The potent and broadly neutralizing human dengue virus-specific monoclonal antibody 1C19 reveals a unique cross-reactive epitope on the bc loop of domain II of the envelope protein

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ABSTRACT Following natural dengue virus (DENV) infection, humans produce some antibodies that recognize only the serotype of infection (type specific) and others that cross-react with all four serotypes (cross-reactive). Recent studies with human antibodies indicate that type-specific antibodies at high concentrations are often strongly neutralizing in vitro and protective in animal models. In general, cross-reactive antibodies are poorly neutralizing and can enhance the ability of DENV to infect Fc receptor-bearing cells under some conditions. Type-specific antibodies at low concentrations also may enhance infection. There is an urgent need to determine whether there are conserved antigenic sites that can be recognized by cross-reactive potently neutralizing antibodies. Here, we describe the isolation of a large panel of naturally occurring human monoclonal antibodies (MAbs) directed to the DENV domain II fusion loop (FL) envelope protein region from subjects following vaccination or natural infection. Most of the FL-specific antibodies exhibited a conventional phenotype, characterized by low-potency neutralizing function and antibody-dependent enhancing activity. One clone, however, recognized the bc loop of domain II adjacent to the FL and exhibited a unique phenotype of ultrahigh potency, neutralizing all four serotypes better than any other previously described MAb recognizing this region. This antibody not only neutralized DENV effectively but also competed for binding against the more prevalent poor-quality antibodies whose binding was focused on the FL. The 1C19 human antibody could be a promising component of a preventative or therapeutic intervention. Furthermore, the unique epitope revealed by 1C19 suggests a focus for rational vaccine design based on novel immunogens presenting cross-reactive neutralizing determinants.

IMPORTANCE With no effective vaccine available, the incidence of dengue virus (DENV) infections worldwide continues to rise, with more than 390 million infections estimated to occur each year. Due to the unique roles that antibodies are postulated to play in the pathogenesis of DENV infection and disease, there is consensus that a successful DENV vaccine must protect against all four serotypes. If conserved epitopes recognized by naturally occurring potently cross-neutralizing human antibodies could be identified, monovalent subunit vaccine preparations might be developed. We characterized 30 DENV cross-neutralizing human monoclonal antibodies (MAbs) and identified one (1C19) that recognized a novel conserved site, known as the bc loop. This antibody has several desirable features, as it neutralizes DENV effectively and competes for binding against the more common low-potency fusion loop (FL) antibodies, which are believed to contribute to antibody-mediated disease. To our knowledge, this is the first description of a potent serotype cross-neutralizing human antibody to DENV.

Dengue viruses (DENVs) have continued to expand in geographic range over the last several decades and are now the most common insect-transmitted virus that targets humans. As a result, the incidence of infections has risen steadily, with more than 390 million infections estimated to occur annually (1), with increasing numbers of the most severe form of dengue disease, dengue hemorrhagic fever (DHF) or shock syndrome (DSS) (2). The mechanisms underlying severe dengue disease remain poorly understood but may involve the pathogenic activities of cross-reactive antibodies (Abs). Following an initial primary infection...
with DENV, lifelong antibody-mediated protection usually develops against the homologous infecting serotype. However, the antibody response against DENV is dominated by a group of cross-reactive antibodies that bind to all four DENV serotypes. These cross-reactive antibodies are weakly neutralizing and generally do not protect against DENV infection when present at physiologic concentrations, although at high concentrations some reduce virus replication in semipermissive animal models. Moreover, the most widely accepted model of pathogenesis of severe dengue disease proposes that with a subsequent infection by a different serotype (known as a secondary infection), serotype cross-reactive antibodies form nonneutralized antigen-antibody complexes that facilitate the efficient entry of the virus into host cells expressing Fc receptors. This enhanced uptake of virus into susceptible cells is proposed to result in increased viral replication and release of cytokines and vasoactive mediators that alter vascular permeability. This process has been termed antibody-dependent enhancement (ADE) of infection and has been demonstrated to occur using human immune serum or monoclonal antibodies (MAbs) in cell culture and in animal models (3–6).

DENVs are members of the Flaviviridae family of single-stranded positive-sense RNA viruses that have pseudoicosahedral symmetry and display 180 copies of the envelope (E) glycoprotein and premembrane/membrane (prM/M) proteins, which are embedded in the lipid bilayer membrane. The immunodominant E glycoprotein is comprised of three structural domains, designated domain I (D1), DII, and DIII; E protein exists as a homodimer in the prefusion state on the mature virus particle and undergoes multiple conformational changes during maturation and fusion. Initial characterization of the targets of neutralizing antibodies was performed using monoclonal antibodies (MAbs) isolated from mice (7–11); however, the ability to translate this information to the human immune response has been limited (12). The major antigenic targets of the neutralizing human antibody response have been studied using polyclonal serum from naturally infected patients (13, 14). Given the polyclonal nature of serum, however, the response to individual antibody epitopes has not been easy to parse out at the molecular level.

A comprehensive understanding of the location of antigenic sites targeted by the protective and/or pathogenic human antibody response is of critical importance to vaccine development efforts against DENV. Over the past 3 years, several panels of human MAbs have been characterized by multiple groups, showing that the human response targets both E and prM protein and is comprised largely of serotype cross-reactive and weakly neutralizing antibodies (15–19). Only a small percentage of antibodies, <10% of those directed against surface-exposed epitopes, are serotype specific or potently inhibitory (neutralizing at a <0.5-μg/ml concentration). This natural scarcity of strongly neutralizing antibodies has made it challenging to map the epitopes targeted by the protective human antibody response. Recently, studies using DENV immune serum depletion and human MAb techniques suggested that the antibodies responsible for serum neutralizing activity following primary DENV infection may be serotype specific and likely target a site outside DIII, with MAbs recognizing epitopes within the hinge region between DII and DIII on the E protein. Many of these antibodies bound quaternary epitopes that existed only on the virion, and not on isolated E proteins (13, 16). The structure of a virion-only binding antibody, in complex with DENV1, was recently determined using cryo-electron microscopy (20). These antibodies are quite potent but generally are serotype specific in their ability to bind DENV and neutralize infection.

It remains unclear if immune humans can also generate antibodies with potent cross-neutralizing properties. Several recent studies suggested that naturally occurring anti-DENV human antibodies with cross-neutralizing activity target the fusion loop (FL) epitope on DII of the E protein (DII-FL) (21–27). These antibodies with cross-neutralizing activity target the fusion loop (FL) epitope on DII of the E protein (DII-FL) (21–27). These antibodies, however, frequently exhibit only weak to moderately neutralizing activity (half-maximal effective concentration needed for neutralization [EC₅₀] of 1 to 5 μg/ml) and frequently display significant ADE activity in vitro and in vivo (25). Sites targeted by potent serotype cross-neutralizing human antibodies have not been described.

In the present study, we assembled a large panel of MAbs, obtained from human subjects following vaccination or primary and secondary infection, to profile the anti-DENV serotype cross-neutralizing antibody response. The majority of cross-reactive

### TABLE 1 Subject demographics and serologic findings from DENV immune subjects studied

<table>
<thead>
<tr>
<th>Type of infection</th>
<th>Infecting virus</th>
<th>Infecting serotype(s)</th>
<th>Subject no.</th>
<th>Location acquired</th>
<th>Yr infected</th>
<th>Time since infection</th>
<th>Reciprocal serum antibody 50% neutralization titer, to indicated DENV serotype (D1)</th>
<th>Reciprocal serum antibody 50% neutralization titer, to indicated DENV serotype (D2)</th>
<th>Reciprocal serum antibody 50% neutralization titer, to indicated DENV serotype (D3)</th>
<th>Reciprocal serum antibody 50% neutralization titer, to indicated DENV serotype (D4)</th>
<th>No. of DENV FL-specific hybridomas obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>Live attenuated</td>
<td>1 49</td>
<td>U.S. NIH vaccine trial</td>
<td>2008</td>
<td>7 mo</td>
<td>37 ND ND ND ND ND</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Natural</td>
<td>Vaccine</td>
<td>1 106</td>
<td>India</td>
<td>2007</td>
<td>2 yr</td>
<td>90 &lt; &lt; &lt; 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>Natural</td>
<td>2 19</td>
<td>Thailand</td>
<td>1997</td>
<td>8 yr</td>
<td>95 &gt; 105</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Field strain</td>
<td></td>
<td>3 118</td>
<td>Nicaragua</td>
<td>2009</td>
<td>1 yr</td>
<td>60 32 980 76 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary</td>
<td>Natural</td>
<td>Multiple</td>
<td>Nicaragua</td>
<td>2009</td>
<td>1 yr</td>
<td>107 220 200 70 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>Field strains</td>
<td>1 115</td>
<td>Sri Lanka</td>
<td>1974–1997</td>
<td>12 yr</td>
<td>90 134 330 165 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>9 184</td>
<td>India–Sri Lanka</td>
<td>2000</td>
<td>5 yr</td>
<td>&gt; &gt; 290 393 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>184 Mexico</td>
<td>Mexico</td>
<td>2006</td>
<td>4 yr</td>
<td>282 209 166 76 16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Subject numbers were assigned in the original clinical studies, as outlined in Materials and Methods.

* Symbols: >, titer greater than 1:1,280; <, titer less than 1:20; ND, not determined.
DII-FL antibodies exhibited weak neutralizing potency and showed strong ADE activity. One unusual clone (1C19), however, exhibited ultrahigh neutralization potency against DENV strains corresponding to all four serotypes. Fine epitope mapping studies revealed that this MAb was distinct from the less potent DII-FL MAbs in that it targeted the conserved bc loop (amino acids [aa] 73 to 79) adjacent to the FL. This antibody has several desirable features, including both the ability to neutralize DENV effectively and the ability to compete for binding against the more common low-potency FL antibodies, which likely contribute to antibody-mediated disease. Therefore, elucidation of the features of this unique epitope may inform the design of vaccines intended to elicit protective immunity with reduced capacity to induce antibodies that enhance infection.

RESULTS

Human MAbs to the DII-FL region of DENV E protein. To study the human antibody response to DENV, we generated several hundred MAbs from subjects following primary and secondary DENV infection as well as recipients of a live attenuated vaccine (25). While characterizing these MAbs, we observed that a large subset of DENV complex-reactive antibodies (those able to bind to viruses from all four serotypes) targeted the DII-FL region, some of which neutralized viruses from multiple DENV serotypes. We assembled a large panel of these MAbs from subjects following natural DENV infection or vaccination for further study (Table 1). MAbs were obtained from subjects with diverse infection histories, including natural primary DENV1, -2, or -3 or secondary infections.

We began to classify our panel of DII-FL MAbs for their binding properties. Using real-time biosensor technology (Octet Red platform), we first performed competitive binding studies, using representative full-length MAbs, on either intact DENV particles or recombinant E80 protein. As can be seen in Fig. 1, all representative MAbs from our panel segregated into one competition group, highlighted in red. No competition was observed between FL MAbs in our panel and antibodies known to bind prM protein or E protein DIII. MAb 1C19 had a unique binding property compared to other MAbs in our panel, as it competed with the virion-only binding MAb 1F4, which recognizes the DI/II hinge region.

Breadth of neutralizing activity of human MAbs. We tested the neutralizing activity of our panel of MAbs against representative DENV strains from the four serotypes, using U937/H11001/DC-SIGN target cells. As shown in Table 2, the neutralization potency varied considerably among MAbs. Many of the MAbs showed...
little or no neutralizing activity toward any serotype, requiring a concentration of >10 µg/ml to neutralize 50% of the virus (EC_{50}) in our assay. Approximately half of the MAbs in our panel displayed moderate neutralization potency (EC_{50} between 1 and 10 µg/ml) to viruses from at least one DENV serotype. Six antibodies (1M4, 1M7, 1N5, 1C19, 1C18, and 4E8) potently neutralized viruses from at least one DENV serotype, with an EC_{50} of <1.0 µg/ml. Two MAbs, 1M7 and 1C19, neutralized at least one serotype below a 0.1-µg/ml concentration. Finally, 1C19 showed the greatest cross-neutralizing potency, neutralizing DENV1, -2, and -3 at or below a concentration of 0.06 µg/ml.

We also assessed the neutralization activity of a subset of our panel using Vero cells. As shown in Table 3, differences in the neutralization potency were observed for each MAb when comparing U937 + DC-SIGN to Vero cells. In most cases, the EC_{50} was 10 to 100 times lower when Vero cells were used. Interestingly, the neutralization potency of 1C19 was unchanged for DENV1 to -3 but was 60 times more potent for DENV4 when Vero cells were used.

Most of the antibodies that we isolated to the DII-FL exhibited

### Table 2: Characteristics of DENV-specific FL-binding human MAbs

<table>
<thead>
<tr>
<th>Type of infection</th>
<th>Subject no.</th>
<th>MAbs</th>
<th>IgG subclass</th>
<th>λ or κ</th>
<th>EC_{50} g/ml, against indicated dengue virus (D) serotype</th>
<th>Fold enhancement of infection, for indicated serotype at 1 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary DENV1 vaccine</td>
<td>49</td>
<td>1M12.2</td>
<td>1</td>
<td>κ</td>
<td>&gt; &gt; &gt; &gt;</td>
<td>8 8 30 1</td>
</tr>
<tr>
<td>Primary DENV1 wild type</td>
<td>106</td>
<td>1M6.2</td>
<td>1</td>
<td>κ</td>
<td>&gt; 8 &gt; &gt;</td>
<td>4 3 29 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2F21</td>
<td>1</td>
<td>κ</td>
<td>&gt; &gt; &gt;</td>
<td>18 7 28 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2I23</td>
<td>3</td>
<td></td>
<td>&gt; &gt; &gt;</td>
<td>5 1 1 1</td>
</tr>
<tr>
<td>Primary DENV2 wild type</td>
<td>10</td>
<td>1M4</td>
<td>1</td>
<td>κ</td>
<td>0.56 0.27 5 2</td>
<td>6 1 56 1</td>
</tr>
<tr>
<td>Primar DENV3 wild type</td>
<td>19</td>
<td>5M22</td>
<td>1</td>
<td>λ</td>
<td>7 9 5 &gt;</td>
<td>11 11 42 11</td>
</tr>
<tr>
<td>Secondary wild type</td>
<td>3</td>
<td>1I17</td>
<td>1</td>
<td>λ</td>
<td>10 8 8 &gt;</td>
<td>7 12 27 7</td>
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<tr>
<td></td>
<td></td>
<td>2A15</td>
<td>1</td>
<td>κ</td>
<td>7 7 5 10</td>
<td>10 6 16 5</td>
</tr>
<tr>
<td>Secondary wild type</td>
<td>118</td>
<td>1M7</td>
<td>1</td>
<td>κ</td>
<td>0.55 0.30 0.02 0.50</td>
<td>1 1 1 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1C19</td>
<td>1</td>
<td>κ</td>
<td>&gt; &gt; &gt;</td>
<td>9 8 11 7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1F12.2</td>
<td>1</td>
<td>κ</td>
<td>&gt; &gt; &gt;</td>
<td>5 2 9 7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1I16</td>
<td>1</td>
<td>λ</td>
<td>&gt; &gt; &gt;</td>
<td>4 1 2 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1K16</td>
<td>1</td>
<td>κ</td>
<td>&gt; &gt; &gt;</td>
<td>5 4 2 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1L4</td>
<td>1</td>
<td>κ</td>
<td>&gt; &gt; &gt;</td>
<td>7 16 11 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1L6</td>
<td>1</td>
<td>λ</td>
<td>&gt; &gt; &gt;</td>
<td>13 16 9 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1N8</td>
<td>1</td>
<td>λ</td>
<td>&gt; &gt; &gt;</td>
<td>6 3 1 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2M11</td>
<td>1</td>
<td>κ</td>
<td>&gt; &gt; &gt;</td>
<td>11 5 2 9</td>
</tr>
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<td></td>
<td>3B4</td>
<td>1</td>
<td>λ</td>
<td>&gt; &gt; &gt;</td>
<td>20 12 36 11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3D18</td>
<td>1</td>
<td>λ</td>
<td>&gt; &gt; &gt;</td>
<td>33 4 9 8</td>
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<td></td>
<td></td>
<td>3G5</td>
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<td>λ</td>
<td>&gt; &gt; &gt;</td>
<td>9 15 7 4</td>
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<td></td>
<td></td>
<td>4E8</td>
<td>1</td>
<td>κ</td>
<td>0.4 0.2 3 &gt;</td>
<td>7 13 14 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5C8</td>
<td>1</td>
<td>κ</td>
<td>1 2 1 3</td>
<td>5 5 4 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5K17</td>
<td>1</td>
<td>κ</td>
<td>2 3 6 5</td>
<td>21 17 33 18</td>
</tr>
</tbody>
</table>

### Table 3: Neutralization potency of human MAbs, as tested in two cell lines

<table>
<thead>
<tr>
<th>MAbs</th>
<th>U937+DC-SIGN cells</th>
<th>Vero-81 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D1</td>
<td>D2</td>
</tr>
<tr>
<td>1M7</td>
<td>0.55</td>
<td>0.3</td>
</tr>
<tr>
<td>1N5</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>1C19</td>
<td>0.06</td>
<td>0.03</td>
</tr>
<tr>
<td>1C18</td>
<td>0.2</td>
<td>&gt;</td>
</tr>
<tr>
<td>1L6</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>3B4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>4E8</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>5C8</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

a Immunoglobulin isotype, subtype, and light chain utilization were determined by ELISA.
b The concentration (µg/ml) at which 50% of virus was neutralized (EC_{50}) is shown for each dengue virus serotype: EC_{50} values between 1.0 and 10.0 µg/ml are shown, 50% neutralization values of <1.0 µg/ml are shown in bold; EC_{50} values of <0.1 µg/ml are shown in bold italic. The “>” symbol indicates no neutralization detected when tested at a concentration as high as 10 µg/ml.

c Enhancement assays were performed for each antibody at a concentration of 1 µg/ml, separately against each DENV serotype, and results are shown as fold enhancement. Enhancement values greater than 20-fold are shown in bold.

### References

evidence of antibody-dependent enhancement (ADE) of infection in Fc receptor-bearing cells, when tested at 1 μg/ml (Table 2). In many cases, the growth of virus was an order of magnitude greater or more in the presence of the MAb tested.

Temperature dependence of neutralization. One possible explanation as to why the DII-FL MAbs neutralized DENV1, DENV2, and DENV3 more efficiently than DENV4 was that the DII-FL epitope was not equivalently accessible on the surface of the DENV4 virion compared to the other DENV serotypes. Recent studies with mouse MAbs and different DENV serotypes have suggested that increasing the temperature of preincubation facilitates exposure of buried epitopes on different virion conformational ensembles, which results in enhanced neutralizing activity of some MAbs (28–31). To assess the effect that temperature has on the ability of our panel of human MAbs to neutralize DENV, we next tested temperature dependence in their titer (FRNT) assays were performed using different DENV strains.

Neutralization assays were performed at 2°C or more in the presence of the MAb tested. Many cases, the growth of virus was an order of magnitude greater or more in the presence of the MAb tested.

Temperature dependence of neutralization. One possible explanation as to why the DII-FL MAbs neutralized DENV1, DENV2, and DENV3 more efficiently than DENV4 was that the DII-FL epitope was not equivalently accessible on the surface of the DENV4 virion compared to the other DENV serotypes. Recent studies with mouse MAbs and different DENV serotypes have suggested that increasing the temperature of preincubation facilitates exposure of buried epitopes on different virion conformational ensembles, which results in enhanced neutralizing activity of some MAbs (28–31). To assess the effect that temperature has on the ability of our panel of human MAbs to neutralize DENV, we next tested temperature dependence in their titer (FRNT) assays were performed using different DENV strains.

Most DII-FL MAbs displayed temperature dependence in their ability to neutralize DENV4 (Table 4), with over 40% of MAbs tested exhibiting at least a 2-fold increase in neutralization potency at 40°C. MAb 1C19, however, did not have a substantial increase in neutralization potency at 40°C compared to 37°C, suggesting that its epitope was not differentially expressed on DENV4 virion structural ensembles.

DII-FL epitope mapping with WNV E proteins. To begin to define the epitope specificity of our panel of MAbs, we next tested them for binding to previously designed wild-type or mutant DII-FL E proteins (Table 5) whose fusion loop is identical in sequence to that of DENV (32). Because of the identity in the fusion loop, we considered it reasonable to use the existing West Nile virus (WNV) mutant E proteins for screening purposes to identify putative epitopes. A caveat for interpretation of such studies is that the E protein scaffold for the FLs differs between WNV and DENV, and it cannot be assumed that a MAb that binds the FL in one context will bind the same FL in another E protein context with equivalent affinity, since residues outside the FL may influence the binding of some MAbs. Most MAbs bound to the wild-type E protein of WNV, with the notable exception of MAb 1C19. As a way to confirm the DII-FL specificity and identify residues important for binding, we next assessed the ability of the MAbs to bind a mutated form of WNV E protein (quadruple [QUAD] mutant) containing four altered residues in the WNV FL (aa 101 and 107) and adjacent bc loop (aa 76 and 77) regions. Each of the MAbs that bound to wild-type WNV E protein also failed to bind the WNV QUAD mutant E protein. Mutation of DII-FL residue 101 alone disrupted binding of all MAbs tested, also establishing the DII-FL region as a primary target of these cross-neutralizing MAbs.

Epitope mapping with yeast library variants. As an alternate strategy to identify residues involved in binding, we screened many of our cross-neutralizing human MAbs for binding to wild-type and mutant DENV2 E proteins (with amino acid point muta-tion W101R, G106R, or L107D) by using yeast surface display (Table 5) (33). Binding was disrupted by each of these three changes for all of the MAbs identified above as binding to the DII-FL region. Again, binding of MAb 1C19 was not disrupted, suggesting that these DII-FL residues are not critical for the interaction of that MAb.

Epitope mapping using shotgun mutagenesis library. We next identified specific residues of the epitopes engaged by our panel of MAbs by screening mutagenized libraries of E proteins for loss of binding of each of the MAbs under study. We completed epitope maps for 29 of the 30 antibodies (Table 5); MAb 2I23 exhibited high nonspecific binding in our library format, so it was not tested. To epitope map each MAb, each of the antibodies was screened on a DENV4 mutation array of human cells expressing E protein clones with alanine mutations at each amino acid position (see Fig. S2 in the supplemental material). Selected MAbs also were screened against a DENV3 mutation array that contains random amino acid substitutions, which are typically more disruptive than alanine mutations. Table 5 shows the critical residues identified by these loss-of-binding experiments (see Table S2 for all critical residue binding data). With the notable exception of 1C19, at least one residue within the conserved FL region (aa 97 to 111) disrupted binding for each MAb. 1C19 was the only MAb in our panel whose binding was not affected by alteration of DII-FL residues. The bc loop residues 73, 78, and 79 were the only critical residues identified by our loss-of-function binding screens for 1C19. Interestingly, in addition to residues within the FL, binding of MAb 2J21 was also disrupted by changes in the neighboring bc loop.

Screening of a small panel of previously isolated highly cross-
reactive murine MAbs also showed that the principal residues targeted in the E protein fall within the DII-FL. Alterations in the bc loop did not alter binding of any of the murine DENV- or WNV-specific MAbs tested (DV2-29 or DV2-52 or WNV E18, WNV E28, WNV E60, WNV E 86, WNV E106, or WNV E119) (see Table S1 and Fig. S1 in the supplemental material). These data suggest that murine DII-FL-specific antibodies, like human antibodies, typically interact with the DII-FL proper and not with the adjacent bc loop. It is of interest that there is a WNV-reactive murine MAB E53 that has been reported to interact with the bc loop of DII (34).

Figure 2 shows a representation of each of the epitope maps grouped according to the phenotype of binding. For 5M22, we identified critical residues in the DENV4 screen in both E and prM proteins; this finding was considered an unusual distribution but was reproducible in repeated assays. The results for this antibody suggest that it interacts with a complex quaternary epitope formed by both prM and E proteins. Screening of a different library (WNV E18, WNV E60, WNV E 86, WNV E106, or WNV E119) (see Table S1 and Fig. S1 in the supplemental material). These data suggest that murine DII-FL-specific antibodies, like human antibodies, typically interact with the DII-FL proper and not with the adjacent bc loop. It is of interest that there is a WNV-reactive murine MAB E53 that has been reported to interact with the bc loop of DII (34).
FIG 2 Epitope mapping of DII-FL region antibodies by shotgun mutagenesis library screening. We performed fine epitope mapping by screening DENV4 alanine scanning and DENV3 shotgun mutagenesis libraries for loss of binding. (A) At the top, a ribbon diagram indicating the structure of the DENV E80 dimer is shown, with color coding for domains of interest, and the inset indicates structures shown in all panels. Critical residues are visualized on the DENV E protein crystal structure (41) (PDB identifier 1UZG) and shown as green spheres. Tables indicate the particular residues affecting binding of each MAb in the left column. At the bottom, critical residues are shown for 1C19 binding using DENV4 alanine scanning and DENV3 shotgun mutagenesis libraries. (B to F) Additional panels show critical residues for MAbs obtained from subjects following DENV1 vaccination (B), natural DENV1 infection (C), natural DENV2 infection (D), natural DENV3 infection (E), or secondary infection (F). *, DENV4 alanine scanning library identified one residue in prM critical for MAb 5M22 binding (data not shown). **, for MAb 2C7, two of the four critical residues that were identified by DENV3 shotgun mutagenesis libraries were also identified using the DENV4 alanine scanning library.
neutralizing antibodies in our panel, MABs 1C19, 1N5, and 1M7. Table S3 in the supplemental material shows the genetic features of these MABs. All three MABs use different germ line antibody variable-region genes and have distinct junctional sequences. None of them display a particularly high degree of somatic hypermutation, each having <20 amino acid changes from their heavy chain variable-region germ line sequence.

Protective efficacy in vivo. We reasoned that the most potent neutralizing MABs should protect against virus replication in vivo. We tested the human MABs 1N5 and 1C19 in AG129 mice (129/Sv mice deficient in alpha/beta and gamma interferon receptors) for protective efficacy. Both antibodies tested reduced the level of viremia after sublethal virus challenge by approximately 10- or 1,000-fold, for DENV1 or DENV2 virus strains, respectively (Fig. 3). MAB 1C19 reduced DENV2 replication at a relatively low dose of 20 μg per mouse (approximately 0.8 mg/kg of body weight).

DISCUSSION

This study describes the characterization of a large panel of human MABs that recognize the DENV DII-FL and possess serotype cross-reactive binding patterns. Most of the MABs in this panel of clones bind to the conventional FL antigenic region, primarily exhibit mostly weak neutralizing potency, and show ADE activity. One ultrapotent MAB, 1C19, however, neutralized viruses from all four DENV serotypes and interacted with the bc loop of E protein DII, adjacent to the FL, without directly binding the FL. This antibody also was found to compete for binding against the more common lower-potency FL antibodies.

Our cross-competition binding assays revealed that 1C19 differed from other cross-reactive MABs in the panel since it competed for binding with all of the MABs in the panel and also competed with a previously described neutralizing antibody, 1F4, which recognizes the DI/II hinge region. None of the other DII-FL MABs that we isolated demonstrated this interference pattern. The ability of 1C19 to block binding of an antibody to the hinge region suggested that the epitopes for these two major classes of potent neutralizing antibodies (bc loop or hinge specific) were distinct but close to each other on the virion particle. We also mapped murine antibodies that target the DII-FL region of DENV and WNV, and we found that the mode of recognition of this epitope of 1C19 also appeared to be distinct from the typical epitope recognized by murine antibodies to this region. Understanding the molecular mode of recognition of the unique epitope targeted by 1C19 is likely of high clinical relevance, as previously identified conventional antibodies that target the FL region enhance disease in vivo (25). Moreover, we note that 1C19 was isolated from a person exposed to a secondary DENV infection (Table 2), indicating that broadly cross-neutralizing antibodies may be a consequence of repeat exposures to DENV. Indeed, people exposed to secondary DENV infections have polyclonal serum antibodies that broadly cross-neutralize different DENV serotypes.

The correlates of immunity to DENV infection are not completely understood. We tested the neutralization potency of this panel of MABs in two cell lines and at two temperatures. Overall, neutralizing potency varied considerably between antibody clones in our panel, with EC50 ranging from >10 μg/ml to 0.01 μg/ml. We also observed considerable variability in potency for particular cross-reactive MABs when tested against viruses from different serotypes or when tested at various temperatures or different cell lines. Many of the MABs bound to similar epitopes (i.e., their epitopes mapped to the same critical residues), and yet they exhibited EC50 values that differed by several orders of magnitude. These data show that comparative studies of the potency of antibodies must consider the conditions of the assays used. The reasons for detecting differing activities of these MABs under various conditions remain unclear but could involve differences in binding affinity, the presence of additional interacting residues that were not detected by our mapping efforts, differences in the accessibility of epitopes as displayed on the surface of virion particles, or differences in the angle of binding of the MABs.

Target epitopes of potently cross-neutralizing antibodies such as 1C19, generated by humans to natural infection, are the best tools that we have for understanding the determinants of human protection. These sites could be the focus of future vaccine design. Epitope mapping for this large panel of MABs was achieved through several independent means, with consensus of the findings in the assays using different approaches. Most of our MABs mapped to the conserved DII-FL (residues 97 to 111), with aa 101, 107, 108, and 111 being the most frequently identified as critical for binding. Critical residues were found in both the FL and bc loop (aa 93 to 97) for MAB 2J21. 1C19, the most broadly potent MAB in our panel, mapped exclusively to the bc loop, without identification of residues within the nearby FL.

With the information provided here and published previously (15, 19), one could develop two distinct but compatible strategies for the rational design of DENV vaccines or antibody therapeutics. One strategy would be to induce high titers of serotype-specific antibodies that target the hinge region between DI and DII. In this case, four antigenic preparations could be developed, with each designed to induce potent protection for one serotype without generation of potentially dangerous, cross-reactive, and nonneutralizing antibodies; we have designated this approach a “four by one” strategy. A second strategy would be to focus on cross-neutralizing epitopes that induce potently inhibitory antibodies to viruses of all four serotypes using a single immunogen, an approach that we have termed a “one by four” strategy. In this case, antigenic preparations would be developed to induce 1C19-

![FIG 3](image-url)
like antibodies through vaccination, targeting this cross-
protective epitope. Eliciting 1C19-like antibodies is attractive, since this is the most broad and potent MAB reported to date that reacts with all four serotypes of DENV and exhibits neutralizing activity. An added benefit of such antibodies is that they might displace other FL-specific antibodies made following previous in-
fection that might otherwise promote disease enhancement. One could even consider a pentavalent strategy combining the “four by one” and “one by four” strategies by including four separate type-
specific hinge region immunogens and one antigen designed to recapitulate the cross-reactive domain II bc loop epitope.

This work also raises the possibility that typically FL-specific MABs may block the 1C19 site and therefore act as “blocking” MABs that actively prevent the development of a more potent immune response focused on the domain II bc loop epitope rec-
ognized by 1C19. This phenomenon might be especially relevant if there are many more FL-specific MABs circulating and/or these are higher affinity than those focused on the 1C19 epitope. It might be possible in future to test whether such MABs block 1C19-like antibodies by immunizing experimental animals with an E protein that lacked the FL, with the hypothesis that such a protein might induce a high frequency of 1C19-type MABs.

MATERIALS AND METHODS

Human subjects and peripheral blood cell isolation. We identified sub-
jects who had acquired DENV infection naturally by screening volunteers with suspected exposure during past travel to, or residence in, regions in which DENV is endemic, as indicated in Table 1. Returned traveler sub-
jects were confirmed to have had DENV infection by testing their serum for the presence of antibodies that neutralized each of the DENV sero-
types. Cases in subjects enrolled in the Pediatric Dengue Cohort Study in Nicaragua were confirmed by RT-PCR, virus isolation, and serology in paired acute and convalescent samples. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient separation on Ficoll. PBMCs also were obtained from subjects who received the live attenuated rDENV1,30 vaccine 28 days following a second vaccine dose (7 months
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Cells. Vero cells (American Type Culture Collection; CCL-81) were
maintained in Dulbecco’s modified Eagle’s medium (DMEM). The U937+DC-SIGN cell line (33, 38) was maintained in RPMI 1640 (In-
vitrogen) supplemented with 50 μM beta-mercaptoethanol. This human monocyte lymphoma cell line was derived from U937 cells but also ex-
presses ectodendritic cell-specific intercellular adhesion molecule-
3-grabbing nonintegrin (DC-SIGN). All media used also were supple-
mented with 5% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin, 0.1 mM nonessential amino acids (Invitrogen), and 2 mM glutamine, and all cells were incubated in the presence of 5% CO2 at 37°C, unless otherwise specified. The 5% FBS was reduced to 2% to make infection medium for each cell line.

Generation of human hybridomas. Cryopreserved PBMC samples were thawed rapidly in a 37°C water bath and washed prior to transfor-
mation with Epstein-Barr virus as described previously (15). Cultures were incubated at 37°C with 5% CO2, for 10 days, prior to screening for antigen-specific cell lines with ELISA. Cells from wells with supernatants reacting in DENV-specific ELISA then were expanded prior to screening by flow cytometric neutralization assay and cytotoxicity with HMAA9,25 nonsecerting myeloma cells, as previously described (13). Following cyto-
fusion, hybridomas were selected by growth in hypoxanthine-
aminoophterin-thymidine (HAT) medium containing ouabain and biolog-
ically cloned.

Human MAB production and purification. Wells containing hybrid-
omas producing DENV-specific antibodies were cloned by three rounds of limiting dilution plating or by use of a ClonePix device (Molecular Devices) per manufacturer’s recommendations. Once clonality was achieved, each hybridoma was expanded until 50% confluent in 75-cm2
flasks. For antibody expression, the cells in 75-cm2 flasks were collected with a cell scrapper; the hybridomas were washed in serum-free medium (Gibco Hybridoma-SFM from Invitrogen; catalog no. 12045084) and split equally among four 225-cm2 flasks (Corning; catalog no. 431082) con-
taining 250 ml serum-free medium. Flasks were incubated for 21 days before medium was clarified by centrifugation and 0.2-μm sterile filtered. Antibodies were purified from clarified medium by protein G chromatog-
raphy (GE Life Sciences; protein G HP columns).

Murine MABs to DENV or WNV E protein. We used purified IgG for six previously isolated WNV-specific murine MABs that cross-reacted with DENV (clones WNV E18, WNV E28, WNV E60, WNV E86, WNV E106, and WNV E119) (33) and two DENV-specific murine MABs (clones DV2-29 and DV2-52) (33) for mapping studies.

Virus and recombinant protein ELISA. For virus capture ELISA, pu-
rified mouse MAB 4G2, prepared in carbonate binding buffer, was used to coat ELISA plates (Nunc; catalog no. 242757) and incubated at 4°C over-
night. After blocking for 1 h, plates were washed five times with phosphate-buffered saline (PBS) and 50 μl of DENV-containing culture supernatant from infected C6/36 cell culture monolayers was added. Plates then were washed 10 times with PBS, and 5 μl of purified human monoclonal antibody (1 μg/ml) was added into 25 μl well of blocking buffer. Plates were incubated at room temperature for 1 h prior to washing five times with PBS. Secondary antibody (goat anti-human Fc; Meridian Life Science; catalog no. W90008A) was applied at 1:5,000 dilution in blocking solution using 25 μl/well, and plates again were incubated at room temperature for 1 h. Following five washes with PBS, phosphate substrate solution (1-mg/ml phosphate substrate in 1 M Tris amino-
methane) (Sigma; catalog no. S0942) was added at 25 μl/well, and plates were incubated at room temperature for 2 h before reading the optical density at 405 nm on a Bio-Tek plate reader.

For recombinant protein capture ELISA using E protein constructs, purified mouse anti-Strep-tag II MAB (StrepMAB-Immo, IBA 2-1517-
001) prepared in carbonate binding buffer was used to coat ELISA plates (Nunc; catalog no. 242757) and incubated at 4°C overnight. After block-
ing for 1 h, plates were washed five times with PBS and 50 μl of recombi-
nant protein construct containing culture supernatant (cultured in insect cells) was added. Plates then were washed 10 times with PBS, and 5 μl of
purified human monoclonal antibody (1 μg/μl) was added into 25 μl/well of block. All other steps were performed the same as described above for the virus capture ELISA.

**Competition assays.** An Octet Red instrument was used for all competition studies. For recombinant E protein competitions, purified mouse anti-Strep-tag II MAb (StrepMAB-Immo, IBA 2-1517-001) was loaded onto anti-mouse IgG Fc Capture tips (ForteBio; catalog no. 18-5088). Recombinant E protein from DENV2, generated previously (36), was added. After a wash step, the first anti-dengue virus MAb was added. Without washing, the second antibody was then added and binding was assessed. For competition assays using crude virion, purified biotinylated mouse anti-dengue virus prM MAb 2H2 was loaded onto streptavidin tips (ForteBio; catalog no. 18-5019). Crude DENV1 WestPac-74 was prepared by centrifuging 250 ml of sterile filtered supernatant from infected C6/36 cell culture monolayers at 10,000 RPM for 12 h. The pellet containing crude virion particles was then suspended in 5 ml PBS and used for capture to MAb 2H2 on biosensor tips. After a washing step, the first anti-dengue virus MAb was added, followed immediately by the second to assess binding interference. The antibodies were judged to compete for the same site if maximum binding of the second antibody was reduced to <25% of its binding in the absence of the first antibody. No competition was achieved if maximum binding of the second antibody was >75% of its binding in the absence of the first antibody.

**DENV Western blotting.** Crude DENV, prepared as described for competition assays, was loaded into a 4 to 12% SDS-PAGE gel run under denaturing nonreducing conditions. After transfer, nitrocellulose membrane was then probed with the purified human MAb in question (diluted 1:1,000) for 1 h at 37°C. The membrane was washed with 3× PBST and incubated with goat anti-human Fc-alkaline phosphatase (AP) secondary antibody (Meridian Life Sciences catalog no. W99008A) for 1 h at 37°C, prior to washing and development using 5-bromo-4-chloro-3-indolyl-phosphate–nitroblue tetrazolium (BCIP-NBT) chromogenic substrate (Invitrogen; catalog no. WP20001).

**Neutralization assay.** The neutralizing potency of MAbs was measured using a flow cytometry-based neutralization assay with the U937 human monocytic cell line stably transfected with DC-SIGN or on Vero cells, as previously described (38, 39).

**Temperature-dependent in vitro neutralization assays.** Focus reduction neutralization titer (FRNT) assays were performed with the different DENV strains and MAbs on Vero cells. Purified MAbs were mixed with 100 focus-forming units (FFU) of different DENV strains (DENV2, NGC; DENV4, 1036) for 1 h at 37 or 40°C. Subsequently, virus-MAb mixtures were added to Vero cell culture monolayers for 1 h, and then a 1% carboxymethyl cellulose overlay was added. Two days later, the overlayers were removed, and monolayers were fixed with 1% paraformaldehyde (PFA) (10 min at room temperature), permeabilized with 0.1% saponin in PBS, and incubated with the cross-reactive mouse WNV E60 MAb (200 ng/ml) (39). Following several washes, wells were incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody (Sigma; 250 ng/ml in saponin buffer) for 1 h at room temperature. Wells were washed, and infectious foci were visualized using TrueBlue substrate (KPL) after a 10-minute incubation at room temperature. Wells were rinsed with water and dried prior to analysis with a Biospot counter (Cellular Technology) using immunocapture software. Neutralization (% reduction in spot numbers in samples preincubated with Ab compared to wells with virus preincubation with medium alone) was calculated.

**ADE assays.** The ability of antibodies to enhance DENV infection was measured using parent U937 cells that lacked expression of DC-SIGN. In the absence of the virus attachment factor, these Fc-γ receptor-bearing cells are susceptible to infection only in the presence of DENV-specific antibodies. The assay was performed as described in detail previously (15). ADE activity was expressed as the percent increase of infected cells in the DENV-specific antibody-treated sample compared to the sample treated with a control antibody.

**Shotgun mutagenesis epitope mapping.** DENV3 (strain CH53489) and DENV4 (341750 strain) prM/E expression constructs were subjected to high-throughput mutagenesis (shotgun mutagenesis) to generate comprehensive mutation libraries. Point mutations were introduced into the DENV3 prM/E polypeptide (strain CH53489) by PCR using a Diversity mutagenesis kit (Clontech Laboratories, Inc., Mountain View, CA) or into the DENV4 prM/E polypeptide using primers designed to mutate each residue to alanine (alanine codons were mutated to serine). In total, 1,400 DENV3 and 660 DENV4 mutants were generated (>97% coverage of each serotype prM/E ectodomain), their sequences were confirmed, and they were arrayed into 384-well plates (one mutation per well). Each of the anti-DENV antibodies was screened on the full DENV4 mutation library containing Ala substitutions at each position, a consolidated DENV4 library containing a subset of the most relevant E protein mutations, or, as needed, a DENV3 mutation library containing random substitutions at each position. The secondary DENV3 screen was conducted for 9 of the MAbs (1M4, 5M22, 1M7, 2C7, 1N5, 1C19, 1C18, 1K16, and 1N8). Each E protein mutant was individually transfected into human HEK-293T cells and allowed to express for 22 h. Cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences), and permeabilized with 0.1% (wt/vol) saponin (Sigma-Aldrich) in PBS plus calcium and magnesium (PBS + ). Cells were stained with purified MAbs (0.1 to 0.6 μg/ml) diluted in 10% normal goat serum (NGS) (Sigma)-0.1% w/v saponin, pH 9. The optimal primary antibody concentration was determined for each antibody using an independent immunofluorescence titration curve against wild-type prM/E to ensure that signals were within the linear range of detection and that signal exceeded background by at least 5-fold. Antibodies were detected using 3.75 μg/ml Alexa Fluor 488-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) in 10% NGS-0.1% saponin. Cells were washed three times with PBS + + 0.1% saponin followed by 2 washes in PBS. Mean cellular fluorescence was detected using the Intellicyt high-throughput flow cytometer (HTTPIC, Intellicyt). Antibody reactivity against each mutant E protein clone was calculated relative to wild-type E protein reactivity by subtracting the signal from mock-transfected controls and normalizing to the signal from wild-type E protein-transfected controls for the serotype tested. Mutations within critical clones were identified as critical to the MAb epitope if they did not support reactivity of the test MAb but did support reactivity of other antibodies. This counterscreen strategy facilitates the exclusion of E protein mutants that are misfolded or have an expression defect. Critical amino acids required for antibody binding were visualized on the DENV E protein crystal structure (40) (PDB identifier 1UZG).Western

**Epitope mapping with WNV chimeras.** For quantification of the E protein specificity of anti-DENV MAbs, plates were coated with 10 μg/ml of recombinant E proteins produced in *Escherichia coli* (wild type, the W101R mutant, or an E-quadruple mutant [T76R M77E W101R L107R]) as described previously (32, 37). Equivalent site density was confirmed by measuring reactivity with a humanized E16 MAb, which recognizes a distinct epitope on the domain III lateral ridge (DIII-LR). Endpoint titers were defined as 3 standard deviations above the background optical density at 450 nm as determined by regression analysis using the Prism program (GraphPad software).

**Epitope mapping with *Saccharomyces cerevisiae* yeast display library variants.** Yeast cells displaying wild-type or mutant DI-DII of DENV2 E proteins on their surface were described previously (33). Yeasts were immunostained with 50 μl of monoclonal antibody solution (2 μg/ml) on ice. After 30 min, yeasts were washed three times with PBS containing 1 mg/ml of bovine serum albumin (BSA). Yeasts then were incubated with a goat anti-mouse secondary antibody conjugated to Alexa Fluor 647 (Molecular Probes) for another 30 min. After fixation in 1% paraformaldehyde (PFA) in PBS, yeasts were analyzed on a Becton-Dickinson BD-FACSArray flow cytometer. Values shown in the data tables were obtained by dividing the total fluorescence product (percent positive population × mean linear fluorescence intensity) of a mutant for each individual antibody by the total fluorescence product of the same
mutant stained with a control antibody (DV2-51) (non-FL binding) × 100.

Murine studies of the protective effect of human MAb IC19 or IN5 treatment on viremia after challenge. The murine studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All procedures were approved and conducted according to UC Berkeley Animal Care and Use Committee guidelines (protocol R252-1013B). All viruses used in the animal studies were propagated in C6/36 cells as previously described (41) and concentrated by centrifugation using 100,000-molecular-weight-cutoff (MWCO) Amicon filters (Millipore). Titration of animal inoculation stocks was performed using a plaque assay on baby hamster kidney cells (BHK21, clone 15) as described previously (42). All procedures were approved and conducted according to UC Berkeley Animal Care and Use Committee guidelines. AG129 mice (129/Sv mice deficient in both alpha/beta and gamma interferon receptors) (43) were administered 20, 50, or 100 μg of MAb IN5 or IC19 or 50 μg of an isotype control (IgG1) intraperitoneally (i.p.) in a total volume of 200 μl, 24 h prior to DENV inoculation. A sublethal dose of DENV1 strain WestPac-74 (5 × 10⁶ PFU) or PFU of DENV2 strain D2S10 was administered intravenously (i.v.) in a total volume of 100 μl. On the third day after virus inoculation, mice were euthanized and serum was obtained from whole blood by centrifugation and stored at −80°C prior to analysis. The viral load in serum was determined by quantitative reverse transcription-PCR (RT-PCR). RNA was extracted from 20 μl of serum using the QiAamp viral RNA minikit (Qiagen), per the manufacturer’s instructions. The ABI Prism Sequence Detection System 7300 was used to perform quantitative RT-PCR (qRT-PCR). Viral copy number was determined using previously published primers and probe sequences (44) and the Verso 1-Step qRT-PCR kit as follows: 2 min reverse transcription, 1 cycle of reverse transcription (3 min at 50°C), 1 cycle of Thermo-Start activation (12.5 min at 95°C), and 40 cycles of denaturation (15 s at 95°C) and annealing-extension (1 min at 60°C). Serum viral load was determined according to the following equation: genome equivalents (GE)/ml = (mean quantity/2 μl) (RNA extraction elution volume/volume of serum per RNA extraction) (1,000 μl/1 ml). Each DENV1 or -2 plate was run using a 10-point standard curve. The limit of detection (LOD) was based on the lowest DENV1 standard detection limit. Statistical analysis was performed using Stata software (Stata statistical software, release 12; StataCorp LP, College Station, TX, 2011) and a two-tailed Wilcoxon rank-sum test.

SUPPLEMENTAL MATERIAL
supplemental material for this article may be found at http://mbio.asm.org

Figure S1, PDF file, 0.5 MB. 
Figure S2, PDF file, 0.4 MB. 
Table S1, PDF file, 0.1 MB. 
Table S2, PDF file, 0.1 MB. 
Table S3, PDF file, 0.1 MB.

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