Mechanistic Study of Broadly Neutralizing Human Monoclonal Antibodies against Dengue Virus That Target the Fusion Loop

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There are no available vaccines for dengue, the most important mosquito-transmitted viral disease. Mechanistic studies with anti-dengue virus (DENV) human monoclonal antibodies (hMabs) provide a rational approach to identify and characterize neutralizing epitopes on DENV structural proteins that can serve to inform vaccine strategies. Here, we report a class of hMabs that is likely to be an important determinant in the human humoral response to DENV infection. In this study, we identified and characterized three broadly neutralizing anti-DENV hMabs: 4.8A, D11C, and 1.6D. These antibodies were isolated from three different convalescent patients with distinct histories of DENV infection yet demonstrated remarkable similarities. All three hMabs recognized the E glycoprotein with high affinity, neutralized all four serotypes of DENV, and mediated antibody-dependent enhancement of infection in Fc receptor-bearing cells at subneutralizing concentrations. The neutralization activities of these hMabs correlated with a strong inhibition of virus-liposome and intracellular fusion, not virus-cell binding. We mapped epitopes of these antibodies to the highly conserved fusion loop region of E domain II. Mutations at fusion loop residues W101, L107, and/or G109 significantly reduced the binding of the hMabs to E protein. The results show that hMabs directed against the highly conserved E protein fusion loop block viral entry downstream of virus-cell binding by inhibiting E protein-mediated fusion. Characterization of hMabs targeting this region may provide new insights into DENV vaccine and therapeutic strategies.

Dengue imposes one of the largest socioeconomic burdens of any mosquito-borne human disease in the world, yet there is currently no available vaccine or specific treatment. Worldwide, there are an estimated 50 to 100 million cases of dengue infection per year, and 2.5 billion people living in regions where dengue is endemic are at risk of infection (1, 2). An estimated 500,000 people, many of them children, are hospitalized annually with severe dengue symptoms, including dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (2, 3). Of note, after an extended absence, dengue has recently reemerged in south Florida (4, 5). Local transmission has also recently been reported in Southern France and Croatia (6, 7).

The four distinct serotypes of dengue virus (DENV) cocirculate in many areas of the world and give rise to sequential epidemic outbreaks when the number of susceptible individuals in the local population reaches a critical threshold and weather conditions favor reproduction of the mosquito vectors Aedes aegypti and Aedes albopictus. Initial infection with one DENV serotype usually generates a protective and long-lasting immune response against reinfection with the same serotype. While antibody cross-reactivity between serotypes is common, cross-serotype protection is only short-lived (8). Low levels of neutralizing antibodies, cross-reactive but nonneutralizing antibodies, or both from previous infections bind virions of other serotypes and target them to Fc receptors on macrophages and certain other cell types, enhancing infection of these cells (9). The presence of these cross-reactive and nonneutralizing antibodies also correlated with severe disease outcome (DHF/DSS) in several studies (10–12). Higher levels of viremia are associated with the development of DHF (12, 13). This antibody-dependent enhancement (ADE) effect may also explain the sequential nature of epidemic outbreaks, as well as the severe disease seen in infants as maternal antibodies wane (10, 11, 14).

Like other members of the genus Flavivirus, DENV has a lipid envelope and a positive-strand RNA genome that codes for a single large polyprotein. This polyprotein is cleaved into separate segments to form the capsid (C), premembrane (prM/M), and envelope (E) structural proteins and enzymatic components required for viral replication and transmission (15). The external E glycoprotein participates in cell recognition and cell entry and is physically arranged in a herringbone pattern as a series of 90 homodimers on the outer surface of the mature virus particle (16). On immature particles, the prM protein lies over the E protein and serves to protect the virus particle from undergoing premature fusion or inactivation within the secretory pathway of the host.
cell, prM is subsequently cleaved by a host protease to release the ectodomain and allow viral maturation (17). The E protein consists of three structural domains (D), DI, DII, and DIII (18, 19). At one end of the molecule is the fusion loop within DII, and at the other end is DIII, which is involved in host cell binding (20). Upon infection and entry of DENV into the acidic environment of the endosome, the E proteins undergo a conformational change and reassemble into 60 trimers with their fusion loops forming the tip of a trimeric spike oriented to insert into the endosomal membrane within the target cell. Subsequent reconfiguration of the E protein trimers results in fusion of the viral membrane and target cell endosomal membrane to facilitate release of the viral contents into the cytoplasm (21–23).

The nature of the human antibody response to DENV is likely to play a dominant role in defining the outcome of infection. A preponderance of antibodies that recognize neutralizing epitopes will lead to virus clearance and reduced symptoms, while an abundance of antibodies that recognize enhancing epitopes will lead to more severe disease. Multiple questions remain about the nature of the antibody balance, including which epitopes are most important for neutralization versus enhancement and whether these are distinct or overlapping epitopes.

Studies with sera from convalescent DENV patients have yielded conflicting information regarding the human antibody response and the epitopes that these antibodies target. Interestingly, while one of the predominant epitopes recognized by human serum antibodies appears to include the fusion loop and adjacent regions (24, 25), one study reported that these fusion loop antibodies are nonneutralizing (24). He et al. tested the ability of patient sera to block binding of DENV serotype 2 (DENV-2) to Vero cells and reported that neutralization occurred primarily by blocking cell attachment, suggestive of a major role for antibodies targeting DIII (26). In contrast, Wahala et al. subsequently reported that human antibodies directed toward epitopes other than DIII (presumably DI/II) are primarily responsible for neutralization (27).

Monoclonal antibodies (mAbs) have been used to further elucidate important epitopes. However, to date, most anti-DENV monoclonal antibodies are of murine origin (mAbs), generated from mice (20, 28, 29). mAbs may not accurately represent the human antibody response to DENV, as mice do not experience human disease other than a transitory viremia and produce an antibody response with more limited diversity and typically lower-affinity antibodies than humans.

Recent studies with human monoclonal anti-DENV antibodies (hMAbs) have highlighted both similarities and major differences between the behavior of sera from convalescent DENV patients and purified hMAbs. In the work of Schieffelin et al., three antibodies that targeted the E protein were isolated from a single donor (30). All three antibodies were cross-reactive with at least two DENV serotypes, one was neutralizing, and all were able to enhance DENV infection. Dejnirattisai et al. reported that in a panel of hMAbs from seven donors, the majority of the antibody response was against prM and was very poorly neutralizing but highly enhancing (31). Beltramello et al. described a wide variety of hMAbs from five DENV patients (32). They included hMAbs against prM, as well as E. However, in contrast to the findings of Dejnirattisai, et al., half of the prM hMAbs reported by Beltramello et al. showed substantial neutralization activity (32). Among the hMAbs recognizing E, Beltramello et al. described antibodies targeting DI/II and DIII. The DIII hMAbs were very highly neutralizing and included serotype-specific and cross-reactive examples. The neutralization activities of the DI/II hMAbs were more diverse and included nonneutralizing, serotype-specific neutralizing, and cross-neutralizing examples. Two of the cross-neutralizing DI/II hMAbs were mapped to the fusion loop using West Nile virus (WNV) E protein mutants.

de Alwis et al. reported that after primary infection most hMAbs were cross-reactive and weakly neutralizing and that many bound to prM (33). Using a modified screening procedure, they were able to detect rare DIII hMAbs that were serotype-specific and strongly neutralizing. Recently, de Alwis et al. reported that the majority of antibodies in human sera bound to intact virions, not nonmonomeric E (34). They found that though abundant in human sera, cross-reactive antibodies did not contribute to neutralization and that type-specific antibodies were responsible for potent neutralization. These findings were confirmed with 3 hMAbs that were isolated by first screening for antibodies that bound to intact virions and then screening for a subset of antibodies that were potently neutralizing. They generated escape mutants and mapped the mutations to the quaternary epitopes containing contacts on two different E proteins in the hinge region between DI and DII.

These studies with hMAbs emphasize the complexity of the human antibody response against DENV and highlight the importance of further examination of the roles of different epitopes in prM, in E protein DI/II (either the fusion loop or the hinge region), and in DIII and the mechanisms by which different antibodies neutralize DENV infection. For instance, an affected stage of viral entry—virus binding to the cell surface versus fusion between the viral envelope and endosomal membrane—has never been identified for any neutralizing hMAb.

In this work, we specifically screened for broadly cross-reactive and neutralizing hMAbs from three patients with distinct histories of DENV infection, and we identified three similar hMAbs that mapped to the conserved epitope containing the E protein DI/II fusion loop. These hMAbs were broadly reactive, high affinity, and conformationally sensitive. With some exceptions, they showed broad but intermediate neutralization activity against all four DENV serotypes and also enhanced all four serotypes. Using a novel assay, we confirmed that these hMAbs inhibited intracellular virus fusion during entry, rather than cell binding, and we provide a mechanistic characterization of these hMAbs.

(This study was presented in part at the 2nd Antivirals Congress, Cambridge, MA, 11 to 13 November 2012 [34a].)

**MATERIALS AND METHODS**

**Cells, viruses, and recombinant E proteins.** The *Macaca mulatta* kidney epithelial cell line LLC-MK, (American Type Culture Collection [ATCC], Manassas VA), used in neutralization assays and to propagate DENV, and the human embryonic kidney cell line HEK-293T (ATCC), used for cloning and expression of hMAbs, were grown in Dulbecco’s modified Eagle medium (DMEM) containing 10% (vol/vol) fetal bovine serum (FBS), 2 mM Glutamax (Gibco, Carlsbad, CA), 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37°C with 5% (vol/vol) CO₂. K-562 human hematopoietic cells (ATCC), used for virus enhancement assays, were grown in RPMI 1640, 10% (vol/vol) FBS, 2 mM Glutamax, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37°C with 5% (vol/vol) CO₂. The *M. mulatta* kidney epithelial cell line MA104 (ATCC) used in intracellular fusion and prefusion assays was grown in advanced DMEM reduced serum medium
obtained from Hawaii Biotech Inc. (Aiea, HI) (18,35). Recombinant strain Schneider 2 cells and purified by affinity chromatography were ing the N-terminal 80% of E protein expressed in Ultra-4 centrifugal filter unit (Millipore, Billerica, MA) in NTE buffer 32,000 rpm for 2ha t4°C, and dialyzed and concentrated using an Amicon 35% potassium sodium tartrate step density gradient in a SW-41ti rotor at min at 4°C, pelleted in a 24% (wt/vol) sucrose cushion using an SW-41ti

ruses in cell culture supernatant were precipitated in 8% (wt/vol) amino acid mixture (BioWhittaker, Lonza Walkersville, Inc., Walkersville, MD) at 37°C with 5% (vol/vol) CO2.

vial, PA) diluted 1:2,000 in PBS containing 0.5% (vol/vol) Tween 20, peroxidase-conjugated affinity-purified anti-mouse IgG (Rockland, Gilbertsville, PA) were coated with ConA at 25

ruses in cell culture supernatant were precipitated in 8% (wt/vol) polyethylene glycol (PEG) 8000 in an SLA–3000 rotor at 9,300 rpm for 51 min at 4°C, pelleted in a 24% (wt/vol) sucrose cushion using an SW-41ti rotor at 32,000 rpm for 90 min at 4°C, equilibrium banded using a 10 to 35% potassium sodium tartrate step density gradient in a SW-41ti rotor at 32,000 rpm for 2 h at 4°C, and dialyzed and concentrated using an Amicon Ultra-4 centrifugal filter unit (Millipore, Billerica, MA) in NTE buffer (120 mM NaCl, 12 mM Tris, 1 mM EDTA, pH 8.0).

Combinant DENV-1, -2, -3, and -4 soluble E protein (se) containing the N-terminal 80% of E protein expressed in Drosophila melanogaster strain Schneider 2 cells and purified by affinity chromatography were obtained from Hawaii Biotech Inc. (Aiea, HI) (18, 35). Combinant DENV-2 N-terminal E protein containing domains I and II (sDII) and DENV-2 E protein domain III (sDIII) expressed in Escherichia coli were obtained from Meridian Life Science (Saco, ME).

Patient samples. The collection and use of human blood samples for this project were reviewed and approved by the institutional review boards of Florida Gulf Coast University, Tulane University School of Medicine, and Tan Tock Seng Hospital. Informed written consent was obtained for all patients. Patient 7B was acquired DENV infection. Patient 7B had acquired DENV while traveling in Myanmar. Blood was drawn from this patient 2 years posthospitalization, as previously described (30). Patient DA003 was hospitalized in Singapore and had blood drawn approximately 4 weeks postinfection. As DENV IgG antibodies were detected, in addition to IgM antibodies, the patient was diagnosed with secondary dengue infection with low disease severity, since no hemoconcentration or bleeding was present. Patient 8C contracted DENV in Jamaica and had blood drawn approximately 3 months postinfection. The patient had fever for 12 days, headache, retro-orbital pain, and blood in sputum on day 10. No information on the type of DENV antibodies present was available from this patient. For all patients, blood was drawn after informed written consent was obtained, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque gradient centrifugation and viably frozen in liquid nitrogen. The patient sera were tested by ELISA and neutralization assays to positively determine infection with DENV.

Epstein-Barr virus transformation. The production of hMAbs by Epstein-Barr virus (EBV) transformation of B cells has been described previously (30, 36–38). Using this method, transformed clonal B cell lines were produced for hMAbs 4.8A (30) and D11C. Briefly, cryopreserved PBMCs were thawed, placed in culture, and inoculated with EBV (strain B95-8). The cells were resuspended in RPMI containing 20% (vol/vol) FBS, Primacyn (InVitrogen, San Diego, CA), and 2 µg/ml CpG 2006 (Midland Certified Reagent Co., Midland, TX) and plated at 104 cells per well in 96-well tissue culture plates.

Cloning and expression of human monoclonal antibodies. To generate hMAbs, transient stimulation of memory B cells was used as an alternative approach to EBV transformation (39). hMAb 1.6D was isol-ated using this method. Cryopreserved PBMCs were thawed and washed as described above and then resuspended in RPMI containing 20% (vol/ vol) FBS, Primacyn, 2.5 µg/ml R848, Toll-like receptor 7 (TLR7) and TLR8 agonist (InvivoGen, San Diego, CA), and 50 U/ml recombinant human interleukin-2 (Roche Diagnostics Corporation, Indianapolis, IN). After incubating for 3 days at 37°C and 5% (vol/vol) CO2, the cells were recounted and plated at 500 to 104 cells per well in 96-well tissue culture plates containing feeder cells.

To generate molecular clones of hMAbs, we constructed linear full-length Ig heavy- and light-chain gene expression cassettes as described previously (40). Molecular clones were constructed for hMAbs 1.6D, from stimulated PBMCs, and D11C, from the transformed B-cell line. Briefly, RNA extracted from hMAB-positive B cells was reverse transcribed and cloned into gene expression cassettes. Purified PCR products of the paired Ig heavy- and light-chain gene expression cassettes were cotransfected into 80 to 90% confluent HEK-293T cells grown in 48-well tissue culture plates (300 ng of each chain per well) using Eugene Transfection Reagent (Roche Diagnostics Corporation, Indianapolis, IN) following the manufacturer’s instructions. Transfection supernatants were tested for hMAB production against all four DENV serotypes by conca-navalin A (ConA) ELISA. Positive cultures were seeded into 96-well plates in DMEM with 10% (vol/vol) FBS and 20 µg/ml Blasticidin S (InvivoGen) to ensure formation of stable hMAB-producing cell lines. Cultures were cloned by serial subculture at progressively lower cell densities in 96-well plates, with repeated antibody screening at each step.

Antibody detection with enzyme-linked immunosorbent assay. B-cell cultures were screened for antibody production using ELISA as described previously (30, 36). Briefly, 96-well plates (Costar; Corning, Corn- ing, NY) were coated with ConA at 25 µg/ml in 0.01 M HEPES (Gibco) for 1 h. The wells were washed (PBS containing 0.1% [vol/vol] Tween 20), and Triton X-100-solubilized DENV produced in serum-free medium was incubated for 1 h. All steps in this ELISA were performed at room temperature. After a wash step, unreacted ConA binding sites in the wells were blocked with RPMI 1640 medium and 10% (vol/vol) FBS for 30 min. Samples from B-cell cultures were transferred to assay plates and incubated for 1 h. The wells were again washed and incubated with peroxidase-conjugated goat anti-human IgG-gamma (Zymed, San Francisco, CA) or peroxidase-conjugated affinity-purified anti-mouse IgG (Rockland, Gilbertsville, PA) diluted 1:2,000 in PBS containing 0.3% (vol/vol) Tween 20, 10% (wt/vol) whey (BiPro, Le Sueur, MN), and 10% (vol/vol) FBS for 1 h. After a final wash step, color was developed with tetramethylbenzidine-peroxide (TMB)-H2O2 as the substrate for peroxidase. The reaction was
stopped after 4 min by adding 1% (vol/vol) phosphoric acid, and color was read as the optical density (OD) at 450 nm.

Confocal microscopy. DENV-infected cells were immunostained with hMABS and imaged using confocal microscopy. LLC-MK2 cells were grown on no. 1.5 Gold Seal coverglass coverslips (Erie Scientific Company, Portsmouth, NH) placed in each well of a 6-well plate overnight to 80% confluence. Wells containing coverslips were infected with DENV in serum-free medium at a multiplicity of infection (MOI) of 0.002 for 1 h at 37°C and aspirated; fresh culture medium was added, and the coverslips were incubated for 3 days at 37°C. Infected cultures were fixed with 10% (wt/vol) formalin overnight at 4°C, permeabilized with 70% (vol/vol) ethanol for 20 min, and rinsed with PBS prior to immunostaining. Virus proteins were detected using 1 μg/ml mAb 4B6, 6A12, or 1B2 (Bio-Rad, Hercules, CA) diluted in 1% (vol/vol) normal goat serum (NGS) and 0.02% (wt/vol) Tween 20. Coverslips were mounted on a confocal microscope (Zeiss) and visualized by using a confocal imaging system (Leica). Infection levels were calculated by using ImageJ (NIH).

Western blotting. Purified DENV-2; DENV-2 sE, produced as described previously (18, 35). Hawa1i Biotech Inc., Aiea, HI); DENV-2 E sD/I; and DENV-2 E sD/I1 (Meridian Life Science, Sacramento, CA) were subjected to SDS-PAGE using 4 to 15% (wt/vol) or 15% (wt/vol) Tris-HCl polyacrylamide gel preparative gels for purification of DENV-2 and soluble recombinant proteins, respectively (Bio-Rad, Hercules, CA). Unless otherwise specified, samples were electrophoresed under nonreducing conditions in 25 mM Tris, 192 mM glycine, 3.5 mM SDS (Sigma-Aldrich, St. Louis, MO) and loaded in a buffer containing 0.7% (wt/vol) SDS. The reduced samples were loaded in a buffer containing 0.05% (wt/vol) SDS and 40 mM dithiotreitol (DTT). A Precision Plus Protein Kaleidoscope ladder was used as a standard (Bio-Rad, Hercules, CA). Proteins were transferred to Amersham Hybond-LFP polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Piscataway, NJ) in 25 mM Tris, 192 mM glycine, and 20% (vol/vol) methanol (Fisher, Pittsburgh, PA). Membrane strips were blocked in 5% (wt/vol) bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO), 0.1% (vol/vol) Tween 20 in PBS and then probed overnight at 4°C with 5 μg/ml of mAbs 4B6, 6A12, and 1B2. Membrane strips were incubated for 3 days at 37°C, 5% (vol/vol) CO₂. The final hMAB concentrations were 0.05, 0.1, 0.2, 0.4, 2, 4, 20, and 40 μg/ml (final concentration of PC and PG, 30 μg/ml) and were loaded in a buffer containing 0.7% (wt/vol) SDS. The reduced samples were loaded in a buffer containing 0.05% (wt/vol) SDS and 40 mM dithiotreitol (DTT). A Precision Plus Protein Kaleidoscope ladder was used as a standard (Bio-Rad, Hercules, CA). Proteins were transferred to Amersham Hybond-LFP polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Piscataway, NJ) in 25 mM Tris, 192 mM glycine, and 20% (vol/vol) methanol (Fisher, Pittsburgh, PA). Membrane strips were blocked in 5% (wt/vol) bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO), 0.1% (vol/vol) Tween 20 in PBS and then probed overnight at 4°C with 5 μg/ml of mAbs 4B6, 6A12, and 1B2. Membrane strips were incubated for 3 days at 37°C, 5% (vol/vol) CO₂. The final hMAB concentrations were 0.05, 0.1, 0.2, 0.4, 2, 4, 20, and 40 μg/ml. Afterward, cells were fixed, and total RNA was isolated using an RNAeasy Mini-kit (Qiagen, Valencia, CA) following the manufacturer’s protocol. A Quantitative reverse transcription (qRT)-PCR was performed on isolated RNA using a universal primer pair. The amplification conditions were 95°C for 5 s, 61°C for 20 s, and 72°C for 30 s.
22°C), Triton X-100 was added to a final concentration of 0.1% (vol/vol) to fully dequench the DiD. The efficiency of fusion is presented as the difference between fluorescence intensities measured after 10 min of co-incubation of labeled virions with liposomes at pH 5.5 and at pH 7.5 normalized to the difference between fluorescence intensities measured for fully dequenched DiD and at pH 7.5. In control experiments, we used dengue virions inactivated by an application of a histidine-modifying reagent, diethylpyrocarbonate (DEPC) (Sigma, St. Louis, MO) (2 mM; 15 min; room temperature).

**Virus intracellular fusion and precellular fusion assays.** DENV-2 virions were labeled with DiD as described above. Virus-endosome fusion events were detected as an increase in cell fluorescence upon DiD dilution (45). MA104 cells (ATCC; ∼10^5 cells/well) were grown overnight in 96-well microtiter plates (Ibidi,Verona, WI). The cells were then incubated for 30 min at 11°C, followed by 5 min at 37°C with 10^5 DI-labeled infectious DENV-2 particles that had been preincubated with hMAbs in 100 μl of serum-free ADMEM for 1 h at room temperature. Unbound DENV-2 and hMAbs were removed by washing twice with 400 μl of serum-free ADMEM, and the cells were incubated for an additional 25 min at 37°C. For each well, we captured images of 5 randomly chosen fields of view using a Zeiss Observer Z1 (oil immersion objective; 40×; Carl Zeiss Microscopy, LLC, Thornwood, NY) and generated maximum-intensity z projections based on 15 z-slices of 0.5 μm each for the subsequent analysis. The projections of the cells were analyzed using ImageJ software to subtract the background and threshold using the software’s Triangle algorithm. For each condition, we averaged fluorescence intensities in 15 fields (5 fields for each of 3 wells). The data are presented as the mean and standard deviation of the mean for the averaged intensities (n = 3) normalized to the averaged intensities measured for the cells incubated with DENV-2 in the absence of hMAbs.

After taking the images for the above-mentioned analysis, we examined the effects of the hMAbs on the total number of cell-associated virions using a novel assay that measured dequenching of DiD incorporated into unfused viral envelopes. MA104 cells incubated without DENV-2, with DENV-2 and 10 μg/ml of heparan sulfate, or with DENV-2 and 100 μg/ml of hMAb 4.8A, D11C, or 1.6D were lysed by a 15-min incubation with 0.1% (vol/vol) Triton X-100 at 37°C. The lysates were cleared by a 5-min centrifugation at 14,000 X g, and 80 μl of each supernatant was mixed with 1,920 μl of a 20 mM HEPES, 150 mM NaCl, pH 7.5, buffer. Using a Fluoromax 4 Horiba Jobin Yvon spectrophotometer (Horiba Scientific, Edison, NJ), we measured the emission fluorescence at 665 nm using an excitation wavelength of 600 nm. The data are presented as the mean and standard deviation of the mean of three independent experiments normalized to the fluorescence intensity measured for DENV-2-infected cells in the absence of hMAbs.

**Antibody binding competition enzyme-linked immunosorbent assay.** hMAbs 4.8A, D11C, and 1.6D were tested for cross-competition with each other to determine whether they recognized overlapping or non-overlapping sites on DEN-1 E protein using an enzyme-linked immunosorbent assay (30, 46, 47). Solubilized dengue E protein in detergent-treated, serum-free culture fluid was immobilized in ConA-coated wells. The plates were washed and blocked for 30 min at room temperature. Purified hMAbs or dilution buffer was incubated in the wells for 30 min at room temperature. Biotinylated hMAbs were then added to the wells at dilutions that gave less than maximal binding and incubated for 1 h at room temperature. Bound biotinylated hMAb was detected with horseradish peroxidase-streptavidin (Vector, Burlingame, CA). After a wash step, color was developed with TMB-H2O2 as the substrate for peroxidase. The reaction was stopped after 4 min by adding 1% (vol/vol) phosphoric acid, and the color was read as the OD at 450 nm.

**Antibody binding competition biolayer interferometry assay.** Real-time competition assays between purified hMAb 1.6D and purified DENV-2 sE were performed using biolayer interferometry with an Octet QK system (Fortebio, Menlo Park, CA). To determine whether the hMAbs recognized overlapping or nonoverlapping sites, we analyzed hMAb 1.6D for competition with itself, as well as with mMAbs 4G2 and 3H5.1. Anti-HIV hMAb 1.7B was used as a negative control. Twenty-five microliters per milliliter of hMAb 1.6D diluted in kinetics buffer containing 1 mM phosphate, 15 mM NaCl, 0.002% (vol/vol) Tween 20, 0.005% (wt/vol) sodium azide, 0.1 mg/ml (wt/vol) BSA, pH 7.4, in PBS was coupled with AHC biosensors (Fortebio, Menlo Park, CA). Unbound hMAb 1.6D was removed from the surfaces of the sensors by incubation in kinetics buffer. sE was preincubated with hMAb or mMAbs at a 1:1 molar ratio. hMAb 1.6D-coupled AHC sensors were then incubated with 50 nM sE, either prebound to antibodies or in kinetics buffer only. Association of sE with the hMAb 1.6D-coupled sensor was measured by light interference.

**Epitope mapping using prM/E mutants.** Mutations were introduced into the prM/E polyprotein of DENV-3 (strain CH53489) by PCR using a Diversity Mutagenesis kit (Clontech Laboratories, Inc., Mountain View, CA), sequenced, and selected to test for hMAb reactivity from a larger library of mutations. Expression plasmids encoding each mutant were transfected into HEK-293 cells, fixed in 4% (vol/vol) paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) 18 h posttransfection, and permeabilized for 45 min with 0.1% (wt/vol) saponin (Sigma-Aldrich) in PBS plus calcium and magnesium (PBS +). Cells were stained for 1 h with hMAbs 4.8A, D11C, 1.6D (0.11 μg/ml in 10% normal goat serum [NGS] [Sigma]-0.1% [wt/vol] saponin), a human polyclonal serum (1:1,000), or the anti-DENV E mMAb 1A1D-2, (1:10,000 mouse ascites fluid, kindly provided by John Roehrig, CDC) (48). Cells were washed three times with PBS + containing 0.1 (wt/vol) saponin, followed by the addition of 0.4 μg/ml horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. Following washes, Femto Substrate (Pierce) was added to each well, and luminescence values were measured after 5 min (Wallac Victor 2; PerkinElmer, Waltham, MA). All incubations were performed at room temperature. Antibody reactivities against each mutant E protein clone were 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-transfected controls. Mutations within critical clones were identified as critical to the hMAb epitope if they did not support reactivity of the test hMAb but supported reactivity of human polyclonal serum and the conformation-dependent mMAb 1A1D-2. The critical residue within critical clones that contained more than one mutation was identified by assessing other clones containing each of those mutations.

**RESULTS**

Broadly reactive anti-dengue virus antibodies were isolated from three different patients. With the goal of understanding the human antibody response in naturally occurring DENV infections, we isolated hMAbs from peripheral blood B cells obtained from patients with distinct histories of DENV infection. These three patients, 7B, 8C, and DA003, contracted DENV in geographically distinct regions, Myanmar, Jamaica, and Singapore, respectively. Cryopreserved PBMC samples were collected at different times postrecovery (approximately 2 years for 7B, 3 months for 8C, and 4 weeks for DA003). Patient DA003 was diagnosed with secondary dengue infection. All three patients were confirmed seropositive to DENV antigens, as shown in Fig. 1A and reported previously for patient 7B (30). From each patient, several hMAbs were produced, either by EBV transformation of B cells (7B and DA003) (30) or by memory B-cell stimulation, followed by molecular cloning (8C) (39, 40). To screen for hMAbs binding to glycosylated DENV proteins, we used a previously described ELISA in which Triton X-100-solubilized DENV proteins were captured in ConA-coated wells of ELISA plates (30). This selection procedure likely biased identification toward cross-reactive hMAbs recognizing the E and prM proteins. Initial selection was
The broad reactivity is illustrated in Fig. 1B for hMAbs D11C and 1.6D and in Schieffelin et al. (30) for hMAb 4.8A. Since we used a number of different methods to isolate these antibodies, we cannot determine what percentage of the total repertoire the antibodies represent. However, since we isolated the antibodies from three out of three patients with different infection histories, we can conclude that they are not uncommon. The three hMAbs were composed of IgG1 heavy chains and kappa light chains.

We further showed that the hMAbs 4.8A, D11C, and 1.6D could bind to DENV antigens expressed in DENV-1, -2, -3, or -4-infected monkey epithelial LLC-MK2 cells, using immunofluorescence assays (Fig. 1C). All three hMAbs exhibited a characteristic crescent-shaped perinuclear staining against all four DENV serotypes under fluorescence confocal microscopy. No staining was observed in uninfected cells. The low virus MOI (0.002) used allowed a clear distinction between staining of virus-infected versus noninfected cells.

Recognition of DENV E protein by human monoclonal anti-dengue virus antibodies. To confirm that the hMAbs recognize DENV E protein, we prepared Western blots using gradient-purified DENV-2 particles under reducing and nonreducing conditions and probed the blot strips with equal amounts (5 μg/ml) of hMAbs 4.8A, D11C, and 1.6D. As shown in Fig. 2A, all three hMAbs recognized a 52-kDa band consistent with the size of DENV-2 E protein in nonreduced samples. No other bands were observed. The 52-kDa band was not present in reduced samples, indicating that all three hMAbs bound to epitopes dependent on disulfide bonds. As a control, an anti-DENV capsid mMAb, D2-C2 (41), recognized bands consistent with the size of DENV-2 capsid protein in both reduced and nonreduced samples. To con-
serotypes of DENV equally well, with equilibrium dissociation constants \( (K_d) \) in the \( 10^{-9} \) to \( 10^{-10} \) M range (Table 1).

**Broadly neutralizing activities of human monoclonal anti-dengue virus antibodies.** We analyzed neutralizing antibodies in patient sera using focus-forming-unit reduction neutralization assays in monkey epithelial LLC-MK2 cells in which serial dilutions of patient sera were incubated with DENV-1, -2, -3, or -4. Sera from patients 8C and DA003 neutralized all four serotypes (Fig. 3A). Serum from patient 7B was previously reported to strongly neutralize DENV-1 and -3 and weakly neutralize DENV-2 and -4 (30). To characterize the neutralizing activities of the hMabs derived from the subjects, we performed neutralization assays with each hMAb (Fig. 3B to D). All three hMabs neutralized DENV-1 through -4 to some extent in a dose-dependent manner. Some of the hMabs were stronger neutralizers than others, whereas some neutralized specific serotypes more strongly than others. For example, the IC\(_{50}\)s of hMabs D11C and 1.6D were 1 \( \mu \)g/ml or below (Fig. 3C and D), whereas hMAb 4.8A did not reach 50% inhibition of infectivity against DENV-2 or DENV-4 over the hMAb concentrations tested (Fig. 3B). The observed neutralization activity of hMAb 4.8A was consistent with patient 7B serum activity (30). Additionally, D11C neutralized DENV-1, -2, and -4 more strongly than DENV-3 (Fig. 3C). hMAb 1.6D neutralized DENV-1 through -4 with similar activity (Fig. 3D).

To determine the neutralization potential of the hMabs against other flaviviruses, we performed neutralization assays using yellow fever virus (YF-17D) and YF-17D pseudotyped with West Nile virus E glycoprotein (Fig. 3B to D). The hMabs neutralized WNV to some extent but did not appreciably neutralize yellow fever virus.

**Antibody-dependent enhancement mediated by human monoclonal anti-dengue virus antibodies.** At certain concentrations and with the proper Fc domain, all anti-DENV antibodies have the potential to mediate antibody-dependent enhancement in Fc receptor-bearing cells *in vitro*. For neutralizing antibodies, this enhancement effect decreases as the antibody concentration increases due to the antibody’s ability to completely coat the virus and effectively neutralize it. However, for nonneutralizing antibodies, the enhancement potential remains high even at high antibody concentrations (30). To determine the antibody-dependent enhancement potential of the three hMabs, we incubated each of the four DENV serotypes with increasing concentrations of hMabs 4.8A, D11C, and 1.6D and infected the Fc receptor II-bearing human macrophage-like cell line K562. Subsequent viral replication was measured by DENV-specific qRT-PCR. In the absence of antibodies that could serve to mediate DENV infection, K562 cells were more permissive to DENV-2 infection than to DENV-1, -3, and -4. As a result, normalized enhancements were

**TABLE 1 Equilibrium dissociation constants of hMabs 4.8A, D11C, and 1.6D bound to DENV-1, -2, -3, and -4 sE**

<table>
<thead>
<tr>
<th>DENV sE bound</th>
<th>4.8A ( K_d (M) ) (mean ± SD)</th>
<th>D11C ( K_d (M) ) (mean ± SD)</th>
<th>1.6D ( K_d (M) ) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENV-1 sE</td>
<td>( 1.2 \times 10^{-9} \pm 1.6 \times 10^{-9} )</td>
<td>( 1.4 \times 10^{-10} \pm 1.2 \times 10^{-10} )</td>
<td>( 1.5 \times 10^{-10} \pm 5.0 \times 10^{-11} )</td>
</tr>
<tr>
<td>DENV-2 sE</td>
<td>( 1.3 \times 10^{-9} \pm 1.1 \times 10^{-9} )</td>
<td>( 1.2 \times 10^{-10} \pm 9.4 \times 10^{-11} )</td>
<td>( 3.5 \times 10^{-10} \pm 4.5 \times 10^{-10} )</td>
</tr>
<tr>
<td>DENV-3 sE</td>
<td>( 7.4 \times 10^{-10} \pm 7.7 \times 10^{-10} )</td>
<td>( 6.2 \times 10^{-10} \pm 3.2 \times 10^{-10} )</td>
<td>( 1.8 \times 10^{-10} \pm 8.0 \times 10^{-11} )</td>
</tr>
<tr>
<td>DENV-4 sE</td>
<td>( 7.6 \times 10^{-10} \pm 5.4 \times 10^{-10} )</td>
<td>( 2.9 \times 10^{-10} \pm 1.5 \times 10^{-10} )</td>
<td>( 2.4 \times 10^{-10} \pm 5.6 \times 10^{-11} )</td>
</tr>
</tbody>
</table>

To confirm that the hMabs bound specifically to E protein, we also prepared Western blots under nonreducing conditions using recombinant DENV-2 sE, which contains the ectodomain of the E protein, and reacted blot strips with hMabs 4.8A, D11C, and 1.6D or anti-DENV capsid mMAb D2-C2 (41) under reducing and nonreducing conditions. Binding of hMabs to DENV-2 proteins on the blot strips was detected at a PMT voltage of 400 V. Protein standards are indicated in kilodaltons. (A) Western blots were prepared with DENV-2 sE, and blot strips were probed with hMabs 4.8A, D11C, 1.6D, and control mMAbs 4G2 and 3H5.1 under nonreducing conditions. Binding of hMabs and mMAbs to DENV-2 sE on the blot strips was detected at a PMT voltage of 220 V.
typically lower for DENV-2 than for the other 3 serotypes. As presented in Fig. 4A to D, each antibody displayed a similar general trend, with a peak enhancement of infection at antibody concentrations of 0.4 to 4 μg/ml, followed by neutralization, resulting in reduced infection at increasing antibody concentrations.

Neutralizing activities of human monoclonal anti-dengue virus antibodies correlate with inhibition of fusion, not binding. Antibodies directed against virus surface proteins are predicted to inhibit an early entry step into target cells. DENV enters through receptor-mediated endocytosis, where the E glycoprotein binds to a cellular receptor on the plasma membrane, followed by endocytosis and fusion of the viral and cellular membranes in the low-pH environment of endocytic vesicles, allowing the viral genome to enter target cells. To determine the details of the mechanism of neutralization, we explored the effects of our antibodies on different stages of viral entry.

To investigate whether hMAbs could inhibit DENV-2 fusion, we used an assay that measures fusogenic activity of DENV particles toward liposomes (45, 52). DENV-2 particles labeled with a self-quenching concentration of a fluorescent lipid, DiD, were pretreated with hMAbs prior to coincubation with liposomes at acidic pH. Lipid mixing between labeled viral and unlabeled liposomal membranes was monitored as an increase in fluorescence, reflecting DiD dilution. As expected, no increase in the fluorescence, and thus no lipid mixing, was observed for virions inactivated by a histidine-modifying reagent, diethylpyrocarbonate (45). In contrast to the negative-control anti-HIV gp120 hMAb EH21, all three anti-DENV E hMAbs strongly inhibited virus-liposome fusion in a dose-dependent manner (Fig. 5A). The relative fusion-inhibiting activities of the hMAbs, with 1.6D being the most potent and 4.8A the least potent, corresponded to their relative neutralization activities (Fig. 3B to D).

Since virus-liposome fusion relies on random collisions between virions and liposomes rather than on E-mediated virion-liposome binding, the ability of hMAbs 4.8A, D11C and 1.6D to inhibit fusion between virions and liposomes suggested that viral entry in vivo might also be inhibited at the fusion stage of the entry. To test this hypothesis, we directly examined the effects of the antibodies on intracellular fusion of DENV-2 and on the prefusion stages of viral entry into rhesus macaque kidney epithelial (MA104) cells. For DENV-2 labeled with DiD at a self-quenching concentration, fusion events along the endocytic pathway dilute DiD and thus lead to an increase in fluorescence signal (45, 53, 54). We quantified the efficiency of intracellular fusion by measuring cell fluorescence with a novel microtiter plate version of the assay described previously (45). Virions were preincubated with the antibodies and then applied to the cells at 11°C for 30 min to permit binding while holding the virions in a temperature-arrested state. The temperature was then raised to 37°C to allow uptake and fusion of the virions. After the first 5 min of incubation at 37°C, we
removed unbound virions and antibodies by rinsing and, after 25 additional minutes, assayed intracellular fusion by fluorescence microscopy (Fig. 5B). Fusion of DiD-labeled virus within endosomes leads to dequenching of DiD and the appearance of brightly fluorescent intracellular structures. Cells were counterstained with DAPI (4',6-diamidino-2-phenylindole) to visualize the nuclei. All three anti-DENV hMAbs inhibited intracellular fusion in a dose-dependent manner (Fig. 5C) corresponding to their relative inhibiting activities in viral neutralization and virus-liposome fusion assays (1.6D was the most potent and 4.8A the least potent) (Fig. 3B to D and 5A). In contrast, a control anti-HIV gp120 hMAb, EH21, did not inhibit intracellular fusion. These results suggest that 4.8A, D11C, and 1.6D directly interfere with the structural transitions required for the virus to fuse to the endosomal membrane.

For virions to reach endosomes and fuse, they must first bind to the cell surface and undergo internalization. In order to test whether our hMAbs inhibited virus-cell binding, the total number of virions associated with cells must be evaluated, including (i) cell surface-bound virions, (ii) internalized but yet unfused virions, and, finally, (iii) fused virions. Note that when we measured fusion after a 30-min incubation at 37°C, fused virions represented only a small fraction of all cell-associated virions (54) and only fused virions were dequenched. After measuring the intracellular fusion efficiency, we lysed the cells and fully dequenched the DiD probe in all unfused virions, using Triton X-100 to disrupt the viral membranes. The level of unquenched DiD fluorescence was therefore proportional to the total number of cell-associated virions and thus can be used to evaluate the effects of different reagents on virus-cell binding (Fig. 5D). As expected, heparan sulfate (10 μg/ml), which inhibits DENV binding to cells (55), dramatically lowered the numbers of cell-associated virions, and consequently, the DiD fluorescence of cell lysates. Preincubation of virions with high concentrations of our hMAbs (100 μg/ml, sufficient to profoundly inhibit intracellular fusion) had no effect on cell lysate DiD fluorescence intensity, indicating that these antibodies do not appreciably affect virus-cell binding. These findings demonstrate that hMAbs 4.8A, D11C, and 1.6D block viral infection downstream of virus-cell binding at the stage of virus-endosome fusion.

Interestingly, hMAb 4.8A did not completely suppress DENV-2 fusion even at very high concentrations, correlating with the observed neutralization activity of this hMAb against DENV-2. The inability of some antibodies to completely neutralize infection and fusion has been previously reported (52, 56–58), suggesting that even at saturation these antibodies only partially neutralize the fusogenic activity of each E protein. Alternatively, the epitopes at some of the viral surface E proteins may be inaccessible, reflecting the heterogeneity of virions and/or E protein chemical environments. For all three hMAbs, inhibition of lipid mixing required somewhat higher concentrations of hMAbs than virus neutralization. This could reflect different conditions (in the neutralization assay, we used 10^2 infectious units versus 10^3 and 10^4 infectious units in liposome and intracellular fusion assays, respectively). This difference may also indicate that for DENV, as for several other viruses (59, 60), early stages of viral fusion (detected as lipid mixing in our assay) require fewer functional fusion proteins and thus are more difficult to inhibit than opening of a fusion pore large enough to release viral RNA, a prerequisite for viral infection. As a result, at neutralizing concentrations of the antibodies, virions may still have enough functional (i.e., not antibody bound) fusion proteins to mediate lipid mixing.
Taken together, our results show that hMAbs 4.8A, D11C, and 1.6D neutralize infection by inhibiting E protein-mediated membrane fusion rather than prefusion stages of viral entry. Targeting of distinct but overlapping fusion loop epitopes. To determine which E protein domain(s) hMAbs 4.8A, D11C, and 1.6D interacted with, we subjected recombinant DENV-2 E proteins sDI/II and sDIII to SDS-PAGE under nonreducing conditions and probed with equal amounts of hMAbs 4.8A, D11C, and 1.6D and control mMAbs 3H5.1 (specific for DENV-2 E DIII) or 4G2 (specific for DENV E DII fusion loop). As illustrated in Fig. 6A, hMAbs 4.8A, D11C, and 1.6D interacted specifically with sDI/II and not with sDIII.

To determine whether hMAbs 4.8A, D11C, and 1.6D bound to overlapping epitopes on E protein, we used an ELISA binding competition assay, as previously reported (30). Unlabeled hMAbs 4.8A, D11C, and 1.6D interacted with, we subjected recombinant DENV-2 E proteins sDI/II and sDIII to SDS-PAGE under nonreducing conditions and probed with equal amounts of hMAbs 4.8A, D11C, and 1.6D and control mMAbs 3H5.1 (specific for DENV-2 E DIII) or 4G2 (specific for DENV E DII fusion loop). As illustrated in Fig. 6A, hMAbs 4.8A, D11C, and 1.6D interacted specifically with sDI/II and not with sDIII.

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tive of the thickness of the antibody-sE complexes. It was anticipated that if an antibody effectively competed for binding to the hMAb 1.6D epitope, sE precomplexed with that particular antibody would not be able to bind to the hMAb 1.6D-coated sensor. In contrast, if a particular antibody bound to a different epitope on sE, the sE-antibody complex would be able to bind to the hMAb 1.6D-coated sensor. Figure 6C illustrates the individual binding curves. As expected, DENV-2 sE bound to the hMAb 1.6D-coated sensor, generating a signal proportional to the thickness of the antibody on the sensor plus the sE protein. When sE was precomplexed with hMAb 1.6D prior to addition (sE plus 1.6D), sE binding to hMAb 1.6D captured on the sensor was profoundly reduced, indicating that hMAb 1.6D can compete very effectively with itself for binding. When mMAb 3H5.1, which binds to DIII, was precomplexed with sE, the sE-3H5.1 complex bound to the hMAb 1.6D-coated sensor, resulting in an increased signal due to the increased thickness of the probe-coupled complex, which consisted of sE plus two antibodies. As a control, when an irrelevant anti-HIV hMAb (1.7B) was added to sE, the binding signal was equivalent to that of sE alone. When mMAb 4G2 was precomplexed with sE, sE bound to the hMAb 1.6D-coated sensor; however, the thickness of the complex was indicative of only sE binding to the sensor with no additional antibody. This result is likely due to effective competition of hMAb 1.6D with the mMAb 4G2 binding epitope, consistent with competition by ELISA (Fig. 6B). The antibody binding competition biolayer interferometry assay further established that hMAbs bound to epitopes on the E protein fusion loop and suggested that these hMAbs may have higher affinities than similar mMAbs.

To more precisely define the epitopes for hMAbs 4.8A, D11C, and 1.6D, we screened a library of DENV-3 E point mutants to identify mutations that reduce hMAb binding. Three residues, W101, L107, and G109, that when mutated significantly reduced 4.8A, D11C, or 1.6D binding compared to wild-type E protein were identified (Fig. 7A). These residues were located directly within the fusion loop (residues 98 to 109) and mapped in close proximity on the structure of the E protein (Fig. 7B). Each E protein mutant reacted to a human polyclonal serum and the conformation-dependent mMAb 1A1D-2 that targets a different epitope (61), confirming that each clone was expressed and was not simply globally misfolded. hMAb 4.8A binding was reduced by mutations at any of the three positions, while D11C and 1.6D binding was reduced by mutations at only W101 or G109. These data suggest that 4.8A, D11C, and 1.6D have overlapping but distinct epitopes in the fusion loop, consistent with their ability to compete with each other and with a fusion loop mMAb.

DISCUSSION

This study focused on the portion of the human antibody response that is broadly neutralizing and potentially protective against all DENV serotypes. Several other classes of DENV-neutralizing hMAbs are primarily serotype specific, including hMAbs that target E protein DIII (31–33) and hMAbs that recognize quaternary epitopes between two E proteins (34, 62). We established the mechanism of action of broadly neutralizing antibodies produced in three human dengue patients. Though the hMAbs were isolated from patients from different countries and diverse ethnic backgrounds, with different infecting viruses, and at different times postrecovery, similar broadly neutralizing hMAbs were produced, suggesting that the target of these hMAbs is a common epitope that plays an important role in DENV infectivity. With the goal of determining the mechanism of neutralization, using a novel assay, we uncoupled DENV binding to target cells from fusion and found that the neutralization activity of the hMAbs...
could not block binding by the hMAbs, indicating that the particular mMAb used either has a lower affinity than the hMAbs or that hMAbs bind to the fusion loop differently, in a manner that allows the hMAbs to displace the mMAb.

The DENV fusion loop is highly conserved, so it is not clear why hMAb 4.8A inhibited DENV-2 and -4 less strongly than DENV-1 and -3 nor why hMAb D11C inhibited DENV-3 less strongly. Additionally, other flaviviruses with nearly identical fusion loop sequences are not inhibited effectively, with hMAbs 4.8A, D11C, and 1.6D achieving only an intermediate level of neutralization against WNV and very poor neutralization against yellow fever virus. It is possible that the fusion loop region may be oriented differently or have altered accessibility in different viruses (56). These hMAbs bind to E under native conditions but do not bind denatured and reduced E protein (Fig. 2A), suggesting that disulfide bridges preserve a structural conformation of the epitopes. Additional nonconserved, fusion loop-adjacent residues may also contribute to antibody binding. Such residues could have a cumulative effect on binding energetics that is not detected when individual residues are mutated in isolation. These potential additional contact residues might be on the same E protein or part of an adjacent E protein on the virus surface. Binding to recombinant sE monomers and dissociated E protein in ELISAs and Western blots is not identical to binding the E proteins as they are arranged on the surface of a virion. E protein dimers are located in distinct symmetry positions on assembled viruses, and steric hindrance may alter the binding of antibodies to these positions, similar to observations with binding to WNV (68).

Both antibody-virus binding (56) and virus-cell binding (45) can be ineffective at 4°C, the temperature that is often used in binding assays (54, 69). Thus, while our virus-cell binding assay based on measuring DiD fluorescence can be used to quantify binding at different temperatures, we measured the efficiency of virus binding at physiological temperature. Note that, like other virus-cell binding assays (for instance, references 69 and 70), our assay does not distinguish between potential nonproductive, nonspecific binding to the cell surface and specific, productive binding between virions and a yet unidentified specific cell surface receptor for DENV, the identity of which is under debate (reviewed in reference 71). We therefore questioned whether our hMAbs might neutralize virus by inhibiting specific virus-receptor binding. We estimated that to be undetectable within the margin of error of our data, any putative specific binding would represent only a small fraction of total binding. Additionally, the hMAbs would need to inhibit only this specific binding and not the nonspecific binding, and only this specific binding would allow productive viral entry and infection. While we cannot exclude the possibility that our hMAbs block viral entry and infection by blocking unknown specific virus-receptor binding yet causing no changes in total virus-cell binding, we consider the conclusion that hMAbs block entry at the fusion stage rather than at the virus-cell binding stage to be the most likely interpretation of our data. The liposome fusion assay results also support this conclusion.

Broadly neutralizing hMAbs can potentially be used in the development of therapeutic treatments. Most previous work in this area has focused on the use of mMAbs (29). The present study has shown that, for binding to E, an anti-DENV hMAb can outcompete an mMAb with a similar epitope. This observation is not surprising, since human antibodies tend to have longer variable regions than mouse antibodies (72, 73). When used therapeuti-
cally in patients, hMABs are also much less likely to provoke an immune response, which can even be directed against the anti-
generically distinct variable regions in humanized mMABs, where the heavy- and light-chain constant regions have been replaced
with human sequences (74). A recent study using a mouse model
of lethal DENV infection showed that hMABs protected mice after
exposure to DENV, highlighting the important role that hMABs
can play in the development of DENV therapeutics (32).

While the neutralization activities reported here are lower than
those of some recently described hMABs (31–33), it is difficult to
compare neutralization potencies between assay systems in differ-
ent laboratories, as the potency can vary depending on the specific
assay used, the serotype and strain of virus, the target cell line, and
the incubation conditions of the assay (48, 75). Despite difficulties
comparing methodologies, neutralization potency alone offers an
incomplete view of the human antibody response. Given our
study and the work of others, there appears to be a wide spectrum
of hMAB responses directed against the DENV surface proteins,
ranging from potently neutralizing, serotype-specific antibodies
to nonneutralizing, cross-reactive antibodies, and many hMABs
falling between these two extremes (30–34). Using vesicular sto-
matitis virus mMABs, Bachmann et al. demonstrated that in vivo
protein was independent of immunoglobulin subclass, avidity,
and in vitro neutralization activity and that above a minimal avid-
ity threshold (≥2 × 10^7 M^{-1}), protection depended simply on a
minimum serum concentration (76). For therapeutic or protec-
tive purposes, whether it would be preferable to have multiple
serotype-specific, highly neutralizing anti-DENV hMABs or a sin-
gle cross-reactive and moderately neutralizing hMAB is currently
unknown.

We have not characterized the extent to which the virus prepar-
ations we used for our neutralization assays contain mature,
immature, or partially mature particles. Thus, we do not know if
hMABs 4.8A, D11C, and 1.6D neutralize infectivity by preferen-
tially binding to completely mature, partially mature, or com-
pletely immature virions. A previous study suggested a structural
basis for the preferential binding of fusion loop antibodies to the
partially exposed fusion loop region on immature flaviviruses (63),
but differences between the mMAB used in that study, which
bound to the bc loop in addition to the fusion loop, and our
hMABs make it difficult to speculate on the role of mature versus
immature virion structure in our results.

One of the most striking outcomes of other recent studies of
hMABs against DENV is the discovery that the response is domi-
nated by broadly reactive but nonneutralizing antibodies directed
against prM and E that serve only to enhance DENV infection in
macrophages and other Fc receptor-bearing cells (30–33). The
majority, if not all, of DENV vaccine candidates approaching or in
clinical trials contain full-length DENV prM and E proteins (77–
86). Full-length DENV prM and E proteins, whether expressed as
part of an attenuated DENV strain or expressed in another man-
ner, may induce a broadly reactive and primarily nonneutralizing
antibody response. Although both neutralizing and nonneutraliz-
ing antibodies can enhance infection, large numbers of broadly
reactive nonneutralizing antibodies could shift the response in
favor of enhancement, which may result in an increased risk of
severe disease in vaccine recipients. However, if immunogens that
present the fusion loop in the proper context can be developed, a
broadly reactive neutralizing response might be possible for a
DENV vaccine. The enhancing activity induced by such an immu-
nogen might be reduced compared to full-length prM and E.

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