Production of a neutralizing antibody against envelope protein of dengue virus type 2 using the linear array epitope technique

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Dengue virus (DENV; genus Flavivirus) contains a positive-stranded RNA genome. Binding of DENV to host cells is mediated through domain III of the viral envelope protein. Many therapeutic mAbs against domain III have been generated and characterized because of its high antigenicity. We have previously established a novel PCR method named the linear array epitope (LAE) technique for producing monoclonal-like polyclonal antibodies. To prove this method could be utilized to produce antibody against epitopes with low antigenicity, a region of 10 aa (V365NIEAEPPFG374) from domain III of the envelope protein in DENV serotype 2 (DENV2) was selected to design the primers for the LAE technique. A DNA fragment encoding 10 directed repeats of these 10 aa for producing the tandem-repeated peptides was obtained and fused with glutathione S-transferase (GST)-containing vector. This fusion protein (GST-Den EIII 10-His 6) was purified from Escherichia coli and used as antigen for immunizing rabbits to obtain the polyclonal antibody. Furthermore, the EIII antibody could recognize envelope proteins either ectopically overexpressed or synthesized by DENV2 infection using Western blot and immunofluorescence assays. Most importantly, this antibody was also able to detect DENV2 virions by ELISA, and could block viral entry into BHK-21 cells as shown by immunofluorescence and quantitative real-time PCR assays. Taken together, the LAE technique could be applied successfully for the production of antibodies against antigens with low antigenicity, and shows high potential to produce antibodies with good quality for academic research, diagnosis and even therapeutic applications in the future.

INTRODUCTION

Dengue virus (DENV; family Flaviviridae, genus Flavivirus) is a considerable threat to public health worldwide. Outbreaks and epidemics caused by DENV infection have become a serious public health issue in many tropical and subtropical countries. DENV is classified into four serotypes (DENV1–4), and is transmitted to humans by mosquito vectors such as Aedes aegypti and Aedes albopictus. Virus infection may cause different kinds of diseases, including dengue fever, with febrile illness, dengue hemorrhagic fever, with plasma leakage syndrome, and dengue shock syndrome. Approximately 100 million cases of dengue fever and 500 000 cases of dengue hemorrhagic fever/dengue shock syndrome occur each year, with a mortality rate of 5% (Gubler, 2002; Wilder-Smith & Gubler, 2008). Therefore, there is an urgent need to develop proper DENV vaccines. In recent decades, there have been several approaches that have attempted to develop DENV vaccines, including live attenuated viruses, chimeric viruses, recombinant subunit antigens, expression vector-based vaccines and DNA vaccines (Chokephaibulkit & Three supplementary figures are available with the online version of this paper.
DENV has a positive-sense ssRNA genome. The RNA genome of 10.7 kb in length has a 5’ cap structure but lacks a 3’ poly(A) tail, and encodes a polyprotein that is cleaved into three structural proteins (core, pre-membrane and envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) by host and/or viral proteases. Among the three structural proteins, the pre-membrane and envelope proteins are the primary antigenic targets of the humoral immune response in humans (Wan et al., 2013). The neutralizing antibodies are usually induced by the envelope protein (Murphy & Whitehead, 2011). The envelope protein consists of 495 aa with a molecular mass of ~60 kDa. It has been suggested that the major role of the envelope protein is host cell attachment and entry (Bhardwaj et al., 2001). The ectodomain of the envelope protein is composed of three domains (domains I–III) (Modis et al., 2003, 2004; Rey et al., 1995). Domain I contains mainly type-specific non-neutralizing epitopes and is thought to be the molecular hinge region involved in low-pH-triggered conformational changes (Roehrig et al., 1998). Domain II is the dimerization domain and is involved in virus-mediated membrane fusion. It contains many cross-reactive epitopes eliciting neutralizing and non-neutralizing mAbs (Rey et al., 1995; Roehrig et al., 1998). Domain III is an immunoglobulin-like structure containing the most distal projecting loops from the virion surface. According to the X-ray crystal structure, domain III contains 10 β-strands (A–G and three small extra sheets, AxCxDx). In addition, domain III carries multiple type- and subcomplex-specific epitopes eliciting neutralizing mAbs that have been hypothesized to block virus binding to the host cell receptor (Bhardwaj et al., 2001; Chiu & Yang, 2003; Modis et al., 2003, 2004; Rey et al., 1995; Sukupolvi-Petty et al., 2007; Volk et al., 2004; Yu et al., 2004). As envelope domain III plays important roles in viral entry, it is a good target for generating neutralizing antibodies.

The linear array epitope (LAE) technique is a method for the preparation of an immunogen containing multiple copies of a defined peptide in a linear alignment. This method was designed to overcome the poor immune responses induced by traditional immunogenic peptide/protein antigens. The tandem-repeated epitopes are able to efficiently induce a strong immune response in vivo (Yi et al., 2006) and prime CD4+ T-cells in vitro (Barratt-Boyes et al., 1999). Immunization of female rabbits with the LAE-produced immunogen that contained the exotoxin receptor-binding domain A of Pseudomonas (PEIa) and 12 copies of gonadotrophin-releasing hormone (GnRH) resulted in the generation of high-titre antibodies specific for GnRH. The anti-GnRH antibodies effectively neutralized GnRH activity in vivo, as demonstrated by the degeneration of the ovaries in the injected rabbits (Hsu et al., 2000). The LAE-produced gastrin-releasing peptide immunogen also induced antibody potentially inhibiting breast cancer cell proliferation in vitro and in vivo (Guojun et al., 2008). A rabbit anti-mouse erythroid Krüppel-like factor (EKLF) antibody induced by a LAE-produced immunogen could be used to detect EKLF in mouse embryonic samples by Western blotting and immunostaining, and was applicable for use in a chromatin immunoprecipitation assay (Shyu et al., 2007).

In this study, we used the LAE technique to produce a tandem-repeated domain III region (V_{365NIEAEPPFG374})\textsubscript{10} of DENV2 envelope protein as the immunogen (referred to as the EIII epitope). We demonstrated that the anti-EIII antibody induced by this novel immunogen could be used to detect the envelope protein either ectopically overexpressed or synthesized by DENV2 infection in cells. Importantly, the antibody could be used successfully to detect DENV virions and block DENV entry into BHK-21 cells.

RESULTS

Epitope selection and the principle of the LAE technique

To test whether the LAE technique could be used to induce antibodies against DENV2 envelope domain III, we chose an epitope (V_{365NIEAEPPFG374}) consisting of β-strand and random-coil secondary structures, and that was not colocalized with previous neutralizing epitopes (Crill & Chang, 2004; Gromowski & Barrett, 2007; Lin et al., 1994; Thullier et al., 2001; Wan et al., 2013). According to the three-dimensional structures of the envelope protein obtained from the Protein Data Bank (http://www.rcsb.org/pdb), this epitope seemed to be localized on the surface of the protein (Fig. S1, available in the online Supplementary Material). To synthesize the repeated EIII epitope fused with glutathione S-transferase (GST), two oligonucleotides (oligo Den E-A and oligo Den E-B) were designed for template-repeated (TR)-PCR. Oligo Den E-A encoded VNIEAEPPFG peptide, and its 5’ and 3’ halves were complementary to those of oligo Den E-B (Fig. 1). The TR-PCR products containing the repeated EIII epitope were used as the templates for the second PCR (adaptor-PCR) to introduce the restriction enzyme sites and stop codon. The products of both TR-PCR and adaptor-PCR showed a ladder pattern in 8% PAGE (Fig. 1d). The lowest band (the major band in Fig. 1d, lane 3) among the ladders was the monomer and the higher bands were multimers. The DNA products were eluted and cloned into T vectors, followed by screening for positive clones. Finally, a clone with 10 tandem-repeated epitopes was obtained.

Preparation of the tandem-repeated antigen for generating rabbit antiserum against domain III of the envelope protein

The DNA fragment with 10 repeats of the EIII epitope was subcloned into two expression plasmids for the production of tandem-repeated antigens, generating GST-Den...
EIII<sub>10</sub>-His<sub>6</sub> and PEIa-Den EIII<sub>10</sub>-His<sub>6</sub> fusion proteins (data not shown) in Escherichia coli BL21(DE3). The induced proteins were purified by Glutathione-Sepharose 4B and nickel affinity columns, respectively. The Coomassie blue staining for purification of GST-Den EIII<sub>10</sub>-His<sub>6</sub> protein is shown in Fig. 2(a). Elution fractions 3–7 were collected as the antigen to immunize rabbits. The harvested antiserum against GST-Den EIII<sub>10</sub>-His<sub>6</sub> protein was named EIII antibody and the antibody titre against recombinant full-length domain III fused with His<sub>6</sub> tag (EIII-His<sub>6</sub>) was monitored by ELISA every 2 weeks. Even as low as 10 ng coated EIII-His<sub>6</sub> could be significantly detected by this antibody; the A<sub>410</sub> values of 10<sup>2</sup>- and 10<sup>3</sup>-fold diluted sera were much higher than those of the control (Fig. 2b). The titre of the antiserum reached a plateau after 6 weeks (data not shown).

**Fig. 1.** The LAE technique includes TR-PCR and adaptor-PCR steps. (a) TR-PCR: oligo Den E-A and oligo Den E-B were used as primers and templates for TR-PCR. (b) Primers designed for TR-PCR. The peptide sequence of the epitope chosen was VNIEAEPPFG. Oligo Den E-A encoded this peptide and oligo Den E-B was partially complementary to oligo Den E-A, as indicated. The 5′ half of oligo Den E-A (shown as a) was complementary to the 5′ half of oligo Den E-B (shown as a′) and the 3′ half of oligo Den E-A (shown as b) was complementary to the 3′ half of oligo Den E-B (shown as b′). (c) Adaptor-PCR: the products of TR-PCR (3 μl) were subjected to adaptor-PCR as templates. Adaptor primers Den E-5b and Den E-3he introduced proper restriction enzyme sites, leading to a BamHI site at the 5′ end, a HindIII/EcoRI site at the 3′ end and two stop codons at the end of the coding region. The PCR products were then subcloned into a T vector. (d) The products of TR-PCR (lane 2) and adaptor-PCR (lane 3) were analysed on 8 % PAGE with ethidium bromide staining. Lanes 1 and 4, 100 bp DNA markers.
Evaluation of the specificity of EIII antibody by various assays

To further confirm the specificity of the harvested EIII antibody, ELISA and Western blot assays were performed. Recombinant EIII-His6 proteins from four serotypes of DENV were used to test whether EIII antibody could recognize EIII protein of all serotypes. In the ELISA, Fig. 2(c) shows that EIII antibody mainly detected EIII-His6 of DENV2 at 10^4-fold dilution, but cross-reacted with EIII-His6 of all four serotypes at 10^2-fold dilution. In the Western blot assay, EIII antibody could recognize purified EIII-His6, PEla-Den EIII10-His6, and GST-Den EIII10-His6, but not the unrelated proteins, such as PEla-His6 (the protein product of the empty pPEla-His6 vector), TAF7-His6 (a transcriptional factor) and NS3-C-His6 (the N-terminal truncated NS3 protein of DENV), suggesting high specificity of the LAE-induced antibody (Fig. 3a). We next examined if EIII antibody could detect full-length DENV2 envelope protein in cells. Fig. 3(b, c) shows that EIII antibody could specifically detect the DENV2 envelope proteins ectopically overexpressed in BHK-21 or HEK293T cells by Western blot analysis and immunofluorescence assay, respectively. Furthermore, we tested if the antibody could be used in immunoprecipitation assays. The cell lysates from

![Image](https://example.com/figure.png)

**Fig. 2.** A novel tandem-repeated antigen was used to generate anti-EIII antiserum from rabbits. (a) Recombinant GST-Den EIII10-His6 protein was expressed in *E. coli* BL21(DE3) and purified using a Glutathione-Sepharose 4B affinity column. Elution fractions were analysed on 10 % SDS-PAGE and stained with Coomassie blue. Lanes: N, total lysates before induction; I, total lysates after 3 h IPTG induction; Wash, wash fraction during column purification. Elution fractions 1–8 indicate fractions eluted by 10 mM glutathione from the affinity column. (b) The titre of anti-EIII antiserum from rabbits was measured by ELISA. Either 10, 100 or 1000 ng EIII-His6 protein from DENV2 PL046 was coated onto ELISA plates and serial dilutions of EIII antibody were added to the plate to measure \( A_{410} \). No antigen (Ag) coated, no primary antibody (1’Ab) and no secondary antibody (2’Ab) were all negative control sets. (c) The titre of anti-EIII antiserum from rabbit was measured by ELISA. Either 10, 100 or 1000 ng recombinant EIII-His6 protein from the four serotypes was coated onto ELISA plates and serial dilutions of EIII antibody were added into the plate to measure \( A_{410} \). No antigen (Ag) coated, no primary antibody (1’Ab) and no secondary antibody (2’Ab) were all negative control sets.
HEK293T cells transfected with vector only or with pCMV-3FLAG-DENV2 envelope were incubated with EIII antibody, resulting in immunoprecipitation of ectopically expressed FLAG-tagged DENV2 envelope proteins (Fig. 3d).

**Application of EIII antibody for detecting virions and blocking viral entry**

As EIII antibody could recognize recombinant envelope proteins in BHK-21 and HEK293T cells, we examined if it could recognize the envelope protein of DENV virions. C6/36 cells were infected with DENV2 for 10–12 days and the cell lysates or the media were collected for Western blot analysis. EIII antibody could clearly detect DENV pre-membrane/envelope heterodimer protein in virus-infected cell lysates as indicated by the filled arrow in Fig. 4(a) (Sukupolvi-Petty et al., 2010; Zhang et al., 2003). EIII antibody also detected envelope protein in the medium fraction of virus-infected culture weakly, as indicated in the open arrow in Fig. 4(a). To further confirm this result, EIII
antibody was used to detect virus-infected BHK-21 cells at different time points after infection. As shown in Fig. 4(b), EIII antibody could detect virus envelope proteins in the infected cells and the signal increased commensurate with the infection time, which correlated with the replication cycle of DENV. As the envelope protein is located on the surface of the virus, the antibody was tested for virion recognition. Different amounts of DENV were coated on ELISA plates and serially diluted EIII antibody was used to detect the virions. The results showed that EIII antibody at 10- to 10^{5}-fold dilutions could detect virions in a dose-dependent manner (Fig. 4c).

Finally, we tested if EIII antibody could be applied to block DENV infection. DENV was pre-incubated with serially diluted EIII antibody and the virus–antibody mixture was used to infect BHK-21 cells. Viruses were tested for NS3 by immunofluorescence and quantitative real-time (qRT)-PCR assays. The immunofluorescence signals were quantified by counting green fluorescence-positive cells in different fields and the results showed that EIII antibody at 10-fold dilution significantly reduced the fluorescence signals (Fig. 5a, b). The levels of NS3 RNA were also decreased by EIII antibody (Fig. 5c), suggesting that viral entry might be blocked effectively. In order to further verify the process, we treated EIII antibody either before or after DENV infection and found that EIII antibody affected viral infection significantly only when in the pre-treated condition, indicating that EIII antibody blocked virus infection through blocking of viral attachment (Fig. 5d). As EIII antibody could block DENV2 infection, we further tested if this antibody would block viral entry of other serotypes. All four serotypes of DENV were used to evaluate the blocking ability (Fig. S3). The results suggested that EIII antibody specifically blocked DENV2 infection, although DENV4 could not be determined (Fig. S3).

![Fig. 4.](image-url)

EIII antibody could recognize viral envelope proteins and virions. (a) C6/36 cells were infected with DENV and the medium was collected 10–12 days post-infection. The cells were lysed using two times sample buffer and the proteins either in the medium fraction (med) or in the cell fraction (cell) were analysed by Western blotting. The first two lanes indicate mock infection. The other six lanes indicate three independent experiments (Exp.1–3) for DENV infection. The filled arrow, open arrow and arrowhead indicate the bands of the pre-membrane/envelope heterodimer, envelope and NS3 proteins, respectively. (b) BHK-21 cells were infected by DENV (m.o.i. 12) and cells were harvested at 12 or 24 h post-infection, followed by Western blot analysis with EIII or NS3 antibody. (c) Virions at different dilution (fold) were coated on ELISA plates and detected by anti-EIII antibody. No primary antibody (1′Ab) and no secondary antibody (2′Ab) were all negative control sets.
DISCUSSION

Infection by DENV is a global concern. However, there is still no approved antiviral treatment available. Several candidate vaccines remain in pre-clinical or clinical evaluation (Durbin & Whitehead, 2010). The main strategy of these candidate vaccines is to induce neutralizing antibodies to block virus infection. The best targets for blocking virus infection are the structural proteins on the virion surface. According to previous studies, many neutralizing antibodies were induced by the envelope proteins. Based on epitope mapping data, many epitopes of the type-specific neutralizing antibodies against individual flaviviruses are localized to domain III, whereas neutralizing mAbs that cross-react with other flaviviruses are localized primarily to domain II (Beasley & Barrett, 2002; O’Rourke, 1977; Huang et al., 2004a, b; Lin et al., 1994; Oliphant et al., 2005, 2006; Stiasny et al., 2006). Some studies further defined contact residues for serotype-specific, subcomplex-specific and cross-reactive mAbs that recognize domain III of the envelope protein in DENV; the epitopes of serotype-specific neutralizing mAbs are localized to the BC-, DE- and FG-loops on the lateral ridge of domain III, whereas subcomplex-specific mAbs recognize the A-strand of domain III (Gromowski & Barrett, 2007; Gromowski et al., 2008; Lok et al., 2008; Rajamanonmani et al., 2009; Sukupolvi-Petty et al., 2007; Thullier et al., 2001). To prove that the LAE technique could improve epitopes of poor antigenicity, we chose the epitope (V365NIEAEPPFG374) localized between the E-strand and F-strand of domain III, which has not been reported to induce any neutralizing antibodies. During the antigen purification process, there were some ladder-like minor bands located under the major bands (Fig. 2a). We speculate that the tandem-repeated sequences might affect antigen translation or degradation. This tandem-repeated antigen indeed induces EIII antibody production, which could be utilized for basic biochemical assays, such as Western blot analysis, immuno-fluorescence and co-immunoprecipitation. In addition, this antibody could specifically detect EIII-His6 (Fig. 3a), ruling out the possibility that it recognized the junction sequences between two repeated epitopes. More importantly, this antibody could also be applied to detect DENV virions and block viral infection in BHK-21 cells. According to a literature search, this is the first report of the application of the LAE technique in virus-related research.

From epidemiological studies, it is generally believed that heterologous antibodies that fail to neutralize the infecting serotype contribute primarily to the development of dengue haemorrhagic fever/dengue shock syndrome in both infants and adults (Halstead, 2003). Those subneutralizing antibodies enhance viral uptake by Fcγ receptor (FcγR)-dependent or FcγR-independent mechanisms, called antibody-dependent enhancement (ADE) (Halstead & O’Rourke, 1977; Huang et al., 2006). To reduce the risk of ADE, the neutralizing antibodies must be protective against each of the four DENV serotypes. This might be achieved by choosing a conserved epitope in different serotypes to produce tetravalent neutralizing antibodies by the LAE technique. Therefore, this is the second reason why we chose a region of 10 aa (V365NIEAEPPFG374) from domain III of the envelope protein in DENV2 as the target epitope. This sequence is 100% conserved in DENV1–3, but has a 2 aa difference in DENV4 (Fig. S2a). Interestingly, even this minor divergence might be reflected in the generated EIII antibody: 2000-fold diluted EIII antibody recognized recombinant EIII-His6 of DENV1–3, but not DENV4 (Fig. S2b). However, 100-fold diluted EIII antibody could detect all recombinant EIII-His6 of all four serotypes (data not shown). Similarly, EIII antibody recognized recombinant EIII-His6 of DENV2 under low concentrations (102-fold dilution), but cross-reacted with EIII-His6 of all four serotypes under high concentrations (104-fold dilution) in ELISA (Fig. 2c). Surprisingly, Figs 5 and S3 also show that EIII antibody specifically blocked DENV2 infection to BHK-21 cells, although the epitopes are identical in DENV1–3. We speculate this specific blockage might derive from higher avidity of EIII antibody with the envelope protein of DENV2, suggesting that the antibody generated by the LAE technique not only recognizes the sequence, but also discriminates between the structural arrangements of the corresponding antigen.

To further delineate the process of blockage, we also examined whether EIII antibody blocked virus infection through blockage of viral attachment to the host receptor (Fig. 5d). Our result is consistent with previous studies, which suggest that domain III also plays important roles in binding to the host cell receptors (Bhardwaj et al., 2001; Chiu & Yang, 2003; Crill & Roehrig, 2001; Hung et al., 2004).

Taken together, the LAE technique can be used to develop antigens with higher antigenicity or antibodies with higher specificity through a simple PCR-based method even without any DNA template. Moreover, the polyclonal antibody produced by the LAE technique recognized the same epitope, displaying the property of a mAb and may be considered as a monoclonal-like polyclonal antibody. Such antigens and antibodies have high potential for use in academic research, diagnosis and therapeutic applications in the future.

METHODS

TR-PCR and adaptor-PCR. KOD Hot Start DNA Polymerase (Novagen) was used in TR-PCR. Two oligonucleotides were used in this reaction: Den E-A, 5′-GGCTTCGATGTTAACACCAAAAGGAGGTTC-3′; and Den E-B, 5′-GGCTTCGATTTAACACCAAAAGGAGGTTC-3′. In TR-PCR, the two primers also functioned as templates. The PCR programmes were 30 cycles of denaturation at 94 °C for 1 min, annealing at 44 °C for 1 min and polymerization at 72 °C for 1 min, followed by a final polymerization step at 72 °C for 10 min. After TR-PCR, the PCR product was then used as template for adaptor-PCR catalysed by Taq polymerase (Fermentas). Two oligonucleotides were used in this reaction: Den E-5b, 5′-GGACTCTCCGTTAACACCAAAAGGAGGTTC-3′; and Den E-3he, 5′-GAATTCATTAGACTTTACCCCAAAAGGAGGTTC-3′. The PCR conditions were five
**Fig. 5.** EIII antibody could block DENV entry. (a) Serial dilutions of purified EIII antibody were pre-mixed with the DENV2 PL046 strain and then the antibody–virus mixtures were used to infect BHK-21 cells. After infection for 48 h, cells were fixed with methanol and then DENV was detected by anti-NS3 (1 : 200) antibody. (b) The signals were quantified by counting the green fluorescence-positive cells/DAPI-positive cells in different fields. The percentage of green fluorescence-positive cells was measured based on >180 DAPI-positive cells examined. The data were analysed by the Mann–Whitney U test (*P < 0.05; NS, no significant difference). (c) As mentioned in (a), RNA from the cells was extracted and analysed by one-step qRT-PCR to detect the levels of NS3. The levels of NS3 RNA were reduced after treatment with diluted antibody. The data were analysed by
cycles of denaturation at 94 °C for 1 min, annealing at 44 °C for 1 min and polymerization at 72 °C for 1 min, followed by 10 cycles of denaturation at 94 °C for 1 min, annealing at 48 °C for 1 min and polymerization at 72 °C for 1 min. Finally, the reaction was terminated with a polymerization step at 72 °C for 10 min. The product of adaptor-PCR was cloned into T vector for sequencing. The correct construct was then subcloned into expression vectors pGEX-His6 or pPELa-His6.

**Antigen and antibody preparation.** Recombinant GST-Den EIII10-His6 protein was expressed in E. coli BL21(DE3) with 1 mM IPTG at 37 °C for 3 h followed by sonication to break the cells. After centrifugation, the soluble protein was purified with Glutathione-Sepharose 4B (Amersham Pharmacia). Either 100 or 500 μg purified GST-Den EIII10-His6 protein was mixed with Freund’s complete adjuvant (Chemicon) and then subcutaneously injected into female New Zealand white rabbits four times at 2 week intervals. Sera were collected from rabbit ears biweekly and titre was tested by ELISA. The sera were purified by the Millipore Montage antibody purification kit.

**ELISA.** The recombinant EIII-His6 proteins were provided courtesy of Dr Chih-Hsiang Leng (National Health Research Institute, Taiwan) (Leng et al., 2009). The consensus sequence for EIII from DENV1 was obtained by alignment of the amino acid sequences from five different isolates of DENV1: Brazil/97-11/1997, Jamaica/CV1636/1977, Nauru/ West Pac/1974, Singapore/S275/1990 and Thailand/AHF 82-80/1980. The consensus sequences for EIII from DENV2–4 were obtained by alignment of the amino acid sequences from different isolates of DENV2: Brazil/97-11/1997, Jamaica/CV1636/1977, Nauru/ West Pac/1974, Singapore/S275/1990 and Thailand/AHF 82-80/1980. From DENV2: 16681-PDK53, China/D2-04, Malaysia M2, Malaysia M3, Peru/IQT2913/1996, Thailand/0168/1979, Puerto Rico/PR159-S1/1969, Tonga/EKB194/1974 P27914, Thailand/PUO- 218/1980, Thailand/16681/84, Thailand/NGS-C/1944 and Thailand/ TH-36/1958 were referenced. Strains for DENV3 included Philippines/ H87/1956, China/0-2/1980, Singapore/8120/1995, Martinique/1243/ 1999 and Sri Lanka/1266/2000. Strains for DENV4 included Philippines/ H241/1956, Dominica/814689/1981, Thailand/0348/1991 Singapore/ 8976/1995 and Thailand/0476/1997. Either 10, 100 or 1000 ng EIII- His6 protein or DENV virions was coated on 96-well plates with coating buffer (10 mM Na2CO3, 35 mM NaHCO3, pH 9.6) at 37 °C for 2 h or 4 °C overnight. After coating, samples were blocked by coating solution (1 % BSA and 0.0375 % saponin in PBS) at 37 °C for 2 h or at 4 °C overnight. The samples were incubated with EIII antibody at 37 °C for 2 h and washed with 120 μl PBS three times. The samples were then incubated with goat anti-rabbit IgG-HRP second antibody at 37 °C for 1 h and washed three times with 120 μl PBS. Finally, substrate solution [ABTS (2,2-azino-diylbenzthiazoline sulfonylic acid) 0.54 mg ml−1, 0.1 M citric acid, pH 4.2, 3 μl 30 % H2O2 ml−1] was incubated with samples at room temperature for 15 min. The signals (A410) were detected by a multiscan ELISA reader (Dynatech).

**Cell cultures, transfection, virus strains and infection.** Baby hamster kidney (BHK-21) cells and human embryonic kidney (HEK293T) cells were cultured at 37 °C with 5 % CO2 in Dulbecco's modified Eagle's medium (DMEM) medium (Gibco) supplemented with 10 % FBS (Biowest). C6/36, a mosquito cell line established from A. albopictus, was grown at 28 °C with 5 % CO2 in RPMI1640 medium (Gibco) supplemented with 10 % FBS. Cells were transfected with pcMV-3FLAG or pcMV-3FLAG-envelope using Turbofect reagent (Fermentas). DENV1 (Myanmar 3886201 strain), DENV2 (PL046, NGC strain), DENV3 (98TW503 strain) and DENV4 (H241 strain) stocks were prepared in C6/36 cells and titrated by plaque assay. In infection experiments, appropriate cells were seeded on culture plates and incubated at 37 °C overnight. The next day, cells were infected at 37 °C for 2 h by adding DENV-containing culture supernatant at m.o.i. 0.02 or 12. After infection, cells were washed with PBS and cultured in fresh medium.

**Western blot and co-immunoprecipitation assays.** Samples and pre-stained marker were subjected to 10 or 12.5 % PAGE. The proteins were then electrophoretically transferred from gels onto PVDF membranes by a semi-dry transfer system (BioBlast). After transferring onto PVDF membranes and subsequent blocking, the blots were incubated with the respective primary antibodies, followed by the secondary antibody conjugated with HRP. Finally, the signal was detected by adding chemiluminescence reagent (PerkinElmer) and visualized using the Chemi Genius2 CG2/D2A machine (Syngene) or traditional X-ray film. For immunoprecipitation, 150 μg cell lysates from HEK293T cells transfected with empty vector or pcMV-3FLAG-envelope plasmid were immunoprecipitated with EIII antibody using the Dynabeads Protein A system (Invitrogen), followed by Western blotting with FLAG antibody.

**Immunofluorescence assay.** In the DENV blocking assay, BHK-21 cells were seeded on coverslips in six-well plates (104 or 3 × 104 cells per well) and incubated at 37 °C overnight. EIII antibody at different dilutions was incubated with DENV at m.o.i. 0.02 at 4 °C for 1 h. The virus–antibody mixture was then added to BHK-21 cells and incubated for an additional 1 h at 4 °C. Negative controls received PBS instead of antibody. After infection, BHK-21 cells were washed three times with 1 ml cold PBS and cultured in DMEM medium supplemented with 10 % FBS. At different time points after infection, the cells on cover slips were then permeabilized with 0.25 % Triton X-100 followed by 100 % methanol fixation at −20 °C for 5 min. After fixation, cells were incubated with rabbit anti-Den NS3 antibody followed by incubation with anti-rabbit IgG–Alexa Fluor 488 (Molecular Probes) secondary antibody. Finally, cover slips were mounted on slides with Vectashield mounting medium (Vector) with DAPI. The slides were observed by fluorescence or confocal microscopy. In transfection experiments, pcMV-3FLAG or pcMV-3FLAG-envelope plasmid was transfected to HEK293T cells by using Turbofect transfection reagent (Fermentas). The primary antibody was EIII or FLAG and the secondary antibody was anti-rabbit IgG–Alexa Fluor 488 or anti-mouse IgG–Alexa Fluor 555 (Molecular Probes) in this experiment.

**qRT-PCR.** Total RNA was isolated from mock- or virus-infected cells by RNeasy C&T reagent (Protech). Either 100 ng or 1 μg RNA was used as template for qRT-PCR using the KAPA SYBR FAST One-Step qRT-PCR kit (Kapa Biosystem) or ABI qRT-PCR kit and ABI StepOne detection system and analysis software (Applied Biosystem). Levels of NS3 were normalized to ß-actin mRNA levels using a Student's t-test (*P<0.05, **P<0.01 versus BHK-21-infected DENV2). (d) DENV2 PL046 strain was pre-mixed with serially diluted purified EIII antibody, followed by infecting BHK-21 cells (m.o.i. 0.02). Alternatively, BHK-21 cells were first infected by DENV, followed by adding serially diluted antibody. In these two procedures, DENV was first incubated at 4 °C for viral attachment followed by incubation at 37 °C for viral entry. At 48 h post-infection, RNA from the cells was extracted and analysed by two-step qRT-PCR to detect the levels of NS3. The levels of NS3 RNA were reduced only in antibody pre-treatment. Three sets of independent experiments were performed and the data were analysed by Student’s t-test (*P<0.05, **P<0.01, ***P<0.001 versus DENV infection only).

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comparative threshold cycle method that converted differences of cycle numbers to test gene/β-actin ratios.

Statistical analysis. The data were analysed using either the Student’s t-test or Mann–Whitney U test and P<0.05 was considered significant.

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