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Persistence of Circulating Memory B Cell Clones with Potential for Dengue Virus Disease Enhancement for Decades following Infection

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Symptomatic dengue virus infection ranges in disease severity from an influenza-like illness to life-threatening shock. One model of the mechanism underlying severe disease proposes that weakly neutralizing, dengue serotype cross-reactive antibodies induced during a primary infection facilitate virus entry into Fc receptor-bearing cells during a subsequent secondary infection, increasing viral replication and the release of cytokines and vasoactive mediators, culminating in shock. This process has been termed antibody-dependent enhancement of infection and has significantly hindered vaccine development. Much of our understanding of this process has come from studies using mouse monoclonal antibodies (MAbs); however, antibody responses in mice typically exhibit less complexity than those in humans. A better understanding of the humoral immune response to natural dengue virus infection in humans is sorely needed. Using a high-efficiency human hybridoma technology, we isolated 37 hybridomas secreting human MAbs to dengue viruses from 12 subjects years or even decades following primary or secondary infection. The majority of the human antibodies recovered were broadly cross-reactive, directed against either envelope or premembrane proteins, and capable of enhancement of infection *in vitro*; few exhibited serotype-specific binding or potent neutralizing activity. Memory B cells encoding enhancing antibodies predominated in the circulation, even two or more decades following infection. Mapping the epitopes and activity of naturally occurring dengue antibodies should prove valuable in determining whether the enhancing and neutralizing activity of antibodies can be separated. Such principles could be used in the rational design of vaccines that enhance the induction of neutralizing antibodies, while lowering the risk of dengue shock syndrome.

Dengue viruses (DENV) are expanding globally with an estimated 50 to 100 million cases of DENV infection occurring worldwide annually, and more than 20,000 associated deaths. It is now estimated that approximately one-fifth of the world population is at risk of infection by DENV (9, 12, 13). Dengue is also threatening the continental United States. More than 25 cases of locally acquired infection were reported in Key West, Florida, in 2009 and 2010 (1). Symptomatic dengue disease ranges in severity from an influenza-like illness to life-threatening hemorrhagic fever or shock. Understanding the pathogenesis of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) is of central importance in the rational development of antiviral for treatment and vaccines to prevent dengue disease (11, 49).

The DENV complex consists of four serotypes. Infection with a single serotype leads to antibody responses that cross-react with all serotypes (14). Despite the cross-reactivity, individuals only develop protective immunity against the serotype responsible for infection (14, 43). Human studies have established clearly that the risk of progressing to DHF is greater during secondary than during primary infection (15). A prevailing theory that explains severe dengue during secondary infection is that preexisting, nonneutralizing dengue-specific antibodies bind to virions and enhance entry and replication in Fc-receptor-bearing cells, which leads to a higher viremia and release of cytokines and vasoactive mediators that increase vascular permeability (15). The molecular process of antibody-dependent enhancement (ADE) of infection has been demonstrated to occur with DENV and antibodies using cells in culture and animal models (16). A better understanding of the molecular, genetic, and structural basis for recognition of DENV by human antibodies is greatly needed and could lead to the rational design of vaccines that enhance the induction of neutralizing antibodies while lowering the risk of DHF/DSS.

DENV are enveloped viruses of the *Flaviviridae* family that display pseudo-icosahedral symmetry, with 180 copies of the envelope (E) glycoprotein and 180 copies of the membrane (M) protein in the lipid bilayer membrane. Previous structural studies have shown that the dengue virus E protein is arranged into 30 rafts of three parallel dimers in mature virion particles (22). Envelope glycoproteins form dimers, and crystal structures of the postfusion or dimeric forms of E protein have been determined for three DENV serotypes (DENV1, DENV2, and DENV3) (27, 29, 30, 51). The E glycoprotein monomer possesses three principal domains, designated domain I (DI), DII, and DIII. DIII is likely the recognition domain for the principal cell receptor (4, 6, 18, 39). DII possesses the fusion loop (28) and an N-linked glycan, which can contribute to cell binding by interaction with DC-SIGN, a dendritic cell-specific adhesion receptor that is a C-type lectin (38). Extensive characterization of neutralizing epitopes on DENV E protein has occurred over the past 10 years, using monoclonal antibodies (MAbs) generated in mice inoculated multiple times with DENV (14, 15, 43, 45). Mapping of epitopes recognized by strongly neutralizing mouse MAbs has identified several major antigenic sites on the E protein (36). Stud-

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ies have shown that sites in the region of the fusion peptide, located at the tip of E protein DII (34, 39) and near the E protein hinge and linker regions between DI and DII, are recognized by cross-reactive, moderately neutralizing mouse MAbs. The most potent neutralizing antibodies are serotype specific, however, and bind epitopes on the lateral surface of DIII of the E protein (29, 33). Investigators also have uncovered specific mechanisms of antibody neutralization of flaviviruses. Some flavivirus antibodies that neutralize probably do so by sterically blocking attachment of the virus to the cell receptor. Other neutralizing antibodies block a step after cell attachment, and such antibodies possibly neutralize by preventing protein conformational changes required for viral fusion in endosomes (19, 32). Some antibodies (for example, the DENV MAb 1A1D-2) appear to bind to hidden epitopes that are transiently exposed on the particle and promote a cascade of E protein rearrangements on the particle (26). These studies have been instrumental in understanding mechanisms for flavivirus neutralization; however, since the antibodies were generated artificially in mice, the ability to translate this information to humans is limited.

The ability of flavivirus MAbs to enhance DENV infection in cell culture and animal models has been studied extensively (2, 8, 10, 16). ADE of infection has been shown to occur with antibodies binding to E or prM proteins (8, 17, 20). This phenomenon results in as much as a 1,000-fold increase in infectivity and is Fc mediated (2, 25). Recent work by Pierson et al., using mouse MAbs against WNV, has shed light on the stoichiometry of this process (35, 37). For each antibody studied, a threshold number of MAbs docked to the virion dictated whether neutralization or enhancement took place. The type of functional activity was determined not only by the location of the epitope but also by the antibody affinity and epitope accessibility. An additional mechanism of ADE has been demonstrated to occur with anti-prM antibodies. Using either mouse or human MAbs, investigators have shown that the presence of anti-prM antibodies causes immature viral particles that have prM on the surface, which are normally noninfectious, to acquire the ability to infect cells efficiently through Fc-mediated pathways (8, 40, 41). The role that ADE plays in the pathogenesis of DENV infection is of significant interest. Characterization of antibodies obtained from humans following natural infection could prove to be an important tool for further investigation.

Until very recently, the isolation of naturally occurring human MAbs has been challenging. Most of our knowledge of the human antibody response to dengue has come from studies using polyclonal sera of naturally infected patients. Using antibody depletion experiments, we have previously demonstrated that E protein DIII binding antibodies make up a small fraction of the antidengue binding and neutralization activity in immune sera (48). Crill et al. confirmed that serotype-specific DIII antibodies formed a very small proportion of the polyclonal response; however, these investigators showed a significant correlation between the presence of such antibodies and DENV neutralizing activity (5). Taken together, these studies suggest that very rare serotype-specific potent neutralizing antibodies may be the primary determinants of protection against severe disease in humans.

Recently, several groups have generated panels of human DENV-specific MAbs using B cells from people exposed to natural DENV infection in order to investigate the humoral response to dengue infection. Schieffelin et al. generated three E protein-reactive human MAbs and showed that all three were at least partially cross-reactive and two lacked neutralizing activity (44). A panel of six human anti-prM MAbs was developed and shown to be entirely cross-reactive and devoid of significant neutralization activity (8). Other investigators have isolated larger panels of human MAbs to dengue virus (3, 7). These studies indicate that most antibodies are serotype cross-reactive and weakly to non-neutralizing. Fewer than 5% of the antibodies displayed moderate to strong neutralization of one or more serotypes.

In the present study, we use a high-efficiency optimized method to generate human hybridomas to make a large panel of MAbs to DENV derived from peripheral blood cells of travelers following natural primary or secondary infections.

MATERIALS AND METHODS

Viruses and recombinant proteins. DENV1 WestPac-74, DENV2 S-16803, DENV3 CH-53489, and DENV4 TVP-360, provided by Robert Putnak (Walter Reed Army Institute of Research, Silver Spring, MD), were used in the present study. These viruses were used to prepare infectious stocks and purified DENV antigens as previously described (48). A pure virus antigen mixture containing equal quantities of each of the four serotypes were used to screen and characterize human hybridomas. Recombinant proteins containing fragments of E or prM were used to determine antigens and domains recognized by human antibodies. Recombinant envelope (rE) proteins (80% of E protein) from the four DENV serotypes were purchased from Hawaii Biotech, Inc. (27). This antigen binds to conformational MAbs, and X-ray crystallography studies have demonstrated that these proteins retained a native-like structure (27, 29). DIII of E protein from each of the four serotypes was expressed as fusion protein with maltose-binding protein (MBP) in *E. coli* and purified as previously described (27). These proteins also bind to conformational MAbs and retain native-like structure (27). Purified DENV2 proteins containing DI and II of envelope and pr peptide of prM were kindly provided by M. Kielian and A. Zheng from Albert Einstein College of Medicine, New York, NY (24, 52).

Human subjects and peripheral blood cell isolation. We identified a panel of 12 dengue-immune subjects in North Carolina by screening volunteers who suspected exposure during past travel to regions where dengue is endemic. We confirmed DENV infection by testing patient sera for the presence of antibodies that neutralized one or more DENV serotypes. In most laboratory-confirmed cases we were able to pinpoint the year and country of infection by taking a detailed travel and clinical history. From dengue-immune subjects ~100 ml of blood was collected by venipuncture and immediately processed to isolate peripheral blood mononuclear cells (PBMCs) by density gradient separation on Ficoll. The cells were immediately cryopreserved and stored in liquid nitrogen. The protocol for recruiting and collecting blood samples from people was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill.

Generation of human hybridomas. Previously cryopreserved samples were thawed rapidly in a 37°C water bath and washed once in 10 ml of prefusion medium (ClonaCell-HY 03801; Stemcell Technologies). The cells were counted, and viability was assessed with trypan blue staining (Gibco 15250-061). For every 4 million viable cells, the following was then added: 13 ml of warmed prefusion medium, 20 μl of CpG stock (2.5 mg/ml; ODN 2006; Invivogen tlrl-hodnb-5), 20 μl of cyclosporine stock (1 mg/ml in ethanol; Sigma catalog no. C1832), 20 μl of Chk2i stock (10 mM; Sigma catalog no. C3742), and 4.5 ml of clarified supernatant from cultures of B95.8 cells (ATCC VR-1492) containing Epstein-Barr virus (EBV). The mixture then was plated into 384-well plates (Nunc catalog no. 164688) at 50 μl/well, and plates were incubated at 37°C with 5% CO_2 for 10 days, prior to screening for antigen-specific cell lines using an enzyme-linked immunosorbent assay (ELISA). Cells from wells with supernatants reacting in a DENV-specific ELISA were then expanded by

collecting all cells in the well and transferring them to a 96-well flat bottom plate (Falcon catalog no. 353072) in 200 μl of prefusion medium containing irradiated human PBMCs, as follows. We used 20 ml of prefusion medium, 20 μl of CpG stock, 20 μl of Chk2i stock, and 8 million heterologous healthy donor PBMCs that had been gamma-irradiated with 3,000 rads. Plates were incubated at 37°C for 4 days before exchanging 100 μl of medium with fresh prefusion medium. The plates then were incubated for an additional 3 to 4 days prior to fusion with HMMA2.5 nonsecreting myeloma cells (kindly provided by Marshall Posner).

Screening ELISA. Gradient-purified DENV prepared in carbonate binding buffer was used to coat ELISA plates (Nunc catalog no. 242757) and then UV inactivated using a calibrated UV light source (Stratalinker; Stratagene) for 10 min prior to incubation at 4°C overnight. The plates then were blocked with 50 μl of blocking solution/well by incubation at room temperature for 1 h. Blocking solution consisted of 10 g of powdered milk, 20 ml of goat serum, 100 ml of 10× phosphate-buffered saline (PBS), and 0.5 ml of Tween (Sigma 7949) mixed to a 1-liter final volume with distilled H_2O. Plates were washed four times with PBS, and 5 μl of supernatant was transferred from one well of a 384-well plate containing EBV-transformed B cell lines, using a pin-tool device (V&P Scientific) into 25 μl of blocking solution/well. The plates were incubated at room temperature for 1 h prior to four additional 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 μl/well, and the plates were again incubated at room temperature for 1 h. After four washes with PBS, phosphatase substrate solution (1 mg of phosphatase substrate/ml in 1 M Tris aminomethane) (Sigma, catalog no. S0942) was added at 25 μl/well, and the plates were incubated at room temperature for 3 h before determination of the optical density at 405 nm on a Molecular Devices plate reader.

Electrofusion of EBV-transformed B cells with myeloma fusion partner. HMMA2.5 cells were counted and suspended as 10 million cells/ml in a microcentrifuge tube in 1 ml of warmed cytofusion medium composed of 300 mM sorbitol (Fisher, catalog no. BP439-500), 0.1 mM calcium acetate (Fisher, catalog no. AC21105-2500), 0.5 mM magnesium acetate (Fisher, catalog no. AC42387-0050), and 1.0 mg/ml of bovine serum albumin (Sigma, catalog no. A2153). After 7 days of expansion in 96-well plates, cells from EBV-transformed B cell wells were pipetted gently into microcentrifuge tubes containing 1 ml of warmed cytofusion medium. Transformed B cells and HMMA2.5 cells were centrifuged at 900 × *g* for 4 min, the supernatants were decanted, and the pellets were resuspended in 1 ml of cytofusion medium. This process was repeated three times to ensure equilibration to the cytofusion medium. The cytofusion medium then was decanted gently from each sample tube such that ~100 μl remained, and the pellet was retained. HMMA2.5 cells were then resuspended in cytofusion medium to achieve a concentration of 10 million cells/ml. Then, 100 μl of HMMA2.5 cell suspension was mixed with each sample tube, and the mixture of HMMA2.5 and EBV-transformed B cells was pipetted into cytofusion cuvettes (BTX, catalog no. 450125). The cuvette then was placed in a modified cytofusion device, using a BTX cuvette holder (BTX Safety stand, model 630B) and a Cyto-Pulse Sciences generator (Cyto-Pulse Sciences, catalog no. PA-4000/PA-101), and the electrical discharge program was run with following settings: a prefusion AC current of 70 V for 40 s, followed by a DC current pulse of 300 V for 0.04 ms and then a postfusion AC current of 20 V for 30 s. After fusion, the cuvettes were incubated at 37°C for 30 min. The content of each cuvette was then added to 20 ml of hypoxanthine-aminopterin-thymidine (HAT) medium containing ouabain, composed of the following: 500 ml of postfusion medium (Stemcell Technologies, catalog no. 03805), one vial 50× HAT (Sigma, catalog no. H0262), and 150 μl of a 1-mg/ml stock of ouabain (Sigma, catalog no. 013K0750). Fusion products then were plated at 50 μl/well into 384-well plates, followed by incubation at 37°C for 21 days before screening hybridomas for antibody production by ELISA.

MAb production and purification. Wells containing hybridomas producing DENV-specific antibodies were cloned biologically by two rounds of limiting dilution plating. Once clonality was achieved, each hybridoma was expanded in postfusion medium (Stemcell Technologies, catalog no. 03805) until 50% confluent in 75-cm^2 flasks (Corning, catalog no. 430641). For antibody expression, the cells in 75-cm^2 flasks were collected with a cell scraper; the hybridomas were washed in serum-free medium (Gibco Hybridoma-SFM; Invitrogen, catalog no. 12045084) and split equally among four 225-cm^2 flasks (Corning, catalog no. 431082) containing 250 ml of serum-free medium. The flasks were incubated for 21 days before the medium was clarified by centrifugation and sterile filtered (0.2-μm pore size). The antibodies were purified from clarified medium by protein G chromatography (GE Life Sciences, protein G HP columns, catalog no. 17-0404-03).

Neutralization assay. The neutralizing potency of antibodies was measured using a flow cytometry-based neutralization assay with the U937 human monocytic cell line stably transfected with DC-SIGN, as previously described (21).

ADE assays. The ability of antibodies to enhance DENV was measured by 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. Test antibodies were diluted to different concentrations and mixed with viruses containing 10^4 FFU in 96-well round-bottom plates and incubated at 37°C for 45 min. Then, 2×10^4 U937 cells were added to the mixture and incubated for 2 h at 37°C. The cells were washed to remove unbound virus-antibody mixtures and incubated with fresh medium at 37°C. The cells were harvested 24 h later, fixed, and processed for flow cytometric analysis to determine the percentages of infected cells. The fold enhancement of ADE activity was expressed as the relative percent increase of infected cells in the DENV-specific antibody-treated sample compared to the sample treated with a control antibody.

RESULTS

Electrofusion technique for generation of human hybridomas. For generation of human hybridomas using PBMCs from individuals who had recovered from natural infection, we used an established electrofusion method but made several alterations to the previously published protocol (50). First, we found that the kinetics and efficiency of transformation of human B cells using EBV with CpG stimulation was improved inhibiting EBV-associated apoptosis with the addition of a pharmacologic inhibitor of CHK2, a protein kinase that is activated in response to DNA damage and is involved in cell cycle arrest (31). Also, after EBV transformation of PBMCs and identification of a well producing a DENV antibody of interest, expansion of EBV-transformed B cells in the presence of irradiated PBMCs, CpG, and CHK2 inhibitor greatly increased transformed B cell numbers, resulting in the greater likelihood of generating the hybridoma of interest by electrofusion. This added step improved the overall efficiency of isolation of hybridomas by at least 2-fold (data not shown). Additional improvements in throughput and efficiency were achieved by adapting a different electroporation cuvette system (BTX catalog no. 45-0207, model 630B) from that previously described for use with a PA-4000/PA-101 electrofusion device (Cyto-Pulse Sciences). This cuvette system allows for the use of standard disposable electroporation cuvettes, permitting cytofusion of two samples at one time. In addition to the convenience of the disposable cuvette, a moderate improvement in the efficiency of hybridoma generation was observed compared to the Cyto-Pulse cuvette system (data not shown). Finally, improvement in consistency was seen with the use of a commercial hybridoma cell culture medium, typically used for generation and growth of mouse hybridomas (ClonaCell-HY Medium, Stemcell Technologies). Together, these modifications resulted overall in a >10-fold increase in the num-

TABLE 1 Subject demographics, serologies, and hybridoma yields from dengue-immune travelers

Type of infection	Serotype	Subject	Geographic location of infection	Yr	No. of yrs since infection	Reciprocal serum antibody 50% neutralization titer to four DENV serotypes[a]				No. of DENV-positive EBV wells/total no. of wells tested	Frequency of DENV-positive B cells in circulation (10^{-4})[b]	No. of hybridomas obtained
						D1	D2	D3	D4			
Primary	2	19	Thailand	1997	8	95	>	20	105	79/1,860	9.3	10
		31	South Pacific	1997	8	20	320	40	20	9/2,232	1.8	1
	3	3	Thailand	2001	4	30	87	338	<	37/744	9.2	7
		5	Puerto Rico	2001	4	<	<	>	<	21/1,860	5.2	1
		103	Nicaragua	1995	14	<	<	278	<	12/1,860	2.5	3
		105	Thailand	2002	7	<	<	210	<	25/2,232	4.5	6
Secondary	Multiple	8	Haiti	1982–1984	21	640	611	213	319	7/744	2.0	1
		9	India/Sri Lanka	2000	5	>	>	290	393	23/2,976	4.2	1
		15	West Indies	1972–1982	23	371	320	288	>	12/2,232	1.6	1
		27	Thailand/Cambodia	1981	24	>	>	>	285	33/1,488	6.3	3
		110	Kuala Lumpur	1998–1999	10	205	>	153	96	49/2,232	9.5	2
		115	Sri Lanka	1974–1997	12	90	134	330	165	6/1,488	1.7	1

[a] >, Titer > 1:1,280; <, titer < 1:20. D1, D2, D3, and D4, DENV1, DENV2, DENV3, and DENV4.

[b] Frequency was estimated based on the total number of B cells plated (an average of 5 % of total PBMCs) with an estimated 10% EBV transformation efficiency, based on colony counts in the transformation plates.

ber of human hybridomas that could be generated using PBMCs from immune individuals compared to historical data in our laboratory.

Demographics and screening of traveler subjects. To better our understanding of the human antibody response to DENV following infection, we initiated a study to identify and obtain blood from people living in North Carolina who had been exposed to DENV during travel or previous residence in regions where this disease is endemic. Subjects with a past clinical history compatible with DENV infection were invited to volunteer. We confirmed a past DENV infection by identifying the presence of DENV neutralizing antibodies in donor serum, testing against each of the four DENV serotypes (Table 1). An individual whose serum mainly neutralized a single serotype was classified as having a history of infection with a single serotype ("primary") (Table 1). Individuals whose serum contained antibodies that broadly neutralized two or more serotypes were considered to have had two or more previous infections ("secondary") (Table 1). For secondary cases, it was not possible to use the neutralization titers to identify the specific serotypes causing infection or the sequences of the infecting viruses, since these sera typically recognized all four serotypes. All DENV-immune subjects filled out a detailed questionnaire about previous travel and clinical history, and this information was used to determine the year and location of likely infection, as well as the interval between infection and sample collection for the current study (Table 1).

To produce DENV-specific human hybridomas, cryopreserved PBMC samples from each subject were thawed and inoculated with EBV in the presence of CpG, CHK2 inhibitor, and cyclosporine, as described in Materials and Methods. As shown in Table 1, between 744 and 2,976 EBV transformed B cell cultures were generated from samples from each of the 12 subjects, who had primary DENV2 (2 subjects) or DENV3 (4 subjects) infection or a secondary infection (6 subjects). Screening for the presence of antibodies to dengue virus in the supernatant of transformed B cell cultures showed that between 0.4 and 5% of wells in the 384-well transformations were positive. Based on the number of positive wells and the number of lymphocytes tested, the frequency of DENV-specific B cells in circulation was estimated for each subject (Table 1). The frequency was based on the total number of B cells plated (estimated as 5% of the total PBMCs), with an estimated 10% EBV transformation efficiency (based on average colony counts in transformed wells). A total of 37 human anti-DENV hybridomas were generated from 12 different subjects, with a yield ranging from 1 to 10 hybridomas per subject. Twenty-eight hybridomas were derived from individuals who had primary infection, and nine were derived from individuals who had suffered from secondary infection. Remarkably, five hybridomas were derived from the cells of subjects who had been infected with DENV 20 years or more prior to PBMC collection. Antibodies secreted by each of the 37 human hybridoma cell clones were purified and characterized to determine their DENV protein specificity. Twenty-nine MAbs bound the E protein, while eight bound the prM protein.

Characteristics of E protein binding human MAbs. Results of the characterization of 29 purified human anti-E MAbs from anti-dengue hybridomas are shown in Table 2. Hybridomas derived from subjects 3, 19, 103, and 105 comprise the majority (23 of the 29 anti-E MAbs generated). Cells were obtained from these subjects following a primary infection. The remaining six anti-E antibodies were derived from six individuals following either primary or secondary infection. Three anti-E MAbs (designated 4G9, 2C2, and 3H4) were IgG2 molecules, while the rest were of the IgG1 isotype. Of the anti-E MAbs generated, 12 used kappa light chains and 17 used lambda light chains.

As seen in Table 2, 29 of the 37 human anti-dengue MAbs bound to E protein in either ELISA or Western blot assay. Of the 29 anti-E antibodies characterized, all but 3 were fully cross-reactive, binding all four dengue serotypes in ELISA at the lowest concentration tested (1 ng/μl). Two antibodies, MAbs 5J20 and 2D7, were considered partially cross-reactive, since they bound to more than one but less than four serotypes. The DENV2 serotype-specific MAb 2D22 bound only to purified DENV2 virus in ELISA. Next, we determined the binding of these MAbs to recombinant E

TABLE 2 Characteristics of envelope (E) protein binding human MAbs[a]

Type of infection	Serotype	Subject	MAb	IgG subclass	λ or κ	Binding to whole virus (ELISA) for serotypes at 1 ng/μl:				Binding to rE protein (ELISA)	Binding to E protein (Western blotting)	Binding to rE protein DIII fragment (ELISA)	Binding to rE protein DI/II fragment (ELISA)	$Neut_{50}$ concn (μg/ml) against four DENV serotypes:				Fold enhancement of infection for DENV serotypes at 1 μg/ml			
						D1	D2	D3	D4					D1	D2	D3	D4	D1	D2	D3	D4
Primary	2	19	1C16	1	λ	+	+	+	+	+	ND	–	+	10	9	9	–	9	18	**53**	11
			5M22	1	λ	+	+	+	+	+	ND	–	+	7	9	5	–	11	11	**42**	11
			1M19	1	λ	+	+	+	+	+	ND	+	ND	–	–	10	–	6	8	**37**	15
			4C23	1	κ	+	+	+	+	+	ND	–	+	–	7	6	7	15	20	**28**	11
			2A10	1	κ	+	+	+	+	+	ND	–	+	7	7	5	8	11	8	**52**	18
			4G9	2	λ	+	+	+	+	+	ND	–	+	9	10	7	–	9	5	18	6
			5J22	1	λ	+	+	+	+	–	+	–	+	–	10	10	–	6	6	22	6
			3F13	1	κ	+	+	+	+	–	–	–	+	–	–	–	–	–	–	**37**	–
			2D22	1	λ	–	+	–	–	–	–	–	–	–	**0.08**	–	–	–	–	–	–
			1B23	1	λ	+	+	+	+	+	ND	+	ND	4	5	2	9	21	11	**67**	7
		31	4N23	1	λ	+	+	+	+	–	+	–	+	–	–	–	–	–	–	19	5
	3	3	2A15	1	κ	+	+	+	+	+	ND	–	+	7	7	5	10	10	6	16	5
			1A15	1	λ	+	+	+	+	+	ND	–	+	–	–	–	–	–	–	7	–
			1C17	1	λ	+	+	+	+	+	ND	+	ND	–	–	7	–	**27**	**26**	**43**	15
			1I17	1	λ	+	+	+	+	+	ND	–	+	10	8	8	–	7	12	**27**	7
			1M23	1	κ	+	+	+	+	+	ND	+	ND	–	–	7	–	9	13	**51**	7
			1L5	1	κ	+	+	+	+	+	ND	–	+	10	10	2	10	18	24	**34**	23
		103	2D7	1	λ	+	+	+	–	+	ND	–	+	3	–	3	–	–	25	12	–
			2C2	2	λ	+	+	+	+	+	ND	–	+	10	1	–	1	6	5	6	–
			2D17	1	κ	+	+	+	+	+	+	–	+	–	–	–	–	13	5	**27**	–
		105	5J20	1	λ	–	–	+	+	+	ND	–	+	–	–	8	–	–	–	**122**	15
			3I6	1	κ	+	+	+	+	+	ND	–	+	–	–	7	–	10	–	15	13
			5J7	1	κ	+	+	+	+	+	ND	–	+	–	–	**0.1**	–	–	–	23	–
			6K5	1	λ	+	+	+	+	+	ND	–	+	7	5	10	–	**40**	5	**29**	13
Secondary	Multiple	8	2J20	1	κ	+	+	+	+	+	ND	+	ND	6	–	**0.5**	–	7	**28**	22	9
		9	2C7	1	λ	+	+	+	+	+	ND	–	+	4	1	4	6	22	5	5	24
		27	3H9	1	κ	+	+	+	+	+	ND	–	+	–	–	–	–	–	–	5	–
		110	6B22	1	κ	+	+	+	+	–	–	–	+	–	–	–	–	12	20	**36**	10
		115	3H4	2	λ	+	+	+	+	+	ND	–	+	10	10	2	10	–	–	–	–

[a] If the antibody did not bind to recombinant E (rE) protein, Western blotting was performed to determine E protein binding as an alternate method. Binding to recombinant DIII or recombinant DI or DII is also indicated. The concentration at which 50% of the virus was neutralized ($Neut_{50}$) is shown for each DENV serotype: a dash (–) indicates a $Neut_{50}$ value of >10 μg/ml, $Neut_{50}$ values between 1.0 and 10.0 μg/ml are given, and $Neut_{50}$ values of <1.0 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: a dash indicates a <5-fold enhancement, 5- to 25-fold enhancement values are given, and >25-fold enhancement values are indicated in boldface. In the absence of antibody, the percentages of U937 cells infected in the ADE assay with DENV1 (D1), DENV2 (D2), DENV3 (D3), and DENV4 (D4) were 0.06, 0.17, 0.31, and 1.31%, respectively. ND, not determined.

protein in ELISA. All but five MAbs bound recombinant E protein in ELISA. E protein-specific MAbs that did not bind recombinant E protein were shown to bind E protein using Western blotting, with the exception of 2D22. DENV2 serotype-specific antibody 2D22 did not bind to structural proteins in Western blot or to recombinant E protein constructs in ELISA in our studies; however, it appears to be E protein specific based on preliminary escape mutant studies (R. de Alwis, unpublished data). Finally, we evaluated the ability of the 29 anti-E MAbs to bind domains I and II (DI/II) or domain III (DIII) fragments of the E protein. Five antibodies bound the DIII portion of the E protein: 1C17, 1M23, 2J20, 1B23, and 1M19. Antibodies that did not bind recombinant domain III of the E protein were tested in ELISA for binding to DI/II of the E protein. All of the anti-E protein antibodies that did not bind DIII (except for 2D22; i.e., 23 of 29 anti-E protein antibodies) bound DI/II.

The ability of these 29 human anti-E protein MAbs to neutralize representative viruses of the four dengue serotypes is shown in Table 2. Interestingly, six antibodies exhibited little or no neutralizing activity (50% virus neutralization concentration [$Neut_{50}$] > 10 μg/ml) against any of the four dengue serotypes. Most of the MAbs, 13 of 29, were found to neutralize viruses weakly for three or four serotypes, with equivalent potency against those viruses. Seven antibodies were found to neutralize only one or two serotypes weakly. Of the 29 antibodies determined to bind E protein, only three—2D22, 5J7, and 2J20—were determined to have moderate to strong ($Neut_{50}$ < 1 μg/ml) neutralizing activity against at least one serotype.

In Fig. 1, the concentration-dependent neutralization activity of two strongly neutralizing human anti-E protein MAbs is presented. The percent neutralizations for the four dengue serotypes are shown in panel A for MAb 5J7. This MAb, which was isolated from an individual who recovered from a primary DENV 3 infection, cross-reacted with all four serotypes and bound to an epitope on DI/II of E protein (Table 1). Despite the serotype cross-reactivity in binding assays, MAb 5J7 neutralized only serotype DENV3 virus. In panel C, the concentration-dependent neutralization activity of MAb 5J7 is shown in greater detail, using a broader range of halving dilutions. MAb 5J7 neutralized 50% of serotype DENV3 at a concentration of 0.1 μg/ml. The percent neutralization for MAb 2D22 against viruses from the four DENV serotypes is shown in Fig. 1B and D. This antibody, which was isolated from a subject exposed to a primary DENV2 infection, bound to DENV2 virions but not to rE protein (Table 1). In panel B, MAb 2D22 is shown to have neutralizing activity only to the DENV2 serotype. MAb 2D22 exhibited 50% neutralization of DENV2 at a concentration of 0.08 μg/ml (Fig. 1D). Interestingly, as can be seen in Fig. 1, the maximum percentages of virus neu-

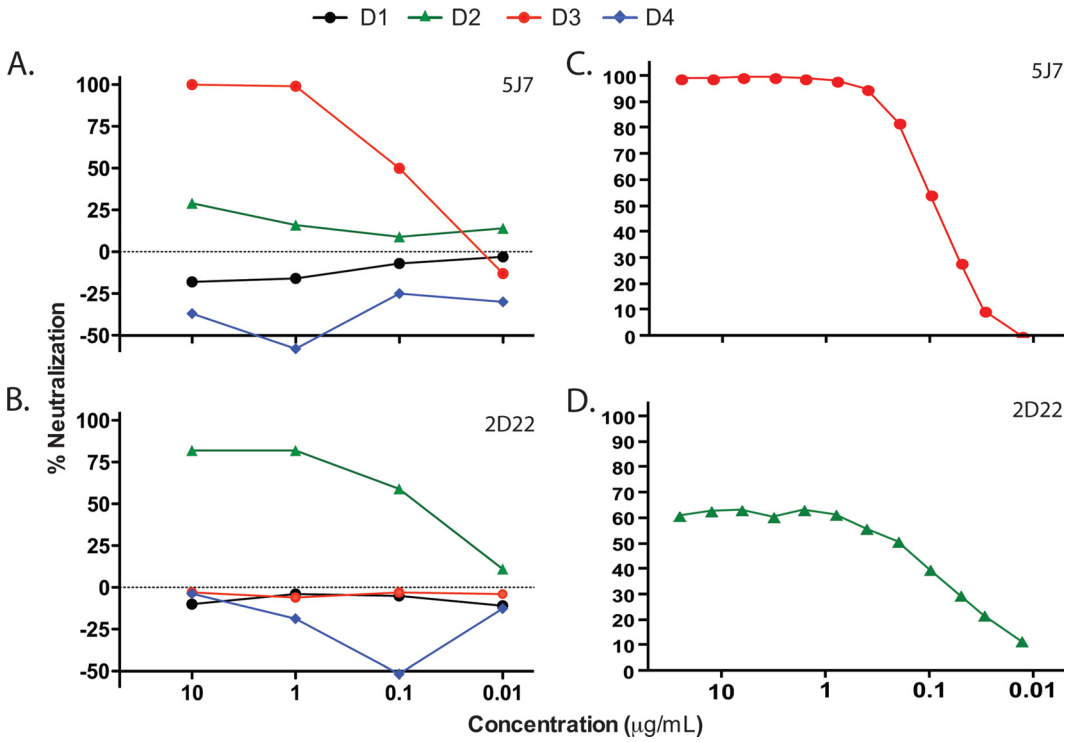

FIG 1 Serotype-specific strongly neutralizing E protein binding human MAbs. (A) The ability of purified human MAb 5J7 to neutralize the four serotypes of DENV is shown over a concentration range. (B) The ability of purified human MAb 2D22 to neutralize the four serotypes of dengue virus is shown over a concentration range. (C) Purified human MAb 5J7 neutralization of serotype 3 virus over a detailed, broader range of halving dilutions. (D) Purified human MAb 2D22 neutralization of serotype 2 virus over a detailed range of halving dilutions. Flow cytometric neutralization assays were performed using U937-DC-SIGN cells.

tralization for MAbs 5J7 and 2D22 were very different. The maximum percentage of neutralization for MAb 5J7 was nearly 100%, whereas MAb 2D22 could neutralize only about 60% of virus in the assay at the highest concentration of antibody tested.

The abilities of the 29 human anti-E MAbs to enhance infection *in vitro* are presented in Table 2. ADE assays were performed at a concentration of 1 μg/ml against a virus from each DENV serotype. Four antibodies did not exhibit enhancing activity (i.e., caused <5-fold enhancement) to three or even four dengue serotypes. Most MAbs, 15 of 29, were found to enhance infection moderately (5- to 25-fold) for viruses from two to four DENV serotypes. Of the 29 antibodies tested, 17 had strong (>25-fold) enhancing activity against virus from at least one serotype. Interestingly, MAb 2J20 strongly neutralized DENV3 but strongly enhanced DENV2 infection.

In Fig. 2, neutralization or enhancement activity against a heterologous DENV4 strain is shown for several representative human anti-E protein MAbs. Flow cytometric neutralization assays were performed using U937-DC-SIGN cells, and enhancement assays were performed with U937 cells lacking DC-SIGN, as described in Materials and Methods. In Fig. 2A, data for fully cross-reactive, weakly neutralizing, and moderately enhancing MAbs 2A15, 1L5, and 2A10 are shown. All three MAbs neutralized 50% of DENV4 at a concentration of ~10 μg/ml. These antibodies enhanced DENV infection moderately, resulting in a 5- to 25-fold peak increase in the titer of DENV4. In Fig. 2B, the results for fully cross-reactive, non-neutralizing and nonenhancing MAbs 3F13, 1A15, and 3H9 are shown. All three antibodies bound DENV4, but they did not neutralize or enhance virus infection effectively. Together, these two principal categories of anti-E protein antibodies represented the majority of the human MAbs we isolated from donors with history of previous primary or secondary DENV infection.

Characteristics of premembrane (prM) protein binding human MAbs. A significant number of the MAbs that we isolated were specific for prM protein. The results of the characterization of purified prM protein-specific human MAbs from anti-dengue hybridomas are shown in Table 3. Hybridomas derived from subjects 27 and 105 comprise four of the eight anti-M MAbs generated. The remaining four antibodies were derived from cells from subjects 3, 5, 15, and 110. In contrast to the findings with anti-E protein MAbs, an equal number of anti-prM protein MAbs was generated from subjects following primary or secondary infection. All of the anti-prM protein MAbs were of the γ1 isotype. Of the anti-prM protein MAbs generated, four had kappa light chains and four had lambda light chains.

As can be seen in Table 3, 8 of the 37 human anti-DENV MAbs did not bind E protein in initial analysis, but instead bound to prM protein in Western blotting analyses. Of the eight anti-prM protein antibodies characterized, all were fully cross-reactive, binding all four dengue serotypes in ELISA at the lowest concentration tested, 1 ng/μl. To further characterize these human anti-prM protein antibodies, we tested their ability to bind the recombinant "pr" portion of the premembrane protein of DENV2 in ELISA. All eight MAbs bound recombinant "pr" in ELISA (Table 3). We

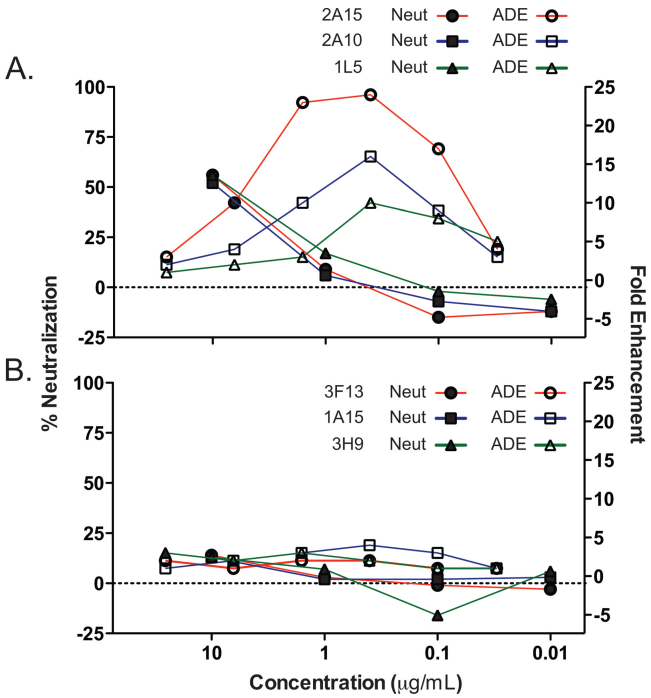

FIG 2 Cross-reactive poorly neutralizing E protein binding human MAbs. (A) The ability of purified human MAb 2A15, 2A10, or 1L5 to neutralize or enhance DENV 4 is shown over a concentration range. These MAbs represent a class of fully cross-reactive, weakly neutralizing, and moderately enhancing antibodies. (B) The ability of purified human MAb 3F13, 1A15, or 3H9 to neutralize or enhance DENV 4 is shown over a concentration range. These MAbs represent a class of fully cross-reactive, non-neutralizing, and nonenhancing antibodies. Flow cytometric neutralization assays were performed using U937-DC-SIGN cells. ADE assays were performed using U937 Fc receptor expressing cells. In the absence of antibody, the percentage of U937 cells infected in the ADE assay with DENV 4 was 2.09%.

tested these eight human anti-prM protein MAbs for their ability to neutralize viruses from each of the four dengue serotypes (Table 3). Interestingly, each exhibited some weak neutralizing activity against virus from at least one of the four dengue serotypes. Surprisingly, MAbs 2M2 and 2H12 showed moderate to strong neutralization activity against virus of a single serotype.

The ability of the eight human anti-prM protein MAbs to enhance infection *in vitro* is shown in Table 3. ADE assays were performed at a concentration of 1 μg/ml against representative viruses from each dengue serotype. All but one of the eight MAbs had strong (>25-fold) enhancing activity for virus from at least one serotype. Interestingly, anti-prM protein MAb 5L20 was nearly devoid of enhancing activity for viruses of any of the dengue serotypes in this assay.

Concentration-dependent neutralization and ADE assays were performed for each of the human anti-prM protein MAbs against viruses from each of the four dengue serotypes. The results for several representative MAbs are shown in Fig. 3. The ability of each anti-prM protein antibody to neutralize and/or enhance infection with DENV1 (Fig. 3A), DENV2 (Fig. 3B), DENV3 (Fig. 3C), or DENV4 (Fig. 3D) is presented. MAbs 4F8, 1G6, and 4E9, each exhibited similar concentration-dependent neutralization curves, with $Neut_{50}$ concentrations of >10 μg/ml against viruses from each dengue serotype. The concentration-dependent neutralization of MAb 5L20, however, differed, exhibiting almost no neutralizing activity. ADE occurred with MAbs 4F8, 1G6, and 4E9 (as well as 5E6, 2M2, 2H12, and 5G22 [data not shown]). Overall, all but one anti-prM protein MAb exhibited enhancing activity. The degree of enhancement varied between 2- and 126-fold, depending on the MAb and the serotype of the virus tested. Interestingly, anti-prM protein MAb 5L20 did not enhance infection in this assay at any concentration tested.

DISCUSSION

These studies reveal that the human B cell response to DENV infection is dominated by cross-reactive antibodies with low or no neutralizing potency and significant potential to enhance infectivity by Fc-mediated mechanisms. Antibodies with enhancing activity were isolated that were directed to both E and prM proteins, thus conferring the ability to enhance infectivity not only with mature infectious particles but also with partially or fully immature particles displaying prM protein that otherwise would be

TABLE 3 Characteristics of premembrane (prM) protein binding human MAbs[a]

Type of infection	Serotype	Subject	MAb	IgG subclass	λ or κ	Binding to whole virus (ELISA) for four DENV serotypes at 1 ng/μl				Binding to r-prM protein (ELISA)	Binding to prM protein (Western blotting)	$Neut_{50}$ concn (μg/ml) against four DENV serotypes:				Fold enhancement of infection for four DENV serotypes at 1 μg/ml			
						D1	D2	D3	D4			D1	D2	D3	D4	D1	D2	D3	D4
Primary	3	3	2H12	1	λ	+	+	+	+	+	ND	–	–	**0.1**	10	14	7	**35**	9
		5	5L20	1	κ	+	+	+	+	+	+	–	–	–	–	–	–	5	–
		105	4E9	1	λ	+	+	+	+	+	+	–	–	1	–	7	–	**30**	21
			5G22	1	λ	+	+	+	+	+	+	–	–	6	–	**40**	12	**94**	**46**
Secondary	Multiple	15	4F8	1	κ	+	+	+	+	+	+	–	–	2	5	13	15	**57**	24
		27	2M2	1	κ	+	+	+	+	+	+	1	**0.5**	2	1	17	8	8	**48**
			1G5	1	κ	+	+	+	+	+	+	–	–	5	–	19	20	**70**	**38**
		110	5E6	1	λ	+	+	+	+	+	+	–	–	6	–	23	15	**36**	13

[a] The pattern of virus binding to purified virus in ELISA at the lowest concentration tested (1 ng/μl) is shown for each serotype. Binding of each antibody to recombinant "pr" fragment of the membrane protein in ELISA, along with binding prM protein by Western blotting, is indicated. Neutralization assays performed for each antibody are shown against each DENV serotype: a dash (–) indicates a $Neut_{50}$ value of >10 μg/ml, $Neut_{50}$ values between 1.0 and 10.0 μg/ml are given, and $Neut_{50}$ values of <1.0 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 shown as the fold enhancement: a dash indicates < 5-fold enhancement, 5- to 25-fold enhancement values are given, and >25-fold enhancement values are indicated in boldface. In the absence of antibody, the percentages of U937 cells infected in the ADE assay with DENV1 (D1), DENV2 (D2), DENV3 (D3), and DENV4 (D4) were 0.06, 0.17, 0.31, and 1.31%, respectively. ND, not determined.

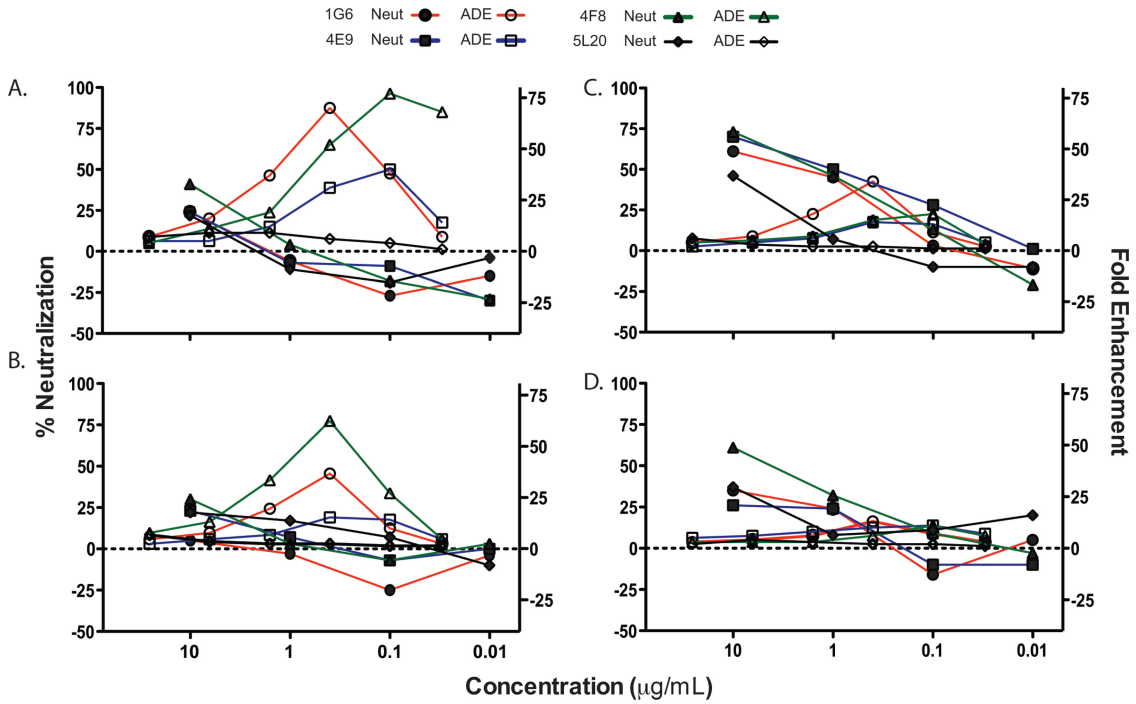

FIG 3 Cross-reactive poorly neutralizing prM protein binding human MAbs. The ability of purified human MAb 1G6, 4E9, 4F8, or 5L20 to neutralize or enhance DENV1 (A), DENV2 (B), DENV3 (C), or DENV4 (D) is shown over a concentration range. Flow cytometric neutralization assays were performed with U937-DC-SIGN cells. ADE assays were performed using U937 Fc receptor expressing cells. In the absence of antibody, the percentages of U937 cells infected in the ADE assay with DENV1, DENV2, DENV3, or DENV4 were 0.06, 0.17, 0.31, and 1.31%, respectively.

poorly infectious or noninfectious. Remarkably, B cells encoding these types of antibodies persisted in the circulation for decades after infection.

Interestingly, however, a few rare, potently neutralizing antibodies that are nearly devoid of enhancing activity were produced naturally by humans in response to infection. Mapping of the epitopes recognized by naturally occurring human DENV-specific antibodies is essential in determining whether these enhancing and neutralizing activities can be separated. Understanding the epitopes and activity of these neutralizing antibodies will be critical for vaccine development, as vaccines that induce high potency neutralizing antibodies that lack enhancing activity are desirable. Ideally, the reactivity of epitopes bound by enhancing antibodies should be reduced or eliminated in candidate antigens during the rational development of a dengue vaccine, so as to discourage such dominant recognition of these antigenic features by the humoral immune response. The goal is that such molecular information could be used in the rational design of dengue vaccines that enhance the induction of protective neutralizing antibodies and reduce the risk of development of severe disease.

DENV E protein was the most common protein target of circulating human memory B cells in the present study. Over three-quarters (29 of 37) of the human anti-dengue antibodies identified here bound to E protein. We screened for virus-specific antibodies using purified whole virus preparations that contained both immature and mature virus particles and all of the virus structural proteins. Therefore, these results confirm that the bulk of the human B cell response to exposed structural antigens is directed toward the E protein. The majority of the anti-E protein antibodies, 23 of 29, were obtained from subjects following primary infection. The E protein has been thought to be the dominant antigen following primary and secondary DENV infection, when evaluated by Western blotting studies using soluble antibodies in polyclonal sera from immune subjects (23). Our studies show that the pattern of specificity of circulating B cells also reflects this predominance. Characterization of the E protein-specific antibodies showed that most were of the IgG isotype. This distribution is typical of the profile of most virus-specific responses in humans and also reflects the predominance of the IgG1 isotype in the circulating human memory B cell population. Light chain usage was split almost evenly at 12 kappa light chains and 17 lambda light chains. Both isotype and light chain usage suggest that isolation of clones using the hybridoma method is unbiased, as expected. Thus, the panel of human antibodies presented here likely reflects the frequency and specificity of the anti-dengue B memory cell pool present in circulation following recovery from primary or secondary infection.

The most striking feature of the anti-E protein antibodies was the predominance of cross-reactivity. Of the 29 anti-E protein antibodies characterized, all but 3 were fully cross-reactive, binding all four dengue serotypes. Considerable attention has been paid to the DIII portion of the E protein for DENV and other flaviviruses (7, 27, 33, 46). The importance of the human antibody response to DIII has been studied extensively, originally using polyclonal sera and more recently using human MAbs (3, 48). However, it is clear that epitopes across other regions of the E protein contribute significantly to human protection (34, 48). The data here provide additional information, specifically a better understanding of the frequency of these antibodies in circulation. Only 5 of the 29 E protein reactive antibodies bound the DIII

portion of E protein. All of the DIII antibodies were cross-reactive, and only one (2J20) had neutralizing activity. Characterization of the 26 fully cross-reactive anti-E protein MAbs showed that most, but not all, were directed toward the DI/II region.

E protein specific human B cells specifying strongly neutralizing antibodies are rare. Of the 29 human anti-envelope antibodies generated, only three (MAbs 5J7, 2J20, and 2D22) met our definition of moderate to strongly neutralizing ($Neut_{50} < 1.0$ μg/ml): two following primary infection and one following secondary infection. Two of the three antibodies, MAbs 5J7 and 2J20, exhibited cross-reactive binding to all four DENVs in an ELISA and yet strongly neutralized virions of only one serotype. 2D22 was DENV2 type-specific both for binding and neutralization. MAb 2J20 bound to DIII and 5J7 to DI/II of the E protein. 2D22 was particularly interesting because it bound to the virus particle but not to any of the recombinant protein constructs used in the present study. There are several plausible explanations for these observations. The 2D22 epitope may be located in a region of E that is not included in the recombinant constructs, given that the membrane- proximal regions were removed in the design of these constructs in order to make them soluble. In addition, the conformation of the epitope may be altered during preparation for ELISA or Western blotting. Alternatively, the epitope recognized by this very interesting human antibody might be very complex, involving the interface of neighboring E proteins. Indeed, human MAbs that only bind a West Nile virus E protein epitope that is preserved on the intact virion but not recombinant protein have recently been described (47). The maximum percentage of virus neutralization for MAbs 5J7 and 2D22 differed greatly. The maximum percentage of neutralization for MAb 5J7 was nearly 100%, whereas MAb 2D22 could neutralize only about 60% at the highest concentration of antibody tested. This finding not only suggests that the MAbs recognize different epitopes but also probably means that they use different mechanisms of neutralization.

ADE activity, not neutralization, was the dominant functional activity noted for the E-protein specific antibodies in the present study. All but two anti-E protein antibodies enhanced infection of at least one serotype at the concentration tested (1 μg/ml). Strongly neutralizing serotype-specific MAb 2D22 and fully cross-reactive weakly neutralizing MAb 3H4 were the only MAbs that had no detectable ability to enhance DENV infection at this initial concentration. Interestingly, further dilution of 2D22, well below the concentration where enhancement occurred with all of the other MAbs, demonstrated enhancement of infection (data not shown). Maximum ADE activity (25-fold) was seen at a concentration of 0.025 μg/ml, compared to between 0.1 and 1.0 μg/ml, seen for other MAbs in our panel. Sixteen antibodies met our definition for mediating strong (>25-fold) enhancement of at least one serotype, including the DIII-binding strongly neutralizing antibody 2J20. Taken together, these data show that the potency of neutralization does not always inversely correlate with the strength of enhancement, since strongly neutralizing antibodies also were strongly enhancing at lower concentrations, whereas MAb 3H4 that was weakly neutralizing did not display enhancing activity. It is important to note that the human antibody response to dengue infection seems to rarely include antibodies that are able to separate neutralization from enhancement, as exemplified by MAb 2D22. This MAb possesses potent serotype-specific neutralization activity without the ability to enhance infection at the typical concentration range seen with weakly neutralizing cross-reactive MAbs. Understanding the specificity of these rare but very important antibodies is critical for the rational design of a dengue vaccine. Preliminary data suggest that this antibody recognizes the E protein but binds to a feature that is only present in the E protein in intact virion particles and not in recombinant soluble forms of E protein or recombinant domains of E.

Interpretation of E protein specific antibody cross-reactivity is complex, since not only do field strains of DENV circulate with serotypic differences, but also there are significant genotypic differences within serotypes. Furthermore, some antibodies to flavivirus E proteins are known to cross-react with diverse flaviviruses (42). Since our subjects acquired DENV infection in Asia and the Americas, the responses theoretically could be affected by undocumented exposure to Japanese encephalitis virus or yellow fever virus or other flavivirus infection or vaccination. However, the subjects studied here did have documented DENV infection histories, and the majority of antibodies cross-react with all four DENV serotypes. Another caveat about these studies of cross-reactivity and ADE is that we used an *in vitro* model of enhanced infection with U937 cells, a human leukemic monocyte lymphoma cell line. It could be of interest in future studies to explore the effect of these antibodies in primary human cells such as monocytes.

Nearly one-quarter, 8 of 37, of the human anti-DENV antibodies identified here were prM protein specific. Compared to the number of anti-E protein antibodies obtained after primary infection versus after secondary infection (i.e., 24 after primary infection and 5 after secondary infection), there appeared to be a greater percentage of anti-prM protein MAbs generated from subjects after secondary infection (17% anti-E, and 50% anti-prM). This finding suggests that the human antibody response to prM protein may be increased relative to the response to E protein following a secondary infection. This observation has been suggested previously by others using Western blotting methods and dengue-immune sera (23). The most prominent characteristic noted in the panel of human anti-prM MAbs here was the degree of cross-reactivity. All eight MAbs were fully cross-reactive, binding all four dengue serotypes in ELISA at the lowest concentration tested (1 ng/μl) using whole virus as antigen. All but one prM protein-specific MAb met our definition for strong (>25-fold) enhancement of infection. Further characterization of these eight MAbs showed that all were directed toward the "pr" portion of the prM protein, since they bound to the recombinant "pr" fragment protein in ELISA. Interestingly, however, two anti-prM protein MAbs met our definition for moderate to strongly neutralizing activity ($Neut_{50} < 1.0$ μg/ml): MAbs 2H12 and 2M2. These two pr-specific MAbs demonstrated serotype-specific neutralizing activity despite being fully cross-reactive. The physiologic significance of potent neutralization of DENV by prM-specific antibodies that should recognize only immature particles is uncertain but bears further exploration.

In summary, the human B cell response encoding DENV-specific antibodies is complex. Human antibody-mediated neutralizing activity is directed to both structural proteins and diverse epitopes in those proteins. Most of the E protein-specific antibodies exhibit ADE activity, whereas only rare clones possess potent neutralizing activity. The antibodies that do neutralize exhibit features that suggest differing mechanisms of neutralization. Most intriguingly, the most potent serotype specific neutralizing antibody identified recognizes an occult epitope that is present in virus

particles but not represented in recombinant antigens. Discovery of the structure and nature of the epitopes for this unique MAb and similar antibodies in the future could point the way toward better rational design of dengue vaccine antigens. Unexpectedly, some prM-specific antibodies exhibited relatively potent neutralizing activity, which is of interest for future studies.

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