Corrections

MEDICAL SCIENCES

The authors note that on page 1555, left column, fourth full paragraph, line 2 “example 17” should instead appear as “example 15.”

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ECOLOGY

The authors note that the accession number 4504979.3 (Iowa corn) should instead appear as 4504797.3 (Iowa corn).

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CELL BIOLOGY

The authors note that on page E589, left column, first paragraph, line 1, “JAX 004781” should instead appear as “JAX 004682.”

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MICROBIOLOGY

The authors note that the following statement should be added to the Acknowledgments: “This research was also supported by the Sunlin and Priscilla Chou Foundation (W.B.M.).”

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Retraction

MICROBIOLOGY

The authors wish to note the following: “We have discovered a significant error that compromises our ability to interpret the in vitro and in vivo data reported in this paper. During recent follow-up studies with the rDENV-3/4 virus, we discovered that the original stocks used to generate the in vitro neutralization data and in vivo primate infection data reported in the manuscript contained contaminating wild-type DENV-4 virus. We have since determined that this low-level contamination was sufficient to confound the chimeric DENV-3/4 results, rendering them uninterpretable. We emphasize that the escape mutant and recombinant protein data reported in the paper are in no way compromised by this contamination and stand on their own. Specifically, mapping of the critical 5J7 antibody epitope residues, shown in Fig. 1, did not utilize any recombinant chimeric DENV. The viral antibody escape mutants were derived from wild-type infectious clones and were sequence verified. The loss of antibody binding studies were conducted by Integral Molecular and used a recombinant protein expression system independent of the chimeric DENV. We deeply apologize for this inadvertent error. Accordingly, we have unanimously decided to retract the manuscript at this time. Given the large number of related constructs that we have subsequently generated in the laboratory, the strategy is sound; however, we cannot interpret the data and results reported in the manuscript.”

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Dengue virus envelope protein domain I/II hinge determines long-lived serotype-specific dengue immunity

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Edited by Michael G. Rossmann, Purdue University, West Lafayette, IN, and approved December 3, 2013 (received for review September 14, 2013)

The four dengue virus (DENV) serotypes, DENV-1, -2, -3, and -4, are endemic throughout tropical and subtropical regions of the world, with an estimated 390 million acute infections annually. Infection confers long-term protective immunity against the infecting serotype, but secondary infection with a different serotype carries a greater risk of potentially fatal severe dengue disease, including dengue hemorrhagic fever and dengue shock syndrome. The single most effective measure to control this threat to global health is a tetravalent DENV vaccine. To date, attempts to develop a protective vaccine have progressed slowly, partly because the targets of type-specific human neutralizing antibodies (NAbs), which are critical for long-term protection, remain poorly defined, impeding our understanding of natural immunity and hindering effective vaccine development. Here, we show that the envelope glycoprotein domain I/II hinge of DENV-3 and DENV-4 is the primary target of the long-term type-specific NAb response in humans. Transplantation of a DENV-4 hinge into a recombinant DENV-3 virus showed that the hinge determines the serotype-specific neutralizing potency of primary human and nonhuman primate DENV immune sera and that the hinge region both induces NAbs and is targeted by protective NAbs in rhesus macaques. These results suggest that the success of live dengue vaccines may depend on their ability to stimulate NAbs that target the envelope glycoprotein domain I/II hinge region. More broadly, this study shows that complex conformational antibody epitopes can be transplanted between live viruses, opening up similar possibilities for improving the breadth and specificity of vaccines for influenza, HIV, hepatitis C virus, and other clinically important viral pathogens.

Significance

Dengue virus is the most important arthropod-borne viral disease of humans worldwide, with an estimated 390 million acute infections annually. The best means to control this global health threat is a vaccine, but dengue vaccine development has progressed slowly, partly because the antigens required to stimulate long-term immunity are not well-defined. Here, we show a specific region on the viral surface (the envelope domain I/II hinge) that is the target of protective antibodies after primary human infections. These results are critically important for dengue vaccine design, because we hypothesize that a successful dengue vaccine will stimulate antibodies that target this region. More broadly, this study establishes a template for similar approaches for improving vaccines for influenza, HIV, hepatitis C virus, and other clinically important viral pathogens.


The authors declare no conflict of interest.

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See Commentary on page 1670.

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Results

Defining the EDI/EDII Hinge. To more fully explore the significance of the EDI/EDII region, we examined the epitope target of the potently neutralizing DENV-3–specific human monoclonal NAb 5J7 (8, 10), which was recovered from a donor (donor 105) previously infected with DENV-3. To identify the 5J7 epitope, we generated a comprehensive library of DENV-3 E proteins, in which every residue was separately mutated (1,400 total mutants). Variant E proteins were expressed individually, and the effect of the point mutations on 5J7 binding to E was analyzed, identifying seven critical residues—Q52, L53, E126, K128, E133, L135, and A203 (Fig. 1A and C)—all localized to the EDI/EDII hinge region.

As a complementary approach, we also passaged DENV-3 in the presence of high concentrations of 5J7 to generate viral escape mutants; we identified three escape mutations—Q269K270insK, L53P, and K128G (Fig. 1A and C)—that are also all localized to the EDI/EDII hinge region. When subsequently tested against donor 105 polyclonal serum, the virus containing the Q269K270insK mutation showed a statistically significant 78% drop in neutralization titer (from 1:379 to 1:83; P < 0.05, ANOVA followed by Dunnett test) (SI Appendix, Fig. S1). One potential explanation for the significant drop in titer is that multiple DENV-3 NAbs in human polyclonal serum target epitopes within the EDI/EDII hinge region.

Construction and Characterization of the Recombinant EDI/EDII Hinge Transplant Virus Recombinant DENV-3/4: Long-Lived Neutralization Responses Against Primary DENV Infections Are Specific to the Serotype Of Infection. To test the hypothesis that the EDI/EDII hinge region is a major target of the human polyclonal neutralization response after primary infection, we first visualized the 5J7
epitope footprint on the DENV-3 E structure (11) by identifying all amino acid residues within 12 Å (i.e., the approximate footprint of an antibody paratope) of the most central escape mutation—K128G. Our intent was to then probe the contribution of this region to polyclonal serum neutralization by extensively mutating it in a DENV-3 infectious clone background (12). Because the EDI/EDII hinge region must remain flexible over the course of DENV infection (13), we replaced the EDI/EDII hinge in a DENV-3 infectious clone with amino acids from the corresponding structures of the DENV-4 EDI/EDII hinge (Fig. 1 B and C). Our hypothesis was that transplanting DENV-4 EDI/EDII hinge residues into the DENV-3 E protein would maintain short-range residue interactions critical to E-protein hinge function but transfer the DENV-4 EDI/EDII hinge serotype-specific epitopes. The resultant recombinant DENV-3/4 hinge virus (rDENV-3/4) contained 25 amino acid changes, showed similar growth kinetics to parental rDENV-3 in Vero and C6/36 insect cells (SI Appendix, Fig. S2), showed similar plaque phenotypes in Vero cells (SI Appendix, Fig. S3), and similarly bound E-specific mAbs that target E epitopes outside the EDI/EDII hinge (SI Appendix, Fig. S4); collectively, these findings show that EDI/EDII hinge function and E tertiary structure were preserved in the chimeric virus.

Transplantation of DENV-4 EDI/EDII Hinge into DENV-3 Leads to Loss of Neutralization by DENV-3 Primary Sera. To assess the impact of EDI/EDII hinge transplantation on polyclonal antibody neutralization, a panel of human (SI Appendix, Table S1) and rhesus macaque (RM) primary DENV-3 convalescent antisera collected >1 y after infection (14) was screened against the parental rDENV-3, rDENV-4, and rDENV-3/4 viruses (Fig. 2). Primary DENV-3 sera potently neutralized DENV-3 and weakly neutralized DENV-4 (Fig. 2 A and B). Remarkably, rDENV-3/4 hinge was rendered insensitive to neutralization by human and primate primary DENV-3 sera (P < 0.05), with a neutralization profile equivalent to DENV-4 sera (Fig. 2 A and B). This near-complete loss of sensitivity to neutralization by primary DENV-3 sera supports the model that the EDI/EDII hinge region contains most or all of the epitope determinants of serotype specificity after natural DENV infection.

Transplantation of DENV-4 EDI/EDII Hinge into DENV-3 Leads to Gain of Neutralization by DENV-4 Primary Sera. If serotype-specific epitopes at the EDI/EDII hinge are the target of human antibodies that neutralize DENV-4 as well, successful transplantation of the DENV-4 EDI/EDII hinge into rDENV-3 should render rDENV-3/4 sensitive to neutralization by primary DENV-4 serum. We then tested rDENV-3, rDENV-4, and rDENV-3/4 virus in neutralization assays against a panel of human (SI Appendix, Table S2) and RM primary DENV-4 antisera (14).

All eight sera effectively neutralized DENV-4 and had much lower neutralization titers against rDENV-3 (Fig. 2 C and D). Significantly, rDENV-3/4 was sensitive to neutralization by DENV-4 antisera, gaining >95% of the DENV-4 neutralizing phenotype for all sera tested. This gain of function or sensitivity to neutralization with the EDI/EDII hinge region transplant strongly supports the model that the EDI/EDII region defines the epitopes critical for serotype-specific neutralization after natural DENV infection.

EDI/EDII Hinge Directs Antibody-Dependent Enhancement in a Serotype-Specific Manner. In secondary DENV infections, cross-reactive non-NAbs from the first infection are thought to contribute to severe disease pathogenesis by Fc receptor-mediated uptake of antibody-bound virus into target cells, a phenomenon called antibody-dependent enhancement (ADE) (15, 16). To test if EDI/EDII hinge transplantation altered human immune sera enhancement in Fc receptor-bearing cells, rDENV-3, rDENV-4, and rDENV-3/4 viruses were characterized in a K562 cell-based ADE assay using primary human DENV-immune sera (17). As expected, when rDENV-3 or rDENV-4 was incubated with physiologically relevant high concentrations of primary sera from each serotype, each virus was enhanced by heterologous but not homologous immune sera (Fig. 3 and SI Appendix, Fig. S5). Thus, at high serum concentrations approximating the levels circulating in people, rDENV-3/4 virus was enhanced by DENV-3 but not DENV-4 immune sera, indicating that the EDI/EDII hinge transplant altered the ADE profile of DENV-3 to a profile similar to DENV-4. When a serum sample had high levels of NAbs to a particular virus (rDENV-3 with primary DENV-3 immune sera), we did not observe enhancement at high serum concentrations (Fig. 3). When NAbs are diluted to subneutralizing levels, they are capable of enhancement (SI Appendix, Fig. S5), which is
unlikely to be of any biological significance at these physiologically irrelevant dilutions of serum.

**EDI/EDII Hinge Directs a Type-Specific Immune Response After Primary Infection.** Primates are the only known vertebrate hosts of DENVs. To study replication and the immunogenic properties of rDENV-3/4 in vivo, we inoculated four dengue-naive RMs with 5 x 10^5 focus forming units (FFUs) virus and followed the animals for viral infection. The RMs did not develop disease but did become viremic for a mean of 4.25 ± 0.957 d (Table 1), similar to days viremia reported for WT DENV at similar doses in RMs (14). At 60 d after rDENV-3/4 inoculation, DENV-4 focus reduction neutralization test (FRNT50) titers were significantly boosted (DENV-3 geometric mean titer, 1:5,531) (Table 1). Because the only DENV-4-specific antigen in rDENV-3/4 was the hinge region itself, this result strongly suggests that long-lived NAb:s that target the EDI/EDII hinge are a major component of protective immunity in vivo.

**Discussion**

DENV infection typically results in a short-lived broad neutralization response that matures over time into a serotype-specific neutralization response. Results presented here show that the EDI/EDII hinge region is the principle target of long-lived serotype-specific NAb:s that develop in humans after natural DENV-3 and DENV-4 infections. Moreover, experimental challenge studies in RMs with the rDENV-3/4 chimera show that the hinge region elicits a serotype-specific NAb response in vivo and strongly suggest that hinge Abs protect against viremia in vivo, although the boosted antibody titers 28 d postchallenge suggest that undetected viral replication may have occurred. The hinge has been shown to play a critical role in the conformational change that E protein undergoes at low pH to fuse with cellular endosomes, allowing viral uncoating and the release of viral RNA into the cellular

**Table 1.** RM prechallenge FRNT50, viremia, and postchallenge FRNT50 following infection with rDENV-3/4

<table>
<thead>
<tr>
<th>RM identification</th>
<th>DENV exposure history</th>
<th>Prechallenge FRNT50 titer</th>
<th>Viremia (log FFU/ML) days postchallenge</th>
<th>Days viremia</th>
<th>60-d Postchallenge FRNT50 titer</th>
<th>28-d Postchallenge FRNT50 titer</th>
</tr>
</thead>
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<tr>
<td>2L7</td>
<td>Naive</td>
<td>&lt;1:20</td>
<td>2.3 2.7 2.8 1.1 1.1 ND ND</td>
<td>5</td>
<td>1:101 1:160 1:343 1:1,536</td>
<td></td>
</tr>
<tr>
<td>2L2</td>
<td>Naive</td>
<td>&lt;1:20</td>
<td>2.7 3.5 1.7 1.1 ND ND</td>
<td>4</td>
<td>1:269 1:99 1:196 1:2,266</td>
<td></td>
</tr>
<tr>
<td>9K8</td>
<td>Naive</td>
<td>&lt;1:20</td>
<td>1.9 2.1 1.8 2.3 1.1 ND ND</td>
<td>5</td>
<td>1:44 1:95 1:167 1:616</td>
<td></td>
</tr>
<tr>
<td>9L4</td>
<td>Naive</td>
<td>&lt;1:20</td>
<td>1.6 2.4 1.1 ND ND ND</td>
<td>3</td>
<td>1:107 1:112 1:224 1:1,047</td>
<td></td>
</tr>
<tr>
<td>6D57</td>
<td>1° DENV-4</td>
<td>&lt;1:20</td>
<td>1:263 ND ND ND ND ND ND</td>
<td>0</td>
<td>1:70 1:112 1:2,248 1:10,197</td>
<td></td>
</tr>
<tr>
<td>6G9</td>
<td>1° DENV-4</td>
<td>1:35</td>
<td>1:149 ND ND ND ND ND ND</td>
<td>0</td>
<td>1:90 1:129 1:734 1:7,147</td>
<td></td>
</tr>
<tr>
<td>6G0</td>
<td>1° DENV-4</td>
<td>&lt;1:20</td>
<td>1:146 ND ND ND ND ND ND</td>
<td>0</td>
<td>1:36 1:41 1:1,163 1:2,897</td>
<td></td>
</tr>
<tr>
<td>6F8</td>
<td>1° DENV-4</td>
<td>&lt;1:20</td>
<td>1:286 ND ND ND ND ND ND</td>
<td>0</td>
<td>1:94 1:273 1:5,559 1:5,814</td>
<td></td>
</tr>
</tbody>
</table>

ND, not detected.
cytoplasm (7). We hypothesize that hinge-targeting NAbs act through mechanisms that block this structural transition, consistent with what has been reported for West Nile virus (20, 21).

Our data are consistent with models where the EDI/EDII hinge of each serotype contains a single or multiple overlapping epitopes targeted by primate NAbs. Indeed, structure studies with human mAbs that bind to the EDI/EDII hinge region of flaviviruses indicate that this region contains overlapping quaternary epitopes (9, 20). Although primary cross-reactive T-cell responses and antibodies do not confer long-term protection against heterologous challenge in primates, it is possible that the EDI/EDII hinge region contains peptides that can be recognized by DENV-specific T cells that also contribute to protection against DENV reinfection. We feel this explanation is less likely given that the most extensive survey of DENV T cell-specific epitopes in humans did not identify any E hinge epitopes among the 25 most immunodominant regions of the DENV proteome (22). Clearly, additional primate challenge and protection studies in different DENV immune backgrounds are needed to more fully decipher the roles of hinge-independent type-specific and cross-protective immunity in vivo and the extent to which changes in epitope display outside the EDI/EDII hinge region also impact antibody recognition of the DENV E glycoprotein.

These results have important implications for development of DENV vaccines. The leading DENV vaccines are live-attenuated flaviviruses expressing DENV E proteins (23). Our results indicate that the success of these live flavivirus vaccines may depend on their ability to elicit antibodies that target the EDI/EDII hinge region. Not only will recombinant virus reagents allow us to provide an EDI/EDII hinge-specific responses, but our results also provide a basis for designing epitope mimicet peptide vaccines that stimulate serotype-specific NAbs and not the cross-reactive antibodies with potential for disease enhancement. More broadly, our demonstration that complex conformational antibody epitopes can be transplanted between live viruses with minimal impacts on growth in vitro and in vivo opens up possibilities for improving vaccine breadth and specificity in other important human viral pathogens with known complex NAb epitopes, including hepatitis C virus (24), influenza (25), and HIV (26).

Materials and Methods

Cells. Mosquito Ae. albopictus C6/36 cells were maintained in MEM (Gibco) media at 32 °C. Human monocyte lymphoma cell line U937 expressing DC-SIGN was maintained in RPMI-1640 (Gibco) at 37 °C supplemented with 5% (vol/vol) FBS. All media used were also supplemented with 5% (vol/vol) FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 1 μM nonosspessent amino acids (Gibco), and 2 mM glutamine, and all cells were incubated in the presence of 5% CO2. The 5% FBS was reduced to 2% to make infection media for each cell line.

ADE Assays. Antibody-dependent enhance assays were conducted as previously described (17) and adopted for K562 cells. Briefly, polyclonal serum samples were diluted twofold from 1:20 and incubated for 1 h at 37 °C with rDENV-3, rDENV-3′, or rDENV-4. K562 cells (5 × 105 cells/well) were added to the antibody–virus mixture and incubated for an additional 2 h at 37 °C. After the 2-h incubation, cells were washed two times with infection media and incubated overnight at 37 °C and 5% CO2. Twenty-four hours after infection, cells were washed, fixed, stained for DENV structural proteins with mAb 4G2, and percent infection-assessed on an EMD Millipore Guava Flow Cytometer.

Binding ELISA. Equal virus quantities of DENV-3 and DENV-3′ (as previously titrated by ELISA) were captured using a mixture of coated anti-PRM and anti-Env antibodies. The capture antibodies used were either mouse or human depending on the species of the primary antibody being tested. The primary antibodies, 4G2 (mouse mAb) and 1NS and 5J7 (human mAbs), were diluted fourfold from 10 to 0.002 μg/ml. Alkaline phosphatase-conjugated secondary antibodies were used to detect binding of primary antibodies with a p-nitrophenyl phosphate substrate, and color change was quantified with spectrophotometry.

Shotgun Mutagenesis Epitope Mapping. A DENV-3 prM/E expression construct (DENV-3 strain CH53489) was subjected to high-throughput mutagenesis (shotgun mutagenesis) to generate a comprehensive mutation library (27). Point mutations were introduced into the DENV-3 prM/E polyprotein by PCR using a Diversity Mutagenesis Kit (Clontech Laboratories, Inc.). In total, 1,400 DENV-3 mutants were generated (~97% coverage of the prM/E ectodomain), sequence confirmed, and arrayed into 384-well plates (one mutation per well). Each E mutant was individually transplanted into HEK-293T cells and allowed to express for 22 h. Cells were fixed in 4% (wt/vol) paraformaldehyde (Electron Microscopy Sciences) and permeabilized with 0.1% (wt/vol) saponin (Sigma-Alrich) in PBS plus calcium and magnesium (PBS+). Cells were stained with purified SJ7 antibody (0.2 μg/ml) diluted in 10% normal goat serum (NGS) (Sigma)/0.1% saponin (pH 9). The optimal primary antibody concentration was determined using an independent immunofluorescence titration curve against WT prM/E to ensure that signals were within the linear range of detection and that signal exceeded background by at least fivefold. Antibody binding was detected using 3.75 μg/ml AlexaFluor488-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) in 10% NGS/0.1% saponin. Cells were washed three times, and mean fluorescence intensity was determined using an acquisition threshold of 200 on a BD Guava Plus Flow Cytometer (Intellicyt). Antibody reactivities against each mutant Env clone were calculated relative to WT Env protein reactivity by subtracting the signal from mock-transfected controls and normalizing to the signal from WT Env-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. The exclusion of Env strategy facilitates the exclusion of Env mutations that are locally misfolded or have an expression defect (28). Critical amino acids required for antibody binding were visualized on the DENV Env crystal structure (Protein Data Bank id 1U2G) (11).

DENV Infection Assays. The four-frame cloning strategy for the recombinant parental rDENV-3 clone was recently described (12). rDENV-4 was submitted to GenBank (accession no. 1683917) and was constructed in a similar manner. In brief, cDNAs were transcribed from a clinical DENV-3 and DENV-4 isolated and subcloned as four separate DNA fragments into stable plasmids (A, B, C, and D fragments). The rDENV-4 A–D plasmids were co-transfected into HEK-293T cells, and cell culture supernatants were passaged one or two times in C6/36 cells, and cell culture supernatants were clarified, supplemented with 20% FBS, and stored at −80 °C.

DENV Immune Sera. Human DENV immune sera were collected from either adult volunteers with histories of DENV infection (29) or anonymous blood donors, or they were provided from a pool of previously characterized DENV immune sera. Sera were initially characterized by flow cytometry at the University of North Carolina (30), FRNT50 at the University of North Carolina, plaque reduction neutralization test (FRNT50) at the National Institutes of Health, or FRNT50 at the Centers for Disease Control and Prevention San Juan to confirm past DENV exposure and also, identify the serotype responsible for primary infections.

Virus Titration and FRNT. The FRNT procedure is based on a method previously described by Durbin et al. (31). Briefly, 24-well plates were seeded with 5 × 104
Vero cells in MEM supplemented with 5% FBS and grown for 24 h. Growth media were removed. For virus titration, virus stocks were diluted serially 10-fold with MEM and 2% FBS in 96-well plates. Cells were overlaid with each dilution and incubated at 37°C for 48 h. The number of foci was counted and titered as 50% tissue culture infectious dose (TCID50).

**Software and Statistics.** Multiple alignments were performed using ClustalX version 1.83 (32). The structural model of the hinge epitope region was generated using MacPyMol (Delano Scientific) and the crystal structure of DENV-3 envelope (Protein Data Bank ID code 1UZG) (6). Alignments and secondary structure figures were generated using ALINE (33). FRNT counts were entered into Graphpad Prism (Version 5.00 for OSX; GraphPad Software). FRNT50 values were calculated by sigmoid dose–response curve fitting, with upper and lower limits of 100 and 0, respectively. All error bars show 95% confidence intervals unless otherwise specified.

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