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

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Abstract

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

Introduction

Dengue viruses (DENVs) are emerging, mosquito-borne flaviviruses and the causative agents of dengue fever (DF) and dengue hemorrhagic fever (DHF). The DENV complex consists of four serotypes designated DENV 1 through 4. A person infected with DENV develops antibodies that cross react with all four serotypes (Roehrig, 2003). However, the antibodies only provide long-term protection against the serotype responsible for the original infection and people can be infected a second time with a different serotype (Halstead, 2002; Rothman, 2004). Individuals experiencing secondary DEN infections face a greater risk of developing severe disease (Halstead, 2002; Rothman, 2004). A leading theory to explain the greater risk

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of severe disease with secondary DEN infection is that pre-existing cross reactive antibodies bind to the virus and enhance infection of Fc-receptor bearing cells (Halstead, 2003). Despite the fact that DEN vaccines are entering large scale clinical testing, we know remarkably little about the relationship between the binding properties of DEN antibodies in human immune sera and the functional outcome of these interactions.

The major target of flavivirus neutralizing antibody is the Envelope (E) protein, although membrane protein (M) and non-structural protein 1 (NS1) antibodies have also been shown to be protective (Roehrig, 2003; Schlesinger, Brandriss, and Walsh, 1987; Vázquez et al., 2002). E protein is responsible for viral attachment to host cells and the low pH fusion of viral and host cell membranes. The crystal structures of E of several flaviviruses have been solved (Modis et al., 2003; Modis et al., 2005; Nybakken et al., 2006; Rey et al., 1995). Individual subunits of E consist of three beta-barrel domains designated E domains I (EDI), II (EDII) and III (EDIII). Native E is a homodimer that lies flat on the surface of the viral membrane.

Our current understanding of the interactions between DENV and antibody is largely based on studies with mouse monoclonal antibodies (Mabs). DENV neutralizing mouse Mabs have been mapped to all three domains of E. In general, strongly neutralizing mouse Mabs are DENV serotype-specific and bind to an epitopes on EDIII that is unique to each serotype (Crill and Roehrig, 2001; Gromowski and Barrett, 2007; Lin et al., 1994; Lok et al., 2008; Roehrig, Bolin, and Kelly, 1998; Sukupolvi-Petty et al., 2007). A DENV type specific epitope on EDIII bound by strongly neutralizing Mabs has been mapped to 4 loops on the lateral face of EDIII (Gromowski and Barrett, 2007; Gromowski, Barrett, and Barrett, 2008; Sukupolvi-Petty et al., 2007). Investigators have also mapped flavivirus cross reactive epitopes on EDIII (Gromowski, Barrett, and Barrett, 2008; Sukupolvi-Petty et al., 2007). Unlike DENV type specific Mabs, cross reactive Mabs that bind to EDIII have moderate to weak neutralizing activity.

Despite the large body of work with mouse Mabs, remarkably little work has been done to characterize the binding properties of human DENV immune sera and to understand the relationship between human antibody binding and neutralization. Convalescent sera from people and horses naturally infected with West Nile virus (WNV), a related flavivirus, had low levels of EDIII-reactive antibody (Oliphant et al., 2007; Sanchez et al., 2007). In WNV immune sera, EDIII-binding antibodies were not primarily responsible for neutralization activity (Oliphant et al., 2007; Sanchez et al., 2007).

People who have recovered from DENV infections also develop EDIII-reactive antibodies (Beasley et al., 2004; Crill et al., 2009; Hapugoda et al., 2007; Holbrook, Shope, and Barrett, 2004; Ludolfs et al., 2002); however, most human antibody appears to be directed towards a flavivirus-cross reactive epitope close to the fusion loop in EDII of DENV (Crill et al., 2009; Lai et al., 2008). To date, no studies have been done to directly test if EDIII-reactive antibodies are primarily responsible for the neutralizing activity of human DENV immune sera. The goal of this study was to measure the level and specificity of EDIII-reactive antibodies in people who have recovered from primary and secondary DENV infections and to determine the contribution of EDIII-reactive antibodies to DENV neutralization.

Materials and Methods

Viruses

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

Viral titers were determined by plaque assay on Vero-81 cells as previously described (Kraus et al., 2007).

Immune sera and Antibodies

Convalescent DENV immune sera were obtained from volunteers who had experienced natural DENV infections during previous travel abroad. The protocol for recruiting and collecting blood from people was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill. Sera from 6 DENV-immune subjects were used in the present study. The properties of these sera are listed in Table 1. We also used eight DENV-reactive mouse Mabs that bind to EDIII. Mab 3H5-1, obtained from Chemicon Co, CA, binds to EDIII of DENV2 only (Gromowski and Barrett, 2007). Mabs 8A1 and 14A4, which bind to EDIII of DENV3, were obtained from Dr. Robert Putnak (Walter Reed Army Institute of Research, Silver Spring, MD). Mab 2Q1899 and 9F16 which bind to EDIII of DENV2 were obtained from United States Biological, Massachusetts. DENV3 EDIII reactive Mab 1H9 was obtained from Dr John G Aaskov (Queensland University of Technology, Australia) (Serafin and Aaskov, 2001) DENV cross reactive EDIII Mab 8A5 and 12C1 were developed in collaboration with the South Eastern Regional Center for Excellence in Biodefence monoclonal antibody core facility at the University of Alabama School at Birmingham.

Purification of DENV antigen for ELISA

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

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

RNA extracted from strain CH53489 of DENV3 was resverse transcribed and PCR amplified to generate a PCR product containing nucleotides coding for the last 15 amino acids of membrane protein and the first 415 amino acids of E protein followed by a 6 histidine tag and a stop codon. This construct was missing the C-terminal amino acids responsible for membrane anchoring of E protein. The PCR product was cloned into pENTR TOPO vector (Invitrogen) and the BaculoDirect baculovirus Expression system (Invitrogen) was used to express recombinant protein according to manufacturer's instructions. Briefly, the Es gene was recombined with BaculoDirect Liner DNA to generate recombinant baculovirus DNA. pENTR/CAT plamsmid was used to produce recombinant baculovirus expressing the chloramphenicol acetyl transferase (CAT) protein, which was later used as a negative control. Sf9 insect cells were transfected with recombinant baculovirus DNA. P1,P2 and P3 recombinant virus stocks were generated according to the manufacturers instruction and expression of protein was confirmed using western blot with mouse Mab 4G2 (for Es) or anti CAT antibodies. P3 baculovirus stocks were used to express Es and CAT proteins.

Expression and purification of DENV EDIII

RNA was extracted from supernatants of cells infected with DENV2 or 3 using QIAmp Viral RNA mini Kit (Qiagen). The nucleotide sequences encoding for EDIII of DENV2 (297-399 AA) and DENV3 (295- 398 AA) were reverse transcribed and PCR amplified. The PCR products were cloned into pMAL c2X vector (NEB) to generate recombinant EDIII (MBP-

EDIII) that is fused to maltose binding protein (MBP) at the N terminus according to the manufacture's instructions. MBP-EDIII from DENV2 and DENV3 were expressed in *Escherichia coli* DH5α (Invitrogen) and purified using amylose resin affinity chromatography (NEB).

Detection of dengue reactive antibody in human immune sera by ELISA

ELISA plates were also coated with 75ng/ per well of purified DENV2 or 3 and the flavivirus cross reactive Mab 4G2 was used to confirm equal binding of each virus to the plate. ELISA plates were coated with 200ng of MBP-EDIII from DENV2 or 3 per well. Rabbit anti MBP sera (NEB) was used to confirm equal binding of MBP-EDIII from both serotypes to ELISA plates. ELISA plates were coated using virus or protein recombinant protein antigen in carbonate buffer at pH 9.6 for 2 hrs at room temperature. The plates were washed 3 times in Tris buffered saline with 0.2% Tween20 (TBST) and incubated with blocking buffer (Tris buffered saline with 0.05% Tween20 containing 3% skim milk and 2% normal goat serum) at 37°C for 1 hr. After washing the plates twice with TBST, human immune serum diluted in blocking buffer was added to each well and incubated at 37°C for 1 hr. Following 3 washes with TBST, alkaline phosphatase-conjugated goat antihuman IgG (Fc-specific) (Sigma) was added to each well for 1 hour at 37°C. After 3 washes with TBST, p-nitrophenyl phosphate substrate (Sigma) was added to each well and the reaction was allowed to develop for 15 minutes before recording optical density at 405nm on a spectrophotometer. In ELISAs with mouse Mabs, the protocol was the same except that alkaline phosphatase-conjugated goat anti-mouse IgG was used as a secondary antibody. To compare binding to DENV2 and DENV3 antigens, we normalized the data by using the serum sample # 24 (secondary dengue) that gave the highest OD with each antigen (DENV2, DENV3, DENV2 EDIII and DENV3 EDIII). For each antigen the maximum OD obtained with serum #24 was defined as an OD of 1. In figures 1 and 4 the Y axis is referred to as relative OD to indicate that the data was normalized using serum sample #24.

As the Es antigen bound poorly to ELISA plates, we used an antigen capture method to compare the binding of whole virus and Es. Plates were coated with 200ng of Mab 8A5 in carbonated buffer at pH 9.6. This antibody binds to E protein from all 4 serotypes. The antibody coated plates were washed and incubated with blocking buffer at 37°C for 1 hr. Next, sufficient DENV3 or Es antigen from DENV3 was added to saturate antigen binding to the antibody coated plates. CAT protein antigen was used as a negative control. The plates were washed again before incubating with serial dilutions of dengue immune human sera. The rest of the assay was performed as described above for the direct antigen coating ELISA.

Depletion of EDIII-reactive antibody in human immune sera

Purified MBP-EDIII was dialyzed against 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA (column buffer) overnight at 4°C. MBP-EDIII (300 ug) was incubated with the amylose resin (NEB) in column buffer containing 3% normal human serum (NHS) and incubated overnight at 4°C. The resin was washed three times with column buffer and 3 more times with PBS to remove unbound MBP-EDIII. The resin was blocked with 5% NHS in PBS before incubating with 1.5mls of human DENV immune serum diluted at 1:10 in PBS for 4 hrs at 37°C. The amylose resin was pelleted and the EDIII antibody depleted human serum was collected. Depletion of EDIII-reactive antibodies was confirmed by ELISA with MBP-EDIII. In addition, each human immune serum sample was absorbed to an amylose resin with MBP alone. MBP absorbed immune sera and MBP-EDIII absorbed NHS were used as negative controls in subsequent neutralization assays

DENV Neutralization assays

DENV neutralizing antibodies was measured by plaque reduction neutralization test (PRNT) or a flow cytometry based neutralization assay. The PRNT was performed as previously described (Kraus et al., 2007). In brief, Vero-81 cells were seeded into 24 well-plates and grown until 80% confluent. Serially diluted sera were mixed with 30 plaque forming units (PFU) of virus and incubated for 1 hr at 37°C. The virus/serum mix was added to the Vero cells and incubated with a nutrient overlay medium (Opti-MEM® with 1% methylcellulose and 10% FBS) for four days at 37°C. The cells were fixed and stained for viral antigen with monoclonal antibody 4G2 as previously described (Kraus et al., 2007). The percentage of neutralization was defined as reduction in the number of foci in the test sera compared to the number of foci in the control wells with normal human serum. The 50% neutralization titers were determined by nonlinear dose-response regression analysis (Prism Package, GraphPad Software, Inc., San Diego, CA). Flow cytometry based neutralization assays were performed in 96-well plates with the U937 human monocytic cell line transfected with DC-SIGN as previously described (Kraus et al., 2007). In brief, immune sera were serially diluted and incubated with sufficient virus to infect 10 to 15% of the cells in the well. The virus/serum mixture was incubated for 1hr at 37° C and then added to the cells for 1hr at 37°C. The cells were washed to remove unbound virus and fresh media was added before incubating cells for 24hrs at 37°C. Cells were fixed, permeabilized and stained with DENV Mabs 4G2 or 2H2, both of which bind to all four serotypes (Kraus et al., 2007). Cells were analyzed with a FACScan flow cytometer (Becton Dickinson) to identify infected cells. The 50% neutralization titers were determined by nonlinear dose-response regression analysis ((Prism Package, GraphPad Software, Inc., San Diego, CA).

Results

Dengue immune human sera were obtained by collecting blood samples from volunteers who might have been infected during foreign travel. Of 35 subjects enrolled in the study, 17 had antibodies that neutralized one or more DENV serotypes. The neutralization patterns of the 17 immune subjects were consistent with past exposures to DENV1 only (one subject), DENV2 only (four subjects), DENV3 only (four subjects) and secondary DENV infections (eight subjects). These sera were also tested for DENV neutralizing antibody by the Centers for Disease Control (CDC) in Fort Collins, CO and the Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, Maryland. The CDC and NIAID laboratories reached the same conclusions as we did about the past infection history of these subjects (unpublished data from Drs Robert Lanciotti, CDC and Steve Whitehead, NIH). For the current study we selected 6 sera representing 2 subjects each who had recovered from primary DENV2, primary DENV3 and secondary DENV infections. The DENV neutralization titers and the most likely year and place of infection of these subjects are listed in Table 1.

DENV Binding Antibodies in Human Immune Sera

Experiments were performed to measure the binding properties of antibodies in the 6 selected immune sera to purified DENV2 and 3. The immune sera were tested at four fold dilutions starting at 1:50. Antibodies in human DENV immune sera cross reacted with both serotypes indicating that the dominant antibodies after primary and secondary infection are serotype cross-reactive (Figure 1). End point virus binding titers were calculated for the 6 sera (Table 2). As expected, subjects with secondary infections had higher titers than subjects with primary infections (Table 2). These results indicate that an ELISA with whole virus as antigen mainly detects serotype cross reactive antibodies and the assay is not predictive of the neutralization properties of the serum sample or past infections history of the subject.

The DENV particle is made up of envelope (E), membrane (M) and capsid (C) proteins. As E protein is the main target of neutralizing antibody, experiments were done to compare the antibody response to E protein and whole virions. As full length E protein alone is not secreted out of cells, we expressed the soluble ectodomain of E (Es) from DENV3 to be used as an antigen. We used immune serum samples # 003 and 011 from primary DENV3 cases and # 009 and 024 from secondary cases and compared binding to DENV3 and Es from DENV3. Antibodies in human immune sera bound well to both Es and virus particles, but greater binding was observed with virus particles compared to Es (Figure 2). These results demonstrate that although the ectodomain of E is a dominant target of antibody, virions contain epitopes that are absent in recombinant Es.

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

Studies with mouse Mabs have demonstrated that most DENV serotype-specific antibodies bind to EDIII (Crill and Roehrig, 2001; Gromowski and Barrett, 2007; Lin et al., 1994; Lok et al., 2008; Roehrig, Bolin, and Kelly, 1998; Sukupolvi-Petty et al., 2007). When using whole virus antigen in an ELISA, the cross-reactive antibodies in human immune sera are likely to dominate and mask signal originating from serotype-specific antibodies. To develop an assay for measuring serotype-specific antibody, recombinant EDIII was expressed as a MBP fusion protein in *E. coli* (Figure 3). Previous studied have demonstrated that EDIII expressed alone or as a MBP fusion protein is folded correctly and displays antibody epitopes present on the virion (Maillard et al., 2008; Volk et al., 2004; Volk et al., 2007; Yu et al., 2004). To confirm that recombinant DENV2 and 3 MBP-EDIII fusion proteins produced in our laboratory were correctly folded, binding assays were performed with eight mouse Mabs that bind to EDIII of DENV2 and/or 3. Mabs 3H5-1, 9F16 and 2Q1899 are antibodies that bind to serotype-specific epitopes on the lateral ridge of DENV2 (Gromowski and Barrett, 2007; Henchal et al., 1985; Sukupolvi-Petty et al., 2007). As predicted, all three antibodies bound to MBP-EDIII from DENV2 but not DENV3 (Table 3). We used mouse Mabs 8A1, 14A4 and 1H9 which are serotype-specific neutralizing antibodies that bind to EDIII from DENV3 only (Serafin and Aaskov, 2001) (unpublished data, Putnak, Wahala and de Silva). Mab 8A1 and 1H9 bind to the lateral ridge of EDIII from DENV3, whereas the 14A4 epitope on DENV3 EDIII has not been mapped yet. Mabs 8A1, 14A4 and 1H9 bound to MBP-EDIII from DENV3 but not DENV2 (Table 3). We also used two neutralizing Mabs that bind to a serotype cross reactive epitopes in EDIII and these two antibodies bound to both recombinant proteins (Table 3). Thus, the type specific and cross reactive neutralizing epitopes on EDIII of DENV are preserved in the recombinant proteins used in the current study.

EDIII-reactive antibodies in human DENV immune sera

After confirming that the recombinant EDIII–MBP fusion proteins expressed appropriate serotype-specific and cross reactive epitopes, the antigens were used to detect EDIII-reactive antibody in our panel of human DENV immune sera. Each immune serum was tested in four-fold dilutions starting at 1:12.5. At high concentrations of serum, antibodies in the two subjects with evidence of past primary DENV2 infections bound to EDIII from DENV2 better than EDIII from DENV3 (Figure 4A and B). Similarly, antibodies in the two subjects with serotype-specific neutralizing antibody to DENV3 bound to EDIII from DENV3 better than EDIII from DENV2 (Figure 4C and D). The two subjects with evidence of past secondary DENV infections had antibodies that bound equally well to both antigens (Figure 4E and F). These results demonstrate that EDIII-reactive antibodies that developed after primary infection were specific to the serotype responsible for infection. The EDIII end point binding titers also displayed serotype-specificity after primary infection (Table 2). In the case of secondary serum samples, the EDIII end point titers were similar for both antigens (Table 2).

We compared the amount of antibody in immune sera directed to the whole virus versus EDIII. To measure relative amounts of available EDIII epitopes on viral and recombinant protein antigens used in the binding assays, end point titers were calculated using MAbs 8A5 and 12C1 (Table 3), which bind to a conserved DENV complex epitope on EDIII. The endpoint titers were 5-10 times higher for recombinant EDIII compared to virus (Table 2). This result was expected because the DENV complex epitope on EDIII has been mapped to the A-B loop, which is poorly exposed on the intact virus but not on recombinant EDIII (Sukupolvi-Petty et al., 2007). Despite the superior binding of 8A5 and 12C1 to recombinant EDIII, human immune sera bound poorly to recombinant EDIII compared to the virus antigen (Table 2). After primary infections the serotype-specific EDIII-reactive antibodies ranged from 0.1 to 8.1% of total virus reactive antibodies (Table 2). Following secondary infections the EDIII-reactive antibodies accounted for 0.2 to 4.0 % of total virus reactive antibodies. These results indicate that subjects who have recovered from DENV infections have low levels of EDIII-reactive antibody and, in the case of primary infections, antibodies are directed to serotype-specific epitope(s) on EDIII.

Role of EDIII-reactive Antibodies in DENV Neutralization

Experiments were performed to determine the contribution of EDIII-reactive antibodies in human immune sera to DENV neutralization. The immune sera were depleted of EDIII binding antibodies by incubating the serum samples with MBP-EDIII bound to an amylose resin. The primary DENV2 and DENV3 immune sera were incubated with MBP-EDIII from DENV2 or DENV3, respectively. The secondary sera were treated with MBP-EDIII from DENV2 (Sample 009) or DENV3 (Sample 024). As depicted in Figure 5, incubation with recombinant MBP-EDIII removed most of the EDIII-reactive antibody. The treatment specifically removed EDIII-reactive antibodies as sera treated with MBP alone were indistinguishable from untreated immune sera (Figure 5). Interestingly, when the secondary sera were depleted using MBP-EDIII from one serotype, most EDIII reactivity to the second serotype was also lost (data not shown) indicating that in secondary immune sera the antibodies are mainly directed against a cross-reactive epitope on EDIII.

As the recombinant EDIII used in above studies was expressed as a MBP fusion protein, it was conceivable that some human antibody epitopes in EDIII were altered or masked by the fusion partner. To determine if MBP fusion partner altered important epitopes on EDIII, binding assays were performed with purified DENV2 EDIII without MBP (kindly provided by Dr. Michael Diamond, Washington University, St. Louis). Dengue immune sera absorbed with recombinant DENV2 EDIIII-MBP were tested for the presence of antibodies that bound to EDIII without MBP. As depicted in Figure 6, immune sera absorbed with DENV2 MBP-EDIII protein failed to bind recombinant DENV2 EDIII without MBP indicating that the MBP fusion partner does not alter or mask the main human antibody epitopes on EDIII.

Next, untreated and EDIII antibody depleted sera were tested in the flow cytometry based DENV neutralization assay with U937 cells expressing DC-SIGN. Figure 7 depicts neutralization curves for primary DENV2, primary DENV3 and secondary DENV immune sera. Serum samples with or without EDIII-reactive antibodies showed similar neutralization patterns (Figure 7). The 50% neutralization titers were ~ 10-15% lower for serum samples depleted with EDIII compared to the MBP treated sera (Table 4). These results indicate that EDIII-reactive antibodies make a minor contribution to the total neutralizing capacity of human DENV immune sera (Table 4).

As it was conceivable that EDIII antibodies might play an important role in DENV neutralization in some cell types but not others, some of the experiments with U937 cells expressing DC-SIGN (Table 4) were repeated with Vero cells. We selected EDIII antibody depleted serum sample # 003 (primary DENV3 immune) and # 009 (secondary DENV

immune) and performed neutralization assays with Vero cells. In the case of sample #003, the neutralization titers for DENV3 were similar for untreated and EDIII antibody depleted serum (50% neutralization titers of 74 and 88 respectively). Similarly, for sample #009, the neutralization titers for DENV2 were similar (50% neutralization titers of 895 and 858 respectively) for untreated and EDIII antibody depleted sera. These results demonstrate that EDIII reactive antibodies were not required to neutralize DENV infection of U937 cells and Vero cells.

Discussion

Despite many publications on interactions between DENV and antibody, surprisingly few studies have been published that on how the binding properties of human antibodies relate to DENV neutralization. The goal of the current study was to characterize the specificity and functionality of antibodies in DENV immune human sera. Here we have demonstrated that EDIII reactive antibodies are present in human immune sera. The EDIII antibodies mainly recognized a type specific epitopes after primary infection and a cross reactive epitope after secondary infection. EDIII binding antibodies were a minor component of the total antibodies in immune sera binding to DENV. Recently Crill and co-workers used DENV2 virus like particles to measure epitopes specific human antibody responses and they also observed low levels of EDIII binding antibodies in human sera (Crill et al., 2009). Our results demonstrate that EDIII binding antibodies make only a minor contribution to the total neutralizing capacity of human immune sera. Thus, the EDIII neutralizing epitopes that have been the focus of much recent work (Gromowski and Barrett, 2007; Gromowski, Barrett, and Barrett, 2008; Sukupolvi-Petty et al., 2007) were not the target of most neutralizing antibody in primary and secondary DENV immune sera.

Several investigators have reported the presence of EDIII-reactive antibodies following natural infection of people and animals with flaviviruses (Beasley et al., 2004; Hapugoda et al., 2007; Holbrook, Shope, and Barrett, 2004; Ludolfs et al., 2002; Sanchez et al., 2007). In human WNV immune sera EDIII-reactive antibodies were present, although at low levels compared to the total antibody binding to virus (Oliphant et al., 2007). Furthermore, investigators have shown that EDIII-reactive antibodies are specific for the infecting virus, unlike antibodies against the whole virus particle, which are highly cross-reactive (Beasley et al., 2004; Hapugoda et al., 2007; Holbrook, Shope, and Barrett, 2004; Ludolfs et al., 2002; Sanchez et al., 2007). Our data reported here indicate that DENV cross reactive antibodies dominate in whole virus binding assays. Our data demonstrate that in subjects who have recovered from primary DENV infection, EDIII-reactive antibodies were mainly directed to an epitope specific for the serotype responsible for infection, whereas in secondary cases EDIII-reactive antibodies bound to a DENV cross-reactive epitope. Recent studies with mouse Mabs have mapped the location of both cross reactive and serotype specific epitopes on DENV EDIII (Gromowski and Barrett, 2007; Gromowski, Barrett, and Barrett, 2008; Sukupolvi-Petty et al., 2007). Although it is reasonable to speculate that the cross reactive and serotype specific epitopes defined by mouse Mabs are also targets of the human antibody response measured here, further studies are needed to confirm this.

Currently, the primary serological assay to identify the DENV serotype responsible for a primary infection is the neutralization test, which is a laborious and time consuming assay. Our results indicate that a simple ELISA with recombinant EDIII as antigen can be used to identify the DENV serotype responsible for primary infection. Our results also demonstrate that the specificity of EDIII-reactive antibodies is preserved in both early (within first year, data not shown) and late (>4 years after infection) convalescent primary sera. In secondary infections, this assay is unlikely to predict responsible serotypes as the response is directed to cross reactive epitope(s) on EDIII. Ludolfs and colleagues also reported similar results using recombinant

EDIII in an immunoblot assay with human immune sera (Ludolfs et al., 2002). Further studies are needed to evaluate the utility of recombinant EDIII as an antigen for identifying DENVs responsible for primary infection. In the case of secondary infections, studies need to address if the EDIII-reactive antibodies simply cross-react with the serotypes responsible for the primary and secondary infections or if they also cross- react with serotypes not responsible for infection.

Studies with mouse Mabs have led to the identification and mapping of a serotype-specific epitope on the lateral ridge of EDIII of several flaviviruses including DENV (Gromowski and Barrett, 2007; Nybakken et al., 2005; Roehrig, 2003; Sukupolvi-Petty et al., 2007). An implicit, but untested assumption has been that antibodies directed to this epitope must play a role in serotype-specific neutralization following natural human infection. Our results demonstrate that DENV-specific EDIII-reactive antibodies play a minor role in neutralization observed with human sera. Studies with immune sera from people and horses naturally infected with WNV have also revealed a variable role for EDIII-reactive antibodies in viral neutralization (Oliphant et al., 2007; Sanchez et al., 2007). In some cases, EDIII-reactive antibody depletion led to a decrease in WNV neutralization whereas in other cases no significant change was observed (Oliphant et al., 2007; Sanchez et al., 2007). Our results do not rule out the possibility of inter domain epitopes involving EDIII making important contributions to neutralization as these epitopes would not be present in the recombinant EDIII proteins used in the current study. We conclude that antibodies directed to inter domain epitopes, epitopes on EDI or II of E protein and, possibly M protein, are mainly responsible for the neutralizing activity of human immune sera.

One potential concern is that the recombinant EDIII MBP fusion proteins used here may be improperly folded and not display important epitopes present on EDIII in the virus particle. Our studies indicated that the recombinant proteins were correctly folded. Eight EDIII-reactive, neutralizing mouse Mabs bound with appropriate specificity to the recombinant EDIII from DENV2 or 3 used here indicating that the main type specific and cross reactive neutralizing epitopes described in the literature were present on our recombinant EDIII proteins (Gromowski and Barrett, 2007; Gromowski, Barrett, and Barrett, 2008; Henchal et al., 1985; Serafin and Aaskov, 2001; Sukupolvi-Petty et al., 2007). Other groups have performed structure studies with EDIII expressed alone or as a MBP-fusion protein and demonstrated that the *E. coli* expressed protein has a structure similar to EDIII in its native form (Volk et al., 2004; Volk et al., 2007; Yu et al., 2004). We also demonstrated here fusing EDIII to MBP did not mask or alter the main human antibody epitopes in EDIII because human sera absorbed with MBP-EDIII failed to bind to EDIII without the MBP fusion portion as well.

In summary, our results indicate that EDIII-reactive antibodies are of minor importance in neutralizing DENV by human DENV immune sera. We propose that the major cross-reactive and serotype-specific neutralizing epitopes targeted by human immune sera are inter-domain epitopes (Goncalvez et al., 2004) and/or located outside EDIII. Currently live attenuated DENV vaccines are being tested in human clinical trials (Edelman, 2007). It is reasonable to assume that the protective antibodies induced by these vaccines will be similar to protective antibodies induced after a natural DENV infection. We predict that the quality and quantity of antibodies against EDIII will not determine the efficacy of live attenuated DENV vaccines. As an alternative approach to live attenuated vaccines, several groups have focused on developing recombinant EDIII vaccines (Babu et al., 2008; Bernardo et al., 2008; Etemad et al., 2008). Given the low levels of EDIII-reactive antibodies detected here in human immune sera, caution is urged in proceeding with EDIII based platforms. We currently do not understand why people develop low levels of EDIII-reactive antibody after natural infection. The human immune system may recognize and react to epitopes on EDI and EDII better than to EDIII. However, with appropriate adjuvants and recombinant protein constructs, it might be possible to stimulate

an effective immune response directed to relevant epitopes on EDIII as well. Such vaccines are likely to neutralize DENV by a mechanism that is different from neutralization observed after natural infection. The topic of flavivirus-antibody interactions has been dominated by studies to identify and characterize epitopes on EDIII. We hope the results reported here will stimulate more work to characterize epitopes on EDI and EDII and their role in DENV neutralization.

Acknowledgments

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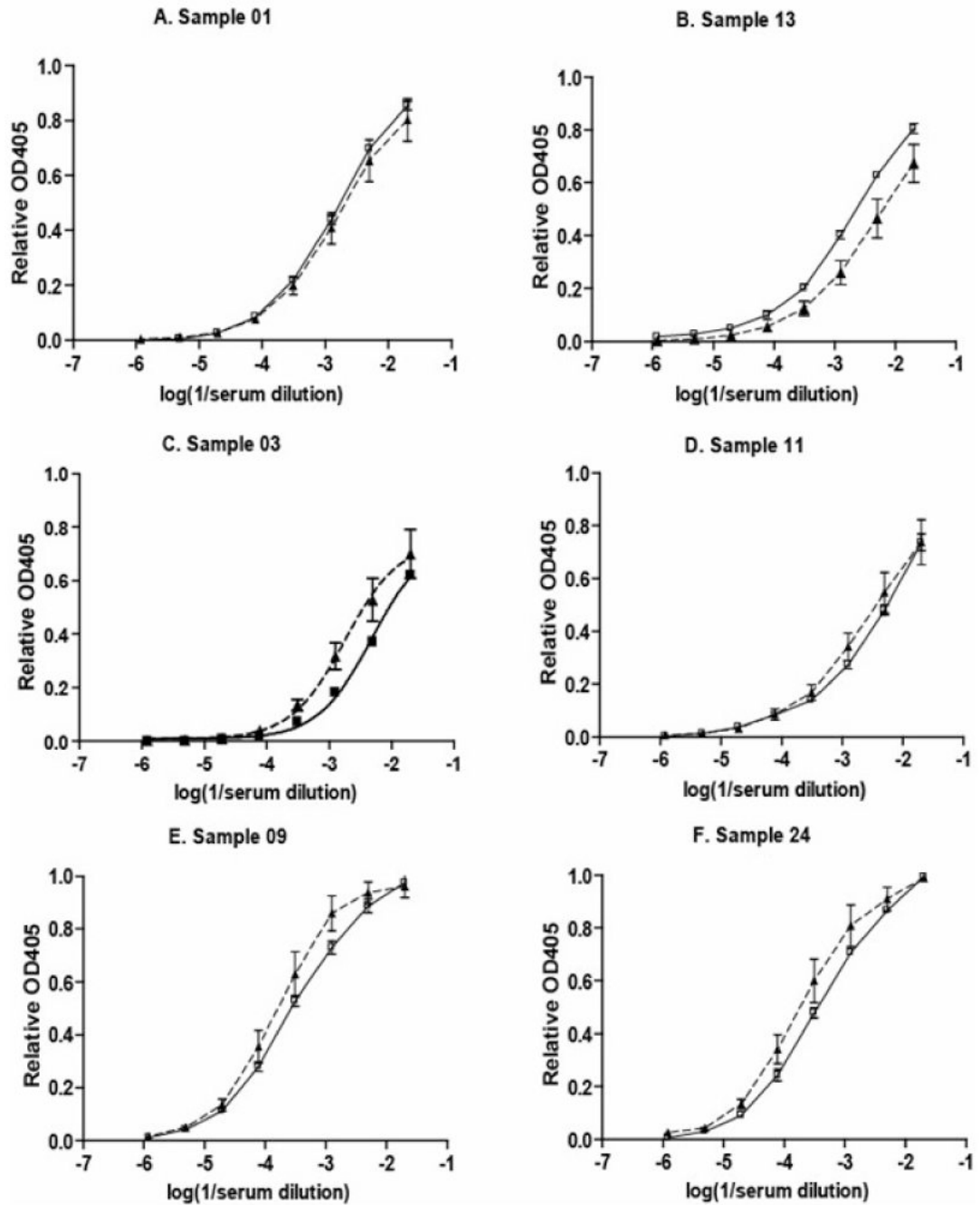
Figure 1.
Binding of human immune sera to purified DENV2 and 3. The binding of antibodies in convalescent sera from patients who have recovered from primary DENV2 infections (A, B), primary DENV3 infections (C, D) and secondary DENV infections (E, F) to purified DENV2 (solid lines) or DENV3 (dashed line) was analyzed by ELISA. The data points represent mean values and the error bars represent the standard error of the mean. The data shows one of two representative experiments.
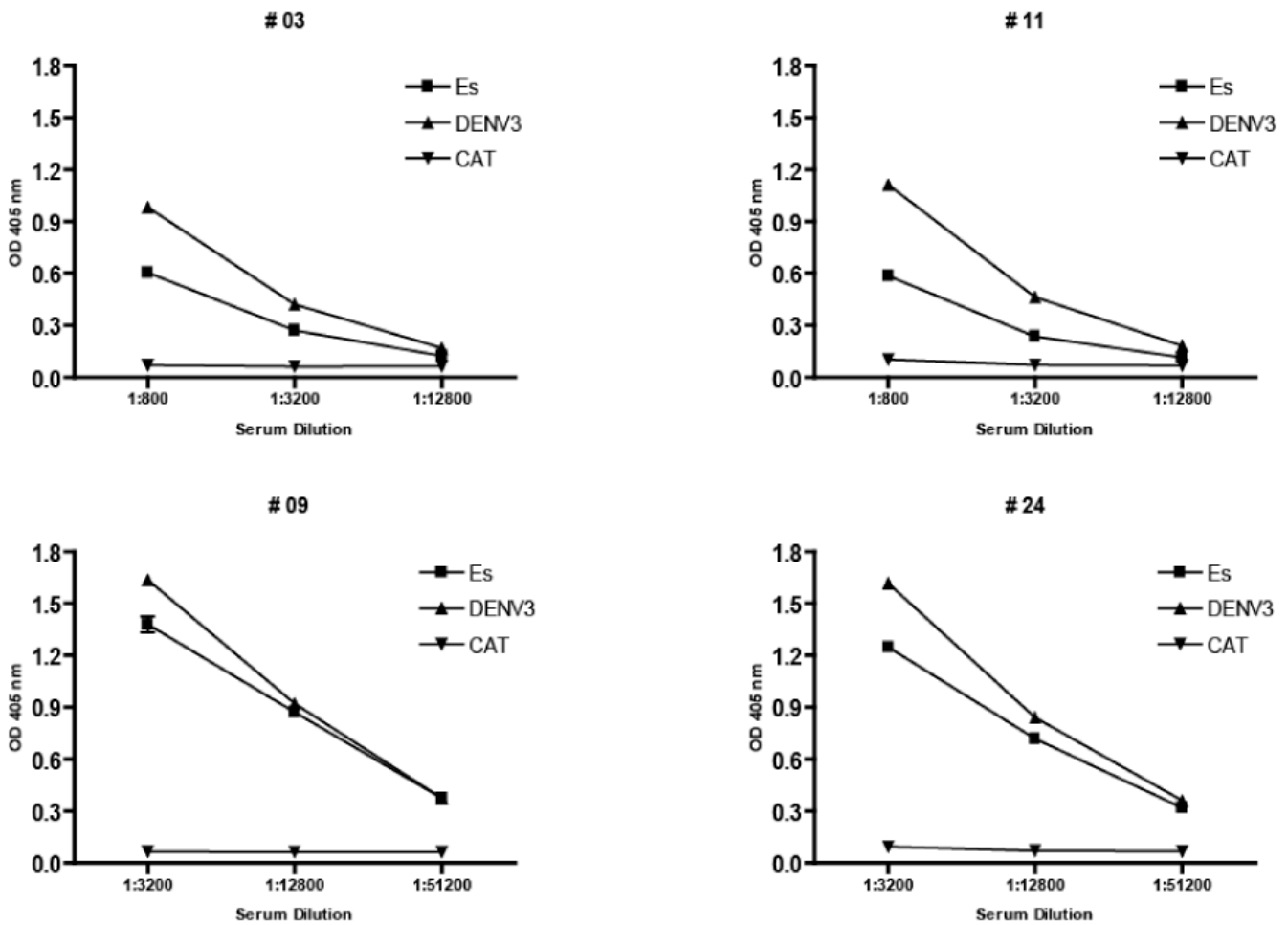
Figure 2.
Binding of human immune sera to purified DENV3 and the ectodomain of E protein. To compare antibody binding to whole virions and E protein, ELISA plates were coated with purified DENV3 or the ectodomain of E protein (Es) from DENV3. As a negative control, plates were coated with chloramphenicol acetyl transferase (CAT) protein. Sera from patients who have recovered from primary DENV3 infections (03 and 011) and secondary DENV infections (09, and 024) were used to measure virus and E protein specific antibody responses. The data shows one of two representative experiments.
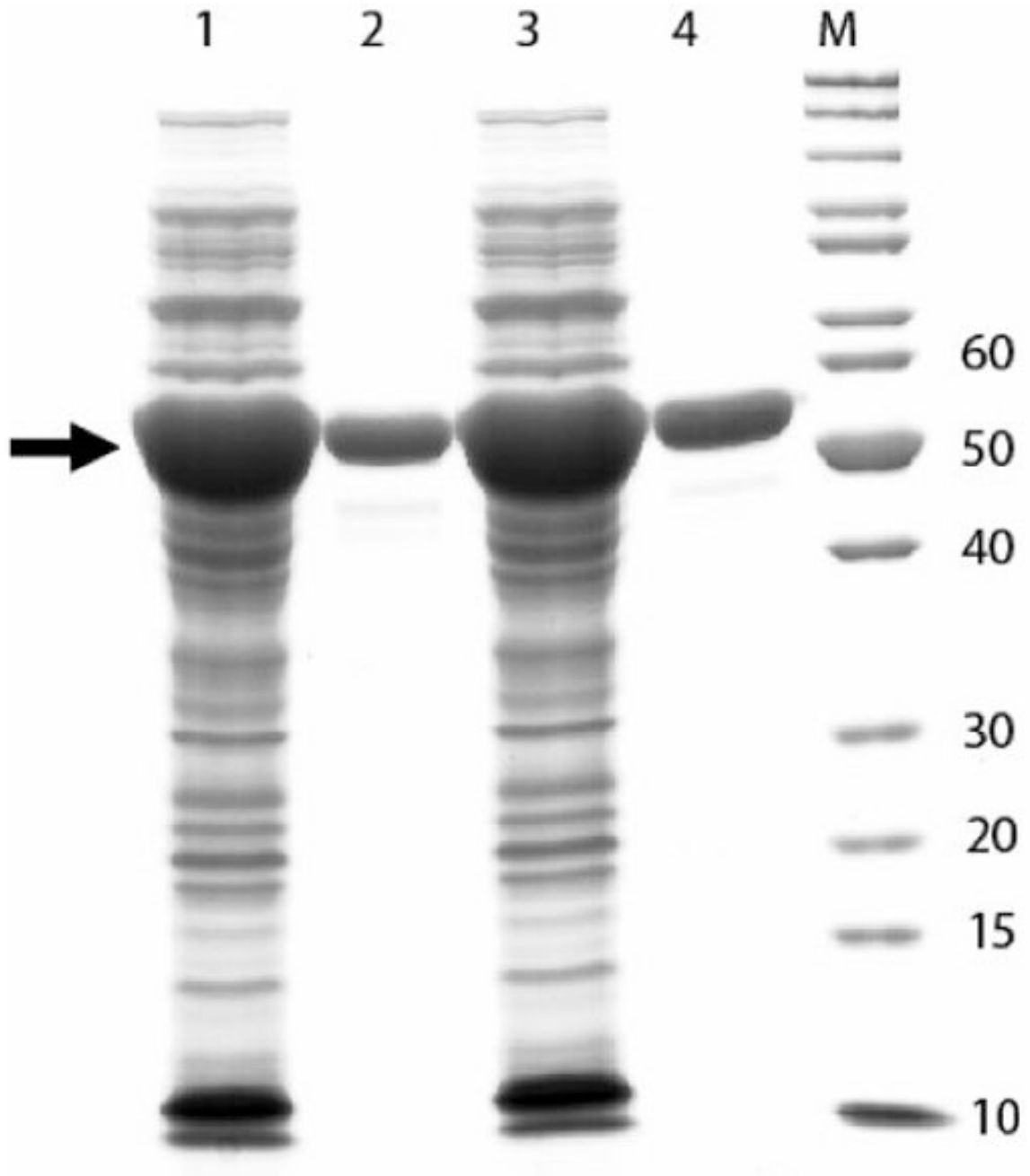
Figure 3.
Purification and characterization of recombinant MBP-EDIII fusion proteins from DENV2 and 3. The recombinant protein expressed in *E. coli* and purified by amylase affinity chromatography. Lanes 1 and 3 depict the DENV2 and DENV3 MBP-EDIII fusion proteins in *E. coli* lysates. Lanes 2 and 4 depict the purified protein obtained after amylase affinity chromatography. The arrow indicated the band corresponding to the 53kD MBP-EDIII fusion protein.
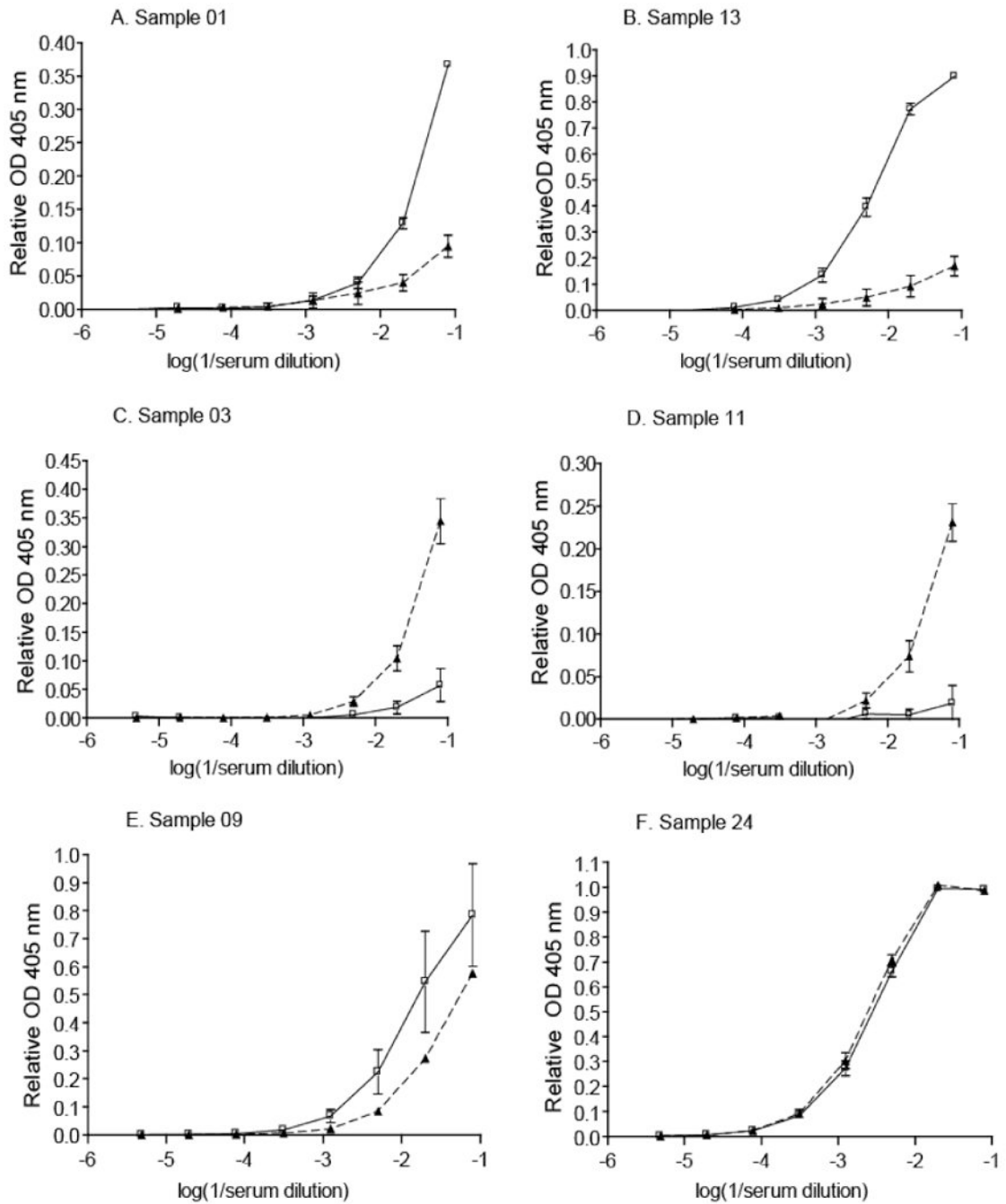
Figure 4.
Binding of human immune sera to MBP-EDIII from DENV2 or 3. Convalescent sera from 2 people each who had recovered from primary DENV2 (A, B), primary DENV3 (C, D) and secondary DENV (E, F) infections were tested for binding to recombinant MBP-EDIII from DENV2 (solid lines) or DENV3 (dashed line). The data points represent the mean values and the error bars represent the standard error of the mean. The data are from one of two representative experiments.
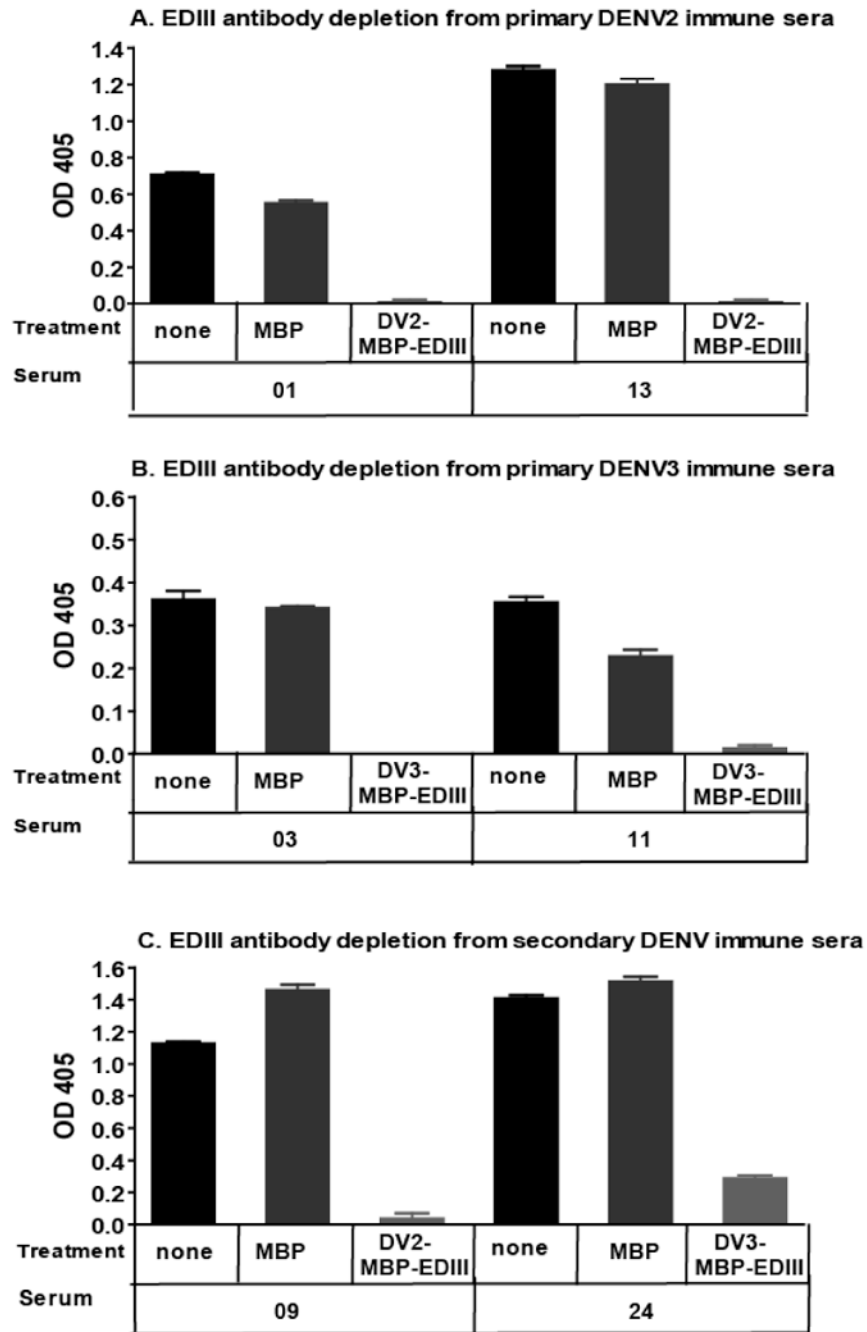
Figure 5.
Depletion of EDIII-reactive antibody from human immune sera. Immune sera were from subjects who had have recovered from primary DENV2 (Panel A), primary DENV3 (Panel B) or secondary DENV (Panel C) infections. The sera were absorbed using MBP alone or recombinant MBP-EDIII-fusion protein from DENV2 (sera # 001, 009 and 013) or DENV3 (sera #003, 011 and 024).

Figure 6.
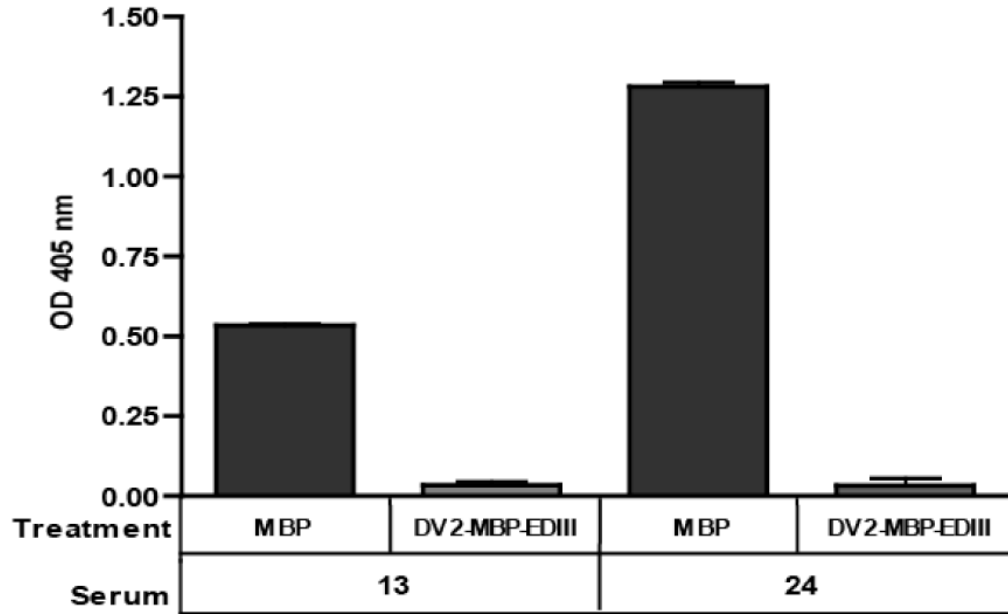Binding of EDIII antibody depleted dengue immune sera to DENV2 EDIII without a MBP fusion partner. Dengue immune sera (# 13, and #24) were absorbed using MBP-EDIII or MBP alone. The absorbed sera were tested for binding to DENV2 EDIII expressed without a MBP fusion partner. The MBP-EDIII absorbed sera failed to bind to EDIII alone indicating that EDIII with or without a MBP expressed similar antibody epitopes.
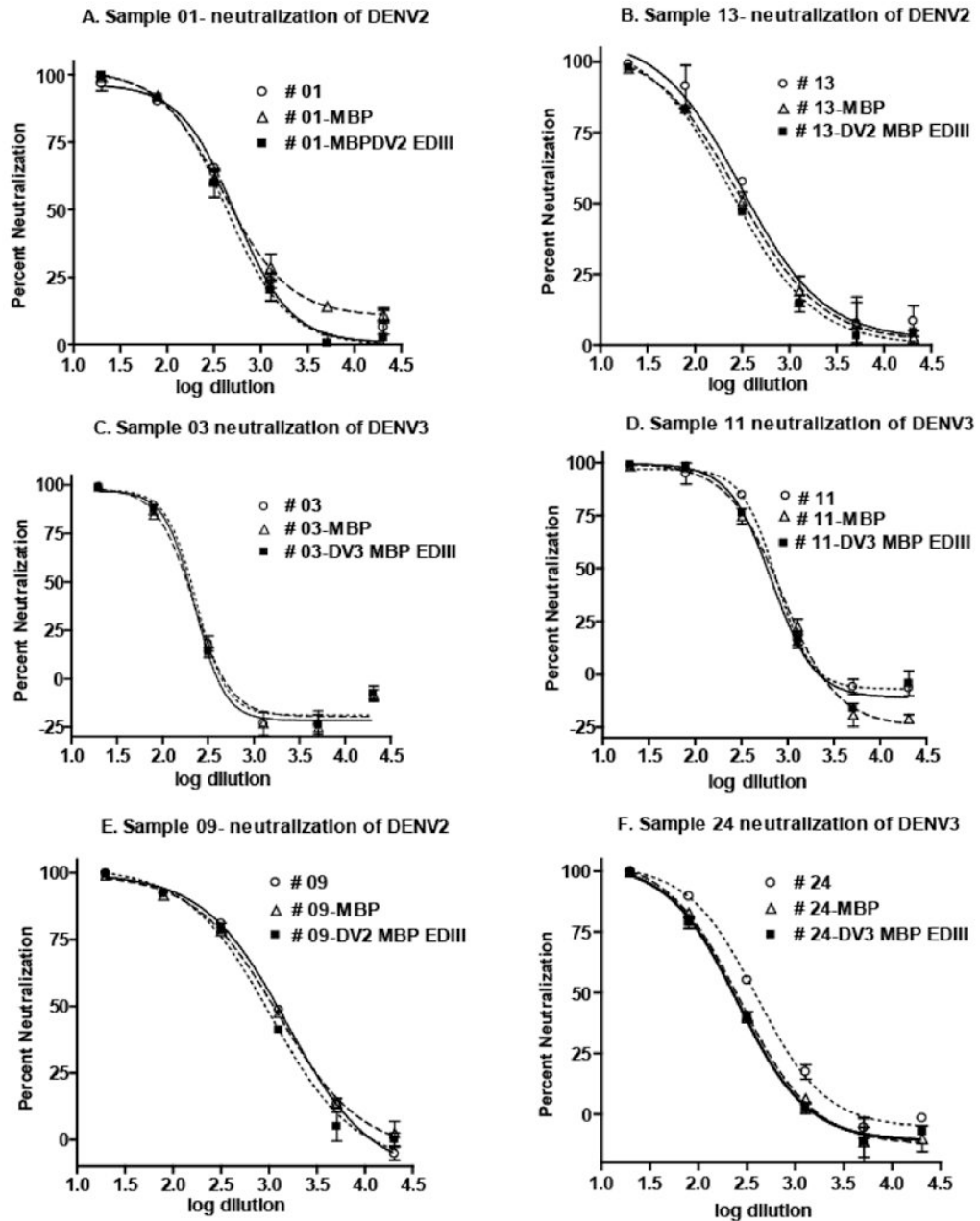
Figure 7.
DENV neutralization by human immune sera depleted of EDIII binding antibodies.
Convalescent sera from 2 people each who had have recovered from primary DENV2 (A, B),
primary DENV3 (C, D) and secondary DENV (E, F) infections were depleted of EDIII binding
antibody and tested for DENV neutralization at different dilutions. Neutralizing antibody was
measured using U937 cells expressing DC-SIGN and flow cytometry. Neutralization curves
are depicted for untreated (open circles), MBP treated (open triangles) and EDIII antibody
depleted (dark squares) sera. The data are from one of two representative experiments.

Table 1

Human DENV immune sera used in study

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

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

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

Table 2

Titer of DENV and EDIII reactive antibody in immune sera

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

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

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

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

[4] Not applicable

Table 3

Binding of DENV neutralizing monoclonal antibodies to recombinant MBP-EDIII fusion proteins

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

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

Table 4

DENY neutralization by immune sera depleted of EDIII reactive antibody

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

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