ANTIBODIES FOR DIAGNOSIS AND TREATMENT OF FLAVIVIRAL INFECTIONS

FIELD

The present invention relates to the fields of medicine, cell biology, molecular biology and biochemistry. In particular, it relates to treatment and diagnosis of diseases, in particular flaviviral diseases such as dengue and West Nile Virus, as well as compositions for such use.

BACKGROUND

Dengue virus, a member of the *Flaviviridae* family, is responsible for over 20,000 deaths per year. Dengue is the most significant mosquito-born viral disease affecting humans. Up to one third of the world's population is at risk of dengue infection. It has been estimated that there are 50-100 million cases of dengue fever and 250,000 to 500,000 cases of dengue haemorrhagic fever each year.

Dengue is a small single-stranded RNA virus of the family *Flaviviridae* comprised of four distinct serotypes (DEN1-4). Its genome consists of a single open reading frame directing the synthesis of a polypeptide which is cleaved by viral and host proteases into ten viral proteins. These include three structural proteins, core (C), envelope (E) and membrane (M), synthesized in precursor form (prM), and seven non-structural (NS) proteins {Rigau-Perez, 1998}.

Infection by dengue virus may result in a spectrum of clinical manifestations. These range from asymptomatic infection through dengue fever (DF) to dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS).

Despite the seriousness of dengue-related disease, a complete understanding of dengue pathogenesis remains elusive. It has been shown that higher virus titres during the early stages of dengue fever correlates with progression to the more severe dengue haemorrhagic fever. However, the lack of proper diagnostics and markers for monitoring the disease progression adds difficulties in predicting the severe outcome. Neither a licensed drug nor a vaccine has been approved to treat severe conditions caused by infection with dengue virus like dengue haemorrhagic fever. Biomarkers for monitoring the disease course and outcome are also critical in determining the proper treatment of the disease.

Other flaviviruses such as West-Nile, Yellow Fever or Japanese Encephalitis viruses are important human pathogens of global concern (Gubler, 2006, Halstead, 2007, Keller et al., 2006).

SUMMARY

We describe an antibody raised against domain III of dengue virus envelope glycoprotein (E), which we designate 9F12.

We demonstrate that antibody 9F12 is capable of neutralizing dengue virus. The antibody is capable of cross-reactivity and is able to effectively neutralize dengue virus from a number of different serotypes such as DENV 1, 2, 3 and 4. The antibody is further capable of binding to West Nile Virus. We disclose the epitope on dengue envelope glycoprotein (E) that is bound by 9F12.

We disclose the variable region of 9F12 and show that Fab fragments, as well as single chain Fv fragments, comprising this variable region, have the same cross-reactive and strongly neutralizing activities. Accordingly, we provide for immunoglobulins based on this variable region for the treatment and detection of flaviviral infections, including dengue and West Nle Virus.

According to a 1st aspect of the present invention, we provide an polypeptide capable of binding to a dengue envelope glycoprotein (E) polypeptide, in which the polypeptide is capable of binding to an epitope bound by antibody 9F12, or a variant, homologue, derivative or fragment thereof.

The polypeptide may comprise an immunoglobulin. The polypeptide may comprise an antibody. The polypeptide may comprise a Fab fragment. It may comprise an single chain Fv.

The epitope may comprise residues K305, K307, K310 and G330 of a dengue envelope glycoprotein (E) sequence, with reference to the position numbering shown as SEQ ID NO: 2.

The polypeptide may comprise the variable region of monoclonal antibody 9F12 (SEQ ID NO: 4, SEQ ID NO: 6).

There is provided, according to a 2nd aspect of the present invention, an polypeptide comprising the variable region of monoclonal antibody 9F12 (SEQ ID NO: 4, SEQ ID NO: 6), or a variant, homologue, derivative or fragment thereof which has at least 90% sequence homology and is capable of binding to a dengue envelope glycoprotein (E) polypeptide.

The polypeptide may be capable of binding to any one or more of envelope glycoprotein (E) from dengue virus serotype I, II, III and IV. The polypeptide may be further capable of binding to envelope glycoprotein (E) from West Nile Virus. The polypeptide may be capable of binding to domain III of envelope glycoprotein (E). It may bind with a EC_{50} binding affinity of 1μm or below. It may bind with an affinity of 100 nm or below. It may bind with an affinity of 90 nm or below or 80 nm or below. It may bind with an affinity of 70 nm or below, 60 nm or below, 50 nm or below, 40 nm or below, 30 nm or below, 20 nm or below, 10 nm or below, 5 nm or below, 4 nm or below, 3 nm or below, 2 nm or below, 1 nm or below. It may bind with an affinity of 0.5 nm or below, 0.4 nm or below, 0.3 nm or below or 0.2 nm or below.

The antibody may be capable of inhibiting a biological activity of envelope glycoprotein (E). The activity may comprise receptor binding activity. The activity may comprise homotrimerization activity. The activity may comprise virus absorbtion to host cells.

The polypeptide may be capable of neutralizing a flavivirus. The polypeptide may be capable of neutralizing dengue virus, such as from serotype I. It may be capable of neutralizing dengue virus from serotype II. It may be capable of neutralizing dengue virus from serotype III. It may be capable of neutralizing dengue virus from serotype IV. It may be capable of neutralizing dengue virus from one or more of serotypes I, II, III and IV. It may be capable of neutralizing dengue virus from each of serotypes I, II, III and IV. The polypeptide may be further capable of neutralizing West Nile Virus. The neutralizing activity may be as measured in a plaque-reduction neutralization assay. The polypeptide may be capable of neutralizing virus with a $PRNT_{50}$ of 10^{-6} or below. It may have a $PRNT_{50}$ of 2×10^{-7} or below. It may have a $PRNT_{50}$ of 10^{-7} or below, $PRNT_{50}$ of 10^{-8} or below or a $PRNT_{50}$ of 10^{-9} or below.

The polypeptide may comprise a monoclonal antibody. It may comprise monoclonal antibody 9F12. It may comprise a humanised monoclonal antibody. It may comprise an Fv. It

may comprise an F(ab'). It may comprise an $F(ab')_2$. It may comprise a single-chain Fv (scFv) fragment. It may comprise a single chain Fv fragment comprising VH sequence (SEQ ID NO: 4) and VL sequence (SEQ ID NO: 6). The polypeptide may comprise the single chain Fv fragment scFv9F12 (SEQ ID NO: 8).

We provide, according to a 3rd aspect of the present invention, a pharmaceutical composition comprising an polypeptide according to the 1st or 2nd aspect of the invention, together with a pharmaceutically acceptable excipient, diluent or carrier.

As a 4th aspect of the present invention, there is provided an polypeptide according to the 1st or 2nd aspect of the invention or a pharmaceutical composition according to the 3rd aspect of the invention for use in: (i) a method of treatment or prevention of a flaviviral infection including dengue and West Nile Virus infection, preferably in which the method comprises administering a therapeutically effective amount of the polypeptide or composition to an individual suffering or suspected of suffering from a flaviviral infection including dengue and West Nile Virus infection; or (ii) a method of diagnosis of a flaviviral infection including dengue and West Nile Virus infection.

We provide, according to a 5th aspect of the present invention, a diagnostic kit comprising an polypeptide according to the 1st or 2nd aspect of the invention or a pharmaceutical composition according to the 3rd aspect of the invention together with instructions for use in the diagnosis of a flaviviral infection including dengue and West Nile Virus infection.

The present invention, in a 6th aspect, provides a polypeptide comprising a sequence shown as SEQ ID NO: 4 or SEQ ID NO: 6, or both, or a variant, homologue, derivative or fragment thereof which is capable of binding envelope glycoprotein (E).

In a 7th aspect of the present invention, there is provided a nucleic acid comprising a sequence shown as SEQ ID NO: 3 or SEQ ID NO: 5, or both and which is capable of encoding a molecule according to the 1st or 2nd aspect of the invention, or a variant, homologue, derivative or fragment thereof which is capable of encoding a polypeptide having envelope glycoprotein (E) binding activity.

According to an 8th aspect of the present invention, we provide a cell comprising or transformed with a nucleic acid sequence according to the 7th aspect of the invention or a descendent of such a cell.

We provide, according to a 9th aspect of the invention, a method of producing an polypeptide according to the 1st aspect of the invention, the method comprising providing a cell according to the 8th aspect of the invention and expressing the polypeptide from the cell.

There is provided, in accordance with a 10th aspect of the present invention, a method of detecting a flavirus-infected cell, such as a dengue-infected cell and West Nile Virus-infected cell, the method comprising exposing a candidate cell to an polypeptide according to the 1st aspect of the invention and detecting expression of envelope glycoprotein (E) polypeptide by the cell.

As an 11th aspect of the invention, we provide a method comprising the steps of providing an polypeptide according to the 1st or 2nd aspect of the invention and allowing the polypeptide to bind to a envelope glycoprotein (E) polypeptide, preferably in which the polypeptide is allowed to bind to a cell expressing envelope glycoprotein (E) polypeptide.

According to a 13th aspect of the present invention, we provide a method of diagnosis of flaviviral infection including dengue and West Nile Virus infection in an individual, the method comprising exposing a biological sample from the individual to an polypeptide according to the 1st or 2nd aspect of the invention and detecting binding between the polypeptide and envelope glycoprotein (E) polypeptide.

There is provided, according to a 14th aspect of the present invention, a method of treatment or prevention of a flaviviral infection including dengue and West Nile Virus infection in an individual suffering or suspected to be suffering from such, the method comprising administering a therapeutically effective amount of an polypeptide according to the 1st or 2nd aspect of the invention or a composition according to the 3rd aspect of the invention to the individual.

We provide, according to a 15th aspect of the present invention, a method of treatment or prevention of flaviviral infection including dengue and West Nile Virus infection in an individual suffering or suspected to be suffering from such, the method comprising diagnosing

flaviviral infection including dengue and West Nile Virus infection in the individual by a method according to the 13th aspect of the invention and treating the individual by a method according to the 14th aspect of the invention.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. See, for example, J. Sambrook, E. F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Books 1-3, Cold Spring Harbor Laboratory Press; Ausubel, F. M. et al. (1995 and periodic supplements; *Current Protocols in Molecular Biology*, ch. 9, 13, and 16, John Wiley & Sons, New York, N.Y.); B. Roe, J. Crabtree, and A. Kahn, 1996, *DNA Isolation and Sequencing: Essential Techniques*, John Wiley & Sons; J. M. Polak and James O'D. McGee, 1990, *In Situ Hybridization: Principles and Practice*; Oxford University Press; M. J. Gait (Editor), 1984, *Oligonucleotide Synthesis: A Practical Approach*, Irl Press; D. M. J. Lilley and J. E. Dahlberg, 1992, *Methods of Enzymology: DNA Structure Part A: Synthesis and Physical Analysis of DNA* Methods in Enzymology, Academic Press; Using Antibodies : A Laboratory Manual : Portable Protocol NO. I by Edward Harlow, David Lane, Ed Harlow (1999, Cold Spring Harbor Laboratory Press, ISBN 0-87969-544-7); Antibodies : A Laboratory Manual by Ed Harlow (Editor), David Lane (Editor) (1988, Cold Spring Harbor Laboratory Press, ISBN 0-87969-314-2), 1855. Handbook of Drug Screening, edited by Ramakrishna Seethala, Prabhavathi B. Fernandes (2001, New York, NY, Marcel Dekker, ISBN 0-8247-0562-9); and Lab Ref: A Handbook of Recipes, Reagents, and Other Reference Tools for Use at the Bench, Edited Jane Roskams and Linda Rodgers, 2002, Cold Spring Harbor Laboratory, ISBN 0-87969-630-3. Each of these general texts is herein incorporated by reference.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Purification of Domain III.

Figure 1A. SDS-PAGE of purified domain III proteins of DENV1 - 4. with Coomasssie Brilliant blue staining.

Figure 1B. CD spectrum of refolded and purified DENV2 domain III. The presence of a positive peak at 198 nm and a negative peak at 217 nm indicate beta strands as the predominant secondary structure.

Figure 1C. Amino-acid sequence alignment of the domain III from DENV1-4 and WNV. Beta strands A to G are marked above the alignment. Loops of N-terminal region (NTR), BC, DE FG forming the epitope surface and the inaccessible AB loop are marked below the alignment (see also Figure 4B).

Figure 2. Virus Neutralization

Figure 2A. Plaque reduction assay with purified mAb 9F12 titrated against the 5 Dengue virus strains DENV1-4.

Figure 2B. Representative example of a Plaque reduction neutralization assay plate. Wells A1 to C1: cell controls for the BHK-21 cells. A2 to C2, A5 to C5 contain the neutralization mixture (the diluted mAb 9F12 and 100 PFU of virus per well). A6 to C6: virus control (no antibody added).

Figure 2C. Fusion inhibition assay: DENV2-infected C6/36 cells were exposed to the mAbs followed by a pH drop (pH 6.0). Cells were stained with Propidium iodide.

Figure 2D. Pre and post adsorption assay. Pre-incubated mixtures of DENV2 with mAb9F12 (open squares) or 4G2 (open circles) were dispensed onto Vero cell monolayers at 4°C for an hour in the pre-adsorption assay. The virus antibody mixture was replaced with diluted virus in the post-adsorption assay, followed by washing and addition of either mAb9F12 (solid squares) or 4G2 (bullets). A naïve mouse serum was used as control (open triangles). Assays were performed in triplicate and error bars indicate standard deviation from the mean.

Figure 3A. Binding of DENV1-4 and WNV to 9F12 by ELISA. Microtitre plates coated with either the recombinant DENV1-4 or WNV domain III were incubated with mAb 9F12. The binding was detected using an anti-mouse antibody conjugated to alkaline phosphatase and revealed using a PNPP substrate.

Figure 3B. Binding of DENV1-4 and WNV to 9F12 by Surface Plasmon Resonance: Typical sensorgrams obtained for the interactions between DENV1-4, WNV domain III with either mAb9F12 or a recombinant scFv9F12. The level of protein immobilization for each serotype was adjusted to avoid mass transfer limitation. The kinetic and affinity constants obtained for the interactions are listed in Table E3.

Figure 4A. Epitope mapping by yeast surface display. Flow cytometry histograms of mAbs binding to yeast expressing wild type and 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 (Sukupolvi-Petty et al., 2007): 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 red. The data are representative of three independent experiments.

Figure 4B. A model of domain III showing the epitope recognized by mAb 9F12 based on the yeast surface display results. Residues forming the mAb 9F12 epitope (dotted spheres - K305, 307 and G330) are labeled. 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.

Figure 5 is a graph showing the presence of antibodies to D2TEd3 in Den 2 infected AG129 mouse sera tested 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) (McBride & Vasudevan, 1995) 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.

Figure 6 are drawings showing *in vivo* neutralization experiments in mice. Three groups of 4 mice each are injected with 0.1mg of mAb 9F12, immune serum (Positive control) or phosphate buffered saline (negative control) one day prior to virus challenge. Mice are subsequently challenged with 2 X 10^6 pfu of strain TSVO1 in 0.4 ml volume of virus suspension intraperitoneally. Plasma and sera are collected and tested for Plaque assay, RT-PCR and NS1. Figure 6A. Plaque titration assay performed on mouse plasma after infection

with DEN2 TSVO1. Figure 6B. Quantitative Real Time Polymerase Chain Reaction (qRT-PCR). Figure 6C. Enzyme Linked Immunosorbent assay (ELISA) performed on the mouse plasma to detect the presence of nonstructural protein 1 (NS1) of Dengue type 2.

Figure 7 is a drawing showing a cytopathicity assay to show the low protection of cells rendered by 9F12 from West Nile virus.

Figure 8 is a drawing showing a comparison of virus binding capacity of 9F12 against standard anti dengue monoclonal antibodies by ELISA (protocol described in methods section below).

Figure 9A, Figure 9B and Figure 9C are drawings showing immunoflurescent staining of dengue virus type-2 infected A-549 cells with mAb 9F12 made fluorescent by using a secondary antibody conjugated to Alexa fluor 488 to give a green fluorescence.

Figure 9A. A-549 cells uninfected (left) and cells infected with 5MOI (3days after infection) reacted with mAb 9F12 (right).

Figure 9B. The same procedure but with 10MOI to show the difference in staining pattern of mAb 4G2 (left), which gives a homogeneous fluorescence whereas that of mAB 9F12 (right) gives a bright speckled fluorescence.

Figure 9C. The same procedure as in Figure 9B but 2days after infection reacted with 4G2 (Left), which is broadly cross reactive to other flaviviruses, mAb 9F12 (middle), cross reactive to Dengue and West nile viruses only, compared to that of mAb 3H5 (right), which is highly specific to dengue virus serotype 2 only. mAb 9F12 shows a comparable fluorescence staining to that of a highly specific mAb, 3H5.

SEQUENCES

SEQ ID NO: 1 shows a reference nucleic acid sequence of envelope glycoprotein (E). SEQ ID NO: 2 shows a reference amino acid sequence of envelope glycoprotein (E).

SEQ ID NO: 3 shows the nucleotide sequence of heavy chain of monoclonal antibody 9F12. SEQ ID NO: 4 shows the polypeptide sequence of heavy chain of monoclonal antibody 9F12. SEQ ID NO: 5 shows the nucleotide sequence of light chain of monoclonal antibody

9F12. SEQ ID NO: 6 shows the polypeptide sequence of light chain of monoclonal antibody 9F12.

SEQ ID NO: 7 shows a nucleic acid sequence of a Single Chain Variable Fragment (ScFV) derived from monoclonal antibody 9F12. SEQ ID NO: 8 shows an amino acid sequence of a Single Chain Variable Fragment (ScFV) derived from monoclonal antibody 9F12.

SEQ ID NO: 9 shows a nucleic acid sequence of a forward primer for DENV-1. SEQ ID NO: 10 shows a nucleic acid sequence of a reverse primer for DENV-1. SEQ ID NO: 11 shows a nucleic acid sequence of a forward primer for DENV-2. SEQ ID NO: 12 shows a nucleic acid sequence of a reverse primer for DENV-2. SEQ ID NO: 13 shows a nucleic acid sequence of a forward primer for DENV-3. SEQ ID NO: 14 shows a nucleic acid sequence of a reverse primer for DENV-3. SEQ ID NO: 15 shows a nucleic acid sequence of a forward primer for DENV-4. SEQ ID NO: 16 shows a nucleic acid sequence of a reverse primer for DENV-4.

SEQ ID No: 17 shows the polypeptide sequence of a synthetic peptide from DENV2 to N- Terminal loop. SEQ ID No: 18 shows the polypeptide sequence of a synthetic peptide from DENV2 to BC loop. SEQ ID No: 19 shows the polypeptide sequence of a synthetic peptide from DENV2 to DE loop. SEQ ID No: 20 shows the polypeptide sequence of a synthetic peptide from DENV2 to FG loop. SEQ ID No: 21 shows the polypeptide sequence of a synthetic peptide from DENV2 to e. SEQ ID No: 21shows the polypeptide sequence of a synthetic peptide from DENV2 to f. SEQ ID No: 22 shows the polypeptide sequence of a synthetic peptide from DENV2 to g. SEQ ID No: 23 shows the polypeptide sequence of a synthetic peptide from DENV2 to h. SEQ ID No: 24 shows the polypeptide sequence of a synthetic peptide from DENV2 to i. SEQ ID No: 25 shows the polypeptide sequence of a synthetic peptide from DENV2 to j. SEQ ID No: 26 shows the polypeptide sequence of a synthetic peptide from DENV2 to k.

DETAILED DESCRIPTION

ANTI-FLAVIRAL ANTIBODIES

Our invention is based on the functional and structural characterization of 9F12, a mouse monoclonal antibody raised against a recombinant Domain III from DENV2. 9F12 has

cross-neutralizing capacity towards five different DENV strains. It is also capable of binding to West Nile virus envelope glycoprotein (E) domain III.

We therefore provide broadly for anti-flaviviral antibodies. The Examples describe the generation and production of antibodies generated from, and which have reactivity against, flaviviral proteins.

The anti-flaviviral antibody may be capable of binding to polypeptides from flaviviruses such as dengue and West Nile Virus. They may be capable of binding polypeptides from other flaviviruses such as Tick-borne Encephalitis Virus and Yellow Fever Virus, etc. Flaviviruses are described in further detail below.

The antibody may be capable of cross-reactivity, i.e., able to bind to more than one polypeptide. For example, the antibody may be capable of binding to two or more variants of a particular polypeptide within a defined group of viruses. The two or more variants may therefore comprise cognate or homologous polypeptides from different types of virus in the group. The antibody may be capable of binding to substantially all of the variants of a particular polypeptide within that group.

The group of viruses may comprise viruses selected from for example (i) members of a virus family, (ii) species from a virus genus and (iii) subtypes or serotypes from a virus species, or any combination of these.

One example is a group of viruses which comprises two or more species of virus. The antibody may be capable of binding to a polypeptide from a variety of flaviviruses, including dengue and West Nile Virus. The antibody may be capable of binding to polypeptides from other flaviviruses such as Tick-borne Encephalitis Virus and Yellow Fever Virus, etc (as set out in detail below).

Another example is a group of viruses that comprises two or more, such as all, serotypes of a particular virus species. The antibody may be capable of binding to polypeptides from a number of serotypes of flavivirus within a particular virus or species. For example, the antibody may be capable of binding a polypeptide from different serotypes such as serotype 1, serotype 2, serotype 3 or serotype 4 of a flavivirus such as dengue virus. The

antibody may be capable of binding to a polypeptides from two, three, four, or more, etc serotypes. The antibody may be capable of binding to all the serotypes.

A further example is a combination of the above, i.e., a group of viruses which comprises two or more species of virus, in which each species independently comprises more than one serotype, such as all serotypes within the species. The antibody may therefore be capable of binding to variants of a polypeptide from more than one, such as all, serotypes of one virus and more than one, such as all, serotypes of another virus. For example, the antibody may be capable of binding a polypeptide from all serotypes of a dengue virus and the cognate or homologous polypeptide from West Nile Virus.

We specifically also disclose an antibody capable of binding to a polypeptide from all serotypes of a dengue virus and the cognate or homologous polypeptide from West Nile Virus, as well as the cognate or homologous polypeptide from other flaviviruses such as Yellow Fever Virus and Tick-borne Encephalitis Virus.

The disclosure of this document enables the production of these antibodies as well as fragments and variants thereof, including humanised and chimeric antibodies, which have one or more similar or identical properties of anti-flaviviral antibodies such as 9F12. Such properties may include binding affinity, binding specificity, cross-reactivity, binding avidity, neutralizing activity, etc, as described in further detail below. The specific antibodies and variants thereof described in this document may be produced by a person skilled in the art from the information disclosed in this document, and employing molecular biology techniques which we also describe in detail.

ANTI-ENVELOPE GLYCOPROTEIN (E) DOMAIN III ANTIBODIES

The polypeptide bound by the anti-flaviviral antibody may comprise a flaviviral protein. The protein may comprise a flaviviral envelope protein. The protein may be a glycoprotein. The protein may comprise a flaviviral envelope glycoprotein (E). The antibody may be capable of binding one or more domains, such as domain III, of envelope glycoprotein (E). We therefore specifically disclose anti-envelope glycoprotein (E) domain III antibodies.

Thus, the term "anti-envelope glycoprotein (E) domain III antibody" should be taken to include monoclonal antibody 9F12 (as well as its humanised counterparts). Also included are polypeptides comprising the variable regions of antibody 9F12 and variants, homologues,

fragments and derivatives thereof. This term should also be taken to include reference to variants, homologues, fragments and derivatives of the anti-envelope glycoprotein (E) domain III antibodies, as described below, where the context permits.

The antibody described here may be capable of binding flaviviral envelope glycoprotein (E), such as domain III of the protein, from a plurality of serotypes, such as all serotypes, of a virus such as dengue. It may be further capable of binding flaviviral envelope glycoprotein (E) from West Nile Virus. It may be capable of binding envelope glycoprotein (E), such as domain III, from other serotypes and viral species. It may be capable of binding to such polypeptides from other genera within the Flaviviridae.

The anti-flaviviral antibodies may be generated against dengue virus, for example domain III of the envelope glycoprotein (E) of this virus. For this reason, the anti-flaviviral antibodies may also be considered anti-dengue antibodies or anti-envelope glycoprotein (E) domain III antibodies.

Monoclonal antibodies and variants thereof including Fab, scFv etc and humanised monoclonal antibodies as well as their properties are described in detail in this document and the Examples. The Examples describe monoclonal antibody 9F12, capable of binding to domain III of dengue virus envelope glycoprotein (E). The Examples also describe Fab fragments from monoclonal antibody 9F12, as well as single chain Fv scFv9F12, capable of binding to domain III of dengue virus envelope glycoprotein (E). Other variants, including humanised versions of each of these antibodies, are also disclosed.

We disclose the sequences of the variable regions of monoclonal antibody 9F12. We further disclose variants, homologues, fragments and derivatives of these variable regions. Using this sequence information, a skilled person may produce antibodies comprising these variable regions or their variants, homologues, fragments and derivatives.

We further disclose the sequences of nucleic acid constructs for expressing these monoclonal antibodies. The sequences of these constructs enable the production of monoclonal antibodies which have identical sequences to 9F12. We further disclose variants, homologues, fragments and derivatives of 9F12.

Finally, we disclose the sequences of constructs capable of expressing the humanised monoclonal antibodies 9F12. We describe methods of expressing the antibodies of interest from cells transfected with the constructs, as well as variants, homologues, fragments and derivatives of these humanised constructs.

Using such sequences and the expression methods, the skilled person may readily transfect relevant host cells and cause them to express the whole monoclonal or humanised anti-flaviviral antibodies, or variants, homologues, fragments and derivatives thereof.

The monoclonal antibodies and variants thereof may comprise the variable region of antibody 9F12, as well as further comprising variable regions from other anti-envelope glycoprotein (E) domain III antibodies known in the art, such as those described in (Crill & Roehrig, 2001), (Nybakken et al., 2005), (Chu et al., 2005, Martina et al., uncorrected proof)., (Lisova et al., 2007), (Gromowski et al., 2008), (Lok et al., 2008)., etc. The anti-envelope glycoprotein (E) domain III antibody may comprise the same or different variable regions in a single antibody molecule. They may comprise one variable region, or more than one variable region. Accordingly, we provide the skilled person with the ability to produce any number of antibodies which comprise the same or similar binding reactivity as antibody 9F12 or other anti-envelope glycoprotein (E) domain III antibodies.

Such antibodies may comprise the full or substantially complete sequences of an antibody (i.e., heavy chain and light chain), or they may comprise a fragment of a whole antibody (such as Fv, F(ab') and $F(ab')_2$ fragments or single chain antibodies (scFv)). The antibodies may further comprise fusion proteins or synthetic proteins which comprise the antigen-binding site of the antibody, as described in detail below.

It will also be evident that such antibodies may be engineered for desirable properties, such as lowered host reactivity, reduced rejection, etc.

The engineering could include "humanisation", by which term we mean the inclusion of (or substitution with) one or more human residues or sequences in an antibody sequence such as a mouse antibody sequence. "Humanisation" in the context of this document includes "chimeric" antibodies, in which the antibody comprises discrete sections of mouse and human sequences, e.g., where one or both of the variable regions comprise mouse sequences, and the remainder of the antibody molecule (such as the constant region) comprises human sequences.

In such chimeric antibodies, the whole of the variable regions of, for example, a mouse or rat antibody may be expressed along with human constant regions. This provides such a chimeric antibody with human effector functions and also reduces immunogenicity (HAMA) caused by the murine Fc region.

Humanisation of 9F12 antibody may be carried out by any suitable means, such as the method described in Hanson BJ, Boon AC, Lim AP, Webb A, Ooi EE, Webby RJ. *Passive immunoprophylaxis and therapy with humanized monoclonal antibody specific for influenza A H5 hemagglutinin in mice.* Respir Res 17:126, 2006.

Generally, a "chimeric antibody" may refer to an antibody having either a heavy and light chain encoded by a nucleotide sequence derived from a murine immunoglobulin gene and either a heavy and light chain encoded by a nucleotide sequence derived from a human immunoglobulin gene.

"Humanisation" also includes CDR grafted or reshaped antibodies. It thus includes engineering at a more discrete level, e.g., antibodies in which the mouse variable region has been mutated to include human residues to reduce immunogenicity. In such an antibody, only the complimentarity determining regions from the rodent antibody V-regions may be combined with framework regions from human V-regions. Such antibodies should be more human and less immunogenic than chimaeric antibodies.

For the avoidance of doubt, where a specific antibody designation is referred to in this document, this should be taken to include a reference to both the mouse monoclonal antibody (as secreted by a hybridoma), as well as to the humanised version of it, unless the context dictates otherwise. Thus, for example, where antibody 9F12 is referred to, this includes both the monoclonal antibody 9F12 (i.e., the mouse hybridoma secreted antibody designated 9F12), as well as a humanised monoclonal antibody 9F12.

ENVELOPE GLYCOPROTEIN (E) DOMAIN III BINDING POLYPEPTIDES

We further provide for polypeptides in general having envelope glycoprotein (E) domain III protein binding activity. Such polypeptides include anti-envelope glycoprotein (E) domain III antibodies. The envelope glycoprotein (E) domain III-binding polypeptides may comprise one or more of the same or similar properties as the monoclonal 9F12. The

polypeptides may be referred to for convenience generally as "anti-envelope glycoprotein (E) domain III antibodies".

It is within the skills of a reader to construct binding molecules which may not be (or may not be described as) antibodies or immunoglobulins but which comprise anti-flaviviral or anti-envelope glycoprotein (E) domain III binding activity as described here. Accordingly, and where the context allows the term "anti-envelope glycoprotein (E) domain III antibodies" should be taken to include any molecule so long as it is capable of binding envelope glycoprotein (E) domain III. Such molecules may include polypeptides, small molecules, as well as antibodies and immunoglobulins, and may be identified through various means known in the art, for example by screening a suitable library for envelope glycoprotein (E) domain III binding activity.

The envelope glycoprotein (E) domain III binding polypeptides (which include anti-envelope glycoprotein (E) domain III antibodies) may comprise similar or identical properties as the monoclonal antibody 9F12. Such similar or identical properties may in particular include binding properties. The envelope glycoprotein (E) domain III binding polypeptides may in general be capable of binding to envelope glycoprotein (E) domain III polypeptides, e.g., envelope glycoprotein (E) domain III from dengue (including serotype 1, 2, 3 and/or 4), envelope glycoprotein (E) domain III from West Nile Virus, etc.

ENVELOPE GLYCOPROTEIN (E) DOMAIN III EPITOPES

The anti-envelope glycoprotein (E) domain III antibodies may have the same or similar binding specificity, binding affinity and/or binding affinity as 9F12. The anti-envelope glycoprotein (E) domain III antibodies may specifically bind to an epitope bound by antibody 9F12.

Methods are known in the art to determine an epitope that is bound by a particular antibody. Such epitope mapping methods are described for example in Hanson et al., (2006). Respiratory Research, 7:126. Furthermore, a skilled person will be able to generate antibodies and screen them for particular properties. A detailed description of such a method is shown in Examples 10 and 17. Accordingly, a skilled person will readily be able to identify anti-envelope glycoprotein (E) domain III antibodies which bind to the same epitope as monoclonal antibody 9F12.

Examples 10 and 17 show that anti-envelope glycoprotein (E) domain III antibody 9F12 binds an epitope comprising residues K305, K307, K310 and G330 of a dengue envelope glycoprotein (E) sequence, with reference to the position numbering shown as SEQ ID NO: 2.

Accordingly, we provide polypeptide including an anti-envelope glycoprotein (E) domain III antibody capable of binding an epitope comprising residues K305, K307, K310 and G330.

POSITION NUMBERING

The numbering of the positions of the epitope residues may be made by reference to the numbering of a dengue virus envelope glycoprotein (E) reference sequence shown below in nucleic acid form (SEQ ID NO: 1) and in amino acid form (SEQ ID NO: 2):

Reference Nucleic Acid Sequence (SEQ ID NO: 1)

```
 861                     atgcgttgca taggaatatc aaatagagac tttgtagaag
 901 gggtttcagg aggaagctgg gttgacatag tcttagaaca tggaagctgt gtgacgacga
 961 tggcaaaaaa caaaccaaca ttggattttg aactgataaa aacagaagcc aaacaacctg
1021 ccactctaag gaagtactgt atagaggcaa agctgaccaa cacaacaaca gattctcgct
1081 gcccaacaca aggagaaccc agcctaaatg aagagcagga caaaaggttc gtctgcaaac
1141 actccatggt ggacagagga tggggaaatg gatgtggatt atttggaaaa ggaggcattg
1201 tgacctgtgc tatgttcaca tgcaaaaaga acatgaaagg aaaagtcgtg caaccagaaa
1261 acttggaata caccattgtg ataacacctc actcagggga agagcatgca gtcggaaatg
1321 acacaggaaa acatggcaag gaaatcaaaa taacaccaca gagttccatc acagaagcag
1381 agttgacagg ctatggcact gtcacgatgg agtgctctcc gagaacgggc ctcgacttca
1441 atgagatggt gttgctgcaa atggaaaata aagcttggct ggtgcacagg caatggttcc
1501 tagacctgcc gttgccatgg ctgcccggag cggacacaca aggatcaaat tggatacaga
1561 aagagacatt ggtgactttc aaaaatcccc atgcgaagaa acaggatgtt gttgttttgg
1621 gatcccaaga aggggccatg cacacagcac tcacaggggc cacagaaatc cagatgtcat
1681 caggaaactt actgttcaca ggacatctca agtgcaggct gaggatggac aaactacagc
1741 tcaaaggaat gtcatactct atgtgcacag gaaagtttaa agttgtgaag gaaatagcag
1801 aaacacaaca tggaacaata gttatcagag tacaatatga aggggacggt tctccatgta
1861 agatcccttt tgagataatg gatttggaaa aaagacatgt tttaggtcgc ctgattacag
1921 tcaacccaat cgtaacagaa aaagatagcc cagtcaacat agaagcagaa cctccattcg
1981 gagacagcta catcatcata ggagtagagc cgggacaatt gaagctcaac tggtttaaga
2041 aaggaagttc tatcggccaa atgattgaga caacaatgag gggagcgaag agaatggcca
2101 ttttaggtga cacagcttgg gattttggat ccctgggagg agtgtttaca tctataggaa
2161 aggctctcca ccaagtttc ggagcaatct atggggctgc cttcagtggg gtctcatgga
2221 ctatgaaaat cctcatagga gtcattatca catggatagg aatgaattca cgcagcacct
2281 cactttctgt gtcactagta ttggtgggag tcgtgacgct gtatttggga gttatggtgc
2341 aggcc
```

Reference Amino Acid Sequence (SEQ ID NO: 2)

```
  1 MRCIGISNRD FVEGVSGGSW VDIVLEHGSC VTTMAKNKPT LDFELIKTEA KQPATLRKYC
 61 IEAKLTNTTT DSRCPTQGEP SLNEEQDKRF VCKHSMVDRG WGNGCGLFGK GGIVTCAMFT
121 CKKNMKGKVV QPENLEYTIV ITPHSGEEHA VGNDTGKHGK EIKITPQSSI TEAELTGYGT
181 VTMECSPRTG LDFNEMVLLQ MENKAWLVHR QWFLDLPLPW LPGADTQGSN WIQKETLVTF
241 KNPHAKKQDV VVLGSQEGAM HTALTGATEI QMSSGNLLFT GHLKCRLRMD KLQLKGMSYS
301 MCTGKFKVVK EIAETQHGTI VIRVQYEGDG SPCKIPFEIM DLEKRHVLGR LITVNPIVTE
```

```
361 KDSPVNIEAE PPFGDSYIII GVEPGQLKLN WFKKGSSIGQ MIETTMRGAK RMAILGDTAW
421 DFGSLGGVFT SIGKALHQVF GAIYGAAFSG VSWTMKILIG VIITWIGMNS RSTSLSVSLV
481 LVGVVTLYLG VMVQA
```

The reference amino acid and reference nucleic acid sequence are derived from the Prototype strain of Dengue virus serotype 2 – New Guinea C sequence having NCBI accession number: D00346.

In the context of the present description a specific numbering of amino acid residue positions in domain III of dengue virus envelope glycoprotein (E) is employed. In this respect, by alignment of the amino acid sequences of various known dengue virus envelope glycoprotein (E) polypeptides it is possible to allot a amino acid position number to any amino acid residue position in any virus envelope glycoprotein (E) polypeptide, the amino acid sequence of which is known. Alignments of sequences may be performed by any of the various means known in the art. For example, pairwise alignment using dot-matrix methods, dynamic programming or word methods may be employed. Multiple sequence alignment methods using dynamic programming, progressive methods, iterative methods or motif finding may also be used. Programs such as FASTA, CLUSTAL-V, CLUSTAL-W, T-Coffee, DALI (distance matrix alignment) and SSAP (sequential structure alignment program) are well known in the art, and may be employed for producing sequence alignments.

An example of such an alignment is shown as Figure 1, and this figure may be referred to for allocation of residue numbers or corresponding amino acids in any virus envelope glycoprotein (E) polypeptide.

Using this numbering system originating from for example the amino acid sequence of dengue envelope glycoprotein (E), aligned with amino acid sequences of a number of other known envelope glycoprotein (E) polypeptides, it is possible to indicate the position of an amino acid residue in an envelope glycoprotein (E) polypeptide.

Therefore, the numbering system, even though it may use a specific sequence as a base reference point, is also applicable to all relevant homologous sequences. For example, the position numbering may be applied to homologous sequences from other flavivirus serotypes or species, or homologous sequences from other organisms.

Examples of homologous sequences include Dengue virus serotype 1- Hawaii (NCBI accession number AAN32773), Dengue virus serotype 3- H87 (NCBI accession number

AAA21187), Dengue virus serotype 4 – H241 (NCBI accession number ACJ65015). 1-774 amino acids are provided in the sequence as the sequence of structural polyprotein. The envelope protein in this sequence is: amino acid number: 279 to 676 = 1 to 397 ("mrcvgvg..... wfrkgss").

Such homologues have 20% or greater, 30% or greater, 40% or greater, 50% or greater, 60% or greater homology, for example 70% or more, 80% or more, 90% or more or 95% or more homology, with the reference sequence SEQ ID NO: 2 above. Sequence homology between proteins may be ascertained using well known alignment programs and hybridisation techniques described herein. Such homologous sequences, as well as the functional equivalents described below, will be referred to in this document as the "envelope glycoprotein (E)" polypeptides.

ANTIBODY BINDING

The anti-envelope glycoprotein (E) domain III antibody may be capable of binding to any one or more of envelope glycoprotein (E) from dengue virus serotype I, II, III and IV. It may further be capable of binding to envelope glycoprotein (E) from West Nile Virus. It may bind to domain III of envelope glycoprotein (E).

The binding between the anti-envelope glycoprotein (E) domain III antibody and its target may be more or less strong or weak, transient, semi-permanent or permanent. The antibody may bind to its target at an EC_{50} binding affinity of 1μm or below, such as 100 nm or below.

It may bind with an affinity of 90 nm or below or 80 nm or below. It may bind with an affinity of 70 nm or below, 60 nm or below, 50 nm or below, 40 nm or below, 30 nm or below, 20 nm or below, 10 nm or below, 5 nm or below, 4 nm or below, 3 nm or below, 2 nm or below, 1 nm or below. It may bind with an affinity of 0.5 nm or below, 0.4 nm or below, 0.3 nm or below or 0.2 nm or below.

The antibody may bind to its target with a K_d of micromolar or nanomolar range. It may bind with a K_d of 10^{-7} M or less, 10^{-7} M or less, 10^{-8} M or less, 10^{-9} M or less or 10^{-10} M or less.

The binding may be measured by any means known in the art, such as ELISA or Surface Plasmon Resonance, both of which are described in detail in the Examples.

Binding of the anti-envelope glycoprotein (E) domain III antibody to the envelope glycoprotein (E) domain III polypeptide may take place within or outside the cell. Such binding may inactivate, inhibit or lower an activity of the envelope glycoprotein (E) domain III polypeptide. The binding may neutralise a envelope glycoprotein (E) domain III activity.

The activity may comprise any biological activity caused by or associated with the envelope glycoprotein (E) domain III polypeptide. The activity may comprise binding to another protein, for example a receptor, a downstream protein or factor. The another protein may comprise envelope glycoprotein (E) domain III itself. The activity may comprise multimerisation activity, such as trimerisation activity or homotrimerisation activity. Binding of anti-envelope glycoprotein (E) domain III antibody to envelope glycoprotein (E) domain III polypeptide may inactivate, inhibit or lower an activity of a receptor, downstream protein or factor. The activity may comprise a biochemical activity or a pathogenic activity. The activity may comprise virus absorbtion to host cells.

The binding may inactivate, inhibit or lower an activity of the virus. It may inactivate or neutralise the virus. The binding between the anti-envelope glycoprotein (E) domain III antibody and the envelope glycoprotein (E) domain III may neutralize dengue virus. It may neutralize dengue virus, from one or more, such as all of serotypes I, II, III and IV. It may neutralize West Nile Virus. The neutralization activity may be measured in a plaque-reduction neutralization assay, which is described in detail in the Examples. The neutralization activity may comprise a $PRNT_{50}$ of 10^{-6} or below, such as 2×10^{-7} or below.

ANTIBODIES

The terms "antibody" and "immunoglobulin", as used in this document, may be employed interchangeably where the context permits. These term include fragments of proteolytically-cleaved or recombinantly-prepared portions of an antibody molecule that are capable of selectively reacting with or recognising envelope glycoprotein (E) domain III or an epitope thereof, such as an epitope of envelope glycoprotein (E) domain III bound by 9F12.

Epitopes of envelope glycoprotein (E) domain III include residues K305, K307, K310 and G330 of a dengue envelope glycoprotein (E) sequence, with reference to the position numbering shown as SEQ ID NO: 2.

Non limiting examples of such proteolytic and/or recombinant fragments include Fab, F (ab') 2, Fab', Fv fragments, and single chain antibodies(scFv) containing a VL and VH domain joined by a peptide linker. These Fvs may be covalently or non-covalently linked to form antibodies having two or more binding sites.

By "ScFv molecules" we mean molecules wherein the VH and VL partner domains are linked via a flexible oligopeptide. A general review of the techniques involved in the synthesis of antibody fragments which retain their specific binding sites is to be found in Winter & Milstein(1991) Nature 349, 293-299.

Whole antibodies, and $F(ab')_2$ fragments are "bivalent''. By "bivalent" we mean that the said antibodies and F(ab') fragments have two antigen combining sites. In contrast, Fab, Fv, ScFv and dAb fragments are monovalent having only one antigen combining site.

The anti-envelope glycoprotein (E) domain III antibody may comprise a high affinity antibody with an binding affinity of 1μm or below, such as 100 nm or below, for example, 0.17 nM to 84 nM.

The term "binding affinity" as used in this document refers to the EC_{50} binding affinity an antibody such as an anti-envelope glycoprotein (E) domain III antibody disclosed here. It may be measured using ELISA or Surface Plasmon Resonnance. A high EC_{50} binding affinity is desirable as it reflects the affinity of an Fab fragment for an antigen.

The term "affinity" may also be defined in terms of the dissociation rate or off-rate (k_{off}) of a an antibody such as an anti-envelope glycoprotein (E) domain III antibody. The lower the off-rate the higher the affinity that an antibody such as an anti-envelope glycoprotein (E) domain III antibody has for an antigen.

The anti-envelope glycoprotein (E) domain III antibody may comprise a peptide *per se* or form part of a fusion protein.

The anti-envelope glycoprotein (E) domain III antibodies described here include any antibody that comprises envelope glycoprotein (E) domain III binding activity, such as binding ability to envelope glycoprotein (E) domain III or binding to the same epitope bound by 9F12 as the case may be, including residues K305, K307, K310 and G330 of a dengue envelope glycoprotein (E) sequence, with reference to the position numbering shown as SEQ ID NO: 2.

The anti-envelope glycoprotein (E) domain III antibodies also include the entire or whole antibody, whether mouse, humanised or human, such antibody derivatives and biologically-active fragments. These may include antibody fragments with envelope glycoprotein (E) domain III binding activity that have amino acid substitutions or have sugars or other molecules attached to amino acid functional groups, etc.

The anti-envelope glycoprotein (E) domain III antibody may comprise isolated antibody or purified antibody. It may be obtainable from or produced by any suitable source, whether natural or not, or it may be a synthetic anti-envelope glycoprotein (E) domain III antibody, a semi-synthetic anti-envelope glycoprotein (E) domain III antibody, a derivatised anti-envelope glycoprotein (E) domain III antibody or a recombinant anti-envelope glycoprotein (E) domain III antibody.

Where the anti-envelope glycoprotein (E) domain III antibody is a non-native anti-envelope glycoprotein (E) domain III antibody, it may include at least a portion of which has been prepared by recombinant DNA techniques or an anti-envelope glycoprotein (E) domain III antibody produced by chemical synthesis techniques or combinations thereof.

The term "derivative" as used in this document includes chemical modification of an anti-envelope glycoprotein (E) domain III antibody. Illustrative of such modifications would be replacement of hydrogen by an alkyl, acyl, or amino group, for example. Thee sequence of the anti-envelope glycoprotein (E) domain III antibody may be the same as that of the naturally occurring form or it may be a variant, homologue, fragment or derivative thereof.

ANTIBODY VARIABLE REGIONS

The term "variable region", as used in this document, refers to the variable regions, or domains, of the light chains (VL) and heavy chains (VH) which contain the determinants for binding recognition specificity and for the overall affinity of the antibody against envelope

glycoprotein (E) domain III (or variant, homologue, fragment or derivative), as the case may be.

The variable domains of each pair of light (VL) and heavy chains (VH) are involved in antigen recognition and form the antigen binding site. The domains of the light and heavy chains have the same general structure and each domain has four framework (FR) regions, whose sequences are relatively conserved, connected by three complementarity determining regions (CDRs). The FR regions maintain the structural integrity of the variable domain. The CDRs are the polypeptide segments within the variable domain that mediate binding of the antigen.

The term "constant region", as used in this document, refers to the domains of the light (CL) and heavy (CH) chain of the antibody (or variant, homologue, fragment or derivative) which provide structural stability and other biological functions such as antibody chain association, secretion, transplacental mobility, and complement binding, but which are not involved with binding a envelope glycoprotein (E) domain III epitope. The amino acid sequence and corresponding exon sequences in the genes of the constant region will be dependent upon the species from which it is derived. However, variations in the amino acid sequence leading to allotypes are relatively limited for particular constant regions within a species. An "allotype" is an antigenic determinant (or epitope) that distinguishes allelic genes.

The variable region of each chain is joined to the constant region by a linking polypeptide sequence. The linkage sequence is coded by a "J" sequence in the light chain gene, and a combination of a "D" sequence and a "J" sequence in the heavy chain gene.

ANTIBODY 9F12 VARIABLE REGION SEQUENCES

The nucleic acid sequence of the heavy chain of the variable region of monoclonal antibody 9F12 is as follows (SEQ ID NO: 3):

```
  1 ctgcagcagt ctggggctga gctggtgagg cctggggctt cagtgaagct gtcctgcaag
 61 gctttgggct acagatttac tgactatgaa atgtactggg tgaagcagac acctgcacat
121 ggcctggaat ggattggagg tattcatcca agaagtggta atactgccta caatcagaag
181 ttcaaggaca aggccacact gactgcagac aaatcctcca gtacagccta catggagctc
241 agcagcctga catctgagga ctctgttgtc tattactgta ccacgtccct ctactggggc
301 caagggacca cggtcaccgt ctcctca
```

The amino acid sequence of the heavy chain of the variable region of monoclonal antibody 9F12 is as follows (SEQ ID NO: 4):

```
  1   L Q Q S G A E L V R P G A S V K L S C K A L G Y R F T D Y E
 31   M Y W V K Q T P A H G L E W I G G I H P R S G N T A Y N Q K
 61   F K D K A T L T A D K S S S T A Y M E L S S L T S E D S V V
 91   Y Y C T T S L Y W G Q G T T V T V S S  109
```

The nucleic acid sequence of the light chain of the variable region of monoclonal antibody 9F12 is as follows (SEQ ID NO: 5):

```
  1 cttggagatc aagcctccat ctcttgcaga tctagtcaga gccttgtaca cagtaatgga
 61 aacacctatt tacattggta cctgcagaag ccaggccagt ctccaaagct cctgatctac
121 agcattttca accgattttc tggggtccca gacaggttca gtggcagtgg atcagggaca
181 gatttcacac tcaaaatcag cagagtggag gctgaggatc tgggagttta tttctgctct
241 caaggtacac atgttccgtg gacgttcggt ggaggcacca acctggaaat caaacgggct
301 gatgctgcac caactgtatc catcttccca ccatccagtg agcagttaac atctggaggt
361 gcctcagtcg tgtgcttctt gaacaacttc taccccaaag acatcaatgt caagtggaag
421 attgatggca gtgaacgaca aaatggcgtc ctgaacagtt ggactgatca ggacagcaaa
481 gacagcacct acagcatgag cagcaccctc acgttgacca aggacgagta tgaacgacat
541 aacagctata cctgtgaggc cactcacaag acatcaactt cacccattgt caagagcttc
601 aacaggaatg agtg
```

The amino acid sequence of the light chain of the variable region of monoclonal antibody 9F12 is as follows (SEQ ID NO: 6):

```
  1 L G D Q A S I S C R S S Q S L V H S N G N T Y L H W Y L Q K
 31 P G Q S P K L L I Y S I F N R F S G V P D R F S G S G S G T
 61 D F T L K I S R V E A E D L G V Y F C S Q G T H V P W T F G
 91 G G T N L E I K R A D A A P T V S I F P P S S E Q L T S G G
121 A S V V C F L N N F Y P K D I N V K W K I D G S E R Q N G V
151 L N S W T D Q D S K D S T Y S M S S T L T L T K D E Y E R H
181 N S Y T C E A T H K T S T S P I V K S F N R N E
```

Anti-envelope glycoprotein (E) domain III antibodies, according to the methods and compositions described here, may be generated from these variable region sequences by methods known in the art. For example, the heavy and light chain sequences may be recombined into a constant sequence for a chosen antibody, through recombinant genetic engineering techniques which are known to the skilled person.

Constant region sequences are known in the art, and are available from a number of databases, such as the IMGT/LIGM-DB database (described in Giudicelli et al, 2006, Nucleic Acids Research 34(Database Issue):D781-D784 and LeFranc et al (1995) *LIGM-DB/IMGT: An Integrated Database of Ig and TcR, Part of the Immunogenetics Database.* Annals of the New York Academy of Sciences 764 (1) , 47–47 doi:10.1111/j.1749-6632.1995.tb55805.x) and the IMGT/GENE-DB database (described in Giudicelli et al, 2005, Nucleic Acids Res. 2005 Jan 1;33(Database issue):D256-61). IMGT/LIGM-DB and IMGT/GENE-DB are part of the ImMunoGeneTics Database located at www.ebi.ac.uk/imgt/.

Methods for combining variable regions with given sequences and constant regions to produce whole antibodies are known in the art and are described in Hanson et al., (2006). *Respiratory Research*, 7:126.

ANTIBODY 9F12 SINGLE CHAIN ANTIBODY SEQUENCES

Fragments of whole antibodies such as Fv, F(ab') and $F(ab')_2$ fragments or single chain antibodies (scFv) may be produced by means known in the art.

The nucleic acid sequence of a Single Chain Variable Fragment (ScFV) derived from monoclonal antibody 9F12, referred to as ScFv9F12, is as follows (SEQ ID NO: 7):

CTGCAGCAGTCTGGGGCTGAGCTGGTGAGGCCTGGGGCTTCAGTGAAGCTGTCCTGCAAGGCT
TTGGGCTACAGATTTACTGACTATGAAATGTACTGGGTGAAGCAGACACCTGCACATGGCCTG
GAATGGATTGGAGGTATTCATCCAAGAAGTGGTAATACTGCCTACAATCAGAAGTTCAAGGAC
AAGGCCACACTGACTGCAGACAAATCCTCCAGTACAGCCTACATGGAGCTCAGCAGCCTGACA
TCTGAGGACTCTGTTGTCTATTACTGTACCACGTCCCTCTACTGGGGCCAAGGGACCACGGTC
ACCGTCTCCTCAGGTGGCGGAGGGAGTGGCGGTGGGGGATCGGGAGGTGGCGGGTCACTTGGA
GATCAAGCCTCCATCTCTTGCAGATCTAGTCAGAGCCTTGTACACAGTAATGGAAACACCTAT
TTACATTGGTACCTGCAGAAGCCAGGCCAGTCTCCAAAGCTCCTGATCTACAGCATTTTCAAC
CGATTTTCTGGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAA
ATCAGCAGAGTGGAGGCTGAGGATCTGGGAGTTTATTTCTGCTCTCAAGGTACACATGTTCCG
TGGACGTTCGGTGGAGGCACCAACCTGGAAATCAAACGGGCTGATGCTGCACCAACT

The GS linker comprising 45 nucleotides is shown underlined in the above sequence. Sequences before the linker are heavy chain (residues 1-327), while sequences after the linker are light chain (315 nucleotides).

The amino acid sequence of a Single Chain Variable Fragment (ScFV) derived from monoclonal antibody 9F12, referred to as ScFv9F12, is as follows (SEQ ID NO: 8) - 229 amino acids:

```
        10          20          30          40          50          60
LQQSGAELVR PGASVKLSCK ALGYRFTDYE MYWVKQTPAH GLEWIGGIHP RSGNTAYNQK

        70          80          90         100         110         120
FKDKATLTAD KSSSTAYMEL SSLTSEDSVV YYCTTSLYWG QGTTVTVSSG GGGSGGGGSG

       130         140         150         160         170         180
GGGSLGDQAS ISCRSSQSLV HSNGNTYLHW YLQKPGQSPK LLIYSIFNRF SGVPDRFSGS

       190         200         210         220
GSGTDFTLKI SRVEAEDLGV YFCSQGTHVP WTFGGGTNLE IKRADAAPT
```

Using the disclosed sequences and the methods described in the literature, for example, the heavy and light chains of the variable region of antibody 9F12, having the sequences shown above, may be transgenically fused to a mouse IgG constant region sequence to produce a mouse monoclonal anti-envelope glycoprotein (E) domain III antibody. The variable region of 9F12 antibody may be engineered with mouse or human IgG constant regions to produce mouse monoclonal or humanized antibodies capable of binding to envelope glycoprotein (E) domain III polypeptide.

POLYPEPTIDE SEQUENCES

It will be understood that polypeptide sequences disclosed here are not limited to the particular sequences set forth in this document, but also include homologous sequences obtained from any source, for example related cellular homologues, homologues from other species and variants or derivatives thereof, provided that they have at least one of the biological activities of an anti-envelope glycoprotein (E) domain III antibody, as the case may be.

This disclosure therefore encompasses variants, homologues or derivatives of the amino acid sequences set forth in this document, as well as variants, homologues or derivatives of the amino acid sequences encoded by the nucleotide sequences disclosed here. Such sequences are generally referred to as a "anti-envelope glycoprotein (E) domain III antibody" sequence.

Biological Activities

In some embodiments, the sequences comprise at least one biological activity of an anti-envelope glycoprotein (E) domain III antibody, as the case may be.

The biological activity may comprise an immunological activity. The anti-envelope glycoprotein (E) domain III antibody may comprise an identical or similar immunological activity as compared to antibody 9F12, or its humanised versions. By "immunological activity" we mean the capability of the anti-envelope glycoprotein (E) domain III antibody to induce a specific immune response in appropriate animals or cells on binding with a envelope glycoprotein (E) domain III antigen.

The biological activity may comprise antigen binding activity. The anti-envelope glycoprotein (E) domain III antibody may bind to envelope glycoprotein (E) domain III or an epitope thereof. The anti-envelope glycoprotein (E) domain III antibody may bind to the same epitope bound by antibody 9F12.

The anti-envelope glycoprotein (E) domain III antibody may bind to the antigen or epitope with the same, a reduced or elevated affinity or avidity. For example, the anti-envelope glycoprotein (E) domain III antibody may bind to the antigen or epitope with at least 10%, such as 20%, such as 30%, 40% 50%, 60%, 70%, 80%, 90% or more, affinity or avidity compared to the cognate antibody, e.g., 9F12 or its humanised counterparts, as the case may be.

The activity may include inhibition of envelope glycoprotein (E) activity as for example measured by reduction of homotrimerization, absorption to cells, viral infectivity, etc, such as for example measured by the assays described in the Examples.

Homotrimerization activity may be assayed by any suitable assay, for example the assay described in Modis, Y., Ogata, S., Clements, D. & Harrison, S. C. (2004). *Structure of the dengue virus envelope protein after membrane fusion.* Nature 427, 313-319.

Viral absorbtion to cells may be assayed by any suitable assay, for example, the assay described in Example 6 below (Adsorption Assays Using Cell-based Flavivirus Immuno-detection (CFI)).

The reduction or inhibition may be conveniently assayed by exposing a test cell or a test animal to a flavivirus such as dengue or West Nile Virus, administering the anti-envelope glycoprotein (E) domain III antibody to the animal (or exposing it to the cell) and determining an effect of the anti-envelope glycoprotein (E) domain III antibody as compared to a similar

control animal or cell that has not been so treated or exposed. The Examples describe such an assay in detail.

The anti-envelope glycoprotein (E) domain III antibody may have such inhibition activity that is the same as, reduced from, or elevated from, the cognate antibody. For example, the anti-envelope glycoprotein (E) domain III antibody may be at least 10%, such as 20%, such as 30%, 40% 50%, 60%, 70%, 80%, 90% or more, effective compared to the cognate antibody, e.g., 9F12 or its humanised counterparts, as the case may be. By this we mean that, say, if the cognate antibody is capable of reducing homotrimerization, viral absorption, infectivity, etc by for example 90%, the anti-envelope glycoprotein (E) domain III antibody may be capable of doing so by below 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, etc, as compared to an untreated animal or cell.

Other assays that detect antibody events can also be used, instead of, or in addition to, the assays described.

Homologues

The anti-envelope glycoprotein (E) domain III antibody polypeptides disclosed include homologous sequences obtained from any source, for example related viral/bacterial proteins, cellular homologues and synthetic peptides, as well as variants or derivatives thereof. Thus polypeptides also include those encoding homologues of anti-envelope glycoprotein (E) domain III antibody from other species including other members of the Flaviviridae, or other animals such as mammals (e.g. mice, rats or rabbits) or humans.

In the context of the present document, a homologous sequence or homologue is taken to include an amino acid sequence which is at least 60, 70, 80 or 90% identical, such as at least 95 or 98% identical at the amino acid level over at least 30, such as 50, 70, 90 or 100 amino acids with a relevant polypeptide sequence, for example as shown in the sequence listing herein. In the context of this document, a homologous sequence is taken to include an amino acid sequence which is at least 15, 20, 25, 30, 40, 50, 60, 70, 80 or 90% identical, such as at least 95 or 98% identical at the amino acid level, such as over at least 15, 25, 35, 50 or 100, such as 200, 300, 400 or 500 amino acids with the sequence of a relevant polypeptide. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present document

homology may be expressed in terms of sequence identity. The sequence identity may be determined relative to the entirety of the length the relevant sequence, i.e., over the entire length or full length sequence of the relevant gene, for example.

Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

% homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues (for example less than 50 contiguous amino acids).

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, the default values may be used when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package (see below) the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A.; Devereux *et al.,* 1984, Nucleic Acids Research 12:387). Examples of other software than can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al.*, 1999 *ibid* – Chapter 18), FASTA (Atschul *et al.,* 1990, J. Mol. Biol., 403-410) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel *et al.*, 1999 *ibid*, pages 7-58 to 7-60). The GCG Bestfit program may be used.

Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). The public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62, may be used.

Once the software has produced an optimal alignment, it is possible to calculate % homology, such as % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

Variants and Derivatives

The terms "variant" or "derivative" in relation to the amino acid sequences as described here includes any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acids from or to the sequence. The resultant amino acid sequence may retain substantially the same activity as the unmodified sequence, such as having at least the same activity as the anti-envelope glycoprotein (E) domain III antibody polypeptides shown in this document, for example in the sequence listings. Thus, the key feature of the sequences – namely ability to bind to envelope glycoprotein (E) domain III polypeptides or reduction in viral infectivity, homotrimerization, viral absorption, etc, as described elsewhere – may be retained.

Polypeptides having the amino acid sequence shown in the Examples, or fragments or homologues thereof may be modified for use in the methods and compositions described here. Typically, modifications are made that maintain the biological activity of the sequence. Amino acid substitutions may be made, for example from 1, 2 or 3 to 10, 20 or 30 substitutions provided that the modified sequence retains the biological activity of the unmodified sequence. Amino acid substitutions may include the use of non-naturally occurring analogues, for example to increase blood plasma half-life of a therapeutically administered polypeptide.

Natural variants of an anti-envelope glycoprotein (E) domain III antibody are likely to comprise conservative amino acid substitutions. Conservative substitutions may be defined, for example according to the Table below. Amino acids in the same block in the second column such as those in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y

Fragments

Polypeptides disclosed here and useful as markers also include fragments of the above mentioned full length polypeptides and variants thereof, including fragments of the sequences set out in the sequence listings.

Polypeptides also include fragments of the full length sequence of any of the anti-envelope glycoprotein (E) domain III antibody polypeptides. Fragments may comprise at least one epitope. Methods of identifying epitopes are well known in the art. Fragments will typically comprise at least 6 amino acids, such as at least 10, 20, 30, 50 or 100 or more amino acids.

Polypeptide fragments of the anti-envelope glycoprotein (E) domain III antibody proteins and allelic and species variants thereof may contain one or more (e.g. 5, 10, 15, or 20)

substitutions, deletions or insertions, including conserved substitutions. Where substitutions, deletion and/or insertions occur, for example in different species, such as less than 50%, 40% or 20% of the amino acid residues depicted in the sequence listings are altered.

Anti-envelope glycoprotein (E) domain III antibody and their fragments, homologues, variants and derivatives, may be made by recombinant means. However, they may also be made by synthetic means using techniques well known to skilled persons such as solid phase synthesis. The proteins may also be produced as fusion proteins, for example to aid in extraction and purification. Examples of fusion protein partners include glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and β-galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences. The fusion protein may be such that it will not hinder the function of the protein of interest sequence. Proteins may also be obtained by purification of cell extracts from animal cells.

The anti-envelope glycoprotein (E) domain III antibody polypeptides, variants, homologues, fragments and derivatives disclosed here may be in a substantially isolated form. It will be understood that such polypeptides may be mixed with carriers or diluents which will not interfere with the intended purpose of the protein and still be regarded as substantially isolated. A anti-envelope glycoprotein (E) domain III antibody variant, homologue, fragment or derivative may also be in a substantially purified form, in which case it will generally comprise the protein in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the protein in the preparation is a protein.

The anti-envelope glycoprotein (E) domain III antibody polypeptides, variants, homologues, fragments and derivatives disclosed here may be labelled with a revealing label. The revealing label may be any suitable label which allows the polypeptide , etc to be detected. Suitable labels include radioisotopes, e.g. ^{125}I, enzymes, antibodies, polynucleotides and linkers such as biotin. Labelled polypeptides may be used in diagnostic procedures such as immunoassays to determine the amount of a polypeptide in a sample. Polypeptides or labelled polypeptides may also be used in serological or cell-mediated immune assays for the detection of immune reactivity to said polypeptides in animals and humans using standard protocols.

The anti-envelope glycoprotein (E) domain III antibody polypeptides, variants, homologues, fragments and derivatives disclosed here, optionally labelled, my also be fixed to a solid phase, for example the surface of an immunoassay well or dipstick. Such labelled and/or immobilised polypeptides may be packaged into kits in a suitable container along with suitable reagents, controls, instructions and the like. Such polypeptides and kits may be used in methods of detection of antibodies to the polypeptides or their allelic or species variants by immunoassay.

Immunoassay methods are well known in the art and will generally comprise: (a) providing a polypeptide comprising an epitope bindable by an antibody against said protein; (b) incubating a biological sample with said polypeptide under conditions which allow for the formation of an antibody-antigen complex; and (c) determining whether antibody-antigen complex comprising said polypeptide is formed.

The anti-envelope glycoprotein (E) domain III antibody polypeptides, variants, homologues, fragments and derivatives disclosed here may be used in *in vitro* or *in vivo* cell culture systems to study the role of their corresponding genes and homologues thereof in cell function, including their function in disease. For example, truncated or modified polypeptides may be introduced into a cell to disrupt the normal functions which occur in the cell. The polypeptides may be introduced into the cell by *in situ* expression of the polypeptide from a recombinant expression vector (see below). The expression vector optionally carries an inducible promoter to control the expression of the polypeptide.

The use of appropriate host cells, such as insect cells or mammalian cells, is expected to provide for such post-translational modifications (e.g. myristolation, glycosylation, truncation, lapidation and tyrosine, serine or threonine phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products. Such cell culture systems in which the anti-envelope glycoprotein (E) domain III antibody polypeptides, variants, homologues, fragments and derivatives disclosed here are expressed may be used in assay systems to identify candidate substances which interfere with or enhance the functions of the polypeptides in the cell.

POLYNUCLEOTIDE SEQUENCES

The variable regions, monoclonal antibody sequences and humanised antibody sequences may comprise polynucleotides. These may comprise DNA or RNA.

They may be single-stranded or double-stranded. They may also be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present document, it is to be understood that the polynucleotides described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of polynucleotides.

Where the polynucleotide is double-stranded, both strands of the duplex, either individually or in combination, are encompassed by the methods and compositions described here. Where the polynucleotide is single-stranded, it is to be understood that the complementary sequence of that polynucleotide is also included.

Variants, Derivatives and Homologues

The terms "variant", "homologue" or "derivative" in relation to a nucleotide sequence described in this document include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleotides from or to the sequence. The resulting sequence may be capable of encoding a polypeptide which has envelope glycoprotein (E) domain III binding activity as described elsewhere in this document.

As indicated above, with respect to sequence identity, a "homologue" has such as at least 5% identity, at least 10% identity, at least 15% identity, at least 20% identity, at least 25% identity, at least 30% identity, at least 35% identity, at least 40% identity, at least 45% identity, at least 50% identity, at least 55% identity, at least 60% identity, at least 65% identity, at least 70% identity, at least 75% identity, at least 80% identity, at least 85% identity, at least 90% identity, or at least 95% identity to a relevant sequence.

There may be at least 95% identity, such as at least 96% identity, such as at least 97% identity, such as at least 98% identity, such as at least 99% identity. Nucleotide homology comparisons may be conducted as described above. A sequence comparison program such as the GCG Wisconsin Bestfit program described above may be used for this purpose. The default scoring matrix has a match value of 10 for each identical nucleotide and -9 for each mismatch.

The default gap creation penalty is -50 and the default gap extension penalty is -3 for each nucleotide.

Hybridisation

We further describe nucleotide sequences that are capable of hybridising selectively to any of the sequences presented herein, such as a 9F12 variable region, antibody and humanised antibody or any variant, fragment or derivative thereof, or to the complement of any of the above. Nucleotide sequences may be at least 15 nucleotides in length, such as at least 20, 30, 40 or 50 nucleotides in length.

The term "hybridisation" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" as well as the process of amplification as carried out in polymerase chain reaction technologies.

Polynucleotides capable of selectively hybridising to the nucleotide sequences presented herein, or to their complement, will be generally at least 70%, such as at least 80 or 90% and such as at least 95% or 98% homologous to the corresponding nucleotide sequences presented herein over a region of at least 20, such as at least 25 or 30, for instance at least 40, 60 or 100 or more contiguous nucleotides.

The term "selectively hybridisable" means that the polynucleotide used as a probe is used under conditions where a target polynucleotide is found to hybridize to the probe at a level significantly above background. The background hybridization may occur because of other polynucleotides present, for example, in the cDNA or genomic DNA library being screened. In this event, background implies a level of signal generated by interaction between the probe and a non-specific DNA member of the library which is less than 10 fold, such as less than 100 fold as intense as the specific interaction observed with the target DNA. The intensity of interaction may be measured, for example, by radiolabelling the probe, e.g. with ^{32}P.

Hybridisation conditions are based on the melting temperature (Tm) of the nucleic acid binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA), and confer a defined "stringency" as explained below.

Maximum stringency typically occurs at about Tm-5°C (5°C below the Tm of the probe); high stringency at about 5°C to 10°C below Tm; intermediate stringency at about 10°C to 20°C below Tm; and low stringency at about 20°C to 25°C below Tm. As will be understood by those of skill in the art, a maximum stringency hybridisation can be used to identify or detect identical polynucleotide sequences while an intermediate (or low) stringency hybridisation can be used to identify or detect similar or related polynucleotide sequences.

We disclose nucleotide sequences that can hybridise to a nucleic acid, or a fragment, homologue, variant or derivative thereof, under stringent conditions (e.g. 65°C and 0.1xSSC {1xSSC = 0.15 M NaCl, 0.015 M Na_3 Citrate pH 7.0}).

Where a polynucleotide is double-stranded, both strands of the duplex, either individually or in combination, are encompassed by the present disclosure. Where the polynucleotide is single-stranded, it is to be understood that the complementary sequence of that polynucleotide is also disclosed and encompassed.

Polynucleotides which are not 100% homologous to the sequences disclosed here but fall within the disclosure can be obtained in a number of ways. Other variants of the sequences described herein may be obtained for example by probing DNA libraries made from a range of individuals, for example individuals from different populations. In addition, other viral/bacterial, or cellular homologues particularly cellular homologues found in mammalian cells (e.g. rat, mouse, bovine and primate cells), may be obtained and such homologues and fragments thereof in general will be capable of selectively hybridising to the sequences shown in the sequence listing herein. Such sequences may be obtained by probing cDNA libraries made from or genomic DNA libraries from other animal species, and probing such libraries with probes comprising all or part of the disclosed sequences under conditions of medium to high stringency.

The polynucleotides described here may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 15, such as at least 20, for example at least 25, 30 or 40 nucleotides in length, and are also encompassed by the term polynucleotides as used herein. Fragments may be less than 500, 200, 100, 50 or 20 nucleotides in length.

Polynucleotides such as a DNA polynucleotides and probes may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques.

In general, primers will be produced by synthetic means, involving a step wise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

Longer polynucleotides will generally be produced using recombinant means, for example using PCR (polymerase chain reaction) cloning techniques. This will involve making a pair of primers (e.g. of about 15 to 30 nucleotides) flanking a region of the sequence which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from an animal or human cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

ENVELOPE GLYCOPROTEIN (E) DOMAIN III POLYPEPTIDES AND NUCLEIC ACIDS

Envelope glycoprotein (E) domain III polypeptide homologues, variants, derivatives and fragments may be defined similarly, as set out in the previous paragraphs.

Where the context permits, a reference to envelope glycoprotein (E) domain III polypeptide should be taken to include reference to a envelope glycoprotein (E) domain III polypeptide homologue, variant, derivative or fragment. Similarly, where the context permits, a reference to an envelope glycoprotein (E) domain III nucleic acid should be taken to include reference to an envelope glycoprotein (E) domain III nucleic acid homologue, variant, derivative or fragment.

ANTI-ENVELOPE GLYCOPROTEIN (E) DOMAIN III ANTIBODY PRODUCTION

The anti-envelope glycoprotein (E) domain III antibody can be produced by recombinant DNA methods or synthetic peptide chemical methods that are well known to those of ordinary skill in the art.

By way of example, the anti-envelope glycoprotein (E) domain III antibody may be synthesized by techniques well known in the art, as exemplified by "Solid Phase Peptide Synthesis: A Practical Approach" E. Atherton and R. C. Sheppard, IRL Press, Oxford England. Similarly, multiple fragments can be synthesized which are subsequently linked together to form larger fragments. These synthetic peptide fragments can also be made with amino acid substitutions at specific locations in order to test for activity *in vitro* and *in vivo*.

The anti-envelope glycoprotein (E) domain III antibody can be synthesized in a standard microchemical facility and purity checked with HPLC and mass spectrophotometry. Methods of peptide synthesis, HPLC purification and mass spectrophotometry are commonly known to those skilled in these arts.

The anti-envelope glycoprotein (E) domain III antibody may also be expressed under *in vitro* and *in vivo* conditions in a transformed host cell into which has been incorporated the DNA sequences described here (such as variable sequences) or allelic variations thereof and which can be used in the prevention and/or treatment of flaviviral related diseases such as dengue and West Nile Virus infection.

The term "vector" includes expression vectors and transformation vectors. The term "expression vector" means a construct capable of *in vivo* or *in vitro* expression. The term "transformation vector" means a construct capable of being transferred from one species to another.

Vectors which may be used for expression include recombinant viral vectors, in particular recombinant retroviral vectors (RRV) such as lentiviral vectors, adenoviral vectors including a combination of retroviral vectors.

The term 'recombinant retroviral vector" (RRV) refers to a vector with sufficient retroviral genetic information to allow packaging of an RNA genome, in the presence of packaging components, into a viral particle capable of infecting a target cell. Infection of the target cell includes reverse transcription and integration into the target cell genome. The RRV carries non-viral coding sequences which are to be delivered by the vector to the target cell. An RRV is incapable of independent replication to produce infectious retroviral particles within the final target cell. Usually the RRV lacks a functional gag pol and/or env gene and/or other genes essential for replication. Vectors which may be used include recombinant pox

viral vectors such as fowl pox virus (FPV), entomopox virus, vaccinia virus such as NYVAC, canarypox virus, MVA or other non-replicating viral vector systems such as those described for example in WO9530018.

Pox viruses may be engineered for recombinant gene expression and for the use as recombinant live vaccines in a dual immunotherapeutic approach. The principal rationale for using live attenuated viruses, such as viruses, as delivery vehicles and/or vector based vaccine candidates, stems from their ability to elicit cell mediated immune responses. The viral vectors, as outlined above, are capable of being employed as delivery vehicles and as vector based vaccine candidates because of the immunogenicity of their constitutive proteins, which act as adjuvants to enhance the immune response, thus rendering a nucleotide sequence of interest (NOI) such as a nucleotide sequence encoding an anti-envelope glycoprotein (E) domain III antibody more immunogenic.

The pox virus vaccination strategies have used recombinant techniques to introduce NOIs into the genome of the pox virus. If the NOI is integrated at a site in the viral DNA which is non-essential for the life cycle of the virus, it is possible for the newly produced recombinant pox virus to be infectious, that is to say to infect foreign cells and thus to express the integrated NOI. The recombinant pox virus prepared in this way can be used as live vaccines for the prophylaxis and/or treatment of infectious disease such as flaviviral infectious disease, including dengue and West Nile Virus.

Other requirements for pox viral vector delivery systems include good immunogenicity and safety. MVA is a replication-impaired vaccinia strain with a good safety record. In most cell types and normal human tissue, MVA does not replicate. Limited replication of MVA is observed in a few transformed cell types such as BHK21 cells. Carroll et al (1997 Vaccine15 : 387-394) have shown that the recombinant MVA is equally as good as conventional recombinant vaccinia vectors at generating a protective CD8+T cell response and is an efficacious alternative to the more commonly used replication competent vaccinia virus. The vaccinia virus strains derived from MVA, or independently developed strains having the features of MVA which make MVA particularly suitable for use in a vaccine, are also suitable for use as a delivery vehicle.

The nucleotide sequence of interest, and of which expression is desired, may operably linked to a transcription unit. The term "transcription unit" as described herein are regions of nucleic acid containing coding sequences and the signals for achieving expression of those coding sequences independently of any other coding sequences. Thus, each transcription unit generally comprises at least a promoter, an optional enhancer and a polyadenylation signal. The term "promoter" is used in the normal sense of the art, e. g. an RNA polymerase binding site. The promoter may contain an enhancer element. The term "enhancer" includes a DNA sequence which binds to other protein components of the transcription initiation complex and thus facilitates the initiation of transcription directed by its associated promoter. The term "cell" includes any suitable organism. The cell may comprise a mammalian cell, such as a human cell.

The term "transformed cell" means a cell having a modified genetic structure. For example, as described here, a cell has a modified genetic structure when a vector such as an expression vector has been introduced into the cell. The term "organism" includes any suitable organism. The organism may comprise a mammal such as a human.

Here the term "transgenic organism" means an organism comprising a modified genetic structure. For example, the organism may have a modified genetic structure if a vector such as an expression vector has been introduced into the organism.

ANTIBODY EXPRESSION

We further describe a method comprising transforming a host cell with a or the nucleotide sequences described in this document, such as 9F12 variable regions, antibody sequences or humanized antibody sequences.

We also provide a method comprising culturing a transformed host cell-which cell has been transformed with a or the such nucleotide sequences under conditions suitable for the expression of the anti-envelope glycoprotein (E) domain III antibody encoded by said nucleotide sequences.

We further provide a method comprising culturing a transformed host cell-which cell has been transformed with a or the such nucleotide sequences under conditions suitable for the expression of the anti-envelope glycoprotein (E) domain III antibody encoded by said

nucleotide sequences; and then recovering said anti-envelope glycoprotein (E) domain III antibody from the transformed host cell culture.

Thus, anti-envelope glycoprotein (E) domain III antibody encoding nucleotide sequences, fusion proteins or functional equivalents thereof, may be used to generate recombinant DNA molecules that direct the expression thereof in appropriate host cells.

By way of example, anti-envelope glycoprotein (E) domain III antibody may be produced in recombinant E. coli, yeast or mammalian expression systems, and purified with column chromatography.

In certain circumstances there are advantages of using antibody fragments, rather than whole antibodies. The smaller size of the fragments allows for rapid clearance, and may lead to improved neutralization of viral activity, infection, progression, etc. Fab, Fv, ScFv antibody fragments can all be expressed in and secreted from *E. coli*, thus allowing the production of large amounts of the such fragments.

The nucleotide sequences encoding the anti-envelope glycoprotein (E) domain III antibody may be operably linked to a promoter sequence capable of directing expression of the anti-envelope glycoprotein (E) domain III antibody encoding nucleotide sequences in a suitable host cell. When inserted into the host cell, the transformed host cell may be cultured under suitable conditions until sufficient levels of the anti-envelope glycoprotein (E) domain III antibody are achieved after which the cells may be lysed and the anti-envelope glycoprotein (E) domain III antibody is isolated.

Host cells transformed with the anti-envelope glycoprotein (E) domain III antibody encoding nucleotide sequences may be cultured under conditions suitable for the expression and recovery of the anti-envelope glycoprotein (E) domain III antibody from cell culture. The protein produced by a recombinant cell may be secreted or may be contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing the

Anti-envelope glycoprotein (E) domain III antibody encoding nucleotide sequences can be designed with signal sequences which direct secretion of the anti-envelope glycoprotein (E) domain III antibody encoding nucleotide sequences through a particular

prokaryotic or eukaryotic cell membrane. Other recombinant constructions may join the anti-envelope glycoprotein (E) domain III antibody encoding nucleotide sequence to a nucleotide sequence encoding a polypeptide domain which will facilitate purification of soluble proteins (Kroll DJ et al(1993) DNA Cell Biol 12:441- 5 3', see also the discussion below on vectors containing fusion proteins).

The anti-envelope glycoprotein (E) domain III antibody may also be expressed as a recombinant protein with one or more additional polypeptide domains added to facilitate protein purification. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals (Porath J (1992) Protein Expr Purif 3-26328 1), protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle, WA). The inclusion of a cleavable linker sequence such as Factor XA or enterokinase (Invitrogen, San Diego, CA) between the purification domain and the anti-envelope glycoprotein (E) domain III antibody is useful to facilitate purification.

The nucleotide sequences described here may be engineered in order to alter a the anti-envelope glycoprotein (E) domain III antibody encoding sequences for a variety of reasons, including but not limited to alterations which modify the cloning, processing and/or expression of the gene product. For example, mutations may be introduced using techniques which are well known in the art, e.g., site-directed mutagenesis to insert new restriction sites, to alter glycosylation patterns or to change codon preference.

In another embodiment, a or the natural, modified or recombinant anti-envelope glycoprotein (E) domain III antibody encoding nucleotide sequences may be ligated to a heterologous sequence to encode a fusion protein. By way of example, fusion proteins comprising the anti-envelope glycoprotein (E) domain III antibody or an enzymatically active fragment or derivative thereof linked to an affinity tag such as glutathione-S-transferase (GST), biotin, His6, ac-myc tag (see Emrich etal 1993 BiocemBiophys Res Commun 197(1): 21220), hemagglutinin (HA) (as described in Wilson et al (1984 Cell 37 767) or a FLAG epitope (Ford etal 1991 Protein Expr Purif Apr; 2 (2):95-107).

The fused recombinant protein may comprise an antigenic coprotein such as GST, beta-galactosidase or the lipoprotein D from *Haemophillls influenzae* which are relatively large co-proteins, which solubilise and facilitate production and purification thereof. Alternatively, the fused protein may comprise a carrier protein such as bovine serum albumin (BSA) or keyhole limpet haemocyanin (KLH). In certain embodiments, the marker sequence may comprise a hexa-histidine peptide, as provided in the pQE vector (Qiagen Inc) and described in Gentz et al (1989 PNAS 86: 821-824). Such fusion proteins are readily expressable in yeast culture (as described in Mitchell et al 1993 Yeast 5:715-723) and are easily purified by affinity chromatography. A fusion protein may also be engineered to contain a cleavage site located between the nucleotide sequence encoding the anti-envelope glycoprotein (E) domain III antibody and the heterologous protein sequence, so that the anti-envelope glycoprotein (E) domain III antibody may be cleaved and purified away from the heterologous moiety. In another embodiment, an assay for the target protein may be conducted using the entire, bound fusion protein. Alternatively, the co-protein may act as an adjuvant in the sense of providing a generalised stimulation of the immune system. The co-protein may be attached to either the amino or carboxy terminus of the first protein.

Although the presence/absence of marker gene expression suggests that the nucleotide sequence for anti-envelope glycoprotein (E) domain III antibody is also present, its presence and expression should be confirmed. For example, if the anti-envelope glycoprotein (E) domain III antibody encoding nucleotide sequence is inserted within a marker gene sequence, recombinant cells containing the anti-envelope glycoprotein (E) domain III antibody coding regions may be identified by the absence of the marker gene function. Alternatively, a marker gene may be placed in tandem with a anti-envelope glycoprotein (E) domain III antibody encoding nucleotide sequence under the control of a single promoter.

Expression of the marker gene in response to induction or selection usually indicates expression of the anti-envelope glycoprotein (E) domain III antibody as well.

Additional methods to quantitate the expression of a particular molecule include radiolabeling (Melby PC et al 1993 J Immunol Methods 159:235-44) or biotinylating (Duplaa C et al 1993 Anal Biochem 229-36) nucleotides, co amplification of a control nucleic acid. and standard curves onto which the experimental results are interpolated.

Quantitation of multiple samples may be speeded up by running the assay in an ELISA format where the anti-envelope glycoprotein (E) domain III antibody of interest is presented in various dilutions and a spectrophotometric or calorimetric response gives rapid quantitation.

Altered anti-envelope glycoprotein (E) domain III antibody nucleotide sequences which may be made or used include deletions, insertions or substitutions of different nucleotide residues resulting in a nucleotide sequence that encodes the same or a functionally equivalent anti-envelope glycoprotein (E) domain III antibody. By way of example, the expressed anti-envelope glycoprotein (E) domain III antibody may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent anti-envelope glycoprotein (E) domain III antibody. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge. solubility, hydrophobicity, hydrophilicity. and/or the amphipathic nature of the residues as long as the binding affinity of the anti-envelope glycoprotein (E) domain III antibody is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid: positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tvrosine.

DIAGNOSTIC KITS

We also provide diagnostic kits for detecting flaviviral infection, including dengue infection and West Nile Virus infection in an individual, or susceptibility to such in an individual.

The diagnostic kit may comprise means for detecting expression, amount or activity of envelope glycoprotein (E) domain III in the individual, by any means as described in this document. The diagnostic kit may therefore comprise any one or more of the following: an anti-envelope glycoprotein (E) domain III antibody, an antibody capable of binding to the same epitope as monoclonal antibody 9F12, monoclonal antibody 9F12, Fab from 9F12, scFv from 9F12, an antibody comprising a variable region of antibody 9F12, or a humanised monoclonal antibody 9F12, etc

The diagnostic kit may comprise instructions for use, or other indicia. The diagnostic kit may further comprise means for treatment or prophylaxis of flaviviral infection, such as any of the compositions described in this document, or any means known in the art for treating flaviviral infection. In particular, the diagnostic kit may comprise an anti-envelope glycoprotein (E) domain III antibody as described, for example obtained by screening.

PROPHYLACTIC AND THERAPEUTIC METHODS

The monoclonal antibody 9F12 may be used for treatment of disease in humans or other animals. We show in the Examples that such anti-envelope glycoprotein (E) domain III antibodies have anti-flaviviral activity. Specifically, the Examples show that the anti-envelope glycoprotein (E) domain III antibodies are capable of treating and preventing viral infection, such as dengue or West Nile Virus viral infection.

We disclose methods of treating flaviviral infection, including dengue infection and West Nile Virus infection. Methods of preventing flaviviral infection (i.e., prophylaxis) also suitably employ the same or similar approaches.

Accordingly, we provide for the use of anti-envelope glycoprotein (E) domain III antibodies in the treatment or prevention of flaviviral disease. The flaviviral disease may comprise dengue or West Nile Virus infection. The anti-envelope glycoprotein (E) domain III antibodies may be used as drugs or therapies to treat dengue or other flaviviral infection. They may be used to prevent such infection or progress of the disease.

In general terms, our methods involve manipulation of cells, by modulating (such as down-regulating) the expression, amount or activity of envelope glycoprotein (E) domain III. The treatment may comprise generally contacting an flaviviral infected cell, or a cell suspected of being a flaviviral infected cell, with an anti-envelope glycoprotein (E) domain III antibody. The methods may involve exposing a patient to an anti-envelope glycoprotein (E) domain III antibody or variant thereof as described here.

It may or in addition be exposed to an anti-flaviviral agent such as an antibody or other molecule known to have effect in preventing or treating a flaviviral disease. Where this is so, the cell may be exposed to both the antibody and the agent together, or individually in sequence. The exposure may be repeated a number of times. Any combination of anti-

envelope glycoprotein (E) domain III antibody and an other agent antibody in whatever amount or relative amount, in whatever timing of exposure, may be used.

We therefore provide for the use of combinations of anti-envelope glycoprotein (E) domain antibodies and anti-flaviviral agents, as described above, in the treatment of a flaviviral disease such as dengue or West Nile Virus infection.

The cell may be an individual cell, or it may be in a cell mass. The cell may be inside the body of an organism. The organism may be one which is known to be suffering from flaviviral infection, or it could be one in which flaviviral infection is suspected, or it could be one which is susceptible to flaviviral infection. The treatment may comprise administering the antibody or antibodies to the organism. As above, a single antibody may be administered, or a combination of anti-envelope glycoprotein (E) domain III antibody and an anti-flaviviral agent may be administered. The administration may be simultaneous or sequential, as described above. Thus, the treatment may comprise administering an anti-envelope glycoprotein (E) domain III antibody simultaneously or sequentially with an anti-flaviviral agent to the individual.

For this purpose, a number of criteria may be designated, which reflect the progress of treatment or prophylaxis or the well-being of the patient. Useful criteria in the case of dengue may include headache, fever, exhaustion, joint and muscle pain, swollen glands (lymphadenopathy) and rash. In the case of West Nile Virus infection, useful criteria may include fever, headache, tiredness, body aches, skin rash (on the trunk of the body) and swollen lymph glands. Symptoms of severe disease (also called neuroinvasive disease, such as West Nile encephalitis or meningitis or West Nile poliomyelitis) include headache, high fever, neck stiffness, stupor, disorientation, coma, tremors, convulsions, muscle weakness, and paralysis, and these may be used as criteria.

Thus, as an example, a treated individual may show a decrease in such a symptom as measured by an appropriate assay or test. A treated individual may for example show a 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% or more decrease in one or more symptoms, compared to an individual who has not been treated.

For example, a patient disease may be defined as being "treated" if a condition associated with the disease is significantly inhibited (*i.e.*, by 50% or more) relative to controls. The inhibition may be by at least 75% relative to controls, such as by 90%, by 95% or 100% relative to controls. By the term "treatment" we mean to also include prophylaxis or alleviation of flaviviral infection.

The antibody approach to therapy involving use of anti-envelope glycoprotein (E) domain III antibodies may be combined with other approaches for therapy of such disorders including conventional drug based approaches.

FLAVIVIRUS

The flavivirus may comprise a tick-borne virus. The flavivirus may comprise a mosquito-borne virus. The flavivirus may comprise a virus with no known arthropod vector.

The flavivirus may comprise a virus within the mammalian tick-borne virus group, such as a Gadgets Gully virus (GGYV), Kadam virus (KADV), Kyasanur Forest disease virus (KFDV), Langat virus (LGTV), Omsk hemorrhagic fever virus (OHFV), Powassan virus (POWV), Royal Farm virus (RFV), Tick-borne encephalitis virus (TBEV) or a Louping ill virus (LIV). The flavivirus may comprise a virus within the seabird tick-borne virus group, such as a Meaban virus (MEAV), a Saumarez Reef virus (SREV) or a Tyuleniy virus (TYUV)

The flavivirus may comprise a virus within the Aroa virus group, such as an Aroa virus (AROAV). The flavivirus may comprise a virus within the Dengue virus group. The flavivirus may comprise a Dengue virus (DENV) or a Kedougou virus (KEDV). The flavivirus may comprise a virus within the Japanese encephalitis virus group, such as a Cacipacore virus (CPCV), Koutango virus (KOUV), Japanese encephalitis virus (JEV), Murray Valley encephalitis virus (MVEV), St. Louis encephalitis virus (SLEV), Usutu virus (USUV), West Nile virus (WNV) or Yaounde virus (YAOV). The flavivirus may comprise a virus within the Kokobera virus group, such as a Kokobera virus (KOKV). The flavivirus may comprise a virus within the Ntaya virus group, such as a Bagaza virus (BAGV), Ilheus virus (ILHV), Israel turkey meningoencephalomyelitis virus (ITV), Ntaya virus (NTAV) or Tembusu virus (TMUV). The flavivirus may comprise a virus within the Spondweni virus group, such as a Zika virus (ZIKV).

The flavivirus may comprise a virus within the Yellow fever virus group, such as a Banzi virus (BANV), Bouboui virus (BOUV), Edge Hill virus (EHV), Jugra virus (JUGV), Saboya virus (SABV), Sepik virus (SEPV), Uganda S virus (UGSV), Wesselsbron virus (WESSV) or Yellow fever virus (YFV).

The flavivirus may comprise a virus within the Entebbe virus group, such as Entebbe bat virus (ENTV) or Yokose virus (YOKV). The flavivirus may comprise a virus within the Modoc virus group, such as Apoi virus (APOIV), Cowbone Ridge virus (CRV), Jutiapa virus (JUTV), Modoc virus (MODV), Sal Vieja virus (SVV) and San Perlita virus (SPV). The flavivirus may comprise a virus within the Rio Bravo virus group, such as Bukalasa bat virus (BBV), Carey Island virus (CIV), Dakar bat virus (DBV), Montana myotis leukoencephalitis virus (MMLV), Phnom Penh bat virus (PPBV) and Rio Bravo virus (RBV).

DENGUE

For the purposes of this description, the terms "dengue", "dengue fever" and "dengue hemorrhagic fever" should be considered synonymous.

Dengue and dengue hemorrhagic fever (DHF) are acute febrile diseases, found in the tropics, with a geographical spread similar to malaria. Caused by one of four closely related virus serotypes of the genus *Flavivirus*, family *Flaviviridae*, each serotype is sufficiently different that there is no cross-protection and epidemics caused by multiple serotypes (hyperendemicity) can occur. Dengue is transmitted to humans by the mosquito *Aedes aegypti* (rarely *Aedes albopictus*).

Signs and Symptoms

The disease is manifested by a sudden onset of fever, with severe headache, joint and muscular pains (myalgias and arthralgias — severe pain gives it the name break-bone fever) and rashes; the dengue rash is characteristically bright red petechia and usually appears first on the lower limbs and the chest - in some patients, it spreads to cover most of the body. There may also be gastritis with some combination of associated abdominal pain, nausea, vomiting or diarrhoea.

Some cases develop much milder symptoms, which can, when no rash is present, be misdiagnosed as a flu or other viral infection. Thus, travellers from tropical areas may inadvertently pass on dengue in their home countries, having not being properly diagnosed at

the height of their illness. Patients with dengue can only pass on the infection through mosquitoes or blood products while they are still febrile.

The classic dengue fever lasts about six to seven days, with a smaller peak of fever at the trailing end of the fever (the so-called "biphasic pattern"). Clinically, the platelet count will drop until the patient's temperature is normal.

Cases of DHF also show higher fever, haemorrhagic phenomena, thrombocytopenia and haemoconcentration. A small proportion of cases leads to dengue shock syndrome (DSS) which has a high mortality rate.

Diagnosis

The diagnosis of dengue is usually made clinically. The classic picture is high fever with no localising source of infection, a petechial rash with thrombocytopenia and relative leukopenia.

Serology and PCR (polymerase chain reaction) studies are available to confirm the diagnosis of dengue if clinically indicated.

Treatment

The mainstay of treatment is supportive therapy. The patient is encouraged to keep up oral intake, especially of oral fluids. If the patient is unable to maintain oral intake, supplementation with intravenous fluids may be necessary to prevent dehydration and significant hemoconcentration. A platelet transfusion is indicated if the platelet level drops significantly.

Prevention

There is no commercially available vaccine for the dengue flavivirus. However, one of the many ongoing vaccine development programs is the Pediatric Dengue Vaccine Initiative (PDVI [1]) which was set up in 2003 with the aim of accelerating the development and introduction of dengue vaccine(s) that are affordable and accessible to poor children in endemic countries.

Primary prevention of dengue mainly resides in eliminating or reducing the mosquito vector for dengue. Initiatives to eradicate pools of standing water (such as in flowerpots) have

proven useful in controlling mosquito borne diseases. Promising new techniques have been recently reported from Oxford University on rendering the Aedes mosquito pest sterile.

Personal prevention consists of the use of mosquito nets, repellents and avoiding endemic areas.

ASSAYS FOR DENGUE

Dengue infection may be assayed by a number of methods, including a method of plaque assay in some embodiments.

Plaque Assay for Dengue

Confluent monolayers of Vero cells are grown in Iscove's medium (HyClone, Logan, Utah) supplemented with 9% heat-inactivated fetal bovine serum (FBS) (HyClone), sodium bicarbonate (0.75 g/liter), penicillin G (100 U/ml), and streptomycin sulfate (100 μg/ml) (indicated as Iscove-9% FBS medium) in 12-well plates at 37°C and 5% CO2. Media containing 4.7% FBS (Iscove-4.7% FBS) or lacking FBS (Iscove-0% FBS) are also used in this study.

Vero cells are seeded into 12-well plates at 5.0 to 5.5 log10 cells per well. Viral infection is performed by aspirating the growth medium from freshly confluent Vero cell cultures, washing the cells sheets twice with 2 ml of Iscove-0% FBS medium, and adding 100 μl of Iscove-0% FBS medium containing dengue virus to deliver a multiplicity of infection (MOI) of 1.0 or 2.0 PFU/cell. Following adsorption of virus for 2 h at 37°C with 5% CO2, the viral inocula are aspirated, the cell sheets are rinsed three times each with 2 ml of PBS, and 1.0 ml of Iscove-0% FBS medium containing the appropriate concentration of P4-PMO is added, followed by incubation of the plates at 37°C with 5% CO2. Except where stated, the replacement media are not changed again for the duration of the growth curve experiment. Controls for these experiments include untreated cells.

At various time intervals, a 20-μl aliquot of medium is removed from each virus-infected well, diluted 1:16 or 1:32 in freezing medium (Iscove-35% FBS medium), and stored at –80°C until plaque titration.

Plaque titrations are performed under agarose overlay in Vero cell monolayers grown in six-well plates as described previously (Butrape et al 2000. J. Virol. 74:3011-3019 and

Miller and Mitchell, 1986, Am. J. Trop. Med. Hyg. 35:1302-1309). In each viral growth curve, the sensitivity limit of plaque titration is indicated by a horizontal line at 1.9 or 2.2 log10 PFU/ml, which resulted from plating 200 µl of the 1:16 or 1:32 dilution of harvested virus in the first well of the six-well plate, respectively.

Cytotoxicity Assay

Cytotoxicity was monitored using fluorescein diacetate (FDA) as previously described (Zhang *et al* 2006).

Other Dengue Assays

Payne et al., 2006, J Virol Methods. 27 describes a method for the quantitation of flaviviruses by fluorescent focus assay. Such an assay may be used instead of, or in combination with, a standard plaque assay. In summary, the assay comprises the following:

Vero cells are plated in 8-well chamber slides, and infected with 10-fold serial dilutions of virus. About 1-3 days after infection, cells are fixed, incubated with specific monoclonal antibody, and stained with a secondary antibody labeled with a fluorescent tag. Fluorescent foci of infection are observed and counted using a fluorescence microscope, and viral titers are calculated as fluorescent focus units (FFU) per ml. The optimal time for performing the fluorescent focus assay (FFA) on Vero cells was 24h for Dengue virus serotypes compared to up to 11 days for a standard Vero cell plaque assay

Other assays which may be used include those described in US 6,855,521, a serotype and dengue group specific flurogenic probe based PCR (TaqMan) assay against the respective C and NS5 genomic and 3' non-coding regions of dengue virus, and US 6,793,488 which describes a Flavivirus detection and quantification assay

DENGUE ENVELOPE GLYCOPROTEIN (E)

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, 2008) and the laminin-binding protein (Thepparit & Smith, 2004, Tio et al., 2005).

Despite this variety of host cell surface receptors identified, one viral component that is 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).

The flavivirus envelope glycoprotein (E), which forms an icosahedral scaffold at the virion surface, is the primary determinant of host-cell tropism and the target of neutralizing antibodies (Rey et al., 1995, Roehrig et al., 2004).

The E protein was found to be structurally similar to the Semliki Forest alphavirus E1 protein, leading to the concept of class II viral fusion glycoproteins (Lescar et al., 2001). X-ray crystallographic studies of soluble fragments of the E protein from DENV-2 and 3, tick-borne encephalitis and West Nile virus revealed a well-conserved elongated structure (Kanai et al., 2006, Modis et al., 2003, Modis et al., 2005, Nybakken et al., 2006, Rey et al., 1995). The molecule consists of three domains. The central domain I bears predominantly serotype-specific non-neutralizing epitopes, the homodimerization domain II contains the fusion loop at its extremity (Allison et al., 2001) and can elicit both neutralizing and non-neutralizing mAbs (Crill & Roehrig, 2001).

Domain III, which is involved in receptor binding (Allison et al., 2001, Bressanelli et al., 2004, Heinz et al., 2003, Rey et al., 1995), is connected to a C-terminal stem anchored to the viral membrane and adopts an immunoglobulin constant domain fold. It contains three exposed loops centered at residues 308, 335 and 387 that project from the surface of the mature virion and are thus largely accessible to antibodies. At endosomal pH, the virion undergoes structural rearrangements leading to homotrimerization of the E protein (Modis et al., 2004).

PHARMACEUTICAL COMPOSITIONS

As disclosed herein, anti-envelope glycoprotein (E) domain III antibodies may be used to treat or prevent flaviviral disease and infection, including dengue infection and West Nile Virus infection.

Anti-envelope glycoprotein (E) domain III antibodies can be administered in a variety of ways including enteral, parenteral and topical routes of administration. For example, suitable modes of administration include oral, subcutaneous, transdermal, transmucosal,

iontophoretic, intravenous, intramuscular, intraperitoneal, intranasal, subdural, rectal, and the like.

In accordance with other embodiments, there is provided a composition comprising an anti-envelope glycoprotein (E) domain III antibody, together with a pharmaceutically acceptable carrier or excipient for the treatment or prevention of flaviviral disease and infection, including dengue infection and West Nile Virus infection.

Suitable pharmaceutically acceptable excipients include processing agents and drug delivery modifiers and enhancers, such as, for example, calcium phosphate, magnesium stearate, talc, monosaccharides, disaccharides, starch, gelatin, cellulose, methyl cellulose, sodium carboxymethyl cellulose, dextrose, hydroxypropyl-p-cyclodextrin, polyvinylpyrrolidinone, low melting waxes, ion exchange resins, and the like, as well as combinations of any two or more thereof. Other suitable pharmaceutically acceptable excipients are described in "Remington's Pharmaceutical Sciences," Mack Pub. Co., New Jersey (1991), incorporated herein by reference.

Pharmaceutical compositions containing an anti-envelope glycoprotein (E) domain III antibody may be in any form suitable for the intended method of administration, including, for example, a solution, a suspension, or an emulsion. Liquid carriers are typically used in preparing solutions, suspensions, and emulsions. Liquid carriers contemplated for use in the practice include, for example, water, saline, pharmaceutically acceptable organic solvent (s), pharmaceutically acceptable oils or fats, and the like, as well as mixtures of two or more thereof. The liquid carrier may contain other suitable pharmaceutically acceptable additives such as solubilizers, emulsifiers, nutrients, buffers, preservatives, suspending agents, thickening agents, viscosity regulators, stabilizers, and the like. Suitable organic solvents include, for example, monohydric alcohols, such as ethanol, and polyhydric alcohols, such as glycols.

Suitable oils include, for example, soybean oil, coconut oil, olive oil, safflower oil, cottonseed oil, and the like. For parenteral administration, the carrier can also be an oily ester such as ethyl oleate, isopropyl myristate, and the like. Compositions may also be in the form of microparticles, microcapsules, liposomal encapsulates, and the like, as well as combinations of any two or more thereof.

The anti-envelope glycoprotein (E) domain III antibody may be administered orally, parenterally, sublingually, by inhalation spray, rectally, or topically in dosage unit formulations containing conventional nontoxic pharmaceutically acceptable carriers, adjuvants, and vehicles as desired. Topical administration may also involve the use of transdermal administration such as transdermal patches or ionophoresis devices. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intrastemal injection, or infusion techniques.

Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-propanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono-or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

Suppositories for rectal administration of the drug can be prepared by mixing the drug with a suitable nonirritating excipient such as cocoa butter and polyethylene glycols that are solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum and release the drug.

Solid dosage forms for oral administration may include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active compound may be admixed with at least one inert diluent such as sucrose lactose or starch. Such dosage forms may also comprise, as is normal practice, additional substances other than inert diluents, e. g., lubricating agents such as magnesium stearate. In the case of capsules, tablets, and pills, the dosage forms may also comprise buffering agents. Tablets and pills can additionally be prepared with enteric coatings.

Liquid dosage forms for oral administration may include pharmaceutically acceptable emulsions, solutions, suspensions, syrups, and elixirs containing inert diluents commonly used

in the art, such as water. Such compositions may also comprise adjuvants, such as wetting agents, emulsifying and suspending agents, cyclodextrins, and sweetening, flavoring, and perfuming agents.

In accordance with yet other embodiments, we provide methods for inhibiting any activity of envelope glycoprotein (E) domain III, in a human or animal subject, the method comprising administering to a subject an amount of an anti-envelope glycoprotein (E) domain III antibody (or composition comprising such compound) effective to inhibit the relevant activity in the subject. Other embodiments provide methods for treating flaviviral infection, including dengue and West Nile Virus, in a human or animal subject, comprising administering to the cell or to the human or animal subject an amount of a compound or composition as described here effective to inhibit a envelope glycoprotein (E) domain III activity in the cell or subject. The subject may be a human or non-human animal subject. Inhibition of protein activity includes detectable suppression of the relevant protein activity either as compared to a control or as compared to expected protein activity.

Effective amounts of the anti-envelope glycoprotein (E) domain III antibody generally include any amount sufficient to detectably inhibit the relevant protein activity by any of the assays described herein, by other assays known to those having ordinary skill in the art or by detecting an alleviation of symptoms in a subject afflicted with flaviviral infection, including dengue infection and West Nile Virus infection.

Successful treatment of a subject in accordance may result in the inducement of a reduction or alleviation of symptoms in a subject afflicted with a medical or biological disorder to, for example, halt the further progression of the disorder, or the prevention of the disorder. Thus, for example, treatment of flaviviral disease and infection, including dengue infection and West Nile Virus infection can result in a reduction in symptoms such as fever, severe headache, joint and muscular pains (myalgias and arthralgias), rashes, gastritis, abdominal pain, nausea, vomiting, diarrhoea, haemorrhagic phenomena, thrombocytopenia, haemoconcentration or dengue shock syndrome (DSS).

The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. It will be understood, however, that the specific dose level for any

particular patient will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, rate of excretion, drug combination, and the severity of the particular disease undergoing therapy. The therapeutically effective amount for a given situation can be readily determined by routine experimentation and is within the skill and judgment of the ordinary clinician.

A therapeutically effective dose will generally be from about 10μg/kg/day to 100mg/kg/day, for example from about 25μg/kg/day to about 20 mg/kg/day or from about 50μg/kg/day to about 2mg/kg/day of an anti-envelope glycoprotein (E) domain III antibody, which may be administered in one or multiple doses.

The anti-envelope glycoprotein (E) domain III antibody can also be administered in the form of liposomes. As is known in the art, liposomes are generally derived from phospholipids or other lipid substances. Liposomes are formed by mono-or multilamellar hydrated liquid crystals that are dispersed in an aqueous medium. Any non-toxic, physiologically acceptable and metabolizable lipid capable of forming liposomes can be used. The present compositions in liposome form can contain, in addition to a compound, stabilizers, preservatives, excipients, and the like. Lipids which may be used include the phospholipids and phosphatidyl cholines (lecithins), both natural and synthetic. Methods to form liposomes are known in the art. See, for example, Prescott, Ed., Methods in Cell Biology, Volume XIV, Academic Press, New York, N. W., p. 33 et seq (1976).

While the anti-envelope glycoprotein (E) domain III antibody can be administered as the sole active pharmaceutical agent, they can also be used in combination with one or more other agents used in the treatment of disorders. Representative agents useful in combination with the anti-envelope glycoprotein (E) domain III antibody for the treatment of flaviviral infection including dengue include, for example, either of the P4-PMO compounds, 5'SL and 3'CS (targeting the 5'-terminal nucleotides and the 3' cyclization sequence region, respectively) described in Kinney et al., 2005, Inhibition of dengue virus serotypes 1 to 4 in vero cell cultures with morpholino oligomers, J Virol. 79(8), 5116-28.

When additional active agents are used in combination with the anti-envelope glycoprotein (E) domain III antibody, the additional active agents may generally be employed

in therapeutic amounts as indicated in the PHYSICIANS'DESK REFERENCE (PDR) 53rd Edition (1999), which is incorporated herein by reference, or such therapeutically useful amounts as would be known to one of ordinary skill in the art.

The anti-envelope glycoprotein (E) domain III antibody and the other therapeutically active agents can be administered at the recommended maximum clinical dosage or at lower doses. Dosage levels of the active anti-envelope glycoprotein (E) domain III antibody in the compositions may be varied so as to obtain a desired therapeutic response depending on the route of administration, severity of the disease and the response of the patient. The combination can be administered as separate compositions or as a single dosage form containing both agents. When administered as a combination, the therapeutic agents can be formulated as separate compositions that are given at the same time or different times, or the therapeutic agents can be given as a single composition.

Bioavailability

The compounds disclosed here (and combinations) are in some embodiments orally bioavailable. Oral bioavailablity refers to the proportion of an orally administered drug that reaches the systemic circulation. The factors that determine oral bioavailability of a drug are dissolution, membrane permeability and metabolic stability. Typically, a screening cascade of firstly in vitro and then in vivo techniques is used to determine oral bioavailablity.

Dissolution, the solubilisation of the drug by the aqueous contents of the gastro-intestinal tract (GIT), can be predicted from in vitro solubility experiments conducted at appropriate pH to mimic the GIT. The anti-envelope glycoprotein (E) domain III antibody may in some embodiments have a minimum solubility of 50 mg/ml. Solubility can be determined by standard procedures known in the art such as described in Adv. Drug Deliv. Rev. 23, 3-25, 1997.

Membrane permeability refers to the passage of the compound through the cells of the GIT. Lipophilicity is a key property in predicting this and is defined by in vitro Log $D_{7.4}$ measurements using organic solvents and buffer. The anti-envelope glycoprotein (E) domain III antibody may have a Log $D_{7.4}$ of -2 to +4 or -1 to +2. The log D can be determined by standard procedures known in the art such as described in J. Pharm. Pharmacol. 1990, 42:144.

Cell monolayer assays such as $CaCO_2$ add substantially to prediction of favourable membrane permeability in the presence of efflux transporters such as p-glycoprotein, so-called caco-2 flux. The anti-envelope glycoprotein (E) domain III antibody may have a caco-2 flux of greater than $2x10^{-6}cms^{-1}$, for example greater than $5x10^{-6}cms^{-1}$. The caco flux value can be determined by standard procedures known in the art such as described in J. Pharm. Sci, 1990, 79, 595-600.

Metabolic stability addresses the ability of the GIT or the liver to metabolise compounds during the absorption process: the first pass effect. Assay systems such as microsomes, hepatocytes etc are predictive of metabolic liability. The compounds of the Examples may in some embodiments show metabolic stability in the assay system that is commensurate with an hepatic extraction of less than 0.5. Examples of assay systems and data manipulation are described in Curr. Opin. Drug Disc. Devel., 201, 4, 36-44, Drug Met. Disp.,2000, 28, 1518-1523.

Because of the interplay of the above processes further support that a drug will be orally bioavailable in humans can be gained by in vivo experiments in animals. Absolute bioavailability is determined in these studies by administering the compound separately or in mixtures by the oral route. For absolute determinations (% absorbed) the intravenous route is also employed. Examples of the assessment of oral bioavailability in animals can be found in Drug Met. Disp.,2001, 29, 82-87; J. Med Chem , 1997, 40, 827-829, Drug Met. Disp.,1999, 27, 221-226.

The term "pharmaceutically acceptable carrier" as used herein generally refers to organic or inorganic materials, which cannot react with active ingredients. The carriers include but are not limited to sugars, such as lactose, glucose and sucrose; starches such as corn starch and potato starch; cellulose and its derivatives such as sodium carboxymethycellulose, ethylcellulose and cellulose acetates; powdered tragancanth; malt; gelatin; talc; stearic acids; magnesium stearate; calcium sulfate; vegetable oils, such as peanut oil, cotton seed oil, sesame oil, olive oil, corn oil and oil of theobroma; polyols such as propylene glycol, glycerine, sorbitol, mannitol, and polyethylene glycol; agar; alginic acids; pyrogen-free water; isotonic saline; and phosphate buffer solution; skim milk powder; as well as other non-toxic compatible substances used in pharmaceutical formulations. Wetting agents and lubricants such as sodium lauryl sulfate, as well as coloring agents, flavoring agents,

lubricants, excipients, tabletting agents, stabilizers, anti-oxidants and preservatives, can also be present.

The term "therapeutically effective amount" as used herein generally refers to an amount of an agent, for example the amount of a compound as an active ingredient, that is sufficient to effect treatment as defined herein when administered to a subject in need of such treatment. A therapeutically effective amount of a compound, salt, derivative, isomer or enantiomer of the present invention will depend upon a number of factors including, for example, the age and weight of the subject, the precise condition requiring treatment and its severity, the nature of the formulation, and the route of administration, and will ultimately be at the discretion of the attendant physician or veterinarian. However, an effective amount of a compound of the present invention for the treatment of disorders associated with bacterial or viral infection, in particular bacterial meningitis, will generally be in the range of about 10 to about 40 mg/kg body weight of recipient (mammal) per day and more usually about 40 mg/kg body weight per day. Thus, for a 70 kg adult subject, the actual amount per day would typically be about 2,800 mg, and this amount may be given in a single dose per day or more usually in a number (such as two, three, four, five or six) of sub-doses per day such that the total daily dose is the same. An effective amount of a salt of the present invention may be determined as a proportion of the effective amount of the compound per se.

The term "treatment" as used herein refers to any treatment of a condition or disease in an animal, particularly a mammal, more particularly a human, and includes: preventing the disease or condition from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it; inhibiting the disease or condition, i.e. arresting its development; relieving the disease or condition, i.e. causing regression of the condition; or relieving the conditions caused by the disease, i.e. symptoms of the disease.

Chemical derivative

The term "derivative" or "derivatised" as used herein includes chemical modification of a compound. Illustrative of such chemical modifications would be replacement of hydrogen by a halo group, an alkyl group, an acyl group or an amino group.

Chemical modification

In one embodiment, the compound may be a chemically modified compound.

The chemical modification of a compound may either enhance or reduce hydrogen bonding interaction, charge interaction, hydrophobic interaction, Van Der Waals interaction or dipole interaction between the compound and the target.

In one aspect, the identified compound may act as a model (for example, a template) for the development of other compounds.

Individual

The compounds are delivered to individuals. As used herein, the term "individual" refers to vertebrates, particularly members of the mammalian species. The term includes but is not limited to domestic animals, sports animals, primates and humans.

EXAMPLES

Example 1. Cells and Viruses

BHK-21 and C6/36 cells (obtained from ATCC, VA) are grown in RPMI-1640 medium (Hybri-Care) containing 10% inactivated fœtal calf serum (iFCS).

Vero cells are grown in DMEM supplemented with 7 % iFCS. Strains of Dengue virus DENV 1 (Hawaii), DENV 2 (New Guinea C and TSVO1), DENV 3 (H87) and DENV 4 (H241) are propagated at 28 °C either in C6/36 or BHK-21 cells, supplemented with 5% iFCS.

Example 2. Cloning, Expression and Purification of Recombinant Domains III from DENV 1-4

The primers used for PCR amplifications are listed in Table E1 below.

Ed3	DENV-1 (291-395)	DENV2 (291-395)	DENV3 (291-395)	DENV4 (291-394)
Mass (kDa)	14370	14669	14495	14392
pI	7.2	7.3	8.5	8.6
Forward* Primers	5'CGTATGCTCGAGAAACTGACCTTAAAAG 3' SEQ ID NO:	5'CATTGCTCGAGAAACTACAGCTCAAA 3' SEQ ID NO:	5'CATATGCTCGAGAAATTGAAACTCACG 3' SEQ ID NO:	5'GCTCATGCTCGAGAAATTGAGAATTAAG 3' SEQ ID NO: 15

	9	11	13	
Reverse Primers	5'GCTCGTCATCGATTTATCCTTTCTTGAACCA 3' SEQ ID NO: 10	5'CGCTGCATCGATTTATCCTTTCTT AAACCA 3' SEQ ID NO: 12	5'CTTATCATCGATTTATCCCTTCCTGTACCA 3' SEQ ID NO: 14	5'CTTATCATCGATTTACCCTTTCCTGAA CCA 3' SEQ ID NO: 16

Table E1. Summary of domain III constructs. *The *XhoI* (forward) and *ClaI* (reverse) restriction sites are underlined respectively.

PCR products are digested and ligated into the Pet16b vector (Novagen). Transformed *E. coli* BL-21(DE3) cells are grown at 37°C until an OD_{600} of 0.8 and protein expression induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 6 hours at 30 °C. Cells harvested by centrifugation at 5000 g for 20 min at 4°C are resuspended in a buffer containing 50 mM Tris HCl (pH 8.5), 200 mM NaCl, 1% Nonidet-P40 and 1% Sodium desoxycholate and disrupted by sonication.

After centrifugation at 48,000 g for 30 minutes, inclusion bodies are solubilized in 8 M urea, 100 mM NaH_2PO4 and 10 mM Tris HCl (pH 8.0). The clarified supernatant is loaded onto a Ni-NTA column (Qiagen) pre-equilibrated with the same buffer and incubated overnight at 4°C. Proteins are eluted at pH 2.4. Refolding is carried out at 4°C by 50 × dilution for 4-5 days in 200 mM Tris HCl (pH 8.2), 200 mM NaCl, 10 mM EDTA, 5 mM and 0.5 mM reduced/oxidized Glutathione and 50 mM L-arginine.

Refolded proteins concentrated by ultrafiltration (Amicon) are purified on a Superdex 75 (HR 10/30) column (GE Healthcare) in 12 mM Tris HCl (pH 8.0), 250 mM NaCl, 0.1mM EDTA and 3 mM DTT. Proper folding is assessed with Circular Dichroism (Chu et al., 2005).

Example 3. Hybridomas

mAb 9F12 is obtained by hyper-immunization of BALB/c mice with DENV2 TSV01 domain III, using standard protocols (Clancy et al., 2007).

Mouse hybridoma cells secreting 9F12 are initially grown in Hybri-Care medium in NUNC flasks and upon confluence, are switched to RPMI-1640, supplemented with 10%

iFCS. Cell supernatants are centrifuged at 800 g for 5 minutes at 22° C and stored at -20° C in 1 M Tris HCl (pH 8.0).

Example 4. Purification of mAb 9F12

A volume of 500 ml of hybridoma supernatants is mixed with sodium sulphate (powder) to a final concentration of 18% (w/v). The resulting precipitate is dissolved in 50 ml of distilled water and mixed with sodium sulphate to a concentration of 14% (w/v). The precipitate is dissolved in 15 ml distilled water and dialyzed against 10 mM sodium phosphate, pH 7.2 (Buffer A) overnight.

Purification is performed through a Cibacron Hi Trap blue column followed by a DEAE resin pre equilibrated with Buffer A at pH 8.0. The mAb is eluted with 25 mM and 40 mM of Buffer A at pH 8.0. Concentrated fractions are purified on a Superdex-200 column (GE healthcare). The mAb 9F12 isotype is determined by ELISA (Pierce).

Example 5. Plaque Reduction Neutralization Assays

The protocol follows Thullier et al., 1999. Briefly, all $PRNT_{50}$ assays are carried out with BHK-21 cell lines at 37°C. Serial dilutions of mAb 9F12 are mixed with 100 PFU/ml of DENV and incubated for 2 hours at 4°C. mAb and virus are incubated with BHK-21 cells at 37°C for 2 hrs.

After incubation, the mixture is replaced with RPMI - 1640 with 1% Carboxy methyl cellulose and appropriate mAb dilution and plates are placed in a 5% CO2 incubator for a period of 4 days for DENV-2, 5 days for DENV-4 and DENV-3 and 6 days for DENV-1. After fixing with 4% Formaldehyde and staining with 1% Methyl violet, plaques are manually counted and the neutralization capacity is estimated as the mAb concentration causing a 50% reduction in PFU.

Example 6. Adsorption Assays Using Cell-based Flavivirus Immuno-detection (CFI)

A 96-well micro culture dish is dispensed with 2×10^4 Vero cells per 100 µl in DMEM supplemented with 2% iFCS and incubated overnight at 37°C in a 5% CO_2 incubator. For the preadsorption assay, tenfold dilutions of either mAb 9F12 or 4G2 is mixed with equal volume of 2×10^4 PFU of DENV2 virus and incubated at 4°C for 1 hour.

The virus and mAb mixture is added to the confluent cell surface and incubated at 4°C for one hour for the virus to get adsorbed onto the cell surface. Negative control received serum free (sf) DMEM in place of the mAb. Cells are washed three times with sfDMEM at 4°C. After incubation with DMEM for 2 days, cells are mixed with the 1:20 diluted 4G2 antibody for immuno-detection. The immune reaction is probed with anti-mouse HRP conjugate, tetra methyl benzidine and stopped with 0.5 N sulphuric acid and absorption followed at 450 nm.

The presence of a uniform number of cells per well is confirmed by staining with Propidium Iodide (PI) (Sigma) in PBS for 10 minutes and the fluorescent signal read at 537-617 nm in a Tecan plate reader. For the postadsorption assay, virus dilution having 1×10^4 PFU of DENV2 is added to the cells directly and incubated at 4°C for 1 hour followed by washes with sf DMEM at 4°C to remove unadsorbed virus. The mAb dilutions are added to cell surface with the adsorbed virus and incubated for1 hour at 4°C. The rest of the assay to detect DENV2 NGC is as described for preadsorption.

Example 7. Membrane Fusion Inhibition Assay

A fusion inhibition assay essentially as described previously (Gollins & Porterfield, 1986, Modis et al., 2004, Stiasny et al., 2007) is standardized at NITD and used for screening fusion inhibitors with 4G2 as a positive control.

Assays are carried out in triplicate for both 4G2 and 9F12. A 96-well micro culture dish is dispensed with a mixture of 1.5×10^5 C6/36 cells and DENV2 at an m.o.i of 0.1 per 100 µl in RPMI -1640 supplemented with 5% iFCS and incubated for 72 hours at 28°C in an air-tight humidified container. Both 9F12 and 4G2, diluted to a final concentration of 10 µM in 95 µl of sf RPMI-1640 are added to the wells and incubated for 1 hour at 28°C.

Subsequently, 5 µl of 0.5M MES, (pH 5.0) is added to the wells and incubated at 37°C for 1 hour to induce syncytia formation. The low pH solution is finally replaced with 0.025mg/ml of Propidium iodide solution made in sf RPMI 1640 and incubated at 28°C for 30 minutes. Plates read at 10 × magnification are visualized with a Nikon Fluorescent microscope.

Example 8. Cloning, Expression and Purification of scFv9F12

A total of 10^7 cells of the mAb 9F12 hybridoma are used for RNA extraction using TRIzol reagents. The V_H chain is amplified with the forward primer (5'-CCA GTT CCG AGC TCG TGA TGA CAC AGT CTC CA-3', SEQ ID NO: 17) and reverse primer (5'-GCG CCG TCT AGA ATT AAC ACT CAT TCC TGT TGA A-3', SEQ ID NO: 18) and the V_L chain is amplified with a light chain primer mix from the cloning module of recombinant phage antibody system (GE healthcare, Sweden).

The final PCR products are purified by agarose gel extraction (Qiagen). DNA sequences obtained are shown below in Example 15. Cloning, expression and purification of the scFv 9F12 is according to (Rezacova et al., 2001).

Example 9. Measurement of Binding Affinities

Binding affinities of mAb 9F12 or scFv9F12 for various DENV domain III serotypes are determined by SPR at 25°C using a Biacore 3000 instrument and by ELISA using standard protocols. Each domain III is covalently immobilized on a carboxymethylated sensor surface (CM5, research grade) using amine coupling chemistry.

The surfaces are activated with 0.2 M EDC (N-ethyl-N'-[3-(diethylamino) propyl] carbodiimide) and 50 mM NHS, (N-hydroxysuccinimide) for 10 minutes. Following covalent binding, surface deactivation is performed using 1 M ethanolamine-HCl (pH 8.5) for 10 min, at a flow-rate of 10 µl/min. The reference surface is treated as the ligand surfaces except that protein injection is omitted.

For the determination of kinetic parameters, either mAb9F12 or the scFv is passed above the reference and protein surfaces in duplicates at five to seven concentrations, in HBS (10mM Hepes buffer, pH 7.4, 150 mM NaCl, 3.4 mM EDTA and 0.005% P-20), at a flow rate of 30 µl/min.

Surfaces are regenerated using the same flow-rate by a 15 µl injection of 50 mM HCl. The level of immobilization for each serotype is specified in the figure legend, and is chosen to avoid mass transfer limitation. For the bivalent mAb, sensorgrams are fitted using the bivalent analyte model.

Example 10. Epitope Mapping

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 is expressed on the surface of yeast as an Aga2 fusion protein as described (Sukulpolvi et al 2007).

DENV-2 Domain III mutants that are generated as part of a random library by error prone mutagenesis in the pYD1 vector are expressed on the surface of yeast as described (Sukulpolvi et al 2007).Wild type or mutant DENV-2 Domain III displayed on yeast are 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 are 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 are analyzed on a FACS Scan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ) using Flo-Jo software. A set of 11 overlapping peptides, each comprising 10 to 12 amino acids from DENV2 domain III is synthesized and purified by HPLC.

ELISA is performed following the protocol of (Thullier et al., 2001). Briefly, microtitre \plates are coated with poly-(L-Lysine) (Sigma) in 0.1M bicarbonate buffer (pH 9.6) followed by incubation with 0.1% glutaraldehyde in PBS. Wells are coated with 100 μl of peptides at concentrations ranging from 10^{-4} M - 10^{-7} M followed by blocking with 50 mM Glycine, PBS-EDTA. A volume of 100 μl of mAb 9F12 at 10^{-8} M is distributed in the wells.

Example 11. Purification and Characterization of Recombinant Domain III

Domain III from DENV1-4 are expressed at high level as inclusion bodies that required refolding. Domain III from WNV is expressed and purified as in Chu et al. (2005). Typically, bacterial culture of 1 litre resulted in 6-8 mg of pure proteins that are refolded as described in the material and methods section.

A typical SDS-PAGE of purified domain III from DENV1-4 is shown in Figure 1A.

For unknown reasons, the stability of DENV4 domain III is much poorer. The identity of the expressed proteins is confirmed by mass spectrometry (not shown). CD spectroscopy is

used to assess the folding of recombinant domain III which elute as monomers as judged by gel filtration profiles.

An example is shown for DENV2 domain III in Figure 1B indicating proper folding with a predominantly β-sheet structure, in agreement with the known 3D structure (Volk et al., 2004).

An alignment of the amino-acid sequences of the various domains III studied in this work is presented in Figure 1C.

Example 12. Preliminary Characterization of Mouse mAb 9F12

As part of an effort to raise antibodies against all four DENV serotypes, we immunized BALB/c mice with 50 μg of purified domain III from DENV2 (TSV01 isolate) using standard protocols.

A panel of mAbs that reacted with DENV2 domain III are identified using ELISA and 9F12 is selected for further studies because it cross-reacted with domain III from DENV1-4 and WNV in a Western Blot assay under denaturing conditions, and also in a dot-blot immunoassay under non-denaturing condition. mAb 9F12 belongs to the IgG1 subtype with *kappa* light chain.

Example 13. *In vitro* Neutralization of DENV by mAb 9F12

Given its broad cross-reactivity towards various recombinant domain III proteins, we tested the neutralizing activity of mAb 9F12 against all four DENV serotypes in a plaque reduction neutralization assay as shown in Figure 2.

Interestingly, purified mAb9F12 neutralized all five DENV strains tested, belonging to serotypes DENV1-4. The $PRNT_{50}$ ranged from 2×10^{-8} to 2×10^{-7} for DENV2, 4 and 1, 3, respectively. This is shown in Figure 2A and Table E2 below.

Feature	9F12
Source	Mouse monoclonal
Isotype	IgG1
Antigen	DENV2 (TSVO1) Envelope Protein domain III
Specificity	Cross reactive
DENV1 (Hawaii)	1.72×10^{-8}M
	$\pm 0.92 \times 10^{-8}$M
DENV2 (NGC)	3.23×10^{-9}M
	$\pm 0.70 \times 10^{-9}$M
DENV2 (TSVO1)	3.61×10^{-9}M
	$\pm 0.87 \times 10^{-9}$M
DENV3 (H87)	8.6×10^{-9}M
	$\pm 1.93 \times 10^{-9}$M
DENV4 (H241)	6.55×10^{-9}M
	$\pm 1.89 \times 10^{-9}$M

Table E2: Characteristics of 9F12 used in the neutralization of Dengue viruses 1-4. Data were determined by Plaque reduction neutralization assay (PRNT) on BHK-21 cells with 100 PFU / well of DENV1 – 4 for the cross reactive 9F12. ± (calculated from 95% Confidence interval with $R^2 = 98 - 99\%$).

The commercially available mouse mAb4G2 is used as the positive control in the neutralization assay and naïve mouse serum is used as the negative control. The 50% neutralization point for the dengue strains tested varied between14 nM and 130 nM of 9F12.

The neutralizing capacity of mAb 9F12 appears to be comparable to that of mAb 4G2 (Positive control). Fab fragments prepared by papain cleavage and recombinant scFv9F12 are

also tested by $PRNT_{50}$ and showed a comparable inhibitory activity against all 5 strains tested indicating that the Fc region is not required for blocking viral infection (data not shown).

Example 14. Mechanism of Neutralization

As demonstrated by cell-based assays shown in Figure 2C and Figure 2D, mAb 9F12 hinders an early event in the virus life cycle, most likely viral adsorption and entry rather than some post cell entry events like fusion.

The C6/36 cells are stained by PI when the cell membranes are fused together to form syncytia, as seen in the virus control in the absence of any antibody or in the presence of mAb 9F12, whereas uninfected cells or the ones treated with mAb 4G2 appear intact.

The mAb 4G2 recognizes the fusion loop on domain II of E protein from all four serotypes hence it does not promote syncytia formation and is a fusion inhibitor. 9F12 on the other hand is a domain III specific that does not block fusion and hence promotes syncytia formation. This suggests that 9F12 neutralization is most likely pre-fusion.

Example 15. Sequencing of mAb 9F12, Cloning and Purification of scFv 9F12

In order to explore the molecular basis of virus neutralization by mab9F12, the VH and VL genes of antibody 9F12 are sequenced and a scFv fragment is cloned to facilitate further structural studies.

PCR amplification yielded products of 440 and 400 bp for its VH and VL variable domains respectively. The nucleotide and conceptually translated amino acid sequences of the V_H and V_L domains of mAb 9F12 are shown below. Residue numbering and indicated hypervariable regions are according to Kabat EA, Wu TT, Perry H, Gottesman K, Foeller C (1991). *Sequences of Proteins of Immunological Interest.* 5th Ed. NIH Publication No. 91-3242, Bethesda, MD.

Nucleotide Sequence of Heavy Chain (SEQ ID NO: 3)

```
  1 ctgcagcagt ctggggctga gctggtgagg cctggggctt cagtgaagct gtcctgcaag
 61 gctttgggct acagatttac tgactatgaa atgtactggg tgaagcagac acctgcacat
121 ggcctggaat ggattggagg tattcatcca agaagtggta atactgccta caatcagaag
181 ttcaaggaca aggccacact gactgcagac aaatcctcca gtacagccta catggagctc
241 agcagcctga catctgagga ctctgttgtc tattactgta ccacgtccct ctactggggc
301 caagggacca cggtcaccgt ctcctca
```

Amino Acid sequence of Heavy Chain (SEQ ID NO: 4)

```
  1   L Q Q S G A E L V R P G A S V K L S C K A L G Y R F T D Y E
 31   M Y W V K Q T P A H G L E W I G G I H P R S G N T A Y N Q K
 61   F K D K A T L T A D K S S S T A Y M E L S S L T S E D S V V
 91   Y Y C T T S L Y W G Q G T T V T V S S  109
```

Nucleotide Sequence of Light Chain (SEQ ID NO: 5)

```
  1 cttggagatc aagcctccat ctcttgcaga tctagtcaga gccttgtaca cagtaatgga
 61 aacacctatt tacattggta cctgcagaag ccaggccagt ctccaaagct cctgatctac
121 agcattttca accgattttc tggggtccca gacaggttca gtggcagtgg atcagggaca
181 gatttcacac tcaaaatcag cagagtggag gctgaggatc tgggagttta tttctgctct
241 caaggtacac atgttccgtg gacgttcggt ggaggcacca acctggaaat caaacgggct
301 gatgctgcac caactgtatc catcttccca ccatccagtg agcagttaac atctggaggt
361 gcctcagtcg tgtgcttctt gaacaacttc taccccaaag acatcaatgt caagtggaag
421 attgatggca gtgaacgaca aaatggcgtc ctgaacagtt ggactgatca ggacagcaaa
481 gacagcacct acagcatgag cagcaccctc acgttgacca aggacgagta tgaacgacat
541 aacagctata cctgtgaggc cactcacaag acatcaactt cacccattgt caagagcttc
601 aacaggaatg agtg
```

Amino Acid Sequence of Light Chain (SEQ ID NO: 6)

```
  1 L G D Q A S I S C R S S Q S L V H S N G N T Y L H W Y L Q K
 31 P G Q S P K L L I Y S I F N R F S G V P D R F S G S G S G T
 61 D F T L K I S R V E A E D L G V Y F C S Q G T H V P W T F G
 91 G G T N L E I K R A D A A P T V S I F P P S S E Q L T S G G
121 A S V V C F L N N F Y P K D I N V K W K I D G S E R Q N G V
151 L N S W T D Q D S K D S T Y S M S S T L T L T K D E Y E R H
181 N S Y T C E A T H K T S T S P I V K S F N R N E
```

The VH region of 9F12 shows highest sequence identity for the heavy chain of the anti-HIV-1 p24 Fab Fragment Cb41, an antibody exhibiting cross-reactivity and polyspecificity (PDB id: 1CFS) (Keitel et al., 1997).

The light chain of 9F12 is most similar to the anti-HIV Protease Fab fragment (PDB id: 1CL7) (Lescar et al., 1999) and to a heteroclitic antibody to lysozyme (PDB id: 1JHL) (Chitarra et al., 1993).

These structures are used to generate a 3D model of the scFv9F12 binding site. The CDR-H3 loop is short (4 amino acids long). Given the prominent role played by the heavy chain in recognizing the antigen –and especially the CDR-H3, which is located at the center of the antibody binding site- and the small size of the H3 region- the shape of the 9F12 antibody binding site is most probably concave.

Example 16. Binding Affinities of mAb 9F12 and scFv9F12 to Various Flavivirus Domain III

Initial binding assays indicated that mAb 9F12 broadly cross-reacts with various flavivirus domain III antigens. We probed the interaction between mAb 9F12 and several recombinant flavivirus domain III using two independent methods. First, the interactions are assessed using ELISA assays as shown in Figure 3A.

The EC_{50} values calculated for the interactions with DENV domain III range from 0.17 nM to 84 nM with the following binding affinities for the antigens: DENV-2 ~ DENV-4 > DENV-1 > DENV-3. Surprisingly, the apparent EC_{50} for WNV domain III is comparable to the immunogen DENV-2 (Figure 3). The observation of heteroclitic binding for mAb 9F12 (higher affinity for an antigen other than the immunogen) is not unprecedented and has been thoroughly analyzed using model antigens such as avian lysozymes (Chitarra et al., 1993; Lescar et al., 1995). It usually occurs when an epitope is shared between evolutionary-related antigens.

To confirm these results, scFv9F12 is expressed, purified and its binding affinities measured using SPR, along mAb 9F12, in order to obtain the kinetic parameters of the various complexes. These are shown in Table E3 below.

	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}	1×10^{-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 x 10^{5}	8.5 x 10^{-3}	4.0 x 10^{-8}	2.2 x 10^{5}	1.8 x 10^{-2}	8.1 x 10^{-8}
WNEd3	5.4 x 10^{4}	4.2 x 10^{-4}	7.8 x 10^{-9}	1.1 x 10^{5}	3.9x 10^{-5}	3.4 x 10^{-10}

Table E3. Kinetic constants and binding affinities of mAb 9F12 and scFv9F12 for the domain III of protein E from the different DENV serotypes and WNV by SPR. *The constants are evaluated from the sensorgrams depicted in Figure 3, using the Langmuir 1:1 model for the scFv and the bivalent analyte model for the mAb. The BIAevaluation 4.1 software was used for data analysis. For the mAb, K_D' is an apparent affinity calculated by the ratio kd1/ka1 obtained from the fit with the bivalent analyte model. For the scFv fitting with a bivalent analyte model gave a slightly better fit suggesting that a part of the scFv molecules were not monomeric (Dolezal et al., 2000, Kortt et al., 1994). In all cases, χ^2 was lower than 5% of Rmax.

Due to the high level of non specific interactions established by domain III proteins with the sensor chip surface, which could not be overcome neither by increasing NaCl concentration nor by adding carboxy methyl dextran to the sample buffer, we immobilized the antigen on the surface and passed the mAb 9F12 and its scFv as "analytes". The binding of the mAb and the scFv is specific.

Figure 2B shows 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.

As shown in Table E3 above, mAb 9F12 binds to all domain III proteins from the different serotypes of DENV and also to WNV, but with different affinities. The kinetic parameters and affinities are evaluated from the sensorgrams obtained for each serotype. An approximation of the active concentrations of mAb 9F12 and scFv9F12 are obtained using its initial binding rates to a high level immobilized surface of domain III of the E protein of DENV-2 in which mass transfer limitation is almost complete (Karlsson et al., 1994).

The highest affinities observed are for the interaction between mAb9F12 and DENV 2, WNV and DENV 2 TSV01 that all bind with the same order of magnitude. For DENV 1 the affinity is one order of magnitude lower and for DENV 3, two orders of magnitude lower than

for the immunogen. The same pattern for the affinities is observed for scFv 9F12 as seen in Table E3 above.

Example 17. Epitope Mapping

To map the epitope recognized by mAb9F12, we used a panel of DENV-2 domain III mutants displayed on the surface of yeast that are previously generated to map a panel of type-specific, subcomplex-specific and cross-reactive mAbs (Sukulpolvi et al 2007).

9F12 showed markedly reduced binding with domain III mutations at residues K305, K307, K310, and G330. This is shown in Figure 4A. Yet it retained binding to variants at residues E383 and P384. This pattern of binding is most consistent with a complex-specific neutralizing epitope centered on the A-strand of domain III, as shown in Figure 4B.

We also synthesized overlapping linear peptides corresponding to DENV 2 domain III sequence and used them for epitope mapping by direct ELISA. Peptides 'a' (298-SYSMCTGKFKVV-309) and 'b' (324-VQYEGDGSPKI-335), showed comparable binding activity whereas peptides 'e' to 'k' along with a peptide from an unrelated protein (from the Coronavirus N protein) showed no appreciable binding, as seen in Table E4 below.

Peptide Representing	Amino Acids	EC_{50}	SEQ ID NO:
N- Terminal loop (SYSMCTGKFKVV)	S298-V309	8.06E-04	SEQ ID NO: 19
BC loop (VQYEGDGSPCKI)	V324-I335	8.24E-07	SEQ ID NO: 20
DE loop (IVTEKSPVN)	I357-N366	4.65E-06	SEQ ID NO: 21
FG loop (IIGVEPGQLKLN)	I379-N390	2.93E-05	SEQ ID NO: 22
Positive control (D2NEd3)	K291-I398	1.19E-09	
Unrelated peptide	Corona virus N	2.61E-03	
e (KLQLKGMSYS)	K291-S300	7.80E-04	SEQ ID NO: 23
f (VKEIAETQHG)	V309-G318	2.82E-05	SEQ ID NO: 24
g (TIVIRVQYEG)	T319-G328	6.30E-03	SEQ ID NO: 25
h (PFEIMDLEKR)	P336-R345	1.91E-04	SEQ ID NO: 26
i (HVLGRLITVNP)	H346-P356	3.02E-03	SEQ ID NO: 27
j (IEAEPPFGDSYI)	I367-I378	3.80E-04	SEQ ID NO: 28
k (LNWKKGSSSI)	L389-I398	3.70E-03	SEQ ID NO: 29

Table E4. EC50 values for 11 different synthetic peptides from DENV2 to mAb9F12 in an ELISA based assay.

Mapping these peptide binding results onto the three-dimensional crystal structure of domain III (PDB id 1k4r) confirms a 9F12 binding epitope involving two of the three exposed loops spanning residues 304-308 of the 'A' β-strand and 326-331 within the BC loop, as shown in Figure 4B.

Example 18. Cytopathicity Assay on West Nile Virus

On Day 1, cells are seeded at a density of 5,000 cells/well, vol = 50μl/well in medium with 2% FBS, 1%P/S (5%FBS if Huh-7 is being used).

On Day 2, cells are infected with DENV at moi=1. Virus is diluted with medium, and 50μl of diluted virus is added to each well.

On Day 5, 10μl of cell-titer glo (Promega) are added to each well. The manufacturer's protocol is followed for reading.

The results of the cytopathicity assay are shown in Figure 7. The results indicate that the antibody exhibits low level of protection against West Nile virus challenge *in vitro*.

Example 18. In Vivo Neutralization Experiments in Mice

Three groups of 4 mice each are injected with 0.1mg of mAb 9F12, immune serum (Positive control) or phosphate buffered saline (negative control) one day prior to virus challenge.

Mice are subsequently challenged with 2 x 10^6 pfu of strain TSVO1 in 0.4 ml volume of virus suspension intraperitoneally. Plasma and sera are collected and tested for Plaque assay, RT-PCR and NS1.

Plaque assay is performed as described in reference (Morens *et al.*, 1985). Briefly, 1.5 x 10^5 numbers of BHK-21 cells per well are seeded in a 24 well multi dish (Nunc) and grown to confluency. Serially diluted plasma samples containing the viruses are overlaid on to the cell surface and incubated for 1 hour at 37°C with 5% CO_2. Then the virus suspension is replaced with 0.8% carboxy methyl cellulose in RPMI 1640. Plates are incubated for 5 days at 37°C with 5% CO_2 at the end of which cells are fixed with 10% Formaldehyde and stained with 1% crystal violet in water for 20 minutes.

The results of the plaque titration assay are shown in Figure 6A. The results show moderate to good protection levels comparable to the immune serum used as positive control in the study.

Example 19. Detection of NS1 Levels in Mice Plasma using PLATELIA-NS1 (BioRad)

Microtitration plates (Maxisorp; Nunc) are coated overnight at 4°C with purified anti-NS1 monoclonal antibodies (mAbs) 17A12 and 4F7. Wells are washed with PBS-Tween 20 (0.05%) and blocked with 3% skimmed milk.

Plasma samples are diluted in PBST and incubated for 1 hour at 37°C, followed by an overnight incubation at 4°C. Wells are washed again and probed for NS1 for 1 hour at 37°C with peroxidase-labeled mAb 12E5. After a final wash, peroxidase activity is detected with 3,

3', 5, 5'-tetramethyl benzidine (TMB) solution (Kirkegaard & Perry Laboratories) and the reaction is stopped by 2.5N sulphuric acid. The colour developed is read at 450 nm.

In this preliminary experiment of mouse protection assay with 9F12 and DENV2 performed in our lab, a significantly reduced amount of the viral NS1 protein is observed, suggesting that the 9F12 antibody may retain neutralization activity *in vivo*.

Example 20. Binding capacity of 9F12 Against Standard Anti Dengue Monoclonal Antibodies by ELISA

An experiment was done to compare virus binding capacity of 9F12 against standard anti dengue monoclonal antibodies by ELISA (protocol described in methods section below).

Whole virus isolates of Dengue virus were inactivated and coated onto a microtitre plate. Monoclonal antibodies diluted to 1:10 with Phosphate buffered saline in 5% skimmed milk and 1% (vol/vol) Tween-20 were reacted with the coated virus.

Secondary antibodies conjugated to Horse radish peroxidase enzyme were used to detect the binding followed by the addition of Tetra methyl benzidine substrate resulting in a blue colour which changes to yellow on addition of 3M HCl. The colour intensity is measured using absorption of 450 nm.

		1:10			**1:10**
D1Eden	**1:10**	**STDEV**	**D2Eden**	**1:10**	**STDEV**
4G2	0.9354	0.01867	4G2	0.6865	0.00156
9F12	0.731	0.00368	9F12	0.6835	0.00354
3H5	0.0108	0.00382	3H5	0.4943	0.01047

		1:10			1:10
D2TSVO1	**1:10**	**STDEV**	**D3Eden**	**1:10**	**STDEV**
4G2	0.8002	0.07255	4G2	0.3505	0.01478
9F12	0.8554	0.01259	9F12	0.2149	0.00495
3H5	0.886	0.07849	3H5	0.0354	0.0046

		1:10
D4Eden	**1:10**	**STDEV**
4G2	0.6206	0.00368
9F12	0.7325	0.03748
3H5	0.0643	0.00672

The results are plotted at Figure 8 with the standard deviation of a duplicate set of measurements. D1-D4: DENV1-4 serotypes. D2TSVO1 – the source of Envelope protein domain III, the immunogen, against which the mAb 9F12 was raised.

The detailed protocol carried out is as follow:

Enzyme Linked Immunosorbent assay (ELISA):

1. Coated Nunc flat 96 well immunoplate with 0.5ug of UV-inactivated DENV1 Hawaii whole virus per well in 50 uL of 50mM bicarbonate buffer (pH9.6) at 4°C overnight.

2. Washed plate twice with PBS

3. Blocked wells with 200uL of 5% (wt/vol) milk in PBS with 1% (vol/vol) Tween-20 for 1 hour at 22°C.

4. Washed plate twice with PBST

5. Added 50uL of inactivated and 1:10 diluted monoclonal antibodies and incubated at 4°C overnight.

6. Washed plate 5X with PBST

7. Added 100uL per well of 1/1000 diluted anti-mouse HRP conjugate and incubated for 1 hour at 22°C

8. Washed thrice with PBST and once with just PBS only.

9. Added 50uL of TMB substrate per well. Covered and incubated at 22°C in dark for a minimum of 5minutes to a maximum of 30 minutes for the blue colour to develop.

10. Added 50uL of 3M HCl to arrest reaction. Colour turns Yellow. The absorbance was read at 450nm with the reference filter set at 602nm using a Tecan plate reader.

Example 21. Immunoflurescent Staining of Dengue Virus Infected Cells with Fluorescent mAb 9F12

An experiment was conducted using immunoflurescent staining of dengue virus type-2 infected A-549 cells with mAb 9F12 made fluorescent by using a secondary antibody conjugated to Alexa fluor 488 to give a green fluorescence.

Figure 9A shows A-549 cells uninfected (left) and cells infected with 5MOI (3days after infection) reacted with mAb 9F12 (right).

Figure 9B shows results from the same procedure but with 10MOI to show the difference in staining pattern of mAb 4G2 (left), which gives a homogeneous fluorescence whereas that of mAB 9F12 (right) gives a bright speckled fluorescence.

Figure 9C uses the same procedure as in Figure 9B but 2 days after infection reacted with 4G2 (Left), which is broadly cross reactive to other flaviviruses, mAb 9F12(middle), cross reactive to Dengue and West nile viruses only, compared to that of mAb 3H5 (right), which is highly specific to dengue virus serotype 2 only. mAb 9F12 shows a comparable fluorescence staining to that of a highly specific mAb, 3H5.

The detailed protocol carried out is as follow:

1. Added 8,000 cells of A-549 per well in 100uL of F12K medium and incubated overnight at 37°C with 5% CO2.

2. Infected with Dengue 2 virus at required MOI and continued incubation at 37°C with 5% CO2.

3. At the end of incubation period, the cells were fixed with a mixture of 60% Acetone + 40% Methanol at -20°C for 10 min.

4. Washed the wells with PBS containing 5% Bovine serum albumin.

5. Added 50 µl of 1:20 diluted monoclonal antibody onto each well.

6. Incubated at 37 0C for 45 mins in a humidified chamber.

7. Washed twice with 1X PBS. Air dried.

8. Added 50µl 1:500 diluted Alexafluor 488 conjugate.

9. Incubated at 37 0C for 45 mins in a humidified chamber. 10. Washed slide by soaking twice with 1X PBS. Air dried.

11. Added 50 µl of buffered glycerol per well. 12. Viewed under fluorescence microscope.

Example 22. Discussion

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 A-strand) and three loops BC (330-333), DE (365-368) and FG (389-391).

Neutralizing mAbs, particularly to DENV, are proposed to bind two structurally distinct epitopes centered either on the FG loop, as in the case of mAb 3H5 (Gromowski et al., 2008), or to the more conserved A strand like mAb 1A1D-2 (Lok et al., 2008). By contrast, several cross-reactive mAbs that bind to residues from the AB loop (313 - 319) are 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 and the BC loop, as shown in Figure 4B. Whether a smaller area of contact between 9F12 with one or more conserved residues at the surface of domain III favors cross-reactivity

must await further structural studies. Comparing the domain III sequences, as shown in Figure 1C, amongst residues that form the epitope recognized by mAb9F12, K305 and G330 are shared by DENV2 and 4 and K307 by DENV2, 1 and WNV.

A recent immunopathological 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. 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.

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Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the claims.

CLAIMS

1. An polypeptidecapable of binding to a dengue envelope glycoprotein (E) polypeptide, in which the polypeptide comprises the variable region of monoclonal antibody 9F12 (SEQ ID NO: 4, SEQ ID NO: 6) capable of binding to an epitope bound by antibody 9F12.

2. An polypeptide according to Claim 1, in which the epitope comprises residues K305, K307, K310 and G330 of a dengue envelope glycoprotein (E) sequence, with reference to the position numbering shown as SEQ ID NO: 2.

3. An polypeptide according to any preceding claim, in which the polypeptide comprises any one or more of the following activities:

(i) binding to any one or more of envelope glycoprotein (E) from dengue virus serotype I, II, III and IV, capable of binding to envelope glycoprotein (E) from West Nile Virus, preferably domain III of envelope glycoprotein (E), with a EC_{50} binding affinity of 1μm or below, or 100 nm or below, 90 nm or below, 80 nm or below, 70 nm or below, 60 nm or below, 50 nm or below, 40 nm or below, 30 nm or below, 20 nm or below, 10 nm or below, 5 nm or below, 4 nm or below, 3 nm or below, 2 nm or below, 1 nm or below, 0.5 nm or below, 0.4 nm or below, 0.3 nm or below or 0.2 nm or below;

(ii) inhibiting a biological activity of envelope glycoprotein (E), selected from the group consisting of receptor binding activity, homotrimerization activity and virus absorbtion to host cells; or (iii) neutralizing dengue virus, from one or more of serotypes I, II, III and IV_5 capable of neutralizing West Nile Virus, as measured in a plaque-reduction neutralization assay, with a $PRNTs_0$ of 10^{-6} or below, or 2×10^{-7} or below.

4. An polypeptide according to any preceding claim which comprises a monoclonal antibody, preferably monoclonal antibody 9F12, a humanised monoclonal antibody, an Fv, F(ab'), $F(ab')_2$ or single-chain Fv (scFv) fragment, preferably a single chain Fv fragment comprising VH sequence (SEQ ID NO: 4) and VL sequence (SEQ ID NO: 6), preferably scFv9F12 (SEQ ID NO: 8).

5. A pharmaceutical composition comprising an polypeptide according to any preceding claim, together with a pharmaceutically acceptable excipient, diluent or carrier.

6. An polypeptide according to any of Claims 1 to 4 or a pharmaceutical composition according to Claim 5 for use in:

(i) a method of treatment or prevention of a flaviviral infection including dengue and West Nile Virus infection, preferably in which the method comprises administering a therapeutically effective amount of the polypeptide or composition to an individual suffering or suspected of suffering from a flaviviral infection including dengue and West Nile Virus infection; or

(ii) a method of diagnosis of a flaviviral disease including dengue and West Nile Virus infection.

7. A diagnostic kit comprising an polypeptide according to any of Claims 1 to 4 or a pharmaceutical composition according to Claim 5 together with instructions for use in the diagnosis of a flaviviral infection including dengue and West Nile Virus infection.

8. A polypeptide comprising a sequence shown as SEQ ID NO: 4 or SEQ ID NO: 6, or both, or a variant, homologue, derivative or fragment thereof which is capable of binding envelope glycoprotein (E) in which the polypeptide comprises the variable region of monoclonal antibody 9F12.

9. A nucleic acid comprising a sequence shown as SEQ ID NO: 3 or SEQ ID NO: 5, or both and which is capable of encoding a molecule according to any of Claims 1 to 4, or a variant, homologue, derivative or fragment thereof which is capable of encoding a polypeptide having envelope glycoprotein (E) binding activity.

10. A cell comprising or transformed with a nucleic acid sequence according to Claim 9 or a descendent of such a cell.

11. An in vitro method of producing an polypeptide according to any of Claims 1 to 4, the method comprising providing a cell according to Claim 10 and expressing the polypeptide from the cell.

12. An in vitro method of detecting a flaviviral-infected cell, including a dengue infected cell and a West Nile Virus infected cell, the method comprising exposing a candidate cell to an polypeptide according to any of Claims 1 to 4 and detecting expression of envelope glycoprotein (E) polypeptide by the cell.

13. An in vitro method comprising the steps of providing an polypeptide according to any of Claims 1 to 4 and allowing the polypeptide to bind to a envelope glycoprotein (E) polypeptide, preferably in which the polypeptide is allowed to bind to a cell expressing envelope glycoprotein (E) polypeptide.