Studies on the inhibition of viral entry of flaviviruses

Ravikumar Rajamanonmani

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STUDIES ON THE INHIBITION OF VIRAL ENTRY OF FLAVIVIRUSES

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Ph. D. Thesis

**School of Biological Sciences
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STUDIES ON THE INHIBITION OF VIRAL ENTRY OF FLAVIVIRUSES

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SCHOOL OF BIOLOGICAL SCIENCES

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS i

TABLE OF CONTENTS iii

LIST OF TABLES..... vii

LIST OF FIGURES viii

ABBREVIATIONS xi

PUBLICATIONS..... xiv

GENBANK ENTRIES..... xv

ABSTRACT 1

1. INTRODUCTION 2

1.1. Viruses 2

1.1.1 *Taxonomy and Classification of viruses* 3

1.2 Flaviviridae 5

1.2.1 *Flaviviruses and flavivirus infections* 6

1.2.2 *Dengue fever and dengue virus classification* 8

1.2.3 *Pathogenesis of dengue virus manifestations* 9

1.2.4 *West Nile virus infection and West Nile virus classification* 10

1.2.5 *Pathogenesis of West Nile virus encephalitis* 13

1.2.6 *Flavivirus life cycle*..... 13

1.2.7 *Flavivirus replication* 14

1.3 Flavivirus proteins 14

1.3.1 *Nonstructural proteins* 17

1.3.2 *Structural proteins* 18

1.3.3. Capsid (C), pre (pr) and Membrane (M) proteins.....	18
1.4 Flavivirus Envelope Protein (E)	20
1.4.1 The Envelope protein domain III (Ed3)	24
1.5 Structure of flavivirus	28
1.6 Interaction with host cell surface receptors	31
1.6.1 Low pH induced endosomal fusion by flavivirus	32
1.6.2 Flavivirus maturation and Budding.....	38
1.7 Viral entry inhibitors.....	40
1.7.1 Anti flaviviral antibodies	41
1.8 Aim of the study	45
2: MATERIALS AND METHODS.....	46
2.1 Molecular cloning	48
2.1.1 Overexpression of rEd3	49
2.1.2 Denaturation and refolding of rEd3	50
2.1.3 Purification of rEd3 by gel filtration chromatography	53
2.1.4 Physicochemical characterization of rEd3.....	54
2.1.5 Enzymatic cleavage of Trx and His tags from rEd3	54
2.1.6 Binding of anti-His or anti-E antibody to rEd3	55
2.1.7 Inhibition of viral entry by rEd3	55
2.1.8 Characterization of mAb 9F12 binding to rEd3 and virus	56
2.2 Maintenance of cell cultures and hybridomas	58
2.2.1 Virus propagation, purification and quantification.....	59
2.2.2 Identification of virus containing fractions from gradients by SDS-	

PAGE, ELISA or Dot-blot	60
2.2.3 Purification of monoclonal antibodies from hybridoma supernatant	61
2.2.4 Plaque reduction neutralization (50%) test (PRNT _{50%})	62
2.2.5 Adsorption assay using cell based flavivirus immuno-detection (CFI)	64
2.2.6 Membrane fusion inhibition assay	65
2.3. Sequencing of mAb 9F12 and construction of recombinant scFv	66
2.3.1 Epitope mapping of mAb 9F12 with peptides and yeast surface display	68
2.4 Synthesis and purification of peptides for virus fusion inhibition	69
2.4.1 Purification of peptides by High Performance Liquid Chromatography	72
2.4.2 Cytotoxicity assay for peptides to be used as fusion inhibitors	74
2.4.3 Assay of peptides as viral entry inhibitors	75
3: FLAVIVIRUS ENTRY INHIBITION BY HOMOLOGOUS RECOMBINANT ENVELOPE PROTEIN DOMAIN III	76
3.1 Cloning of Ed3 constructs	76
3.2 Expression and purification of rEd3 from DENV1-4 and WNV	77
3.2.1 Recombinant Ed3s were stable when expressed in pET 16b	80
3.3 Inhibition of DENV1-4 with homologous rEd3 from DENV1-4 and WNV	83
3.4 Discussion	86
4: FLAVIVIRUS FUSION INHIBITION BY PEPTIDES COMPETING WITH THE STEM ANCHOR REGION	89
4.1 Synthetic peptide purification and mass profile	89
4.2 Cytotoxicity and entry inhibition by synthetic peptides	91
4.3 Discussion	92

5: FLAVIVIRUS NEUTRALIZATION BY SPECIFIC MOUSE MONOCLONAL ANTIBODY AND ITS FRAGMENTS AGAINST THE ENVELOPE PROTEIN DOMAIN III	96
5.1 Purification profile of mAb 9F12	96
5.2 Dengue virus purification and quantification	97
5.3. Plaque Reduction neutralization Test (PRNT ₅₀).....	99
5.4. Mechanism of neutralization by mAb 9F12	101
5.5 Sequencing of mAb 9F12 and construction of recombinant scFv	102
5.6 Binding of mAb 9F12 to free rEd3 or on virus surface	106
5.7 Epitope mapping by synthetic peptides as mimotopes	107
5.8 Discussion	110
Annexe 1: Binding affinity of mAb 9F12 and scFv to rEd3s by SPR.....	117
6: SUMMARY AND PERSPECTIVES	120
6.1 Future Perspectives	121
REFERENCES.....	122

LIST OF TABLES

Table 1.1 Classification of viruses based on nucleic acid	4
Table 2.1 Details of primers for Ed3 constructs from DENV1-4 & WNV	46
Table 2.2 Details of rEd3 expression conditions	47
Table 2.3 Summary of biochemical characteristics of rEd3 proteins	52
Table 2.4 Details of cell cultures and hybridomas used in the study	58
Table 2.5 Details of peptides for fusion inhibition assay and epitope mapping	72
Table 4.1 List of synthetic peptides synthesized and purified in the study	89
Table 4.2 Inhibition of DENV2 NGC by peptides from the stem anchor region studied by Hrobowski et al., (2005)	93
Table 5.1 Nucleotide and conceptually translated amino acid sequences of the V _H and V _L domains of mAb 9F12	103
Table 5.2 Kinetic constants and binding affinities of mAb 9F12 and scFv9F12 to rEd3 from DENV1-4 serotypes and WNV by SPR	119

LIST OF FIGURES

Figure.1.1 The family <i>Flaviviridae</i>	5
Figure. 1.2 Flavivirus classification	7
Figure.1.3 Feeding and transmission cycle of mosquito-borne flaviviruses	7
Figure.1.4 Geographical distribution of dengue and dengue haemorrhagic fevers.....	9
Figure.1.5 <i>Culex</i> species of mosquito and Approximate world distribution of WNV and Kunjin virus, a subtype of WN virus	11
Figure.1.6 Phylogenetic tree of WNV based on sequence of the E protein	12
Figure.1.7 The flavivirus life cycle.....	15
Figure.1.8 Schematic diagram of the flavivirus polyprotein (DENV)	16
Figure.1.9 Membrane topology of the flavivirus structural proteins	19
Figure.1.10 Comparison of class II fusion proteins from alpha and flaviviruses.....	21
Figure 1.11 The Dengue virus E protein.....	23
Figure.1.12 Organization of E protein dimers in the recombinant subviral particles of TBE virus.....	24
Figure.1.13 Folding diagram of TBE Domain III	26
Figure.1.14 Electrostatic exposed surface of Ed3.....	27
Figure.1.15 Structure of flaviviruses	30
Figure.1.16 The dengue virus structure	31
Figure.1.17 Pseudoatomic model of Dengue virus.....	32
Figure.1.18 Domain rearrangements in the Dengue sE monomer during fusion.....	33
Figure.1.19 The Dengue sE trimer.....	34
Figure.1.20 Schematic representation of fusion by class II fusion protein.....	35

Figure.1.21 Secondary structure alignment of the flavivirus fusion proteins.....	36
Figure.1.22 A model of the flavivirus maturation pathway.....	39
Figure.1.23 Antigenic complexity of the Flavivirus E protein.....	42
Figure 2.1 Vector maps of pET 16b constructs of recombinant flavivirus rEd3.....	48
Figure 2.2 Secondary structure alignment of ED3 from DENV1-4 and WNV.....	53
Figure. 2.3 Construct map of the recombinant scFv in the vector pEt 24a(+)......	67
Figure 2.4 Scheme for Solid phase peptide synthesis using Fmoc chemistry	69
Figure 3.1 Cloning the constructs exemplified with WNV Ed3.....	76
Figure 3.2 Induction profile of recombinant Ed3	78
Figure 3.3 Purification profiles of DENV1-4 Ed3 in pET 32b (+) vector.....	79
Figure 3.4 Affinity purification and refolding profile of rEd3	81
Figure 3.5 Chromatogram of low molecular weight calibration markers.....	81
Figure 3.6 Recombinant Ed3 from DENV1-4 and WNV in pET 16b vector.....	83
Figure 3.7 Competitive inhibition of DENV 1-4 entry with homologous rEd3	84
Figure 4.1 Purification of peptides by HPLC	90
Figure 4.2 Cytotoxicity and virus inhibition by synthetic peptides).....	91
Figure 5.1 Purification of mAb 9F12	96
Figure 5.2 DENV purification and quantitation by plaque assay	98
Figure 5.3 Plaque reduction neutralization test (PRNT _{50%}).....	100
Figure 5.4 Mechanism of neutralization by mAb 9F12.....	101
Figure 5.5 Amplification of heavy and light chain sequences of mAb 9F12	102
Figure 5.6 Sequence and construct details of recombinant scFv	105
Figure 5.7 Expression profile of scFv.....	105

Figure 5.8 Binding of mAb 9F12 to Ed3 by ELISA and immunofluorescence 107

Figure 5.9 Epitope mapping of mAb 9F12 109

Figure 5.10 Demonstration of antibodies to DENV2ED3 in infected mice 112

Figure 5.11 Mapping of epitopes of mAb 9F12 and 3H5 on DENV E protein..... 113

Figure 5.12 Mapping of mAb 9F12 epitopes on whole virus structure of DENV .. 114

Figure 5.13 Sensograms from the interaction of rEd3 with mAb 9F12 and scFv ... 118

ABBREVIATIONS

3D model	Three dimensional model
aa	Amino acid
ADE	Antibody dependent enhancement (of infection)
ATCC	American type culture collection
BHK 21	Baby hamster kidney tissue fibroblast cell line, clone 21
BOP	Benzo-triazolyl-oxytris(dimethylamino)phosphonium hexafluorophosphate
bp	basepair
BSA	Bovine serum albumin
BSL-2	Biosafety level-2
C	Nucleocapsid protein
CD	Circular Dichroism
CDR	Complementarity determining region (of antibody)
CPE	Cytopathic effect
Cryo EM	Cryo electron microscopy
C terminus	The carboxy terminus of an amino acid
DCM	Dichloromethane
DC-SIGN	Dendritic cell ICAM3-grabbing non-integrin
DENV	Dengue virus
DENV (1-4)	Dengue virus serotypes 1-4
DF	Dengue fever
DHF	Dengue haemorrhagic fever
DIEA	Diisopropylethylamine
DMF	N,N'-Dimethyl formamide
DNA	Deoxyribonucleic acid
DNAase	Deoxyribonuclease
DSS	Dengue shock syndrome
DTT	Dithiothreitol
E	Envelope Glycoprotein
Ed3	Envelope protein domain III
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme Linked Immuno Sorbent Assay
ER	Endoplasmic reticulum
Fc	Fragment crystallizable (part of immunoglobulin)

FCS	Fœtal Calf Serum
FDA	Food and Drug Administration (of USA)
FITC	Fluorescent isothiocyanate
Fmoc	9-Fluorenylmethoxycarbonyl
FRW	Framework (1-3 of an antibody molecule)
GAG	Glycosaminoglycan
(Gly ₄ S) ₃	Linker sequence of amino acids Glycine and Serine
HA	Haemagglutinin
HBSS	Hank's balanced salt solution
HCV	Hepatitis C virus (a flavivirus)
HeLa	Human cervical carcinoma cell line
HIV	Human immunodeficiency virus
HR	Heptad repeats (1&2)
HRP	Horse radish peroxidase
HSHS	Highly sulfated heparan sulfate
IBV	Infectious bronchitis virus
ICAM	Inter cellular adhesion molecule
iFCS	Inactivated fœtal calf serum
IgC	Immunoglobulin constant domain
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
JEV	Japanese encephalitis virus
Kb	Kilobase
KDa	Kilodalton
LBA	Luria Bertani agar
LB broth	Luria Bertani broth
M	Membrane or Matrix protein
mAb	monoclonal antibody
MBHA	4-methylbenzhydramine
mM	Millimolar (10 ⁻³ M) concentration (of a solute)
μM	Micromolar (10 ⁻⁶ M) concentration (of a solute)
MVE	Murray valley encephalitis
MWCO	Molecular weight cutoff
NCR	Non coding region
nM	Nanomolar (10 ⁻⁹ M) concentration (of a solute)
NMR	Nuclear magnetic resonance
NP 40	Nonidet P40 – nonionic detergent
NS	Nonstructural protein
N terminus	Amino terminal end of an amino acid

NUS	National University of Singapore
OD ₆₀₀	Optical density at 600nm wavelength
ORF	Open reading frame
PCR	Polymerase chain reaction
PDB	Protein data bank
PFU	Plaque forming unit
PMSF	Phenyl methyl sulphonyl fluoride
prM	Precursor M protein
PRNT 50	Plaque reduction neutralization test (50%)
rEd3	Recombinant envelope protein domain III
RGD motif	Arginine-Glycine-Aspartic Acid
RNA	Ribonucleic acid
rpm	Rotations per minute
RSP	Recombinant subviral particle(s)
SARS CoV	Severe acute respiratory syndrome coronavirus
scFv	Single chain fragment variable
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
sE	Soluble ectodomain (of E protein)
SFV	Semliki Forest virus
SPPS	Solid phase peptide synthesis
SPR	Surface plasmon resonance
TBE	Tick Borne encephalitis
TGN	Trans-Golgi network
TLR	Toll-like receptor
TM	Trans-membrane domain
USUV	Usutu virus
UTR	Untranslated region
Vero	African green monkey kidney tissue continuous cell line
V _H	Variable heavy chain of the immunoglobulin molecule
V _L	Variable light chain of the immunoglobulin molecule
WNV	West Nile virus
WNV sf	West Nile virus (Sarafend strain)
wt WNV	Wild type West Nile virus
WN Ed3	West Nile virus E protein domain III
YF	Yellow fever

PUBLICATIONS RELATED TO THIS WORK

1. Chu, J. J. H*, **Rajamanonmani, R***, Li, J., Bhuvanakantham, R., Lescar, J., and Ng, M. L. (2005). Inhibition of West Nile virus entry by using a recombinant domain III from the envelope glycoprotein. J Gen Virol 86, 405-412. PubMed ID: 15659760. (Received 44 citations recorded by SCOPUS and two citations in a text book and a WHO report).

* Equal contributory authors

2. **Rajamanonmani, R.**, Nkenfou, C., Clancy, P., Yau, Y. H., Shochat, S. G., Sukupolvi-Petty, S., Schul, W., Diamond, M. S., Vasudevan, S. G., and Lescar, J. (2009). On a mouse monoclonal antibody that neutralizes all four dengue virus serotypes. J Gen Virol 90, 799-809. PubMed ID: 19264660.

PUBLICATIONS NOT RELATED TO THIS WORK:

1. Xiaohong Gao, Kim Pin Yeo, Siqi Sharon Aw, Claudia Kuss, Jayasree K. Iyer, Saraswathy Genesan, **Ravikumar Rajamanonmani**, Julien Lescar, Zbynek Bozdech, Peter R. Preiser (2008). Antibodies Targeting the PfRH1 Binding Domain Inhibit Invasion of *Plasmodium falciparum* Merozoites. PLoS Pathog 4(7): e1000104. doi:10.1371/journal.ppat.1000104. PubMed ID: 18617995.

GENBANK ENTRIES

1. The sequences elucidated in this study are deposited in the Genbank with the following accession numbers. Mus musculus mAb 9F12 immunoglobulin gamma 1 heavy chain mRNA, partial cds. ACCESSION FJ493472
2. Mus musculus mAb 9F12 immunoglobulin gamma 1 kappa light chain mRNA, partial cds. ACCESSION FJ493471.

ABSTRACT

Dengue virus serotypes 1-4 (DENV1-4) cause serious febrile illness in humans, including Dengue haemorrhagic fever (DHF) and Dengue shock syndrome (DSS). Presence of avid neutralizing antibodies during clinical illness is critical in the outcome of clinical disease and severity of manifestations. Presently there are no specific anti-flaviviral therapeutic agents or an effective vaccine available for clinical use. In the current study, the possibilities of using the DENV envelope protein domain III (Ed3), as it possesses receptor binding activity, as a possible competitor to whole virus for entry into host cell receptor was explored. As the Carboxy terminal stem anchor region contributes the maximum to post trimeric conformational rearrangement of the envelope protein leading to fusion of the virus membrane to the host, a key event in successful infection, the binding pocket of two of the helices were targeted with specific peptides but with limited success. Following the successful prevention (>90%) of West Nile virus (WNV) infection and to a lesser extent of DENV infection with a single polyclonal serum from mice raised against the envelope protein domain III of WNV, another flavivirus, a mouse monoclonal antibody (mAb) 9F12 was found to be neutralizing all four serotypes of DENV at relatively low concentrations of the mAb. The antibody bound to soluble recombinant Ed3 from DENV1-4 and also to the whole virus. These findings led to the extensive characterization of the antibody for the binding kinetics, possible epitopes along with the nucleotide sequence and generation of a recombinant single chain fragment variable (scFv) engineered to avoid the murine components but to retain the immunologic characteristics of the antibody in this study.

INTRODUCTION

1. INTRODUCTION

1.1 Viruses

Viruses are ultramicroscopic filterable mobile genetic elements requiring host cell machinery for its replication. Evolutionary origin of viruses is one of the much speculated areas in life sciences since there is no evidence from past as fossils for larger animals. They are too small and probably too fragile to have withstood the processes that led to fossilization, or even to preservation of short stretches of nucleic acid sequences in leaf tissues or insects in amber. The only acceptable hypothesis so far is the co-evolutionary origin of viruses along with the hosts with occasional crossovers due to challenging survival. A wide variety of animal viruses were found out during the year 1900-1930 and were characterized according to their size, physico-chemical properties, pathogenic effects and mode of transmission. Depending upon the host cell being infected, these viruses are bacteriophages (infecting bacteria), animal or plant viruses.

Viruses, as obligatory intracellular parasites having either DNA or RNA as their genome, usually carry the message for the structural proteins and the nonstructural proteins required for the multiplication and assembly of the virus. In 1956, Watson and Crick proposed the theory of 'identical subunits' according to which the viral genome is covered by a shell made up of multiple copies of a single protein so that genetic economy is maintained in the virus. The second part of their proposal concerned the way in which the subunits must be packed in the protein shell or capsid. On general grounds, it was expected that subunits would be packed so as to provide each with an identical environment. This is possible only if they are packed symmetrically. Through the various technical advances in ultra-structural evaluation, it is evident that the virus architectural symmetry falls into two major categories *viz.* the helical and icosahedral symmetry.

Linear viral capsids have RNA genomes that are encased in a helix of identical protein subunits exemplified by Tobacco mosaic virus. The length of the helical viral nucleocapsid is determined by the length of the nucleic acid. Icosahedral symmetry allows the nucleic acid packaged in a more ordered manner that each protein subunit is placed in an almost identical environment which formed the basis for the theory of 'quasi-equivalence' proposed by Caspar and Klug in the year 1962. The icosahedron itself has 20 equilateral triangular facets with three fold symmetry in each facet and therefore requires $60T$ structure units where T is the Triangulation number. A virion, a complete virus particle, with all the components of the envelope and capsid is capable of injecting and getting the viral genome inside the host target cell.

1.1.1. Taxonomy and Classification of viruses

The International Committee on Taxonomy of Viruses (ICTV) recognizes about 1,550 virus species but some 30,000 virus strains and isolates are being tracked by virologists in different fields of biology. The ICTV is the "international court" of experts that rules on names and relationships of all viruses, but only to the level of species. There are currently 23 families of viruses that infect humans now available in ICTV database. The primary criteria used to differentiate viruses are:

- relatedness of genome sequence
- natural host range / cell and tissue tropism
- Pathogenicity and cytopathology
- Mode of transmission
- Physicochemical properties of virions
- Antigenic properties of viral proteins

The most commonly used classification of viruses proposed by David Baltimore is based on the type of nucleic acid the virus contains and its mode of replication that divided the viruses into seven classes (**Table 1.1**). The most recent classification cited includes the two important human pathogens viz. the Coronaviruses (under *Nidovirales*) and the Marburg and Ebola viruses (under *Mononegavirales*) (Büchen-Osmond, 2007).

Table. 1.1. Classification of viruses based on nucleic acid.

Class	Nucleic Acid	Examples	Envelope	Genome size (kb)
I	dsDNA	Herpes virus	Yes	120-220
		Poxvirus	Yes	130-375
		Adenovirus	No	3.0-4.2
		Papillomavirus	No	5.3-8.0
II	ssDNA	Adeno-associated virus	No	5
III	dsRNA	Reovirus	No	18-31
IV	(+)ssRNA	Togavirus	Yes	9.7-11.8
		Poliovirus	No	7.4
		Foot-and-mouth disease virus	No	7.5
		Hepatitis A virus	No	7.5
		HepatitisC virus	Yes	10.5
V	(-)ssRNA	Flavivirus	Yes	11
		influenza virus	Yes	15-Dec
VI	(Reverse) RNA	HIV	Yes	9.7
VII	(Reverse) DNA	Hepatitis B Virus	Yes	3.1

Table 1.1. Classification of viruses based on nucleic acid. Nucleic acid type ss = single strand; ds = double strand. (+) RNA acts as mRNA for the synthesis of proteins. (-) RNA cannot function as mRNA. Reovirus and influenza virus have segmented RNA genomes; the total length is shown here. The most recent classification cited includes the two important human pathogens viz. the Coronaviruses (under *Nidovirales*) and the Marburg and Ebola viruses (under *Mononegavirales*) (Büchen-Osmond, 2007).

1.2 Flaviviridae

Flaviviridae is a large family of related positive strand RNA viruses consisting of three genera: the flaviviruses, the pestiviruses and the hepaciviruses. An additional group of GB agents are awaiting a formal classification within the family. Members of this family exhibit diverse biological properties, as exemplified with the analysis on the helicase sequences in figure (Fig. 1.1A), and lack serologic cross-reactivity, although they share similarities in virion morphology, genome organization and presumed RNA replication strategy (Simons *et al.*, 1995).

Figure. 1.1. (A) The family *Flaviviridae*. Phylogenetic tree based on analysis of NS3 helicase regions. Shown are members of the flavivirus genus: yellow fever virus (YFV), dengue-1 (DENV1), dengue-2 (DENV2), West Nile virus (WNV) and Japanese encephalitis virus (JEV); the pestivirus genus: bovine viral diarrhea virus (BVDV) and classical swine fever virus (CSFV); several hepacivirus (HCV) isolates, including GBV-B and the unclassified viruses GBV-A and GBV-C (Knipe & Howley, 2007). **(B).** Schematic diagram of the genomic RNA. It is capped at the 5' end but lacks a poly-(A) tail at its 3'-end. It contains untranslated regions (UTR) 5' and 3' to the open reading frame (ORF). The 5'UTR is about 100 nucleotides in length and the 3' UTR is 400 to nearly 800 nucleotides in length. The structural proteins are encoded at the 5' one third of the genome followed by the non-structural proteins. More detailed review on the function of these UTR's are provided in the reference (Markoff, 2003). The genome organization in a single ORF and its expression into a single polyprotein are the common features that bring the three genera under the family *Flaviviridae*.

The increasing significance of *Flaviviridae* as human and animal pathogens emphasizes that their study remains no less pertinent than earlier. The organization of the genome RNA is similar for all genera in the family. The RNA carries a single open reading frame (ORF) encoding a polyprotein (**Fig. 1.1B**) that is translated in a cap-dependent manner at the rough endoplasmic reticulum (rER).

1.2.1. Flaviviruses and flavivirus infections

The flavivirus genus consists of nearly 80 viruses, many of which are arthropod-borne human pathogens. The flaviviruses are widely distributed throughout the world, although a specific flavivirus may be geographically restricted to a continent or a particular part. Representing a relatively large and mixed group of viruses, flaviviruses include both those that have remained almost untouched by human influence and those whose epidemiology and evolution have been significantly influenced by humans. This human influence will continue and it is therefore likely that flavivirus, as evidenced with DENV, WNV and Usutu virus (USUV), will assume larger importance in disease emergence in the future.

Phylogenetic and epidemiological analyses suggest that the more virulent genotypes are now displacing those that have lower epidemiological impact (Gould *et al.*, 2003). The salient features of flavivirus taxonomy are illustrated (**Fig. 1.2**).

Flaviviruses can be transmitted biologically by haematophagous arthropods, mainly the ticks and mosquitoes, or directly from vertebrate host to vertebrate host. By far the mosquitoes are the most important vectors of human and domestic animal infections. The transmission cycle is represented below (**Fig. 1.3**).

Figure. 1.2. Flavivirus classification. The relationships between selected flaviviruses are shown in the left of the dendrogram. The serological (serocomplex) and phylogenetical (clade and cluster) classifications of these flaviviruses are shown on the right side of the Figure (Lindenbach, 2001).

Figure. 1.3. Feeding and transmission cycle of mosquito-borne flaviviruses. Viremia usually occurs at levels that can infect mosquitoes for 2 to 6 days. After a blood meal it takes 8 to 14 days for the virus to reach the salivary glands to be ready to become infective. This is the extrinsic incubation period. The incubation period relates to the time taken from infection to onset of symptom in the vertebrate host (Centres for Disease Control and Prevention, Atlanta, USA).

Flaviviruses cause a variety of diseases including fever, encephalitis and haemorrhagic fever. Flavivirus infections of regional endemic concern include Kyasanur Forest disease (KFD), Murray Valley encephalitis (MVE), St. Louis encephalitis (SLE), tick-borne encephalitis (TBE) and West Nile (WN) encephalitis. Entities of major global

concern include dengue fever with its associated dengue haemorrhagic fever (DHF) and shock syndrome (DSS), Japanese encephalitis and yellow fever. Decreases in mosquito control efforts during the later part of 20th century, coupled with societal factors such as increased transportation and dense urbanization, have contributed to the re-emergence of flaviviruses such as DENV in South and Central America. Neurotropic flaviviruses can produce severe destructive central nervous system disease with serious sequelae. In 1999, WNV was isolated for the first time in the Western hemisphere during an outbreak in the New York city that was responsible for several human cases of encephalitis including four deaths (Mostashari *et al.*, 2001) . The West Nile virus found in U.S. has become established in Canada, parts of Central and South America and the Caribbean, and it is continuing to spread (Kramer *et al.*, 2008).

1.2.2. Dengue fever and dengue virus classification

Dengue fever is caused by Dengue virus transmitted by *Aedes* mosquitoes to humans. The World Health Organization estimates that there may be 50-100 million cases of dengue virus infections worldwide every year, which result in 250,000 to 500,000 cases of DHF and 24,000 deaths each year (WHO, 2000). The growing urbanization of populations combined with the failure to control the mosquito vector has resulted in the spread of the disease. Today, about two and a half billion people live in areas, where viral transmission of one of the four serotypes of DENV occurs, therefore, making dengue an increasingly important public health concern (Gubler, 1998). Dengue is the most common cause of arboviral disease and also it is a relatively common cause of fever in travellers to the tropics (Fig. 1.4), but severe disease is rare (Gibbons & Vaughn, 2002).

Figure. 1.4. Geographical distribution of dengue (light shading) and dengue fever plus dengue haemorrhagic fever (dark shading) (Gibbons and Vaughn, 2002).

There are four dengue virus serotypes, called DENV-1, DENV-2, DENV-3, and DENV-4 under the genus *flavivirus* and the family *Flaviviridae*. Dengue viruses have a single stranded RNA genome of positive polarity which is about 11,000 nucleotides long (**Fig. 1.1B**). They have 60-80% amino acid sequence homology between each other. Presence of common group epitopes on the envelope protein results in extensive cross reactivity in serological tests. Infection with one dengue serotype provides lifelong immunity to that virus, but there is no cross-protective immunity to the other serotypes. Thus, persons living in an area of endemic dengue can be infected with three, and probably four, dengue serotypes during their lifetime (Gubler, 1998).

1.2.3. Pathogenesis of dengue virus manifestations

Dengue virus infection in humans causes a spectrum of illness ranging from unapparent or mild febrile illness to severe and fatal haemorrhagic disease. Infection with any of the four serotypes causes a similar clinical presentation that may vary in severity, depending on a number of risk factors. The incubation period varies from 3 to 14 days

(average, 4 to 7 days). Important risk factors influencing the proportion of patients who have severe disease during epidemic transmission include the strain and serotype of the infecting virus and the immune status, age, and genetic background of the human host (Gubler, 1998).

Classic dengue fever is primarily a disease of older children and adults. Haemorrhagic manifestations are not uncommon in Dengue fever patients but generally self limiting and rarely fatal. The characteristic manifestations of DHF are plasma leakage, thrombocytopenia and haemoconcentration. A more severe and fatal form of the disease is DSS. Rashes are seen all over the body and on the face accompanied by prolonged shock with a 40% mortality rate (Gibbons & Vaughn, 2002).

The pathogenesis of DHF and DSS is still controversial. The most accepted hypothesis, known as the secondary infection or antibody-dependent enhancement (ADE) implies that patients experiencing a second infection with a heterologous DENV serotype have a significantly higher risk for developing DHF and DSS. Pre-existing heterologous dengue antibody recognizes the infecting virus and forms an antigen-antibody complex, which is then bound to and internalized by immunoglobulin Fc receptors on the cell membrane of leukocytes, especially macrophages. Because the antibody is heterologous, however, the virus is not neutralized and is free to replicate once inside the macrophage and thus, enhances the infection and replication of DENV in cells of the mononuclear cell lineage (Brandt *et al.*, 1982).

1.2.4. West Nile virus infection and West Nile virus classification

Based on early observations, human WNV disease seemed to fall in two categories: a mild, self limited, febrile disease called West Nile fever seen mostly in

children and young adults and rare cases of West Nile meningitis or encephalitis seen mainly in older patients (Smith & Edward, 2006).

West Nile virus was first isolated from the blood of a febrile patient in the West Nile district of Northern Uganda in 1937 and was subsequently found in other African and Asian countries. Enzootic, and ultimately, epidemic transmission of WNV depend on a bird-mosquito-bird pattern of transmission.

Figure. 1.5. (A) *Culex* species of mosquito. (B) Approximate worldwide distribution (shown in cyan and pink) of WNV along with HCV and DENV respectively (Campbell *et al.*, 2002; (Watowich, 2009).

After early recognition that mosquitoes are the vectors for WNV, ecologic studies of WNV in Egypt revealed the enzootic cycle involving mosquito vectors and bird amplifying hosts, and the occurrence of dead-end-infections in humans and horses. Mosquito vectors for WNV include largely ornithophilic species in the genus *Culex* (Fig. 1.5A) as well as largely mammalophilic species in the genus *Aedes*. WNV, *sensu lato*, is indigenous to Africa, Asia, Europe and Australia and was recently introduced to North America (Fig. 1.5B).

Cities with relatively poor economic and infrastructural conditions, and those that lack effective arbovirus surveillance systems and vector mosquito control programmes, are particularly vulnerable (Campbell *et al.*, 2002).

West Nile virus is grouped in the Japanese encephalitis (JE) antigenic complex of viruses that includes major human pathogens like JEV, MVEV and SLEV (Smith & Edward, 2006). Phylogenetic analysis of worldwide isolates, based on their E glycoprotein, reveal two distinct lineages of WNV (**Fig. 1.6**).

Figure. 1.6. Phylogenetic tree of WNV based on sequence of the E protein. Two distinct lineages are apparent. All WNVs isolated from recent epidemics are closely related with 97% or greater homology and belong to a single clade.

Those in lineage 1 have a worldwide distribution, ranging from West Africa to the Middle East, eastern Europe, North America, and Australia (Kunjin virus); whereas lineage 2 consists of enzootic strains from Africa. The 11kb genome of WNV consists of a single ORF with the structural and nonstructural proteins responsible for replication and assembly (Campbell *et al.*, 2002).

1.2.5. Pathogenesis of West Nile virus encephalitis

Many species of vertebrates can be infected by WNV-carrying mosquitoes. The commonly reported estimates (1 in 5 infected patients develops fever and 1 in 150 infected patients develops severe neurological disease) come from serologic surveillance data from the New York epidemic (Mostashari *et al.*, 2001). Of the cases reported in 2007, approximately 64% were reported as West Nile fever (milder disease) and 36% were reported as West Nile meningitis or encephalitis. The human incubation period of WNV is 2 to 14 days followed by fever and headaches. West Nile virus can be recovered from the blood only during the first few days of illness. A detailed review on the interactions between WNV pathogenesis and the innate and adaptive host immune responses is provided in the references (Gea-Banacloche *et al.*, 2004; Samuel & Diamond, 2006; Smith & Edward, 2006).

1.2.6 Flavivirus life cycle

The molecular biology of flaviviruses is best understood in the context of the viral life cycle (Lindenbach & Rice, 2003). Flaviviruses replicate in a variety of cells of insect, mammal, or avian origin. Cell receptors specific for flaviviruses have not been identified, but it is believed that virus binding to the cell may be promoted through the initial interaction of E protein with highly sulfated heparan sulfate (HS) residues present on the surface of a wide variety of cells. Since flaviviruses are transmitted between insect and vertebrate hosts during their natural transmission cycle, it is likely that the cell receptor(s) they utilize is a highly conserved protein. Recently, it has been suggested that a short segment of E protein (amino acids 306-314) present in a hydrophilic sequence in domain III of dengue E protein may be critical for virus infectivity (Thullier *et al.*, 2001).

This sequence and amino acid 306 of JEV were shown to be associated with protein binding to cell surface glycosaminoglycans. Entry of WN/KUN viruses into host cells is believed to occur via receptor-mediated endocytosis in coated pit vesicles (Chu & Ng, 2004b). Flavivirus replication occurs in the perinuclear region of the endoplasmic reticulum (ER) of cells that become vacuolated and hypertrophic (Deubel *et al.*, 2001).

1.2.7. Flavivirus replication

The intracellular life cycles of the flaviviruses are very similar. Infection with one of the arthropod-borne flaviviruses begins when the vector takes a blood meal and the virus is introduced into the host.

Viral proteins are translated using the input template RNA resulting in a polyprotein, which later gets cleaved into individual structural and nonstructural proteins. Progeny virions are assembled from structural proteins and viral RNA and are exported through the Golgi compartment and secreted. A schematic representation of flavivirus replication is exemplified by Dengue virus (Fig. 1.7).

1.3. Flavivirus proteins

The positive sense genomic RNA, infective by itself without the rest of the viral proteins, once released from the flavivirus nucleocapsid into the cytoplasm of the host cell, is translated into a single polyprotein (Fig. 1.8). The viral serine protease NS2B-NS3, and several cell proteases then cleave the polyprotein at multiple sites to generate the mature viral proteins. The viral RNA-dependent RNA polymerase (RdRp), NS5, in conjunction with other nonstructural proteins and possibly cell proteins, copies complementary minus strands from the genomic RNA template and these (-) RNA strands in turn serve as templates for the synthesis of new genomic RNAs (Brinton, 2002).

Both the structural and functional aspects of flavivirus proteins are so extensively studied that a promising therapeutic intervention is not too far.

Figure. 1.7. The flavivirus life cycle. (A) Virions bind to cell-surface attachment molecules and receptors and are internalized through endocytosis. (B) In the low pH of the endosome, viral glycoproteins mediate fusion of viral and cellular membranes, allowing disassembly of the virion and release of RNA into the cytoplasm. (C) Viral RNA is translated into a polyprotein that is processed by viral and host cellular proteases. (D) Viral non-structural proteins replicate the genomic RNA. (E) Virus assembly occurs at the ER membrane, where capsid protein and viral RNA are enveloped by the ER membrane and prM/E glycoproteins to form the immature virus particles. (F) Immature virus particles are transported through the secretory pathway. In the low pH of the trans-Golgi network (TGN), furin-mediated cleavage of prM drives maturation of the virus. (G) Mature virus is released into the extracellular environment. Numbers shown in colored boxes refer to the pH of the respective compartments (Perera *et al.*, 2008).

1OKE, 2VBC, 2PID, 2J7U, respectively) (Egloff *et al.*, 2002; Li *et al.*, 2008; Luo *et al.*, 2008; Ma *et al.*, 2004; Modis *et al.*, 2003; Yap *et al.*, 2007). Structures are currently not available for the proteins denoted in red.

1.3.1. Nonstructural proteins

The N-terminal one fourth of the polyprotein encodes structural proteins (C-prM-E) (**Fig. 1.8**), followed by nonstructural proteins (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5). The nonstructural proteins are involved widely in the early stage of virus life cycle. Mutations in NS1 have been shown to affect the initiation of minus-strand synthesis. The NS2A is a small hydrophobic protein that directs the replication complex to the membrane organelles. NS3 possesses RNA helicase, nucleotide triphosphatase and NS2B mediated protease activity. NS4A associates itself with NS1 during replication (Rice *et al.*, 1985). The highly hydrophobic NS4B is a membrane protein that associates with NS3 and viral RNA suggesting a possible role in viral replication complex. Also it is shown to be an antagonist of the interferon-induced antiviral response (Miller *et al.*, 2006). NS5 functions as an RNA-dependent RNA polymerase along with its methyl transferase activity which is required for the capping of the progeny viral genomes.

Among the seven nonstructural proteins, flavivirus NS1 protein can elicit high-titered antibody in flavivirus-infected or immunized animals. Though the antibodies are found to be non-neutralizing, animals immunized with NS1 or passively transferred with anti-NS1 antibodies are protected against subsequent virus challenge (Gould *et al.*, 1986; Schlesinger *et al.*, 1986; Schlesinger *et al.*, 1987).

1.3.2. Structural proteins

According to the best structural model, virions contain 180 copies of the E protein, an unknown amount of prM and a single copy of the viral genome. The cryo-EM visualization of mature dengue virus has revealed 180 copies of M protein in the lipid envelope. Thus, translation of the viral genome must occur at least 180 times for every nascent genome that is produced and packaged. Also, a single input genome can give rise to multiple daughter genomes. Thus, utilization of the flavivirus genome as a template for translation is probably more efficient than its use as a template for replication (Lindenbach & Rice, 2003; Zhang *et al.*, 2003a).

1.3.3. Capsid (C), pre (pr) and Membrane (M) proteins

Capsid is a highly basic protein of 11kDa. Charged residues, probably mediating the RNA interaction, are clustered at the N and C termini, separated by a short internal hydrophobic domain that mediates membrane association. The structure of C protein from DENV and Kunjin virus, subtype of WNV, showed common features in that both form a dimer in solution and contain four α helices which are organized into tetramers with highly positively charged surfaces (Dokland *et al.*, 2004; Kuhn *et al.*, 2002; Ma *et al.*, 2004; Zhang *et al.*, 2003a); (Zhang, 2003b).

Less is known about the C protein as an antigen or the protective nature of the antibody response. A recombinant fusion protein of E domain III-capsid chimeric protein, produced in *E. coli* which is highly aggregated by the addition of oligonucleotides, induced a functional humoral response and cell mediated immunity against DENV2 and conferred a significant protection in mice (Valdes *et al.*, 2009).

Figure. 1.9. Membrane topology of the flavivirus structural proteins. The flavivirus structural proteins are translocated and anchored in the ER by various signal sequences and membrane anchor domains. The predicted orientation of the structural proteins across the endoplasmic reticulum (ER) membrane is shown. Transmembrane helices are indicated by cylinders, arrows indicate the sites of post-translational cleavage and the cleavage sites of specific enzymes are indicated by different colours. E, envelope; NS1, non-structural protein 1; prM, precursor to membrane protein (Mukhopadhyay *et al.*, 2005).

The 26 kDa prM, precursor protein of M is translocated into the ER lumen by the C-terminal hydrophobic domain of C protein (**Fig. 1.9**). On the luminal side of the ER, the prM and E proteins form a stable heterodimer within a few minutes of translation and prM is required for proper refolding of E protein (Amberg *et al.*, 1994; Lindenbach & Rice, 2003). Cleavage of prM into 'pr' and 'M' is the key process towards maturation of the virus, the sequence of which is well characterized with structural details (Li *et al.*, 2008) along with the conformational changes in the E protein (Lin & Wu, 2005); Vazquez and colleagues., (2002) have shown that a synthetic peptide mimicking the most exposed region of prM (aa3-31) with linear epitopes induced both neutralizing and protective antibodies in mice. More recently, recombinant subviral particles using native

prM and E proteins from all four DENV serotypes have been produced (Wang *et al.*, 2009).

1.4. Flavivirus Envelope Protein (E)

The E protein, a 53 kDa glycoprotein with 495 amino acid (Figs. 1.10 & 1.11A) residues and twelve conserved cysteine residues resulting in six disulfide bonds is the major envelope protein forming the icosahedral assembly on the viral surface (Fig. 1.11C). Significant progress has been made towards the understanding of the structure, organization and thereby the host viral interactions of flavivirions ever since the first X-ray crystal structure of the envelope glycoprotein of TBE virus was solved (Rey *et al.*, 1995) at a resolution of 2 Å. This structure of a soluble dimeric fragment, known as the ectodomain isolated by trypsin digestion of purified TBE, showed an elongated dimeric molecule with the fusion peptide and the transmembrane domain at opposite ends. The TBEV E protein demonstrated a new paradigm for a virus membrane protein, since its structure and biology strongly suggested that it is lying down on the virus surface rather than standing upright as in the “spike proteins” of viruses such as influenza (Kielian, 2002). Similar soluble ectodomains generated by proteolytic cleavage of an alphavirus family member Semliki Forest virus (SFV) E1 protein, when crystallized, revealed a remarkable similarity in their secondary and tertiary structures (Kielian, 2006; Lescar *et al.*, 2001). The paradox that the ectodomains of the two fusion proteins from alpha and flaviviruses lack detectable amino acid sequence conservation, therefore suggests that they have a common origin of evolution. More importantly, their structures predominantly contained β strand secondary structure in contrast to the well exemplified influenza virus haemagglutinin, a class I fusion protein, which refold during fusion to

form a six helix bundle containing a central α -helical coiled coil domain (Skehel & Wiley, 1998).

The striking similarity between the structures of the two fusion envelope proteins from alpha and flaviviruses is evident from **Fig. 1.10**. Both proteins appear to play an important role in virion assembly by providing lateral contacts that contribute to the icosahedral arrangement of the envelope. Thus, the alphavirus E1 and the flavivirus E fusion proteins were proposed as the inaugural members of the “class II fusion proteins” (Lescar *et al.*, 2001).

Figure. 1.10. Overall fold of E1 from SFV and comparison with TBEV E Protein. 3D diagram of the folded structure of SFV, an alphavirus E1 (top) and of TBEV, a flavivirus E (bottom) in which the three structural domains are displayed in different colors. Disulfide bridges are drawn in green. The conserved stretch of amino acids (83–100 in SFV-E1 and 98–111 in TBE-E), containing the putative fusion peptide, is colored orange. The amino and carboxyl termini are indicated, respectively, by N and C. A yellow triangle marks the glycosylation site (Asn141 in SFV-E1 and Asn154 in TBE-E). (Adapted from (Lescar *et al.*, 2001)).

Following the two pioneering structure elucidations, a series of flavivirus E proteins, either as soluble ectodomains or recombinantly expressed and purified proteins

from DENV2 or 3 (Modis *et al.*, 2003; 2004; Modis *et al.*, 2005), WNV (Kanai *et al.*, 2006; Nybakken *et al.*, 2006; Nybakken *et al.*, 2005) have been characterized either alone or in complex with a ligand or antibody fragment. The crystal structure of the soluble E dimer of DENV2 elucidated by Modis and colleagues, (2003), unveiled a hydrophobic pocket lined by residues that influence the pH threshold for fusion. The pocket, which accepts a hydrophobic ligand, opens and closes through a conformational shift in a β -hairpin at the interface between two domains. These features point to a structural pathway for the fusion-activating transition and suggest a strategy for finding small-molecule inhibitors of dengue and other flaviviruses.

The flavivirus E protein is typically an elongated structure of β sheets, forming head-to-tail homodimers that most likely lie parallel with the virus membrane (**Fig. 1.11B**). Each E protein subunit is composed of three domains (**Fig. 1.11 A & D**): centrally located domain I, which forms a β barrel and bears the N-glycosylation site; domain II, which projects along the virus surface between the transmembrane regions of the homodimer subunits and bears the fusion loop that targets the host membrane during a pH dependent fusion; and domain III, which maintains an immunoglobulin constant region-like fold, with receptor-binding property (Mandl *et al.*, 2000). The domain III is followed at the stem anchor region by two helices namely the H1 and H2, the C terminus of which runs through the membrane via the trans-membrane segments called the TM1 and TM2. A common feature of class II proteins is that low pH converts them from a homo-dimeric native state into a trimeric fusogenic complex.

Figure 1.11. The Dengue virus E protein. (A) The organization of the three domains of dengue sE. Domain I is red, domain II in yellow and domain III is blue. (B) The sE dimer. This is the conformation of E in the mature virion and in solution above the fusion pH. The three domains in both the units are marked as I, II, III and I', II' and III' (colour coded as in 'A'). The N and C termini are also marked. A putative receptor binding loop on domain III (residues 382-385) is marked with a triangle. The glycans on domain I and II are marked with ball and stick. (C) Packaging of E on the surface of the virus. EM image reconstructions show that 90 E dimers pack in an icosahedral lattice (Modis *et al.*, 2004). (D) Schematic drawing of domains I, II, and III together with the stem-anchor region of the flavivirus E protein. H1^{pred} and H2^{pred}, predicted α -helical regions; CS, conserved sequence element; TM1 and TM2, transmembrane segments (Adapted from (Allison *et al.*, 1999)).

In the case of flaviviruses, lateral envelope protein interactions appear to be sufficient to generate capsidless subviral particles that have an icosahedral arrangement (Fig. 1.12A & B). Infected cells also release a subviral particle containing the envelope proteins but lacking the nucleocapsid. These particles are smaller than virions and possess haem-agglutinating activity (Russell *et al.*, 1980).

Similar secreted particles derived from several different flaviviruses (Schalich *et al.*, 1996) have been produced in recombinant form by co-expression of DENV prM and E alone in cell culture or with the C protein, in the yeast *Pichia pastoris* (Tan *et al.*, 2007), demonstrating that these proteins are intrinsically capable of forming specific particulate structures in the absence of other viral components (Ferlenghi *et al.*, 2001).

The flavivirus E protein has a dual function: as a receptor binding protein, it is the primary determinant of host range, tissue tropism, virulence and is a major antigen in eliciting neutralizing antibodies during the immune response (Roehrig *et al.*, 2003).

Figure. 1.12. (A) Organization of E protein dimers in the recombinant subviral particles (RSP) of TBE virus as deduced from fitting the high-resolution sE structure into an image reconstruction of cryoEM densities. Reveals a T=1 structure accommodating 60 units of the soluble E protein dimers. (B) Detailed view of the site of lateral interactions near the five fold axis in the icosahedral lattice of E protein dimers in the TBE virus RSP. Image shows the contact between domain II of one subunit and domain III of an adjacent dimer (Ferlenghi *et al.*, 2001).

1.4.1. The Envelope protein domain III (Ed3)

Among the three domains of the E protein, the C-terminal domain III, with a predominant β -strand secondary structure as the other two domains, (Fig. 1.13A & B), can fold independently into a stable conformation allowing the determination of the solution structure of recombinant Ed3 from JE virus (Wu *et al.*, 2003), WNV (Volk *et al.*,

2004), TBE (Langat) (Mukherjee *et al.*, 2006), Omsk haemorrhagic fever (Volk *et al.*, 2006), DENV4 (Volk *et al.*, 2007) and the X-ray crystallographic structures for WNEd3 (Nybakken *et al.*, 2005) and DENV2Ed3 with an antibody fragment (Lok *et al.*, 2008). It is joined to the central domain through a connecting segment and anchored to the end of the segment by a disulfide bridge.

Although the flavivirus Ed3s share only 40-60% amino acid homology as a group, nine β strands are regularly found in these subunit proteins. Strands β 1 - β 4 are nearly identical in all the flavivirus structures, although β 3 was not observed in the NMR structures of JEV and WNV, both members of the JE serocomplex. Beta strands β 8 and β 9 (blue arrows above the sequence alignment marked 'F' and 'G' respectively in Fig. 1.21) are nearly consistent across all of the structures, although they are both slightly longer in both the WN NMR and X-ray crystal structure. In contrast, a large variability is observed in β strands β 5, β 6 and β 7. As a whole, this region of the protein is more variable and dynamic than the rest of the Ed3 subunit.

Despite the similarity of the flavivirus Ed3 secondary structures and overall fold, each virus displays a unique surface. The electrostatic surfaces of the DENV2, 3 and 4 Ed3 proteins as viewed from the centre of the virion five-fold pore, show dramatic surface differences among these viruses (Fig. 1.14).

Figure. 1.13. A. Folding diagram of TBE Domain III (on the left) from the first flavivirus E protein structure, showing the β sheets GFC (blue) and ABED (grey) forming an IgC-like module with a small additional β sheet ($A_xC_xD_x$ in blue) at one edge. There is no inter-sheet disulfide in this domain (Adapted from [Rey et al, 1995](#)). Ribbon representation of the WNEd3 (on the right) to show the designation of β strands. The side chains of several amino acids important for antigenic differences between WNV strains are also shown. **B.** Ribbon diagrams of several flavivirus Ed3 proteins illustrating their backbone structural differences near the β strand five. The $\beta 5a$ is always present and $\beta 5b$ is sometimes present, which are shown in cyan, while the same region is coloured orange when the β sheet is not present ([Volk et al, 2007](#)). It is to be noted that there are two different nomenclatures to represent the same secondary structures. For reasons of clarity both should be unified.

Figure. 1.14. Electrostatic exposed surface of Ed3. Blue indicates positive charge while red indicates negative charge. (A–C) Electrostatic surface of the DEN2, DEN3 and DEN4 viruses as viewed from within the 5-fold virion pore. The virion surface is located at the top of these images. (D–F) Electrostatic view of the DEN2, DEN3 and DEN4 Ed3 proteins as viewed from above the virion surface (Volk *et al*, 2007).

DENV2Ed3 has significantly more negative charges near the bottom compared to DENV3 and 4, while the DENV3Ed3 displays more negative charges near the upper surface of the protein. DENV4Ed3 distinctly has greater amount of uncharged surface area. The differences in electrostatic charge on the surfaces of Ed3s were predicted to be important determinants of differences in antigenicity and other biological properties of the viruses (Volk *et al.*, 2007). Also it is hypothesized that the dengue viruses originally existed in a sylvatic cycle involving mosquitoes and monkeys in the absence of human disease, but evolved into a human pathogen with a cycle involving the *Aedes* mosquitoes. The same study on analyzing the E sequences, identified 9 amino acid differences of

which 5 in the Ed3, (K340R, M342V, I335T, I364V and A382V) attributing to the emergence of DENV4 as human virus from a sylvatic cycle. Also, changes in cell receptor-binding properties of Ed3 have been implicated in the emergence of DENV as human virus. Whether there is a probable hint of emergence of newer subtypes or even serotypes by further host-viral interactions both by modified environmental and host factors indicate the need for urgent intervention and prophylactic measures.

Functionally, the Ed3 is directly involved in receptor binding (Huerta *et al.*, 2008; Hung *et al.*, 2004) by virtue of its structure resembling an IgC like fold and its position on the whole virus where the former projects above the convex surface allowing access to potential receptor molecules and neutralizing antibodies. Also, the Ed3 plays a major role in providing the necessary 'horse power' required for the fusion machinery which is evident *vide infra*.

1.5. Structure of flavivirus

The structure of Dengue virus, initially with a 24 Å (Kuhn *et al.*, 2002) and later with a resolution of 12 Å -14 Å (Zhang *et al.*, 2003a; Zhang, 2003b; Zhang *et al.*, 2004) had been determined by using a combination of cryoelectron microscopy (**Fig. 1.15A & B**) and fitting of the known structure of glycoprotein E into the electron density map (**Fig. 1.15C & D**). The three E monomers per icosahedral asymmetric unit do not have quasi equivalent symmetric environments.

A cross-sectional view (**Fig. 1.16A**) shows concentric layers of density indicating the multilayer organization of the virus. The outermost layer contains the highest density and corresponds to the E and M transmembrane proteins. The 35 - 40 Å thick lipid bilayer appears non-spherical with the transmembrane components of the M and E

proteins visible. The cryo-EM reconstruction of dengue virus shows a relatively smooth outer shell and the E protein dimers arranged in a “herringbone” pattern (**Fig. 1.15D & 1.16C**), made up of sets of three closely apposed dimers in a striking, almost parallel array.

Though the overall fold of the fusion proteins from both alpha and flaviviruses are similar, there are differences as to their core structures and the readiness of the latter to form subviral particles leading to issues in fusion, maturation and budding. Based on the similarity between WNV and DENV E proteins (49% identity), a homology model of the WNV E protein was constructed and fitted into the outer layer of the cryo-EM density using the program EM Fit. The general arrangement of WNV and the fitted E glycoprotein is similar to that of DENV; however, several features unique to each virus can be seen in the difference density map. Positive difference density corresponds to the carbohydrate moieties of the glycosylation sites at E residues 67 and 153 in DENV. Negative density corresponds to the carbohydrate moiety found at E residue 154 in WNV. There is a significant positional shift between the densities contributed by the glycosylation sites of dengue E 153 and WNVE 154. This is likely due to the difference in the glycosylation site or because of an addition of five amino acids around this site in WNV (Mukhopadhyay *et al.*, 2003).

The atomic structure of E protein from WNV was recently solved (Kanai *et al.*, 2006), where slight differences between the dengue and West Nile virus cryoEM structures in the E₀F₀ loop region (Mukhopadhyay *et al.*, 2003) are attributed to the additional bulk of the loop and its α -helix, rather than to the shift in the position of the glycan.

Figure. 1.15. Structure of flaviviruses. **(A)** A surface shaded view of the cryo-EM reconstruction of immature DENV-2 at a resolution of 12.5 Å showing the spiky surface features (Zhang *et al.*, 2004). **(B)** A surface shaded view of the cryo-EM reconstruction of mature DENV-2 at a resolution of 14 Å showing the relatively smooth surface features (Zhang *et al.*, 2003a). **(C)** Fit of the atomic coordinates of the E protein Ca residues into the immature virus (Li *et al.*, 2008) and **(D)** mature virus (Zhang *et al.*, 2003a), showing the arrangement of the E proteins on the surface of the virion. Each monomer of the E protein is colored with domains I, II, and III in red, yellow, and blue, respectively. The cyan indicates the density of prM covering the fusion tip of the domain II of the E protein on the immature virus in (C). Note the difference in orientation of the E proteins as well as the surface packing of the E proteins in the immature and mature viruses (Perera *et al.*, 2008).

Figure. 1.16. The dengue virus structure. **(A)** A central cross section looking down an icosahedral three-fold axis, showing the polygonal shape of the membrane. The darkness of the shading is proportional to the magnitude of the cryo-EM density. Viral components are labeled. **(B)** Maximum density heights are plotted below on a relative scale as a function of radius. **(C)** A radial cryo-EM density section highlighting the herring bone arrangement of 3 E dimers. The density is indicated in gray scale, with the highest density being the blackest. The limit of an icosahedral unit is marked by a brown triangle. The dimer on the two-fold axis is marked red and the ones in general position are marked in green and blue. The transmembrane helices are seen in cross section and are marked for the green monomer. The E-T1 and E-T2 are the E transmembrane anchors and the M-T1 and M-T2 are the M transmembrane anchors respectively. (Figures adapted from (Zhang *et al.*, 2003a).

1.6. Interaction with host cell surface receptors

West Nile virus is found to bind to Toll like receptor-3 (TLR-3) (Wang *et al.*, 2004) and $\alpha\beta 3$ integrins (Chu & Ng, 2004a). Several primary receptors and low affinity co-receptors of cellular attachment molecules have been proposed for DENV including the highly sulfated heparan sulfate (Chen, 1997), the dendritic cell-specific ICAM3-grabbing-non-integrin (DC-SIGN) on immature dendritic cells (Pokidysheva *et al.*, 2006; Tassaneetrithep *et al.*, 2003), the L-SIGN (Navarro-Sanchez *et al.*, 2003), the mannose binding receptor on macrophages (Miller *et al.*, 2008) and the laminin-binding protein (Thepparit & Smith, 2004; Tio *et al.*, 2005). Several other cell surface putative receptors

have been proposed for flavivirus binding (Bielefeldt-Ohmann, 1998; de Lourdes Munoz, 1998; Kimura *et al.*, 1994; Marianneau *et al.*, 1996; Ramos-Castaneda *et al.*, 1997; Salas-Benito & del Angel, 1997). In addition, opsonization with immunoglobulins enhances virus particle binding and infection of cells expressing immunoglobulin Fc receptors (Peiris & Porterfield, 1979; Schlesinger *et al.*, 1983). Despite this variety of host cell surface receptors identified for entry, the only one viral component that was consistently shown to bind directly to cellular receptors is the Ig-like domain III of the E protein (Beasley & Barrett, 2002; Hung *et al.*, 2004; Lin & Wu, 2003; Thullier *et al.*, 2001). Also, on the icosahedral surface of the matured virion, the domain III forms the pore at the five-fold axis (Fig. 1. 17).

Figure. 1.17. Pseudoatomic model of Dengue virus. The icosahedral scaffold made up of the envelope protein of Dengue virus. The five fold, three fold and two fold axes are marked as 5, 3 and 2 respectively. The domain III (coloured blue) forms the pore at the fivefold axis and occupies the three fold axes. (Model was generated in Pymol (version 0.98) using the coordinates of PDB id: 1k4r).

1.6.1. Low pH induced endosomal fusion by flavivirus

Upon binding to the receptors, virions enter the host cells through the clathrin coated pits via receptor mediated endocytosis (Gollins & Porterfield, 1985; Nawa, 1998).

Once brought into the prelysosomal endocytic compartment, extensive structural rearrangement (**Fig. 1.18**) of the E protein homodimer occurs to form a homotrimer (**Fig. 1.19**) and this leads to the exposure of the fusion loop at the domain II raising it perpendicular to the virus surface.

Figure. 1.18. Domain rearrangements in the Dengue sE monomer during the transition to trimer during the process of fusion. (A) An sE monomer in its prefusion conformation. This is the structure adopted in mature virus particles and in solution at pH >7, when sE is a dimer. (B) Schematic representation of the secondary structure of domain I and the links to domain II and III in the prefusion conformation. (C) An sE monomer in its post fusion conformation as seen in sE trimers. The three domains have rotated and shifted with respect to each other, bringing the C-terminus 39Å closer to the fusion loop (orange). The fusion loop retains essentially the same conformation before and after fusion. (D) Secondary structure of domain I and its links to domains II and III in the trimeric post fusion conformation. The domain I – II linker inserts between strands A₀ and C₀. The C-terminal region of A₀ flips out, switches to the other β-sheet, and creates an annular trimer contact (◄) with the two other A₀ in the trimer (Modis *et al* 2004).

The multiple events (**Fig. 1.20**) leading to the fusion of the virus through the fusion tip with the endosomal membrane are well characterized and summarized in the references given (Bressanelli *et al.*, 2004; Harrison, 2005; Harrison, 2008; Modis *et al.*, 2004; Mukhopadhyay *et al.*, 2005; Weissenhorn *et al.*, 2007). Formation of trimer contacts spreads from the fusion loops at the trimer tip to domain I at the base. Domain III shifts and rotates, folding the C terminus of sE back towards the fusion loop. The length of the interdomain linker permits independent rotation of individual domains III, allowing for the spontaneous symmetry-breaking required at this point.

Figure. 1.19 (A). The Dengue sE trimer. The hydrophobic residues in the fusion loop are exposed at the tip. The expected position of the hydrocarbon layer of the fused membrane is shown in green. Representative lipids are shown to scale. A chloride ion (black sphere) binds near the fusion loop. **(B)** Surface representation of the trimer. The dashed grey arrow (Δ) indicates the most likely location of the stem. An extended cavity is visible near the tip of the trimer; access to the cavity will probably be occluded by the stem. The glycan on Asn 67 and representative lipids are shown in space filling representation. **(C)** H1 helix of the stem. Close-up view on the side of the closed conformation of the low pH induced trimer as a surface representation. The subunit in the foreground is colored and those in the background are grey. The domain III / stem linker coloured purple, ends at a groove at the interface between the domain IIs in the trimer. Helix H1 is modeled as a pink ribbon occupying the groove (Sequences and secondary structures of the H1/H2

and the membrane anchor regions are marked in **Fig. 1.21**). **(D)** Same as **(C)** but in the ribbon representation. Note that the images **(C)** and **(D)** are inverted forms of Figure **(A)** and **(B)**. (Modified from (Bressanelli *et al.*, 2004; Modis *et al.*, 2004).

Figure. 1.20. Schematic representation summarizing the steps involved in the fusion process of class II fusion protein. **(A)** The dimeric E protein is on the virus surface with the fusion peptide (green tip) buried in the dimer. **(B)** The protein binds to a receptor and is internalized into the endosome. Under low pH conditions the domain II (yellow) swings out towards the host cell membrane, presumably at the domain I-II hinge region. **(C)** This conformational change allows the E proteins to rearrange laterally. The fusion peptide inserts itself into the outer leaflet of the host cell membrane, enabling the trimerization of the E protein. **(D)** Domain III (greyblue) of the E protein folds back onto itself and in the process brings the viral membrane towards the fusion peptide and host cell membrane. **(E)** As domain III moves close to domain II, hemifusion of the lipid membranes occurs. **(F)** Final form of trimer where the stem, transmembrane regions get docked along with the domain IIs and fusion peptides in close proximity. X-ray crystallographic evidences are available only to the first and last steps and the intermediates have been studied by biochemical analyses (Mukhopadhyay *et al.*, 2005; Stiasny *et al.*, 2007).

Figure. 1.21. Secondary structure alignment of the class II membrane fusion proteins of Flaviviruses (aligned and numbered according to TBE virus strain Senzhang: Genbank accession number: [AAN77871](#)) to show the DIII stem linker region (purple), stem and anchor regions (grey) (denoted by solid bars below the alignment) which get docked along with the domain IIs and fusion peptides in close proximity (as shown in Fig1.20F). The secondary structure, domain nomenclature and colour coding follow the definitions used for the TBEV sE dimer (Bressanelli *et al.*, 2004; Rey *et al.*, 1995). A colour coded bar below the sequence indicates domains I, II and III in red, yellow and blue respectively. Highly conserved residues are given on a red background.

The grey bar below the C-terminal segments denotes that they are not part of the crystal structures. The numbers 1-6 along the bars indicate the position of 6 pairs of Cysteine residues engaged in disulfide bonding. The secondary structure elements are indicated above the sequence, with arrows and helices for β strands and α helices, respectively. In the stem region, the predicted α -helices (H1, H2, TM2 and TM2) are indicated in grey above the sequence, and only apply to the TBEV sequence. Other virus sequences used in the alignment are DENV1 (Hawaii, Accn. No: [EU848545](#)); DENV2(New Guinea C, Accn. No:[BAA00255](#)); DENV3 (H87, Accn. No: [AAA21187](#)); DENV4 (H241, Accn. No: [AY947539](#)); WNV (US, Accn. No: [AAN85090](#)); KUNV (MRM61C) (Accn. No: [D00246](#)); JEV (Vellore P20778, Accn.No: [AAC29474](#)); MVE (Accn. No: [X03467](#)) and YF (17D, Accn. No: [X03700](#)) (Aligned using ESPript2.2 version 3.06 ([Gouet et al., 2003](#))).

Domain III undergoes the most significant displacement in the dimer-to-trimer fusion transition. It rotates by about 70° , and its centre of mass shifts by $36\text{\AA}$ towards domain II. This folding-over brings the C terminus of domain III (residue 395) $39\text{\AA}$ closer to the fusion loop. The way in which the stem is likely to fold back suggests that peptides derived from stem sequences could block completion of the conformational change, by interacting with surfaces on the clustered domains II. This would interfere with the final stage of the conformational change, whereas targeting the detergent occupying hydrophobic pocket beneath the 'kl' loop would interfere with the first stage. Inhibitors developed from these two strategies could thus act in synergy ([Modis et al 2004](#)).

1.6.2. Flavivirus maturation and budding

An assembling flavivirus particle is believed to acquire its envelope by budding through the membrane of the endoplasmic reticulum or an intermediate compartment of the early secretory pathway. In this compartment, membrane-bound heterodimers of E and prM interact with the nucleocapsid components (the C and RNA) to assemble into

mature virions. These particles are transported through the Golgi, where furin-mediated cleavage of prM to M converts the immature particles to the mature form (**Fig. 1.22**), primed to undergo a fusion-activating transition (Stadler *et al.*, 1997). The prM appears to protect the fusion loop of the E protein preventing the latter from fusing with the host membrane during release.

Figure. 1.22. A model of the flavivirus maturation pathway. (A) The events during conformational changes of the virus particles in the secretory pathway. Immature particles bud into ER as spiky virions and are transported through Golgi into the TGN, where the acidic pH induces a conformational change of the virion. Furin cleavage takes place in the TGN, and pr remains associated until the virion is released into the extracellular milieu. The approximate luminal pH values of the specified cellular compartment are indicated. (B) Configuration of the glycoproteins on the surface of the virion during maturation. The structure of the E protein in the secretory pathway is largely unchanged, except for movements at the hinge between domains I and II. In contrast, the oligomerization states of the glycoproteins are critically dependent on pH. The fusion loops are indicated by red stars. (Modified from (Li *et al.*, 2008; Yu *et al.*, 2008). (C) Schematic of a flavivirus in its immature and mature form. On the immature virion, the prM and E form a heterodimer which on maturation by host cell furin mediated cleavage releases the 'pr' and leads to the formation of a homodimer with two units of the E protein arranged in a head to tail manner. At this stage, if the virus is subjected to limited proteolysis, releases a soluble dimeric ectodomain (sE) which was crystallized (Rey *et al.*, 1995) to establish the first atomic structure of a viral protein. This mature virion when it enters the host cell endosome, the acidic compartment, the E protein undergoes fusion activated transition to form a homotrimer with 3 units of the E protein (Heinz *et al.*, 2003).

1.7. Viral entry inhibitors

The main difficulty in chemotherapeutic antiviral drug discovery is that, after studying millions of small molecule compounds, only a handful of the drugs show desired activity and unfortunately, many compounds out of these few, have toxic side effects, or are poorly absorbed. Therefore, eventually 1/10,000 of the compounds studied are developed into drugs.

Viral proteins or their subunits with critical functions in the virus lifecycle, have been shown to serve as exogenous dominant negative inhibitors of either viral entry as in the case of envelope protein domain III from alpha and flaviviruses (Liao & Kielian, 2005), or multiplication, as exemplified by the protease enzyme (PR) from Human Immunodeficiency Virus (HIV) (Miklossy *et al.*, 2008).

So far the most clinically advanced entry inhibitor peptide is T20, a fusion inhibitor of HIV, which has been shown to potently inhibit virus replication both *in vitro* and *in vivo* (Greenberg *et al.*, 2004). T20 is a 36-amino acid synthetic peptide, the sequence of which is derived from gp41 HR2. Approval of this drug as Enfuvirtide by FDA in 2003 for clinical use was followed by similar approaches extended to HCV (Liu *et al.*, 2010) and SARS CoV (Ujike *et al.*, 2008).

There are no antiviral drugs active against flaviviruses. Vaccines against JE, Yellow fever and TBE viruses are the only commercially available vaccines to residents of areas of endemicity and to travelers. These vaccines though expensive, need to be improved in terms of safety, efficacy and manufacturing cost. Dengue vaccine development has been hampered by the lack of good animal models and the need to create a tetravalent vaccine that produces neutralizing antibodies against each dengue virus serotype, to avoid problems with antibody-dependent enhancement (Whitehead *et*

et al., 2007). An extensively characterized YFV17D-based chimeric vaccine candidate, ChimeriVax™, against DENV, WNV and JEV awaits large-scale efficacy trials and registration (Guy *et al.*, 2010).

1.7.1. Anti-flaviviral antibodies

Humoral immunity is a vital part of the host response to flaviviruses that controls dissemination of infection, and in case of flaviviral encephalitis, arrests viral replication in the central nervous system. Antibodies are believed to protect against flavivirus infection through multiple mechanisms, including (1) direct neutralization of receptor binding, (2) inhibition of viral fusion, (3) Fc- γ -receptor-dependent viral clearance, (4) complement-mediated lysis of virus or infected cells, and (5) antibody-dependent cytotoxicity of infected cells (Pierson *et al.*, 2008).

In a study involving AG129 mice, passive transfer of DENV-immune serum was found to be protective against replication of heterologous challenge virus in all tissues of the mice, whereas adoptive transfer of DENV-immune cells significantly protected the mice from replication of the challenge virus, only when a lower inoculum was administered. Passive transfer of 300 μ L of polyclonal anti-DENV2 sera (PRNT50 titer against DENV2=1:1225) has given complete protection in mice from DENV2 replication in spleen ($p=0.0369$) and serum and also substantially reduced the levels of viral replication in the lymph nodes (Kyle *et al.*, 2008). Furthermore, mice lacking B cells are found to be more vulnerable to flavivirus infection (Chambers *et al.*, 2008; Diamond *et al.*, 2003; Halevy *et al.*, 1994; Pierson *et al.*, 2008; Roehrig *et al.*, 2001).

The use of hybridoma technology (Kohler & Milstein, 1975) has facilitated the generation and selection of highly specific and avid antibodies from immune B cells

conferred with endless proliferative property *in vitro*, for both diagnostic and therapeutic purposes.

Figure. 1.23. Antigenic complexity of the flavivirus E protein. All three domains of the E protein contain epitopes recognized by neutralizing antibodies. Domains of the WNV E protein are colour coded as in Fig. 1.13. Residues that impact antibody binding (shown in magenta) were identified using a yeast-display mapping approach for a large panel of antibodies, identifying structurally distinct epitopes in E-DIII (blue), E-DI (red), and E-DII (yellow) (Pierson *et al.*, 2008).

The highly complex antigenic structure of the E protein, offered partly by its changing conformations at different cellular compartments, from all four serotypes has resulted in the identification and generation of an array of antibodies with varying specificities and neutralization efficacies. Based on these two properties, the flaviviral antibodies have been characterized as type-, sub complex-, and complex-specific or cross reacting antibodies to indicate the level of specificities of these antibodies. Epitopes recognized by neutralizing antibodies have been identified in all three domains of the E protein (**Fig. 1.23**); however, many of the most potent mAbs are virus-type specific and bind epitopes within E-DIII (reviewed in (Gromowski & Barrett, 2007; Roehrig *et al.*, 2003; Sukupolvi-Petty *et al.*, 2007) and references therein). One of the well-characterized strongly neutralizing mAb, the anti-WNV E16 antibody, bound to four discontinuous

loops on the lateral ridge of WNV E-DIII (E-DIII-LR) (Oliphant *et al.*, 2005) and blocked viral fusion. This promoted viral clearance by delivering virus to the lysosome for destruction (Thompson *et al.*, 2009). The high affinity antibody proved its therapeutic potential with its ability to disrupt transneuronal spread, neutralize at low virion occupancy (Pierson *et al.*, 2007) and to protect mice (Oliphant *et al.*, 2005) and remarkably in hamster, when administered after neuronal infection (Morrey *et al.*, 2006). These promising results with other supportive evidences for passive protection when combined with contributory immune modulators like the complement components, as in the case of WNV infections (Klein & Diamond, 2008), provide the basis for generation of more and more antibody libraries against Dengue and other flaviviruses.

Methods to characterize the antibodies for their anti-viral, physicochemical and structural properties range from the old classical plaque reduction neutralization tests (PRNT) to a variety of newer innovative tools like the yeast surface display of a variety of epitopes. An extensively characterized potential neutralizing antibody becomes a prospective candidate for developing into therapeutics.

Early clinical trials exclusively with murine mAbs posed the risk of inducing anti-murine antibodies in the recipient. Also the murine Fc part of the mAb was less efficient in recruiting human effector functions. Thirdly, the biological half-life of murine mAbs is less than a day as compared to a week in the case of human antibodies. These hurdles are overcome by using either fragments of antibodies like Fab portions obtained by limited proteolysis or the minimal antibody called the single chain fragment variable (scFv), which is a product of the two hyper-variable domains one each from the heavy and light chain of mAb linked by an engineered link to maintain the binding conformation of

paratopes. The recombinant DNA technology application to mAb has led to generation of large quantities of specific scFv in *Escherichia coli* making it economic and easily available for therapeutic use. Today a large number of antibody-based clinical trials are being performed for a variety of indications including viral infections. This particular therapeutic modality has grown to the point where it constitutes approximately 30% of all clinical trials (excluding vaccine and gene therapy) registered by the FDA in the USA (Glennie & Johnson, 2000). Humanizing these antibodies by grafting the complementarity determining regions (CDR1-3) into human frame works (FRW1-3) results in better effector functions like complement-mediated cytotoxicity (Riechmann *et al.*, 1988). One such humanized mAb raised against the respiratory syncytial virus glycoprotein F, Palivizumab, has been approved for therapeutics by the FDA.

The associated threat of antibody-dependent enhancement (ADE) of flaviviral infection can be avoided by administration of neutralizing avid antibodies early enough during viremia to reduce viral load which otherwise may lead to complications like DHF. Alternatively, the minimally reactive engineered antibody fragments or humanized antibodies with high neutralizing efficacy against all four serotypes would prevent the complications of ADE.

1.7 Aim of the study

The important roles of flavivirus E protein domain III in:

- virus-host receptor binding and fusion
- eliciting neutralizing antibodies which are efficient blockers of viral entry and
- the C-terminal helical regions of domain III occupy the hydrophobic groove formed by trimerization of envelope protein by endosomal acidic pH, led to the present study with the following aims:

1. To study the effect of recombinant flavivirus envelope protein domain III (rEd3), the receptor binding region, to compete with the whole virus in entry process into host cells.
2. To synthesize peptides spanning the C-terminal helical region of domain III to block viral fusion to host membrane.
3. Identify and characterize a mouse monoclonal antibody raised against the rEd3 for its binding, neutralizing potentials with its epitopes mapped using synthetic mimotopes.
4. To generate recombinant antibody fragments with comparative immunobiological properties of the antibody to match the different serotypes so as to avoid complications of antibody-dependent enhancement (ADE) otherwise elicited by heterologous Fc portion as that of mice.

MATERIALS AND METHODS

2. MATERIAL AND METHODS

This chapter gives the details of the various constructs, viruses, cell cultures and other logistics used in the study with the procedures followed to obtain pure recombinant proteins, viruses, peptides and antibody. Methods adopted to characterize the individual components like the recombinant protein, peptides or antibody and in the inhibition of virus replication are also described in respective sections.

Table. 2.1. Details of primers for generation of domain III constructs for DENV1-4 & WNV

No	Construct (aa)	Plasmid	Primers	Restriction sites	Comments/ Source
1	D1Ed3* 291-395	pET32b	F-5' AAGGCCATGGCTAAACTGACCTTAAAA-3' R-5' TGGTGCTCGAGTTATCCTTTCTTGAACCA-3'	<u>NcoI</u> <u>XhoI</u>	DENV1 Hawaii
2	D2TEd3 291-395	pET32b	F-5' TAAGGCCATGGCTAAACTACAGCTCAAA-3' R-5' AAAGCTCGAGTTATCCTTTCTTAAACCAAGTTG-3'	<u>NcoI</u> <u>XhoI</u>	DENV2 TSVO1
3	D2NEd3 291-395	pET32b	F-5' TAAGGCCATGGCTAAACTACAGCTCAAA-3' R-5' AAAGCTCGAGTTATCCTTTCTTAAACCAAGTTG-3'	<u>NcoI</u> <u>XhoI</u>	DENV2 NGC
4	D3Ed3 291-395	pET32b	F-5' CAAGGCCATGGCTAAATTGAACTCAAG-3' R-5' GGTGCTCGAGTTATCCCTTCCTGTACCA-3'	<u>NcoI</u> <u>XhoI</u>	DENV3 H87
5	D4Ed3 291-394	pET32b	F-5' GGTGCTCGAGTTATCCCTTCCTGTACCA3' R-5' TGGTGCTCGAGTTACCCCTTCCTGAACCA3'	<u>NcoI</u> <u>XhoI</u>	DENV4 H241
6	D1Ed3 291-395	pET16b	F-5' CGTATGCTCGAGAAACTGACCTTAAAAG-3' R-5' GCTCGTCATCGATTATCCTTTCTTGAACCA-3'	<u>XhoI</u> <u>ClaI</u>	DENV1 Hawaii
7	D2TEd3 291-395	pET16b	F-5' CATTGCTCGAGAAACTACAGCTCAAA-3' R-5' CGCTGCATCGATTATCCTTTCTTAAACCA-3'	<u>XhoI</u> <u>ClaI</u>	DENV2 TSVO1
8	D2NEd3 291-395	pET16b	F-5' CATTGCTCGAGAAACTACAGCTCAAA-3' R-5' CGCTGCATCGATTATCCTTTCTTAAACCA-3'	<u>XhoI</u> <u>ClaI</u>	DENV2 NGC
9	D3Ed3 291-395	pET16b	F-5' CATATGCTCGAGAAATTGAACTCAG-3' R-5' CTTATCATCGATTATCCCTTCCTGTACCA-3'	<u>XhoI</u> <u>ClaI</u>	DENV3 H87
10	D4Ed3 291-394	pET16b	F-5' GCTCATGCTCGAGAAATTGAGAATTAAG-3' R-5' CTTATCATCGATTATCCCTTCCTGAACCA-3'	<u>XhoI</u> <u>ClaI</u>	DENV4 H241
11	WNEd3 299-401	pET16b	5' -CAGCTCGAGGGAACAACATATGGTGTATGCTC-3' 5' -ACCGGATCCTTACCCAGATTGTGCCAGTG G-3'	<u>XhoI</u> <u>BamHI</u>	WNV Sarafend

* The recombinant proteins expressed from and virus strains used in this study:

DENV 1 – Hawaii, ATCC VR-71

DENV 2 - New Guinea C, ATCC VR-1255

DENV2 - TSV01, (McBride & Vasudevan, 1995) Genbank accession AY031176
 DENV 3 - H87, ATCC VR-1256
 DENV 4 - H241, ATCC VR1490
 WNV (Sarafend strain), Genbank accession AY688948 (Li *et al.*, 2006).

Table. 2.2. Details of domain III expression conditions

Construct (aa)	Plasmid	Tag details	Expression Host/Cleavage protease	Expression condition	Antibiotic
D1Ed3 291-395	pET32b	N-terminal Trx & His ₆	BL-21(DE3)/ Enterokinase	1mM IPTG at 30°C/6 hrs	Ampicillin
D2TEd3 291-395	pET32b	N-terminal Trx & His ₆	BL-21(DE3)/ Enterokinase	1mM IPTG at 30°C/6 hrs	Ampicillin
D2NEd3 291-395	pET32b	N-terminal Trx & His ₆	BL-21(DE3)/ Enterokinase	1mM IPTG at 30°C/6 hrs	Ampicillin
D3Ed3 291-395	pET32b	N-terminal Trx & His ₆	BL-21(DE3)/ Enterokinase	1mM IPTG at 30°C/6 hrs	Ampicillin
D4Ed3 291-394	pET32b	N-terminal Trx & His ₆	BL-21(DE3)/ Enterokinase	1mM IPTG at 30°C/6 hrs	Ampicillin
D1Ed3 291-395	pET16b	N-terminal His ₁₀	BL-21(DE3)/ Factor Xa	1mM IPTG at 30°C/6 hrs	Ampicillin
D2TEd3 291-395	pET16b	N-terminal His ₁₀	BL-21(DE3)/ Factor Xa	1mM IPTG at 30°C/6 hrs	Ampicillin
D2NEd3 291-395	pET16b	N-terminal His ₁₀	BL-21(DE3)/ Factor Xa	1mM IPTG at 30°C/6 hrs	Ampicillin
D3Ed3 291-395	pET16b	N-terminal His ₁₀	BL-21(DE3)/ Factor Xa	1mM IPTG at 30°C/6 hrs	Ampicillin
D4Ed3 291-394	pET16b	N-terminal His ₁₀	BL-21(DE3)/ Factor Xa	1mM IPTG at 30°C/6 hrs	Ampicillin
WNEd3 299-401	pET16b	N-terminal His ₁₀	BL-21(DE3)/ Factor Xa	1mM IPTG at 30°C/6 hrs	Ampicillin

Figure. 2.1. Vector maps of pET 16b constructs of recombinant flavivirus envelope protein domain III.

2.1. Molecular cloning

The details of recombinant plasmid constructs are summarized in Table 2.1. Briefly the target DNA was amplified with Advantage II polymerase enzyme (Clontech) in an Applied Biosystems PCR machine with the following cycle:

Denaturation step - 94° C/2.5 min

Primer annealing - 55° C/30 sec

Strand extension 68° C/3 min - 30 cycles

Final extension 68° C/3min followed by 4° C for cooling down.

Amplified products were purified using QIAquick (Qiagen inc) kit or by agarose gel purification and digested by respective restriction enzymes. The products were ligated using the T4 ligase (Roche) at 16° C overnight and cloned into the respective vectors. The constructs were transformed into chemically competent *Escherichia coli* DH5α cells. Sequences of positive transformants were confirmed.

2.1.1. Overexpression of rEd3

Confirmed plasmid constructs from DH5α cells were transformed into chemically competent expression hosts of BL21(DE3). Twenty five milliliters of LB broth was inoculated with a single colony of *E. coli* transformant cells and incubated at 37° C overnight to obtain the seed culture. One litre of LB broth with 100 µg/ml of ampicillin was seeded with this suspension and incubated at 37° C in an orbital shaker until the OD₆₀₀ reached 0.8-1.0. An aliquot of 1 ml was retained as an uninduced control. IPTG (isopropyl-β-D-1-thiogalactopyranoside) was added to a final concentration of 1mM and incubated at 30° C with constant shaking of 230 rpm for 5 hours.

Detergent lysis buffer:

50mM Tris-HCl (pH 8.0)

200mM NaCl

1% Sodium deoxycholate

1% NP40

0.1mM EDTA

After induction

1. The bacterial suspension was centrifuged at $6000 \times g$ (Beckmann, Avanti J series) for 20 min. at 4°C .
2. The supernatant was removed and the pellet was resuspended in detergent lysis buffer, with a ratio of 1 volume of buffer to 5 volumes of culture suspension. (For a volume of 250 mL of broth culture, 50 mL of detergent lysis buffer was used).
3. The bacterial suspension was mixed by vortexing and centrifuged at $48,000 \times g$ for 20 min. at 4°C .
4. The supernatant was removed and the pellet was resuspended again in detergent lysis buffer. The washed bacterial pellet was lysed in a French Pressure Cell at 1200 psi at 4°C or disrupted by sonication for 30 min. with 6 sec 'ON' and 6sec 'OFF' cycle at 20% amplitude in a Vibracell sonicator. The lysed suspension was centrifuged at $48,000 \times g$ for 30 min. at 4°C .
5. The supernatant was separated from the pellet otherwise the inclusion body. All fractions starting from the un-induced cell culture to processed inclusion body were run on SDS-PAGE gel (15%) to identify fractions containing the expressed protein.
6. The inclusion body was stored in a -20°C freezer for less than 4 weeks, and for a longer period, kept in a -80°C freezer.

2.1.2. Denaturation and refolding of rEd3

9M Urea buffer

After dissolving 540 grams of urea in 950 ml of distilled water, the solution is mixed with resin AG 501X8 (D) (Biorad) and stirred overnight. The resin removes calcium and magnesium cations, which make up about 1M of the 9M urea.

Urea buffer (Working Buffer)

100mM NaH₂PO₄

10mM Tris-HCl (pH8.5)

8M urea

Refolding buffer (to refold rWNEd3) (Chu & Rajamanonmani *et al.*, 2005)

50mM Tris/HCl (pH8.0)

1mM EDTA

20% glycerol

100mM NaCl and

3mM DTT

Refolding buffer (to refold rD1-D4Ed3) (Rajamanonmani *et al.*, 2009)

200mM Tris-HCl (pH8.0)

10mM EDTA

5mM Reduced Glutathione

0.5mM Oxidized Glutathione

50mM L-Arginine

1. Proteins were denatured in urea. The inclusion body was denatured in urea buffer at pH 8.5 and centrifuged at 48,000 ×g force to obtain supernatant containing the rEd3.

2. The supernatant was mixed with Ni-NTA beads overnight at 4° C on a rotor, after which the suspension was loaded on an econocolumn. Urea buffer with stepwise lowering of pH^{**} was added to the column. The final pH was 2.4, at which the protein eluted from the column. The eluted protein at a concentration of ~2mg/ml from Nickel column was diluted with the refolding buffer to 10µg/ml quantity with constant stirring on a magnetic stirrer at 4° C. The pH of the refolding buffer was maintained away from the pI of the protein throughout the refolding step.
3. The diluted and refolded protein was concentrated and exchanged with refolding buffer in a concentrator at 4° C with constant observation to avoid concentration dependent aggregation.

Table. 2.3. Summary of biochemical characteristics of recombinant domain III (rEd3) proteins.

Ed3	DENV-1 (291-395)	DENV2 (291-395)	DENV3 (291-395)	DENV4 (291-394)	WNV (299-401)
Mass (kDa)	14370	14669	14495	14392	13891
pI	7.2	7.3	8.5	8.6	7.31
Elution pH from Ni-NTA column	2.4	2.4	2.4	4.5- 2.4	2.4
pH of refolding bufer	8.2	8.2	7.8	7.8	8.0

4. The refolded protein was centrifuged at 3000 ×g for 20 min. at 4° C to remove any aggregation. The clarified protein was concentrated and purified by gel filtration chromatography in a Superdex 75 (HR 10/30) column (GE Healthcare) using a buffer (12mM Tris HCl (pH8.0), 250mM NaCl, 0.1mM EDTA and 3mM DTT) to avoid the formation of intermolecular disulphides.

5. The protein was purified by preparatory gel filtration chromatography.

** The stepwise pH elution was done using the buffer: 100mM NaH₂PO₄, 10mM Tris, 8M urea at pH 8.0, 6.3, 5.9, 4.5 and 2.4.

Figure. 2.2. Secondary structure alignment of envelope protein domain III from the six viruses used in the study (DENV1-4 and WNV). The two strains from DENV2 serotype (NGC and TSV01) differ in their domain III only at position E390 (N $\rightarrow$ S).

2.1.3 Purification of domain III by gel filtration chromatography

Refolded proteins concentrated by ultrafiltration (Amicon) were purified on a Superdex 75 (HR 10/30) column (GE Healthcare) using the buffer containing 12mM Tris/HCl (pH 8.0), 250mM NaCl, 0.1mM EDTA and 3mM DTT.

2.1.4. Physicochemical characterization of Ed3

The CD spectra were recorded on a Jasco J810 spectro-polarimeter using three accumulations of data at 0.1 nm intervals and were smoothed using the noise reduction routines provided with the instrument, including solvent background subtraction. The buffer contained 12mM Tris/HCl, 10mM sodium phosphate, 2mM DTT at pH 8.0 and the protein concentration was 0.1 mg /ml. Deconvolution of the CD far UV-spectrum was carried out with the CDNN and CONTIN software (Sreerama & Woody, 2000). Mass spectrometry analysis using a matrix-assisted laser desorption ionization time of flight mass spectrometer (MALDI-TOF) was employed to determine the molecular mass of the sample.

Denaturing SDS-PAGE under reducing and non-reducing conditions was performed to check the size of the target protein. The protein was transferred to the PVDF membrane and analyzed using commercial anti-His antibody and horseradish peroxidase (HRP)-conjugated anti-mouse antibody as the primary and secondary antibodies, respectively, to demonstrate the expression of the protein of interest. The protein induced from the pET32b/DEN2E₂₉₁₋₃₉₅ construct was cleaved after refolding with enterokinase to remove both the His- and Trx-tags or factor Xa to remove the N-terminal His-tags. The first ten residues at the N-terminal sequence of the protein were identified by Edman degradation after cleaving the N-terminal His – tags at the Institute of Pasteur, France.

2.1.5 Enzymatic cleavage of Trx and His tag

The recombinant Ed3 proteins were cleaved with enterokinase (EK) to remove the thioredoxin and His tags. One µg of the enzyme was reacted with 500 µg of the

recombinant protein. The cleavage reaction was performed in EK buffer (50mM Tris-HCl pH 8.0, 50mM NaCl, 2mM CaCl₂, 0.1% Tween-20). Inhibition of enterokinase activity was done using 20X protease inhibitor cocktail. Cleavage with Factor Xa (Qiagen) and removal with Factor Xa removal resin (Qiagen) were performed following the manufacturer's guidelines. The recombinant Ed3 upon cleavage of the N-terminal His tag was subjected to N-terminal protein sequencing by Edman degradation at the Institute Pasteur, France to confirm the identity of the protein expressed.

2.1.6. Binding of mouse anti-His or mouse anti-E antibody to rED3 by Western blot

Presence of His tag in the recombinant fusion protein was confirmed by mouse anti- His antibody (Qiagen) by Western Blot. Mouse anti-E antibody, D2B7/9F12-2-1, was a kind gift from Prof. S.G.Vasudevan, Head, Dengue unit, Novartis Institute for Tropical Diseases, Biopolis, Singapore. Western blots using D2B7/9F12-2-1 as the primary antibody were performed on DENV Ed3s resolved under reducing and non-reducing conditions and later transferred to PVDF membrane. The secondary antibody used was HRP-conjugated anti-mouse antibody.

2.1.7 Inhibition of viral entry by rED3

Purified soluble domain III from DENV 1-4 as a His tagged fusion protein or an unrelated 14 kDa recombinant protein belonging to the nucleocapsid of IBV, a chicken coronavirus (Fan *et al.*, 2005) as a negative control, for the matching mass and His tag, at concentrations of 100, 10, 1, 0.1, 0.05 and 0.01µM was incubated in duplicate with 1×10^5 of C6/36 cells/well in a 6-well plate with RPMI-1640 supplemented with 5% FCS and 25mM Hepes (maintenance medium) at 4° C for 1hr. Unbound protein molecules were removed by washing the cells three times with Hank's balanced salt solution (HBSS).

The pretreated cells were then incubated with homologous DENV 1-4 in maintenance medium at 30° C for 1hr. Unadsorbed virus is removed and cell surface received 2ml/well of maintenance medium to continue the incubation at 30° C for 60 hrs. At the end of incubation period the cell supernatant was collected, centrifuged at 1000 ×g force for 10minutes and the virus was quantified by plaque assay on BHK-21 cells.

Inhibition of WNV entry by recombinant WNEd3 was carried out in collaboration with the Flavivirology laboratory, Department of Microbiology, National University of Singapore.

2.1.8 Characterization of mAb 9F12 binding to recombinant Ed3 and virus

Purified Ed3 at a concentration of 0.2 µg per well in carbonate-bicarbonate buffer was coated to 96 well plate (Maxisorp, Nunc) at 4° C for overnight. Purified virus at a concentration of 0.8 µg per well was used in triplicate as a positive control. After blocking with 1% of BSA in PBS (w/v), the plates were incubated with tenfold dilutions of mAbs for 3 hrs at room temperature (RT). The reaction was probed with anti-mouse antibody conjugated with alkaline phosphatase for an hour at RT followed by addition of p-nitro phenyl phosphate (Pierce), and the optical density was measured at 405 nm (OD₄₀₅) after arresting the reaction with 50 µl of 2M NaOH. The OD from antibody containing wells was normalized against the Ed3 coated wells without the primary antibody to eliminate the background noise. Normalized OD₄₀₅ against the varying logarithmic dilutions was fitted using Graphpad Prism (version 5.0) using nonlinear curve fitting following the guidelines of the software. The kinetics of recombinant domain III binding to mAb 9F12 and to the recombinant single chain Fv were evaluated by surface

plasmon resonance (SPR) in collaboration with Dr. Susana Geifman Shochat, School of Biological Sciences, NTU.

The interaction of mAb 9F12 to whole virus present in the infected cells was studied by immuno-fluorescence. Monolayer cells of A-549 grown on glass cover-slips were infected with DENV-2 TSV01 at an MOI of 5. Cells were fixed with 80% acetone at -20° C for 30 min and allowed to react with 50 µl of 10⁻⁶M in 9F12 or 4G2 (as positive control) in PBS with 0.7% BSA (w/v) for 1 hr at room temperature. The A-549 cells were stained with anti-mouse-Alexafluor 488 for 1 hour at 4° C and observed under 10 × objective of an Olympus fluorescent microscope.

2.2. Maintenance of cell cultures and hybridomas

Table. 2.4. Details of cell cultures and hybridomas used in the study

No	Cell culture	Source/Accession Number	Medium used	Atmosphere/ Temperature	Application
1	C6/36	<i>A. albopictus</i> insect cell/ATCC CRL1660	RPMI-1640 + 25mMHepes + 10% Foetal calf serum (Gibco)	Atmospheric Oxygen / 28°C	Monolayer*
2	C6/36	<i>A. albopictus</i> insect cell/ATCC CRL1660	RPMI-1640 + 25mMHepes + 5% Foetal calf serum + Penicillin-Streptomycin (Gibco)	Atmospheric Oxygen / 28°C	Virus amplification
3	BHK-21	Baby hamster Kidney fibroblast/ATCC CCL-10	RPMI-1640 + 10% Foetal calf serum (Gibco)	5% CO ₂ / 37°C	Growth medium for Monolayer
4	BHK-21	Baby hamster Kidney fibroblast/ATCC CCL-10	RPMI-1640 + 2% Foetal calf serum (Gibco)	5% CO ₂ / 37°C	Maintenance medium-Virus titration or neutralization
5	A-549	Human lung carcinoma/ATCC CCL-185	F12-K medium + 2% Foetal calf serum	5% CO ₂ / 37°C	Virus growth
6	Vero	African Green monkey kidney fibroblast/ATCC CRL-1587	Dulbecco's Modified Eagle's medium + 7% Foetal calf serum	5% CO ₂ / 37°C	Monolayer
7	Vero	African Green monkey kidney fibroblast/ATCC CRL-1587	Dulbecco's Modified Eagle's medium + 2% Foetal calf serum	5% CO ₂ / 37°C	WNV Virus propagation or DENV Adsorption assay
8	4G2	Mouse myeloma cells/ ATCC HB-112	Hybricare (ATCC) + 10% FCS + 1.5gm of NaHCO ₃ (pH7.2) filter sterilization	5% CO ₂ / 37°C	Growing Hybridoma for Dengue cross reactive mAb production
9	3H5	Mouse myeloma cells/ ATCC HB-46	Hybricare (ATCC) + 10% FCS + 1.5gm of NaHCO ₃ (pH7.2) filter sterilization	5% CO ₂ / 37°C	Growing Hybridoma for Dengue-2 specific mAb production
10	D2/B7-9F12	Mouse myeloma cells (Subhash G. Vasudevan, NITD, Singapore)	Hybricare (ATCC) + 10% FCS + 1.5gm of NaHCO ₃ (pH7.2) filter sterilization	5% CO ₂ / 37°C	Growing Hybridoma - <u>mAb characterised.</u>

* Monolayer cell cultures were grown to confluence and trypsinized for further passaging and virus propagation. Suspension cell lines were centrifuged at 900 ×g force in a Bio-contained centrifuge to sediment cells and washed once with growth medium or serum free medium for further passaging or for weaning from serum, respectively. All cell lines and viruses were handled in a Biosafety level-2 cabinet following the safety guidelines provided by the Office of safety, Health and Environment (OSHE). All biological wastes generated were treated with 0.5% Melsept (Braun Medical AG) for not less than 1 hour and

finally autoclaved in containers with loosened caps before discarding to the centralized waste disposal unit.

2.2.1. Virus propagation, purification and quantification

Monolayers of C6/36 or Vero cells were infected with a multiplicity of infection (MOI) of 0.1 with respective virus. When Dengue viruses produced 90% syncytium (cytopathic effect; CPE), the cells along with the medium were harvested and centrifuged at 1000 $\times$ g force to sediment cells and debris to collect the supernatant as source of virus for further concentration by density gradient and titration by plaque assay. West Nile virus produced lysis of cells and when CPE reached 75 – 80%, the cells with medium were centrifuged to collect the supernatant as source of virus. Viruses were stored at -80° C bio-contained deep freezers.

A volume of 80 ml of virus supernatant was concentrated in an Amicon concentrator (100 kDa MWCO) using 3000g force to a volume of 2 ml and loaded onto preformed sucrose (11-55%) or glycerol-potassium tartrate gradient (10-35%) in Beckmann ultra-clear ultracentrifuge tubes (13.2 ml). The tubes are placed inside the SW41-Ti bucket and closed by screw caps under the BSL-2 and centrifuged at 75,000 $\times$ g force for 18 hrs at 4° C. Fractions of 1.0 ml were collected and analysed by SDS-PAGE gel for protein bands, enzyme linked Immunosorbent assay (ELISA) or dot blot to identify the virus containing fractions.

Confluent monolayers of either BHK-21 or Vero cells in a 24 well tissue culture multi-dish (NUNC) were challenged with 200 μ l of serial tenfold dilution of the purified and concentrated virus fractions in triplicate followed by incubation at 37° C for 1hr. Cells were overlaid with 1.6% of carboxy-methyl cellulose (CMC) premixed with 2X cell culture medium after washing the cell surface. The multi-dishes were incubated at 37° C

with 5% CO₂ in a CO₂ incubator for 5-6 days and stained with 1% crystal violet after fixing the cells with 3.7% formaldehyde to visualize the plaques. Number of plaques in the wells with the highest dilution of the virus was counted and the plaque forming units were calculated as exemplified below.

- Number of plaques in triplicate wells = 13 + 9 + 14 = 36
- Average = 36/3 = 12
- Highest dilution showing plaques = 10⁻⁵
- Reciprocal of the dilution = 10⁵

Virus titre = 12 × 10⁵ × 5 (conversion factor for per ml since the volume added per well is only 200 µl)

Virus titre = 6.0 × 10⁶ PFU/ml

2.2.2. Identification of virus containing fractions from gradients by SDS- PAGE, ELISA or Dot-blots

Fractions from sucrose gradients were analysed on denaturing SDS-PAGE gels of 12% to identify the major protein bands of the virus purified. Since samples from tartrate gradients precipitate with SDS-PAGE gel loading buffer, the former were diluted in tenfold dilution and was coated to wells of a 96 well Maxisorp (Nunc) plate in carbonate – bicarbonate buffer (pH 9.0) at 4° C for overnight and was probed with a cross reactive antibody, 4G2 as primary antibody and anti-mouse antibody conjugated with alkaline phosphatase (AP) (My Bio source) followed by addition of p-nitrophenyl phosphate (Pierce). The optical density was measured at 405 nm (OD₄₀₅) in a Tecan multimode microplate reader (Infinite 200, Tecan group limited, Switzerland) with 602 nm as

reference wavelength. The entire procedure was carried out in the BSL-2 with biosafety precautions.

Multiple samples from same virus were screened by Dot-blots on a membrane without the need for a BSL-2 since upon immobilization of the fractions on a nitrocellulose membrane, the membrane was dried and inactivated by ultraviolet. Inactivated membranes were probed with the same set of primary and secondary antibodies but with chemiluminiscent substrate (GE healthcare) and exposing to X-ray film which was further developed in a Kodak X-OMAT automated film processor. The purity and integrity of the final purified virus population was confirmed by transmission electron microscopy in collaboration with the Electron Microscopy unit at Department of Microbiology, NUS.

2.2.3 Production, concentration and purification of monoclonal antibodies from hybridoma supernatant

Hybridoma supernatants were collected, centrifuged at 1000 ×g force for 30 min at 4° C in sterile containers and filtered through 0.2 µm membrane. The sterile supernatant was purified according to (Rajamanonmani *et al.*, 2009). A volume of 500 ml hybridoma supernatants was mixed with sodium sulphate (powder) to a final concentration of 18% (w/v) at room temperature. The resulting precipitate was dissolved in 50 ml distilled water and mixed with sodium sulphate to a concentration of 14% (w/v) at 4° C. The precipitate was dissolved in 15 ml distilled water and dialyzed against 10mM sodium phosphate, pH 7.2 (buffer A) overnight. Purification was performed through a Cibacron Hi Trap blue column (GE healthcare) followed by a Diethylaminoethyl (DEAE) sepharose pre-swollen resin (GE healthcare) pre-equilibrated

with buffer A at pH 8.0. The mAb was eluted with 25mM and 40mM buffer A at pH 8.0. Concentrated fractions were purified on a Superdex-200 column (GE healthcare) through an FPLC (Akta, GE healthcare). The mAb 9F12 isotype was determined by ELISA (Pierce).

2.2.4 Plaque reduction neutralization (50%) test (PRNT_{50%})

Day -1:

- BHK21 cells were seeded into 24 well plates at 2×10^5 cells per well in 500 μ l volume and the plates were incubated overnight.
- Antibody-Methylcellulose (MCA) overlay was prepared from carboxy methyl cellulose (CMC) as follows: from a stock of 2×10^{-4} M concentration of antibody

2×10^{-5} M	Mixed 9 ml of CMC with 1000 μ l 10^{-4} M stock Ab to get 10 ml overlay
2×10^{-6} M	Mixed 9 ml of CMC with 1000 μ l 10^{-5} M diluted Ab to get 10 ml overlay
2×10^{-7} M	Mixed 9 ml of CMC with 1000 μ l 10^{-6} M diluted Ab to get 10 ml overlay
2×10^{-8} M	Mixed 9 ml of CMC with 1000 μ l 10^{-7} M diluted Ab to get 10 ml overlay. Further dilutions were done in the same manner.

Day 0:

The antibody was diluted as follows: from a stock of 10^{-4} M concentration of antibody

2×10^{-5} M	100 μ l stock Ab with 900 μ l of 2%FBS-RPMI 1640 to get 1 ml volume
2×10^{-6} M	100 μ l 10^{-5} M Ab with 900 μ l of 2%FBS-RPMI 1640 to get 1 ml volume
2×10^{-7} M	100 μ l 10^{-6} M Ab with 900 μ l of 2%FBS-RPMI 1640 to get 1 ml volume
2×10^{-8} M	100 μ l 10^{-7} M Ab with 900 μ l of 2%FBS-RPMI 1640 to get 1 ml volume. Further dilutions were done in the same manner

- Diluted the virus in 2% FBS-RPMI on ice to get a concentration of 300 pfu/ml
- Co-incubation:

Mixed 310 μ l of the different dilutions of antibody with equal volume of fixed concentrations of virus (310 μ l + 310 μ l = 620 μ l). Examples of dilutions required for the 4 serotypes (based on their original titre)

D1	1:20
D2	1:10000
D3	1:150
D4	1:3000

- The mixture was incubated at 4°C for 2 hrs.
- Added 200 μ l of each virus-Ab mixtures to triplicate wells of BHK21 monolayer in 24 well plates and incubated at 37°C for 1 hr.
- Removed the mixture from each well and added 0.5 ml of the various concentrations of Ab-overlay per well.
- Incubated the plates at 37°C with 5% CO₂ for 4-6 days

Day 4

- For DENV2, the cells were fixed at 88-90 hr after incubation, and stained the plates with 1% Crystal violet to count the plaque numbers and calculated the plaque reduction ratio.
- For DENV4, the cells were fixed at 94-96 hr after incubation, and stained the plates with 1% Crystal violet to count the plaque numbers and calculated the plaque reduction ratio.

Day 5

- For DENV3, the cells were fixed at 116-118 hr after incubation, and the plates were stained with 1% Crystal violet to count the plaque numbers and calculated the plaque reduction ratio.

Day 6

- For DENV1, the cells were fixed at 138-140 hr after incubation, and the plates were stained with 1% Crystal violet to count the plaque numbers and calculated the plaque reduction ratio.

- The formula for calculation of % of plaque reduction is:

- % of Plaque reduction =

$$\frac{\text{Average of triplicate of plaques in (Virus control wells – test wells)}}{\text{Average of triplicate of plaques in virus control wells}} \times 100$$

- eg: in 3 wells with 10^{-8} M of antibody, the number of plaques = 22, 29, 19 **I**
In 3 wells with no antibody (virus control), the number of plaques = 54, 48, 60. **II**
- Then average in **I** = $22 + 29 + 19 = 70 / 3 = 23.3$
- Average in **II** = $54 + 48 + 60 = 162 / 3 = 54$
- % of reduction = $\frac{54 - 23.3}{54} \times 100 = \frac{30.7}{54} \times 100 = 56.8\%$
- % of reduction with 10^{-8} M = 56.8%

2.2.5. Adsorption assay using cell-based flavivirus immuno-detection (CFI)

The adsorption assay was performed according to an earlier publication (Crill & Roehrig, 2001) but with modifications. A 96-well micro culture dish was dispensed with 2×10^4 Vero cells $(100 \mu\text{l})^{-1}$ in DMEM supplemented with 2% iFCS and incubated overnight at 37°C in a 5% CO_2 incubator. For the pre-adsorption assay, tenfold dilutions

of either mAb 9F12 or 4G2 was mixed with an equal volume of 2×10^4 PFU of DENV-2 virus and incubated at 4°C for 1 hr. The virus and mAb mixture was added to the confluent cell surface and incubated at 4°C for an hour for the virus to get adsorbed onto the cell surface. Negative control received serum free (sf) DMEM in place of the mAb. Cells were washed three times with sf MEM at 4°C . After incubation with DMEM for 2 days, cells were mixed with the 1: 20 diluted 4G2 antibody for immuno-detection. The immune reaction was probed with anti-mouse HRP conjugate, tetramethyl benzidine and stopped with 0.5 N sulphuric acid; absorption was then measured at 450 nm. The presence of a uniform number of cells per well was confirmed by staining with propidium iodide (PI) (Sigma) in PBS for 10 min and the fluorescent signal was read at 537 nm (excitation) – 617 nm (emission) in a Tecan plate reader. For the post-adsorption assay, a virus dilution containing 1×10^4 PFU. of DENV2 was added to the cells directly and incubated at 4°C for 1 hr followed by washes with sf DMEM at 4°C to remove unadsorbed virus. The mAb dilutions were added to cell surface with the adsorbed virus and incubated for 1 hr at 4°C . The rest of the assay to detect DENV2 NGC was as described for pre-adsorption.

2.2.6 Membrane fusion inhibition assay.

A fusion inhibition assay based on syncytia formation by C6/36 *Aedes albopictus* cells at low pH (Randolph & Stollar, 1990) was used for screening fusion inhibitors. The antibody 4G2 was used as a positive control as it prevents syncytia formation (Summers *et al.*, 1989). Assays were carried out in triplicate for both 4G2 and 9F12. Briefly, a 96-well micro culture dish was dispensed with a mixture of 1.5×10^5 C6/36 cells and DENV-2 at an m.o.i of 0.1 per 100 μl in RPMI 1640 supplemented with 5% iFCS and incubated

for 72 hr at 28° C in an air-tight humidified container. Both 9F12 and 4G2, diluted to a final concentration of 10 μ M in 95 μ l sf RPMI 1640, were added to the wells and incubated for 1 hr at 28° C. Subsequently, 5 μ l of 0.5M (N-morpholino) ethanesulfonic acid (pH 5.0) was added to the wells and incubated at 37° C for 1 hr to induce syncytia formation. The low pH solution was finally replaced with 0.025 mg PI ml⁻¹ solution made in sf RPMI 1640 and incubated at 28 °C for 30 min in order to stain non-viable cells/syncytia. Plates read at $\times$ 10 magnification objective were visualized with a Nikon fluorescence microscope.

2.3. Sequencing of mAb 9F12 and construction of recombinant scFv

First the DNA sequence of mAb 9F12 was obtained by sequencing the PCR amplified product followed by cloning the sequence of the variable heavy and light chains linked by a (Gly₄S)₃ linker by a two step PCR method.

A total of 10⁷ cells of the mAb 9F12 hybridoma were used for RNA extraction using TRIzol reagents. The V_H chain was amplified with the forward primer (5'- GAG GTC CAG CTC GAG CAG TCT GGA CC -3') and reverse primer (5'- GGC AGC GAT CCA GGG GCC AGT GG - 3') and the V_L chain was amplified with a light chain primer mix from the cloning module of recombinant phage antibody system (GE Healthcare). The final PCR products were purified by agarose gel extraction (Qiagen). DNA sequences obtained have been deposited with GenBank (accession nos FJ493471 and FJ493472 for the heavy and light chain, respectively).

Figure. 2.3. Construct map of the recombinant single chain fragment variable (scFv) in the vector pEt 24a(+).

Cloning, expression and purification of the scFv9F12 in pET 24a (+) vector with the gene of interest cloned in between XhoI and NdeI restriction sites followed by a C-terminal non cleavable 6X His tag was performed according to the protocol of (Rezacova *et al.*, 2001). The variable segments of the heavy and light chains were amplified using the Heavy forward (*NdeI*): 5'-CTTTAAGAAGGAGATATACCATATGCTGCAGC-3' Heavy linker: 5'-TCCCCCACC GCCACTCCCTCCGCCACCTGAGGAGACGGTGA CCGT-3'; Light linker: 5'-AGTGGCGGTGGGGGATCGGGAGGTGGCGGGT CACTT GGAGATCAAGCC-3'; Light reverse (*XhoI*): 5'- CTGATGCTGCACCAACTCTCGAG CACCACC -3' to introduce *NdeI* and *XhoI* restriction sites (underlined), respectively. Conditions for PCR amplification were as follows: six cycles of denaturation at 94° C for 1 min, annealing at 65° C for 2 min, and extension at 72° C for 4 min. After the reaction, the overlapped scFv DNA fragments were amplified using the V_H sense and the V_L antisense primer. PCR amplified products were purified, treated with NdeI/XhoI, and then cloned into pET-24a (+). Clones were subjected to DNA sequencing to verify the assembly of the variable heavy and light chains. The recombinant scFv protein was under

Kanamycin as selectable marker. The protein was induced at 30° C with 1mM IPTG for 6 hours and the protein was expressed as inclusion body that was denatured and refolded as described in section 2.1.2 for denaturation and refolding of rEd3.

2.3.1 Epitope mapping of mAb9F12 with peptides and yeast surface display

Peptide mimotopes synthesized (in section 2.4 below) were used to map the epitopes of mAb 9F12 by ELISA following the protocol of (Thullier *et al.*, 2001). Briefly, microtitre plates were coated with poly-(L-lysine) (Sigma) in 0.1M bicarbonate buffer (pH 9.6) followed by incubation with 0.1% glutaraldehyde in PBS. Wells were coated with 100 µl of peptides at concentrations ranging from 10^{-4} to 10^{-7} M followed by blocking with 50mM glycine in PBS-EDTA. A volume of 100 µl mAb 9F12 at 10^{-8} M was distributed in the wells. The binding was probed with 1:2000 diluted anti mouse antibody conjugated with alkaline phosphatase (AP) (My Bio source) followed by addition of p-nitrophenyl phosphate (Pierce). The optical density was measured at 405 nm (OD_{405}) in a Tecan multimode microplate reader (Infinite 200, Tecan group limited, Switzerland) with 602nm as reference wavelength.

Yeast surface display was carried out according to (Sukupolvi-Petty *et al.*, 2007) in collaboration with Department of Molecular Microbiology, Pathology and Immunology, Washington University School of Medicine, St Louis, USA, using DENV-2 domain III mutants that were generated as part of a random library by error-prone mutagenesis in the pYD1 vector and were expressed on the surface of yeast.

2.4 Synthesis and purification of peptides for virus fusion inhibition

Fmoc derivatives are cleaved by organic bases with the formation of soluble co-products which are easily eliminated by simple washing in Solid Phase Peptide Synthesis (SPPS). Full range of 9-Fluorenylmethoxycarbonyl (Fmoc)-amino acids and Rink amide-MBHA Resin 100-200 mesh 0.3~0.8 mmol/g 1% cross linked with Divinyl Benzene (DVB) were purchased from GL Biochem(Shanghai) Ltd, China.

Figure 2.4. Scheme for Solid phase peptide synthesis (SPPS) using Fmoc chemistry. The steps involved are shown in table 2.3.1 as a log sheet. Modified from <http://www.sigmaaldrich.com/life-science/custom-oligos/custom-peptides/learning-center/solid-phase-synthesis.html>

Table. 2.4.1. Steps involved in SPPS as a log sheet

Fmoc Solid phase peptide synthesis - log sheet		Aminoacids from C terminus to N terminus														
No.	Procedure	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	Fmoc Rinkamide MBHA resin 1gm yields 0.3mM peptide															
2	Swelling by DCM - soak for 20 min															
3	Drain DCM - soak with DMF- 2 min															
4	Deprotect with 20% Piperidine in DMF - 30 min															
5	Weigh the amino acid, BOP in the same bottle															
6	First washing starts - DMF wash 3X 2 min each															
7	DCM wash 3X 2 min each															
8	Methanol wash 1x - 2min															
9	DCM wash 1X 2 min															
10	Ninhydrin test = Resin + Sol A + Sol B = Positive (heat solution mix at 110 °C for 5 min. purple pink colour)															
11	Dissolve amino acid + BOP in DMF; add DIEA - 5 min															
12	Coupling the amino acid - 1 hour															
13	II washing starts: DMF 2x 2 min each															
14	DCM 2x - 2min each															
15	Methanol + DCM wash 1x - 2min															
16	DCM wash 1x 2 min															
17	Ninhydrin test = Resin + Sol A + Sol B = Negative (Blueish)															
18	Proceed to deprotection with a prewash of DMF (Step 4)															

Fmoc: 9-Fluorenylmethoxycarbonyl; MBHA: 4-methylbenzhydrylamine;
 DCM: Dichloromethane; DMF: N,N-Dimethyl Formamide; BOP: Benzo-
 triazolyl-oxytris(dimethylamino)phosphonium Hexafluorophosphate;
 DIEA: Diisopropylethylamine
 Ninhydrin test:
 Solution A: 33.3g phenol + 500mg ninhydrin in 10mL ethanol
 Solution B: 0.0002M KCN in pyridine

The following were important note to be pondered during SPPS.

1. In Fmoc chemistry, the peptide is synthesized from C-terminus to N-terminus. Care was taken to confirm the identity of amino acid when determining the amino acid order of addition.
2. Piperidine is a highly flammable poisonous liquid that causes potential and permanent damage to skin or eyes when contacted with. Therefore care was taken to handle the chemical in the fume hood.

3. Many of the reagents and solvents can dissolve or corrode plastics. Therefore all lab wares used in the peptide laboratory were glass and organic solvent compatible pipettes.
4. KCN is insoluble in pure pyridine. To make the solution, an excess amount of KCN was dissolved in water and then diluted the solution to the desired concentration using pyridine.

The whole procedure was carried out under the fume hood following chemical safety and personal protection guidelines. The synthesized peptide with the resin was

- Washed with DMF followed by
- Wash with DCM and
- cleaved from the Fmoc resin for 2-3 hrs using the cleavage mixture:

%	Component	Volume added
90	Tri fluoro acetic acid	450µl
5	Thioanisole	25 µl
3	Ethylene dithiol (EDT)	15 µl
2	Anisole	10 µl
100	Total	500 µl

- Added the cleavage mixture to the peptidyl-resin.
- Mixed the solution periodically.
- The cleaved peptide was filtered through polypropylene filter to be delivered drop wise into cold ether at -20°C. The peptide gets precipitated in cold ether.

- The precipitate was centrifuged and washed with ether for 3 times to remove other impurities.
- The precipitate was dried under desiccation to be weighed, dissolved in buffer B (section 2.3.2) and purified by HPLC.

2.4.1 High Performance Liquid Chromatography (HPLC) for purification of peptides

Using a C8 analytical column (Zorbax) of 4.6 × 150 mm in a HPLC (Shimadzu) the peptide was analysed for its elution profile.

The buffer A: 0.045% of Trifluoroacetic acid (TFA) in water and the

Buffer B: 60% Acetonitrile with 0.045% of TFA in water.

The volume of peptide injected was 40 µl containing 0.2 mg of peptide.

The molecular mass of purified peptides was confirmed by Nanospray ionization.

Purified peptide was lyophilized and preserved in powder form in -20° C until used.

Table 2.5 Details of peptides used in fusion inhibition assay and in epitope mapping.

Region of E protein [@]	Sequence of peptide	Molecular mass
K291-300	K L Q L K G M S Y S (10mer)	1154.6238
S298-V309	S Y S M C T G K F K V V (12mer)	1349.6592
V309-G318	V K E I A E T Q H G (10mer)	1111.5742
T319-G328	T I V I R V Q Y E G (10mer)	1177.6575
V324-I335	V Q Y E G D G S P C K I (12mer)	1295.5936
P336-R345	P F E I M D L E K R (10mer)	1277.6558

H346-P356	H V L G R L I T V N P (11mer)	1218.7317
I357-N366	I V T E K D S P V N (10mer)	1101.5786
I367-I378	I E A E P P F G D S Y I (12mer)	1337.6259
I379-N390	I I G V E P G Q L K L N (12mer)	1280.7572
L389-I 398	L N W F K K G S S I (10mer)	1179.652
Peptides for fusion inhibition		
K406 – A425 (H1 helix of WNV(sf) stem)	K A F T T T L R G A Q R L A A L G D T A (20mer)	2061.13
K406 – A415	K A F T T T L R G A (10mer)	1046.93
Q416 – A425	Q R L A A L G D T A (10mer)	1014.2
G433- S452 (H2 helix of WNV(sf) stem)	G V F T S V G K A I H Q V F G G A F R S (20mer)	2064.09
G433 – A442	G V F T S V G K A I (10mer)	959.54
H443 – S452	H Q V F G G A F R S (10mer)	1104.55
G395 – L415 (H1 helix of DENV2 NGC)	G S S I G Q M F E T T M R G A K R M A I L (21mer)	2284.15
L425 – 448 (H2 helix of DENV2 NGC)	L G G V F T S I G K A L H Q V F G A I Y G A A F (24mer)	2423.30
R411 – H437 (Part of H1 + linker + H2 helices Of DENV2 NGC)	R M A I L G D T A W D F G S L G G V F T S I G K A L H (27mer)	2819.44

@ The sequence of envelope protein domain III from DENV 2 NGC (Alphanumeric in blue in the table) served as the template for the mimotopes. The sequence (alpha numeric in black in the table) of the stem

anchor region following the domain III of both DENV2 NGC and WNV marked as H1 and H2 in gray in Fig 1.21 were selected for fusion inhibition assay. The boxes shaded in light pink and grey form the peptides from the H1 and H2 helices respectively of WNV E and the boxes coloured cyan contain the peptides from the same region but belonging to DENV 2 NGC.

2.4.2. Cytotoxicity assay for peptides to be used as fusion inhibitors

- All peptides for cell based assay were dissolved in 90% DMSO and 10% of the growth medium to start with a 100X stock concentration. Peptides were diluted 1 in 100 (100 μ M) dilution from a stock of 10mM to have a concentration of 1% of DMSO or less so that there is no cytotoxicity due to high concentrations of DMSO.
- BHK-21 cells (1×10^4 cells in 50 μ l per well) in flat bottomed 96-well plates were added with 1 in 100 diluted peptides (100 μ M) in 10 μ l/well of RPMI-1640 with 10% FCS were grown overnight in a humidified atmosphere (37 °C with 5% CO₂). Three wells were included for cells with DMSO control.
- Reserved three wells as a background control (culture medium without cells) along with other appropriate controls (Ribavirin at a concentration of 800 μ M was used as a positive control for cytotoxicity).
- Added 10 μ l of premixed WST-1 Cell Proliferation Reagent (Clontech Laboratories, Inc.) to each well (1:10 final dilution).
- Plate was incubated for 2 hrs at 37° C in a humidified atmosphere maintained at 5% CO₂.
- Plate was shaken thoroughly for 1 min on a shaker to mix the water soluble red colour of the Formazan dye developed evenly.

- The absorbance of colour developed was measured at 440 nm with a reference filter at 690nm using a multi-well plate reader.

2.4.3 Assay of peptides as viral entry inhibitors

- Seeded 0.5×10^5 cells of BHK-21/well in 0.5 of complete growth medium for 24-well plate. Incubated overnight at 37°C in 5% CO₂ incubator.
- The stock concentration of each peptide was diluted in maintenance medium to have 1 in 100 dilution of each peptide stock solution in a volume of 100 µl.
- The peptide transfecting agent Chariot (Active Motif, Japan) stock was diluted in maintenance medium: 2 µl of 10-fold diluted Chariot reagent to 50 µl of maintenance medium per well.
- Fifty microlitre of the '1 in 100' diluted peptide were mixed with equal volume of Chariot reagent of fixed concentration and incubated at RT for 30 min.
- The mixture (100 µl) was overlaid on to the washed cell surface and 100 µl of maintenance medium was added to prevent the cells from drying (200 µl total volume per well). Incubated for 1hour at 37° C with 5% CO₂ so that the peptide was taken into the cells by the Chariot reagent.
- Added 250 µl of maintenance medium (Total volume is 450 µl).
- DENV2NGC virus was diluted to 500 PFU/well and a volume of 50 ul was added to wells to make the final volume as 500 µl to continue to incubate for 2 hrs at 37° C with 5% CO₂. The final concentration of the peptides in the wells was 1 in 500 dilution of the stock peptide concentration.
- Aspirated the medium containing peptide and virus from cells.
- Added 0.5 ml of CMC overlay and incubated at 37° C, 5% CO₂.
- Examined the plate for plaques after staining with 1% Crystal violet.

**FLAVIVIRUS ENTRY INHIBITION BY
HOMOLOGOUS RECOMBINANT
ENVELOPE PROTEIN
DOMAIN III**

3. FLAVIVIRUS ENTRY INHIBITION BY HOMOLOGOUS RECOMBINANT ENVELOPE PROTEIN DOMAIN III

Several receptors on various host cell surfaces have been proposed to interact with flavivirus E protein for entry. Among the three domains, the central and dimerization domains are highly interconnected both by secondary structures and by an inter domain disulfide bond, whereas the Ig-like domain III, which is free and folds itself independently, is a potential competitor to the whole virus for entry into host cells by blocking the host receptor. This chapter presents the results that were obtained from such inhibition studies using the different recombinant E protein domain III from flaviviruses.

3.1. Cloning of rEd3 constructs

Figure. 3.1. Cloning the constructs exemplified with WNV Ed3 (299 – 401 aa). **(A)** WNEd3 PCR product of ~ 400 bp fragment. **(B)** Shows 3 recombinant plasmids digested with restriction enzyme *PstI*. The recombinant construct acquires an additional *PstI* restriction site (position 552 of the construct in the WNEd3 construct map on Fig.2.1) through the insert and thereby with a single enzyme the former is cut into two fragments of ~4.8 and 1.2 kb size confirming the presence of the gene of interest. The exact DNA sequences of these constructs were confirmed by gene sequencing.

The cloning of Ed3 fragment into pET 16b expression vector is exemplified by WNV Ed3 (299-401aa) sequence. The amplified fragment (**Fig. 3.1A**) was inserted in between the *Xho I* and *Bam HI* restriction sites of the pET 16b vector. The recombinant plasmid, when digested with *Pst I* restriction enzyme, yielded two DNA fragments of 4.8 and 1.2 kb each (**Fig. 3.1B**), since the enzyme cut the vector as well as the inserted fragment. The sequence of the construct was confirmed by gene sequencing.

3.2. Expression and purification profiles of rEd3 from DENV1-4 and WNV

Among the 4 serotypes of DENV, two strains from DENV2 (NGC and TSV01) were chosen for the following reasons:

- All the four strains chosen belong to prototypes and have to be included as the standard practice to evaluate a new antibody but
- DENV2 –TSVO1 is a clinical isolate which was the origin of the antigen that elicited the mAb 9F12 characterized in the study.

Induction with 1mM IPTG resulted in the over expression of the rEd3 protein as a fusion protein of ~ 14 kDa (**Fig. 3.2**), when expressed in pET 16b vector or of ~29 kDa (**Fig. 3.3A**) with His and Trx tags, when expressed in pET 32b vector.

Recombinant domain III from DENV 1-4, when expressed in pET 32b (+) vector with N-terminal thioredoxin and 6X His tag, was partly in the soluble fraction and most of it was present in the inclusion body. The protein from the inclusion body was subjected to denaturation, affinity purification, refolding and finally gel filtration chromatography.

Figure. 3.2. Induction profile of recombinant E domain III exemplified by WNEd3. M- Molecular weight marker (2 lanes). UI – uninduced *E.coli* BL-21. Ind – Induced *E.coli* BL21. P – Cell pellet. IB – detergent washed inclusion body. Black arrow indicates the position of ~14kDa His tagged fusion protein of E domain III.

During gel filtration, the fusion proteins were eluted in a single peak but with a shoulder (**Fig.3.3A**). Also the fractions from the gel filtration chromatography, especially the peak fraction, showed a concentration dependent aggregation (the gel images in **Fig. 3.3A**).

The His/Trx fusion tag, present in the pET 32b vector, was followed by a D-D-D-D-K amino acid sequence susceptible to cleavage by the enzyme enterokinase. Cleavage of fusion tags from purified fusion proteins originating from both the soluble and insoluble sources required high concentrations of the enzyme enterokinase (each 500 μ g of fusion protein required 0.05 μ g of the enzyme). Despite high concentration of the enzyme, the cleavage was incomplete leaving behind ~20% of the fusion protein un-cleaved (lane marked 'C' in both the gel and blot images **Fig. 3.3B**). The fusion protein and cleavage products were analyzed by western blot using a specific mouse monoclonal antibody, mAb 9F12, which is characterized in the later part of this study. The antibody bound to both the fusion protein and the cleaved fraction but not to the His/Trx tag (**Fig. 3.3B** left panel).

Figure. 3.3. (A) Purification profiles of DENV1-4 Envelope protein domain III in pET 32b (+) vector. Most of the protein was in the insoluble fraction which was solubilized by denaturation followed by affinity chromatography, refolded and purified by gel filtration chromatography. Domain III fractions of ~29kDa protein (lanes marked 1, 2 and 3) from DENV1, 3 and 4 showed concentration dependant aggregation (markedly in lane 3 of all three serotypes). Domain III from DENV2 TSV01 (lanes 2 and 3) and from DENV4 (lane 4 and 5) showed a conspicuous dimer. **(B)** The Enterokinase cleavage of Thioredoxin tag from the expressed domain III is exemplified with Ed3 from DENV2 NGC. The gel insert on the left shows the His-Trx tag of ~18 kDa and the ~12kDa D2NED3 (lane marked 'C') from the ~29kDa fusion protein (lane marked 'F'). The western blot on the right shows the cleaved products (lane 'C') and the fusion protein (lane 'F') both of which are stained with a specific mouse monoclonal antibody, 9F12, characterized in the later

part of this study. The antibody did not detect the His/Trx tag. When the cleaved protein was subjected to Gel filtration chromatography after passing through NI-NTA column to remove the cleaved tags, the protein, though was eluted in a single peak, could not be recovered.

After cleavage, the His/Trx tag was removed by Ni-NTA affinity purification, followed by gel filtration chromatography. Though the cleaved protein was eluted in a single peak, the fractions were aggregated resulting in irrecoverable loss of protein. Therefore the construct was changed to pET 16b (+) vector with relatively smaller N-terminal 10X His tag cleavable with factor Xa, as compared to the big His/Trx tag (31kDa) of pET 32b vector, and the resulting expression of the protein in the insoluble pellet warranting an *in vitro* refolding.

3.2.1. Envelope protein domain IIIs were stable when expressed in pET 16b

Envelope protein domain III was expressed in the inclusion body (lane 'IB' in **Fig. 3.4**), which was treated with 8M urea buffer for denaturation and affinity chromatography. The protein bound to the Ni-NTA resin at 4°C for overnight. The protein was eluted (lane 'Ni' in **Fig. 3.4**) under similar conditions as that of the fusion protein from pET 32b. The refolded and concentrated protein (lane 'GF' in **Fig. 3.4**), purified finally by gel filtration chromatography, was stable as a monomer.

The His tag from the purified fusion protein was cleaved using factor Xa to obtain the ~12kDa protein which was calibrated against low molecular weight calibration markers mixed (lane 1 in the SDS-PAGE gel insert in **Fig. 3.5**) and loaded on to a Superdex 75 HR 10/30 Gel filtration column.

Figure 3.4 Affinity purification and refolding profile of recombinant E domain III exemplified by WNEd3 in a Coomassie blue stained (left) 15% SDS – PAGE gel and the detection of His tag with anti-His antibody by Western blot (Right). **IB** – detergent washed inclusion body. **Ni** – Eluted from Nickel column at pH 2.4. **M**- Molecular weight marker. **Ref** – Refolded and concentrated. **GF** – Protein after Gel filtration chromatography.

Figure 3.5 Chromatogram of low molecular weight calibration markers (left) and the refolded WNEd3 gel filtration profile (right). A mixture of low molecular weight calibration markers (Amersham) along with blue dextran 2000 (lane 1 of the gel image insert) was applied on to a Superdex 75 HR 10/30 Gel filtration column and was eluted using 0.05 M PBS with 2mM DTT at a flow rate of 0.1 ml per minute. Peaks A to E correspond to Blue Dextran 2000 (MW 2000 000), Bovine serum albumin (MW 67000), Ovalbumin (MW 43000), Chymotrypsinogen (MW 25000) and Ribonuclease A (13700) respectively. The 18% SDS – PAGE gel picture shows the lane 1 with the mixture of low molecular weight calibration markers (Amersham); lanes 2 to 6 corresponding to the elution fractions from peaks A to E. Lanes 7 and 8 were loaded with the FPLC fractions of WN Ed3 of ~12kDa (after cleavage of His tags by factor Xa)

from peak F (bottom figure) eluted under same conditions. Lane 9 shows the molecular weight marker.

The elution profile was found to correlate with monomeric mass of the protein, which is ~12 kDa. The N-terminal sequencing results confirmed the first ten amino acid residues of the protein (Personal communication from Dr. Jacques d'Alayer, Pasteur Institute, France, to Dr.Lescar).

In vitro refolding has been the method of choice for recombinant proteins expressed as insoluble aggregates in *E.coli*. All the six recombinant proteins which were refolded successfully, varied in their final yield. Maximum yield of protein was obtained with WNEd3 (12-15 mg/L) followed by DENV1Ed3, DENV2NEd3 and DENV2TEd3 (6-8 mg/L) followed by DENV3Ed3 and DENV4Ed3 (1-3mg/L) (**Fig. 3.6A**).

Successful refolding requires the confirmation of appropriate content of secondary structure by physicochemical and biological methods. In the present study, the refolded, cleaved and purified protein was subjected to circular dichroic spectrum (CDS) which revealed a predominant β sheet as the secondary structure (**Fig. 3.6B**). The spectrum was deconvoluted using standard software programs like CDNN or CONTIN and was found to have 45% of β sheet content (Chu & Rajamanonmani *et al.*, 2005) and the rest were unordered. This result is in accordance with a content of 45.3% of residues in a β -strand conformation as observed in the crystallographic 3D structure of the C-terminal domain of TBE virus, a related flavivirus (residues 301–395) (Rey *et al.*, 1995; PDB code 1svb). This is slightly higher than the 35% (residues 301–401) reported for the closely related rED3 protein of JE virus determined by NMR ((Wu *et al.*, 2003); PDB code 1pjw) and the 40% (residues 296-399) of β -sheet content of the DENV1 Ed3 crystal

structure ((Nelson *et al.*, Unpublished data) PDB code 3irc). The rest of the secondary structure content was found to be unordered in these structures reported.

Figure 3.6. (A) Envelope protein domain III from DENV1-4 and WNV in pET 16b (+) vector were expressed as inclusion bodies, denatured, refolded and purified by gel filtration chromatography. The purified and concentrated fractions of Ed3 fusion proteins (~14 kDa) are shown in the 18% SDS-PAGE gel stained with Coomassie brilliant blue. The two strains from DENV2, NGC and TSV01 are marked as N and T respectively below the image of the gel. (B) Appropriate refolding and secondary structure content were confirmed by circular dichroic spectrum (CDS) which is exemplified by domain III from WNV here. Deconvolution of the spectrum with CDNN and CONTIN revealed 45% of β sheet as the major secondary structure content of the protein which is in agreement with X-ray crystallographic data of Ed3 (Chu & Rajamanonmani *et al.*, 2005).

Murine polyclonal serum that was raised against the biophysically characterized WNVED3, generated in this study, neutralized 80% of WNV and 50% of DENV2 in a study carried out at National University of Singapore (Chu & Rajamanonmani *et al.*, 2005). This result evidences the conformational integrity of the refolded protein.

3.3. Inhibition of DENV1-4 with homologous rED3 III from DENV1-4 and WNV

Virus inhibition studies on DENV1-4 by competition with homologous recombinant Ed3 showed considerable level of inhibition (**Fig.3.7**) against DENV 2

(NGC and TSV01) with homologous recombinant Ed3 following a sigmoidal trend with logarithmic dilutions.

Figure 3.7. Competitive inhibition of DENV1-4 viral entry with homologous recombinant Ed3 quantified by plaque assay. While the Ed3's showed considerable inhibition of viral entry of DENV1-4, a negative control with the unrelated recombinant His tagged Nucleocapsid protein from IBV, a corona virus, showed no inhibition. While a sigmoidal dose response was seen with inhibition of DENV1-2 by homologous Ed3,

DENV3 and 4 showed a linear response. Error bars indicate the standard deviation of an average of triplicate.

The negative control used in this study, a purified recombinant fusion protein of the nucleocapsid from an unrelated virus (with <20% aminoacid sequence identity by alignment) namely IBV, a chicken corona virus, expressed in pET 16b had an N terminal His Tag (MW ~ 14 kDa) to match the DENVEd3 proteins with similar tag. Insignificant virus entry inhibition, that was shown by the IBV protein, rules out any possible contributions from the His tag. The DENV1 showed 92% inhibition with D1Ed3 following the similar trend as DENV2, whereas DENV3 and 4 showed almost a linear trend of inhibition. Effective concentration-50% (EC_{50}) values could be obtained for D1 ($21.3 \pm 0.06\mu\text{M}$), D2NGC ($0.13 \pm 0.04\mu\text{M}$) and D2TSVO1 ($0.11 \pm 0.02\mu\text{M}$) as the data could be fitted whereas the same for DENV3 and DENV4 could not be obtained. Recombinant Ed3 from all DENV1-4 were confirmed of their structural integrity by CDS as exemplified by WNEd3 before being tested for their competitive inhibition. While the sigmoidal dose response seen with DENV1 and 2 Ed3 inhibiting the respective viruses indicates a saturation of the host receptors by respective receptor binding domains, the linearity of DENV3 and 4 indicate lack of saturation of host receptor occupancy. This might be a result of possible degradation of a certain percentage of the soluble recombinant Ed3 proteins by cellular proteases *in vivo* leaving the former unsuitable for receptor binding. Also, viruses, if were present as large aggregates in the test, would be preferred to single soluble smaller molecules of the exogenous Ed3, by the host receptor since the whole purpose of host-virus interaction through a primary weak binding receptor is to concentrate viruses at the cell surface rather than individual host-virus interaction for a successful infection.

3.4. Discussion

Envelope protein, as purified aggregates by itself when expressed in insect cells through baculovirus expression system (Kelly *et al.*, 2000), or as virus-like particles when expressed in combination with other structural proteins as in yeast expression system (Sugrue *et al.*, 1997), have been found to be immunogenic in animals eliciting neutralizing antibodies. Since the first report in 2001 on the successful immunization of mice with tetravalent subunit vaccine (Simmons *et al.*, 2001), until a recent update on the state of the art of DENV vaccines (Swaminathan *et al.*, 2010), there are increasing evidences that recombinant flavivirus Ed3 could serve as a promising subunit vaccine. Some formulations were found to be more applicable to diagnostics rather than prophylaxis. Though the immunoglobulin-like domain is preferred for its small molecular mass with considerable immunogenicity when introduced into host system, so far no single formulation has been evaluated clinically.

Flavivirus Ed3, as the only receptor binding protein interacting with several of the host cell surface receptors, should be able to compete with the whole virus and thus can prevent viral entry. Application of envelope protein domain III subunits as antagonists is one of the possibilities in preventing flavivirus infections for they block the most crucial step of viral entry, the receptor-binding. In the present study, a dose response inhibition obtained with exogenous recombinant DENV1 and DENV2 Ed3s indicate competition of these proteins with viruses for receptors. Similar studies carried out by collaborators (at National University of Singapore) with the homologous recombinant Ed3, resulted in 60% inhibition of WNV and 30% of DENV entry into Vero cells (Chu & Rajamanonmani *et al.*, 2005) and further work from there with DENV1 and 2 but in

C6/36 and HepG2 cells have shown almost comparable results of 90% and 100% inhibition of DENV entry respectively (Chin *et al.*, 2007). Therefore competitive inhibition of viral entry exhibited by soluble recombinant Ed3 in this study serves direct evidence to the receptor binding property of flavivirus E protein domain III.

In a prospective study, recombinant Ed3 from DENV2 that included helix 1 of the E protein has been shown to block entry of DENV2 and also DENV1 by specifically inhibiting virus fusion at the cell surface and within the normal endosomal entry pathway and prevented both complete fusion and lipid mixing. A similar domain from the E1 protein of an alphavirus, SFV, did not inhibit DENV2 entry but inhibited the entry of SFV (Liao & Kielian, 2005). The authors suggested that the exogenous Ed3 proteins could function as inhibitors of class II fusion mechanisms and thus provide proof of principle of a dominant-negative inhibitor strategy. Among the three important targets on the flavivirus E protein: the β -OG binding pocket, the E protein rafts in the mature virus and the E protein homotrimer, this study demonstrated the key role of the domain III–core trimer interaction in virus fusion and infection as a target. Also there was cross-inhibition within alphaviruses and flaviviruses suggesting the conservation of the domain III contacts within the group. More detailed study on the key intermediate states of trimer formation of DENV2 E protein (Liao *et al.*, 2010) identified that the H1 of the stem region is essential for the fusion inhibitory function of the exogenous Ed3. The exogenous Ed3 alone did not show any fusion inhibition in the study.

Our observation of inhibition of viral entry by exogenous DENV Ed3 was based on the fact that the soluble domain competes with whole virus particle for the host receptor and thereby preventing the very early step of virus-host interaction rather than

the step of trimerization of the E protein leading to fusion with the endosomal membrane, a later event. While the successful fusion inhibition using rEd3 with H1 by Liao *et al*, (2001 & 2010), as a lead, opens up possible generation and screening of a library of structural homologues for fusion inhibitors, our results from the rEd3 can form the basis for a DENV entry inhibitor with exogenous domain III as a lead.

FLAVIVIRUS FUSION INHIBITION BY PEPTIDES COMPETING WITH THE STEM ANCHOR REGION

4. FLAVIVIRUS FUSION INHIBITION BY PEPTIDES COMPETING WITH THE STEM ANCHOR REGION

The series of events following the trimeric fusion of flavivirus envelope protein with the host cell endosomal membrane involve the helical regions of the stem anchor region (Fig. 1.19, 1.20 and 1.21 with the legends for details). Results of the efforts targeting these helical peptides to serve as potential lead compounds are described here.

4.1. Synthetic peptide purification and mass profile

Table. 4.1. List of synthetic peptides synthesized and purified in the study.

K406 – A425 (H1 helix of WNV(sf) stem)	K A F T T T L R G A Q R L A A L G D T A (20mer)	2061.13
K406 – A415	K A F T T T L R G A (10mer)	1046.93
Q416 – A425	Q R L A A L G D T A (10mer)	1014.2
G433- S452 (H2 helix of WNV(sf) stem)	G V F T S V G K A I H Q V F G G A F R S (20mer)	2064.09
G433 – A442	G V F T S V G K A I (10mer)	959.54
H443 – S452	H Q V F G G A F R S (10mer)	1104.55
G395 – L415 (H1 helix of DENV2 NGC)	G S S I G Q M F E T T M R G A K R M A I L (21mer)	2284.15
L425 – 448 (H2 helix of DENV2 NGC)	L G G V F T S I G K A L H Q V F G A I Y G A A F (24mer)	2423.30
R411 – H437 (Part of H1 + linker + H2 helices Of DENV2 NGC)	R M A I L G D T A W D F G S L G G V F T S I G K A L H (27mer)	2819.44

(A)

(B)

Figure. 4.1. (A) HPLC purification of synthesized peptides is exemplified here with H443-S452 (10mer peptide) along with the mass spectrometry result showing the major peak with the mass as 1103.5Da (upper pannel) and the same results for K406-A425 (20mer peptide) with a mass of 2062.2735Da. (B) Among the peptides synthesized a peptide

from DENV2, R411 – H437 (27mer), incorporating a part of H1 and entire H2 helices with the connecting linker region showed a predominant secondary content of alpha helical structure.

The Fmoc-SPPS resulted in a relatively pure form of the peptide as evidenced from the HPLC elution profile and mass spectrometry results (**Fig. 4.1A**). The helical propensity of the peptide R411-H437 (27mer) was shown by CDS (**Fig. 4.1B**). A positive peak at 190nm and two negative absorptions at 206 and 222 nm are suggestive of predominant α -helical structure (Castiglioni, 2001).

4.2. Cytotoxicity and entry inhibition by synthetic peptides

Figure. 4.2. Cytotoxicity^s and virus inhibition[#] by synthetic peptides.

^s Percentage of viable cells is proportionate to the intensity of colour developed by the conversion of tetrazolium salt by mitochondrial dehydrogenase to water soluble Formazan dye to give a red color. The highest concentration tested was 1 in 100 (100 μ M) diluted stock of the peptide solution (10mM) to have less than 1% of the final concentration of DMSO in the test wells, from which the percentage of viable cells has been recorded.

[#] Percentage of virus inhibition by the peptides was measured by plaque reduction assay with DENV2NGC only. Control wells with cells, cells

with peptide transfecting agent, Chariot, did not show any remarkable difference from the cell control.

The results of cell based assays to study the effect mimotopes resembling the stem anchor region from two of the flaviviruses, namely WNV and DENV2 (**Fig. 4.2**), reveal that the mimotopes from WNV have less cytotoxicity when compared to those from DENV2 at a concentration of 100 μ M. The drug Ribavirin, at a concentration of 100 μ M, exhibited 64% of cell survival (data not shown). The CC₅₀ of Ribavirin with BHK-21 cells is found to be 87 μ M (Noueiry *et al.*, 2007) which justifies the cytotoxicity observed with 100 μ M of the drug used in this study.

Peptide K406-A425 (20mer), belonging to the H1 helical region of WNV E stem-anchor, showed a 40% inhibition of DENV2NGC. The corresponding sequences from both WNV and DENV2NGC share a homology of 40% between themselves and with other members of flaviviruses might explain the low level of inhibition in the assay. Peptide R411-H437 from the H1 helical region of DENV2NGC, with a helical propensity by itself without the rest of the sequence of the native molecule, exhibited maximum cytotoxicity possibly indicating the cell permeability of the peptide. The protection rendered by other peptides included in the study were $\leq 20\%$ which require further confirmation by other assays like fusion inhibition assays.

4.3. Discussion

Michael's group identified a peptide DN59 (33mer) with high inhibitory activity against DENV at a concentration of 10 μ M. Also this peptide showed less toxicity and more specificity as it was not protective against Sindbis virus, an alpha virus but has a similar class II fusion protein (Hrobowski *et al.*, 2005). The success of this peptide and the failure of a shorter peptide in the current study, a 27mer R411-H437 (**Fig. 1.21**), can

probably be attributed to the absence of the C-terminal part of the H2 helix of DENV 2 stem anchor region. This view is supported by the fact that when a peptide with a scrambled sequence of the same stem region was tested for inhibitory activity, there was no inhibition confirming the sequence specific nature of the target helical binding pocket of the H1- H2 helix.

Table. 4.2. Inhibition of DENV 2 NGC by peptides from the stem anchor region studied by Hrobowski *et al* (2005)

Peptide	Sequence	Location ^a	Concentration (μM)	% Inhibition +/- SD
DN80	MVDRGWGNHAGLFGKGSIV	386-400	49.9	17 +/- 10
DN57	ANLVHTQWFLDLPLFWLPGADTQGSNNI	485-503	30.6	7 +/- 4
DN81	ANLVHRCWFLDLPLFWLPG	485-512	42.6	25 +/- 8
DN59	MAILGDTAWDFGSLGGVFTSIGKALHQVPGAIIY	692-724	29.0	93 +/- 2
WN82	VVDRGWGNAGLFGKGSID	396-410	52.5	4 +/- 13
WN53	TFLVHREWFMDLNLFWSSAGSTVWR	500-518	98.7	56 +/- 5
WN83	TFLVHREWFMDLNLFWSSA	500-524	128.0	70 +/- 2

^a numbering from the beginning of the E polypeptide in either DENV or WNV.

Studying the role of the membrane proximal helix and the stem region of flavivirus fusion using a set of recombinant proteins to mimic the low pH triggered E protein trimerization events in vitro, (Liao *et al*, 2010), it was shown that DIII and the stem region fold back to pack against the core trimer, locking the linker into position and preventing reversal of DI contacts. Concomitantly, these interactions generate the stable hairpin and drive membrane fusion. The results from this study have generated a model for the steps in rearrangement of the dengue virus E protein during membrane fusion. Another study (Schmidt *et al.*, 2010) employed a trypsin susceptibility assay on liposomal membrane fusion resulting in content mixing and a pyrene based fluorescence assay as a measure of hemifusion, revealed that the peptides containing the C-terminal half of the DENV2 stem could bind the sE postfusion trimer, while those derived exclusively from the N-terminal half could not. Peptide sequence 419–447 of DENV2

(DENV2^{419–447}) has the lowest K_d (~150 nM). Inhibition studies carried out with the same set of peptides that included the entire “helix II” segment were particularly found to be active. DENV2^{413–447} and DENV2^{419–447} were the strongest inhibitors; neither scrambled version, DENV2^{413–447(scram)} or DENV2^{419–447(scram)} had no effect on viral titre. The extent of binding correlated with the extent of inhibition. Also the authors have speculated how the binding of these stem peptides inhibit fusion despite their specific binding sites are not exposed until acidification in an endosome, that the peptides bind to transiently exposed sites as the E protein fluctuates among an ensemble of conformations. Epitope mapping of flavivirus group reactive antibodies 2C5.1 and 4G2, using synthetic peptides spanning the E protein, (Falconar, 2008) showed positive binding against the 101-WGNGCGLFG-109 peptide from the flavivirus-conserved fusion sequence with better binding when the Cysteine 105 was replaced with a Glycine. The W101, L107 and F108 are proven to be critical for membrane insertion and stabilization of fusion trimers. Substitution of the W101 with Alanine abolished the binding of fusion peptide to membranes (Melo *et al.*, 2009). This peptide, along with another peptide epitope sequence 393 to 401, was suggested to be better candidate for diagnostic or cross protection assays (Falconar, 2008).

In the present study, the synthetic peptides tested though were well tolerated by cells, the inhibitory effect were not remarkable. From the above studies cited, it is quite evident that the helical groove (**Fig. 1.19C & D**) is highly conformation specific and the peptides designed were not complete to occupy the entire groove to efficiently inhibit the trimerization. Other possible reason could be that the peptides might have been aggregated once dissolved into the medium from 100% DMSO which has been

eliminated by Schmidt *et al* (2010), by providing the peptide with a ‘solubility tag’ of sequence RGKGR at the C-terminus. Also the CMC overlay was not incorporated with the same concentration of the peptide which requires large quantities of the peptide resulting in the escalation of cost of therapeutics. Also the assay requires to be performed in a relevant cell line of human origin, like monocytes or dendritic cells, to be correlated clinically. More experiments are required with the peptides, to improve their permeability into cell, by tracking their target inside host cells by attaching fluorescent probes to the peptides. Also the synergistic effect of the peptides from this region in conjunction with other small molecules or among themselves, (eg. E101-109 and 393 -401) would probably be an objective for a prospective study.

**FLAVIVIRUS NEUTRALIZATION
BY SPECIFIC MOUSE MONOCLONAL
ANTIBODY AND ITS FRAGMENTS
AGAINST THE ENVELOPE
PROTEIN DOMAIN III**

5. FLAVIVIRUS NEUTRALIZATION BY SPECIFIC MOUSE MONOCLONAL ANTIBODY AND ITS FRAGMENTS AGAINST THE ENVELOPE PROTEIN DOMAIN III

Antibodies to flavivirus E proteins include many type, subtype-specific and cross reacting ones with decreasing potentials to neutralize virus infectivity in the same order as listed. Type specific antibodies against E domain III are considered the most efficient blockers of viral entry. This chapter focuses on the cross neutralizing potential along with other biochemical characterization of a mouse monoclonal antibody, 9F12 that was originally raised against E protein domain III from DENV 2 TSV01 strain, a clinical isolate from Townsville, Australia.

5.1. Purification profile of mAb 9F12

The results of the purification steps of the mAb 9F12 are provided in (Fig. 5.1).

Figure. 5.1. Purification of mAb 9F12. (A) Break through fractions of mAb 9F12 from DEAE (benchtop econocolumn) after precipitation with sodium sulphate on an 8% SDS-PAGE gel stained with Coomassie brilliant blue (B) Purified and concentrated 9F12 after gel filtration chromatography, run on a 12% SDS-PAGE gel and (C) Western blot of 9F12 under reducing (R) and non reducing (NR) condition probed with anti-mouse monoclonal antibody HRP conjugate followed by chemiluminescence detection.

The yield and purity of monoclonal antibody, as evidenced from Figures (5.1A & B), was found to be improved by the introduction of two steps in the protocol that eliminated the associated bovine serum albumin present in the tissue culture medium; the precipitation with 18% (w/v) Sodium sulphate powder where the majority of BSA was precipitated and the subsequent purification through Cibacron Hi Trap blue column (GE healthcare). The final concentration of mAb was about 9-12 mg from 1 litre of culture supernatant.

5.2. Dengue virus purification and quantification

DENV 2 passaged through C6/36 usually showed a titre of 10^6 PFU/ml. Upon concentration by either sucrose or glycerol-tartrate gradient, a viral titre of 10^8 PFU/ml was obtained. The results of purification steps are given in (Fig. 5.2).

The gradient purified virus through glycerol-tartrate gradient required an immediate buffer exchange through an Amicon concentrator (MWCO 100,000 kDa) with RPMI-1640, before proceeding to immune detection. The purified virus containing fractions in RPMI- 1640 could be stored frozen at -80°C for as long as 6 months without considerable loss in infectivity.

(A)

Figure. 5.2. DENV purification and quantitation by plaque assay. **(A)** Concentrated virus preparation from Glycerol-Potassium tartrate gradient was analyzed by Transmission electron microscopy after staining with either Uranyl acetate (left) or Phosphotungstic acid (middle and right). Field shows integral viral particles. **(B)** One ml fractions from the gradient numbered from 1-14 were subjected to ELISA or dot immunoassay. The plot shows the peak fractions (11-13) with high signals which correlate with the dot assay shown in (C). **(C)** Dot blot assay of the fractions diluted to a maximum of 10^{-6} show peak intensity with fractions 11-13 as seen with ELISA. The fractions correspond to 30% gradient. **(D)** Peak fractions were pooled together and quantified by plaque assay showed a titre in the order of 10^8 PFU/ml. Multidish of 4 X 6 wells with BHK-21 were used in the assay. Top three rows included tenfold dilutions of the virus (the log of dilutions marked on top of the plate as -3 to -8), each dilution was performed in triplicate per column and the lowest row incorporated both cell and virus controls in triplicates.

5.3. Plaque Reduction neutralization Test (PRNT 50%)

The PRNT50 results are shown in (Fig. 5.3). The 50% neutralization concentrations of 9F12 against the four dengue serotypes 1-4 were tested. The lowest to the highest concentrations required to neutralize the virus by 50% was in the order: DENV2 NGC ($3.23 \times 10^{-9} \text{M} \pm 0.70 \times 10^{-9} \text{M}$) $\approx$ DENV2 TSV01 ($3.61 \times 10^{-9} \text{M} \pm 0.87 \times 10^{-9} \text{M}$) < DENV 4 ($6.55 \times 10^{-9} \text{M} \pm 1.09 \times 10^{-9} \text{M}$) < DENV 3 ($8.6 \times 10^{-9} \text{M} \pm 0.93 \times 10^{-9} \text{M}$) < DENV1 ($1.72 \times 10^{-8} \text{M} \pm 0.12 \times 10^{-8} \text{M}$). The neutralization efficacy of 9F12 was comparable to that of mAb 4G2, the standard for cross neutralizing antibody but raised against E protein domain II especially to the fusion tip, following the same order of virus serotypes; DENV2 NGC ($5.82 \times 10^{-9} \text{M} \pm 0.41 \times 10^{-9} \text{M}$) $\approx$ DENV2 TSV01 ($6.02 \times 10^{-9} \text{M} \pm 0.81 \times 10^{-9} \text{M}$) < DENV 4 ($7.24 \times 10^{-9} \text{M} \pm 2.1 \times 10^{-9} \text{M}$) < DENV 3 ($9.42 \times 10^{-9} \text{M} \pm 1.79 \times 10^{-9} \text{M}$) < DENV 1 ($3.27 \times 10^{-8} \text{M} \pm 0.6 \times 10^{-8} \text{M}$).

Cell based adsorption assays were performed to identify the mode of neutralization adopted by 9F12 using Vero cells. Level of protection rendered by mAb 9F12 when introduced prior to adsorption was comparable to that of 4G2 which protected the cells both pre- and post-adsorption phases (indicating that the latter interferes a later phase than entry as compared to 9F12 which seems to block the very early step of viral entry to the host cell) (Fig. 5.4A). The fact that 4G2 is raised against the envelope protein domain II especially to the fusion tip, favours it to be an efficient fusion blocker (Summers *et al.*, 1989).

(A)

(B)

Figure. 5.3. Plaque reduction neutralization test (PRNT50%). (A) An example of a PRNT plate is shown here. The first column is a triplicate of cell control and columns 2 – 5 have the various tenfold molar dilutions of the antibody 9F12 with 100 PFU/well number of virus. The last column shows the virus control with 100 PFU/well. More than one plate had been used per virus wherever required. (B) Plot of the PRNT titres of the 5 strains of DENV tested in this study are shown here.

5.4. Mechanism of neutralization by mAb 9F12

Figure. 5.4. Mechanism of Neutralization by 9F12.

(A) Pre and post adsorption assay. The mAb 4G2 protected cells during and after virus challenge whereas mAb 9F12 protected the cells only when they were introduced prior to challenge. The naïve mouse serum included as a negative control showed no remarkable level of protection.

(B) Fusion inhibition assay using c6/36 cells. Mosquito cells produce syncytium (indicated by black arrows on the left pannel) when infected with DENV which makes the cells permeable to Propidium Iodide (PI) (indicated by black arrows on the right pannel) whereas intact healthy cells are not permeable to PI. Uninfected cells with definite cell borders (1) and mAb 4G2 (4) show no syncytium whereas 9F12 (3) and the virus infected cells (2) show extensive fusion.

(B)

In a cell based membrane fusion inhibition assay using c6/36 mosquito cells, the two antibodies, 4G2 and 9F12 were clearly differentiated as in (Fig. 5.4B). Uninfected or protected cells, as in the case of 4G2, with intact cell membrane are not permeable to Propidium iodide dye; whereas cells which have lost their cell membrane by fusion allow the dye to accumulate (extensive areas of syncytium are marked with black arrows in the image).

5.5. Sequencing of mAb 9F12 and construction of recombinant scFv

Figure. 5.5. Amplification of heavy and light chain sequences of mAb 9F12. Lanes 1 and 2 from left have molecular size markers of 1 kb and 100 bp lengths. Lanes marked L and H show the amplified products of light and heavy chains. The lane cDNA show the product of reverse transcription used as the template to amplify the heavy and light chain products.

The heavy and light chains were amplified and the sequence was obtained (Table. 5.1, 5.5 and Fig. 5.6). The amplified V_H and V_L genes were assembled into the scFv gene using a linker sequence. The scFv fragment has a $(Gly_4Ser)_3$ linker and also $VH-(Gly_4Ser)_3-VL$ orientation as shown in the schematic representation in the figure (Fig. 5.6C). The amplified products were cloned into pET 24a (+) vector and transformed into BL-21 *Escherichia coli* for expression of the recombinant single chain fragment variable (scFv) region.

Table. 5.1. Nucleotide and conceptually translated amino acid sequences of the V_H and V_L domains of mAb

(A) Heavy Chain

[illegible]

(B) Light Chain

[illegible]

Table. 5.1. Nucleotide and conceptually translated amino acid sequences of the V_H and V_L domains of mAb 9F12 (A) Heavy Chain - Nucleotides corresponding to the first three amino acid residues of the V_H domain have not been sequenced. (B) Light Chain- Nucleotides corresponding to the first 14 amino acid residues of the V_L domain have not been sequenced. Residue numbering and indicated hypervariable regions are according to (Kabat & Wu, 1991).

ctgcagcagctcgtgggctgagctggtaggcctggggcttcagtgaaagctgtcctgcaaggcttgggctacagatttactgactatgaaat
gtactgggtgaagcagacacctgcacatggcctggaatggattggaggtattcatccaagaagtggtaatactgctacaatcagaagttc
aaggacaaggccacactgactgcagacaaatectccagtcagcctacatggagctcagcagcctgacatctgaggactctgtgtctatt
actgtaccacgtccctctactggggccaagg
gaccacggtcaccgtctctca (Heavy chain)

ggggcggaggaggagtcggcgtgggggatcgggaggtggcgggtca (Glycine Serine Linker)

cttgagatcaagcctccatctcttgcagatctagtcagagcctgtacacagtaatggaaacacctatttacattggtagctgcagaag
ccaggccagctccaaagctctgatctacagcattttcaaccgattttctggggtccagacaggttcagtgccagtggtcaggagc
agatttcacactcaaaatcagcagagtgaggctgaggatctgggagtttattctgtctcaaggtacacatgttccgtggacgttcgg

(A)

10	20	30	40	50	60
LQQSGAELVR	PGASVKLSCK	ALGYRFTDYE	MYWVKQTPAH	GLEWIGGIHP	RSNGTAYNQK
70	80	90	100	110	120
FKDKATLTAD	KSSSTAYMEL	SSLTSEDSV	YYCTTSLYWG	QGTTVTVSSG	GGGSGGGGSG
130	140	150	160	170	180
GGGSLGDQAS	ISCRSSQSLV	HSNGNTYLHW	YLQKPGQSPK	LLIYSIFNRF	SGVPDRFSGS
190	200	210	220		
GGGSGGGGSG	GGGSGGGGSG	GGGSGGGGSG	GGGSGGGGSG	GGGSGGGGSG	GGGSGGGGSG

(B)

(C)

Figure. 5.6. Sequence and construct details of the recombinant single chain fragment variable incorporating a serine-glycine (SG₄)₃ linker. (A) Nucleotide sequence of heavy chain (1-327 nucleotides *italicized*) followed by 45 nucleotides coding the 15 aminoacid serine glycine linker (underlined) which ends with the sequence from the light chain fragment (sequence in bold). (B). The nucleotide sequence of the scFv conceptually translated into Aminoacid sequence. (C) The fused PCR product (~800bp) of heavy and light chains with Glycine-Serine (G₄S)₃ linker as shown in the schematic representation of the scFv gene on the right. The amplified and fused PCR product is inserted into pET 24a (+) shown in the construct map of **Fig. 2.3**.

Figure. 5.7. Expression profile of recombinant scFv with a final concentration of 1 mM IPTG. UI – uninduced cell suspension; I – Induced cell suspension; R - refolded protein after denaturation; P – purified fraction of scFv by gel filtration chromatography; M - Molecular weight marker.

Transformant clones when checked for expression in *E.coli*, had the protein in the insoluble compartment (**Fig. 5.7**) and therefore was subjected to denaturation and refolding.

5.6 Binding of mAb 9F12 to recombinant Envelope protein domain III or on virus surface

The ELISA results yielded the effective concentration (50%) when the data were fitted with Prism (Graphpad Prism version 5.0) (**Fig. 5.8A.**). Binding of soluble Ed3 from DENV2, 4 and remarkably WNV showed saturation with a sigmoidal response, Ed3 from DENV1 and 3 did not reach saturation implying the low affinity interaction of these two with the antibody. Immuno-fluorescent staining of infected cells (**Fig. 5.8B**) with 9F12 showed intensely ‘speckled and granular’ fluorescence whereas those with 4G2 showed bright, homogeneous and compact fluorescence indicating the distribution of envelope protein, and thereby the whole virus, mostly in the perinuclear region.

(A)

Figure. 5.8. Binding of mAb 9F12 to recombinant envelope protein domain III. **(A)** By ELISA. Microtitre plates coated with either the recombinant DENV1-4 or WNV domain III were incubated with tenfold dilutions of mAb 9F12. The binding was detected using an anti-mouse antibody conjugated to alkaline phosphatase and revealed using a PNPP substrate. The assay was carried out in triplicate and the data represents the mean with error bars indicating the standard deviation from mean. **(B)** By Immuno fluorescence. Cells of A549 were infected with DENV2 TSVO1 strain and fixed cells were probed with either mAb 9F12 (1 and 2) or 4G2 (3 and 4) stained with antimouse Alexafluor 488 conjugate. (1) Infected cells probed with 9F12 show granular 'speckled staining' (white arrows in (1)). Infected cells probed with 4G2 show bright and compact fluorescence (yellow arrows in (3)). Uninfected cells of A549 show no fluorescence with either 9F12 (in (2)) or with 4G2 (in (4)).

5.7. Epitope mapping by synthetic peptides as mimotopes

Synthetic mimotopes, spanning the entire length of the E domain III of DENV 2 NGC, was titrated against mAb 9F12 by ELISA. The results are presented in (**Fig. 5.9A**).

The blocking with 50 mM glycine in PBS-EDTA was preferred to blocking with BSA since the peptides were too small that virtually no signal was developed upon addition of secondary antibody and appropriate substrate. After a few trials with the procedure, a concentration of 10^{-5} M of peptides was found to be optimal to differentiate a positive (OD of 0.8) from the negative (OD of 0.1) reaction of peptides.

(A)

Figure. 5.9(A). Epitope mapping by peptide mimotopes. Binding of mAb 9F12 to DENV2 Ed3 and peptides covering the Ed3 sequence K291-I398. ELISA plates coated with either Ed3 or the peptides were reacted with 9F12. The experiment was carried out in triplicate and the error bars show the standard deviation from the mean absorbance. The insert is a model of domain III showing the epitopes recognized by mAb 9F12 based on the yeast surface display results. Residues forming the mAb 9F12 epitope (dotted spheres - K305 and 307) and shared by the mAb 1A1D-2 (Lok *et al*, 2008) are labeled in blue. The one extra residue G330 which is recognized by the mAb 9F12 is marked in black. Loops BC, DE and FG and the N-terminal region (NTR) are colored in blue. The solvent-inaccessible loop AB (in the context of the viral particle) is marked with a black arrow.

(B)

Figure. 5.9(B). Epitope mapping by yeast display and peptide mapping. Flow cytometry histograms of mAbs binding to yeast expressing wild type or mutant domain III. The following antibodies were used as negative and positive staining controls, respectively for each of the indicated yeast based on data from a prior publication : Wild type: WNV E16 and 3H5-1; K305E: 1A1D-2 and 3H5-1; K307Q: 1A1D-2 and 3H5-1; K310E: 1A1D-2 and 3H5-1; P384A: 3H5-1 and 5A2-7; G330D: 6B6-10 and 3H5-1. In each case, staining with 9F12 is shown in blue. The data are representative of three independent experiments.

The above results could identify the segments containing the epitopes for 9F12 but in an effort to confirm the residues contributing to the binding, a collaborative approach was sought with the Departments of Medicine, Molecular Microbiology, Pathology & Immunology, Washington University School of Medicine, St. Louis, USA for screening their epitope library display on yeast surface (Sukupolvi-Petty *et al.*, 2007).

Yeast surface display of DENV-2 Domain III mutants: The DNA fragment encoding amino acid residues 294 to 409 (Domain III) of DENV-2 E protein was

expressed on the surface of yeast as an Aga2 fusion protein as described (Sukulpolvi *et al* 2007). DENV-2 Domain III mutants that were generated as part of a random library by error prone mutagenesis in the pYD1 vector were expressed on the surface of yeast as described (Sukulpolvi *et al* 2007). Wild type or mutant DENV-2 Domain III displayed on yeast were harvested, washed with PBS supplemented with BSA (1 mg/ml) and stained with 50 µl of diluted mAbs (9F12, 3H5-1). After 30 minute incubation on ice, yeast were washed in PBS with BSA and then stained with a goat anti-mouse IgG secondary antibody conjugated to Alexa Fluor 647 (Molecular Probes, Invitrogen Carlsbad CA). After fixation with 1% para-formaldehyde in PBS, yeast cells were analyzed on a FACS Scan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ) using Flo-Jo software. The results of epitope mapping by Yeast surface display are presented in (Fig. 5.9B).

5.8. Discussion

The presence of virus-specific antibodies *in vivo* can inhibit virus entry and spread through several mechanisms, including the direct neutralization of virus infection. Such potent neutralizing antibodies are not present during clinical infection for unknown reasons that the antibody response is dominated by non-neutralizing and less potent antibodies. To this end, a preliminary screening of a group of 8 DENV infected mice sera were screened for the presence of anti domain III antibodies with a control group of uninfected mice sera (Fig. 5.10 & legend). Sera from 5 mice showed significant levels of anti DENV2Ed3 antibodies on day 10 by when the viremia is already cleared in the primary infection. Immuno-pathological study of WNV (Oliphant *et al.*, 2007) suggested that highly neutralising virus-specific antibodies of the IgG subtype do not appear in detectable levels during primary infection of mice until days 10-15, by the time the

viremic phase is completed. This phase is very critical during the clinical infection since presence of such type specific antibodies become unworthy or deleterious when the individual develops infection with a second serotype leading to complications of DF into DHF or DSS. Moreover, analysis of convalescent serum samples from WNV-infected humans showed that a significant proportion of individuals never developed antibodies to the neutralizing epitope located on the domain III-lateral ridge. Likewise, potent neutralizing human mAbs to domain III are rarely isolated from WNV-infected patients (Throsby *et al.*, 2006). The antibody responses thus appear to be skewed toward the induction of less neutralising antibodies particularly to the fusion loop. Overall, the IgG response to domain III appears variable and comprises only a small fraction of the whole antibody response. Thus, for unclear reasons, the development of neutralizing antibodies that bind WNV domain III appears to be an uncommon event in humans, in contrast to experimental infections in C57BL/6J mice. In a preliminary experiment of mouse protection assay with 9F12 and DENV2 performed in our lab, a significantly reduced amount of the viral NS1 protein was observed (data not shown), suggesting that the 9F12 antibody may retain neutralization activity *in vivo*.

In the present study, the antibody 9F12 was able to bind to its immunogen, envelope protein domain III either directly to the soluble recombinant protein or when it is still present on the virus surface, seen by immunofluorescence, indicating that the epitopes are readily accessible for interaction. The difference in the pattern of fluorescence between the mAbs 4G2 and 9F12 could probably due to the fact that the former detects epitopes on E domain II on mature virus only and the latter detects the epitopes from E domain III of both mature and immature virus particles. Also the

efficiency of binding was evident through its relatively low values of concentrations required for both binding and neutralization *in vitro* against all the 4 serotypes tested in this study. To obtain more insight into the binding kinetics, surface plasmon resonance (SPR) was performed in collaboration with Asso. Prof. Geifman Shochat, at the Drug Discovery Centre of School of Biological Sciences, NTU (Results in Annexe 1 for comaparison with ELISA results).

Figure. 5.10. Infected mouse sera were tested for the presence of antibodies to D2TEd3 by ELISA. Sera from a group of 8 mice collected on day 2, 4, 6, 8 and 10 of post infection with DENV-2 (TSVO1) along with a group of 8 naive mice as negative control were included in the study. D2TEd3 was coated on plate and the mice sera were added at a dilution of 1 in 10 in duplicate and probed with anti-mouse secondary antibody conjugate and PNPP substrate. Sera from five mice show the presence of antibody at significant levels on day 10.

Several cellular attachment molecules or receptors have been proposed for DENV including the highly sulfated heparan sulfate (Chen, 1997), the dendritic cell-specific ICAM3-grabbing-non-integrin (DC-SIGN) on immature dendritic cells (Pokidysheva *et al.*, 2006; Tassaneetrithep *et al.*, 2003), the L-SIGN (Navarro-Sanchez *et al.*, 2003), the

mannose binding receptor on macrophages (Miller *et al.*, 2008) and the laminin-binding protein (Thepparit & Smith, 2004; Tio *et al.*, 2005). Despite this variety of host cell surface receptors identified, the one viral component that was consistently shown to bind directly to cellular receptors is the Ig-like domain III of the E protein (Beasley & Barrett, 2002; Hung *et al.*, 2004; Lin & Wu, 2003; Mandl *et al.*, 2000; Thullier *et al.*, 2001). Subtle structural variations at the surface of domain III are likely to influence the interactions of the virus with attachment molecules (cell tropism) and also with antibodies from the host that define serotypes.

Blocking the virus-receptor interaction is an important mechanism of neutralization, but clearly not the only one. The results of adsorption assay experiments in the present study indicate that mAb 9F12 blocks virus entry by binding to epitopes, which directly or indirectly are involved in receptor binding. The epitopes identified on the secondary structure of the Ed3 by 9F12 show that the epitopes are accessible on the virion surface as seen in the quaternary structure (Fig. 5.11 & 5.12).

Figure. 5.11. Mapping the epitopes of mAb 9F12 and 3H5 on the tertiary structure of DENV E protein dimer (PDB ID: 1OAN). The epitope residues recognized by 9F12 confirmed by Yeast display, marked in black alphabets with their numbers in the E protein sequence, are represented by green spheres in the cartoon. The ribbon diagram of E protein (red) drawn in Pymol is shown in side view with the bottom side facing the viral membrane. The mAb 9F12 epitopes are present away from the membrane and protrude on the virion surface. The blue spheres on the left of the image point the major epitopes recognised by the type specific 3H5:

residues G304, E383 and P384, which are inaccessible as they are closer to the membrane.

Figure. 5.12. Mapping of Mab 9F12 epitopes on the quaternary structure of DENV E protein dimer showing the five, three and two fold axis of icosahedral symmetry (PDB ID: 1K4R). The epitope residues confirmed by Yeast display are marked in dark blue on the domain III which is lightblue. The C-alpha tracing of E protein scaffold (pink) of half virus structure (for reasons of clarity) drawn in Pymol is shown from top view at the five fold axis with the other side facing the viral membrane. The accessibility of the epitopes is clearly evident at the periphery of this half virus structure.

Several neutralizing antibodies to flavivirus domain III proteins (Lisova *et al.*, 2007) have already been identified: their epitopes cluster onto the top lateral surface of the Ig-like domain. A structurally well-characterized interaction involves mAb E16 with domain III from WNV (Nybakken *et al.*, 2005). mAb E16 binds to an epitope composed of residues 302-309, (the N-terminal part of the lateral ridge marked NTR in **Fig. 5.9A** insert) and three loops BC (330-333), DE (365-368) and FG (389-391). Neutralizing mAbs, particularly to DENV, were proposed to bind two structurally distinct epitopes centered either on the FG loop (E383 and P384 in **Fig. 5.11**) , as in the case of mAb 3H5 (Gromowski *et al.*, 2008), or to the more conserved A strand like mAb 1A1D-2 (Lok *et*

et al., 2008). By contrast, several cross-reactive mAbs that bind to residues from the AB loop (313 - 319) were found to be poorly neutralising as these epitopes are not exposed at the surface of the E protein dimer and point inward toward the lipid bilayer in the mature viral particle. The relatively strong neutralizing capacity of mAb 9F12 can thus be partly attributed to the fact that it binds an epitope centered at the solvent exposed and easily accessible 'A' strand (K305 and K307 marked in **Fig. 5.11**) and the BC loop (marked in **Fig. 5.9A** insert) involving the residue G330 marked in **Fig. 5.11**. Whether a smaller area of contact between 9F12 with one or more conserved residues at the surface of domain III, does favor cross-reactivity must await structural studies. Also the epitope screening has been carried out against peptide sequences or Ed3 epitopes of DENV2 only. Whether the antibody recognizes any other epitope residues on Ed3 from other serotypes need further characterization. Comparing the domain III sequences between the two strains of DENV2, the change N390S (NGC→TSVO1) rather than the commonly seen N390D is noteworthy as this residue has been implicated in host cell receptor binding and blockage of this receptor with an antagonist prevented infection (*Leitmeyer et al.*, 1999). As the epitope cluster of mAb 9F12 does not involve this residue, N390S which is located on the end of 'G' strand (marked with a blue arrow above the alignment in **Fig. 1.21** and the β sheet structure drawn in green colour in **Fig. 5.9A** insert), both strains of DENV2 included in this study had a comparable binding and neutralization efficacy. Amongst residues of Ed3 that form the epitope recognized by mAb9F12, K305 and G330 are shared by DENV2 and 4 and K307 by DENV2, 1 and WNV. The mutations of these residues did not seem to affect binding of domain III-lateral ridge antibodies on yeast (*Sukulpolvi et al* 2007). Studies by (*Gromowski et al.*, 2008), have used analogous mutations with recombinant domain III implying that the changes are more likely local

rather than with long range effects. In the case of mAbs E16 and 1A1D2, the major epitopes were revealed earlier by yeast surface display and was later confirmed by crystallography (Lok *et al.*, 2008; Oliphant *et al.*, 2005). Thus, more insight into the interactions of 9F12 to domain IIIs would require crystallization of the complex.

The potential of an avid cross reactive antibody such as 9F12 can be applied to the development of therapeutic intrabodies. Intracellular antibodies, or intrabodies, are antibody fragments that are expressed in the cell and targeted to certain compartments of the cell, where they perform inhibitory functions by targeting specific proteins. There are several mechanisms of inhibition by intrabodies – they disturb molecular interactions like protein-protein interactions. Antibody fragments in the form of scFv derived from hybridoma cells producing mAbs directed against HCV core protein were intracellularly expressed in ER of cells (Heintges *et al.*, 1999). Direct or steric inhibition of viral core protein functions (multimerization, RNA packaging) by such intrabodies could thus interfere with virus replication.

The availability of a single high affinity antibody that could efficiently neutralize all four DENV serotypes early enough during the viremic phase may rapidly reduce the viral load and possibly prevent progression to severe vascular leakage syndrome, which occurs at a later phase during the natural history of infection in a subset of dengue patients. In the absence of a fully validated tetravalent vaccine and given the potential risks posed by antibody-dependent enhancement effects, treatment via passive immunotherapy appears as a possible alternative, provided an early-enough diagnostic of infection by dengue can be made. In this respect, the mouse mAb 9F12 that cross-reacts

with all four serotypes of DENV and strongly neutralizes virus infection, is a possible candidate for further rounds of affinity maturation and humanization.

Annexe 1: Binding affinity of mAb 9F12 or its scFv to rEd3s by surface plasmon resonance (SPR)

Binding affinities of mAb 9F12 or scFv9F12 for various DENV domain III serotypes were determined by SPR at 25°C using a Biacore 3000 instrument. Each domain III was covalently immobilized on a carboxymethylated sensor surface (CM5, research grade) using amine coupling chemistry. The surfaces were activated with 0.2M EDC (N-ethyl-N'-[3-(diethylamino) propyl] carbodiimide) and 50mM NHS, (N-hydroxysuccinimide) for 10 minutes. Following covalent binding, surface deactivation was performed using 1M ethanolamine-HCl (pH 8.5) for 10 min, at a flow-rate of 10µl/min. The reference surface was treated as the ligand surfaces except that protein injection was omitted. For the determination of kinetic parameters, either mAb9F12 or the scFv was passed above the reference and protein surfaces in duplicates at five to seven concentrations, in HBS (10mM Hepes buffer, pH 7.4, 150mM NaCl, 3.4mM EDTA and 0.005% P-20), at a flow rate of 30 µl/min. Surfaces were regenerated using the same flow-rate by a 15 µl injection of 50mM HCl. The level of immobilization for each serotype is specified in the figure legend, and was chosen to avoid mass transfer limitation. For the bivalent mAb and scFv, sensorgrams were fitted using the bivalent analyte model and the results are presented in (**Fig. 5.13**) and (**Table. 5.2**).

Figure. 5.13. Examples of sensorgrams obtained for the interactions between domain III of E protein of DENV 1, 2, 3 and WNV with either mAb9F12 or scFv9F12 and the fit for their respective interactions. All interactions with mAb and scFv were best fitted with the bivalent analyte model from Biaevaluation software 4.1. This model describes the binding of a bivalent analyte to immobilized ligand, where one analyte molecule can bind to one or two ligand molecules. Binding to the first ligand molecule is described by a single set of rate constants, so that the two sites on the analyte are equivalent on the first step. Binding of the second ligand molecule is described by a second set of rate constants allowing the model to take cooperative effects into account. The affinity is an approximation since it is calculated from the ratio of the kinetic constants k_{d1}/k_{a1} which describes the main part of the interaction. For the mAb 9F12 this model is an obvious choice. For the scFv, the bivalent analyte model gave a better fit suggesting that a large part of the scFv molecules were not monomeric (Dolezal *et al.*, 2000; Kortt *et al.*, 1994). Indeed gel filtration analysis of scFv showed that the protein elutes as a dimer (data not shown), and the bivalent analyte model describes the interaction in a more accurate way than the 1:1 Langmuir model.

Table 5.2. Kinetic constants and binding affinities of mAb 9F12 and scFv9F12 for the rEd3 from the different DENV serotypes and WNV by SPR.

	scFv			mAb 9F12		
Serotype	k_a^* ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)	k_{a1} ($M^{-1}s^{-1}$)	k_{d1} (s^{-1})	K_D' (M)
D1Ed3	3.5×10^4	3.0×10^{-3}	8.7×10^{-8}	2.2×10^5	3.7×10^{-3}	1.6×10^{-8}
D2NEd3	4.0×10^4	3.9×10^{-5}	1.0×10^{-9}	1.1×10^5	1.0×10^{-4}	1.0×10^{-9}
D2TEd3	N.D.	N.D.	N.D.	2.5×10^5	1.2×10^{-3}	4.9×10^{-9}
D3Ed3	2.2×10^5	8.5×10^{-3}	4.0×10^{-8}	2.2×10^5	1.8×10^{-2}	8.1×10^{-8}
WNEd3	5.4×10^4	4.2×10^{-4}	7.8×10^{-9}	1.1×10^5	3.9×10^{-5}	3.4×10^{-10}

*The constants were evaluated from the sensorgrams depicted in Fig 5.10, using the bivalent analyte model for both the mAb and the scFv. The BIAevaluation 4.1 software was used for data analysis.

K_D' is an apparent affinity calculated by the ratio k_{d1}/k_{a1} obtained from the fit with the bivalent analyte model. In all cases, χ^2 was lower than 5% of Rmax.

SUMMARY AND PERSPECTIVES

6. SUMMARY AND PERSPECTIVES

The results of the present study indicate that

- Envelope protein domain III is the receptor binding domain and that soluble recombinant domain III was able to compete with whole virus for entry into host cells.
- The coiled-coil stem anchor region following the envelope protein domain III is present in a highly specific binding pocket for designing competitive inhibitors to prevent late events of entry like fusion.
- The mAb 9F12 proves to be a relatively potent antibody with efficient binding and neutralization capacities independent of the Fc portion as shown by the binding of recombinant scFv to soluble domain III.
- The mAb 9F12 has accessible surface epitopes on both the soluble recombinant E domain III and the same on viral surface.
- The paratopes of mAb 9F12 forms a concave surface of interaction with its epitopes by virtue of its short CDR3 stretching to clamp the residues, a possible explanation to the relatively high binding affinity of the former.
- Highly potent neutralizing antibodies are not available during experimental infection; a situation parallel to clinical infection and the late appearance of such antibodies require to be replaced with avid cross neutralizing antibodies or its fragments to avoid the dangers of DENV infections through antibody dependent enhancement.

- With its relatively low concentrations required for neutralization, mAb 9F12 is a potential candidate for affinity maturation and humanization.

6.1. FUTURE PERSPECTIVES

The results of the current study require further extensions on the following aspects:

- Structural information regarding the interaction between the mAb 9F12 or its fragments with the E domain III by co-crystallization of the two and X-ray crystallography will open more venues for application of the antibody into clinical research.
- As a potential candidate, mAb 9F12 require further affinity maturation and humanization to avoid the complications of administering mouse components into human system.
- The effectiveness of this antibody needs to be characterized in vivo neutralization of DENV infections by using animal models e.g. AG- 129 mice.
- Since the mAb 9F12 identifies the E domain III on host cells, an earlier component to interact with the host when compared to the 4G2 recognizing the fusion tip, a later component in infection, the former may find its application into the laboratory diagnosis of early DENV infection of peripheral blood mononuclear cells without requiring much modifications of the antibody but simple conjugation to fluorophores.

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