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Structure and Function Analysis of Therapeutic Monoclonal Antibodies against Dengue Virus Type 2▽†

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Dengue virus (DENV) is the most prevalent insect-transmitted viral disease in humans globally, and currently no specific therapy or vaccine is available. Protection against DENV and other related flaviviruses is associated with the development of antibodies against the viral envelope (E) protein. Although prior studies have characterized the neutralizing activity of monoclonal antibodies (MAbs) against DENV type 2 (DENV-2), none have compared simultaneously the inhibitory activity against a genetically diverse range of strains *in vitro*, the protective capacity in animals, and the localization of epitopes. Here, with the goal of identifying MAbs that can serve as postexposure therapy, we investigated in detail the functional activity of a large panel of new anti-DENV-2 mouse MAbs. Binding sites were mapped by yeast surface display and neutralization escape, cell culture inhibition assays were performed with homologous and heterologous strains, and prophylactic and therapeutic activity was evaluated with two mouse models. Protective MAbs localized to epitopes on the lateral ridge of domain I (DI), the dimer interface, lateral ridge, and fusion loop of DII, and the lateral ridge, C-C′ loop, and A strand of DIII. Several MAbs inefficiently inhibited at least one DENV-2 strain of a distinct genotype, suggesting that recognition of neutralizing epitopes varies with strain diversity. Moreover, antibody potency generally correlated with a narrowed genotype and serotype specificity. Five MAbs functioned efficiently as postexposure therapy when administered as a single dose, even 3 days after intracranial infection of BALB/c mice. Overall, these studies define the structural and functional complexity of antibodies against DENV-2 with protective potential.

Dengue virus (DENV), a member of the *Flaviviridae* family of RNA viruses, is related to several other human pathogens of global concern, including yellow fever and tick-borne, West Nile, and Japanese encephalitis viruses. DENV infection in humans occurs after *Aedes aegypti* or *Aedes albopictus* mosquito inoculation and results in clinical disease, ranging from a febrile illness (dengue fever [DF]) to a life-threatening hemorrhagic and capillary leak syndrome (dengue hemorrhagic fever [DHF]/dengue shock syndrome [DSS]). Globally, there is significant diversity among DENV strains, including four distinct serotypes (DENV type 1 [DENV-1], DENV-2, DENV-3, and DENV-4) that differ at the amino acid level by 25 to 40%. Additional complexity occurs within each serotype, as genotypes vary from one another by up to 3% at the amino acid level (21, 49). No approved antiviral treatment is currently available, and several candidate tetravalent vaccines remain in clinical development (reviewed in reference 11). Because of the increased geographic range of its mosquito vectors, urbanization, and international travel, DENV continues to spread worldwide and now causes an estimated 50 to 100 million infections and 250,000 to 500,000 cases of DHF/DSS per year, with 2.5 billion people at risk (68).

DENV is an enveloped icosahedral virus with a single-stranded, positive-polarity RNA genome. The 10.7-kb genome is translated as a single polyprotein, which is cleaved into three structural proteins (capsid [C], premembrane/membrane [prM/M], and envelope [E]) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) by host and viral proteases. The mature DENV virion is ~500 Å in diameter, with a highly organized outer protein shell, a 50-Å lipid membrane bilayer, and a nucleocapsid core (26). Mature DENV virions are covered by 90 anti-parallel E protein homodimers, arranged flat along the surface with quasi-icosahedral symmetry. The immature virion, which lacks cleavage of the prM protein, has a rough surface with 60 spikes each composed of three prM-E heterodimers (7, 73). Exposure to mildly acidic conditions in the *trans*-Golgi network promotes virus maturation through a structural rearrangement of the

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flavivirus E proteins and cleavage of prM to M by a furin-like protease (29, 66, 69, 70). The ectodomain of DENV E protein is comprised of three discrete domains (34–36, 39). Domain I (DI) is a central, eight-stranded β-barrel, which contains a single N-linked glycan in most DENV strains. DII is a long, finger-like protrusion from DI, with the highly conserved fusion peptide at its distal end and a second N-linked glycan that recognizes DC-SIGN (37, 38, 46, 59). DIII, which adopts an immunoglobulin-like fold, has been suggested to contain cell surface receptor recognition sites (5, 64, 71). Several groups have recently defined contact residues for type-specific, subcomplex-specific, and cross-reactive monoclonal antibodies (MAbs) that recognize DIII of DENV-2 (16, 17, 31, 47, 57, 61). Type-specific MAbs with neutralizing activity against DENV-2 localized to the BC, DE, and FG loops on the lateral ridge of DIII, whereas subcomplex-specific MAbs recognized an adjacent epitope centered on the connecting A strand of DIII at residues K305, K307, and K310.

To date, no study has compared the *in vitro* inhibitory activity of MAbs in cells against a genetically diverse range of DENV-2 strains and their protective capacity in animals. Here, we had the goal of generating strongly neutralizing MAbs that would recognize virtually all DENV-2 strains and function as a possible postexposure therapy. Twenty-four new anti-DENV-2 mouse MAbs were generated with moderate or strong neutralizing activity against the homologous virus in cell culture assays. Binding sites were mapped for the majority of these by yeast surface display, identifying distinct epitopes in regions in DI (lateral ridge), DII (dimer interface, lateral ridge, and fusion loop), and DIII (lateral ridge, C-C′ loop, and A strand). Several MAbs failed to neutralize efficiently at least one DENV-2 strain of a distinct genotype, suggesting that antibody recognition of neutralizing epitopes varies among DENV-2 genotypes.

To begin to assess the utility of this new panel of inhibitory MAbs as possible therapeutics against DENV-2, we evaluated their protective capacity in a stringent intracranial challenge model in BALB/c mice. Among the 16 neutralizing MAbs tested in mice, most were protective when given as prophylaxis. Seven of these had postexposure therapeutic activity when administered as a single dose by intraperitoneal route even 3 days after intracranial infection. For the MAbs with the greatest therapeutic potential, protection was confirmed with an antibody-enhanced vascular leakage mouse model (2, 72) of DENV-2 infection.

MATERIALS AND METHODS

Cells and viruses. BHK21 and HEK-293T cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Omega Scientific) and antibiotics (penicillin G and streptomycin). Raji-DC-SIGN-R cells were cultured in RPMI-1640 medium supplemented with 10% FBS and antibiotics. DENV-2 strains used in this study included 16681 (Southeast Asian genotype), C0477 (Southeast Asian genotype), New Guinea C (NGC) (Southeast Asian genotype), D2S10 (Southeast Asian genotype), ArA6894 (Indian genotype), IQT2913 (American genotype), and PM33974 (West African genotype). DENV strains from other serotypes were obtained from colleagues (A. de Silva, University of North Carolina, and R. Tesh, University of Texas Medical Branch) and included 16007 (DENV-1), 16652 (DENV-3), and 1036 (DENV-4). All viruses were propagated in C6/36 *Aedes albopictus* cells according to described protocols (57).

E gene sequencing. The virus nucleotides from positions 616 to 2578 were amplified by reverse transcription (RT)-PCR using a high-fidelity polymerase and RNA extracted directly from infected C6/36 cells. Each amplicon was sequenced on both strands by conventional capillary sequencing on an Applied Biosystems 310 genetic analyzer using previously reported primers (28). The resulting sequence reads were assembled, and the E gene sequences aligned using the Lasergene suite (DNASTAR).

Generation, purification, and labeling of anti-DENV-2 MAbs. To generate anti-DENV-2 MAbs, alpha/beta interferon receptor-deficient (IFN-α/βR$^{-/-}$) C57BL/6 mice were infected with 10^5 PFU of a mixture (1:1) of DENV-2 strains 16681 and NGC via an intraperitoneal route and rechallenged 2 weeks later with the same strains. Mice with serum showing the highest binding titer to Raji-DC-SIGN-R cells infected with DENV-2 strain 16681 (~1:10,000) were immunized with purified DIII (50 μg, strain 16681 [31]) in phosphate-buffered saline (PBS) as a final intravenous boost. Three days later, splenocytes were fused to P3X63Ag8.6.5.3 myeloma cells using polyethylene glycol 1500 (20). Hybridomas producing anti-DENV-2 MAbs were subcloned by limiting dilution, isotyped using an enzyme-linked immunosorbent assay (ELISA) kit (Southern Biotech) and purified by protein A or protein G immunoaffinity chromatography. For *in vivo* studies, MAbs were purified by a second size exclusion chromatography step and then concentrated using a Centricon centrifugal concentrator (Millipore). For some experiments, MAbs were labeled directly with Alexa Fluor 647 or Alexa Fluor 488 (Invitrogen) according to the manufacturer's instructions.

***In vitro* neutralization assays.** Three virus neutralization assays were used. (i) Plaque reduction neutralization titer (PRNT) assays were performed with the different DENV-2 strains and MAbs on BHK21 cells as described previously (10, 57). Fifty-percent PRNT ($PRNT_{50}$) values were determined using nonlinear regression analysis and expressed as nanograms per milliliter of antibody. (ii) To assay whether MAbs inhibited at a pre- or postattachment step, a PRNT assay was performed with the following modifications. In the postattachment assay, after cells and solutions were prechilled to 4°C, 10^2 PFU of DENV-2 was added to cells and viral adsorption was allowed for 1 h at 4°C. Wells were washed thrice with cold media to remove unbound virus, and MAb was added at the specified concentrations. Virus-antibody complexes were allowed to form for 1 h at 4°C and then were washed three times with cold media. Cells were warmed to 37°C, and the PRNT assay was completed. For the preattachment assay, a PRNT assay with all cells and solutions at 4°C, in which MAb DENV-2 were mixed for 1 h at 4°C or 37°C prior to addition to cells, was performed. (iii) DENV-2 reporter virus particles (RVPs) were produced as described previously (1). Briefly, HEK-293T cells were transfected with plasmids encoding DENV-2 strain 16681 C-prM-E and a West Nile virus (WNV) replicon to generate DENV-2 RVPs. In some experiments, the C-prM-E plasmid was mutated using the QuikChange site-directed mutagenesis kit (Stratagene) to introduce amino acid substitutions in the E gene. Neutralization assays were performed as described previously (1, 45), and infection was measured by flow cytometry at 48 h after infection. The 50% effective dose (EC_{50}) of each antibody was calculated using nonlinear regression analysis.

Domain and fine epitope mapping by yeast surface display. The DNA fragments encoding amino acid residues 1 to 292 (DI-DII) and 293 to 409 (DIII) of the DENV-2 E protein were amplified from DENV-2 strain 16681 by RT-PCR with KpnI and XhoI or BamHI and XhoI sites added at the 5′ or 3′ end, respectively. The PCR product was cloned as a downstream fusion to Aga2 and Xpress epitope tag genes in the yeast surface display vector pYD1 (Invitrogen) and transformed into *Saccharomyces cerevisiae* strain EBY100 (Invitrogen) to generate yeast that expressed DENV-2 DI-DII or DIII as described previously (42, 57).

To generate random variant libraries, DENV-2 DI-DII or DIII was mutated by error-prone PCR, using the GeneMorph II random mutagenesis kit (Stratagene). The libraries were ligated into the pYD1 vector and transformed into XL2-Blue ultracompetent *Escherichia coli* cells (Stratagene). The colonies were pooled and transformed into yeast. For mapping of each individual MAb, the relevant DENV-2 DI-DII or DENV-2 DIII library was screened according to a previously described protocol (42, 57). For individual clones that lost binding to the desired MAb, the mutant pYD1-DV2 DI-DII or DIII plasmids were recovered, transformed into XL1-Blue-competent cells (Stratagene), purified using a QIAprep spin miniprep kit (Qiagen), and sequenced. In some cases, variants with multiple mutations were isolated. To determine which mutation conferred the phenotype, single mutations were engineered using a QuikChange II mutagenesis kit (Stratagene).

Immunostaining of DENV-infected cells. To assess binding of DENV-2 MAbs to different DENV strains, Raji-DC-SIGN-R cells were infected at a multiplicity of infection (MOI) of 0.5 or 1. Depending on the strain, Raji-DC-SIGN-R cells were harvested 48, 72, or 96 h after infection. Cells were washed, fixed in PBS

with 1% paraformaldehyde, permeabilized, incubated with MAbs, and processed by flow cytometry as described previously (53).

Generation of MAb-resistant virus escape mutants. DENV-2 (16681 strain, 5×10^5 PFU) was incubated with 25 μg/ml of MAb DV2-48 or DV2-67 for 1 h at 37°C in DMEM. The mixture was added to 5×10^5 BHK21 cells in a 6-well plate. After infection for 2 h at 37°C, wells were washed thrice with DMEM, and fresh media containing 25 μg/ml of MAb were added. Virus growth under antibody selection proceeded for 72 h at 37°C. At each passage, half of the supernatant was mixed 1:1 with 50 μg/ml of MAb for 1 h. The remaining half was stored at −80°C. After three to six passages under MAb selection, virus-containing supernatants were tested by plaque reduction assay for escape from neutralization of DV2-48 or DV2-67. After the escape phenotype was confirmed, an aliquot of the supernatant was used for a BHK21 cell plaque assay under MAb selection. Plaque-purified virus was amplified further under MAb selection (25 μg/ml) overnight at 37°C. BHK21 cells were scraped from wells, and total cellular RNA was isolated using an RNeasy kit (Qiagen). cDNA was amplified with random hexamers as well as primer DENV-2 3756R (5′-TCAAGAGTAGTCC AGCTGCAAA-3′) using a Superscript III first-strand synthesis system for RT-PCR (Invitrogen) and served as a template for PCR amplification using forward (609F, 5′-GCAGAATGAGCCAGAAGACAT-3′) and reverse (2784R, 5′-TGC TTTGCCCCATGTTTTC-3′) primers. Amplicons were sequenced, and the neutralization escape mutant sequence was compared to that of the parent virus stock that was passaged in parallel in the absence of MAb selection.

Mouse experiments. All mouse studies were approved and performed according to the guidelines of the Washington University School of Medicine Animal Safety Committee or the University of California, Berkeley Animal Safety Committee. IFN-α/βR$^{-/-}$ mice on the C57BL/6 background were a gift of Jonathan Sprent (The Scripps Research Institute). The IFN-α/βR$^{-/-}$ × γR$^{-/-}$ mice on a 129 Sv background (AG129) have been described previously (52). BALB/c mice were purchased commercially (Jackson Laboratories). All mice were housed in pathogen-free barrier facilities. In prophylaxis experiments, mice were administered a single dose of individual MAbs via an intraperitoneal route 1 day before infection. BALB/c and AG129 mice were challenged with DENV-2 NGC (Southeast Asian genotype, 6×10^4 PFU) intracranially or DENV-2 D2S10 (Southeast Asian genotype, 10^5 PFU) intravenously, respectively, and mortality was monitored. In postexposure therapeutic experiments, a single dose of MAb was administered by intraperitoneal injection 2 or 3 days after infection.

Statistical analysis. All data were analyzed using Prism software (GraphPad Prism, San Diego, CA). Kaplan-Meier survival curves were analyzed by the log rank test. For neutralization assays, an unpaired Student *t* test was used to determine significance.

Nucleotide sequence accession numbers. Two new E gene sequences, C0477 and ArA6894, were deposited in GenBank with accession numbers HM234641 and HM234642.

RESULTS

Generation of MAbs against DENV-2. Previous experiments identified type- and subcomplex-specific MAbs of DENV-2 that neutralized infection in cell culture and localized to DIII of the E protein (16, 17, 57). However, these studies did not assess the *in vivo* efficacy of these antibodies nor evaluate their ability to neutralize a diversity of DENV-2 strains. As a first step toward generating antibody therapeutics that protect against genetically diverse DENV-2 strains, we generated a larger panel of DENV-2-specific MAbs by infecting mice with a mixture of 16681 (Southeast Asian genotype) and NGC (Southeast Asian genotype) DENV-2 strains and boosting with homologous virus or recombinant DIII. After screening more than 3,000 hybridoma clones as part of four independent fusions, 57 new MAbs that recognized cells infected with DENV-2 were isolated (see Table S1 in the supplemental material).

All MAbs were tested by ELISA for binding to recombinant E protein or Western blot immunoreactivity against DENV-2-infected cell lysates and for binding to permeabilized BHK21 cells propagating a subgenomic replicon that expressed only the nonstructural proteins (41). We identified 33 new MAbs that recognized E protein and 21 that recognized nonstructural proteins; the remaining MAbs (DV2-16, DV2-34, and DV2-47) recognized DENV-2-infected cells but could not be assigned to a specific protein, as they failed to bind to purified recombinant proteins by ELISA or Western blot analysis (data not shown).

MAbs that recognized the DENV-2 E protein were tested semiquantitatively for neutralization of DENV-2 16681 by single endpoint plaque reduction assay using undiluted hybridoma supernatant. Of the 33 MAbs that mapped to the E protein, 7 showed no neutralization activity (0% neutralization), 2 had modest inhibitory activity (50 to 90% neutralization), and 24 were strongly neutralizing (>90% neutralization). MAbs also were screened for E protein domain recognition using yeast that expressed DENV-2 DI-DII, DIII, or the entire ectodomain of E (DI-DII-DIII) on their surface (see Table S1 in the supplemental material): 13 bound to yeast expressing DI-DII and 17 recognized DIII on yeast. A total of 13 of the DI-DII-specific MAbs and 11 of the DIII-specific MAbs showed strong inhibitory activity.

Cross-reactivity with other serotypes. To begin to determine the breadth of the inhibitory potential of the MAbs, we assessed cross-reactivity of binding using Raji-DC-SIGN-R cells infected with different serotypes of DENV. Three DI-DII-specific (DV2-29, DV2-30, and DV2-52) and one DIII-specific (DV2-77) strongly neutralizing MAb cross-reacted with all other serotypes of DENV, with some recognizing distantly related flaviviruses (e.g., WNV). Additionally, two DI-DII specific (DV2-40 and DV2-53) and two DIII-specific (DV2-76 and DV2-94) neutralizing MAbs were subcomplex-specific and recognized a subset of DENV serotypes. Eight DI-DII-specific and eight DIII-specific strongly neutralizing MAbs were type specific and reacted only with DENV-2-infected cells (see Table S1 in the supplemental material).

Genotype-specific neutralization. Globally, there is diversity among DENV strains, including distinct serotypes (DENV-1, -2, -3, and -4) that differ at the amino acid level by 25 to 40%, and variation within each serotype, with genotypes varying by *in vitro* and *in vivo* phenotypes (21, 49). We examined the neutralizing potential of anti-DENV-2 MAbs against individual strains that corresponded to heterologous DENV-2 genotypes: ArA6894 (Indian genotype), IQT2913 (American genotype), and PM33974 (West African genotype). We initially assessed the inhibitory activity of neat hybridoma supernatants of the 24 neutralizing MAbs by a standard plaque reduction assay. With the exception of DV2-67, all MAbs showed strong neutralization activity (75% or greater) against the Indian and American genotypes (data not shown). Nonetheless, we observed decreased neutralization activity by several MAbs against the West African genotype (PM33974). This virus strain is classified as a sylvatic DENV that is transmitted primarily between nonhuman primates and mosquitoes; viruses belonging to this genotype have not been associated with outbreaks of severe disease in humans (48).

Although the semiquantitative neutralization results were informative, we expanded on them by purifying 18 MAbs and performing a dose-response analysis to define the concentration of MAb that blocked plaque formation by 50% (the $PRNT_{50}$, here expressed as ng/ml of antibody) (Table 1). In addition to strains corresponding to the homologous and het-

TABLE 1. MAb neutralization of homologous and heterologous DENV-2 strains[a]

MAb	Domain	$PRNT_{50}$[b]					
		16681	NGC	C0477	ArA6894	IQT2913	PM33974
DV2-29	DI-DII	852	182	1,189	154	NT	NT
DV2-30	DI-DII	190	500	500	321	71	996
DV2-36	DI-DII	1,212	>5,000	>5,000	614	NT	NT
DV2-40	DI-DII	869	4,500	>5,000	1,204	NT	NT
DV2-44	DI-DII	204	280	324	91	13	231
DV2-46	DI-DII	2,069	1,500	634	126	NT	NT
DV2-48	DI-DII	731	267	1,440	1,254	NT	NT
DV2-51	DI-DII	1,176	936	>5,000	419	NT	NT
DV2-52	DI-DII	876	491	296	204	NT	NT
DV2-58	DI-DII	2,467	>5,000	200	653	>5,000	2,351
DV2-70	DIII	168	214	133	375	NT	NT
DV2-73	DIII	92	2,800	827	453	NT	NT
DV2-76	DIII	29	185	303	92	9	72
DV2-77	DIII	200	311	32	280	NT	NT
DV2-87	DIII	76	200	111	232	11	486
DV2-96	DIII	67	106	34	69	146	1,648
DV2-104	DIII	76	228	79	320	81	232
DV2-106	DIII	311	60	79	192	1	2,061

[a] Neutralizing activity was determined by plaque reduction assay on BHK21 cells with increasing concentrations of purified MAbs and 10^2 PFU of the indicated DENV-2 genotype (16681, Southeast Asian; NGC, Southeast Asian; C0477, Southeast Asian; ArA6894, Indian; IQT2913, American; and PM33974, West African). The data were derived from two or three independent experiments performed in duplicate.

[b] $PRNT_{50}$ values were calculated by nonlinear regression analysis and are expressed as ng/ml of antibody. NT, not tested.

erologous genotypes mentioned above, we performed experiments with a low-passage Southeast Asian genotype strain (C0477) and with NGC, as this Southeast Asian strain was used for immunization and therapeutic studies *in vivo* (see below). In general, DIII-specific MAbs showed higher neutralization activity against the homologous strain (16681) than DI-DII-specific MAbs, with most (6 of 8 MAbs) having $PRNT_{50}$ values below 200 ng/ml. Neutralization levels of other strains (C0477 and NGC) of the homologous Southeast Asian genotype strains were generally comparable, with some exceptions: DV2-58 and DV2-77 showed stronger (6- to 12-fold) inhibitory potential against C0477, DV2-106 had stronger (11- to 14-fold) neutralizing activity against NGC and C0477, and DV2-73 was less potent (9- to 30-fold) against NGC and C0477.

In the analysis of neutralization of heterologous DENV-2 genotypes, most MAbs, with the exception of DV2-36, DV2-40, DV2-48, and DV2-58, neutralized the Indian genotype strain (ArA6894) efficiently ($PRNT_{50}$ below 600 ng/ml). Somewhat surprisingly, DV2-46 neutralized ArA6894 to a greater extent (5- to 16-fold) than the homologous Southeast Asian genotype strains against which it was raised. A subset of the strongly neutralizing MAbs (DV2-30, DV2-44, DV2-58, DV2-76, DV2-87, DV2-96, DV2-104, and DV2-106) was tested for neutralization of strains corresponding to the American and West African genotypes (Fig. 1 and Table 1). With the exception of DV2-58, the remainder of the MAbs strongly neutralized infection by the American genotype strain (IQT2913), with $PRNT_{50}$ values less than 150 ng/ml. Remarkably, DV2-106 had >60-fold greater neutralizing activity against IQT2913 than any of the strains of the Southeast Asian genotype. However, several MAbs (DV2-30, DV2-87, DV2-96, and DV2-106) inhibited infection of the West African genotype strain (PM33974) less efficiently (up to 25-fold-lower neutralization potency) than other genotypes.

Epitope mapping of neutralizing MAbs using yeast display. To understand in greater detail the disparity in inhibitory potential of the neutralizing MAbs, we mapped the amino acid residues required for MAb binding using yeast surface display (Table 2). Two approaches were used. (i) For forward genetics, a library of DI-DII and DIII variants of strain 16681 was generated using random mutagenesis for flow cytometry-based screens to identify residues that comprise MAb epitopes. (ii) For reverse genetics, based on prior studies that mapped DIII-specific neutralizing MAbs against DENV-2 (16, 17, 31, 57), mutations were engineered (S300T, K307E, V309K, K310E, D329G, G330D, K361E, E383R, P384N, and K393R) on residues of the BC, DE, and FG loops and the A β-strand (A strand) of DIII of DENV-2 strain 16681, and these variants also were displayed on the surface of yeast.

(i) DI-DII-specific MAbs. The highly conserved fusion loop in DII has been identified as a recognition site for moderately and weakly neutralizing cross-reactive MAbs (7, 8, 15, 43, 58). Consistent with this, four of our DI-DII-specific neutralizing MAbs localized to sites within this loop of DII (Fig. 2A and Table 2). Mutation of W101, G106, or L107 reduced binding of cross-reactive (DV2-29)-, complex (DV2-30 and DV2-52)-, and subcomplex (DV2-36)-specific neutralizing MAbs. The yeast display screening of variant DI-DII also identified the dimer interface on DII as a second epitope for neutralizing DENV-2 MAbs. Binding of MAbs DV2-30, DV2-40, DV2-44, DV2-46, and DV2-58 was reduced or abolished by mutations at residue K88, Q233, or H244. We also identified by yeast surface display one type-specific neutralizing MAb (DV2-51) that mapped to a site in DI at residue E184.

(ii) DIII-specific MAbs. The amino acid residues K305, K307, and K310 on the A strand of DIII have been defined as recognition sites for some subcomplex- and type-specific MAbs (17, 57). In agreement with this, several of the newly generated

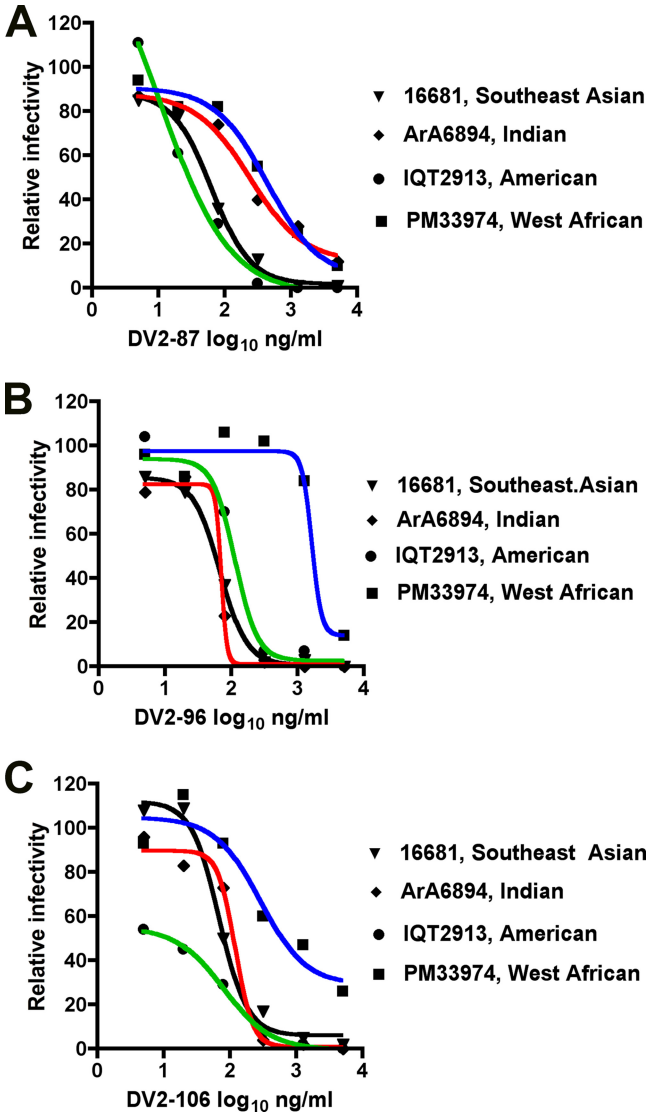

FIG. 1. MAb neutralization of different DENV-2 genotypes. Increasing concentrations of purified DV2-87 (A), DV2-96 (B), and DV2-106 (C) were mixed with 10^2 PFU of DENV-2 strains corresponding to all four genotypes, and neutralization was assessed by a standard PRNT assay in BHK21 cells. Graphs were generated after regression analysis using statistical software. The data are representative of at least three independent experiments.

DIII-specific neutralizing MAbs localized to residues along the A strand (Fig. 2B and Table 2). The subcomplex-specific neutralizing MAb DV2-76 showed moderately reduced binding after mutation of the A-strand residue K310. Both type-specific (DV2-70, DV2-96, and DV2-106) and subcomplex-specific (DV2-76) MAbs were affected by residue changes at positions K305 and K307, which lie at the junction of the N-terminal linker and the A-strand regions. Several MAbs (DV2-70, DV2-76, and DV2-96) also lost binding when residue V309 was changed. Although the type-specific neutralizing MAbs DV2-70 and DV2-96 map to residues in the conserved A strand (K305, K307, or V309) in a manner analogous to that of the subcomplex-specific MAbs, they were distinguished

TABLE 2. Summary of MAb binding to DENV-2 DI-DII or DIII mutants expressed on the surface of yeast[a]

MAb	T55A	E71K	K88E	W101R	G106R	L107D	E184D	Q233H	H244Q	Y299N	S300T	M301G	T303Y	K305E	K307E	V309K	K310E	E311R	I312F	T315G	H317R	R323E	E327R	D329G	G330D	S331Y	K334N	P336H	E338D	M340K	H346P	H346Q	T359I	K361E	P364A	N366T	I367A	D375E	E383R	P384N	L389A	N390Y	W391A	K393R
DI-DII variants																																											
DV2-29	66	61	74	**9**	**6**	**6**	89	75	73																																		
DV2-30	**3**	**11**	54	64	56	**4**	100	100	**3**																																		
DV2-36	45	44	49	41	19	17	88	83	88																																		
DV2-40	29	24	**15**	78	60	52	40	**5**	46																																		
DV2-44	58	47	**20**	100	87	65	53	**8**	88																																		
DV2-46	63	53	2	99	89	78	66	**8**	70																																		
DV2-51	100	59	80	100	97	100	**8**	100	95																																		
DV2-52	82	68	88	**17**	91	29	94	87	100																																		
DV2-58	30	28	**7**	100	64	51	29	**6**	65																																		
DIII variants																																										
DV2-70	70	100	86	38	**<1**	**<1**	**<1**	76	74	86	81	72	99	**11**	75	31	55	100	100	57	69	95	97	52	67	85	87	84	72	100	85	**5**	52	76	93
DV2-73	85	91	64	90	82	58	100	93	78	94	100	71	100	100	89	89	92	95	**3**	93	**<1**	**3**	**3**	82	90	84	95	100	86	100	86	94	85	93	93
DV2-76	89	67	100	70	**8**	**10**	**7**	26	53	100	74	51	96	38	98	64	100	100	100	100	90	100	100	90	75	97	97	93	95	100	89	58	79	99	100
DV2-77	85	100	42	49	78	77	90	100	85	93	75	73	95	84	78	61	55	93	**<1**	73	**<1**	**<1**	**<1**	85	81	100	88	91	88	100	92	77	100	99	87
DV2-87	78	88	70	94	79	72	90	93	100	98	92	89	97	82	82	87	83	90	**2**	100	**<1**	**4**	**3**	79	72	97	100	79	93	100	92	100	73	99	93
DV2-96	83	73	100	34	**2**	**<1**	**<1**	93	82	91	87	40	94	**20**	100	51	63	96	100	100	88	100	100	64	71	95	94	57	97	99	86	**16**	77	80	95
DV2-104	84	98	74	100	84	73	84	92	90	98	76	100	93	94	82	100	80	95	**<1**	97	**<1**	**<1**	**<1**	100	70	99	92	76	90	100	93	99	73	90	90
DV2-106	83	62	97	24	**<1**	**<1**	29	86	48	79	91	95	94	**14**	92	**17**	55	100	100	98	99	100	100	42	83	97	91	23	100	100	89	**<1**	75	71	95

[a] Values shown were achieved by dividing the total fluorescence product (percent positive population × mean linear fluorescence intensity) of a mutant for each individual antibody by the total fluorescence product of the wild-type DI-DII or DIII × 100. Values in bold indicate reductions in MAb binding of greater than or equal to 80% for a given mutation. Underlined values show a reduction between 60 and 79%. The results are the average of between three and five independent experiments for each mutant and each antibody.

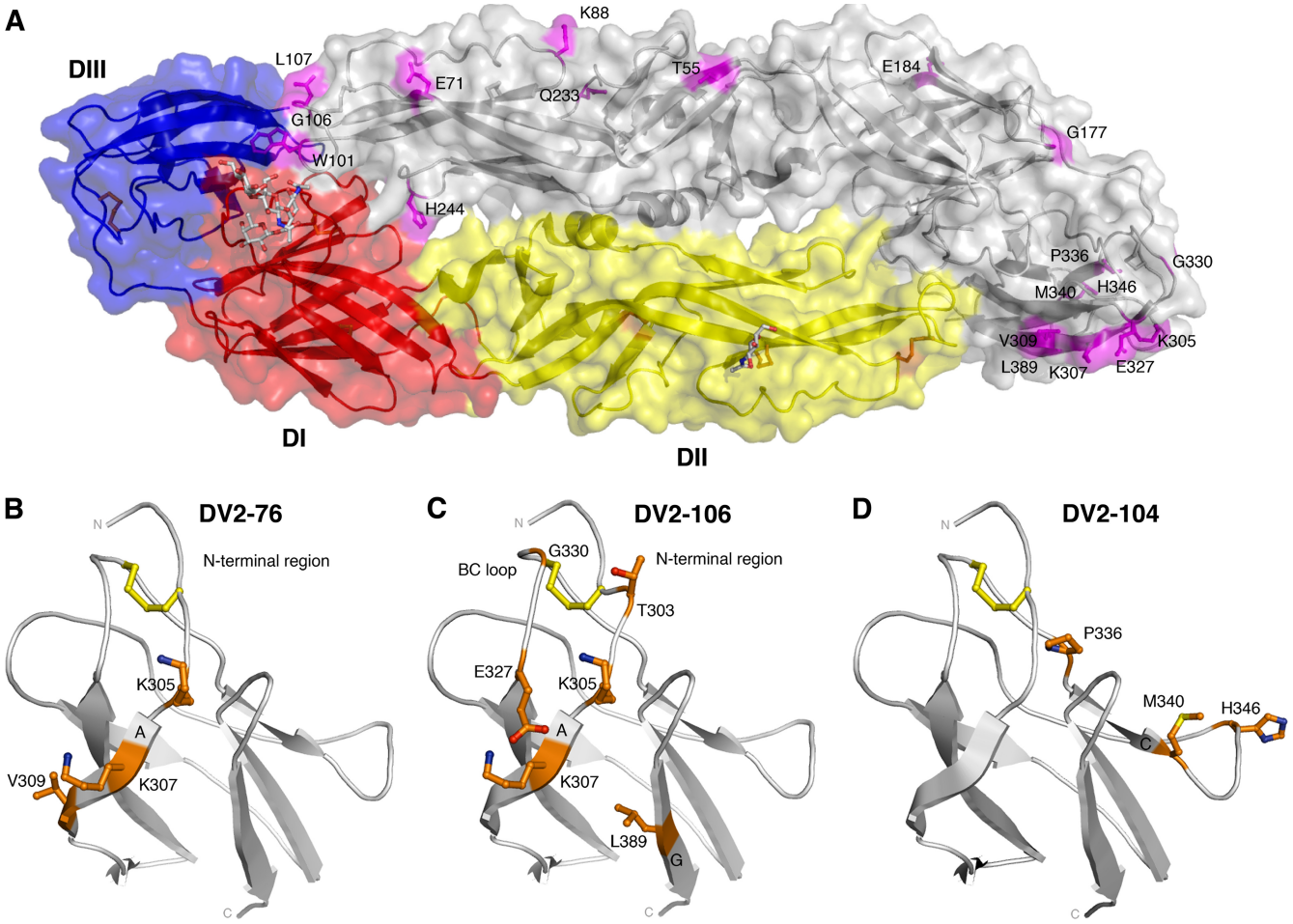

FIG. 2. Epitope mapping of anti-DENV-2 MAbs on the E protein. (A) Localization of neutralizing epitopes on DENV-2 DI-DII as determined by yeast surface display. Structure of DENV-2 DI-DII (strain 16681), with amino acid residues that significantly affect binding of indicated neutralizing MAbs marked. (B to D) Localization of neutralizing epitopes on DENV-2 DIII as determined by yeast surface display. Structure of DENV-2 DIII (strain 16681), with amino acid residues that affect binding of representative neutralizing MAbs marked in orange. (B) DV2-76 (A strand); (C) DV2-106 (lateral ridge); (D) DV2-104 (C-C′ loop).

by an additional recognition site in the BC loop at position E327. Thus, some type-specific neutralizing MAbs recognize an epitope comprised of amino acids in the N-terminal linker, BC loop, and A strand (Fig. 2C), whereas subcomplex neutralizing MAbs localize more directly to amino acids on the A strand.

The amino acid residues E383 and P384 in the FG loop were previously identified as binding sites for type-specific neutralizing DENV-2 MAbs that mapped to the DIII-lateral-ridge epitope (16, 57). Surprisingly, none of the newly generated strongly neutralizing MAbs lost binding when these residues were changed. Instead, two groups of type-specific neutralizing MAbs that recognized distinct regions were identified in this study. DV2-70, DV2-96, and DV2-106 bound to sites along the lateral ridge of DIII, including the N-terminal linker, the BC loop, the top region of the A strand, and a residue in the G strand. A second group of neutralizing MAbs (DV2-73, DV2-77, DV2-87, and DV2-104) showed loss of binding, with mutations in three residues that have not been described previously as sites for neutralizing antibody recognition (Fig. 2D). The P336 residue is found at the junction of the BC loop and the C strand and is conserved among all DENV serotypes. M340 and H346 are located in the C strand and C-C′ loop, respectively, and are unique to the DENV-2 serotype.

Epitope mapping of MAbs using neutralization escape and reverse genetics. As two MAbs (DV2-48 and DV2-67) recognized a determinant on DENV-2 that was not present on yeast-displayed forms of E, we generated neutralization escape mutants to define their epitopes. After sequential virus passage on BHK21 cells under DV2-48 or DV2-67 selection, these MAbs lost the ability to neutralize DENV-2 infection (Fig. 3A). To identify the mutations that conferred the escape phenotype, the viral sequence from plaque-purified escape variants was compared to that for virus passaged in parallel in the absence of MAb selection. All (4 of 4) sequences from the DV2-48 escape variants contained the same single nucleotide change encoding a G177D mutation in the E protein. In contrast, of the DV2-67 escape variants that were sequenced, three contained a single mutation (M196V), and one contained two amino acid changes (K291R and K307R).

To establish which amino acid substitutions conferred the

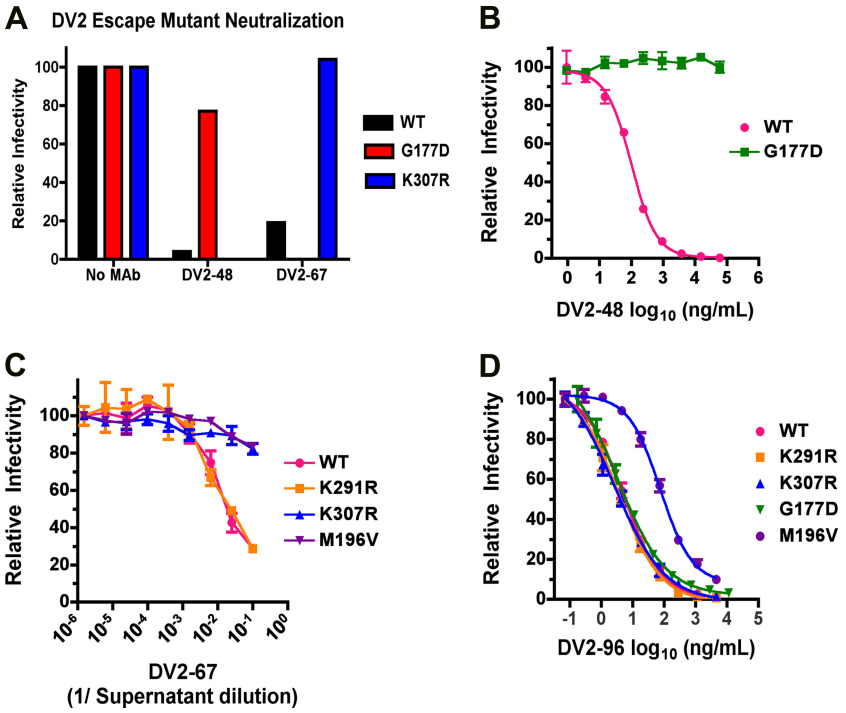

FIG. 3. Characterization of neutralization escape mutants. (A) A neutralization assay was performed with selected escape variants (G177D or K307R) after plaque purification. These escape variants were obtained after three to six passages of DENV-2 (strain 16681) under selection with either DV2-48 or DV2-67 on BHK21 cells. Note that the G177D and K307R variants are resistant to DV2-48 and DV2-67, respectively. Data are representative of two independent experiments performed in duplicate. (B to D) Confirmation of resistant phenotype with DENV-2 RVPs. Mutated RVPs (G177D, K291R, K307R, or M196V) were used to confirm that single amino acid substitutions in the E protein reduce or eliminate neutralization by the selecting MAb (DV2-48 [B]; DV2-67 [C]; and DV2-96 [DR] control DIII MAb). In each series, the reduced neutralizing capacity of the indicated MAb is compared to the neutralizing capacity of wild-type DENV-2 RVP generated in parallel. The data are representative of two or three separate experiments performed in triplicate. Error bars indicate standard error within a single experiment. Lines represent curve fits generated by nonlinear regression analysis. For panel C, diluted hybridoma supernatant was used instead of purified antibody.

neutralization escape phenotypes, we utilized a reverse-genetics approach. DENV-2 reporter virus particles (RVPs; strain 16681) composed of E proteins encoding single amino acid mutations were produced and analyzed for MAb neutralization (1). Whereas DV2-96 neutralized the wild type and G177D, K307R, and K291R variants equivalently, RVPs composed of E proteins with the G177D mutation were resistant to neutralization by DV2-48, even at concentrations of 60 μg/ml of MAb (Fig. 3B). Correspondingly, K307R RVPs were resistant to neutralization by DV2-67, whereas K291R RVPs remained sensitive and behaved similarly to wild-type RVPs (Fig. 3C). Interestingly, M196V RVPs resulted in altered neutralization curves for DV2-67, DV2-96, and several other MAbs compared to wild-type RVP, suggesting a global change in E protein structure or arrangement (Fig. 3C and D and data not shown). We subsequently mapped the residues that conferred neutralization escape onto the DENV-2 E crystal structures (34, 35). G177D is solvent-accessible in DI and localizes in proximity to the E184D epitope identified for DV2-51 (Fig. 2A), whereas K307R maps to the middle of the A strand of DIII.

Structural analysis of genotypic variation of DENV-2 E protein. Amino acid sequences of the ectodomain of the E protein of six DENV-2 strains were aligned, representing strains of the Southeast Asian, American, Indian, and West African genotypes (see Fig. S1 and S2 in the supplemental material), with 33 sites of variation observed. PM33974, a sylvatic strain of the West African genotype, showed great sequence diversity compared to the consensus DENV-2 sequence, with 14 and 3 amino acid differences in DI-DII and DIII, respectively. The IQT2913 strain of the American genotype also had significant variation, with nine amino acid changes detected. The other genotypes had limited diversity in the E protein, ranging from two to five changes for strains of the Southeast Asian and Indian genotypes. Sequence alignment with other DENV serotypes or flaviviruses (e.g., WNV) revealed that 16 of the sites of genetic variation among the genotypes were unique to the DENV-2 serotype, with six of them occurring only in the sylvatic strain.

There are 12 conservative substitutions and 13 nonconservative substitutions within DI and DII among the genotypes of DENV-2 (see Fig. S1 in the supplemental material). Of the conservative substitutions, four are solvent exposed or located along the dimer interface (I/M6, S/T81, V/I129, and S/L247), whereas eight are buried and likely contribute to domain stabilization and structure (Y/F59, K/R93, I/V139, I/V141, I/V162, I/V164, V/I181, and L/I277) (Fig. 4A). We identified 11 nonconservative substitutions that are solvent exposed and two nonconservative substitutions that are proximal to the viral membrane. Nine of the 11 nonconservative substitutions occur in DII, with the *e* strand alone containing four solvent-accessible genotypic variants (T/R120, K/L122, N/K124, and K/E126). The remaining variation in DII was in the *b* strand

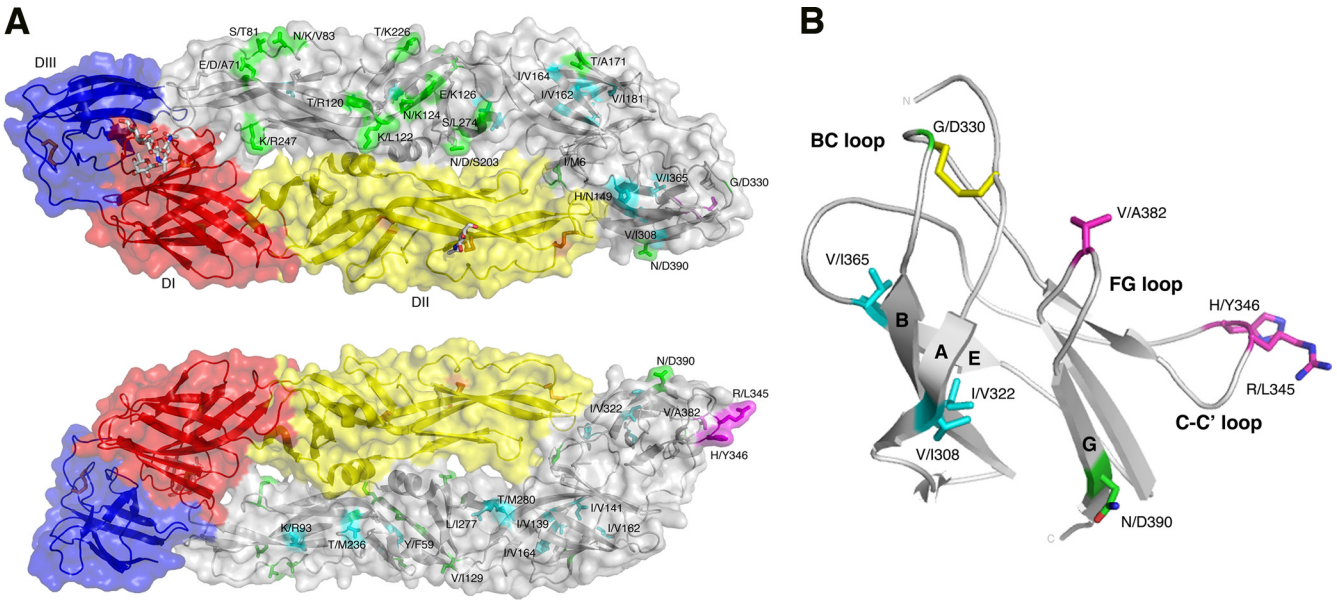

FIG. 4. Structural analysis of genotypic variation. (A) Diagram of DI-DII (top, top view; bottom, bottom view) showing sequence variation of different DENV-2 genotypes. Amino acid sequences that differ among DENV-2 genotypes are indicated, with solvent-accessible or -inaccessible residues depicted in green and cyan, respectively. (B) Ribbon diagram of DENV-2 DIII with β-strands and loops labeled accordingly from published X-ray crystallographic structure. Amino acid sequences that vary among the different DENV-2 genotypes are indicated, with solvent-inaccessible and -accessible residues depicted in blue and green, respectively. Magenta coloring indicates residues that are not predicted to be solvent accessible in the mature virion based on cryo-electron microscopic reconstruction (26) but may be accessible during other stages of the virus life cycle.

(E/D/A71) and the *ef* (N/K/V83), *fg* (N/D/S203), *hi* (T/K226), and *jk* (S/L274) loops. The remaining solvent-exposed residues that varied were present in DI at amino acid positions 149 (H/N) and 171 (T/A); however, residue 149 is likely shielded by the conserved N-linked glycan at asparagine 153. T/K236 and T/M280 are nonconservative substitutions in DII that localize proximal to the lipid bilayer of the virion.

Alignment of the DIII amino acid sequences revealed eight sites of variation among the DENV-2 genotypes (see Fig. S2 in the supplemental material). Using the pseudo-atomic model of the mature DENV-2 virion (26), we assessed the solvent accessibility of these variable amino acids. For four of the residues that had conservative substitutions (V/I308, I/V322, K/R345, and I/V365) in the A strand, B strand, C-C′ loop, and E strand, the amino acid side chains are predicted to be partially or completely solvent inaccessible (Fig. 4B). Similarly, two of the nonconservative substitutions (A/V382) at the junction of the FG loop and in the C-C′ loop (H/Y346) appear buried in the mature virus. In contrast, the nonconservative substitution D/G330 is located at the apex of the BC loop and is fully solvent accessible. Consistent with this, changes in this residue were associated with altered MAb (DV2-70 and DV2-106) binding and genotype-specific neutralization. The final nonconservative substitution (N/D390) in the G strand appears solvent accessible but did not alter the function of any of the MAbs in this study. Although substitutions in the C-C′ or FG loops at residues 345, 346, or 382 are not surface exposed in the structure of the mature DENV-2 virion, they may become surface accessible as part of the ensemble of transitional states (31), which likely reflect "breathing" of the virion. This may explain why substitutions at position H346 reduced binding of several type-specific neutralizing antibodies (DV2-73, DV2-77, DV2-87, and DV2-104).

Prophylaxis in mice. To define the relationship between neutralization in cell culture and protection *in vivo* and to explore the possibility for antibody therapy, we evaluated the efficacy of inhibitory MAbs against DENV-2 NGC in BALB/c mice. This mouse model was selected because (i) NGC efficiently causes lethal infection in BALB/c mice after an intracranial challenge (12, 54); (ii) large numbers of BALB/c mice can be obtained commercially, facilitating comparison of many MAbs in a short period of time; and (iii) although intracranial DENV infection of BALB/c does not recapitulate pathogenesis in humans, it is a stringent protection model, as small amounts of MAb cross the blood-brain barrier and accumulate in the central nervous system (22).

A single dose (500 μg) of 16 different neutralizing MAbs was administered as prophylaxis 1 day prior to intracranial infection with 6×10^4 PFU of DENV-2 NGC. With the exception of DV2-36, all neutralizing MAbs showed significant protection against lethal DENV-2 NGC infection at a 500-μg dose (Table 3). Among those tested, 11 of 16 (DV2-30, DV2-44, DV2-46, DV2-48, DV2-52, DV2-58, DV2-76, DV2-77, DV2-87, DV2-96, and DV2-104) enhanced survival rates beyond 77%. Four of sixteen neutralizing MAbs (DV2-29, DV2-51, DV2-70, and DV2-106) protected less efficiently yet significantly, with survival rates of 37 to 63% ($P \leq 0.02$).

To begin to gauge *in vivo* potency, we administered 5- and 25-fold-lower doses (100 μg and 20 μg) 1 day prior to intracranial infection of BALB/c mice with DENV-2 NGC (Table 3). The 20-μg dose of DV2-30 provided little protection against lethal infection, with no difference in average survival

TABLE 3. Pre- and postexposure protection of BALB/c mice from DENV-2 NGC infection with neutralizing MAbs[a]

MAb	Dose (μg/mouse)	Survival[b]	*P* value[c]
Prophylaxis studies administered 1 day before infection			
PBS control	0	0/31	
WNV-E16	500	0/9	NS
DV2-29	500	5/8	<0.0001
DV2-30	500	5/5	<0.0001
DV2-30	100	5/5	<0.0001
DV2-30	20	0/5	NS
DV2-36	500	2/5	NS
DV2-44	500	5/5	<0.0001
DV2-44	100	5/5	<0.0001
DV2-44	20	3/5	0.005
DV2-46	500	7/9	<0.0001
DV2-46	100	4/5	0.0002
DV2-48	500	4/5	<0.0001
DV2-48	100	2/5	0.03
DV2-51	500	3/8	0.02
DV2-52	500	5/5	<0.0001
DV2-52	100	4/5	0.0004
DV2-52	20	3/5	0.0004
DV2-58	500	9/10	<0.0001
DV2-58	100	5/5	<0.0001
DV2-58	20	2/5	0.007
DV2-70	500	5/9	<0.0001
DV2-76	500	9/10	<0.0001
DV2-76	100	3/5	0.005
DV2-76	20	3/5	0.005
DV2-77	500	9/10	<0.0001
DV2-87	500	5/5	<0.0001
DV2-87	100	3/5	0.007
DV2-87	20	2/5	NS
DV2-96	500	8/9	<0.0001
DV2-96	100	4/4	<0.0001
DV2-96	20	3/5	0.0004
DV2-104	500	4/5	0.0006
DV2-104	100	3/5	0.004
DV2-104	20	2/5	NS
DV2-106	500	3/5	0.001
Therapeutic studies administered at day 2 after infection			
PBS		0/22	
DV2-30	500	4/5	0.002
DV2-44	500	4/5	0.007
DV2-46	500	8/12	<0.0001
DV2-52	500	7/11	<0.0001
DV2-58	500	4/5	0.004
DV2-76	500	4/5	0.007
DV2-77	500	9/13	<0.0001
DV2-87	500	4/5	0.004
DV2-96	500	4/5	0.004
DV2-104	500	4/5	0.007
DV2-106	500	3/5	0.04
Therapeutic studies administered at day 3 after infection			
PBS		0/10	
DV2-30	500	3/12	0.03
DV2-44	500	10/11	<0.0001
DV2-58	500	5/12	0.04
DV2-76	500	6/11	<0.0001
DV2-87	500	4/12	0.04
DV2-96	500	9/12	<0.0001
DV2-104	500	10/12	<0.0001

[a] Three-week-old BALB/c mice were infected with 6×10^4 PFU of DENV-2 NGC by an intracranial route. At day −1, day +2, or day +3, a single dose of the indicated MAbs was administered via an intraperitoneal route. Mice were monitored for survival for 21 days after infection. Of note, intracranial DENV infection of BALB/c mice is a highly stringent protection model, as low concentrations (~0.1% of serum levels) of MAb cross the blood-brain barrier and accumulate in the central nervous system (22).

[b] Survival refers to the number of animals surviving compared to the total number infected over a period of 21 days.

[c] *P* values were calculated using the log rank test of the Kaplan-Meier survival curve by comparing the no-antibody and antibody-treated mice. NS represents values that were not statistically different ($P > 0.05$).

TABLE 4. Prophylaxis of neutralizing MAbs against antibody-enhanced, lethal infection with DENV-2 D2S10[a]

Enhancing antibody (description)	MAb prophylaxis	No. of mice that survived/total no. of mice[b]	*P* value[c]
98J serum (25 μl; anti-DENV-1 polyclonal immune serum)	PBS	0/5	
	DV2-44	5/5	<0.003
	DV2-76	5/5	<0.003
	DV2-104	5/5	<0.003
4G2 (20 μg; DII-fusion loop-specific MAb)	PBS	0/5	
	DV2-44	5/5	<0.003
	DV2-76	5/5	<0.003
	DV2-104	5/5	<0.003
IgG2a (20 μg; nonenhancing)	PBS	5/5	<0.003
NMS (nonenhancing)	PBS	5/5	<0.003

[a] Six- to eight-week-old AG129 mice were administered by an intraperitoneal route either 25 μl of anti-DENV-1 98J polyclonal immune or naïve mouse serum (NMS) in 200 μl PBS, or 20 μg of the flavivirus cross-reactive fusion loop MAb 4G2 or an isotype control IgG2a MAb. Immediately after, mice were given 50 μg of the indicated anti-DENV-2 MAb. Twenty-four hours after antibody transfer, mice were infected with 10^5 PFU of DENV-2 D2S10 intravenously. Morbidity and mortality were measured for 10 days.

[b] The antibody-enhanced mortality measured between days 0 and 10 is due to a vascular leakage syndrome, as previously described (2, 72).

[c] The *P* value was calculated using the log rank analysis of a Kaplan-Meier survival curve compared to mice treated with PBS.

times observed. In comparison, 40 to 60% of mice survived infection after receiving a single dose of 20 μg of any of seven different MAbs (DV2-44, DV2-52, DV2-58, DV2-76, DV2-87, DV2-96, and DV2-104).

Epidemiological data and cell culture experiments have suggested that poorly neutralizing cross-reactive anti-DENV antibodies contribute to severe disease in humans by an antibody-dependent enhancement (ADE) of infection mechanism (reviewed in reference 19). Recently, a disease model of ADE of DENV infection that causes a vascular leakage syndrome was developed in AG129 immunocompromised mice (2, 72): passive transfer of anti-DENV antibodies enhanced disease after infection with a mouse-adapted DENV-2 Southeast Asian genotype strain (D2S10). To further define the protective activity of anti-DENV-2 MAbs *in vivo*, we selected three of the promising MAbs from the BALB/c studies and evaluated their activity in this ADE disease model. Fifty micrograms of MAb (DV2-44, DV2-76, or DV2-104) or PBS was administered concurrently with enhancing concentrations of either anti-flavivirus MAb 4G2 (which recognizes the highly conserved fusion loop in DII) or polyclonal anti-DENV-1 cross-reactive immune serum. One day later, mice were infected with a sublethal dose (10^5 PFU) of DENV-2 D2S10 by an intravenous route. Mice receiving 4G2 or DENV-1 immune sera and PBS or an isotype control MAb succumbed to lethal DENV infection within 5 days (Table 4 and data not shown). Among mice treated with anti-DENV-2 MAbs (DV2-44, DV2-76, or DV2-104), only one mouse, a recipient of DV2-76, demonstrated any sign of morbidity. All other animals receiving neutralizing DENV-2 MAbs remained healthy throughout the experiment.

Therapeutic activity of neutralizing MAbs in mice. To assess the potential therapeutic efficacy of neutralizing anti-DENV-2 MAbs, BALB/c mice were infected intracranially with DENV-2 NGC, and 2 days later, a single 500-μg dose of

MAb (DV2-30, DV2-44, DV2-46, DV2-58, DV2-76, DV2-77, DV2-87, DV2-96, DV2-104, and DV2-106) was administered by an intraperitoneal route (Table 3). Treatment with any of nine different MAbs (DV2-30, DV2-44, DV2-46, DV2-58, DV2-76, DV2-77, DV2-87, DV2-96, and DV2-104) protected 80% of mice ($P \leq 0.0007$). When a single dose was given 3 days after intracranial infection, seven of the MAbs again protected significantly ($P \leq 0.03$), with four (DV2-44, DV2-76, DV2-96, and DV2-104) showing survival rates between 55 and 91%.

For most MAbs, there was a correlation between neutralizing activity against DENV-2 NGC in BHK21 cells and therapeutic efficacy *in vivo* in mice. As an example, the DIII-specific MAbs DV2-76 and DV2-96 had $PRNT_{50}$ values of 100 to 200 ng/ml against DENV-2 NGC (Table 1). In contrast, for the DI-DII-specific MAbs DV2-46 and DV2-58, protective activity was associated with relatively poor intrinsic neutralizing activity ($PRNT_{50}$ of 1.5 to 7.2 μg/ml) against DENV-2 NGC. Thus, relative neutralization potency in BHK21 cells did not necessarily predict the protective efficacy of MAbs *in vivo* in this model.

Strongly protective MAbs inhibit infection at a postattachment step. Antibody neutralization may occur by inhibiting receptor attachment, internalization, and/or endosomal fusion (44). To begin to identify mechanistic correlates of protection, we performed pre- and postattachment neutralization assays (23, 63) with several of the therapeutic MAbs (DV2-44, DV2-58, DV2-76, DV2-77, DV2-87, and DV2-104). For comparison, we also tested DV2-29, a DII fusion loop-specific MAb with more-limited activity *in vivo*, as a control. A saturating (50-μg/ml) concentration of MAb was incubated with DENV-2 (strain 16681) before or after virus binding to a monolayer of BHK21 cells, and infection was measured by a plaque reduction assay. As expected, all MAbs neutralized DENV-2 infection when premixed with virus at 37°C (Fig. 5A). However, DV2-29 showed reduced inhibitory activity when the preincubation step was performed at 4°C (Fig. 5B). Temperature sensitivity of MAb neutralization was observed previously with MAbs that recognize the A strand of DIII and may be due to a requirement for dynamic motion on the virion surface to enhance epitope accessibility (31). All MAbs with therapeutic activity *in vivo* potently inhibited DENV-2 infection when added after virus adsorption to the cell surface (Fig. 5C), indicating that at least part of their neutralizing activity was at a postattachment step of the viral life cycle, similar to that observed for highly therapeutic anti-WNV MAbs (60, 63). This included several MAbs (DV2-73, DV2-77, and DV2-87) that mapped to the C strand and C-C′ loop epitope on DIII. As expected, DV2-29, which neutralized inefficiently after a 4°C preincubation, failed to inhibit in the postattachment assay.

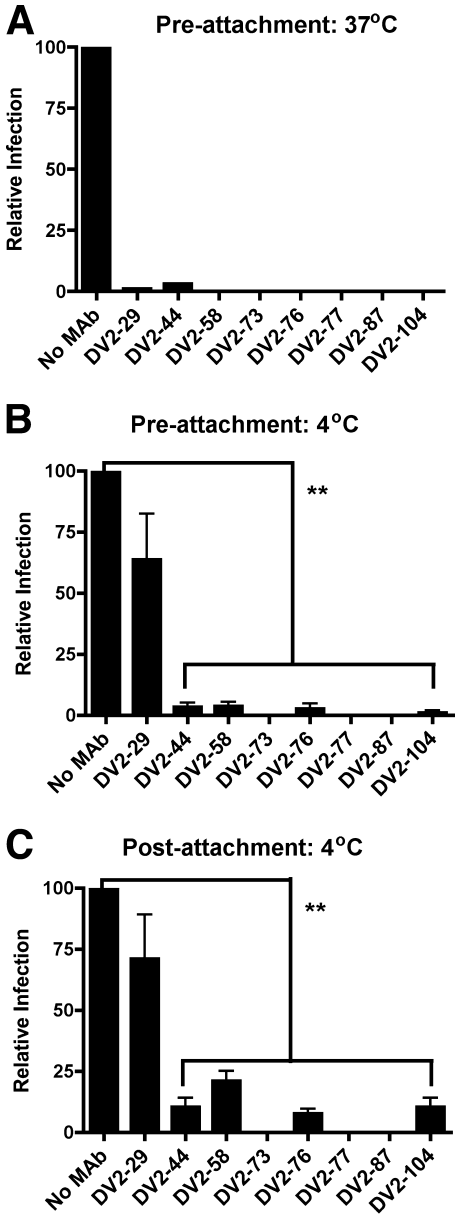

FIG. 5. Strongly neutralizing DENV-2 MAbs inhibit at a postattachment stage. To determine whether the MAbs neutralize DENV-2 infection after cellular attachment, BHK21 cells were prechilled and 10^2 PFU of DENV-2 (strain 16681) was added to each well for 1 h at 4°C. (C) After extensive washing at 4°C, saturating concentrations of the MAbs (50 μg/ml) were added for 1 h at 4°C, and then the neutralization assay was completed. In comparison, a standard preincubation neutralization test with all steps performed at 37°C (A) or 4°C (B) is shown for reference. (A and B) In this case, virus and MAb are incubated together for 1 h at 37°C or 4°C, prior to addition to cells. Data shown are the average of three independent experiments, with error bars representing standard deviation. Asterisks indicate statistically significant differences. Note that DV2-29 loses some of its neutralization potency when preincubated at 4°C compared to 37°C.

DISCUSSION

In a prior study, we localized the epitopes of 14 type-, subcomplex-, and complex-specific MAbs that reacted with DENV-2, had different inhibitory properties against a single DENV-2 strain in cell culture, and localized to DIII of the E protein (57). Despite acquiring a detailed understanding of the structural basis of neutralization by DIII-specific MAbs against DENV, these studies did not assess the *in vitro* inhibitory activity in cells against a genetically diverse range of DENV-2 strains or their protective capacity in animals. Moreover, in our original study and those by others (16, 17), evaluation *in vivo* was not performed, which is essential for identifying MAbs

with therapeutic activity as possible treatment against DENV-2 infection in humans.

Neutralizing antibodies against DENV-2 were generated against all three domains of the E protein, consistent with previous studies of other flaviviruses (3, 4, 14, 15, 27, 42, 43, 50, 53, 58). We observed disparate neutralizing activity of individual MAbs against strains corresponding to different DENV-2 genotypes, results analogous to those of studies of DENV-1 and DENV-3 (53, 65). In comparative studies of strains from all DENV-2 genotypes, many MAbs showed reduced inhibitory potency values against heterologous genotypes. This heterogeneity in neutralizing activity for different DENV-2 genotypes is likely explained by the ~3% amino acid variation between individual genotypes, with up to 7% of the amino acids being variable across the four genotypes of DENV-2. Alternatively, variable neutralization could reflect genotype- or strain-specific differences in virus maturation, which can affect epitope accessibility (40).

Previous studies identified the lateral ridge and A-strand epitopes in DIII as targets of type- and subcomplex-specific MAbs against DENV-2 with strongly neutralizing activity (16, 17, 30, 33, 47, 57, 61) and the fusion loop in DII as a recognition site for cross-reactive MAbs with weaker inhibitory activity (8, 9, 15). Our report expands on the mapping of inhibitory anti-DENV MAbs by identifying epitopes in the C strand and C-C′ loop of DIII, within DI, and at the interfaces of adjacent E protein dimers on DII. The discovery of type-specific neutralizing MAbs against DENV-2 in DI (DV2-48 and DV2-51 at residues G177 and E184, respectively) is consistent with an earlier report that localized a protective DENV-4-specific chimpanzee MAb (5H2) to amino acid K174 in DI (27). Similarly, neutralizing MAbs that map to sites along the dimer interface have been identified for other flaviviruses (8, 32, 43, 63). Because some of these MAbs inhibited virus fusion in a liposome assay *in vitro* (56, 63), this class of antibodies may neutralize efficiently by disrupting the pH-dependent E protein structural transitions required for membrane fusion.

This study is the first to examine the genotypic variation of DENV-2 in the context of antibody neutralization. Genotypic variation was observed at 25 different amino acid sites in DI and DII. The diversity clustered in DII with twice the frequency of variation per residue compared to DI or DIII. The majority of changes were nonconservative and on solvent-accessible surfaces, suggesting that DII may accommodate more variability and contribute significantly to antigenic variation. However, and as seen previously with DENV-1 MAbs in DIII (53), the mapping of neutralizing MAbs in DI and DII did not correspond directly to the residues of genotypic variation. The sylvatic West African strain had more genotypic variation than any other, with a total of 17 substitutions, 15 of which were unique among the DENV genotypes. As sylvatic strains circulate between nonhuman primates and arboreal mosquitoes of the *Aedes* genus (62), they are not under the same evolutionary constraints that bottleneck variation between human DENV strains and *Aedes aegypti* mosquitoes. Thus, sylvatic strains may accumulate substitutions that would normally disrupt transmission of human strains.

For DIII of DENV-2, genotypic variation was limited to eight amino acids. Two surface-accessible residues (positions 330 and 390) had nonconservative substitutions among different DENV-2 genotypes. The G→D change at position 330 in the sylvatic West African strain was associated with the decreased neutralizing potential of DV2-106, which showed reduced binding to the same variant when displayed on yeast. In contrast, changes at position 390 did not affect binding of any of the neutralizing MAbs in this study. This amino acid is a known contact residue for the subcomplex neutralizing MAb 1A1D-2 (31), but it is unknown whether variation at this position affects neutralization of strains of the American genotype (e.g., IQT2913). Changes at amino acid 390, however, have been associated with altered virulence, as a D→N change in Mexican strains of the American genotype caused a small plaque phenotype and decreased virulence in mice (51). Substitutions in the C-C′ loop of DIII at positions 345 and 346 are not surface exposed, based on the DENV-2 mature virion structure, but may be accessible in transition states (31). However, MAbs that mapped to the C strand and C-C′ loop did not show significant temperature sensitivity of neutralization, suggesting that this epitope is accessible on a significant fraction of DENV-2 virions. Genotypic variation in this region is significant because it could affect binding of type-specific neutralizing MAbs (DV2-73, DV2-77, DV2-87, and DV2-104) that map to this epitope. Indeed, neutralization of the C0477 strain, which contained an H→Y substitution at position 346, was associated with the decreased neutralizing potential of DV2-73.

Although the plaque reduction neutralizing activity for DIII-specific MAbs correlated with *in vivo* protective activity, this relationship was less clear for DI-DII MAbs, which generally neutralized less well but, in several cases, still retained efficacy *in vivo*. DV2-46 and DV2-58, which had relatively poor neutralizing activity against DENV-2 NGC in BHK21 cells, still had significant postexposure therapeutic activity in the intracranial BALB/c mice challenge model. This poor correlation between neutralizing activity *in vitro* and protection *in vivo* is analogous to that observed in DENV-2 vaccine trials in which prechallenge neutralization titers did not predict protection in nonhuman primates (55). The PRNT assay with BHK21 cells, while a convenient method to measure inhibitory antibodies *in vitro*, by itself, does not provide a complete picture of protection *in vivo*, at least for MAbs that bind outside DIII.

Our experiments describe a postexposure therapeutic effect by several MAbs that map to distinct epitopes in DI-DII (DV2-30, DV2-44, and DV2-58) and DIII (DV2-76, DV2-77, DV2-87, DV2-96, and DV2-104) on DENV-2. While prior studies have demonstrated that, as prophylaxis, anti-E MAbs protect rodents and nonhuman primates against DENV infection (6, 24, 25, 27, 58), few reported MAbs have efficacy when added after infection. Until recently, the concept of MAb therapy against DENV seemed implausible due to the concern for ADE. As subneutralizing concentrations of antibody augment infection of Fcγ receptor-expressing cells likely by enhancing the efficiency of virus entry (13, 18), administration of virus-specific MAbs could adversely impact the outcome of DENV infection. However, we reported recently that highly neutralizing DIII-specific anti-DENV-1 MAbs had therapeutic activity in AG129 mice even when administered as a single dose 4 days after infection (53). Additionally, DII-specific fusion loop MAbs that were modified to eliminate Fcγ receptor interactions and ADE also functioned as postexposure therapy

against the D2S10 strain of DENV-2, which causes a fatal vascular leakage syndrome in AG129 mice (2). In our studies using the D2S10 strain and AG129 mice, three neutralizing MAbs (DV2-44, DV2-76, and DV2-104) functioned efficiently as prophylaxis to prevent antibody-dependent enhanced disease.

In summary, our results suggest that antibodies raised against one genotype within the DENV-2 serotype may have decreased inhibitory potency against heterologous genotypes. Given that this paradigm also appears true for DENV-1 (53) and DENV-3 (65), it may be critical to assess whether polyclonal antibody responses generated against vaccine strains, which are inherently attenuated compared to natural infection, neutralize strains of the more divergent heterologous genotypes within a serotype in a durable manner (67).

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