EVALUATION OF RECOMBINANT DENGUE VIRAL ENVELOPE B DOMAIN PROTEIN ANTIGENS FOR THE DETECTION OF DENGUE COMPLEX–SPECIFIC ANTIBODIES

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Abstract. To increase the specificity of dengue (DEN) diagnosis based on antibody detection, we have evaluated recombinant proteins as antigens that incorporate most of the B domain of the DEN virus envelope protein fused to the trpE protein of *Escherichia coli* (trpE-DEN). A pooled antigen consisting of trpE-DEN proteins representing all four serotypes of DEN virus was used in an indirect ELISA for the detection of IgG or IgM antibody. This assay was compared with a standard IgG indirect ELISA and an IgM-capture ELISA using DEN virus–infected cell culture pooled antigens. The results indicated that the trpE-DEN antigens and the cell culture antigens were equally sensitive for detecting IgM and IgG antibodies in convalescent sera from Peru and Indonesia representing virus isolation–confirmed primary and secondary DEN infections, respectively. Forty-one day postinfection IgG antibody-positive sera obtained from individuals infected with DEN-1 virus who had been vaccinated with other flaviviruses were more strongly reactive with the cell culture antigen than with the recombinant antigen, but by day 21 postinfection, a strong antibody response to the trpE-DEN antigens was present. These results suggested that the early antibody response was directed predominantly towards shared flavivirus group antigens that were not detected with the trpE-DEN antigens. Comparison of the trpE-DEN-1 recombinant antigen with a DEN-1 virus–infected cell lysate antigen for the detection of IgG antibody in sera from a cohort of 55 individuals from Peru who seroconverted over a one-year period indicated greater specificity for the recombinant antigens. Also, sera from individuals with no known DEN infections that had been sequentially vaccinated with yellow fever and Japanese encephalitis reacted with the DEN virus cell culture antigen in the IgG ELISA, but did not react with the trpE-DEN pooled antigens. Similarly, YF IgM antibody positive samples that showed cross-reactivity with the DEN virus cell culture antigens, did not react with the trpE-DEN pooled antigens. These results indicated that the trpE-DEN pooled antigen provided a more specific diagnosis of dengue infections than DEN virus-infected cell culture antigen and avoided the biohazards associated with handling live virus during the preparation of diagnostic reagents. The trpE-DEN pooled antigen should permit a better approach to distinguish between past DEN and other flavivirus infections in epidemiologic surveys, and also increase the specificity of serologic diagnosis of acute DEN infections.

Dengue (DEN) viruses (types 1, 2, 3, and 4) cause millions of human infections each year resulting in a spectrum of illness ranging from mild fever to severe hemorrhagic fever and shock. The DEN viruses are classified within the family Flaviviridae, genus Flavivirus, which consists of more than 60 arboviruses, including other important human pathogens such as yellow fever (YF) and Japanese encephalitis (JE) viruses. Because the DEN viruses occur throughout tropical and subtropical areas of the world, their distribution overlaps with other human pathogenic flaviviruses. This can complicate the interpretation of serologic results for DEN infections since all flaviviruses share antigenic determinants that induce cross-reactive antibody.

The ELISA and the hemagglutination-inhibition (HI) test are commonly used to detect DEN IgG antibody as an indication of past infection using infected cell culture or animal tissue preparations as viral antigens. Even though these assays detect flavivirus group-reactive antibody as well as DEN-specific antibody, they can be used to diagnose DEN infection based on comparative end-point antibody titers against all of the flaviviruses that occur in the area, provided an individual has not also previously been infected with any other flavivirus. Once an individual has been infected by two different flaviviruses, the ELISA and HI tests formatted to detect total or IgG antibody using the standard viral antigen preparations show very broad cross-reactivity among members of the genus. The neutralization test is the most specific serologic assay for the diagnosis of past DEN infections, but is more difficult to perform and not as amenable to testing large numbers of sera compared with the ELISA and HI test. These assays also can be used to diagnose acute DEN infections based on a four-fold or greater increase or decrease in antibody titers between appropriately paired acute and convalescent serum samples from the same individual, but the same problems with cross-reactivity remain.

In recent years, acute DEN cases frequently have been diagnosed based on the detection of IgM antibody by a capture ELISA. Since DEN IgM antibody is usually only detectable for 2–3 months following an acute infection it is possible to make a presumptive diagnosis based on a single serum sample. Unfortunately, when using infected animal tissue or cell culture-derived antigens in the IgM antibody-capture ELISA, cross-reactivity to other flaviviruses still can occur. As a result, to make a definitive diagnosis requires the inclusion of antigens from other flaviviruses that are present in an area as well as DEN antigen for comparison of reactivity patterns.

Previous studies have shown that recombinant proteins engineered in *Escherichia coli* as trpE fusion products with the B domain of the DEN virus envelope could be used as antigens in the ELISA to detect antibody. These antigens showed reactivity with homologous DEN virus polyclonal mouse immune ascitic fluid, but no or only minimal cross-reactivity with polyclonal mouse immune ascitic fluids prepared to other flaviviruses. Because of the difficulties in making a specific diagnosis of DEN infections associated with the current antigens used in serologic assays, this study was conducted to evaluate these recombinant DEN B do-
main fusion proteins as DEN complex-specific antigens in the ELISA for detecting antibody in human sera.

**MATERIALS AND METHODS**

**Test sera.** All of the human sera used in this study had been collected from volunteers with informed voluntary consent under protocols previously approved by Committees for Protection of Human Subjects from the collaborating institutions or collected for the diagnosis of acute febrile illnesses by attending physicians. The DEN antibody-positive samples included 1) nine sera from residents of Peru that had been collected 2–4 weeks after virus isolation–confirmed primary DEN-1 infections (Hayes CG, unpublished data), 2) five convalescent sera from Indonesian residents with virus isolation–confirmed secondary DEN-1, -2, or -3 infections (Graham R, unpublished data), 3) acute and serial follow-up convalescent sera from three individuals with virus isolation–confirmed DEN-1 infections who had known histories of vaccination against YF virus and tick-borne encephalitis (TBE) virus, and 4) end of study follow-up serum samples from 55 residents of Peru enrolled in a one-year prospective study who had been diagnosed as having experienced a DEN virus infection during the past year.

As a control for cross-reactive IgG antibodies induced by past exposures to flaviviruses other than DEN, eight sera were available from individuals who had been vaccinated against both YF and JE, but had no history of a DEN infection and had never lived in a DEN endemic area. Sera used to test for IgM antibody cross-reactivity included convalescent samples from six Peruvian residents who had been diagnosed as acute YF cases based on clinical presentation and a comparison of DEN and YF viral IgM antibody titers in a standard antibody-capture ELISA (Watts DM, 1995, unpublished data).

Specificity of the DEN viral antigens was assessed by testing several existing collections of sera that were shown to be negative for flaviviral IgM and IgG antibodies by either the indirect IgG ELISA or the IgM capture ELISA using cell culture–derived DEN, YF, and JE viral antigens. Eight of these sera were known to have high IgG antibody levels, and two were known to have high IgM antibody levels to human pathogenic viruses unrelated to DEN (Integrated Diagnostics Inc., Baltimore, MD).

**Plasmids, cell lines, virus stocks.** The PATH plasmids containing DEN cDNA have been described and were provided by Dr. P. W. Mason (Yale University School of Medicine, New Haven, CT). Briefly, separate PATH plasmids were constructed for each DEN serotype consisting of an in-frame fusion of the B domain of the viral envelope gene with the trpE gene of *E. coli* (trpE-DEN). The DEN virus envelope envelope amino acid sequences expressed from the pHAT plasmids were 293-412 for DEN-1, 298-400 for DEN-2, 297-398 for DEN-3, and 298-400 for DEN-4. The pHAT1 plasmid that expressed only the trpE protein, and the mouse ascitic fluids, LLC-MK2 cells, and Vero cells were obtained from the American Type Culture Collection (Rockville, MD). The virus strains used to prepare antigens or for performing the plaque-reduction neutralization test (PRNT) were DEN-1 (Western Pacific), DEN-2 (New Guinea C), DEN-3 (CH53489), DEN-4 (341750), JE (SA-14-14-2), and YF (17D and French Neurotrophic).

**Preparation of trpE-DEN fusion proteins.** Inclusion bodies containing the trpE-DEN fusion proteins or the trpE protein only were isolated from tryptophan-starved *E. coli* HB 101 cells harboring the recombinant PATH plasmids. Expression of the trpE protein and the trpE-DEN fusion proteins was initiated by the addition of 10 μg/ml of indole-acrylic acid (Sigma, St. Louis, MO) to early log phase cells grown in M9 minimal medium at 37°C in the presence of 100 μg/ml of ampicillin. After growth for 4 hr, the cells were harvested by centrifugation, and the pellet was resuspended in lysis buffer (50 mM Tris-Cl pH 7.5, 0.1 M EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 2 mg/ml of lysozyme) and incubated on ice for 2 hr. Sodium chloride and Nonidet P-40 were added to yield final concentrations of 1 M and 2%, respectively, and incubated for an additional 15 min at room temperature. The insoluble fraction was briefly sonicated, centrifuged, and washed once with 10 mM Tris-Cl, pH 7.5, 1 M NaCl and resuspended in 2 ml of 10 mM Tris-Cl, pH 7.5. The samples were prepared in nonreducing gel electrophoresis sample buffer (0.5 M Tris-HCl, 2% glycerol, 10% sodium dodecyl sulfate [SDS] [w/v], 0.1% bromophenol blue, pH 6.8), boiled for 5 min, and fractionated on a 10% preparative SDS-polyacrylamide gel. Part of the gel was stained with Coomassie brilliant blue and the fusion protein band was excised and eluted from the gel using a Centrifor (Amicon, Beverly, MA). Electroelution occurred in 0.5x electrophoresis buffer (0.0125 M Tris, 0.096 M glycine, 0.5% SDS, pH 8.3) at room temperature for 3 hr at 20 mA. The eluted fusion protein was dialyzed for 24 hr against several changes of phosphate-buffered saline (PBS) and concentrated with a centriprep 30 (Amicon), aliquoted, and frozen at −20°C. For use as antigen in the indirect ELISA to detect DEN IgM or IgG antibody, the individual recombinant trpE-DEN fusion proteins were mixed to form a pool of all four DEN virus serotypes (trpE-DEN pool). The optimal concentration of each trpE-DEN protein to use in the antigen mixture was determined by titration against hyperimmune mouse ascitic fluid (HMAF) to the homologous DEN virus serotype.

**Preparation of cell lysate and cell supernatant antigens.** The DEN viral antigens were prepared in Vero cell cultures as previously described. Briefly, the growth medium of Vero cells was Earle’s minimum essential medium containing 1% nonessential amino acids, 1% sodium pyruvate, 1% L-glutamine, 1% penicillin-streptomycin, 0.5% Fungizone, and 20% fetal bovine serum. Confluent culture monolayers were inoculated with 10 plaque-forming units/ml of each DEN virus serotype, or YF virus, or JE virus incubated at 37°C and observed for cytopathic effects (CPEs). Cells were harvested after 7–9 days when 50–75% of the cells were detached from the monolayer. For the cell supernatant antigen used in the IgM capture ELISA, the supernatant of infected and uninfected (control) cells was harvested and centrifuged at 9,000 rpm for 15 min at 4°C, aliquoted, and stored at −70°C. Equal volumes of undiluted supernatant from cell cultures infected with DEN-1, -2, -3, or -4 viruses were mixed to prepare the antigen pool used to detect DEN IgM antibody. This dilution was determined to be optimal based on titrations using a known dengue IgM.
antibody–positive pool from human sera. For the cell lysate antigen used in the IgG ELISA, infected cells and uninfected (control) cells were scraped from the flasks with a cell scraper and centrifuged at 9,000 rpm for 15 min at 4°C. The supernatant was discarded and the cells were resuspended in 2 ml of borate saline, pH 9.0, and centrifuged again at 9,000 rpm for 15 min at 4°C. This was repeated once, and the final pellets were resuspended in 5 ml of borate saline containing 1% Triton X-100 and sonicated at a power setting of 4.5 for 25 sec. The sonicated preparation was then centrifuged at 9,000 rpm for 10 min at 4°C, and the supernatant was aliquoted and frozen at −20°C. As with the trpE-DEN fusion proteins, the individual cell lysate antigens for each DEN virus serotype were titrated against homologous HMAF to determine optimal concentrations and pooled for use in the indirect IgG ELISA. Protein concentrations of the recombinant trpE-DEN proteins as well as the cell lysate antigens were determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL).

**Indirect IgG and IgM ELISA.** Immulon II microtiter-plates (Dynatech, Chantilly, VA) were coated with 100 µl per well of recombinant trpE alone or trpE-DEN pool (275 ng/well for IgG and 650 ng/well for IgM detection), DEN virus–infected cell lysate pool or uninfected cell lysate (890 ng/well), YF virus–infected and uninfected cell lysate (140 ng/well), or JE virus–infected and uninfected cell lysate (100 ng/well) diluted in PBS. The plates were blocked by incubation in PBS, 0.01% Tween 20, 5% nonfat dry milk (Difco, Detroit, MI) at 37°C for 1 hr. After the plates were washed five times with wash buffer (PBS, 0.1% Tween 20, 0.01% thimerosal), 100 µl of human sera or mouse ascitic fluid diluted 1:100 in PBS, 0.01% Tween 20, 5% nonfat dry milk was added and incubated at 37°C for 1 hr. Serum samples for the indirect IgM assay were absorbed using Rapi-Sep-M obtained from Integrated Diagnostics Inc. for the removal of IgG antibodies. Human sera tested with the recombinant trpE antigens were diluted ahead of time and absorbed for 1 hr at 37°C (during the blocking step) with 60 µg/100 µl of boiled *E. coli* extract consisting of the unfused trpE protein and other *E. coli* proteins and then added to microtiter plates. The plates were washed again five times with wash buffer, and antibodies were detected by adding 100 µl/well of horse-radish peroxidase-conjugated anti-human IgG or anti-human IgM monoclonal antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD) in PBS, 0.01% Tween 20, 5% nonfat dry milk. After incubation for 1 hr at 37°C, the plates were washed five times with PBS, 0.1% Tween 20, 0.1% thimerosal, once with PBS only, and then incubated with 100 µl/well of 2,2'-azino-di-(3-ethyl-benzthiazoline sulfonate) (ABTS) substrate solution at 37°C for 30 min. Absorbance (optical density [OD]) was read at 410 nm using a Dynatech MR 5000 microplate reader.

**IgM antibody capture ELISA.** Goat anti-human IgM (Tago, Burlingame, CA) diluted 1:300 in PBS was used to coat Linbro/Titertek (ICN Biomedicals, Aurora, OH) microtiter plates at a concentration of 100 µl/well at 4°C overnight. The plates were then washed five times with wash buffer containing PBS, 0.1% Tween 20, 0.01% thimerosal and blocked using 100 µl of blocking buffer containing PBS, 3% nonfat dry milk, 0.5% bovine serum albumin, 0.01% thimerosal. After incubation at 37°C for 1 hr, the plates were washed and incubated with patient sera diluted 1:100 in dilution buffer containing PBS, 5% nonfat dry milk, 1% Tween 20, 1% normal human serum, 0.01% thimerosal at 37°C for 1 hr. After washing with wash buffer, each serum was incubated with 100 µl of infected cell culture supernatant antigen as well as negative antigen preparation in dilution buffer and incubated at 37°C for 1 hr. The plates were washed as before and antigen was detected using DEN virus HMAF followed by goat anti-mouse IgG, IgA, and IgM (heavy plus light chains) conjugated with horseradish peroxidase (Kirkegaard and Perry Laboratories) in dilution buffer at 37°C for 1 hr. A final washing step was followed by a wash with PBS and flavivirus-specific IgM was detected with ABTS substrate solution by incubation at 37°C for 1 hr. Absorbance was measured at 410 nm using a Dynatech MR 5000 microplate reader.

**Determination of seropositivity by ELISA.** Each test serum was reacted in duplicate wells on the microtiter plate coated with recombinant or cell culture–derived viral antigen and duplicate wells were coated with the negative control antigen consisting of unfused purified trpE protein or uninfected Vero cell lysate or supernatant as appropriate. The net OD values were determined by subtracting the absorbance of each test serum with the negative control antigen from the absorbance value of the viral antigen. Negative and positive control sera were included in each assay. The cut-off OD value for test serum seropositivity in both the indirect and antibody-capture ELISA was set at ≥ 0.10 since the mean adjusted OD + three standard deviations for the negative control sera was consistently below this value.

**Plaque-reduction neutralization assay.** The PRNTs were performed in LLC-MK2 cells as previously described. Briefly, confluent cell monolayers were prepared in six-well plates ( Falcon; Becton Dickinson, Franklin Lakes, NJ) and maintained at 37°C in a CO₂ incubator. Sera were tested using two-fold dilutions starting at 1:10. Plaque-forming units (PFU) were visualized on day 7 by staining with 0.02% neutral red in normal saline. Each serum was tested in duplicate and the number of PFU were recorded as the average of the number observed in each of the two cultures. The 50% reduction of PFU by the test samples was estimated on the basis of the average number of PFU observed in cultures inoculated with mixtures of antibody negative sera and the control virus dose.

**RESULTS**

Sera considered to be flavivirus IgG antibody negative based on lack of reactivity (adjusted OD < 0.10) in the indirect IgG ELISA with DEN, JE, and YF virus cell culture lysates antigens were tested in the same assay with the trpE-DEN pooled antigen. One of 32 of these samples was reactive (adjusted OD = 0.140) with the trpE-DEN pooled antigen giving a specificity of 96.9%. The 10 sera selected as true anti-flavivirus IgM antibody negatives based on lack of reactivity (adjusted OD < 0.10) in the antibody-capture ELISA with DEN and YF virus cell culture supernatant antigens were all negative in the indirect IgM ELISA using the trpE-DEN pooled antigen.

All of the convalescent sera from the virus isolation–confirmed primary DEN-1 cases from Peru were positive for
TABLE 1
Comparison of trpE-dengue (DEN) pooled antigen with DEN virus–infected cell lysate pooled antigen, yellow fever (YF) virus–infected cell lysate antigen, and Japanese encephalitis (JE) virus–infected cell lysate antigen for the detection of IgG antibodies in the indirect ELISA*

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Primary†</th>
<th>Secondary‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>trpE-DEN</td>
<td>1.61 ± 0.05</td>
<td>1.63 ± 0.09</td>
</tr>
<tr>
<td>DEN cell lysate</td>
<td>0.66 ± 0.06</td>
<td>1.69 ± 0.04</td>
</tr>
<tr>
<td>YF cell lysate</td>
<td>0.10 ± 0.02</td>
<td>NT</td>
</tr>
<tr>
<td>JE cell lysate</td>
<td>NT</td>
<td>1.19 ± 0.07</td>
</tr>
</tbody>
</table>

* Values are the mean of ±SEM optical density. NT = not tested.
† Convalescent serum samples (n = 9) from Peru collected after virus isolation–confirmed primary DEN-1 infections.
‡ Convalescent serum samples from two DEN-1, two DEN-2, and one DEN-3 virus isolation–confirmed secondary DEN cases from Indonesia.

The three individuals with virus isolation–confirmed DEN-1 virus infections who had previously been vaccinated against either YF or YF and TBE all showed a pattern of seroconversion when their sera were tested in the indirect IgG ELISA with the trpE-DEN antigen pool (Figure 1). The level of IgG antibody reactivity detected with the trpE-DEN antigen for two of these individuals, however, was lower for the earliest convalescent samples compared with the level of antibody detected by the DEN or YF virus–infected cell culture lysate antigens (Figure 1A and B), but in the later convalescent samples, IgG antibody reactivity was equivalent. Both of these individuals had received an experimental live DEN-1 vaccine and the earliest convalescent samples represented day 14 postinfection sera. A similar pattern was not seen for DEN IgM antibody. The IgM reactivity with the trpE-DEN antigen pool in the indirect ELISA for the 14 day postinfection sera for these individuals (OD values = 1.36 and 0.76) was greater than the reactivity with the DEN virus cell culture supernatant pooled antigen in the antibody-capture ELISA (OD values = 0.21 and 0.57). For the third individual who experienced a natural DEN 1 virus infection (Figure 1C), the level of IgG antibody reactivity with the
DEN virus cell lysate and trpE-DEN antigen pools was approximately equivalent in both the acute and convalescent samples, and remained detectable by both antigen preparations over a one-year period.

The trpE-DEN recombinant antigen alone was used to test sera from the 55 Peruvians who acquired DEN IgG antibody during a one-year prospective study. The seroconversions had originally been demonstrated by testing paired baseline and one-year follow-up sera from each study subject with the indirect IgG ELISA using DEN-1 virus cell culture lysate antigen. Seven of the samples that were positive with the DEN virus cell lysate antigen were negative with the trpE-DEN recombinant antigen by the indirect IgG ELISA (Figure 2). These seven sera were further tested in the PRNT to confirm the pattern of reactivity in the ELISA (Table 2). Four of the samples were negative for DEN-1 virus neutralizing antibody, but one had a neutralizing antibody titer of ≥ 1:40 against YF virus. The remaining three had low-titered DEN-1 virus neutralizing antibody. The highest neutralizing antibody titer against DEN-1 virus was 1:20, but this sample also had a YF virus neutralizing antibody titer ≥ 1:40.

To determine the cross-reactivity of the trpE-DEN pooled antigen with IgG antibody from related flaviviruses, the eight sera from individuals who had been previously vaccinated against YF and JE viruses were tested in the indirect IgG ELISA (Figure 3). All of the sera were antibody positive with the YF and JE virus cell culture lysate antigens. With the DEN virus cell lysate pooled antigen, six (75%) of the sera were positive, but none of the sera reacted with the trpE-DEN pooled antigen. Yellow fever virus neutralizing antibodies were confirmed in all six of these samples, but DEN virus neutralizing antibodies were not detected (Table 3).

The nine convalescent samples from virus isolation–con-
Table 3

Plaque-reduction neutralization test (PRNT) titers of six individuals vaccinated against yellow fever (YF) and Japanese encephalitis viruses with no known exposure to dengue (DEN) viruses that were positive in the IgG indirect ELISA using DEN virus cell lysate antigens but were negative with the trpE-DEN recombinant antigens*.

<table>
<thead>
<tr>
<th>Serum no.</th>
<th>PRNT titers</th>
<th>DEN-2</th>
<th>YF</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>&lt;1:10</td>
<td>1:120</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>&lt;1:10</td>
<td>1:40</td>
<td></td>
</tr>
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<td>8</td>
<td>&lt;1:10</td>
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</tr>
<tr>
<td>11</td>
<td>&lt;1:10</td>
<td>1:70</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>&lt;1:10</td>
<td>1:240</td>
<td></td>
</tr>
</tbody>
</table>

* ELISA results are shown in Figure 3.

To determine cross-reactivity of the trpE-DEN antigen with IgM antibody against a related flavivirus, the seven samples from the suspected acute YF cases from Peru were tested. All of these samples were positive with YF virus–infected cell culture supernatant antigen as well as with DEN virus cell culture supernatant pooled antigen in the IgM-capture ELISA; however, none of the sera reacted with the trpE-DEN pooled antigen in the indirect IgM ELISA (Figure 4).

Table 4

Comparison of indirect IgM ELISA using trpE–dengue (DEN) pooled antigen with the IgM antibody-capture ELISA using DEN virus–infected cell supernatant pooled antigen*.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Primary†</th>
<th>Secondary‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>trpE-DEN</td>
<td>1.93 ± 0.03</td>
<td>0.47 ± 0.17</td>
</tr>
<tr>
<td>DEN virus cell supernatant</td>
<td>1.61 ± 0.07</td>
<td>0.58 ± 0.19</td>
</tr>
</tbody>
</table>

* Values are the mean ± SEM optical density.
† Samples are convalescent sera (n = 9) from DEN-1 virus isolation–confirmed primary DEN cases from Peru.
‡ Convalescent serum samples from two DEN-1, two DEN-2, and one DEN-3 virus isolation–confirmed secondary DEN cases from Indonesia.

DISCUSSION

Based on convalescent sera collected from DEN-1, -2, or -3 virus isolation–confirmed infections, the sensitivity of the trpE-DEN pooled antigen for detecting anti-DEN virus IgG was equivalent to the sensitivity of the DEN virus–infected cell lysate pooled antigen in the indirect IgM ELISA for both primary and secondary DEN virus infections. Although we did not test the trpE-DEN pooled antigen with sera from DEN-4 virus–confirmed human infections, results from comparative studies using HMAF suggested that it would be as sensitive as the DEN virus cell lysate pooled antigen (Simmons M, unpublished data).

The DEN-1 virus isolation–confirmed cases from Peru were classified as primary DEN virus infections. Dengue-1 virus was the only serotype known to be endemic in Peru at the time the samples were collected. Also, all nine samples had been shown by PRNT to possess high-titered DEN-1 virus neutralizing antibody (> 1:320), but were negative (< 1:10) for YF virus neutralizing antibody (Simmons M, unpublished data). The absent or very low level of reactivity with the YF virus–infected cell culture lysate antigen in the IgG indirect ELISA also supports this conclusion.

The cases from Indonesia where all four serotypes of DEN virus cocirculate had been classified as secondary DEN virus infections based on clinical data and HI antibody titers $\geq$ 1:1,280 against DEN virus (Graham R, unpublished data). The comparable level of reactivity in the indirect IgG ELISA of these presumptive secondary DEN sera with both the trpE-DEN pooled antigen and the DEN virus cell lysate pooled antigen is reassuring since immunologic memory would be expected to drive the early antibody response predominantly against antigenic epitopes shared by the different DEN virus serotypes causing subsequent infections. Previous ELISA studies with the individual trpE-DEN recombinant proteins using HMAFs showed that they had a lower
level of serotype cross-reactivity than the individual DEN virus–infected cell culture lysate antigens, but were not absolutely serotype specific (Simmons M, unpublished data).\textsuperscript{12} Previous epitope mapping studies also have shown the presence of both DEN virus type-specific and DEN virus complex/subcomplex-reactive epitopes within the region of the DEN virus envelope used to construct the trpE-DEN recombinant proteins.\textsuperscript{19} The results with these presumptive secondary DEN virus infection cases from Indonesia suggest that within the time frame that the sera were collected following the onset of DEN illness, an anamnestic antibody response to shared DEN virus complex/subcomplex antigens does not result in a loss of sensitivity with the recombinant antigens.

Of potentially greater concern with these recombinant antigens, for the same reason related to memory response, is the early detection of DEN virus-specific IgG antibody in individuals with acute DEN virus infections who have previously been exposed to a non-DEN flavivirus. This scenario could result from sequential natural infections since the geographic distribution of the DEN viruses overlaps with other human pathogenic flaviviruses in many areas of the tropics or from previous vaccination against YF, JE, or TBE, followed by a natural DEN virus infection.\textsuperscript{2} Earlier studies with the recombinant DEN virus antigens showed no or very minimal cross-reactivity with HMAF against flaviviruses not belonging to the DEN virus complex.\textsuperscript{12} The antibody response patterns of the two volunteers who had previously been vaccinated against other flaviviruses and subsequently received a DEN-1 virus live vaccine (Figure 1A and B) support the concern that the initial IgG antibody response is directed mostly at shared flavivirus group antigenic epitopes not contained or at least not recognized on the recombinant proteins. The 14 day post DEN-1 virus infection samples for both volunteers showed substantially stronger IgG reactivity against the DEN virus cell lysate pooled antigen than the trpE-DEN pooled antigen; although, at 21 days the reactivity to both antigen preparations was about equal. The equal or greater reactivity of the day 14 post DEN-1 virus infection sera IgM antibody with the trpE DEN antigen compared with the DEN virus cell supernatant antigen is not surprising since IgM antibody probably reflects a primary immune response, instead of a memory B cell response.\textsuperscript{21} The IgG antibody response in the acute-phase serum from the remaining case did show the same pattern as the vaccinees (Figure 1C). This individual had a natural DEN-1 virus infection several years after being vaccinated for YF, and the first sample positive for DEN virus antibody was taken 10 days after the patient reported the onset of illness. Since the natural incubation period of dengue is thought to last 5–8 days, this sample probably was taken 15–18 days after infection. Results from this case also show that the IgG antibody that reacts with the epitopes on the trpE-DEN pooled proteins are long-lasting, indicating that this antigen could be used retrospectively to determine past DEN virus exposures in a population.

The usefulness of the trpE-DEN antigen for detecting IgG antibody from past DEN virus infections was further evaluated with the 55 seroconversion samples from the Peru one-year incidence study. The initial comparative ELISA results suggested that the trpE-DEN antigen was not as sensitive as the DEN virus cell lysate antigen because seven sera that were classified as positive in the indirect ELISA with the latter antigen were negative with the trpE-DEN antigen. However, subsequent neutralizing antibody results with these samples suggested that the antibody detected with the DEN virus cell lysate antigen in the ELISA could be cross-reactive antibody from other flaviviruses. Since at the time these samples were collected, DEN-1 was the only serotype of dengue virus known to be circulating in Peru, it is unlikely that the antibody detected with the DEN-1 virus cell lysate antigen in the ELISA represents cross-reactive antibody from a different serotype of dengue virus. However, two of these samples showed higher reactivity against YF virus than against DEN-1 virus in the PRNT. Yellow fever virus is known to be epizootic among the subhuman primates and to occasionally infect humans in the Amazon region of Peru in which these samples were collected.\textsuperscript{2} The remaining samples did not neutralize DEN-1 virus or were only positive at a 1:10 dilution. These samples were also negative for YF virus neutralizing antibody but could still represent infections with other flaviviruses such as Bussaquara or Ilheus. Although these viruses have not been isolated in Peru, they are known to occur in the tropical forests of surrounding countries.\textsuperscript{2} The failure of the trpE-DEN antigen to react with these samples in the indirect IgG ELISA, particularly with the two YF virus neutralizing antibody-positive samples, further suggests that flavivirus cross-reactive epitopes are not present on the recombinant protein. This observation is supported by the absence of reactivity of the trpE-DEN pooled antigen with the sera from individuals that had been vaccinated against both YF and JE viruses. Although most of these samples did react in the indirect IgG ELISA with the DEN virus cell lysate pooled antigen, they were negative for DEN virus neutralizing antibody, thus confirming the DEN virus complex specificity of the trpE-DEN pooled antigen.

The results with the DEN virus isolation–confirmed convalescent samples from Peru and Indonesia showed that the trpE-DEN pooled antigen also can be used in an indirect ELISA to detect anti-DEN virus IgM antibody in both primary and secondary DEN virus infections. These samples were passed through a disposable protein G column to remove any competing anti-DEN virus IgG, which could theoretically reduce the sensitivity of anti-DEN virus IgM antibody detection.\textsuperscript{23, 24} The lack of reactivity of the trpE-DEN antigen with the samples from suspected cases of YF from Peru supports the previous IgG results that cross-reactive flavivirus group epitopes are not present on these recombinant proteins. Although these were not virus isolation–confirmed cases of YF, they were collected during the course of a YF epidemic in Peru from clinically suspected patients. These samples also had previously been shown to possess four-fold or greater IgM antibody titers against YF virus compared to DEN virus in an IgM antibody-capture ELISA using infected mouse brain–derived antigens (Watts DM, unpublished data).

These studies show that the trpE-DEN pooled recombinant antigens can be used in an ELISA to detect anti-DEN virus IgM antibody in convalescent serum samples from acute primary and secondary DEN virus infections as well as for the retrospective determination of DEN virus infections. Dengue virus IgM antibody also was readily detected in an indirect ELISA using the recombinant proteins as an-
tigens. The greater DEN virus complex specificity of the recombinant antigens compared with the infected cell culture–derived antigens in the ELISA indicates that they may be useful for the definitive serologic diagnosis of DEN virus infections in areas where other flaviviral infections occur among human populations. Although our results with the recombinant antigens are very promising, clearly additional acute- and convalescent-phase samples from patients infected with each of the four dengue virus serotypes and with other flaviruses should be evaluated to determine the full utility of these reagents for the detection of specific dengue virus antibody.

Acknowledgments: We thank I. A. Phillips for performing the DEN virus IgM capture ELISA. We also express our gratitude to Dr. S. B. Halstead for reviewing this manuscript.

Financial support: This research was supported by Naval Medical Research and Development Command work unit # 61102AM0011511.

Disclaimer: The opinions and assertions herein are not to be construed as official or as reflecting the views of the Navy Department or the naval service at large.

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