



# Dengue Virus prM-Specific Human Monoclonal Antibodies with Virus Replication-Enhancing Properties Recognize a Single Immunodominant Antigenic Site

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## ABSTRACT

The proposed antibody-dependent enhancement (ADE) mechanism for severe dengue virus (DENV) disease suggests that non-neutralizing serotype cross-reactive antibodies generated during a primary infection facilitate entry into Fc receptor bearing cells during secondary infection, resulting in enhanced viral replication and severe disease. One group of cross-reactive antibodies that contributes considerably to this serum profile target the premembrane (prM) protein. We report here the isolation of a large panel of naturally occurring human monoclonal antibodies (MAbs) obtained from subjects following primary DENV serotype 1, 2, or 3 or secondary natural DENV infections or following primary DENV serotype 1 live attenuated virus vaccination to determine the antigenic landscape on the prM protein that is recognized by human antibodies. We isolated 25 prM-reactive human MAbs, encoded by diverse antibody-variable genes. Competition-binding studies revealed that all of the antibodies bound to a single major antigenic site on prM. Alanine scanning-based shotgun mutagenesis epitope mapping studies revealed diverse patterns of fine specificity of various clones, suggesting that different antibodies use varied binding poses to recognize several overlapping epitopes within the immunodominant site. Several of the antibodies interacted with epitopes on both prM and E protein residues. Despite the diverse genetic origins of the antibodies and differences in the fine specificity of their epitopes, each of these prM-reactive antibodies was capable of enhancing the DENV infection of Fc receptor-bearing cells.

## IMPORTANCE

Antibodies may play a critical role in the pathogenesis of enhanced DENV infection and disease during secondary infections. A substantial proportion of enhancing antibodies generated in response to natural dengue infection are directed toward the prM protein. The fine specificity of human prM antibodies is not understood. Here, we isolated a panel of dengue prM-specific human monoclonal antibodies from individuals after infection in order to define the mode of molecular recognition by enhancing antibodies. We found that only a single antibody molecule can be bound to each prM protein at any given time. Distinct overlapping epitopes were mapped, but all of the epitopes lie within a single major antigenic site, suggesting that this antigenic domain forms an immunodominant region of the protein. Neutralization and antibody-dependent enhanced replication experiments showed that recognition of any of the epitopes within the major antigenic site on prM was sufficient to cause enhanced infection of target cells.

Infections due to the four dengue virus (DENV) serotypes (DENV1 to DENV4) continue to increase globally in both frequency and severity (1, 2). There is currently no licensed vaccine or approved drug treatment for dengue infection. Each of the dengue virus serotypes is associated with disease, ranging in severity from a febrile flu-like illness to life threatening hemorrhagic fever or shock. Gaining a better understanding of the pathogenesis of severe dengue, especially in the setting of enhanced disease during secondary infection, is of central importance for the development of and testing of experimental DENV vaccines. The most accepted model of how severe dengue disease develops proposes that during secondary DENV infection preexisting cross-reactive antibodies, induced following a previous primary DENV infection, form infectious virus-antibody complexes that efficiently enter and infect cells expressing Fc receptors, resulting in increased viral replication and the release of cytokines and vasoactive mediators that increase vascular permeability, culminating in severe dengue disease (3). This process has been termed antibody-dependent enhancement (ADE) of

infection and has been studied extensively using cell culture and various animal models (4–7).

The *Flaviviridae* family comprises enveloped viruses possessing a genome that is a single-stranded positive-sense RNA mole-

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cule, covered with capsid protein. The viral envelope has 180 copies each of the envelope (E) and membrane (M) glycoproteins. E protein binds to receptors on target cells and mediates low-pH-induced fusion between the viral and cellular membranes required for viral entry. Each of the two protomers in the E protein dimer possesses three principal domains, designated domains I, II, and III (DI, DII, and DIII) (8). DENV binds to cellular receptors via an N-linked glycan on DII, as well as other regions on E protein that have not been well defined. The hydrophobic fusion loop is located at the tip of DII. E protein forms homodimers that lie flat on the surface of the mature virus. Units of three parallel E dimers form 30 stable raft-like structures that cover the viral surface (9).

DENVs bud into the lumen of the endoplasmic reticulum (ER) as immature virions, which subsequently mature during secretion out of cells. Immature virions have E and prM protein heterodimers that arrange into 60 trimeric spikes (10). During secretion, immature virions enter an acidic *trans*-Golgi compartment in which the cellular protease furin cleaves prM to generate E protein dimers that lie flat on the viral surface. "The cleaved pr-protein portion remains associated with the particle, overlying the fusion loop of the E protein, which is likely to prevent formed virus from fusing with intracellular membranes of the host producer cell" (10). Upon release of the virus into the extracellular environment, a neutral pH allows for the disassociation of the pr-protein, and the mature virus then is able to bind to, and fuse with, a new cell (10). The intracellular cleavage of prM by furin is often inefficient, resulting in various degrees of immature virus being present in cell culture-generated DENV preparations (11). Fully immature particles, which continue to possess 180 copies of prM protein, are considered noninfectious. However, when bound by anti-prM antibodies, immature and partially mature DENV can be taken up into and infect Fc receptor-bearing cells such as monocytes (12). The extent to which immature or partially immature DENVs are generated *in vivo* during a human infection remains unclear. The human antibody response to infection, however, contains antibodies directed toward the prM protein (13, 14).

Studies of the human antibody response using monoclonal antibodies (MABs) developed following natural primary and secondary DENV infections show that the majority, >95%, target cross-reactive epitopes (15–19). A substantial proportion of human antibodies that target surface-exposed proteins are directed toward the prM protein (15, 16). These serotype cross-reacting antibodies do not exhibit neutralizing properties *in vitro*, nor do they demonstrate protection when studied in animal models of DENV infection. In fact, several groups have shown that anti-prM MABs exhibit significant infection enhancing properties in cell culture and in animal models of DENV infection (15, 16, 18, 20). Moreover, immune sera from people exposed to primary DENV infections contain prM antibodies that enhance heterologous serotypes in cell culture and animal models of DENV infection (20). Thus, anti-prM antibodies are likely to play an important role in the pathogenesis of dengue disease in humans and may be a key component of the proposed ADE mechanism of infection hypothesized to be at the center of the development of severe DENV disease.

Mapping the epitopes targeted by the anti-prM protein antibody response is important for our basic understanding of the pathogenesis of DENV infection and disease. Most studies of prM human antibody reactivity to date have been conducted using

polyclonal sera. One murine anti-prM MAB designated 4D10 has been reported, which was mapped using phage display technology to residues 14 to 18 of DENV1-4 prM protein (21). The clonal basis for recognition of prM by human antibodies is not understood.

We sought here to define the landscape of the human anti-prM protein response at the clonal level in order to determine the number of antigenic sites on prM, identify the dominant epitope targets, and explore how the enhancing potencies exhibited by different MABs relate to epitope specificity. We used B cells from subjects with diverse serotypes of infection or vaccination to isolate 25 human MABs to prM. The results show that all of the antibodies recognize a single major antigenic site on the prM protein. Competition binding assays showed that only a single antibody molecule can engage a prM protein molecule at any given time. Using alanine scanning-based shotgun mutagenesis mapping, it became clear that diverse antibodies exhibited minor differences in fine specificity, since they target several different but overlapping epitopes. Antibody-mediated virus neutralization and infection enhancement experiments showed, however, that recognition of any of the epitopes with the immunodominant site could enhance replication.

## MATERIALS AND METHODS

**Human subjects and peripheral blood cell isolation.** We identified subjects in North Carolina and Tennessee who had acquired DENV infection naturally by screening volunteers with suspected exposure during past travel to regions where dengue is endemic. Subjects were confirmed to have had DENV infection by testing their serum for the presence of antibodies that neutralized each of the DENV serotypes. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient separation on Ficoll. The cells were cryopreserved immediately and stored in liquid nitrogen until study. The protocol for recruiting and collecting blood samples from subjects was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill and the Vanderbilt University Medical Center. Cryopreserved PBMCs from a repository of cells from subjects previously infected in Nicaragua were kindly provided by Eva Harris (University of California, Berkeley). Cryopreserved PBMCs from a repository of cells from subjects vaccinated with two doses of a live attenuated rDEN1Δ30 vaccine were kindly provided by Anna Durbin (Johns Hopkins University) and Stephen Whitehead (National Institutes of Health [NIH]). Informed consent was obtained from all subjects.

**Generation of human hybridomas.** Previously cryopreserved samples were thawed rapidly in a 37°C water bath and washed prior to transformation with Epstein-Barr virus as described previously (15). Cultures were incubated at 37°C with 5% CO<sub>2</sub> for 10 days prior to screening for antigen-specific cell lines with enzyme-linked immunosorbent assay (ELISA). Cells from wells with supernatants reacting in DENV-specific ELISA were then expanded prior to screening by flow cytometric neutralization assay and cytofusion with HMMA2.5 nonsecreting myeloma cells, as previously described (15). After cytofusion, hybridomas were selected by growth in hypoxanthine-aminopterin-thymidine (HAT) medium containing ouabain and biologically cloned. Wells containing hybridomas producing DENV-specific antibodies were cloned biologically by three rounds of limiting dilution plating, by using a ClonePix device (Molecular Devices) according to the manufacturer's recommendations or using flow cytometric sorting with a single cell collection device. Once clonality was achieved, each hybridoma was expanded until 50% confluent in 75-cm<sup>2</sup> flasks. For antibody expression, the cells in the 75-cm<sup>2</sup> flasks were collected with a cell scraper; the hybridomas were washed in serum-free medium (Gibco Hybridoma-SFM from Invitrogen, catalog no. 12045084) and split equally among four 225-cm<sup>2</sup> flasks (Corning, catalog no. 431082) containing 250 ml of serum-free medium. Flasks were incu-

bated for 21 days before the medium was clarified by centrifugation and 0.2- $\mu$ m-pore-size sterile filtered. Antibodies were purified from clarified medium by protein G chromatography (GE Life Sciences, protein G HP columns).

#### Characterization of antibody isotype, subclass, and variable genes.

The isotype and subclass of secreted antibodies were determined by ELISA. Nucleotide sequences of variable gene segments were determined by Sanger sequencing from cloned cDNA generated by PCR with reverse transcription amplification of cellular mRNA, using variable gene-specific primers designed to amplify antibody genes from all gene families (22). The identities of the gene segments and mutations from the germ line sequences were determined by alignment using the ImMunoGeneTics database (<http://imgt.cines.fr>) (23).

**Viruses and recombinant proteins.** DENV1 WestPac-74, DENV2 S-16803, DENV3 CH-53489, and DENV4 TVP-376 virus strains, provided by Robert Putnak (Walter Reed Army Institute of Research, Silver Spring, MD), were used in the present study for both binding ELISA and neutralization assays. Virus-containing supernatant used in virus capture ELISAs was prepared in C6/36 mosquito cells grown in complete minimal essential medium (Gibco, catalog no. 51985-034).

Recombinant proteins representing fragments of E or prM-protein were used to determine antigens and domains recognized by human MABs. Recombinant DENV proteins were constructed using the sequences of the above strains. Sequence optimization, gene synthesis, and molecular cloning of all recombinant DENV protein constructs for expression in baculovirus was performed by GenScript USA, Inc. The amino acid residues for the E-protein constructs (rE) were as follows: DENV1 rE (1 to 397), DENV2 rE (1 to 397), DENV3 rE (1 to 395), and DENV4 rE (1 to 397). The amino acid residues for the Pr-protein constructs (rPr) were as follows: DENV1 rPr (1 to 86), DENV2 rPr (1 to 86), DENV3 rPr (1 to 86), and DENV4 rPr (1 to 86). Protein production and purification were described previously (24).

**ELISA using virus or recombinant protein antigens.** For virus capture ELISA, purified mouse MAB 4G2, prepared in carbonate binding buffer, was used to coat ELISA plates (Nunc, catalog no. 242757) and incubated at 4°C overnight. After blocking for 1 h, plates were washed five times with phosphate-buffered saline (PBS), and 50  $\mu$ l of DENV-containing culture supernatant from infected C6/36 cell culture monolayers was added. The plates then were washed ten times with PBS, and 5  $\mu$ l of purified human MAB (1  $\mu$ g/ $\mu$ l) was added to 25  $\mu$ l of blocking solution/well. The plates were incubated at room temperature for 1 h prior to five washes with PBS. Secondary antibody (goat anti-human Fc; Meridian Life Science, catalog no. W99008A) was applied at a 1:5,000 dilution in blocking solution using 25  $\mu$ l/well, and the plates again were incubated at room temperature for 1 h. After repeat PBS washing (five times), phosphatase substrate solution (1 mg/ml phosphatase substrate in 1 M Tris aminomethane) (Sigma, catalog no. S0942) was added at 25  $\mu$ l/well, and the plates were incubated at room temperature for 2 h before the optical density was read at 405 nm on a BioTek plate reader.

For recombinant protein capture ELISA using E protein or prM constructs, purified mouse anti-Strep-tag II MAB (StrepMAB-Immo, catalog no. IBA 2-1517-001) prepared in carbonate binding buffer was used to coat ELISA plates (Nunc, catalog no. 242757), followed by incubation at 4°C overnight. After blocking for 1 h, the plates were washed five times with PBS, and 50  $\mu$ l of recombinant protein construct containing culture supernatant (cultured in insect cells) was added. The plates were then washed ten times with PBS, and 5  $\mu$ l of purified human MAB (1  $\mu$ g/ $\mu$ l) was added to 25  $\mu$ l of blocking solution/well. All other steps were performed as described above for the virus capture ELISA.

**DENV Western blotting.** The blots were performed using crude whole DENV antigen or purified whole DENV antigen. The crude DENV2 (strain S-16803) antigen was harvested from infected cell culture medium, concentrated by centrifugation, and loaded onto a 4 to 12% SDS-PAGE gel run under denaturing reducing conditions or nonreducing conditions. After transfer, the nitrocellulose membrane was probed

with the purified human MAB in question (diluted 1:1,000) for 1 h at 37°C. The membrane was washed three times with PBS-Tween and then incubated with goat anti-human Fc-AP secondary (Meridian Life Science, catalog no. W99008A) for 1 h at 37°C prior to washing and development using BCIP/NBT chromogenic substrate (Invitrogen, catalog no. WP20001). E-protein-specific MABs were included as controls. Purified whole DENV2 (strain NGC) antigen was purchased from a vendor (Microbix, Canada; catalog no. EL-22-02). A total of 1  $\mu$ g of the pure antigen was loaded onto 12% SDS-PAGE gels under denaturing, nonreducing conditions (without DTT). We blotted and tested the samples with three different concentrations (0.5, 1, or 2.0  $\mu$ g/ml) of each of the prM antibodies. We used goat anti-human horseradish peroxidase as the secondary antibody.

**Neutralization assay.** The neutralizing potency of MABs was measured using a flow cytometry-based neutralization assay with the U937 human monocytic cell line stably transfected with DC-SIGN, as previously described (25, 26).

**ADE assays.** The ability of antibodies to enhance DENV infection was measured using U937 cells that had not been engineered to express DC-SIGN. In the absence of the virus attachment factor, these Fc receptor-bearing cells are only susceptible to infection in the presence of DENV-specific antibodies. The assay was performed as described in detail previously (15). ADE activity was expressed as the percent increase of infected cells in the DENV-specific antibody-treated sample compared to the sample treated with a control antibody.

**Epitope mapping using antibody competition.** A biosensor instrument using biolayer interferometry (Octet Red; ForteBio) was used for all competition-binding studies. For competition assays, DENV2 virions were produced in cell culture and immunoaffinity purified on the biosensor tips. Briefly, purified biotinylated mouse anti-dengue virus prM MAB 2H2 was loaded onto streptavidin tips (ForteBio, catalog no. 18-5019). Crude DENV2 S-16803 was prepared by centrifuging 250 ml of sterile-filtered supernatant from infected C6/36 cell culture monolayers at 10,000 rpm for 12 h. The pellet containing crude virion particles was then suspended in 5 ml of PBS and captured on the biosensor tip using MAB 2H2. After a washing step, the first human anti-DENV MAB was added, followed immediately by a second human MAB to assess binding interference. The antibodies were judged to compete for the same site if maximum binding of the second antibody was reduced to <25% of its binding in the absence of the first antibody. No competition was achieved if maximum binding of the second antibody was >75% of its binding in the absence of the first antibody.

**Shotgun mutagenesis epitope mapping.** DENV3 (strain CH53489) and DENV4 (341750 strain) prM/E expression constructs were subjected to high-throughput mutagenesis ("shotgun mutagenesis") to generate comprehensive mutation libraries. Random mutations were introduced into the DENV3 prM/E polyprotein by PCR using a diversity mutagenesis kit (Clontech Laboratories, Inc., Mountain View, CA), and for the DENV4 construct primers were used to mutate each prM/E polyprotein residue to alanine (and alanine codons to serine). In total, 1,400 DENV3 and 660 DENV4 mutants were generated (>97% coverage of each serotype prM/E ectodomain), sequence confirmed, and arrayed into 384-well plates (one mutation per well). Each anti-DENV antibody was screened on the full DENV3 mutation library or a consolidated DENV3 library containing a subset of the most relevant E protein mutations. Four MABs (1H7.2, 2H21, 2K2, and 5M22) were also screened on the DENV4 mutation library. For screening, vectors for each DENV protein mutant were transfected individually into human HEK-293T cells and allowed to express for 22 h before the cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) and permeabilized with 0.1% (wt/vol) saponin (Sigma-Aldrich) in PBS plus calcium and magnesium (PBS++). The cells were incubated with purified MABs (0.1 to 2.0  $\mu$ g/ml) diluted in 10% NGS (Sigma)–0.1% saponin (pH 9). The optimal primary antibody concentration was determined for each antibody using an independent immunofluorescence titration curve against wild-type prM/E to ensure that signals

TABLE 1 Subject demographics and serum serologies<sup>a</sup>

Type of infection and serotype(s)	MAB	Subject	Geographic location of infection	Date (yr)	Time (yr) since infection	Reciprocal serum antibody 50% neutralization titer to various DENV serotypes				Study reference
						D1	D2	D3	D4	
Primary										
DENV1	2J9, 2H21, 2G3, 2B17, 1H7.2, 1E23	106	India	2007	2	90	15	13	11	24
DENV2	5M22	19	Thailand	1997	8	95	>	20	105	15
DENV3	1E16	118	Nicaragua	2009	1	60	32	980	76	19
	1C6*	5110	Nicaragua	2010	1					
	2H12	3	Thailand	2001	4	30	87	338	<	15
	5G22 and 4E9	105	Thailand	2002	7	<	<	210	<	15
Vaccine										
rDENV1Δ30	1O6	39	NA	2009	0.6	189	ND	ND	ND	24
	1L13	53	NA	2009	0.6	39	ND	ND	ND	24
Secondary										
DENV1 and DENV3	1K20*, 1B22, 1H10, 1I12, 2K2, 4G21, 5E15	184	Mexico	2006	4	282	209	166	76	19
Multiple	1G10*	1089	Nicaragua	2009	1	107	220	200	70	
	4F8	15	West Indies	1972–1982	23	371	320	288	>	15
	1G6 and 2M2	27	Thailand/Cambodia	1981	24	>	>	>	285	15

<sup>a</sup> >, titer > 1:1,280; <, titer < 1:20. \*, Unpublished; NA, not applicable; ND, not determined.

were within the linear range of detection and that the signal exceeded the background by at least 5-fold. Antibodies were detected using 3.75 µg/ml Alexa Fluor 488-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) in 10% NGS–0.1% saponin. The cells were washed three times with PBS+ +/0.1% saponin, followed by two washes in PBS. The mean cellular fluorescence was detected using a high-throughput flow cytometer (Intellicyt). Antibody reactivity against each mutant protein clone was calculated relative to wild-type protein reactivity by subtracting the signal from mock-transfected controls and normalizing that value to the signal from wild-type protein-transfected controls for the serotype tested. Mutations within clones were identified as critical to the MAb epitope if they did not support reactivity of the test MAb but did support reactivity of other antibodies. This counterscreen strategy facilitates the exclusion of DENV protein mutants that are misfolded or have an expression defect. Critical amino acids required for antibody binding were visualized on the DENV2 prM/E protein crystal structure PDB ID 3C6E (27).

## RESULTS

**Human MABs to the DENV prM protein.** We isolated a panel of human anti-prM protein MABs from otherwise healthy human subjects with diverse histories of DENV infection or vaccination. A total of 25 human MABs were isolated; details about the donors for the MABs are shown in Table 1. MABs were obtained from individuals following DENV1, DENV2, or DENV3 primary natural infections or after vaccination with a monovalent DENV1 live attenuated virus. We also obtained anti-prM MABs from individuals following secondary DENV infections, including one whose secondary infection had been determined previously to be a result of DENV1 and DENV3 exposures. The length of time from when the subject experienced the infection until we obtained peripheral

blood samples for isolation of prM-specific B cells ranged from 1 to 24 years.

**DENV antigen binding by ELISA or Western blot.** We tested the ability of each member of this panel of MABs to bind to antigens from each of the four DENV serotypes in a virus capture ELISA. As can be seen in Table 2, all 25 human anti-prM protein MABs isolated were serotype cross-reactive, binding to all four dengue serotypes. We next tested binding against a recombinant “pr” portion of the prM protein. All MABs tested showed binding to this recombinant protein, representing the cleaved portion of the prM protein. We also tested binding of these prM-reactive MABs to recombinant DENV E protein in ELISA. Surprisingly, two MABs, designated 2M2 and 5M22, bound to soluble E protein in ELISA, in addition to binding to prM. We subjected MABs to Western blot analysis using whole virus preparations with or without DTT reducing reagent. As expected, most of the MABs tested bound to a protein with the expected apparent molecular weight of prM in the Western blot, as can be seen in Fig. 1. Five MABs did not show binding, or exhibited variable binding, in Western blot analysis, likely due to SDS denaturation of the epitope structure during the procedure. MAB 2M2 and to a lesser extent 5G22 bound to both prM protein and to a protein with the apparent molecular weight of E protein (Fig. 1); MAB 5M22 also exhibited this pattern (not shown). None of the MABs bound when tested under reducing conditions.

**Functional properties of human anti-prM protein MABs.** We tested the functional properties of this panel of MABs by performing both neutralization and enhancement assays against prototypic viruses from each of the DENV serotypes. As can be seen in

TABLE 2 Functional features of prM-reactive human MAbs<sup>a</sup>

MAb	Binding to whole virus in ELISA, for the indicated serotype, at 1 µg/ml				Binding to protein				50% neutralization concn (µg/ml) against the indicated serotype				Fold enhancement of infection, for the indicated serotype, at 1 µg/ml				
	D1	D2	D3	D4	ELISA		Western blotting		D1	D2	D3	D4	D1	D2	D3	D4	
					rE protein	rPr protein	E protein	PrM protein									
1B22	+	+	+	+	-	+	-	+	-	-	-	-	2	1	1	3	
1C6	+	+	+	+	-	+	-	+	-	9	4	-	18	22	35	32	
1E16	+	+	+	+	-	+	-	-	-	-	-	-	5	1	3	17	
1E23	+	+	+	+	-	+	-	+	-	-	-	-	4	1	5	4	
1G6	+	+	+	+	-	+	-	+	-	-	5	-	19	20	70	38	
1G10	+	+	+	+	-	ND	-	-	-	-	-	-	18	22	34	31	
1H7.2	+	+	+	+	-	+	-	+	-	-	-	-	5	2	3	5	
1H10	+	+	+	+	-	+	-	+	-	-	-	-	16	23	31	30	
1I12	+	+	+	+	-	+	-	+	-	-	-	-	3	2	2	6	
1K20	+	+	+	+	-	ND	-	+	-	-	-	-	16	23	27	36	
1L13	+	+	+	+	-	+	-	+	-	-	-	-	6	2	3	11	
1O6	+	+	+	+	-	+	-	-	-	-	-	-	17	23	32	29	
2B17	+	+	+	+	-	+	-	+	-	-	-	-	3	3	4	3	
2G3	+	+	+	+	-	+	-	+	-	-	-	-	3	1	3	9	
2H12	+	+	+	+	-	+	-	+	-	-	8	-	14	7	35	9	
2H21	+	+	+	+	-	+	-	+	-	-	-	-	3	1	1	4	
2J9	+	+	+	+	-	+	-	+	-	-	-	-	3	1	2	9	
2K2	+	+	+	+	-	+	-	+	-	-	2	-	17	26	25	30	
2M2	+	+	+	+	+	+	+	+	+	1	0.5	2	1	17	8	8	48
4E9	+	+	+	+	-	+	-	+	-	-	1	-	7	-	30	21	
4F8	+	+	+	+	-	+	-	+	-	-	2	5	13	15	57	24	
4G21	+	+	+	+	-	+	-	+	-	-	-	-	18	22	32	0.5	
5E15	+	+	+	+	-	+	-	+	-	-	-	-	20	20	61	24	
5G22	+	+	+	+	-	+	+/-	+	-	-	6	-	40	12	94	46	
5M22	+	+	+	+	+	ND	+	+/-	7	9	5	-	11	11	42	11	

<sup>a</sup> Binding to each DENV serotype and recombinant E and pr protein constructs are shown. The concentration (µg/ml) at which 50% of virus was neutralized (neut<sub>50</sub>) is shown for each DENV serotype: a dash indicates neut<sub>50</sub> value > 10 µg/ml, neut<sub>50</sub> values between 1.0 and 10.0 µg/ml are shown, neut<sub>50</sub> values ≤ 0.5 µg/ml are indicated in boldface. ADE assays were performed for each human antibody (at a concentration of 1 µg/ml) against each DENV serotype and are shown as the fold enhancement: ≥25-fold enhancement values are indicated in boldface. -, Negative or none. ND, not done.

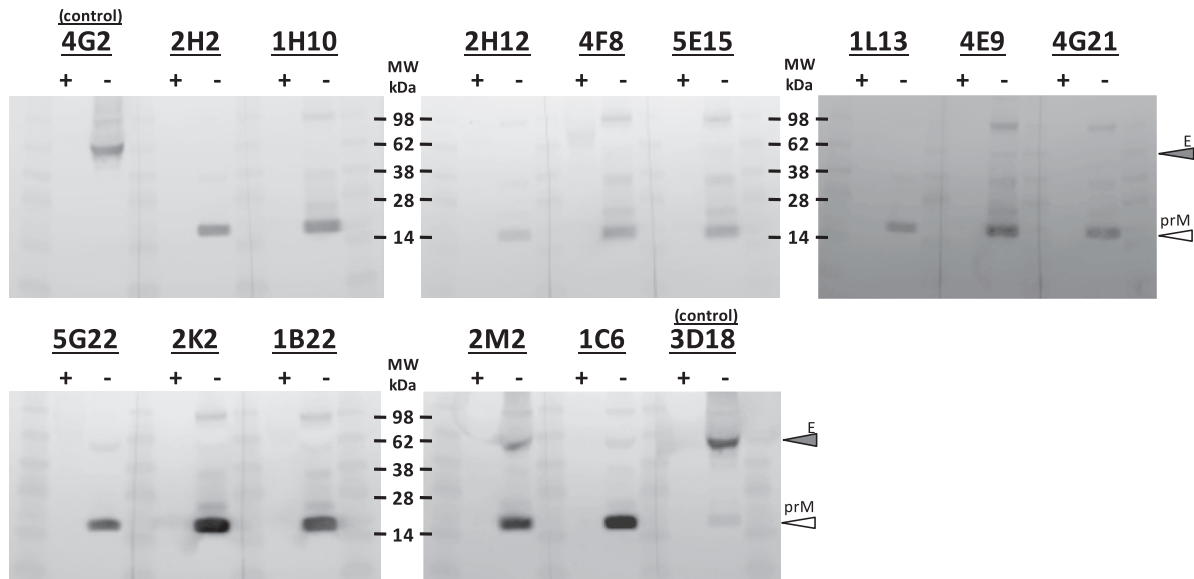
Table 2, with one exception, the members of this panel of human anti-prM protein MAbs showed little or no neutralizing activity against viruses from the four DENV serotypes. MAb 2M2, however, which bound to both E and prM protein in ELISA, did weakly neutralize viruses of all four serotypes. MAb 5M22, which also bound to both E and prM proteins, showed weak neutralizing activity against DENV serotypes 1, 2, and 3.

We also evaluated the panel of MAbs for their ability to enhance the virus infection of FcR-bearing cells in culture. Each MAb was tested at a 1-µg/ml concentration against viruses of each of the four DENV serotypes. Each of the MAbs showed some degree of infection-enhancing properties. A large proportion (15 of 25; 60%) of the MAbs exhibited very potent (>25-fold) enhancement of replication for virus of at least one serotype. Four of the MAbs (designated 1G6, 4F8, 5E15, and 5G22) demonstrated >50-fold enhancement of at least one DENV serotype at this concentration. MAb 5G22 showed the greatest degree of enhancement, increasing DENV3 infectivity by a remarkable 94-fold at the 1-µg/ml concentration tested.

**Epitope mapping using antibody competition-binding experiments.** To better understand the antigenic landscape recognized by the human prM antibody response, we performed MAb competition-binding assays using DENV particles captured on a biosensor tip and analyzed by biolayer interferometry. This experimental strategy allowed real-time determination of antibody

binding to captured virus particles. Each of the human MAbs in this panel fell into the same competition-binding group (see Fig. S1 in the supplemental material), suggesting that all of them bind to the same single major antigenic site on the prM protein. In other words, the binding of any one antibody in the panel blocked the binding of any other. As a control reagent we used the human MAb 3D18 that recognizes the DENV E protein fusion loop (28) when determining whether any of the MAbs in the panel could bind exclusively to the portion of the E protein around the fusion loop, which is in the area underlying prM in immature particles (28). For several of the MAbs in the panel, we generated Fab fragments to determine whether competition for binding could be reduced or eliminated if the steric footprint was smaller. Again, with an anti-prM protein Fab bound to virus particles, none of the full-length anti-prM pairs of MAbs that were tested could bind simultaneously.

**Epitope mapping using alanine scanning based shotgun mutagenesis.** We next set out to define the prM protein residues that are critical for binding by the MAbs in the panel (see Fig. S2 in the supplemental material). As seen in Fig. 2 and Table S1 in the supplemental material, critical residues were determined for each of the MAbs in the panel, with the exception of 2M2 and 4E9. In several instances, critical residues were defined using mutant libraries based on both DENV3 and DENV4 prM/E proteins. Despite all MAbs falling into one competition-binding group, the



**FIG 1** Western blot of human MAbs probed against DENV antigens. Centrifuge-concentrated crude DENV was loaded into a 4 to 12% SDS-PAGE gel run under denaturing nonreducing conditions. After transfer, the nitrocellulose membrane then was probed with the indicated human MAb. E-protein-specific MAbs were included as controls. The “+” or “-” symbols indicate samples tested with or without the addition of dithiothreitol reducing agent. MW, molecular weight in thousands.

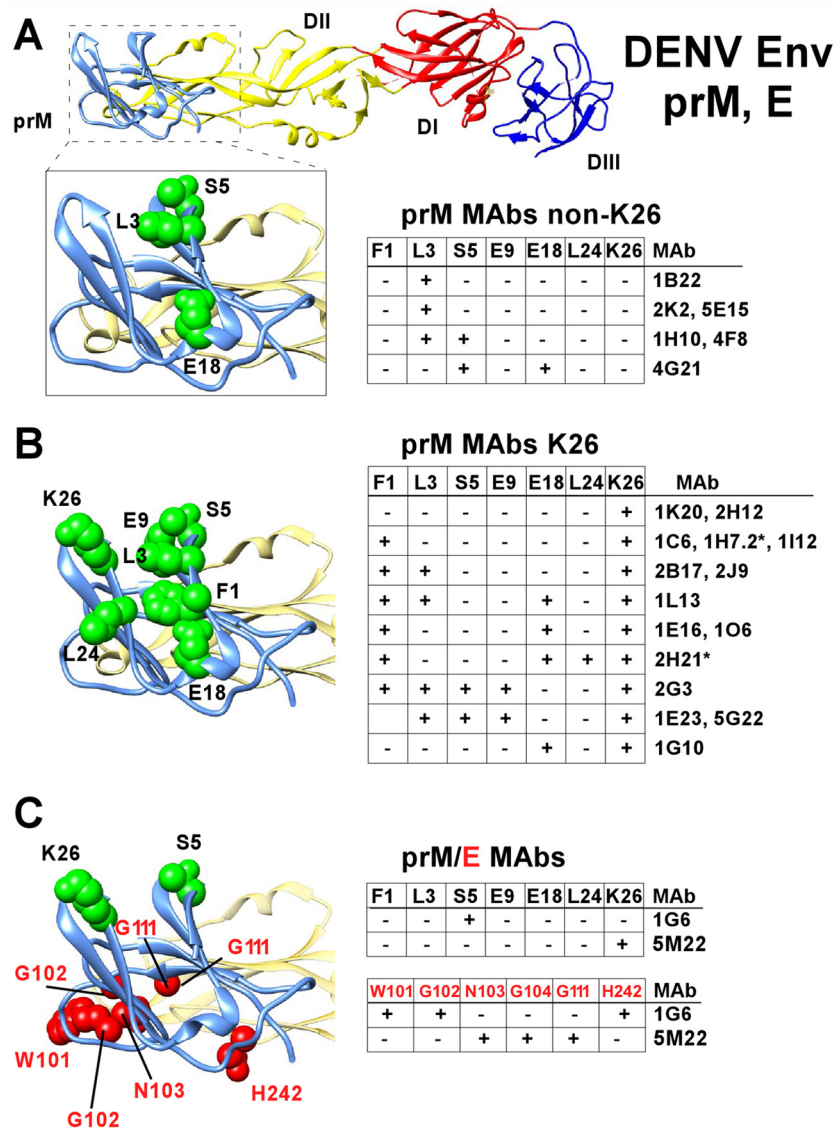
results of this fine epitope mapping work demonstrated that regions of the prM protein that are required for the binding of some of the human MAbs differ. Seven commonly recognized residues were identified (F1, L3, S5, E9, E18, L24, and K26), and there were residues critical for the binding of over 50% of the MAbs in the panel. Residue K26 was critical for the binding of 71% of the MAbs. F1 or L3 residues also were found to be necessary for the binding of 42 or 46% of the MAbs, respectively. We identified critical residues within both prM and E protein for MAb 1G6. [Figure 3](#) provides a prM protein map indicating the frequency of residues participating in MAb epitopes; the reduction of binding for critical residues is shown in [Table S2](#) in the supplemental material. We analyzed the distribution of residues recognized by MAbs from donors with primary versus secondary infection and did not detect major distinguishing patterns. Specifically, F1, L3, S5, E18, and K26 contributed to the binding for MAbs derived from primary and secondary cases; E9 contributed to the binding of 3 of 14 (21%) MAbs from primary and 0 of 11 (<9%) MAbs from secondary cases.

**Genetic features of the antibodies.** Given that each of the MAbs isolated recognized a single major antigenic site, it was possible that these diverse individuals were using a single dominant antibody gene to encode a canonical antibody that is inherently fit for binding to prM in the germ line configuration. To determine whether such unique genetic features were characteristic of human anti-prM protein antibodies, we obtained heavy- and light-chain-variable gene sequences for 23 clones and analyzed them for variable gene usage and amino acid motifs ([Table 3](#)). We noted that each of the antibodies was encoded by distinct variable gene sequences using diverse  $V_H$ , D, and  $J_H$  gene segments. The  $V_H$ 1-69 segment was moderately over-represented, being found in 5 of 23 clones (22%). The mean HCDR3 length of 18.3 was about 2 amino acids longer than the typical mean of 16 in human B cells. Consistent with this, many of the longer HCDR3 regions were encoded

by the longest D gene segments (D2 and D3 family members). We also determined the light-chain usage and heavy-chain isotype/subtype by ELISA to determine whether any of these features were common in the prM panel. We found that all of the clones were of the IgG1 isotype. Interesting, the lambda light chain was highly over-represented, being found in 20 of 23 (87%) of clones. The variable gene sequences did contain somatic mutations consistent with a memory cell phenotype, but the number of mutations was not unusual or excessive. Thus, there appears to be a lambda light-chain bias in the response against prM; otherwise, there are no distinguishing genetic features in the prM-specific repertoire.

## DISCUSSION

We sought to improve our understanding of the molecular landscape of the human anti-prM protein antibody response by determining the number of antigenic sites, the dominant epitope targets, and how these epitopes relate to the enhancing potencies exhibited by different MAbs. The results show that human MAbs target several overlapping epitopes on the surface of prM protein, but their footprints fall into a single major antigenic site. We did not identify any particular relationship between the infection enhancing properties of the antibodies and the fine specificity of the epitope. In fact, every prM antibody enhanced infection with viruses from each of the four serotypes, no matter what the exposure history of the donor or the fine epitope specificity. The findings suggest that, as a class, prM antibodies mediate ADE using essentially a single mode of action. All of the MAbs we isolated were IgG1 subclass. It was also remarkable that this class of antibody was common to every individual studied, no matter what the type of DENV exposure history. This panel of human MAbs was obtained from human subjects following diverse types of DENV infection, including DENV serotype 1, 2, or 3 primary or secondary dengue virus infections, or following DENV1 live attenuated virus vaccination. We did not detect any major differences in the anti-



**FIG 2** Epitope mapping of prM region antibodies by shotgun mutagenesis library screening. We performed fine epitope mapping by screening for loss of binding to a DENV3 random mutagenesis library, and in some cases a DENV4 alanine scanning library. Critical residues are visualized as green spheres on the DENV2 prM/E protein crystal structure (PDB identifier 3C6E; 28). (A) Anti-prM MAbs that did not have residue K26 as critical for binding; (B) anti-prM MAbs that have residue K26 as critical for binding; (C) MAbs that had critical residues in prM and in E. MAbs marked by asterisks were mapped on both the DENV3 and DENV4 libraries (see Table S1 in the supplemental material).

bodies or the epitopes recognized by prM-specific MAbs based on infecting serotype or primary versus secondary infection status.

Despite the common functional profile of these antibodies and recognition of a single antigenic site, the members of the panel of human MAbs exhibited a diversity of features that showed that there are many antibodies and recognition features that target human MAbs to this site on both the antibody and the virus side of the interface. We considered whether there was a canonical antibody in humans forming a public clonotype across individuals that accounts for this common recognition pattern. Several antibody heavy-chain-variable ( $V_H$ ) genes have been associated specifically with recognition of structurally distinct viral epitopes, for instance the association of  $V_H1-46$  gene use with rotavirus VP6-specific antibodies (29–31),  $V_H1-69$  gene use in influenza hemag-

glutinin stem region antibodies (32, 33), or  $V_H1-02$  or  $V_H1-46$  gene use in human MAbs specific for the CD4 supersite on HIV envelope protein (34). These gene associations generally derive from optimal interacting amino acids encoded by antibody heavy-chain complementarity region 2 (HCDR2). Other canonical amino acid motifs have been identified in the antibody HCDR3, encoded by the  $V_H$ , diversity (D) and joining (J) genes, for instance, the HIV neutralizing antibodies PG9 and PG16 (35). We did not identify any such antibody gene associations in this panel of antibodies to prM, even though they all recognize a single major antigenic site. These data suggest that it is likely that most if not all individuals can make prM-specific ADE antibodies, and they can do so using a diversity of antibody clones from a wide variety of naive B cells.

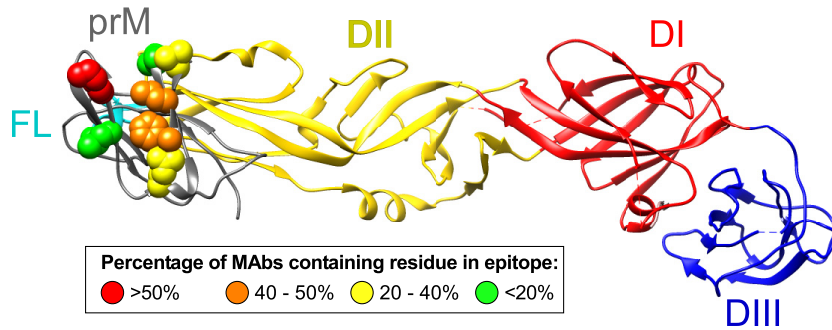


FIG 3 Percentage of MABs containing specific prM region residues in the epitope. Colors show the percentage of MABs for which the indicated residue contributes to the epitope, as determined by the loss of binding in fine epitope mapping studies. Residues are visualized on the DENV2 prM/E protein crystal structure (PDB identifier 3C6E).

Diversity in mode of recognition by these antibodies also was suggested by features of their binding characteristics. First, epitope mapping studies identified seven different residues necessary for binding of antibodies in the panel. There was a clear hierarchical pattern, with certain prM residues most commonly involved in the interaction. Residue K26 was involved in the binding of 17 human antibodies, while residues F1 and L3 were critical for binding of nearly half of our MABs. These residues, along with E9, E18, and L24, are highly conserved across the DENV complex. This finding is the likely explanation for why this class of MABs is so universally serotype cross-reactive, since they target areas of the prM protein that are highly conserved. Interestingly, critical residues were also identified in the E protein for MAb 1G6. These residues were focused within the fusion loop and neighboring E protein domain II. Interpreting the role of critical residues in E protein for 1G6 antibody binding is complex. The point muta-

tion in the prM/E antigen on the surfaces of cells in the saturation mutagenesis studies could destabilize or alter the fine structure of prM and cause loss of binding or, alternatively, the MAB may bind a quaternary epitope with contact residues in both prM and E. In the ELISA, the prM and E components were tested separately, and the MAB bound only to prM. Likely 1G6 binds a quaternary prM/E epitope in which most of the contact residues are in prM, and this component of the epitope is sufficient for binding in ELISA. Clearly, the mode of binding for antibodies that bind to both prM and E is complex, with molecular recognition of quaternary epitopes that would be found only on virus particles. This recognition pattern has been described previously for a human anti-prM MAB that was isolated by phage display (36). The significance of recognition of a quaternary epitope involving both E and prM protein residues for these human anti-prM protein antibodies is unclear, but

TABLE 3 Genetic features of prM-reactive human MABs

MAB	IgG subclass	Light chain	Heavy-chain-variable genes			Junctional protein sequence	Heavy-chain CDR3 length (amino acids)	No. of somatic mutations in heavy chains	
			V <sub>H</sub>	D	J <sub>H</sub>			Nucleotides	Amino acids
1B22	IgG1	λ	1-69	2-2	6	CATLSLFCDSASCYHDPTSMVDVW	21	34	18
1C6	IgG1	λ	1-18	2-2	5	CAKMEDCNSTSCYGGTNWFDPW	20	23	10
1G6	IgG1	κ	1-3	3-22	4	CARVNGDSAYYYGAPDYW	16	30	15
1G10	IgG1	λ	3-15	2-2	6	CTDLPLEYQLFYDYMDVW	18	17	7
1H7.2	IgG1	λ	3-23	2-15	4	CAKMGLCSSGGSCYTGFIHFW	18	31	17
1H10	IgG1	λ	4-4	3-3	3	CARVEFGFFGVVAKGIDLW	17	27	14
1I12	IgG1	λ	3-74	3-3	5	CARGVNYDLWSAYSTDENWFDPW	21	12	9
1K20	IgG1	λ	4-31	2-15	5	CARGRYCNDDSCYSESAIWFDPW	22	25	17
1L13	IgG1	λ	4-31	2-21	5	CATESYCRGNCCYPTVIDPW	19	16	10
1O6	IgG1	λ	1-69	2-2	5	CARATDCSTTSCYSSWFDPW	19	21	14
2B17	IgG1	λ	3-72	2-2	6	CAREGSCGSSTSCYADHYGMDVW	22	13	9
2G3	IgG1	λ	1-69	3-22	4	CATDTSGNLDFW	10	24	14
2H12	IgG1	λ	3-49	3-3	4	CTQTPYCSGDKCYPVFFDSW	19	17	11
2H21	IgG1	λ	3-15	3-9	5	CATVEYCDATSCYNDEAWFDPW	21	30	19
2J9	IgG1	λ	3-49	2-15	5	CTRVVDCSGVNCYPMGWDPW	19	15	7
2K2	IgG1	λ	3-21	5-12	6	CARDRDTLPRDYHYHYGMDVW	19	20	9
2M2	IgG1	κ	5-51	3-10	6	CGRHLYYFGSGKALLHGADVW	19	24	15
4E9	IgG1	λ	3-11	2-15	1	CARGPEGYCSGNCCYPAEYFQHW	21	17	9
4F8	IgG1	κ	4-59	2-8	3	CAISLGYCTGGKCHSGLGTFDIW	21	18	14
4G21	IgG1	λ	1-69	5-12	4	CATDSGYVYFYGW	12	7	4
5E15	IgG1	λ	3-30	2-2	3	CTGGLGYCSSSSCYLGAFDVW	19	28	18
5G22	IgG1	λ	3-53	1-26	4	CARGGSFYDPFDYW	12	8	4
5M22	IgG1	λ	1-69	2-2	5	CARETSCSSSSCYGTNWFDPW	19	19	10



both 2M2 and 5M22 are some of the few neutralizing MABs that recognize prM.

Our shotgun mutagenesis approach identifies epitopes irrespective of MAB neutralization status or viral fitness and so is not limited to inhibitory MABs or to mutations that are compatible with virus replication. Nonetheless, this strategy cannot detect the contribution of alpha carbons to an interaction, mutations to other substitutions could result in different results, and the ability to differentiate direct from indirect or allosteric effects on MAB interactions depends on the number and diversity of other available control MABs. However, the concordance of mutagenesis, competition binding, ELISA, and Western blot data provides confidence that the epitope residues identified here are accurate. Other residues may also be involved in each epitope, but their mutation may have resulted in misfolding of the protein, or they did not disrupt the energetics of binding significantly enough to be identified as critical.

The number of antibodies that can bind one prM protein molecule is one factor that may contribute to the complexity of antibody-dependent enhancement. Only one antibody binding group was identified by MAB competition-binding assays using whole virus particles. These data suggest that, despite the overall complexity of the human anti-prM protein antibody response, only a single antibody molecule is bound to one molecule of the prM protein in immature particles at a given moment in time. Therefore, anti-prM antibodies must be in a state of competition with one another for binding to the single accessible site. This dynamic is also complicated by the fact that the occupancy of antibodies on the population of prM molecules on a single particle may not be complete, and most particles likely contain a mixture of cleaved or uncleaved prM/E protein. In the case of the polyclonal antibody response in serum, the antibody concentration, affinity, and epitope availability are likely to play a critical role in determining which anti-prM antibodies are most successful in engaging the virus.

In summary, the human antibody response encoding DENV prM protein-specific antibodies is complex in terms of their genetic origins and fine specificity, but these antibodies exhibit consistent functional properties. These antibodies are cross-reactive, possess very limited neutralizing properties, and are potent at infection enhancement *in vitro*. The human prM protein-specific antibody response targets diverse epitopes while competing for binding to a single antigenic site. Continued discovery of the structure and nature of the epitopes for this unique group of MABs in the future could point the way toward rational design of DENV vaccine antigens to minimize the cross-reactive disease-enhancing antibody profiles of those receiving the vaccine. Development of vaccine preparations that have reduced tendencies to induce prM-specific antibodies, such as viruses or particles lacking the immunodominant K26 residue, could be considered.

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