2-46, and DV2-58) (35) (Fig. S2). We also titrated the amount of rE protein on the beads required to deplete rE-binding Abs efficiently from immune sera (Fig. S3). Both untreated and control-depleted immune sera bound to rE from all four serotypes, but the binding was greatest for the homologous serotype (Fig. 2 A and B). Depletion of primary immune sera using homologous rE ablated binding to rE from each of the four serotypes (Fig. 2 A and B). Successful depletion of rE-binding Abs was also confirmed by Western blot, where rE and solubilized virions were used as the antigen on the blot (Fig. 2C). By Western blot, we could not detect binding to rE protein (which is missing 20% of the native protein at the C terminus) or to full-length E protein from the virus (Fig. 2C). These results established that beads coated with the rE from the homologous serotype efficiently removed all Abs recognizing purified rE protein. We also measured the relative proportion of virion-binding Abs in human immune sera that bound to rE by comparing the binding of untreated, control-depleted, and rE-depleted sera with the homologous virus by ELISA. Results demonstrated an approximate 45 ± 7% reduction in DENV binding following the removal of rE-binding Abs (Fig. S4 and Table S2), indicating that approximately half of the DENV-specific Abs in primary immune sera recognized the intact virus but not rE protein.

Next, we assessed the neutralizing activity of six immune sera depleted of rE-binding
Abs. Unexpectedly, four of the six immune sera displayed no loss of neutralization potency after removal of rE-binding Abs (Fig. 2 D and E and Table 2). One of the three primary DENV2-immune sera and all three of the primary DENV3-immune sera tested displayed no significant loss of neutralization against the homotypic virus after removal of rE-specific Abs. In contrast, two of the three primary DENV2-immune sera displayed a statistically significant two- to threefold drop (P <0.05) in the 50% neutralization (Neut50) titer when rE-specific Abs were removed (Table 2). Sera from rhesus macaques (Macaca mulatta) immunized with Venezuelan equine encephalitis virus (VEEV) replicons expressing DENV3 E85 protein were used as a positive control in these experiments. These animals should develop neutralizing Abs that bind to rE protein; accordingly, rE-coated beads removed >98% of the neutralizing Abs from these vaccine sera (Fig. 2F). We conclude that although there was some variation among human immune sera in the contribution of rE-reactive Abs to homotypic DENV neutralization, a large fraction of DENV neutralizing Abs in humans consists of neutralizing Abs that bind to intact virions but not the rE protein.

**Characterization of hMAbs That Strongly Neutralize DENV**

As an alternate approach to identify neutralizing viral epitopes targeted by DENV-immune individuals, we generated a panel of hMAbs that strongly neutralized DENV. These Abs were generated by transforming memory B cells from DENV-immune subjects with EBV and generating hMAbs by electro-fusion as previously described (33). Because strongly neutralizing hMAbs comprise a minor fraction of the total hMAbs isolated from immune subjects (2, 6, 7), we used a two-step screen to isolate strongly inhibitory Abs: We first
identified Abs that bound to DENV virions and then tested them for neutralizing activity. We isolated three strongly neutralizing type-specific hMAbs (Neut\textsubscript{50} value <0.2 µg/mL), designated 1F4, 2D22, and 5J7, that inhibited infection of DENV1, DENV2, and DENV3, respectively. Two of these hMAbs bound to the intact virus but not to rE (Table 3).

\textit{Generation of DENV Mutants That Escape Neutralization by hMAbs}

To map the epitopes engaged by neutralizing hMAbs, we subjected the appropriate DENV serotype to Ab pressure and selected for neutralization escape mutant viruses in vitro. DENV1, DENV2, or DENV3 was passaged several times under varying concentrations (0.2–10 µg/mL) of the neutralizing hMAb 1F4, 2D22, or 5J7, respectively. The original WT virus was passaged in parallel in the absence of hMAb treatment. Structural genes of the mutant and WT viruses were sequenced and compared to identify the mutation(s) responsible for neutralization escape. We successfully isolated two escape mutants against DENV1 type-specific MAb 1F4, with two independent single-nucleotide mutations resulting in amino acid changes at position 274 (G→E) in the DI-DII hinge and 47 (K→E) in DI of the E protein (Fig. 3 A and D) that conferred loss of neutralization. K47 and G274 are located 13.2 Å apart and likely comprise part of the same 1F4 epitope. For the DENV2-specific neutralizing hMAb 2D22, we isolated one mutant with an EDIII mutation at residue 323 (R→G) that resulted in neutralization escape (Fig. 3 B and E). Selection with the neutralizing DENV3 specific hMAb 5J7 resulted in an escape mutant with a lysine insertion in the E DI-DII hinge region between the amino acid residues Q269 and N270 (Fig. 3 C and F). All the mutated residues are surface exposed on the structure of the E protein dimer and within the footprint of a complex epitope described for an hMAb (CR4354) that strongly neutralized West Nile
virus (WNV) (14, 34) (Fig. 3G and H and Table 3).

3.5 Discussion

Although it has been known for several decades that humans develop strongly neutralizing Abs following DENV infection, the relevant Abs and epitopes on the virus have not been identified. This is a major gap in knowledge, given the important roles postulated for Abs in clearing DENV or enhancing infection and disease. Here, the results from seven DENV-immune individuals demonstrate that the DENV-specific human Ab response consists of distinct populations of serotype cross-reactive and type-specific Abs. The type-specific Abs were responsible for potent neutralization of DENV. Although cross-reactive Abs were abundant in human immune sera, their contribution to neutralization was relatively negligible.

DENV serotype-specific neutralizing Abs generated in mice bind to epitopes that are present on the soluble form of rE protein (3, 6, 7, 11, 31, 33, 39). We report here that a substantial fraction of DENV-reactive Abs in human immune sera, including type-specific neutralizing Abs, bound to the intact virion but not to rE protein. Some of these virion-specific Abs likely bind to prM or M proteins, which also are displayed on the virion surface. However, prM/M is unlikely to be the primary target of neutralizing Abs, because several studies have shown that human anti-prM Abs are highly cross-reactive and weakly neutralizing (2, 6, 7). The rE protein used in this study was only 80% of the full-length E protein and lacked the amphipathic helices, conserved stem anchor, and transmembrane regions. Cryo-EM reconstructions of mature virions demonstrate that the amphipathic helices, stem anchor, and transmembrane segments are not exposed on the surface, and
therefore are unlikely to be the targets of type-specific neutralizing Abs (16, 22, 44). Based on the results of Ab depletion studies with human immune sera, we suggest that the packing of E proteins on the virion surface creates unique epitopes involving two or more E protein molecules in adjacent symmetry groups, and that these quaternary epitopes are targets of the human neutralizing Ab response. Similar complex quaternary epitopes that are recognized by potently neutralizing hMAbs have been described for several other viruses, including WNV and HIV (8, 14, 34).

Our studies with hMAbs also confirmed that humans can produce strongly neutralizing Abs that bind a quaternary epitope expressed on intact virus particles but not on rE protein. The strongly neutralizing hMAbs 1F4 and 5J7 selected for viruses that escaped neutralization with mutations or insertions either on or near the E DI-DII hinge region. Consistent with this, other MAbs that strongly neutralize flaviviruses also have been reported to recognize the hinge region between EDI and EDII (9, 14, 21, 26, 38). Additionally, we identified a mutation at position 323 on EDIII that resulted in neutralization escape from the anti-DENV2 hMAb 2D22. This Ab likely also recognized a structurally complex neutralizing epitope, because it bound to DENV2 particles but not to EDIII or rE from DENV2. Collectively, the location of all four escape mutations identified for hMAbs 1F4, 2D22, and 5J7 (representing Abs that neutralize 3 different serotypes in a type-specific manner) map to a region that overlaps with an epitope recently described for the CR4354 hMAb that strongly neutralized WNV in vitro and in vivo (14, 38), and bound to virions but not to soluble E protein. Cryo-EM studies revealed that the CR4354 footprint spanned the DI-DII hinge from one E protein dimer and DIII of the adjacent E dimer (14). Abs that bind to this epitope were postulated to be strongly neutralizing for two reasons: (i) each mature virion should have 120
of these epitopes available for interaction, of which 90% could be simultaneously occupied by Ab (14), and (ii) Abs binding to this EDI–EDII complex epitope could cross-link adjacent E dimmers and inhibit trimer formation, which is a prerequisite for viral fusion. Because all the escape mutants that we identified localized within the footprint of CR4354, we propose that this region is also a target of human neutralizing Abs against DENV. Structure studies are in progress to further define the relationships of 1F4, 2D22, and 5J7 with one another and with CR4354.

The EDI/II hinge region is not the only target of neutralizing hMAbs. Strongly neutralizing hMAbs that bind to EDIII (including A strand and lateral ridge epitopes recognized by mouse Abs) have been isolated from memory B cells of DENV-immune subjects (2, 6, 7, 33). Further studies are needed to estimate the variety and frequency of DENV-specific memory B cells encoding for strongly neutralizing Abs. Such estimates are not possible from the existing DENV hMAb literature because of the different methods used to screen for DENV-specific memory B cells and the different criteria used to select B-cell clones and hMAbs for in-depth study (2, 6, 7, 33). Previous studies have demonstrated that human immune sera depleted of EDIII Abs retained most of their neutralizing activity (42). Similar to WNV, recombinant DENV with mutations in EDIII lateral ridge and A-strand epitopes remained sensitive to neutralization by human immune sera (25, 30, 41). In the current study, we report that depletion of Abs that bind homologous rE also had more modest effects on neutralization potency, whereas removal of Abs that bound intact homologous virus resulted in a large decrease in neutralization titer. Collectively, these observations suggest that Abs in human immune sera that recognize the intact virus only account for the majority of the inhibitory activity relative to those binding EDIII and rE protein.
Our findings reporting on a target of neutralizing human Abs after natural infection are relevant to the development of DENV vaccines and the evaluation of vaccines currently under development. The leading dengue vaccine candidates currently being tested in clinical trials consist of tetravalent formulations of live-attenuated dengue or dengue/yellow fever chimeric viruses (20, 27). Studies are needed to determine if these live viral vaccines also induce neutralizing Abs that bind to quaternary epitopes expressed on the intact virion but not on soluble E protein.

3.6 Supporting Information

SI Methods

Serum Samples

Human blood donor recruitment and sample collection were in compliance with the Institutional Review Board of the University of North Carolina at Chapel Hill. All individuals were informed, and written consent was obtained before blood donation. The rhesus macaques (~7 y of age) were vaccinated with a VEEV replicon particle (VRP-rE) expressing amino acids 1–424 of DENV3 E ectodomain (85% of full-length E protein, also designated as E85), boosted at 7 wk. The serum used for the present experiments was collected at 3 wk after the boost.

Depletion of DENV-Specific Abs from Human Immune Sera

Beads were washed three times with 0.1 M borate buffer (pH 8.5) and incubated with the relevant purified DENV in borate buffer overnight at room temperature (RT). Control beads were incubated overnight with an equivalent amount of BSA. The control and virus-
adsorbed beads were blocked with BSA (10 mg/mL) in borate buffer for 30 min at RT three times and washed six times with PBS. Human immune sera were depleted of virus-specific Abs by incubating sera with virus-adsorbed beads for 2 h at 37 °C with end-over-end mixing. Each immune serum was subjected to at least three sequential rounds of depletions before confirming successful removal of the respective Abs by coated (antigen directly coated on plate) and capture (antigen captured by the mouse MAb 4G2) ELISA.

Depletion of DENV rE-Specific Abs from Human and Monkey Immune Sera

Cyanogen bromide (CNBr)-activated beads were covalently conjugated with rE protein following the manufacturer’s protocol (Sigma). CNBr beads were washed four times with distilled water, followed by three additional washes with coupling buffer [0.1M NaHCO₃, 0.5 M NaCl (pH 8.5)]. The relevant DENV rEprotein diluted in coupling buffer was incubated with CNBr-activated beads for 2 h at RT. Control beads were incubated longer with the blocking reagent instead of rE protein. The unreacted groups on the rE-conjugated beads and control beads were blocked and incubated with 0.2 M glycine (pH 8.0), washed three times with coupling buffer, and then washed four times with PBS. Human and rhesus macaque immune sera were incubated with rE conjugated beads for 2 h at 37 °C. Each serum sample was subjected to at least three sequential rounds of Ab depletion before confirming successful removal of the respective Abs detectable by coated or capture ELISA.

Detection of DENV or rE-Binding Abs by ELISA

ELISA plates were coated with either 50 ng per well of intact purified virus or 100 ng
per well of rE protein in carbonate buffer (pH 9.6) for 2 h at RT. Plates were blocked with 3% (vol/vol) normal goat sera in Trisbuffered saline (TBS) containing 0.05% (vol/vol) Tween 20 (blocking buffer). Undepleted, control-depleted, and antigen-depleted immune serum were diluted in blocking buffer and incubated on plates for 1 h at 37°C. Sera were used at dilutions of 1:40 and 1:25 for the depletion confirmation ELISAs in the virus and rE depletion experiments, respectively. DENV or rE reactive Abs were detected using an alkaline phosphatase-conjugated goat anti-human IgG secondary Ab and paranitrophenyl phosphate substrate as previously described (5).

Detection of rE-Binding Abs by Western Blot

Purified DENV (700 ng per well) and DENV rE protein (500 ng per well) were diluted with nonreducing SDS sample buffer, loaded onto a 12% polyacrylamide SDS/PAGE gel, and electrophoresed. Viral proteins were transferred onto polyvinylidene fluoride membranes and blocked overnight at 4°C with 5% (wt/vol) dried nonfat milk. Membrane was then probed with immune sera (diluted 1:1,000) for 1 h at 37°C, washed three times with TBS containing 0.2% (vol/vol) Tween-20, incubated with a goat anti-human IgG-HRP secondary for 1 h at 37°C, washed three times, and developed using ECL substrate.

Measuring DENV neutralization by immune sera and monoclonal antibodies

Neutralizing activity of both immune sera and monoclonal antibodies were measured using a flow cytometry-based neutralization assay with U937 monocytic cells stably transfected with dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) as previously described (15). Briefly, virus and antibody mixtures were
pre-incubated for 1 hr at 37°C, prior to the addition of DC-SIGN expressing U937 cells (U937+DC-SIGN). After 2 hrs of incubation at 37°C with virus-antibody immune complexes, cells were washed twice with infection media. Cells were fixed and permeabilized 24 hrs after infection, probed with 2H2 (anti-prM antibody) conjugated to 488 and infected cells quantified using a Guava flow-cytometer (Milipore).

Focus reduction neutralization assays (FRNT) were conducted using Vero-81 cells as described previously (15). Briefly, virus and serially diluted serum were pre-incubated for 1 hr at 37°C, incubated with Vero-81 cells (grown to 80% confluency) for 2 hrs at 37°C and then overlaid with methylcellulose containing nutrient media. The cells were fixed at either day 3 (for DENV2 and DENV4) or day 4 (for DENV1 and DENV3) and stained for foci using the anti-E MAb, 4G2, and goat anti-mouse HRP and True blue substrate.

*Generation of Anti-DENV hMAbs*

Supernatants from EBV-transformed lymphoblastoid cell lines were screened for binding to DENV by ELISA and, in some cases, tested for neutralization of DENV using a flow cytometry-based assay. Positive wells were fused with HMMA2.5 myeloma cells to generate hybridoma lines as previously described (33, 43). Hybridoma lines then were biologically cloned and grown in serum-free medium (no. 12045084, Gibco Hybridoma-SFM; Invitrogen), and hMAbs were purified using protein G chromatography.

*Generation and Characterization of hMAb Neutralization Escape*
Mutant Viruses. Virus-Ab mixtures were added to Vero cells and passaged every 3–5 days in the presence of Ab to enrich for escape mutant viruses. Virus growth in the presence of Ab was monitored by quantitative RT-PCR and by immunofluorescent detection of DENV antigens in cell monolayers. Following four to six passages under Ab selection, the capsid, prM, and E genes of the escape variants were amplified by RT-PCR and sequenced to identify mutations associated with the Ab escape phenotype.

Statistical Analysis

Sigmoidal binding and neutralization curves were compared between undepleted, control-depleted, and virus-depleted or rE-depleted groups using a one-way ANOVA analysis, followed by a Tukey multiple comparison test at P < 0.05. The one-dilution binding data (represented in bar charts) for control-depleted and virus-depleted or rE-depleted samples were compared using an unpaired Student t test of means. All statistical analyses were conducted using GraphPad Prism4.
Figure 3.1 Binding and neutralization properties of primary DENV-immune sera depleted of total or cross-reactive DENV-binding Abs. Total DENV-specific Abs were removed from DENV3 primary immune serum (e.g., subject 011) using polystyrene beads coated with purified DENV3 and tested for DENV binding (A) and neutralization (B). The sera depleted with the homologous serotype did not bind to any of the four DENVs and failed to neutralize DENV3. Similar results were observed for four other primary immune sera (two
DENV2 and two DENV3 sera) depleted with the homologous serotype responsible for infection. Primary DENV2 (C and D) and DENV3 (E and F) immune sera were depleted of cross-reactive Abs using beads coated with virus of a heterologous serotype and tested for DENV binding (C and E) and neutralization of the homologous serotype (D and F). Immune sera depleted of cross-reactive Abs contained type-specific Abs that bound to virus from the homologous serotype only. Immune sera depleted of cross-reactive Abs were as potently neutralizing as undepleted or control-depleted sera. Results presented here for cross-reactive Ab depletions are representative of data obtained with four primary DENV2 and three primary DENV3 human immune sera (Table 1). *P < 0.001 by an unpaired Student t test of mean binding values.
Figure 3.2 Binding and neutralization properties of primary DENV-immune sera depleted of rE-binding Abs. DENV rE from the homotypic strain was coupled covalently to agarose beads and incubated with the relevant DENV-immune sera to deplete DENV rE-specific Abs. (A and B) Binding of immune sera to rE protein. Primary DENV2 (A) and DENV3-immune (B) sera were depleted with DENV2 and DENV3 rE proteins, respectively, and binding to rE protein from each of the four serotypes was measured by ELISA. Depletion with the rE from the homologous serotype led to a loss of binding to rE protein from each of
the four serotypes. (C) Successful removal of all rE-reactive Abs from sera (e.g., primary DENV3-immune subject 003) also was confirmed by Western blot analysis. Purified homotypic DENV (700 ng per well) and rE protein (500 ng per well) were electrophoresed, transferred to nitrocellulose membrane, and probed with undepleted, control-depleted, or rE-depleted sera (at a 1:1,000 dilution). (D and E) Neutralization of the homologous DENV by rE-depleted sera was measured using a U937+DC-SIGN flow cytometry-based assay. Homologous DENV neutralization by primary DENV2 (D; subject 031) and primary DENV3 (E; subject 003) human immune sera depleted of rE-binding Abs was tested. No reduction in neutralization potency was observed following removal of rE-binding Abs from either of these two serum samples. A total of six primary immune sera were depleted of rE-binding Abs and tested (Table 2). (F) Nonhuman primates vaccinated with rE develop neutralizing Abs that can be depleted with rE antigen. Rhesus macaques (M. mulatta) were vaccinated and boosted with an alphavirus vector expressing DENV3 E ectodomain, and sera were collected 10 wk post-vaccination. Depletion of rE-binding Abs from sera of vaccinated animals (e.g., M630) removed greater than 98% (value estimated by comparing Neut50 values between control-depleted and rE-depleted sera) of the neutralizing Abs. Data are representative of two vaccinated rhesus macaque controls.
Figure 3.3 Epitope mapping of escape mutants generated from type-specific neutralizing hMAbs. Neutralization profiles of respective WT and escape mutants against 1F4 (A), 2D22 (B), and 5J7 (C). Neutralization escape by the mutant viruses was confirmed using U937 + DC-SIGN cells in a flow cytometry-based neutralization assay for 1F4 and 2D22, and by focus reduction neutralization assay (FRNT) for 5J7. Display enlarged views indicate the positions of the original amino acids of the escape mutations on EDIII and the EDI-EDII hinge region for 1F4 (D), 2D22 (E), and 5J7 (F). Images were generated with
DENV1, DENV2, and DENV3 E dimer structures, respectively. The DENV2 and DENV3 E dimer structures [Research Collaboratory for Structural Bioinformatics (RCSB) accession nos. 1OAN and 1UZG, respectively] (22, 23) were modeled using the UniProt protein database viewer and PyMOL (Schrödinger) to generate structures for DENV1 and DENV3 (Thai 95) E dimers. (G) Alignment of E protein segments from DENV and WNV identified in the neutralizing hMAb-binding epitope of CR4354. Mutations leading to escape from 1F4 (blue), 2D22 (green), or 5J7 (pink) are highlighted on relevant regions of the aligned DENV E protein sequences. A portion of the CR4354 epitope that overlaps with the corresponding DENV escape mutations described here is highlighted in bold on the aligned WNV (New York 2000) sequence. (H) Escape mutations were mapped onto the E polymeric structure generated for TBEV (RCSB accession no. 1K4R) (16). The positions of escape mutations generated from 1F4, 2D22, and 5J7 are highlighted on the structure in blue (Gly274, K47), green (Arg323, His282, Asp362), and pink (Gln271, Asn272) (i.e., residues surrounding the lysine insertion), respectively. The footprint of the anti-WNV CR4354 hMAb that spans E protein dimers is circled with a white line. Note that all escape mutations for 1F4, 2D22, and 5J7 fall within the CR4354 footprint. *Neut_{50} values for each escape mutant differed significantly from the respective WT virus (P < 0.0001).
Table 3.1 Homologous DENV serotype neutralization titers of immune sera depleted of cross-reactive antibodies from subjects following primary infection.

<table>
<thead>
<tr>
<th>Infection Serotype</th>
<th>Sample ID</th>
<th>Reciprocal of 50% neutralization titer against the homologous virus (SEM)\textsuperscript{a,b}</th>
<th>Undepleted</th>
<th>Control depleted</th>
<th>Cross-reactive Ab depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary DENV2</td>
<td>001</td>
<td>2600 (2040-2700)</td>
<td>1650 (1100-1650)</td>
<td>1412 (1060-1600)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>013</td>
<td>350 (260-470)</td>
<td>320 (260-380)</td>
<td>420 (370-550)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>019</td>
<td>1202 (1000-1550)</td>
<td>1047 (930-1580)</td>
<td>1000 (800-1420)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>031</td>
<td>1150 (1000-1310)</td>
<td>790 (650-950)</td>
<td>640 (540-740)</td>
<td></td>
</tr>
<tr>
<td>Primary DENV3</td>
<td>003</td>
<td>250 (230-350)</td>
<td>210 (160-260)</td>
<td>300 (250-360)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>011</td>
<td>320 (265-390)</td>
<td>300 (260-380)</td>
<td>252 (211-300)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>118</td>
<td>628 (510-770)</td>
<td>720 (610-860)</td>
<td>618 (500-750)</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}Data is representative of experiments repeated at least thrice for each serum sample. The flow-based neutralization assay using U937 cells stably expressing DC-SIGN (U937+DC-SIGN) was used to generate the reciprocal Neut\textsubscript{50} values. The Neut\textsubscript{50} values of undepleted, control depleted and cross-reactive antibody depleted sera were compared for each serum by one-way ANOVA analysis. No statistical significance was found between control depleted and cross-reactive depleted groups for any of the tested sera.

\textsuperscript{b}Standard error of mean (SEM) for reciprocal Neut\textsubscript{50} values were calculated from the sigmoidal neutralization curves using GraphPad Prism4 and given in parenthesis.
Table 3.2 Homologous DENV serotype neutralization titers of primary immune sera depleted of rE-binding antibodies.

<table>
<thead>
<tr>
<th>Infection Serotype</th>
<th>Sample ID</th>
<th>Reciprocal of 50% Neutralization titer against the homologous virus (SEM)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Undepleted</td>
</tr>
<tr>
<td>Primary DENV2</td>
<td>001(^b)</td>
<td>660</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(510-840)</td>
</tr>
<tr>
<td></td>
<td>019(^c)</td>
<td>1250</td>
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<td></td>
<td></td>
<td>(1010-1500)</td>
</tr>
<tr>
<td></td>
<td>031</td>
<td>1020</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(790-1300)</td>
</tr>
<tr>
<td>Primary DENV3</td>
<td>003</td>
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<td>(140-235)</td>
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<td>118</td>
<td>1580</td>
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<tr>
<td></td>
<td></td>
<td>(1180-2020)</td>
</tr>
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</table>

\(^a\)Data is representative of experiments repeated at least thrice for each serum sample. Standard errors of mean (SEM) for reciprocal Neut\(_{50}\) values were calculated from the sigmoidal neutralization curves using GraphPad Prism4 and are given in parenthesis.

\(^b\)There was a statistically significant difference between the undepleted/control depleted and rE depleted groups for sample by a one-way ANOVA analysis followed by a Tukey’s multiple comparison test at P <0.01.

\(^c\)There was a statistically significant difference between the undepleted and rE depleted groups when analyzed by a one-way ANOVA at P<0.05.
Table 3.3 Binding and neutralization properties of isolated strongly neutralizing human MAbs

<table>
<thead>
<tr>
<th>MAbs</th>
<th>Virus Specific</th>
<th>rE</th>
<th>EDIII</th>
<th>prM</th>
<th>Binding (2 µg/ml)⁵</th>
<th>Neut50 titer (µg/ml)⁴</th>
<th>Escape mutant</th>
<th>Escape Mutation</th>
<th>Mutation Location</th>
<th>Analogous residue in WNV (Comparison to CR4354 epitope)⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>1F4</td>
<td>Type-Specific</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.11</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>1</td>
<td>G274E</td>
</tr>
<tr>
<td>2D22b</td>
<td>Type-Specific</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&gt;10</td>
<td>0.08</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>2</td>
<td>K47E</td>
</tr>
<tr>
<td>5J7b</td>
<td>Type-Specific</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>0.10</td>
<td>&gt;10</td>
<td>4</td>
<td>Q269_N270insK</td>
</tr>
</tbody>
</table>

⁵Binding of Human MAbs (at 2 µg/ml) to DENV antigens were measured by ELISA.

⁶Binding and neutralization properties of 2D22 and 5J7 were taken from previous study (32).

⁷Binding to prM was determined by western blot analysis.

⁸Neut50 values were generated using the flow-based neutralization assay with U937-DC-SIGN cells. Values in bold indicate the lowest Neut50 concentration and the most neutralization sensitive serotype for each MAb.

⁹Comparison of the escape mutations generated against 1F4, 2D22 and 5J7 to the CR4354 epitope in WNV (14, 38).
Figure 3.S1 Comparison of neutralization properties of primary DENV-immune human sera depleted with either one heterologous virus or all three heterologous viruses. (A) Depletion of primary DENV2-immune sera (e.g., subject 019) with DENV3, or DENV1, DENV3, and DENV4 had no statistically significant effect on the Neut$_{50}$ against the homotypic virus, DENV2. (B) Depletion of primary DENV3-immune sera (e.g., subject 003) with DENV2 or DENV1, DENV2, and DENV4 had no statistically significant effect on the Neut$_{50}$ against DENV3. Data are representative of single experiments conducted in duplicate. The Neut$_{50}$ values of undepleted, control-depleted, and cross-reactive depleted sera were compared for each serum using one-way ANOVA analysis followed by a Tukey multiple comparison test at $P < 0.05$. 
Figure 3.S2 Confirmation of the DENV2 rE structure on the CNBr-activated beads using mouse MAbs. Mouse MAbs 9F16 (E DIII-specific) (36); 4G2 (fusion loopspecific); DV2-30, DV2-46, and DV2-58 (dimer interface-specific) (35); and hMAbs 2D22 (virus-specific) were incubated three consecutive times with either control beads or DENV2 rE-conjugated beads for 2 h at 37 °C. The depleted samples were tested for binding to DENV2 rE by capture ELISA. (A) All previously mapped mouse MAbs that bound epitopes on DENV rE protein were successfully depleted with rE protein covalently conjugated to beads. (B) Virus-specific hMAb 2D22 was not depleted by rE protein. Data are representative of individual experiments conducted in duplicate. *$P < 0.001$ by an unpaired Student $t$ test of mean binding values.
Figure 3.S3 Titration of DENV rE quantities that were covalently conjugated to beads.

CNBr-activated beads were mixed with varying quantities (i.e., 0, 0.02, 0.2, 2, 10, 20, and 40 µg/mL) of DENV2 and DENV3 rE protein and were then incubated with primary DENV2 (i.e., subject 001) and DENV3 (i.e., subject 003) sera, respectively. The remaining supernatant was tested for the presence of homologous rE-binding Abs by capture ELISA. The gray arrow represents the amount of rE added to beads during ensuing rE depletion experiments with immune sera.
Figure 3.S4 Binding properties of rE-depleted sera to the homotypic DENV. Removal of rE binding Abs from primary DENV2 (A) and DENV3 (B) sera resulted in a statistically significant ($P < 0.05$) 45 ± 7% decrease in binding ($EC_{50}$) to the homologous virus. Statistical analysis was conducted using a one-way ANOVA. Data are representative of three primary DENV2 and three primary DENV3 human immune sera (Table S2).
Table 3.S1 A panel of late convalescent DENV-immune sera from individuals with past primary DENV2 or DENV3 infections.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Location of Infection</th>
<th>Year of Infection</th>
<th>Interval between infection and sample collection</th>
<th>DENV Neutralization&lt;sup&gt;b&lt;/sup&gt; (FRNT&lt;sub&gt;50&lt;/sub&gt; reciprocal titer)</th>
<th>Infecting Serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Sri Lanka</td>
<td>1996</td>
<td>9 years</td>
<td>&lt; 20</td>
<td>271</td>
</tr>
<tr>
<td>013&lt;sup&gt;a&lt;/sup&gt;</td>
<td>South Pacific</td>
<td>1997</td>
<td>8 years</td>
<td>178</td>
<td>&gt;1280</td>
</tr>
<tr>
<td>019</td>
<td>Thailand</td>
<td>1997</td>
<td>8 years</td>
<td>95</td>
<td>&gt;1280</td>
</tr>
<tr>
<td>031</td>
<td>South Pacific</td>
<td>1997</td>
<td>8 years</td>
<td>28</td>
<td>&gt;320</td>
</tr>
<tr>
<td>003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Thailand</td>
<td>2001</td>
<td>4 years</td>
<td>30</td>
<td>87</td>
</tr>
<tr>
<td>011&lt;sup&gt;a&lt;/sup&gt;</td>
<td>El Salvador</td>
<td>1998</td>
<td>7 years</td>
<td>84</td>
<td>124</td>
</tr>
<tr>
<td>105</td>
<td>Thailand</td>
<td>2002</td>
<td>8 years</td>
<td>&lt; 20</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>118</td>
<td>Nicaragua</td>
<td>2009</td>
<td>1.5 years</td>
<td>60</td>
<td>32</td>
</tr>
</tbody>
</table>

<sup>a</sup>The FRNT<sub>50</sub> values for these serum samples were reported in a previous study (42).

<sup>b</sup>FRNT<sub>50</sub> values in bold signify the highest 50% neutralization reciprocal titers for each serum sample.
Table 3.S2 Binding properties of DENV-immune sera with and without rE-binding antibodies to the homologous virus serotype.

<table>
<thead>
<tr>
<th>Infection Serotype</th>
<th>Sample ID</th>
<th>50% Reciprocal binding titers to the homologous virus (SEM)$^a$</th>
<th>Homotypic rE Depleted$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Undepleted</td>
<td>Control depleted</td>
</tr>
<tr>
<td>Primary DENV2</td>
<td>001$^b$</td>
<td>280 (260-290)</td>
<td>310 (290-335)</td>
</tr>
<tr>
<td></td>
<td>031$^b$</td>
<td>445 (415-475)</td>
<td>455 (435-475)</td>
</tr>
<tr>
<td></td>
<td>003$^b$</td>
<td>216 (205-225)</td>
<td>190 (180-200)</td>
</tr>
<tr>
<td>Primary DENV3</td>
<td>105$^b$</td>
<td>60 (53-66)</td>
<td>63 (60-68)</td>
</tr>
<tr>
<td></td>
<td>118$^c$</td>
<td>440 (400-490)</td>
<td>350 (325-375)</td>
</tr>
</tbody>
</table>

$^a$Data representative of experiments repeated at least twice for each serum sample. Standard error of mean for each reciprocal 50% reciprocal binding titer (EC$_{50}$) given in parentheses.

$^b$There is a statistically significant difference between the control depleted and rE depleted groups by a one way ANOVA analysis followed by a Tukey’s multiple comparison test at P <0.001.

$^c$There is a statistically significant difference between the control depleted and rE depleted group when analyzed by a one way ANOVA at P<0.05.

$^d$The rE-reactive antibodies account for about 45±7 % of the total homotypic virus binding antibodies.
References


CHAPTER FOUR

Further characterization of the neutralizing human antibody response to dengue virus.

4.1 Overview

At present, numerous tetravalent vaccine platforms are being developed in hopes of eliciting long-term, balanced neutralizing antibody responses against each of the four DENV serotypes. We recently mapped several strongly neutralizing, type-specific human MAbs to the EDI-DII hinge region. In the present chapter, we map the EDI-DII hinge epitope in further detail and confirm that this region is important for long-term type-specific protection in humans. Furthermore, we studied the mechanistic contribution of EDI-DII hinge for antibody neutralization. Finally, we extend some of our in vitro observations to experiments in vivo, in the AG129 mouse model.

4.2 Introduction

The antibody response is the primary immune mechanism of protection against DENV infections in humans. Until recently, data with mouse MAbs indicated that protection from DENV infections was due to neutralizing antibodies targeting the DIII region of the DENV E protein (19, 20, 33, 57, 58). Studies with human polyclonal sera have indicated that humans make very little EDIII antibodies, and this minor fraction of
EDIII-specific antibodies do not contribute significantly to long-term protection in humans (42, 44, 51, 64, 65).

Discrepancies in DENV-specific antibody responses in mouse versus humans, led to increased focus on the antibody response in humans. Recent studies (including data in chapter 2) have shown that the majority of antibodies produced by humans are cross-reactive, weakly neutralizing and bind to either prM or E protein (4, 15, 17, 53). It was observed that potently neutralizing antibodies were rare and difficult to isolate. Work described in Chapter 3 of this dissertation lead to the successful isolation and mapping of several strongly neutralizing, type-specific MAbs derived from human PBMCs (16). Similar to the previously mapped WNV-specific human MAb (27, 63), CR4354, these strongly neutralizing MAbs were mapped to a complex, quaternary epitope that focused around the flexible DI-DII hinge of the E protein (16). However, it is unclear whether the EDI-DII hinge is also the target of type-specific protection by DENV-immune human polyclonal sera.

Four peptide linkers connecting DI and DII of the E protein forms the EDI-DII hinge. During the life cycle of DENV, this hinge region undergoes angular changes of close to 40° and plays important roles during maturation and fusion (7, 29, 35-37, 47, 54, 55). A low pH-induced E trimer to dimer conformational change in the TGN during virus maturation induces almost a 30° angular change in the EDI-DII hinge (45, 70, 72). During viral fusion, the low pH-induced E dimer to trimer conformational change in the endosomes forces a 40° angular change in the EDI-DII hinge (35, 47, 54, 55). Interestingly, single mutations in the EDI-DII hinge region lead to changes in the pH-threshold of viral fusion, indicating that this hinge also plays an important role in maintaining the pH-threshold of fusion (3, 7, 22, 26, 31, 35, 40). It is possible that antibodies binding the EDI-DII hinge efficiently neutralize the
virus, not by blocking attachment, but by blocking the large conformational change that leads to viral fusion.

Despite the evidence of broadly cross-protective long-term immunity after a secondary DENV infection (reviewed in (66)), non-EDIII-binding, strongly cross-neutralizing antibodies have not been described in literature. Two regions on the DENV structural proteins show high protein sequence conservation across the four serotypes, 1) the fusion loop in the distal end of EDII, and 2) the cleavable pr portion of prM. Studies have shown that both primary and secondary infections induce large quantities of fusion loop-specific MAbs (30, 32, 44), while secondary infections also elicit large amounts of anti-prM antibodies (10, 62). The physiological relevance of prM-specific antibodies, capable of binding to only immature or partially mature virus during a natural infection, is yet unknown. Unfortunately, previously mapped anti-fusion loop and anti-prM antibodies (from both humans and mice) have only been weak to moderately cross-neutralizing MAbs (4, 8, 12, 15, 17, 48, 53). Therefore, the potently cross-protective epitope(s) targeted by the human antibody response is yet to be discovered.

The present chapter attempts to further characterize the EDI-DII hinge epitope with MAbs and DENV-immune sera. Since EDI-DII hinge region plays a critical role in viral fusion, we also investigated the mechanism of neutralization of isolated neutralizing MAbs and primary DENV-immune human sera. Additionally, in an attempt to map cross-protective immunity, we mapped several strongly cross-neutralizing human MAbs to a non-fusion loop region on EDII. Furthermore, several in vitro results were tested in vivo using the AG129 mouse model.
4.3 Methods

Virus and cell lines

The WHO reference strains of DENV1 (West Pacific 74), DENV2 (S-16803), DENV3 (CH53489) and DENV4 (TVP-360) were used in the present study. Working virus stocks for neutralization assays were grown in the mosquito cell line, C6/36, as previously described in Wahala, W.M.P.B et al, 2009 (65). Briefly, C6/36 cells at 80% confluency were infected with DENV (MOI=0.01), incubated at 28°C (under 5% CO₂), harvested at 6-8 days, and stored at -80°C until use. DENV viruses were originally obtained by Dr. Robert Putnak (Walter Reed Army Institute of Research, Silver Spring, MD).

Vero-81 and C6/36 cells were obtained from ATCC. U937 cells stably expressing DC-SIGN were kindly provided by Mark Heise Lab (UNC Chapel Hill).

Human DENV-immune sera and MAbs

DENV-immune sera were obtained from individuals approximately 2 to 10 years after experiencing a DENV infection during travel to a DENV endemic region. Blood donations were taken in compliance with the Institutional Review Board of the University of North Carolina at Chapel Hill.

The DENV-specific human MAbs, 1F4, 2D22, 5J7, 1C19 and 1N5, used in this study were isolated, generated, and purified from human memory B-cells by Scott Smith in the laboratory of Dr. James Crowe at Vanderbilt University (VU) as previously described (16, 53). Briefly, these MAbs were isolated by a two step screening process, where human memory B cells obtained from DENV-immune subjects were first tested for binding to purified DENV antigens (i.e. DENV1-4 at a 1:1:1:1 mixture) and then tested for protection.
using the \textit{in vitro} flow-based U937+DC-SIGN neutralization assay. 1F4, 2D22 and 5J7 were isolated from a secondary DENV-immune, primary DENV2-immune, and primary DENV3-immune subjects, respectively, while both 1C19 and 1N5 were isolated from subjects with secondary DENV3 infections. The molecular Fab fragments of 1F4, 2D22 and 5J7 were generated by sequencing heavy and light chains from the hybridoma, inserting the genes into expression plasmids, and co-transfecting the plasmids into 293F cells. The expressed Fab was then purified using a lambda select or Kappa select column (GE). All Fab fragments were generated by Scott Smith in the laboratory of Dr. James Crowe at VU.

\textit{Generation of neutralization escape mutants}

Neutralization resistant mutants against 5J7, 1C19 and 1N5, were generated as previously described (15, 16). Briefly, DENV3 (CH53489) was passaged five times in Vero cells under increasing 5J7 concentrations. Similarly, DENV2 (S-16803) was passaged under increasing 1C19 or 1N5 antibody pressure. Growth of virus under antibody pressure was monitored through cytopathic effect on Vero cells, Q-RT-PCR of virus in supernatant, or focus assay of infected cells for detection of viral antigen. The C, prM and E genes of prospective neutralization escape mutants were amplified by RT-PCR and sequenced to determine the mutation(s) responsible for neutralization escape.

\textit{Epitope mapping using mutant prM-E proteins}

Epitope mapping studies of human MAbs were conducted using a prM-E mutant panel as described previously (12). Briefly, mutations were introduced to prM-E genes from DENV3 (CH54389) or DENV4 (TVP-360). Mutant prM-E proteins were expressed in HEK-293 cells, cells fixed and permeabilized, stained with MAb being tested, followed by staining
with a secondary antibody conjugated to horse peroxidase (HRP), incubation with HRP substrate and measurement of resulting luminescence. Antibody reactivities to mutant prM-E proteins were assessed by comparing binding to the wild type prM-E protein. Using the above technique, epitope mapping data for 5J7 and 1C19 was generated by Kristen Kahle and Jen Pfaff from Integral Molecular, Inc.

*Mapping antibodies using an infectious clone*

We used the previously described DENV3 infectious clone made with the parent clone UNC3001 (DENV3 genotype III), which is a 1986 DENV3 clinical isolate from Sri Lanka (34). The DENV3 genome was cloned as a set of four cDNA fragments (named DEN A through D, with sizes 2.0 kb, 1.1 kb, 3.9 kb, and 3.7 kb, respectively). DEN A plasmid encoding the DENV3 E gene with 26 amino acid mutations in the EDI-DII hinge region was synthesized by Bio Basic Inc (Ontario, Canada). Plasmids were amplified in *E.coli*, purified, ligated and transfected into Vero cells. Vero cells were incubated at 37°C under CO2 for 4 days. Resulting virus was passaged as described in Messer, W.B. et al, 2012 (34), and frozen at 80°C until use. The EDI-DII hinge mutant DENV3 virus (UNC3001-A12) had 26 amino acids mutated to the corresponding DENV4 amino acid in the same region (more specifically, K51Q, E52Q, V53L, L55T, T58K, Y59L, K124R, T126E, N128K, L129V, M199L, K200T, K203N, T205A, L207M, K210R, L214F, A222S, V270I, D271Q, S272N, G273S, D274G, N276T, H277S, M278I). The WT DENV3 (UNC3001) and EDI-DII hinge mutant DENV3 virus (UNC3001-A12) were generated by William B. Messer and Jeremy P. Huynh in Raph Baric lab at UNC Chapel Hill.
**ELISA binding assays**

(i) **Binding of 1N5 and 1C19 to DENV antigen and recombinant proteins.** ELISA analysis of 1N5 and 1C19 binding to purified DENV antigen and recombinant proteins (rE, EDI/DII, EDIII and pr) were conducted as previously described (15, 16, 53). Briefly, DENV virus antigen or recombinant proteins were coated on to ELISA plates in carbonate buffer (pH 9.6) or captured by a DENV-specific mouse MAb (2H2 for pr/prM and 4G2 for E protein). Plates were then blocked with 1x TBS (with 0.05% Tween20) buffer containing 3% normal goat serum. 1N5 and 1C19 were tested at 2 µg/ml in blocking buffer. Secondary antibody (anti-human HRP) was used at 1:2500, followed by para-nitrophenylphosphate (pNPP) substrate. Purified DENV virus antigen and recombinant proteins (rE, EDI/DII, EDIII and pr) were generated as previously described (15, 16, 53).

(ii) **Binding analysis of 1N5 and 1C19 to mutant DENV virus.** Binding curves of 1N5 and 1C19 with WT and respective DENV2 escape mutants were generated using an ELISA protocol similar to the one described above. The only differences are, that equal amounts of WT and escape mutant virus were captured onto the plate with the mouse MAbs, 4G2 and 2H2. And the human MAbs, 1N5 and 1C19, were used over a concentration range of 2 µg/ml to 0.002 µg/ml.

**DENV neutralization assays**

(i) **Flow-based U937+DC-SIGN cell assay** was conducted as described previously (15, 28, 65).

(ii) **Flow-based Vero cell assay** was conducted as described previously (15, 65).
(iii) **Pre- and post-attachment neutralization assay** was conducted as previously described (13, 25, 63). Briefly, as shown in Figure 4.6A, Vero cells were seeded in 24 well plates 48 hours prior to infection. On the day of the experiment, cells, antibodies and media were chilled by incubating at 4°C for 30 mins. For the *pre-attachment* neutralization assay, virus and antibodies were incubated at 4°C for 1 hr, then added to chilled vero cells and incubated for another 1 hr at 4°C. Cells were then washed three times with cold 1x PBS (4°C), and overlay media containing 1% methylcellulose was added to each well. Plate was then incubated at 37°C (under CO₂) about 4-5 days post-infection, after which cells were fixed and stained. For the *post-attachment* neutralization assay, pre-chilled virus was added to pre-chilled vero cells and incubated at 4C for 1 hr. Cells were then washed twice with cold 1x PBS (4°C), and incubated with diluted antibodies for another hour at 4°C. Cells were then washed once with chilled 1x PBS and incubated at 37C (under CO₂), until cells are fixed and stained. Cells were fixed and stained as previously described (28, 65), where fixing with 80% methanol is followed by blocking with 5% non-fat dried milk in 1x PBS, staining with 4G2 (for detection of DENV2-4) or 2H2 (for detection of DENV1), and anti-mouse HRP conjugated secondary antibody. Infection was detected as foci using a trypan blue substrate.

**In vivo neutralization experiments with AG129**

All *in vivo* experiments in AG129 mice were conducted by Katherine Williams in the laboratory of Dr. Eva Harris (UC, Berkley) using a previously described protocol (67). AG129 mice were administered human sera diluted to a final volume of 400 µl exactly 24 hrs prior to intra-venous (i.v.) challenge with DENV2 D2S10 (10³ pfu). Between four to six hours prior to DENV2 infection, 100 µl per mouse of whole blood was extracted through a
small retro-orbital or sub-mandibular bleed, serum isolated by high speed centrifugation and serum used in the in vitro U937+DC-SIGN neutralization assay. After four days of infection, the mice were sacrificed, and several organs (i.e. spleen, bone marrow, liver and serum) were harvested, and viremia measured by quantitative-reverse transcription PCR (Q-RT-PCR).

4.4 Results

Further characterization of the 5J7 epitope

Prior escape mutant studies mapped several strongly neutralizing trype-specific human MAbs, including 5J7, to the DI-DII hinge of the E protein. A highly rare lysine insertion between amino acids 269 and 270 in the E protein enables DENV3 to significantly escape neutralization by 5J7 (16). Further passaging of WT DENV3 virus under 5J7 antibody pressure, generated two additional mutants (L53P and K128G) that were found to significantly escape neutralization by 5J7 (shown in Figure 4.1A). When the mutant residues were mapped on to the DENV3 E protein structure (RCSB 1UZG), all three escape mutation positions were in the EDI-DII hinge region. Furthermore, the three mutation positions were spatially situated within 20Å of each other (K128G-L53P = 11.4 Å; K128G-Q269_N270insK =14.4 Å; L53P-Q269_N270insK= 14.2 Å) and could easily form the footprint of an antibody epitope.

Unlike 1F4 and 2D22, 5J7 binding was not restricted to an epitope created only when E proteins are assembled into the mature virion particle (16). Since 5J7 was found to bind the soluble recombinant E protein (rE), an attempt was made to expand the 5J7 epitope using a high throughput binding assay developed by Integral Molecular, Inc (12). The technique has been previously used to successfully map a cross-reactive fusion-loop binding MAb (12).
This binding assay involved the testing of 5J7 binding against a mutant panel of intracellularly expressed recombinant prM-E proteins. As indicated in Figure 4.1B, seven E protein mutants (Q52L, Q52R, L53P, L53Q, E126K, K128R and E133K) resulted in <20% reactivity with 5J7 as compared to the WT prM-E protein. Not so surprisingly, the amino acid positions 52, 53, 126, 128 and 133 fall within the EDI-DII hinge region, and are within 20Å of each other. These mutation positions both confirm our escape mutant data and expand the footprint of the 5J7 epitope.

*The EDI-DII hinge region is a major target of type-specific protection in humans*

As shown in chapter 3, several potently neutralizing type-specific antibodies directed at the EDI-DII hinge region were isolated from human memory B-cells of individuals with past natural DENV infections (16). However, antibodies of this nature were rare, making it difficult to gain insight into the dominant viral target of protective antibodies in DENV-immune humans. Therefore, we utilized our knowledge of the 5J7-binding site, and compared the variable amino acid residues between DENV3 and DENV4 that fell within 12Å of the 5J7 epitope on the EDI-DII hinge region in the E protein structure. As shown in Figure 4.2A, twenty-six variable amino acid sites were identified as possible critical binding sites within this epitope. We inserted twenty-six DENV4 residues (i.e. K51Q, E52Q, V53L, L55T, T58K, Y59L, K124R, T126E, N128K, L129V, M199L, K200T, K203N, T205A, L207M, K210R, L214F, A222S, U270I, D271Q, S272N, G273S, D274G, N276T, H277S, M278I) in to the EDI-DII hinge of the DENV3 infectious clone (UNC3001), and termed the hinge mutant virus as UNC3001-A12. It is important to note that the prediction of critical amino acid residues on the EDI-DII hinge region and the insertion of these 26 mutations into
the DENV3 infectious clone were work conducted in the laboratory of Ralph Baric (specifically by William B. Messer, Jeremy Huynh and Boyd Young).

Insertion of 26 mutations into the EDI-DII hinge of DENV3 gave a productive, live virus (UNC3001-A12) that grew to titers similar to the WT virus (UNC3001) in C6/36 mosquito cells (replication kinetic data not shown). The non-EDI-DII hinge binding anti-EDII MAb, 1N5, was used to ensure that mutation of the EDI-DII hinge region did not lead to complete misfolding of EDII (Figure 4.2B and D). Additionally, neutralization assays of WT and UNC3001-A12 against 5J7 were also used as a control to confirm complete loss of neutralization due to mutation of the EDI-DII hinge (Figure 4.2C and E). Neutralization experiments with a panel of four primary DENV3-immune human sera showed that mutation of the EDI-DII hinge significantly (P<0.001) reduced the neutralizing capacity of the sera (Table 4.1). Similar data was also generated by William B. Messer in the laboratory of Ralph Baric (data not shown). This implies that humans infected with primary DENV3 mount a protective antibody response that target the EDI-DII hinge. Similar neutralization assays with two primary DENV4-immune human sera showed that mutation of the EDI-DII hinge in the DENV3 virus to DENV4 residues was sufficient to significantly (P<0.001) increase the neutralization capacity of the sera (Table 4.1). This gain of neutralization phenotype indicated an unexpected, successful transplant of a DENV4 neutralizing epitope into the DENV3 virus background. This indicates that antibodies that target the EDI-DII hinge region contribute substantially to long-term type-specific protection after primary infections in humans.

In an attempt to gain insight into the epitopes targeted after secondary infections, we also used the WT and UNC3001-A12 to conduct preliminary neutralization assays against
convalescent serum from individuals who had been exposed to secondary infections. As shown in Table 4.1, no significant differences were observed between neutralization of WT versus UNC3001-A12. Therefore, although the EDI-DII hinge region is a dominant target of type-specific neutralizing antibodies, it may not necessarily be the target of cross-protective antibodies.

Potently cross-protective human MAbs target domain II of the E protein

Cross-protective neutralization after DENV infections in humans is observed during two main situations, 1) transiently after primary DENV infections, and 2) long-term after secondary infections (2, 14, 50). It is thought that transient cross-protection after primary DENV infections maybe due to large quantities of low affinity, weakly cross-neutralizing antibodies. However, conserved antigenic epitopes in secondary infections may stimulate affinity maturation of weakly cross-neutralizing antibodies into higher affinity antibodies that provide cross-protection long-term. Apart from moderately cross-neutralizing anti-fusion loop antibodies, no potently cross-neutralizing DENV-specific mouse or human MAb have been described in the literature (12). Therefore, very little it known about the viral epitope(s) that cross-protective human antibodies target.

To investigate the cross-protective epitopes of targeted by the human antibody response, we isolated two potently cross-neutralizing human MAbs, 1N5 and 1C19. Both 1N5 and 1C19 were isolated from PBMCs donated by two individuals with a secondary DENV3 infection. These PBMCs were kindly given by the laboratory of Dr. Eva Harris (UC, Berkley) to the laboratory of Dr. James Crowe (VU) for MAb isolation. As shown in Table 4.2, 1N5 neutralized all four serotypes with Neut$_{50} \leq 0.5$ $\mu$g/ml, while 1C19 potently neutralized DENV1, 2 and 3 (Neut$_{50} < 0.1$ $\mu$g/ml) and only moderately neutralized DENV4
(5 µg/ml <Neut₅₀ > 1 µg/ml). Both antibodies bound virus and rE protein from all four serotypes, but did not bind prM by ELISA or Western blot analysis (Table 4.2). Neither 1N5 nor 1C19 bound EDIII, but as expected bound recombinant EDI/DII protein. This indicated to us that unlike the cross-protective mouse MAbs described in literature that were mapped to the A strand epitope of EDIII (20, 33, 58), both 1C19 and 1N5 bound to a novel cross-protective epitope within EDI/DII.

In an attempt to map the viral epitopes targeted by cross-protective human antibodies, DENV2 (S-16803) was passaged under 1N5 or 1C19 selection pressure, and neutralization escape mutants with at least a ten-fold reduction in neutralization were generated against the antibodies (Figure 4.3A and B). Two escape mutants with the point mutations M118K and E126K were raised against 1N5, while three escape mutants with the single mutations E62Q, M118K and T120K were generated against 1C19 (Table 4.3). Mapping the mutated residues on the DENV2 E protein structure (RCSB 1OAN) revealed that the escape mutations for both human MAbs fell in the middle of EDII (Figure 4.3E). Both 1C19 and 1N5 were found to bind equally to their respective escape mutants and WT DENV2 virus (Figure 4.3C and D), indicating that although these mutations led to significant escape from neutralization, it did not ablate binding to the virus. Surprisingly, the 1C19 MAb was mapped by the intracellular prM-E mutant panel to residues R73 and G78 on the DENV E protein, which unlike the escape mutant residues lie on a loop above the fusion loop (Figure 4.4). Presently, the R73A and G78A mutations that ablated 1C19 binding is being inserted into a DENV2 infectious clone for further conformation and characterization.

At this moment, it is unclear whether the mapping of 1C19 by neutralization escape mutants or by the mutant prM-E panel was more accurate. However, it is worth mentioning
that two of the three WT DENV2 samples passaged in Vero cells in the absence of 1C19 selection also raised neutralization escape viruses with similar mutations (data not shown). Further analysis of the neutralization escape mutants revealed that the mutations increased the positive charge on the virus. Studies have shown that increase in positive charge in this region increases binding of DENV to heparin sulfate on the cell surface (46). It is possible that these escape mutations also increased binding to heparin sulfate and thereby, presented an indirect mechanism of neutralization escape from 1C19 by increasing infectivity of Vero cells. The possible role of heparin sulfate in the neutralization escape from 1C19 in Vero cells is being presently investigated.

**Insights into the mechanism of protection of strongly neutralizing human antibodies**

Since two of the three potently neutralizing type-specific antibodies were non-rE protein binding, cryo-EM studies are being conducted by Shee Mei Lok Lab (National University of Singapore) for further detailed characterization of the binding epitopes. The Fab fragments of 1F4, 2D22 and 5J7 were produced for mapping of these antibodies by cryo-EM. As shown in Figure 4.5F, Fab fragments lack both bivalency and the Fc portion of whole antibodies. Interestingly, the neutralization potency of some antibodies is dependent on the bivalency of the antibody. Therefore, to gain some insight into the mechanism of neutralization, we compared the neutralization properties of Fab versus MAbs of 1F4, 2D22 and 5J7. As shown in Table 4.4, there was about a ~10 fold difference in Neut$_{50}$ between Fab versus MAb for both 1F4 and 2D22. This difference in Neut$_{50}$ was observed regardless of whether the neutralization assay was conducted in Vero cells or the FcγR-bearing cells, U937+DC-SIGN. Therefore, bivalency is not required for DENV neutralization by 1F4 and
2D22, and the minor difference in Neut_{50} is probably due to a decrease in affinity in the Fab fragment.

Comparing neutralization profiles of 5J7 Fab versus MAb showed interesting differences. Although both Fab and MAb of 5J7 was capable of neutralizing DENV3 to 100% in Vero cells (Figure 4.5A and B), saturating concentrations of 5J7 Fab were only able to neutralize less than 50% of DENV3 in U937+DC-SIGN cells (Figure 4.5 C and D). Past studies have shown that the presence of FcγR can have a drastic impact on DENV neutralization by certain groups of antibodies (9, 38, 39, 68). However, blocking the FcγR on U937+DC-SIGN cells using 1% normal human sera had no effect on DENV3 neutralization by 5J7 Fab or MAb (Figure 4.5E). At the moment, the reason for the cell-specific observance of the lack of complete DENV3 neutralization with excess 5J7 Fab is unclear, and further experiments are presently being conducted to investigate this unusual phenomenon.

Most DENV-specific antibodies either neutralize by blocking attachment of DENV to host receptor(s) (i.e. pre-attachment blocking) or by inhibiting a step after attachment, and thereby preventing viral fusion (i.e. post-attachment blocking). Thus, as shown in Figure 4.6, we conducted pre- and post-attachment neutralization assays to investigate the mechanism of neutralization of the type-specific neutralizing and cross-neutralizing human MAbs that we had isolated. The type-specific MAbs, 1F4, 2D22 and 5J7 strongly neutralized DENV1, DENV2 and DENV3, respectively at 4°C (Figure 4.6B, C and D). As shown on Figure 4.6, there is no statistical difference in the pre versus post-attachment Neut_{50} titers of 1F4, 2D22 and 5J7 (Figure 4.6 B, C and D, respectively), signifying that these three type-specific MAbs neutralize primarily at a post-attachment step. The pre- and post-attachment neutralization assays were inconclusive for the cross-neutralizing human MAbs, 1N5 and 1C19, since
neutralization by these MAbs were temperature sensitive and did not efficiently neutralize DENV2 at 4°C (Figure 4.6 E and F). In an attempt to extend mechanistic studies to DENV-immune human polyclonal sera, we conducted pre- and post-attachment neutralization assays with a panel of eight primary DENV immune, late convalescent human sera. We found that only four out of the eight sera (i.e. 50%) primarily neutralized DENV at a post-attachment step (Table 4.5). Although this observation must be extended to a larger panel of primary DENV-immune sera, it seems that type-specific DENV neutralization after natural infections is not limited to post-attachment blocking mechanisms.

*Antibodies that do not target the structural DENV proteins are also protective in vivo*

Several *in vitro* experiments were extended to *in vivo* experiments with the AG129 mouse model. Human sera were sent from our laboratory to the laboratory of Dr. Eva Harris, and all *in vivo* experiments in AG129 mice were conducted by Katherine Williams at the laboratory of Dr. Eva Harris (UC, Berkley). Neutralization analysis of pre-challenge bleeds from AG129 mice 24 hrs after passive transfer of human sera, confirmed prior observed *in vitro* results (in chapter 3) by showing that the removal of cross-reactive antibodies from primary DENV2-immune human sera did not significant affect Neut$_{50}$ titers (Figure 4.7A). Challenge studies with the mouse-adapted DENV2 D2S10 strain showed that removal of cross-reactive antibodies from primary DENV2-immune sera did not significantly affect viral titers in serum, spleen or bone marrow (Figures 4.7B, C and D).

Depletion of DENV2-binding antibodies from primary DENV2-immune human sera was used as a control to show successful depletion of DENV neutralizing antibodies. *In vitro* analysis of DENV2-depleted primary DENV2-immune sera showed a removal of greater
than 95% of the serum neutralizing potency. Similarly, neutralization analysis of pre-
challenge bleeds from AG129 mice indicated statistically similar Neut\textsubscript{50} titers between naïve
human sera and DENV2-depleted primary DENV2 immune sera (Figure 4.7A). However, it
was observed in the viral challenge studies that viral titers in serum, spleen and bone marrow
were significantly different between mice with passively transferred naïve human sera and
mice with DENV2-depleted primary DENV2-immune sera (Figure 4.7 B, C and D).
Surprisingly, removal of DENV virus-binding antibodies from serum, did not completely
remove protection against DENV challenge. Further investigations are being conducted to
determine the source of this significant residual protection observed \textit{in vivo}.

4.5 Discussion

Until recently, very little was known about the viral epitopes targeted by the
protective antibody response in humans. Improvements in the development of human
hybridoma technology have enabled the isolation and characterization of numerous DENV-
specific human antibodies (4, 5, 15, 17, 21, 53, 61, 71). Several recent studies have found
that the human antibody response is predominantly cross-reactive and weakly neutralizing,
while strongly neutralizing antibodies make up a minor population of the entire antibody
response (4, 15, 17, 53). We previously mapped three strongly neutralizing type-specific
antibodies to a complex, quaternary epitope that includes the EDI-DII hinge (16). Similarly,
another recent study mapped a DENV1-specific, strongly neutralizing human MAb
(HM14c10) by cryo-EM to the EDI-DII hinge (59).

In the present study we further mapped the EDI-DII hinge epitope and mutated
residues in the DENV3 EDI-DII hinge region into DENV4 residues. We showed that this
hinge mutant (UNC3001-A12) virus not only significantly escapes neutralization from primary DENV3-immune human sera, but also gains neutralization by primary DENV4-immune sera. Therefore, we successfully mapped the dominant DENV epitope responsible for type-specific protection in humans. Furthermore, this is the first time in the DENV field that a successful epitope transplant has been conducted, and that too against human polyclonal sera.

Comparison studies of the E protein structure between DENV2 and DENV3 emphasize that in addition to amino acid differences in the EDI-DII hinge, there was also a 10° angular difference between the serotypes (35, 37). It is possible that serotype-specificity is not only achieved by the differences in amino acid residues in the EDI-DII hinge, but also by the angular difference between serotypes. Cryo-EM and X-ray crystallography studies comparing the WT (UNC3001) and the hinge mutant (UNC3001-A12) should be conducted to investigate this.

It is important to take note that several of the WT DENV passaged in the absence of neutralizing antibody pressure led to mutations in the EDI-DII hinge region that led to neutralization escape from 1F4 and 5J7 (data not shown). Several published studies have indicated the EDI-DII hinge as being one of the areas that accumulated mutations when DENV is repeatedly passaged in cell culture (31). It is shown that mutations in the EDI-DII hinge changes the pH-threshold of fusion, and better adapts the virus to pH changes in cell culture (3, 7, 22, 26, 31, 35, 40). Furthermore, it’s also shown that mutations in this region also adapt the virus to mice, leading to an encephalitic phenotype (22, 40).

The mechanism of virus neutralization by flaviviral antibodies is generally accepted as blocking either attachment (13, 23, 43), or a step post-attachment (18, 49, 56, 60, 63).
Many flaviviral-specific mouse MAbs that bind the EDIII or the fusion loop has been found to neutralize by blocking fusion or a step post-attachment (49, 56, 60). The EDI-EDII hinge plays an important role in viral fusion. The EDI-DII hinge undergoes a large conformational change during viral fusion as E protein dimers disengaged and form E trimers (reviewed in (41)). As expected, we found that the EDI-DII hinge binding antibodies all neutralized DENV by blocking a post-attachment step. Investigation with polyclonal sera, showed that primary DENV-immune human sera is more complicated and does not necessarily always neutralize through a post-attachment blocking mechanism. The EDI-DII hinge binging MAb, HM14c10, was found to neutralize predominantly by blocking virus attachment to cells (59). Therefore, EDI-DII hinge binding antibodies can neutralize by blocking either attachment or a step post-attachment.

*In vitro* analysis of antibody neutralization is often complicated by the cell line being used to conduct the assay. The presence of FcγR-bearing cell lines can distinguish between homotypic neutralization and heterotypic neutralization by human immune-sera (9, 38, 39, 68). Chan, et al 2011 (9), provided the molecular mechanism of this observation; weakly neutralizing antibodies at high concentrations form aggregates, which then binds to the inhibitory FcγRIIB on the surface of monocytes or macrophages. At low concentrations, these antibodies promote ADE of the virus that it binds (9). We initially hypothesized that maybe the difference in 5J7 neutralization in the two cell lines (Vero and U937+DC-SIGN) was due to the presence of FcγR in U937+DC-SIGN cells (Figure 4.5). Neutralization experiments in the presence and absence of blocked FcγR showed no difference in neutralization. This was not unexpected since the cell-specific 5J7 neutralization difference was only observed with the 5J7 Fab and not with the whole MAb. Unpublished data by our
group show that virus with higher partially immature states cannot infect Vero cells, while infection in FcγR-bearing cells (such as U937+DC-SIGN cells) is not very sensitive to different states of virus maturity. It is possible that the 5J7 Fab is especially sensitive to virus maturity, and hence is unable to neutralize a fraction of the virus population. This hypothesis is presently being tested.

Cross-protection is observed transiently following a primary DENV infection and long-term after a secondary DENV infection (1, 2, 14, 50). The only cross-protective MAbs described in literature to date are weakly to moderately neutralizing human MAbs. These MAbs either bind the highly conserved fusion loop or the prM protein (4, 12, 15, 17, 53). In the present study, we described two strongly cross-neutralizing human MAbs, 1N5 and 1C19. Although there is some discrepancy in the mapping data using escape mutants or mutant E proteins, 1N5 and 1C19 does not map to the same region (i.e. fusion loop or prM protein) as cross-neutralizing MAbs described in literature.

The AG129 mouse model is traditionally used as a lethal enhancing model for investigating ADE of DENV (reviewed in (73)). However, the AG129 model has also been optimized for studying the neutralization properties of DENV-specific antibodies (6, 74). We showed using in vivo experiments that cross-reactive antibodies do not contribute to long-term protection of the homotypic virus after a primary DENV infection. Surprisingly, removal of >95% of in vitro neutralization in a primary DENV-immune sera using the homotypic virus, did not ablate protection in vivo. Depletion with DENV virus only removes antibodies specific to the structural proteins, E and prM. Primary DENV infections also produce detectable amounts of NS1-specific antibodies. Although there is some controversy around the role of anti-NS1 antibodies, there are several studies that show that NS1-specific
antibodies can offer some protection against DENV challenge \textit{in vivo} (11, 24, 52, 69).

This chapter attempts to further characterize the human antibody response. To date, natural DENV infections have resulted in the most successful DENV immunizations in humans. We are hoping that the identified immune and mechanistic role of the EDI-DII hinge in long-term, type-specific protection will help current vaccine developers to assess the quality and epitopes targeted by their vaccines. Furthermore, we realize the complexity of antibody neutralization, and caution basic scientists and vaccine developers to take into consideration the effects of different cell lines, \textit{in vivo} components and NS1-specific antibodies when assessing neutralization properties of antibodies \textit{in vitro}. 
Figure 4.1 Additional mapping data for 5J7.  

A) Neutralization assays showed the generation of two additional escape mutants (with mutations L53P or K128G). B) An intracellular prM-E-binding assay was used to further characterize the binding epitope of 5J7. Proper folding of the prM-E proteins being tested were confirmed using a panel of mapped MAbs, such as D004 (anti-fusion loop) and D178 (anti-EDII) The binding assays were conducted by Kristen Kahle and Jen pfaff from Integral Molecular, Inc.
Figure 4.2 Twenty-six amino acid residues on the DI-DII hinge of DENV3 E protein was successfully mutated to the corresponding residues in DENV4. A) The 26 mutated positions highlighted on the WT DENV3 E protein dimer structure. As expected, the non-EDI-DII hinge-binding control MAb, 1N5, neutralizes both WT (UNC3001) and EDI-DII
hinge mutant (UNC3001-A12) similarly, in both B) U937+DC-SIGN and D) Vero cells. Mutating the EDI-DII hinge in DENV3, significantly ablates neutralization by the EDI-DII hinge-binding MAb, 5J7, in both C) U937+DC-SIGN and E) Vero cells.
Figure 4.3 Mapping 1N5 and 1C19 through neutralization escape mutants. EDII mutants show significant neutralization escape, but not binding escape from 1C19 (A & C) and 1N5
(B & D). E) All the escape mutations selected against 1C19 and 1N5 mapped to the middle of EDII. The binding data of 1C19 and 1N5 to the neutralization escape mutants were generated by Jennifer McGraw.
Figure 4.4 Epitope mapping of 1C19 using a DENV3 prM-E mutant library. A) Show that the mutations R73A and G78A significantly ablates binding of 1C19 as compared to WT DENV. B) Amino acid residues R73 and G78 are on a loop situated above the fusion peptide in EDII. The binding assays were conducted by Integral Molecular, Inc.
Figure 4.5 Neutralization differences of 5J7 fAb in Vero versus U937+DC-SIGN not due to Fcγ receptors. Both 5J7 whole Ab (A) and fAb (B) neutralizes DENV3 to 100% in Vero cells. Unlike the whole Ab (C), the neutralization of 5J7 fAb (D) against DENV3 saturates before 100%. (F) Apart from bivalency, the main difference between whole Ab and fAb is
the absence of an Fc portion in the fAb. (E) However, this difference in neutralization between U937+DC-SIGN and Vero cells is not due to the presence of FcγR, because pre-blocking the FcγR in U937+DC-SIGN cells with 1% normal human sera did not affect neutralization.
Figure 4.6. Pre-attachment and post-attachment neutralization analysis of strongly neutralizing human monoclonal antibodies. A) As shown, pre-attachment and post-attachment neutralization assays were conducted at 4°C. The type-specific MAbs, 1F4 (B),
2D22 (C), and 5J7 (D), can neutralize the respective DENV serotype at a step after viral attachment to cells. DENV neutralization by the cross-reactive MAbs, 1C19 (E) and 1N5 (F), is sensitive to temperature and difficult to assess by this assay.
Figure 4.7 Neutralization analysis of virus-depleted human serum in AG129 mice. Mice were administered 50 µl of each human serum diluted to a final volume of 400 µl, 24 hrs prior to challenge with DENV2 D2S10. A) About 4-6 hrs prior to DENV infection, small retro-orbital or sub-mandibular bleeds were taken and analyzed for circulating pre-infection neutralization titers. Four days post-infection, mice were sacrificed, organs harvested, and
viral load in the serum (B), bone marrow (C) and spleen (D) assessed by Q-RT-PCR. Data collected by Katherine Williams (in the laboratory of Dr. Eva Harris). Data representative of experiments conducted with two different primary DENV2-immune sera.
Table 4.1 Effects of swapping the DENV3 EDI-DII hinge with DENV4 residues on neutralization by DENV-immune human sera.

<table>
<thead>
<tr>
<th>Infection type</th>
<th>Serum Sample ID</th>
<th>WT (UNC 3001)</th>
<th>Hinge Mutant (3001-A12)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary DENV3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRB 003</td>
<td>812</td>
<td>98*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(1200-550)</td>
<td>(115-85)</td>
<td></td>
</tr>
<tr>
<td>DT 105</td>
<td>602</td>
<td>60*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(900-400)</td>
<td>(190-20)</td>
<td></td>
</tr>
<tr>
<td>DT 118</td>
<td>8500</td>
<td>600*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(13,250-5500)</td>
<td>(1200-325)</td>
<td></td>
</tr>
<tr>
<td>DT 125</td>
<td>3390</td>
<td>102*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4700-2450)</td>
<td>(150-70)</td>
<td></td>
</tr>
<tr>
<td><strong>Primary DENV4</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DT 100</td>
<td>60</td>
<td>590*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(115-30)</td>
<td>(760-460)</td>
<td></td>
</tr>
<tr>
<td>DT 102</td>
<td>56</td>
<td>630*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(90-35)</td>
<td>(840-475)</td>
<td></td>
</tr>
<tr>
<td><strong>Secondary</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRB 000</td>
<td>470</td>
<td>380</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(600-370)</td>
<td>(560-260)</td>
<td></td>
</tr>
<tr>
<td>IRB 027</td>
<td>590</td>
<td>860</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(930-370)</td>
<td>(1200-600)</td>
<td></td>
</tr>
</tbody>
</table>

*Hinge mutant Neut_{50} statistically different from WT Neut_{50} at P<0.001
Table 4.2 Binding and neutralization characteristics of two broadly neutralizing human MAbs that were isolated.

<table>
<thead>
<tr>
<th>MAb</th>
<th>Donor</th>
<th>Virus</th>
<th>rE</th>
<th>EDI/DII</th>
<th>EDIII</th>
<th>Pr</th>
<th>Neut\textsubscript{50} titer (\textmu g/ml)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1N5\textsuperscript{b}</td>
<td>Secondary Complex (All four DV)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>0.14</td>
<td>0.20  0.50  0.40</td>
</tr>
<tr>
<td>1C19</td>
<td>Secondary Complex (All four DV)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>0.06</td>
<td>0.03  0.04  2.74</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Neutralization assay for represented neutralization data was conducted using U937+DC-SIGN cells.

\textsuperscript{b}Pre-inoculation with 1N5 was observed to significantly reduce viremia in the liver, spleen, bone marrow and serum of AG129 mice challenged with D2S10.

\textsuperscript{c}1N5 and 1C19 did not bind to viral prM by Western blot analysis either.
Table 4.3 Successful generation of several DENV2 neutralization escape mutants against 1C19 and 1N5.

<table>
<thead>
<tr>
<th>Human MAb</th>
<th>Serotype</th>
<th>Mutant #</th>
<th>Escape Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1C19</td>
<td>DENV2</td>
<td>1</td>
<td>M118K</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>E126K</td>
</tr>
<tr>
<td>1N5</td>
<td>DENV2</td>
<td>1</td>
<td>E62Q</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>M118K</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>T120K</td>
</tr>
</tbody>
</table>
Table 4.4 Neutralization properties of DENV by whole MAb versus fAb in two different cell lines.

<table>
<thead>
<tr>
<th></th>
<th>U937+DC-SIGN cells</th>
<th>Vero cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole MAb [nM]</td>
<td>SEM (nM)</td>
</tr>
<tr>
<td>1F4</td>
<td>0.19</td>
<td>(0.17-0.21)</td>
</tr>
<tr>
<td>2D22</td>
<td>1.47</td>
<td>(1.35-1.62)</td>
</tr>
<tr>
<td>5J7</td>
<td>0.57</td>
<td>(0.49-0.68)</td>
</tr>
</tbody>
</table>

*Neutralization curve of 5J7 fAb against DENV3 virus saturated before reaching a 100%.
Table 4.5. Pre- and post-attachment neutralization assays with a panel of late convalescent primary DENV-immune human sera.

<table>
<thead>
<tr>
<th>Infection type</th>
<th>Serum Sample ID</th>
<th>Pre-attachment 4°C</th>
<th>Post-attachment 4°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary DENV1</td>
<td>IRB 006</td>
<td>300 (200-430)</td>
<td>90* (53-140)</td>
</tr>
<tr>
<td></td>
<td>DT 106</td>
<td>310 (235-400)</td>
<td>25* (20-35)</td>
</tr>
<tr>
<td></td>
<td>GL 010</td>
<td>1100 (725-1660)</td>
<td>580</td>
</tr>
<tr>
<td></td>
<td>GL 024</td>
<td>880 (560-1350)</td>
<td>200* (135-300)</td>
</tr>
<tr>
<td>Primary DENV2</td>
<td>IRB 001</td>
<td>155 (130-180)</td>
<td>87 (52-145)</td>
</tr>
<tr>
<td></td>
<td>DT 110</td>
<td>1000 (830-1200)</td>
<td>575</td>
</tr>
<tr>
<td>Primary DENV4</td>
<td>DT 100</td>
<td>70 (40-125)</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>DT 102</td>
<td>200 (120-330)</td>
<td>330 (250-560)</td>
</tr>
</tbody>
</table>

*Post-attachment Neut$_{50}$ statistically different from pre-attachment Neut$_{50}$ at P<0.05
References


CHAPTER FIVE

Mapping Enhancing Antibodies Produced by the Human Immune Response After Primary Dengue Virus Infections.

5.1 Overview

Antibody dependent enhancement (ADE) is the leading theory for why some people develop severe disease following a secondary dengue virus (DENV) infection. The viral epitopes recognized by enhancing antibodies after natural infections have not been fully defined. We used antibody-depletion techniques to remove heterotypic DENV-specific and rE-specific antibodies from primary DENV-immune sera. The depletion effects of specific antibodies on ADE of heterotypic virus infection were tested both in vitro (K562 cells) and in vivo (AG129 mouse model). Removal of cross-reactive antibodies ablated enhancement of heterotypic virus infection. Furthermore, we observed that both rE-specific and prM-specific antibodies in primary DENV-immune sera significantly contribute to enhancement of heterotypic DENV infection. We hope that the identification of enhancing epitopes would facilitate the development of safe, non-enhancing vaccines against DENV.

5.2 Introduction

Primary infections with DENV produce a cross-reactive immune response in humans, but protection is only against the serotype of infection. Due to the phenomenon of antibody dependent enhancement (ADE), these weakly neutralizing, cross-reactive antibodies lead to a
higher probability of severe disease (DHF/DSS) during secondary infections (10, 11, 32). During ADE, cross-reactive antibodies bind virus at sub-neutralizing concentrations, and enter host cells (such as monocytes and macrophages) through FcγR-mediated endocytosis (reviewed in (30)). Productive DENV infections through ADE (as compared to the conventional route of entry) have been found to result in higher viremia and a suppressed host antiviral state (3, 4, 9, 12, 15, 23, 30). The possibility of exacerbating DENV infections through ADE presents vaccine developers with a challenging task. Therefore, it is imperative that we identify the viral epitopes targeted by enhancing populations of antibodies in humans.

Development of a suitable small animal model for investigation of DENV infection and antibody responses have been severely hindered by the low or lack of DENV replication in wild type immune-competent mouse models. The earliest mouse models consisted of intracranial DENV challenges in immune-competent suckling mice. Unfortunately, these models resulted in death through non-human-like DENV-induced disease, such as neurological disease and paralysis (21, 25). Replication in a rodent model was first shown in the IFN α/β and γ receptor deficient mouse mode, AG129 (13). It was further discovered that the AG129 mouse model also presents a human-like vascular leakage lethal syndrome when challenged with DENV in the presence of DENV-specific antibodies (1, 26, 36). Therefore, the AG129 is presently the most suitable animal model available for testing ADE of DENV infections.

Recent studies (including chapter 2) investigating the memory B-cell response after natural DENV infections have revealed that the antibody response in humans is dominated by cross-reactive, weakly neutralizing antibodies (2, 5, 7, 28). These cross-reactive antibodies were found to efficiently enhance DENV infection, and usually over a wide range
of concentrations (2, 5, 7, 28). A study analyzing the memory B-cell response after human immunization with a leading DENV vaccine candidate, observed a similar dominantly cross-reactive, weakly neutralizing and highly heterotypic virus-enhancing antibody response (27). A majority of these cross-reactive human MAbs mapped to either the prM or the E protein (2, 5, 7, 28).

Any DENV-specific antibody, regardless of neutralization potency, will at some concentration enhance infection in FcγR-bearing cells. To investigate the viral epitopes targeted by antibodies responsible for enhancement of secondary infection, it is insufficient to analyze enhancement properties of isolated human MAbs. We need to look at the antibody repertoire in circulation prior to infection, and conduct ADE assays at Ab concentrations that approximate physiological concentrations in circulation. Therefore, the present study investigates the antibody groups responsible for enhancement of heterotypic serotypes by studying late convalescent human sera following natural primary DENV infections. The proposed studies will be conducted both in vitro (using the FcγR-bearing cell line, K562) and in vivo (using the AG129 mouse model).

5.3 Methods

Virus and cell lines

All in vitro assays were conducted with the DENV WHO reference strains, i.e. DENV1 West Pac 74, DENV2 S-16803, DENV3 CH54389 and DENV4 TVP-360. The WHO reference viruses were initially obtained from Dr. Robert Putnak (Walter Reed Army Institute of Research, Silver Spring, MD). All in vivo assays in the AG129 mice were conducted using the widely used mouse-adapted DENV2 D2S10 stain (26). All viruses were
infectious assays were grown as described previously in chapter 4 using the mosquito cell line, C6/36 (33). All viruses for antigen purification were grown in the mammalian cell line, Veros. In vitro ADE assays were conducted using K562 and/or U937 cells. Cell lines were obtained from ATCC.

*Human sera and Fabs*

As described in chapter 4, blood donations were obtained from individuals who had travelled to DENV endemic regions and experienced a primary DENV2 or DENV3 infection. These human samples were obtained with informed consent and approximately 2 to 10 yrs after DENV infection. All donations were taken in compliance with the Institutional Review Board of the University of North Carolina at Chapel Hill. The whole blood samples were processed to serum for use in the following experiments.

The MAbs 2D22 (DENV2-specific, EDI-DII hinge binding), 1C19.2 (cross-reactive, EDIII-binding), 1B22 (cross-reactive, prM-binding) and 2K2 (cross-reactive, prM-binding) were generated in collaboration with Scott Smith in the laboratory of Dr. James Crowe (VU). The Fab fragments of 2D22 (2D22 Fab), 1C19.2 (1C19 Fab), 1B22 (1B22 Fab) and 2K2 (2K2 Fab) were generated through enzymatic digestion of the respective MAbs.

*Depletion of virus-specific antibodies from human sera*

Human sera were depleted of virus-specific antibodies as previously described in chapter 3 (6). Briefly, heterotypic virus was grown in vero cells and purified using ultracentrifugation, sucrose cushion and Opti-prep gradients as described previously (6, 33). The highly purified DENV virus was then passively adsorbed to polystyrene beads (4.5 µm),
and incubated with human sera at 37°C to remove the appropriate DENV-specific antibodies. Successful depletion was assessed using a virus-binding ELISA.

Depletion of rE-specific antibodies from human sera

Human sera were depleted of rE-binding antibodies as previously described in chapter 3 (6). Briefly, purified rE protein from all four serotypes were bought from Hawaii Biotech. Purified rE was covalently conjugated to cyanogen bromide (CNBr) activated beads using amine chemistry. The rE conjugated beads were then incubated with human sera at 37°C to remove rE-specific antibodies. Successful removal of all cross-reactive rE-specific antibodies was confirmed using a rE-binding ELISA.

ELISA binding assays

Binding assays of depleted sera to purified DENV virus and/or rE protein was conducted as previously described in chapter 3 (6). DENV Virus or rE were either directly coated or captured by the anti-E protein mouse MAb, 4G2, blocked with 1% normal goat serum, incubated with human serum diluted to 1:20, and binding detected with an alkaline phosphatase-conjugated anti-human secondary antibody.

ADE assay in K562 cells

In vitro ADE assays were conducted in K562 cells as previously described (35). Human sera were diluted 2-fold starting from 1:20, then incubated for 1 hr at 37°C with virus at an MOI of 1.0. Approximately 5x10⁴ cells were added to each well containing virus-
antibody mixtures and then incubated for 2 hrs at 37°C, after which cells were washed 2x with fresh media and incubated at 37°C for another 22 hrs. Cells are fixed 24 hrs post-infection, stained for infection and run on a flow-cytometer to measure percent infections.

**ADE assay in AG129 mice**

In vivo ADE assays in AG129 mice were conducted in Eva Harris Lab as previously described (35). AG129 mice were administered with DENV-immune sera or naïve sera in a final volume of 400 ul, about 24 hrs prior to i.v. challenge with a sub-lethal $10^4$ pfu dose of DENV2 D2S10. Mice were then observed over a 10 day period, and scored for morbidity and mortality.

5.4 Results

*ADE of DENV in K562 cells and the AG129 mouse model*

First, we optimized the ADE assay with the human erythromyeloblastoid leukemia cell line, K562, for investigation of enhancing antibodies in human sera. As shown in Figure 5.1, primary DENV-immune human sera enhanced the heterotypic serotypes at very high serum concentrations (i.e. $\geq 1:80$), while homotypic serotypes (as expected) were only enhanced at low serum concentrations (i.e. $\leq 1:640$). Homotypic serotypes are neutralized at high serum concentrations; therefore understandably, no enhancement can be seen at these serum concentrations.

Similarly, human sera (both naïve and DENV-immune) were titrated in the AG129 mouse model to optimize the serum concentration of peak DENV enhancement. The AG129
mice were passively administered with human immune sera 24 hrs prior to challenge with the mouse-adapted virus, DENV2 D2S10 (Figure 5.2A). As shown in Figure 5.2B, the homotypic immune human sera (i.e. primary DENV2-immune) protects the mice from a lethal DENV2 challenge, while passive transfer of the same quantity of heterotypic human serum (i.e. primary DENV1, DENV3 or DENV4-immune sera) leads to an ADE-induced mortality.

Depletion of cross-reactive antibodies ablated ADE of heterotypic DENV virus

Next we used the *in vitro* and *in vivo* models to identify specific Ab populations in polyclonal sera that drive ADE. Several primary DENV2-immune sera were depleted with the heterotypic virus, DENV3, while several primary DENV3-immune sera were depleted with the heterotypic virus, DENV2. As shown in Figure 5.3A, successful virus-specific depletion was confirmed using a virus-binding ELISA. Further characterization of DENV binding and neutralization by heterotypic virus-depleted primary DENV-immune sera is described in chapter 3 (6). *In vitro* ADE studies with heterotypic-virus depleted sera, showed that removal of heterotypic DENV virus-binding antibodies from human sera, completely ablated enhancement of heterotypic serotypes (Figure 5.3).

Heterotypic virus-depleted DENV-immune sera were then passively transferred into AG129 mice to indirectly assess the role of cross-reactive antibodies in DENV enhancement *in vivo*. Passive transfer of primary DENV3-immune sera into AG129 showed significant morbidity and mortality when challenged with a sub-lethal dose of DENV2 D2S10 (Figure 5.4). However, removal of all heterotypic virus-binding antibodies from primary DENV3-immune sera decreased morbidity and percent survival to levels similar to mice administered
with naïve human sera (Figure 5.4). Thus, cross-reactive antibodies binding to the virion (and therefore the structural proteins) are the main antibody component in human immune sera responsible for heterotypic DENV enhancement.

_Depletion of rE-specific antibodies decreased ADE of heterotypic virus_

We then investigated the role of rE-binding antibodies in heterotypic DENV virus enhancement. Primary DENV-immune human sera was depleted of cross-reactive rE-binding antibodies using purified heterotypic rE protein (purchased from Hawaii Biotech). Removal of cross-reactive rE-binding antibodies from primary immune serum significantly reduced binding to homotypic rE as well, indicating that a majority of the rE-binding antibodies in DENV serum were cross-reactive (Figure 5.5A). Furthermore, _in vitro_ ADE investigations showed that removal of cross-reactive rE-binding antibodies significantly reduced enhancement of heterotypic DENV serotypes by shifting the ADE curve about 0.5-1.0 logarithmic values (Figure 5.5B and C).

The DENV enhancement properties of cross-reactive rE-depleted human sera was then tested in the AG129 mice. Depletion of cross-reactive rE-binding antibodies resulted in survival of 50% of the mice (Figure 5.6A) and was significantly different from the undepleted serum sample (P<0.01). Similar results were observed for two different primary DENV3-immune sera. Therefore, we took a closer look at the morbidity scores of each individual mouse in the control depleted primary DENV3 serum group versus rE-depleted serum group. Interestingly, compared to the control depleted primary DENV3 serum group, there was greater variability in morbidity scores within the rE-depleted sera group (Figure 5.6B and C). It is surprising that the removal of rE-specific antibodies did not lead to partial morbidity of all the mice in that group, but rather led to a consistently decreased morbidity.
and mortality of 50% of the group (in comparison to the control depleted DENV3 serum group). It seems that DENV rE-binding antibodies in primary DENV-immune human sera are only partially responsible for ADE of heterotypic DENV infections.

*Competition ADE assays with prM-binding Fab indicates a role for prM-binding Abs in ADE*

Since we found that E-binding antibodies were only partially responsible for enhancement of heterotypic serotypes, we investigated the role of prM-binding antibodies. The prM protein is a transmembrane protein that is expressed at very low quantities in cell culture. Therefore, we are presently still in the process of optimizing expression and purification of the prM protein (and recombinant versions of the prM protein). However, we still probed the importance of prM-binding antibodies by conducting competitive ADE assays with primary DENV-immune sera and Fabs that bound to prM. Collaborators in the laboratory of Dr. James Crowe generated Fabs from the prM-binding MAbs, 1B22 and 2K2. As shown in Figures 5.7, both 1B22 Fab and 2K2 Fab did not neutralize DENV1 or DENV2 at the concentrations used in the competitive ADE assay. Primary DENV3-immune sera enhanced infection of DENV1 and DENV2 to approximately 40% in the presence of a negative control binding Fab, 2D22 Fab (DENV2-specific) and 1F4 Fab (DENV1-specific) respectively. However, addition of 1B22 Fab or 2K2 Fab competed for virus-binding with DENV-specific antibodies in immune sera and reduced enhancement of heterotypic virus infection by 25-50% (Figure 5.7). Although further experiments need to be conducted with a larger panel of Fabs to confirm the above results, it seems that prM-binding antibodies in primary immune sera also play a role in ADE of heterotypic DENV serotypes.
5.5 Discussion

The cause of severe disease in secondary DENV infections has been a topic of much controversy, and has been attributed to either the phenomenon of ADE or “cytokine storm” (22). It has also been shown that NS1-specific antibodies contribute to severe disease by cross-reacting with human platelets and endothelial cells (18). However, our present results show that ADE of heterotypic serotypes both in vitro and in vivo are caused by cross-reactive antibodies that bind the virus particle (i.e. antibodies that bind the E protein and/or the prM protein). As expected, both our in vitro and in vivo results also indicate that heterotypic virus enhancement is at high serum concentrations, as compared to homotypic virus enhancement.

Several groups have shown that the DENV-specific antibody response generates a large quantity of cross-reactive E-specific antibodies capable of enhancing DENV infection (2, 5, 7, 27, 28). In the present study, we show that rE-specific antibodies in primary immune sera contribute significantly to enhancement of heterotypic virus infection. Several studies with immune sera from individuals who were naturally infected with DENV have indicated that greater than 90% of the DENV-specific antibody response is to the highly conserved fusion loop within the DENV E protein. However, these studies were conducted using either western blot analysis or binding to subviral particles (17, 19). Proteins rarely preserve their tertiary and quaternary structures during western blotting, and subviral particles lack the 5-3-2 axis of symmetry in whole mature DENV viral particles. Comparison of virus-specific and rE-specific antibodies has shown that less than 35% of the DENV-specific antibody response is rE-specific in humans after natural primary infections (5). Furthermore, an interesting study has shown that LALA variants of fusion loop-specific MAbs can be used to
therapeutically prevent enhancement by anti-DENV-immune mouse sera (34). Therefore, although our present studies indicate the significant contribution of rE-specific antibodies in ADE of heterotypic DENV viruses, further experiments need to be conducted to determine the contribution of fusion loop-specific antibodies to enhancement of heterotypic DENV.

In addition to E-specific antibodies, several groups have recently isolated many weakly neutralizing, cross-reactive prM-binding antibodies from human PBMCs from individuals after natural primary infections (2, 5, 7, 27, 28). However, western blot analysis indicates that very little prM-binding antibodies are present in primary DENV-immune human sera as compared to immune sera after secondary DENV infections (2, 31). Therefore, till now the role of prM-antibodies in ADE of secondary infections has been unclear. Our preliminary results indicate that prM-specific antibodies contribute to 25-50% of the in vitro heterotypic DENV enhancing capabilities of primary immune sera.

Although the currently presented work further establishes the AG129 mouse model as appropriate for studying DENV enhancement with human immune serum, this model does have its limitations. For example, despite many labs are working on generating several mouse adapted DENV strains, at present we are limited to conducting our ADE experiments in AG129 with the best characterized DENV2 strain, D2S10 (26). Furthermore, there is much criticism in the DENV field that the lack of IFNα/β and γ receptors in AG129 severely compromise its ability to produce a full humoral and cellular immune response to viral infections. Therefore, it is unclear whether E and prM-binding antibodies would have a different effect in a fully immune-competent DENV model.

Severe disease during DENV infections has been found to be multi-factorial. In addition to sub-protective pre-immunity to DENV, numerous host factors (such as female
sex, human leukocyte antigen (HLA) class I alleles, variant of the DC-SIGN receptor gene, polymorphism in the tumour necrosis factor (TNF) gene and AB blood group, race, and polymorphisms in the Fcγ receptor and vitamin D receptor genes) also increase the risk of severe disease (8, 14, 16, 20, 24, 29). Therefore, it is important to expand our panel of human sera before we firmly establish the role of prM and E targeting antibodies in ADE of heterotypic DENV infections.
Figure 5.1 Primary DENV immune sera enhance heterotypic serotypes at high serum concentrations. The above primary DENV3-immune human serum enhances the heterotypic serotypes, DENV1 (A), DENV2 (B) and DENV4 (D) at very high serum concentrations, while it enhances the homotypic DENV3 (C) virus at a much lower serum concentration. K562 cells were used in this assay. DENV infects K562 through FcγR mediated endocytosis. Data representative of 8 serum samples.
Figure 5.2 In the AG129 mouse model, heterotypic sera enhance DENV2 infection and causes ADE-induced lethality, while homotypic sera protects from DENV challenge. A) Human sera was passively transferred 24 hrs prior to challenge with the DENV2 mouse adapted strain, D2S10. The mice were then scored for morbidity and mortality. B) All heterotypic sera (primary DENV1, primary DENV3 and primary DENV4 sera) caused significantly lethality compared to naïve human sera, while the same quantity of homotypic sera protected the mice from ADE-induced mortality. Data collected by Kate Williams (Eva Harris Lab, UC Berkley). α-DENV1 = primary DENV1, α-DENV2 = primary DENV2, α-DENV3 = primary DENV3, α-DENV4 = primary DENV4, NHS = naïve human sera.
Figure 5.3 Removal of cross-reactive antibodies from primary DENV-immune human sera, removes enhancement of heterotypic DENV infection in vitro. A) Binding ELISA to DENV virus show that depletion of a primary DENV3 sera with DENV2 virus removes binding to heterotypic DENV2 and DENV4, while significantly reducing binding to heterotypic DENV1. Depletion of cross-reactive antibodies from primary DENV3-immune serum, ablated enhancement of heterotypic DENV1 (B) and DENV2 (C), while significantly reduced enhancement of homotypic DENV3 virus (D). Data representative of both primary DENV2 and DENV3-immune human sera.
Figure 5.4 Removal of cross-reactive antibodies from primary DENV3-immune sera significantly protected AG129 mice from ADE of DENV2. Depletion of primary DENV3-immune human sera with heterotypic DENV virus (DENV1, DENV2 and DENV4) significantly increased percent asymptomatic (A) and percent survival (B) in AG129 mice. Percent asymptomatic and percent survival of virion depleted anti-DENV3 significantly different from control depleted anti-DENV3 (P<0.001). Data collected by Kate Williams and Michael Schmidt (Eva Harris Lab, UC Berkley). Data is representative of two primary DENV3-immune human sera.
Figure 5.5 Recombinant E-binding cross-reactive antibodies make up a significant portion of the heterotypic virus-enhancing antibodies. A) Primary DENV3 serum was successfully depleted of DENV1,2 and 4 rE-binding antibodies. Removal of cross-reactive rE-specific antibodies from a primary DENV3-immune human serum significantly reduced enhancement of the heterotypic viruses, DENV1 (B) and DENV2 (C), but did not similarly effect enhancement of the homotypic virus, DENV3 (D). Data representative of a serum panel including both primary DENV2 and DENV3-immune human sera.
Figure 5.6 DENV recombinant E-binding antibodies in human serum are only partially responsible for ADE of heterotypic DENV infections in AG129 mice. A) Removal of E-specific antibodies from a primary DENV3-immune human sera resulted in partial phenotype, where 50% of the mice survived. A closer observation of the morbidity scores
indicated that although morbidity was consistent within the mice injected with control depleted DENV3-immune sera (B), there was high variability within the mice group injected with cross-reactive rE-depleted DENV3-immune sera (C). Data collected by Kate Williams, Michael Schmidt and Sarah Killingbeck (Eva Harris Lab, UC Berkley). Data is representative of two different primary DENV3-immune human sera.
Figure 5.7 Between 25-50% of heterotypic virus enhancement by primary DENV-immune sera can be attributed to prM-binding antibodies. The Fab fragments of the prM-specific human antibodies, 1B22 and 2K2, do not neutralize DENV1 (A) and DENV2 (B) in the concentration range used in the competition ADE assays. Anti-prM Fab from 1B22 and 2K2 competes with a primary DENV3-immune human serum (at peak enhancement concentration) and reduces 25-50% of enhancement of the heterotypic serotypes, DENV1 (C) and DENV2 (D).
References


CHAPTER SIX

Discussion

6.1 A snapshot of the human antibody response to DENV

Recent findings that the mouse antibody response to DENV is different from that of the human response instigated several groups to investigate the human long-lived memory plasma and B-cell response after natural DENV infections (40, 41, 47, 58, 59). Our depletion experiments concluded that primary DENV-infections in humans stimulate circulating plasma cell-secreted antibodies that can be divided into two groups; 1) a large fraction of cross-reactive, weakly neutralizing antibodies responsible for heterotypic virus enhancement, and 2) a small fraction of strongly neutralizing antibodies responsible for type-specific protection (10). As a parallel approach, PBMCs were isolated and in depth analysis was conducted on the DENV-specific memory B-cell response in individuals that had had natural DENV infections. Surprisingly, in several individuals over 9% of the EBV-transformed B-cells were found to be DENV-positive even 8 years after DENV infections (2, 50). This is further evidence of life long antibody responses stimulated by natural DENV infections. One study primarily characterized the memory B-cell response in individuals very early (i.e. within 2 weeks of day of defervescence) after infection and estimated about 40-70% of the virionDENV structural protein-specific antibodies as being directed against the prM protein (11). These antibodies were found to be highly cross-reactive and weakly neutralizing
Similar prM-binding antibodies were described put forward in chapter 2 and other studies that characterized the late convalescent memory B-cell response following primary or secondary DENV infections (2, 9, 49, 50). A large proportion of the DENV-specific antibodies also bound E protein, and several of these cross-reactive, weakly neutralizing antibodies were mapped to the highly conserved fusion loop.

Since weakly neutralizing, cross-reactive antibodies dominated the human antibody response, neutralizing antibodies were infrequently isolated (less than 5% of the DENV virus-specific response) (50). Weakly neutralizing, cross-reactive antibodies capable of ADE of DENV infection further explains why primary DENV-immune sera offer type-specific protection and heterotypic virus enhancement. It is interesting that weakly DENV neutralizing memory B-cells are kept in circulation years after infection. It is possible that these same weakly neutralizing, cross-reactive antibodies at higher numbers are responsible for the transient cross-protection observed right after primary infections. As of yet, in depth analysis of the human memory B-cell response has not been conducted for other flaviviral infections. Therefore, it is difficult to postulate whether this dominant weakly neutralizing, cross-reactive response is a trend across flaviviruses, or whether it’s DENV-specific.

6.2 Lessons learnt from protective antibodies following natural DENV infections in humans.

Role of EDI-DII hinge-specific Abs in type-specific protection

Prior antibody mapping studies using mouse mAbs had identified EDIII as the target of neutralizing antibodies (16, 17, 33, 53, 54). Screening memory B cells from naturally DENV-infected individuals with DENV rE or EDIII successfully isolated EDIII-specific
antibodies (2, 9). However, EDIII-specific antibodies were found to be rare in DENV-immune individuals, and did not contribute to significant protection in humans (40, 41, 47, 58, 59). Therefore, surprisingly, DENV EDIII is not the major target of protective antibodies in humans (40, 41, 47, 58, 59). We therefore, we isolated potently neutralizing, type-specific human mAbs, and mapped them to a complex quaternary epitope that focused around the DI-DII hinge of the DENV E protein (10). Cryo-EM studies mapped a type-specific, strongly neutralizing anti-DENV1 human mAb to the same region and further solidified our findings (55). Our studies with the EDI-DII hinge mutant (UNC3001-A12) and human DENV-immune sera firmly establish the importance of the EDI-DII hinge region in protection following primary infections. It seems that other flaviviral infections in humans may also elicit protective antibodies that target the EDI-DII hinge. Escape mutant analysis have mapped strongly protective mAbs raised in mice or chimpanzee to residues in the EDI-DII hinge of JEV and Murray Valley encephalitis virus (14, 18, 19, 26, 27, 35). Since several of these complex JEV EDI-DII hinge-binding antibodies were generated in mice, it is evidence that mice are able to generate these types of complex neutralizing antibodies (18, 26, 27). Therefore, the difference in the mouse and human antibody responses to DENV maybe attributed to the lack of DENV replication and growth in immune competent mice. Additionally, cryo-EM studies also mapped a potently neutralizing anti-WNV human antibody (CR4354) to a complex quaternary epitope, which included the EDI-DII hinge of one WNV E dimer and EDIII of the adjacent WNV E protein dimer (23, 57). Therefore, it seems that protection through antibody binding to complex quaternary epitopes focused around the EDI-DII hinge may be a common trend within the flaviviruses.
Flaviviruses are not the only enveloped viruses to elicit protective antibodies against conformational, complex epitopes. Recent X-ray crystallography structures mapped a rare Sudan virus (genus *Ebolovirus*) neutralizing human mAb to an epitope created by the close proximity of two glycoproteins (GP1 and GP2) on the virion particle (12). Furthermore, despite the lack of detailed structural information, Hepatitis C virus (HCV) has also been found to induce strongly neutralizing antibodies that recognize complex, conformational epitopes (24, 25, 45). In the HIV (retroviridae) field, several potently neutralizing human antibodies have been mapped to what has been termed the quaternary neutralizing epitope (QNE); these antibodies do not bind monomeric pg120, instead recognize the gp120 trimeric form of the mature envelope spike (15, 51). Therefore, it seems that antibodies targeting complex, quaternary conformational epitopes play a large role in human protection against enveloped viruses.

*DENV EDI-DII hinge-specific Abs and mechanism of neutralization*

Binding the EDI-DII hinge offers antibodies several distinct advantages towards successful neutralization. The flaviviral EDI-DII hinge plays vital roles in virus maturation, entry and fusion (reviewed in (22)). During the DENV life cycle, the EDI-DII hinge angle undergoes over a 35° change, which effects the relative positioning of DII and DIII regions of the DENV E protein (36-38). A majority of the antibodies that bind the EDI-DII hinge block steps post-attachment, such as entry and fusion (Chapter 4 & (23, 57)). DENV is observed to infect several different cell lines using various host attachment receptors (such as C-type lectins, glycosaminoglycans and phosphatidylserine receptors (5, 8, 42)). Therefore, neutralization by attachment-blocking antibodies is restricted to the cell type expressing the
relevant host attachment factor. However, since DENV entry and fusion mechanism are universal and to a large extent cell line independent, antibodies that block steps post-attachment would be capable of neutralization in any DENV-susceptible cell line.

The EDI-DII hinge region has also been attributed to play a role in maintaining the pH-threshold of fusion (1, 3, 18, 20, 31, 36, 39). Continuous passage of flaviviruses in cell culture leads to point mutations in this region that changes the pH at which fusion events are triggered (31). It is important to keep in mind that although there is fairly high amino acid sequence variability in the EDI-DII hinge sequence between serotypes attributing to type-specificity, the sequence variability within serotypes is relatively conserved (57). Therefore, accumulation of mutations in the EDI-DII hinge region of cell culture-passaged virus is probably due to pH changes that occur in cell culture settings, and hence probably only a cell culture phenomenon.

The EDI-DII hinge region is fairly well exposed in both the mature and immature virion (28, 36-38, 61), enabling EDI-DII hinge specific antibodies to efficient neutralize DENV irrespective of virus maturity. The dynamic nature of both the DENV virion structure and the EDI-DII hinge region increases the accessibility of antibodies to this region. Furthermore, it is highly unlikely that an excess of weakly neutralizing anti-fusion loop antibodies would interfere with EDI-DII hinge-specific antibodies, as was recently shown for neutralizing EDIII-specific antibodies (60). Therefore, a DENV vaccine platform that stimulates protective EDI-DII hinge-specific antibodies might be the solution to a successful DENV vaccine.
Role of DII-binding Abs in cross-reactive protection

Very little is known about the role of cross-protective antibodies and the epitopes that they bind. Several studies have mapped cross-reactive antibodies to prM and the E fusion loop (2, 4, 6, 7, 9, 11, 30, 32, 41, 43, 50, 56). However, these antibodies were moderately neutralizing at best (2, 4, 7, 9, 11, 43, 50). Our work in chapter 4 identifies a novel cross-protective epitope in EDII. The 1N5 and 1C19 antibodies that mapped to this region were isolated from secondary DENV infected individuals. A recent study also isolated strongly cross-neutralizing antibodies from PBMCs of individuals experiencing acute secondary DENV infections (48). Furthermore, long term cross-protection has been observed in humans only after secondary or tertiary DENV infections. It seems that these long-lived potently cross-neutralizing antibodies are produced only after secondary or tertiary infections.

6.3 ADE, protection and implications for vaccine design

The cause of severe disease (DHF/DSS) during DENV infections is multi-factorial. Several host factors that increase the risk of severe disease include female sex, human leukocyte antigen (HLA) class I alleles, variant of the DC-SIGN receptor gene, polymorphism in the tumour necrosis factor (TNF) gene and AB blood group, race, and polymorphisms in the Fcγ receptor and vitamin D receptor genes (13, 21, 29, 34, 46, 52). ADE has been attributed to be a risk factor for severe disease during secondary infections. Therefore, the characterization of the antibodies responsible for ADE of heterotypic DENV virus during natural infections is vital for developing safe vaccines.

As characterized in this dissertation and recent papers, the human antibody response to DENV is dominated by weakly neutralizing, cross-reactive antibodies that are capable of
efficient ADE of DENV infections (2, 10, 11, 49, 50). Unlike protective antibodies, antibodies responsible for DENV enhancement bind to regions on both prM and the E protein. The amino acid sequence of both prM and the fusion loop of E protein are more than 70% conserved among the four DENV serotypes. A recent study analyzing the memory B-cell compartment of individuals who had received the live-attenuated DV1 vaccine (DENV1Δ30) versus individuals who had had natural DENV1 infections, showed similar antibody profiles (49). Therefore, it is highly likely that other leading DENV vaccines also elicit a dominant weakly neutralizing, enhancing antibody repertoire.

Using the results in this dissertation and presently available knowledge of the human antibody response to natural DENV infections, there seems to be two ways of designing a successful DENV vaccine. The first approach is the same approach that current vaccine developers are trying to achieve, i.e. balanced, life long protective immunity against each of the four DENV serotypes. For this vaccine approach to be successful, it is important to elicit strongly neutralizing immunity capable of overcoming the inherently produced weakly neutralizing, cross-reactive, DENV-enhancing antibodies. Since the most potently neutralizing type-specific antibodies were found to recognize complex, quaternary epitopes that are only preserved on the virion particle (10, 23, 55, 57), it is understandable that the most successful, leading vaccine platforms are live-attenuated vaccines (as opposed to subunit vaccines). Surprisingly, the leading live DENV vaccine candidate (i.e. the yellow fever dengue chimeric vaccine) in phase IIB clinical trials failed to produced balanced protective responses against the four DENV serotypes (44). Therefore, it seems important that vaccine developers assess the viral epitopes recognized by the neutralizing antibodies produced by the vaccine recipients. The second approach for a successful DENV vaccine is
to present the human immune system with only the viral epitopes responsible for potently neutralization. More specifically, the complex, quaternary epitope, including the EDI-DII hinge can be re-created either in an unrelated protein, or a distantly related flavivirus. This approach removes conserved regions in the immunogen that may otherwise produce cross-reactive antibodies that have the potential to cause ADE of DENV. At the same time, this vaccine approach presents the body with an epitope that can elicit life-long, potently neutralizing antibodies.

The findings in this dissertation expand our knowledge of the human antibody response to DENV. It also reveals novel viral epitopes responsible for type-specific protection, and attempts to characterize the mechanism of neutralization. Furthermore, this dissertation investigates the viral epitopes that lead to ADE of heterotypic DENV infections. Finally, we hope the findings in this dissertation will contribute to the development of safe and successful DENV vaccine designs.
References


