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Antibodies to Envelope Glycoprotein of Dengue Virus during the Natural Course of Infection Are Predominantly Cross-Reactive and Recognize Epitopes Containing Highly Conserved Residues at the Fusion Loop of Domain II[▽]

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The antibody response to the envelope (E) glycoprotein of dengue virus (DENV) is known to play a critical role in both protection from and enhancement of disease, especially after primary infection. However, the relative amounts of homologous and heterologous anti-E antibodies and their epitopes remain unclear. In this study, we examined the antibody responses to E protein as well as to precursor membrane (PrM), capsid, and nonstructural protein 1 (NS1) of four serotypes of DENV by Western blot analysis of DENV serotype 2-infected patients with different disease severity and immune status during an outbreak in southern Taiwan in 2002. Based on the early-convalescent-phase sera tested, the rates of antibody responses to PrM and NS1 proteins were significantly higher in patients with secondary infection than in those with primary infection. A blocking experiment and neutralization assay showed that more than 90% of anti-E antibodies after primary infection were cross-reactive and nonneutralizing against heterologous serotypes and that only a minor proportion were type specific, which may account for the type-specific neutralization activity. Moreover, the E-binding activity in sera of 10 patients with primary infection was greatly reduced by amino acid replacements of three fusion loop residues, tryptophan at position 101, leucine at position 107, and phenylalanine at position 108, but not by replacements of those outside the fusion loop of domain II, suggesting that the predominantly cross-reactive anti-E antibodies recognized epitopes involving the highly conserved residues at the fusion loop of domain II. These findings have implications for our understanding of the pathogenesis of dengue and for the future design of subunit vaccine against DENV as well.

Dengue virus (DENV) belongs to the genus *Flavivirus* in the family *Flaviviridae*. The four serotypes of DENV (DENV1, DENV2, DENV3, and DENV4) are the leading cause of arboviral diseases in the tropical and subtropical areas (15, 17, 60). It has been estimated that more than 2.5 billion people in over 100 countries are at risk of infection, and more than 50 million dengue infections occur annually worldwide (15, 17, 60). While most DENV infections are asymptomatic or result in a self-limited illness, dengue fever (DF), some people may present with the severe and potentially life-threatening diseases dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (15, 17, 60).

DENV contains a positive-sense, single-stranded RNA genome of about 10.6 kilobases. Flanked by the 5′ and 3′ untranslated regions, the single open reading frame encodes a polyprotein precursor, which is cleaved by cellular and viral protease into three structural proteins, the capsid (C), precursor membrane (PrM), and envelope (E), as well as seven nonstructural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (34). The E protein, a glycoprotein of approximately 55 kDa, contains 12 strictly conserved cysteine residues forming six disulfide bridges and is present as a heterodimer with PrM protein before the maturation of the virion. E protein participates in virus entry and is the major target of both neutralizing and enhancing antibodies (34). X-ray crystallographic studies of the ecotodomain of E protein have revealed three distinct domains (37, 38, 44). Domain I is located in the center. Domain II, an elongated domain, contains an internal fusion loop at the tip and participates in the membrane fusion and dimerization of E protein. Domain III, an immunoglobulin-like domain, is believed to be involved in receptor binding (7, 34). Domains I, II, and III have been shown to correlate with previously described antigenic domains C, A, and B, respectively (35, 37, 44, 45). Domain II (A) contains mainly the flavivirus group and subgroup cross-reactive epitopes, whereas domains I (C) and III (B) contain mainly subcomplex- and type-specific epitopes.

Although transient cross-protection against heterologous serotypes has been documented, primary infection by one serotype of DENV provided primarily lifelong protection against that serotype, which generally correlated with the appearance of neutralizing antibody to the homologous serotype (14, 48).

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On the other hand, epidemiological observations revealed that individuals experiencing secondary infection with a heterologous serotype had a significantly higher risk of developing DHF/DSS than those with primary infection (4, 18, 50). In the presence of cross-reactive nonneutralizing anti-E antibody, DENV has been shown to replicate to higher titers in human monocytes in vitro, the so-called antibody-dependent enhancement phenomenon (22, 23, 39, 40). This has led to the hypothesis that the cross-reactive antibodies from a previous infection may enhance DENV infection in vivo by increasing the numbers of DENV-infected cells, thus leading to profound immune activation and subsequent immunopathological processes in DHF/DSS (21). Therefore, antibodies against E protein play a critical role in both protection from and enhancement of disease (21, 22, 39).

The PrM protein, a glycoprotein of about 19 kDa, contains six highly conserved cysteine residues forming three disulfide bridges and is cleaved to Pr and M proteins by furin or furin-like protease during maturation (34). Immunization with PrM and M proteins has been reported to confer protection in mice (3). The NS1 protein, also a glycoprotein of about 40 kDa, contains 12 highly conserved cysteine residues forming six disulfide bridges and is present intracellularly, on the cell surface, and outside of the cells (34). Immunization with NS1 protein and passive transfer of anti-NS1 antibodies have been reported to confer protection against DENV2 in mice (11, 25, 43, 51). Recently, it was shown that anti-NS1 antibodies can bind to endothelial cells, induce the expression of cytokines and chemokines, and cause apoptosis (9, 32, 33). Moreover, anti-PrM antibody was reported to enhance DENV infection in vitro (27). These observations suggested that antibodies to PrM and NS1 proteins are also involved in the pathogenesis of disease.

Most studies of antibody responses after DENV infection employed assays that incorporated the whole DENV as antigen, such as the hemagglutination inhibition assay, enzyme-linked immunosorbent assay (ELISA), and plaque-reduction neutralization test (PRNT), and therefore provided little information regarding antibody responses to individual DENV proteins. Only a few studies utilized Western blot analysis to investigate antibody responses to individual DENV proteins (5, 30, 52, 58). Since the infecting serotypes of patients in most of these studies were not clear and DENV proteins of only one or two serotypes were examined, the extent of homologous and heterologous antibody responses to DENV proteins of different serotypes remains unknown. In this study, we examined antibody responses to E, PrM, C, and NS1 proteins of all four serotypes in confirmed DENV2 patients with different levels of disease severity (DF and DHF) and immune status (primary and secondary infections) by Western blot analysis. Our findings revealed that the rates of antibody responses to PrM and NS1 proteins were higher in patients with secondary infection than in those with primary infection. Antibodies to PrM, E, and NS1 were predominantly conformation sensitive. The majority of anti-E antibodies after primary infection were cross-reactive, and only a minor proportion were type specific, which may account for the type-specific neutralization activity. Moreover, the predominantly cross-reactive anti-E antibodies recognized epitopes involving the highly conserved residues at the fusion loop of domain II.

MATERIALS AND METHODS

Patients. The patients were all confirmed DENV2 cases from an outbreak in Kaohsiung, a metropolitan city in southern Taiwan, in 2002 as described previously (59). DF and DHF were diagnosed according to the WHO case definitions (60). With informed consent, acute and convalescent-phase blood samples were collected, and sera were prepared within 6 h of collection and stored at −80°C until use (59). Primary or secondary infection was determined by a previously described NS1-specific immunoglobulin M (IgM) and IgG capture ELISA (53, 59). A Japanese encephalitis virus-NS1 IgM ELISA was used to exclude Japanese encephalitis virus infection (53). Day 1 was defined as the day of onset of fever (oral temperature ≥ 38°C). Convalescent-phase sera (≥day 7) from a total of 69 patients, including 19 with primary infection (16 with DF, 3 with DHF) and 50 with secondary infection (23 with DF, 27 with DHF), were examined by Western blot analysis. The analysis of convalescent-phase sera after day 7 was justified by the finding in several sequential samples from patients with primary and secondary infection that, despite an increase in intensity, there was no increase in the number of DENV protein bands recognized by sera after day 7, from day 14 to day 109 in most cases (see Fig. 2).

Western blot analysis. Briefly, C6/36 cells mock infected or infected with DENV1 (Hawaii strain), DENV2 (NGC strain), DENV3 (H87 strain), or DENV4 (H241 strain) were lysed when cytopathic effects were observed in 50% of cells, and the lysates were subjected to sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis and Western blot analysis using patients' sera (1:5,000 dilution) or mouse monoclonal antibody (MAb) as described previously (31). Loading of similar amounts of four DENV antigens was verified by Western blot analysis using mixed sera consisting of a pool of nine sera from DENV2 patients with secondary infection and anti-NS1 MAb DB29-1, which recognized NS1 protein of four serotypes similarly well (data not shown). The mouse MAbs included anti-E MAbs FL0232, FL0231, and FL0251 (Chance Biotechnology, Taipei, Taiwan; 3H5 and 4G2 (American Type Culture Collection, Manassas, VA) (24), anti-NS1 MAbs DB29-1 and D2-8-2, anti-C MAb DB32-40-30; and anti-PrM MAb 70-21 (27). For reducing gel, the nonreducing buffer (2% sodium dodecyl sulfate, 0.25 M Tris, pH 6.8, 10% glycerol, 0.001% bromophenol blue [final concentrations]) was supplemented with β-mercaptoethanol (0.36 M, final concentration). The specificity of the binding of the anti-E MAbs to four DENV serotypes was demonstrated by Western blot analysis (data not shown).

Antibody blocking experiment. Based on the initial observation of antibody excess in our Western blot analysis using patient serum at 1:5,000 dilution (data not shown), serum was further diluted to facilitate efficient blocking in the blocking experiment. Briefly, one aliquot of diluted serum (1:50,000 to 1:2,000,000), which was preblocked with a nitrocellulose membrane (Hybond-c super; Amersham Biosciences, United Kingdom) containing excessive amounts of lysates (the same antigen loaded in five lanes, 6 μl for each lane) derived from either DENV1-, DENV3-, or DENV4-infected C6/36 cells at room temperature for 1 h, was subjected to Western blot analysis side by side with another aliquot of nonblocked serum using nitrocellulose membranes containing lysates (1 μl antigen loaded in each lane) derived from mock-infected, DENV2-infected, or other-serotype-infected C6/36 cells. In the case of blocking with DENV1, the intensities of E protein bands of DENV2 and DENV1 were analyzed by the UVP (Upland, CA) Bio Imaging system and the UVP software Work 4.5. The completeness of blocking was demonstrated by the background level of the intensity of the E protein band of the blocked serotype (DENV1) detected by the blocked serum. The ratio of the intensity of the DENV2 E protein band detected by the blocked serum to that of the band detected by the nonblocked serum was determined as the DENV2-specific E-binding activity relative to the blocked serotype (DENV1; in percent). The E-binding activity cross-reactive to the blocked serotype (DENV1) was equal to 100% minus the percentage of DENV2-specific E-binding activity relative to DENV1.

Neutralization assay. The PRNT was performed as described previously with minor modifications (29). Briefly, 50 PFU of DENV1 (Hawaii strain), DENV2 (NGC strain), DENV3 (H87 strain), and DENV4 (H241 strain) were preincubated with sera of different dilutions (1:10 to 1:40 or higher) at 4°C for 15 h and inoculated into BHK-21 cells, which were seeded in a 24-well plate (1×10^5 cells) in minimum essential medium (HyClone, Logan, CT) with 10% fetal calf serum 1 day earlier, in duplicate, at 37°C for 2 h. Following the addition of minimum essential medium containing 1% methyl cellulose (Sigma, St. Louis) and 2% fetal calf serum, the plates were incubated at 37°C for 5 days (DENV1 and DENV2) to 7 days (DENV3 and DENV4). After fixation with 3.7% formaldehyde, removal of methyl cellulose, and staining with 1% crystal violet solution in 20% methanol, plaques were counted. $PRNT_{70}$ was the titer that caused greater than 70% reduction in plaque numbers. Monotypic DENV2 neutralization was defined by a $PRNT_{70}$ only to DENV2 of ≥10 or by a $PRNT_{70}$ to more than one

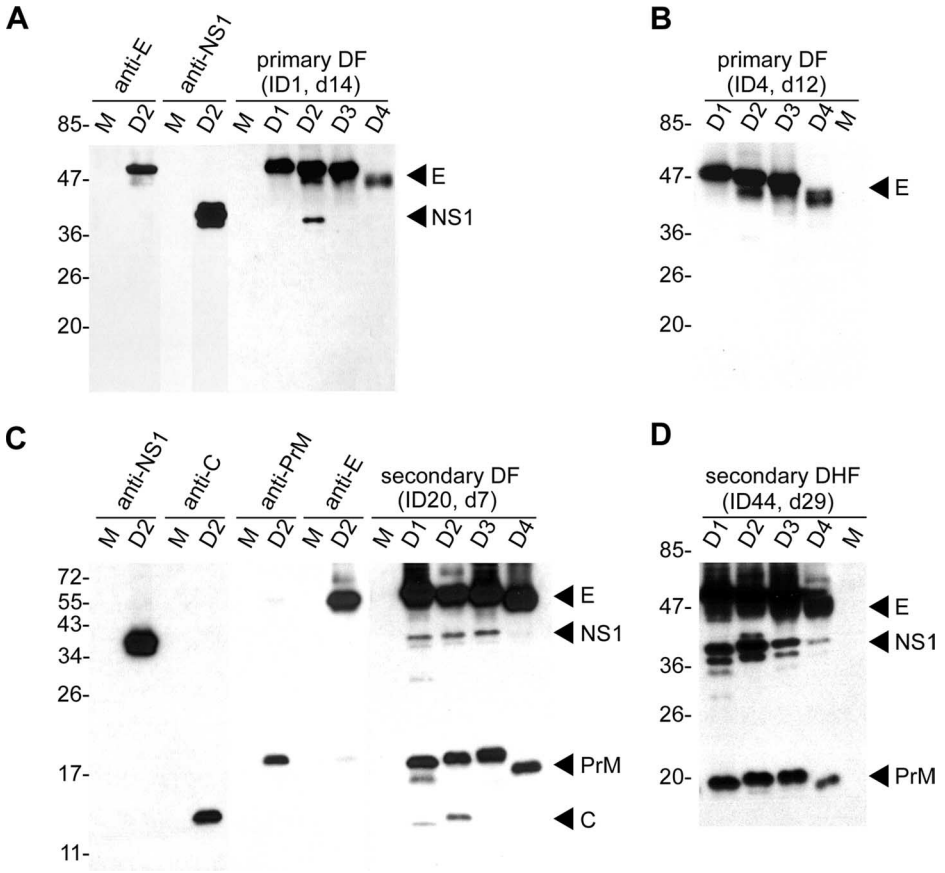


FIG. 1. Antibody responses to different DENV proteins of four serotypes in patients with primary and secondary DENV2 (D2) infections. Convalescent-phase sera from two patients with primary infection (A and B) and two with secondary infection (C and D) as well as anti-E (4G2), anti-PrM (70-21), anti-C (DB32-40-30), and anti-NS1 (DB29-1) MAbs were subjected to Western blot analysis using lysates derived from mock (M)-, DENV1 (Hawaii strain)-, DENV2 (NGC strain)-, DENV3 (H87 strain)-, or DENV4 (H241 strain)-infected C6/36 cells. DF and DHF were classified according to the WHO case definition (60). Primary or secondary infection was determined as described previously (53, 59). Day 1 was defined as the day of onset of fever. Arrowheads indicate the E, PrM, C, and NS1 proteins recognized. Molecular size marker units are kDa. d14, day 14.

serotype of ≥10 with a $PRNT_{70}$ only to DENV2 of ≥80, as described previously, except that $PRNT_{70}$ rather than $PRNT_{50}$ was used as a more stringent cutoff (4, 8).

E mutant constructs. The plasmid constructs expressing the PrM/E proteins of DENV1 (Hawaii strain), pCB-D1 and the fusion loop mutant pCB-D1-107LD, have been described previously (26). To generate other E mutant constructs, site-directed mutagenesis was performed by using pCB-D1 as the template and two-step PCR mutagenesis as described previously (26). The exposed residues at the fusion loop or nonfusion loop of domain II were chosen according to the prediction based on E protein structure (36, 38) (Helix system at NIH [http://helix.nih.gov]). The PCR primers were d1E2XcmIA, d1E150DraIIIB, d1E101WAB1 (5′-ACAGCCATTGCCCGCGCCTCTGTCCAC-3′), d1E101WAA2 (5′-GTGGACAGAGGCGCGGGCAATGGCTGT-3′), d1E108FAB1 (5′-GCTACCTTTTCCGGCAAGCCCACAGCC-3′), d1E108FAA2 (5′-GGCTGTGGGCTTGCCGGAAAAGGTAGC-3′), d1E209HAB1 (5′-AAACCATTGTTTGGCGACAAGCCATGA-3′), d1E209HAA2 (5′-TCATGGCTTGTCGCCAAACAATGGTTT-3′), d1E258-GAB1 (5′-AGTGTGCATTGCTGCTTCTTGTGATCC-3′), d1E258GAA2 (5′-GGATCACAAGAAGCAGCAATGCACACT-3′), d1E262TAB1 (5′-TCCGGTCAACGCAGCGTGCATTGCTCC-3′), d1E262TAA2 (5′-GGAGCAATGCACGCTGCGTTGACCGGA-3′), d1E265TAB1 (5′-TTCTGT CGCTCCGGCCAACGCAGTGTG-3′), and d1E265TAA2 (5′-CACACTGCGTTGGCCGGAGCGACAGAA-3′). After the second PCR, the 444-bp product was digested with XcmI and DraIII and cloned into the respective sites of pCB-D1. All mutants were confirmed by sequencing the entire 444-bp region of the insert to exclude a second site mutation.

Transfection. 293T cells (1×10^5 cells) were transfected with 10 μg of each plasmid DNA by the calcium phosphate method. At 48 h posttransfection, cells were washed with 1× phosphate-buffered saline and lysed with 1% NP-40 lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.5% Na orthovanadate) containing protease inhibitors (Roche Diagnostics, Indianapolis, IN), followed by centrifugation at 14,000 rpm (851 rotor; Thermo Electron Corporation, Waltham, MA) at 4°C for 30 min to obtain cell lysates for Western blot analysis by using mixed sera, an individual patient's serum, and MAbs (26, 31).

Antibody binding to different mutant E proteins. The intensities of E protein bands of the wild type (pCB-D1) and mutants were analyzed as described above. The recognition index of a serum or MAb for a mutant E protein is given by the following formula: (intensity of the mutant E band/intensity of the wild-type E band [for a serum or MAb]) × (intensity of the wild-type E band/intensity of the mutant E band [for mixed sera]), as described previously (56). The nonparametric Mann-Whitney U test, in the software SPSS base 8.0 (SPSS Inc., Chicago, IL) was used to compare the recognition indices for each mutant E protein between sera from patients in primary and secondary infection groups.

RESULTS

Higher rates of antibody responses to PrM and NS1 in patients with secondary infection than in those with primary infection. Since identification of antibodies to different DENV proteins in previous Western blot analyses relied primarily on the predicted molecular weight of the protein, rather than using known MAbs or recombinant proteins as controls, reac-

tivities to certain protein bands cannot be convincingly demonstrated (5, 52, 58). To investigate antibody responses to E, PrM, C, and NS1 proteins, Western blot analysis was carried out using patients' sera side by side with MAbs to each of these proteins. Representative examples of antibody responses in two patients with primary infection and two with secondary infection were shown in Fig. 1. While antibodies to E proteins of four serotypes were found in patients with primary infection, antibodies to E and PrM proteins of four serotypes as well as NS1 proteins of three or four serotypes were found in patients with secondary infection (Fig. 1). The antibody responses to E, PrM, C, and NS1 proteins of four serotypes in 69 patients, including 19 cases of primary infection (16 of DF, 3 of DHF) and 50 cases of secondary infection (23 of DF, 27 of DHF), were summarized in Table 1. In agreement with previous reports that the anti-E response was the major antibody response, anti-E antibodies were found in all patients, followed by anti-NS1 and anti-PrM antibodies (5, 52, 58). This was further supported by analysis of sequential samples from several cases with either primary or secondary infection, in which strong anti-E antibodies appeared first, followed by anti-NS1 or anti-PrM antibodies (Fig. 2). Anti-C antibodies were found in only a few cases with secondary infection (Table 1). Based on the early-convalescent-phase sera and the dilution tested, there was no difference in the rates of antibody responses to E, PrM, C, and NS1 proteins of each serotype between DF and DHF patients with either primary or secondary infection ($P > 0.05$; Fisher's exact two-tailed test). In contrast, the rates of antibody responses to PrM and NS1 proteins of each serotype were significantly higher in patients with secondary infection than in those with primary infection for either the DF or DHF group or the two groups together ($P < 0.01$; Fisher's exact two-tailed test) (Table 1).

Antibodies to E, PrM, and NS1 are predominantly conformation sensitive. Previous studies of mouse anti-E and anti-NS1 MAbs have shown that most of these MAbs lost reactivity under reducing conditions on treatment with β-mercaptoethanol and therefore were sensitive to the conformation provided by disulfide bridges (10, 45). To investigate whether human antibodies to these proteins were also conformation sensitive, lysates derived from four serotypes of DENV-infected cells were subjected to Western blot analysis under both nonreducing and reducing conditions. As the reagent controls, flavivirus group-reactive mouse anti-E MAb 4G2, which was previously reported as β-mercaptoethanol sensitive, completely lost its binding to all four E proteins under reducing condition (Fig. 3A, top). In contrast, DENV2-specific anti-E MAb 3H5, previously reported as partially resistant to β-mercaptoethanol, showed decreased binding to E protein under reducing conditions (Fig. 3A, bottom). Similarly, DENV2-specific anti-NS1 MAb D2-8-2 can recognize NS1 protein under reducing conditions, in which the band decreased in intensity and migrated more slowly (Fig. 3B, bottom). Another anti-NS1 MAb, DB29-1, can recognize NS1 proteins of all four serotypes under both nonreducing and reducing conditions with a slight decrease in intensity (Fig. 3B, top). The antibody response in a patient with primary infection was shown in Fig. 3C, in which the anti-E antibodies lost reactivity to all four serotypes under reducing conditions, suggesting that polyclonal anti-E antibodies recognized epitopes that were sensitive to β-mercaptoeth-

TABLE 1. Summary of antibody responses to E, PrM, C, and NS1 proteins of four DENV serotypes in patients with DENV2 infection

Patient group[a] (*n*)	Response (no. positive/no. tested [% positive]) for indicated DENV serotype[d] to:															
	E				PrM				C				NS1			
	D2	D1	D3	D4	D2[b]	D1[b]	D3[b]	D4[c]	D2	D1	D3	D4	D2[b]	D1[b]	D3[b]	D4[b]
Primary DF (16)	16/16 (100)	16/16 (100)	16/16 (100)	14/16 (87.5)	1/16 (6.3)	4/16 (25)	0/16 (0)	0/16 (0)	0/16 (0)	0/16 (0)	0/16 (0)	0/16 (0)	10/16 (62.5)	3/16 (18.8)	2/16 (12.5)	0/16 (0)
Primary DHF (3)	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)	1/3 (33.3)	1/3 (33.3)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	0/3 (0)	2/3 (66.7)	1/3 (33.3)	1/3 (33.3)	0/3 (0)
Secondary DF (23)	23/23 (100)	23/23 (100)	23/23 (100)	23/23 (100)	22/23 (95.7)	22/23 (95.7)	18/23 (78.3)	18/23 (78.3)	1/23 (4.3)	3/23 (13)	1/23 (4.3)	1/23 (4.3)	23/23 (100)	22/23 (95.7)	21/23 (91.3)	17/23 (73.9)
Secondary DHF (27)	27/27 (100)	27/27 (100)	27/27 (100)	27/27 (100)	27/27 (100)	25/27 (92.6)	24/27 (88.9)	21/27 (77.8)	0/27 (0)	3/27 (11.1)	0/27 (0)	0/27 (0)	27/27 (100)	25/27 (92.6)	27/27 (100)	23/27 (85.2)

[a] DF and DHF were classified according to the WHO case definition (60). Primary and secondary infections were determined as described previously (53, 59).
[b] $P < 0.01$, primary DF versus secondary DF, primary DHF versus secondary DHF, and primary total versus secondary total (Fisher's exact two-tailed test).
[c] $P < 0.01$, primary DF versus secondary DF and primary total versus secondary total; $P < 0.025$, primary DHF versus secondary DHF (Fisher's exact two-tailed test).
[d] D2, DENV2.

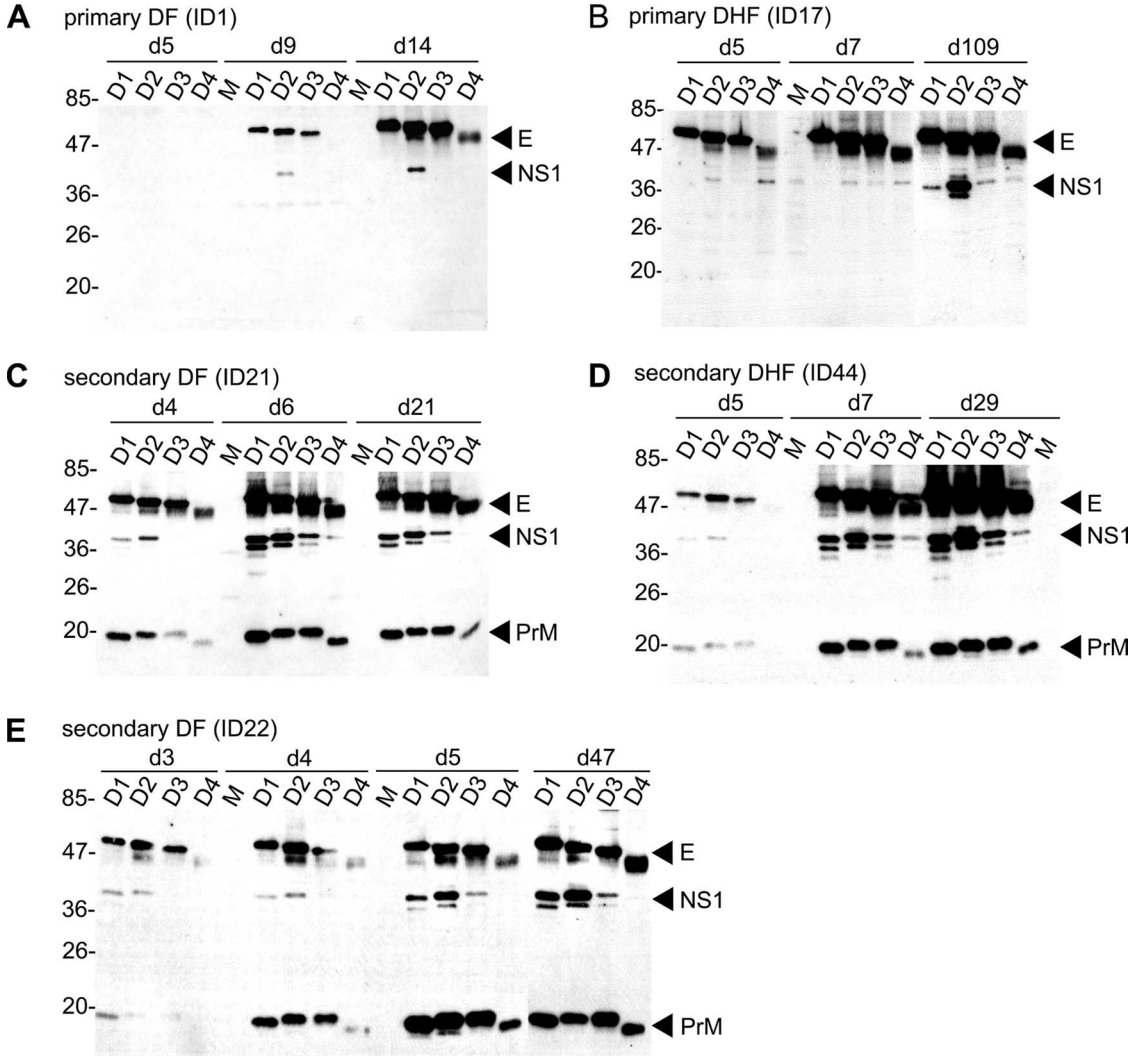
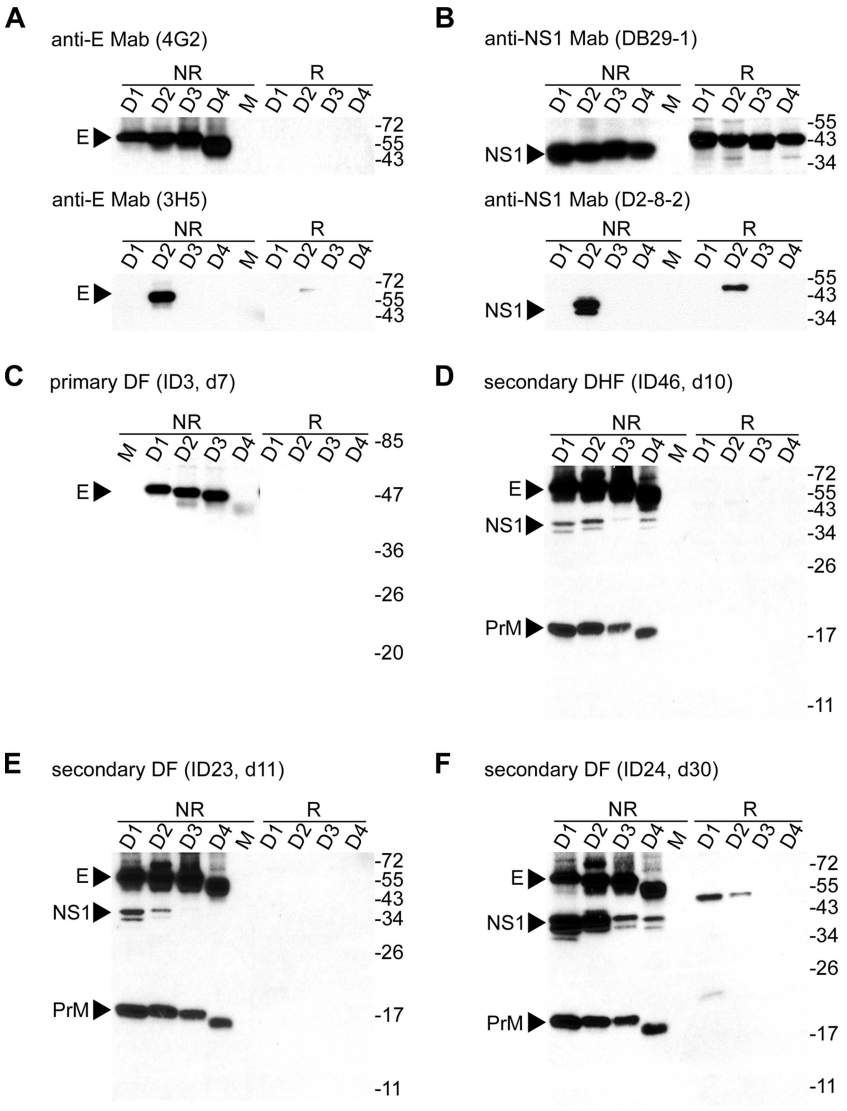


FIG. 2. Antibody responses to different DENV proteins of four serotypes in sequential sera from patients with primary (A and B) and secondary (C to E) DENV2 (D2) infection. Sera were subjected to Western blot analysis, and the data are presented as described for Fig. 1. Arrowheads indicate the E, PrM, and NS1 proteins recognized. Molecular size marker units are kDa. d5, day 5.

anol. Similarly, the reactivity of anti-E and anti-PrM antibodies to four serotypes was completely undetectable under reducing conditions in three patients with secondary infection (ID46, ID23, and ID24), whereas the anti-NS1 antibodies retained weak reactivity to DENV1 and DENV2 under reducing conditions in one patient (ID24) (Fig. 3D to F). The anti-E, anti-PrM, and anti-NS1 antibody responses under nonreducing and reducing conditions in 15 cases with primary infection and 10 cases with secondary infection were summarized in Fig. 3G. Under reducing conditions, anti-E antibodies completely lost reactivity in all 15 cases with primary infection and retained faint reactivity in 4 of the 10 cases with secondary infection, suggesting that the epitopes recognized by anti-E antibodies generated during the natural course of infection were predominantly conformation sensitive. Similarly, anti-PrM and anti-NS1 antibodies, though present only in some cases with primary infection, lost reactivity under reducing conditions in most cases with primary infection and retained faint reactivity in 20 to 50% of cases with secondary infection.

The majority of primary anti-E antibodies are cross-reactive and nonneutralizing against heterologous serotypes. Since anti-E antibodies cross-reactive to all four serotypes were generated in nearly all cases with primary infection (Table 1), we next investigated the relative amounts of homologous and heterologous anti-E antibodies in patients with primary infection by using an antibody blocking experiment. As shown in Fig. 4A, after blockage with lysates derived from DENV1-infected cells, the anti-DENV1 E reactivity for one case (ID17) was completely abolished and, surprisingly, the anti-DENV2 E reactivity was also abolished, with only a very faint band left, of which the intensity was 0.6% of the anti-DENV2 E intensity for the nonblocked serum. Thus, the DENV2-specific E-binding activity relative to DENV1 was 0.6% and the E-binding activity cross-reactive to DENV1 was 99.4% in this case, suggesting that the majority of anti-DENV2 E antibodies generated during primary DENV2 infection cross-reacted with DENV1 E protein and that only a minor proportion were DENV2 specific. Similarly, anti-DENV2 E reactivity in this



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	anti-E		anti-PrM		anti-NS1	
	NR	R	NR	R	NR	R
primary infection cases (n=15)	15/15	0/15	3/15	0/15	7/15	2/15
secondary infection cases (n=10)	10/10	4/10	10/10	2/10	10/10	5/10

FIG. 3. Antibody responses to different DENV proteins of four serotypes under nonreducing and reducing conditions in patients with primary and secondary DENV2 (D2) infections. Anti-E MAbs (4G2 and 3H5 in panel A) and anti-NS1 MAbs (DB29-1 and D2-8-2 in panel B), as well as convalescent-phase sera from patients with primary (C) or secondary infection (D to F) were subjected to Western blot analysis under nonreducing (NR) and reducing (R) conditions as described in Materials and Methods. DF and DHF were classified according to the WHO case definition (60). Primary or secondary infection was determined as described previously (53, 59). Day 1 was defined as the day of onset of fever. Arrowheads indicate E, PrM, and NS1 proteins. Molecular size marker units are kDa. d7, day 7. (G) Summary of the results for 25 patients, including 15 with primary infection and 10 with secondary infection. One representative experiment of more than two is shown.

case greatly diminished after blockage with lysates derived from DENV3- or DENV4-infected cells, with DENV2-specific E-binding activity of 1.6% relative to both DENV3 and DENV4 (Fig. 4A). The results of the blocking experiment for the other two cases were shown in Fig. 4B and C. Of note, the blocking experiment using different dilutions of sera in these cases revealed very similar DENV2-specific E-binding activity (data not shown). Table 2 summarized the E-binding activity specific to DENV2 and cross-reactive to DENV1, DENV3, or DENV4 in seven patients with primary DENV2 infection.

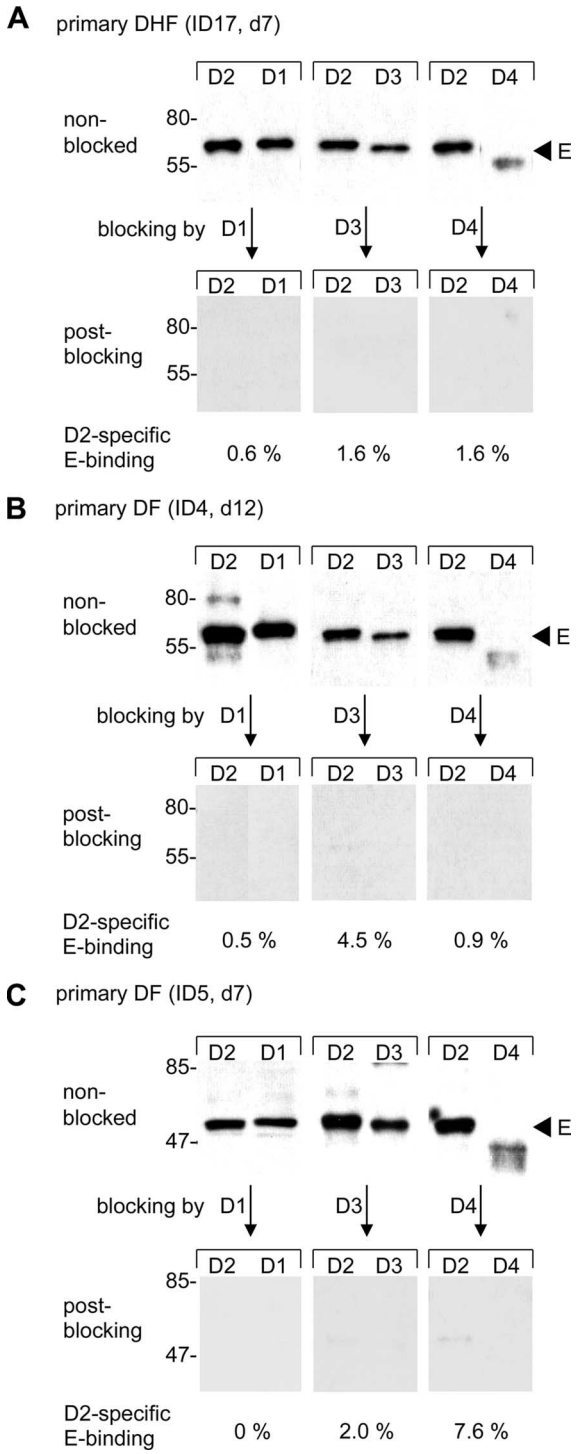

FIG. 4. Antibody blocking experiment to determine the DENV2 (D2)-specific E-binding activity relative to DENV1, DENV3, and DENV4 in sera of three patients with primary DENV2 infection, including (A) ID17, (B) ID4, and (C) ID5. The antibody blocking experiment was as described in Materials and Methods. The ratio of the intensity of the anti-DENV2 E protein band from postblocking serum to that of the band from nonblocked serum (in percent) was determined as the DENV2-specific E-binding activity for that serotype. Primary infection was determined as described previously (53, 59). Day 1 was defined as the day of onset of fever. Arrowheads indicate E proteins. Molecular size marker units are kDa. One representative experiment of more than three was shown. d12, day 12.

With one exception, the DENV2-specific E-binding activity ranged from 0% to 8.6% and the E-binding activity cross-reactive to other serotypes ranged from 91.4% to 100%, suggesting that the majority of anti-E antibodies generated during primary infection were cross-reactive. The one exception was patient ID6, for whom DENV2-specific E binding was 21.5% after blockage with DENV4 E protein, suggesting that less DENV4-cross-reactive anti-E antibodies were generated in this case than in others.

To further investigate the neutralization activity to four serotypes of DENV in these sera, a previously described PRNT was carried out using $PRNT_{70}$ as the cutoff (29). In agreement with previous reports, a predominantly monotypic neutralization pattern for DENV2 was seen in all seven cases with primary DENV2 infection (Table 2) (1, 29). Of note, the neutralization titers were generally low, probably due to the relatively early convalescent phase of these sera tested (median, day 12; range, days 7 to 14). For ID5's early serum at day 7, the monotypic DENV2 neutralization pattern was observed only when $PRNT_{50}$ was measured.

Cross-reactive anti-E antibodies recognized epitopes containing highly conserved residues at the fusion loop of domain II. Previous studies have reported that the binding sites of several broadly flavivirus cross-reactive MAbs from mice and a Fab antibody from chimpanzees involved some highly conserved residues at the fusion loop (amino acid residues 98 to 109) of domain II (6, 12, 54). To investigate whether the predominantly cross-reactive anti-E antibodies generated during primary DENV2 infection recognize the highly conserved residues at the fusion loop, site-directed mutagenesis was carried out to replace two residues at the fusion loop, tryptophan at position 101 and phenylalanine at position 108, with alanine (38) (mutants designated 101WA and 108FA), in the DENV1 PrM/E-expressing construct pCB-D1 (26). For these DENV2-infected patients, the construct expressing DENV1 PrM/E proteins was chosen for mutational study to measure the cross-reactive anti-E antibodies. Residues 101W and 108F were predicted to be exposed based on the E protein structure (36, 38). Another previously described fusion loop mutant, with leucine at position 107 (107LD), was also included in the analysis (26). Alanine substitutions were also introduced to the other four domain II residues predicted to be exposed (36), including histidine at position 209, glycine at position 258, threonine at position 262, and threonine at position 265 (designated 209HA, 258GA, 262TA, and 265TA). After transfection of the wild type, pCB-D1, and mutant constructs to 293T cells, lysates were subjected to Western blot analysis using anti-E MAbs and patients' sera. Compared with that derived from pCB-D1, comparable amounts of E protein bands derived from each mutant can be detected by mixed sera, suggesting that the mutations introduced did not affect the expression and global conformation of the E protein (Fig. 5A). Consistent with previous reports, the flavivirus group-reactive MAb 4G2 could not bind to E proteins containing three individual mutations at the fusion loop (101WA, 107LD, and 108FA), whereas its binding to four mutant E proteins with mutations at the nonfusion loop residues of domain II was only mildly affected compared with the binding to the wild type (Fig. 5B). Similarly, the binding of two other flavivirus group-reactive MAbs, FL0231 and FL0232, was abolished by muta-

TABLE 2. E-binding activity and neutralization activity to four serotypes of DENV in sera from patients with primary DENV2 infection

Patient[a]	Sampling day[b]	E-binding activity[c] (%)				Neutralization ($PRNT_{70}$) result[d]			
		Specific to D2	Cross-reactive to:						
			D1	D3	D4	D2	D1	D3	D4
ID17	7	0.6–1.6	99.4	98.4	98.4	1:10	—	—	—
ID4	12	0.5–4.5	99.5	95.5	99.1	1:10	—	—	—
ID1	14	0–2.3	100	97.7	99.8	1:160	—	—	1:10
ID5	7	0–7.6	100	98.0	92.4	— (1:10)	— (—)	— (—)	— (—)
ID2	9	0–8.6	98.8	100	91.4	1:10	—	—	—
ID6	13	0–21.5	100	96.9	78.5	1:40	—	1:10	—
ID7	12	0–0.4	100	100	99.4	1:160	—	1:40	—

[a] Primary infection was determined as described previously (53, 59). ID17 was a DHF case and others were DF cases according to the WHO case definition (60).
[b] Day 1 was defined as the day of onset of fever.
[c] E-binding activity specific to DENV2 (D2) and E-binding activity cross-reactive to DENV1, DENV3, or DENV4 were as described in Materials and Methods.
[d] $PRNT_{70}$ was described in Materials and Methods. —, no reduction in plaque numbers greater than 70% at 1:10 dilution. Parentheses indicate the results for $PRNT_{50}$.

tions at two or three fusion loop residues but not by mutations at the four nonfusion loop residues (Fig. 5B). In contrast, the binding of DENV1 type-specific MAb FL0251 was reduced by mutations of the nonfusion loop residues (especially 258GA and 262TA) at the dimer interface of domain II but not by mutations of the two fusion loop residues (101WA and 107LD) (Fig. 5B). FL0251 probably recognized an epitope similar to a recently identified epitope at the same region (263H), which was recognized by West Nile virus (WNV) type-specific MAb E100 (42).

We next examined sera from seven patients with primary infection (Table 2). While the binding of these sera to E proteins containing mutations at the four nonfusion loop residues was mildly affected compared to that to the wild-type E protein, the binding to E proteins with mutations at the fusion loop residues was greatly reduced (101WA in all cases, followed by 108FA and 107LD to a lesser extent), suggesting that the predominantly cross-reactive polyclonal sera generated during primary infection recognized epitopes that involved some highly conserved residues at the fusion loop of domain II (Fig. 6A). For comparison, polyclonal sera from seven patients with secondary infection were also examined. Compared to the pattern observed in patients with primary infection, the binding of these sera to mutant E proteins with mutations at the fusion loop residues was only moderately reduced in most cases (Fig. 6B).

Since the 14 sera examined above were largely from the early convalescent period (day 7 to day 14) of infection, we further examined three and four sera from patients with primary and secondary infections, respectively, at a later time point (day 16 to day 109). For three of the seven patients (ID17, ID49, and ID21), early-convalescent-phase sera were also analyzed. As shown in Fig. 6C, the binding for three convalescent-phase sera at later time points from patients with primary infection was greatly reduced by the fusion loop mutations, a pattern similar to that for the early-convalescent-phase sera (Fig. 6A). In contrast, the binding pattern for four convalescent-phase sera at later time points from patients with secondary infection was only moderately affected by the fusion loop mutations (Fig. 6D).

The intensities of wild-type and mutant E protein bands were further quantified, and the recognition indices of each MAb or serum for different mutant E proteins were calculated (56). Consistent with the pattern observed in Fig. 6A and C, the mean recognition indices of 10 sera from patients with primary infection were greatly reduced by mutations of the fusion loop residues (101WA and 108FA), whereas they were slightly affected by mutations of the nonfusion loop residues (Fig. 6E). This was similar to the pattern of three flavivirus group-reactive MAbs (4G2, FL0231, and FL0232) (Fig. 5C). By contrast, the mean recognition indices of 11 sera from patients with secondary infection were moderately affected by mutations of the fusion loop residues (Fig. 6F). The recognition indices for two fusion loop mutants, 101WA and 108FA, were significantly lower for sera from patients with primary infection than for sera from those with secondary infection ($P = 0.001$ and 0.007, respectively; Mann-Whitney U test, two tailed), whereas the recognition indices for other mutant E proteins were not different between the two groups ($P > 0.05$; Mann-Whitney U test, two tailed).

DISCUSSION

In this study, we examined antibody responses to E, PrM, C, and NS1 proteins of four serotypes of DENV during the natural course of DENV2 infection. Based on the early-convalescent-phase sera and the dilution tested, there was no difference in the rates of antibody responses to E, PrM, C, and NS1 proteins between DF and DHF patients with either primary or secondary infection. The rates of antibody responses to PrM and NS1 proteins were significantly higher in patients with secondary infection than in those with primary infection (Table 1), suggesting that anti-PrM or anti-NS1 antibody responses may be used to distinguish primary and secondary infections. The difference in the rates of anti-NS1 or anti-PrM antibody responses to the infecting (homologous) serotype and heterologous serotypes in patients with primary or secondary infection suggested the need for a larger sample size at different time points of infection to develop an assay to distinguish primary and secondary infections. In the case of anti-NS1 antibody, it was reported recently that NS1 serotype-specific IgG capture ELISA together with sampling day data can distinguish primary and secondary infections conveniently (53).

Previously, several linear epitopes located at different re-

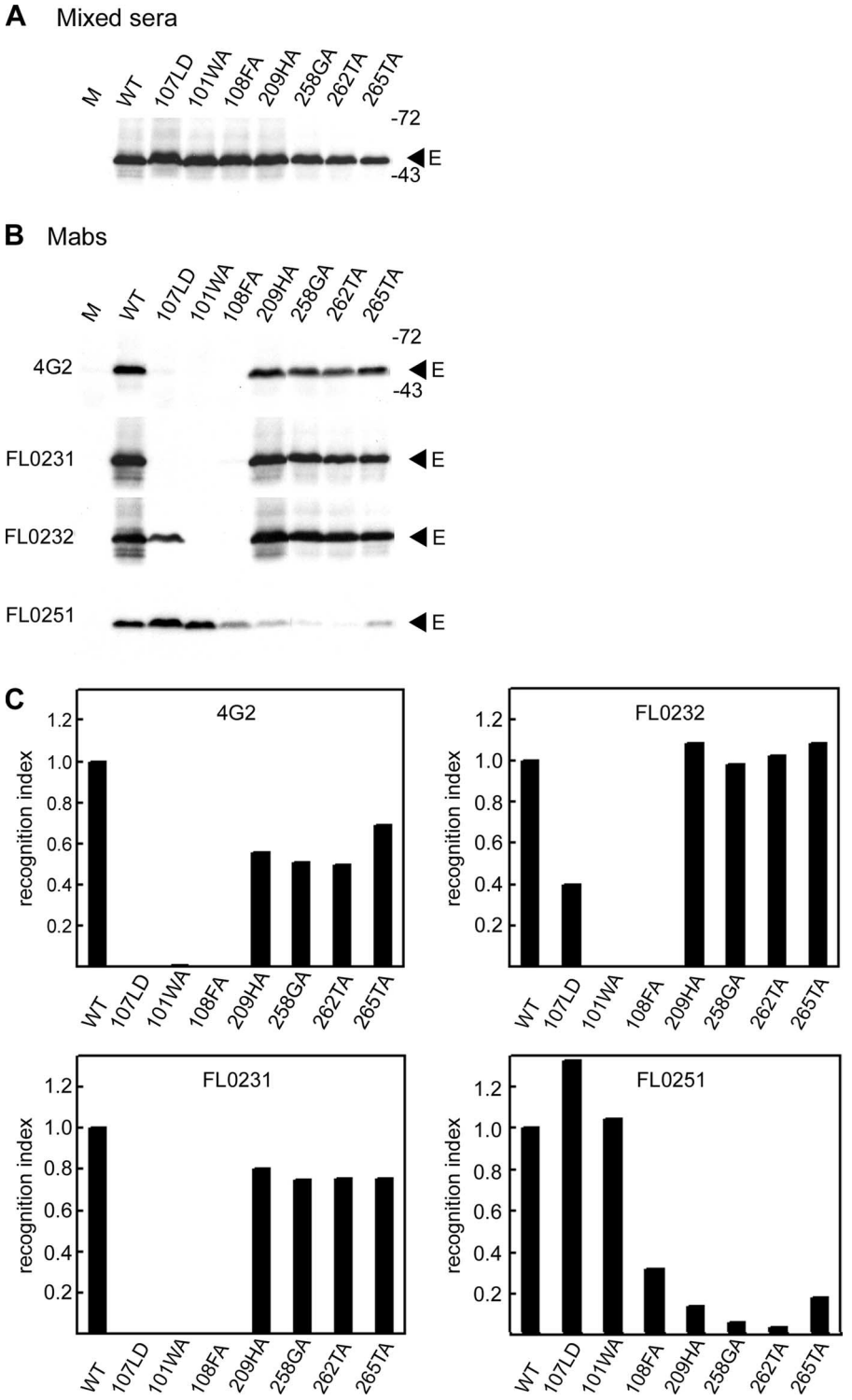

FIG. 5. MAb binding to DENV1 E proteins with mutations within (101WA, 107LD, and 108FA) and outside (209HA, 258GA, 262TA, and 265TA) the fusion loop of domain II. Cell lysates derived from 293T cells transfected with mock (M), wild-type (WT; pCB-D1), and mutant E constructs were subjected to Western blot analysis by using mixed sera (A) and four anti-E MAbs (B) (26). Mixed sera consisted of a pool of nine sera of DENV2 patients with secondary infection (59). Arrowheads indicate E proteins. Molecular size marker units are kDa. One representative experiment of more than two was shown. (C) The intensities of E protein bands of wild type and mutants were analyzed as described in Materials and Methods. The recognition index of a MAb to a mutant E protein is given by the following formula: (intensity of the mutant E band/intensity of wild-type E band [by MAb]) × (intensity of wild-type E band/intensity of mutant E band [by mixed sera]).

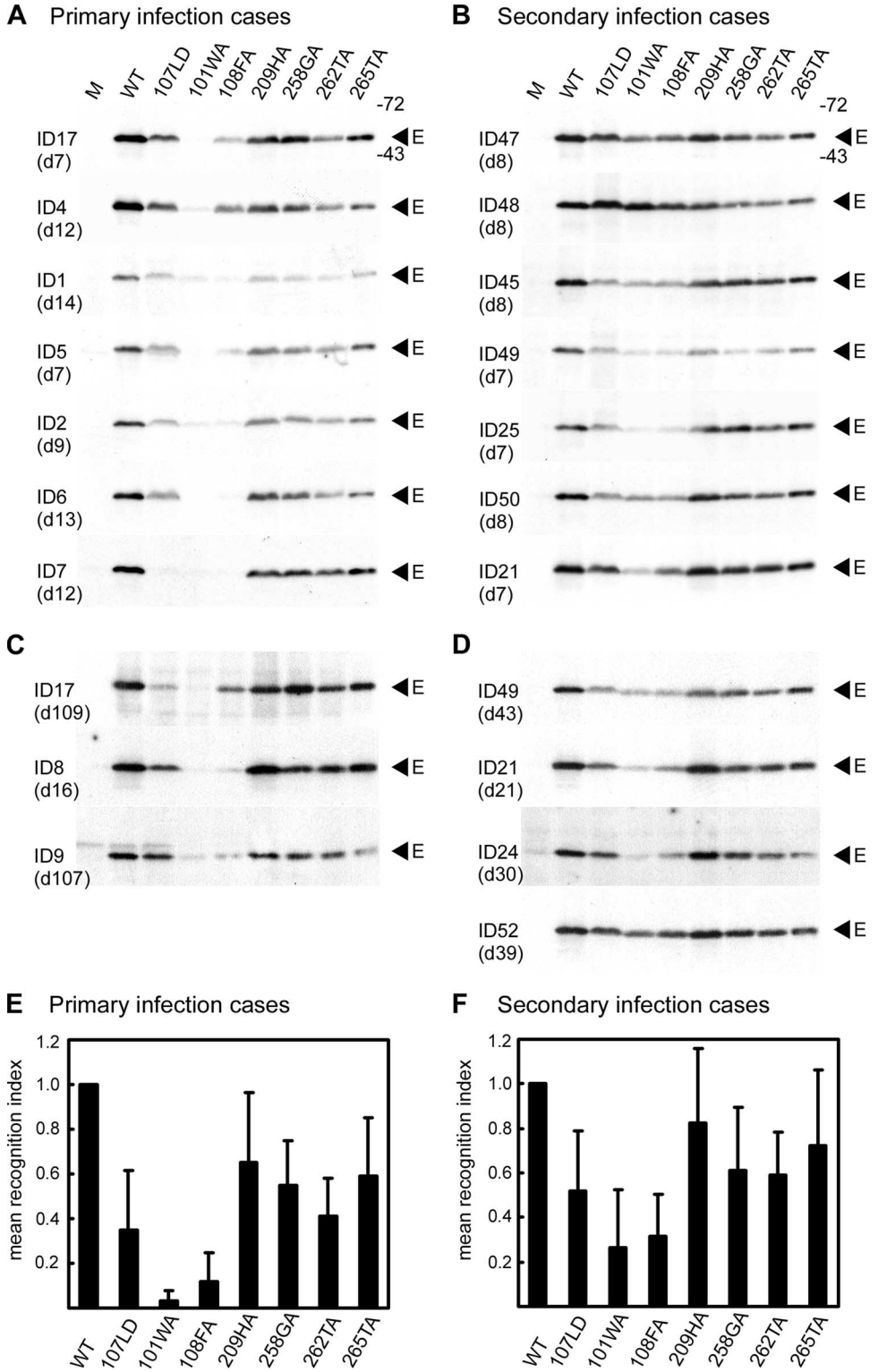

FIG. 6. Binding of convalescent-phase sera to DENV1 E proteins with mutations within (101WA, 107LD, and 108FA) and outside (209HA, 258GA, 262TA, and 265TA) the fusion loop of domain II. Cell lysates derived from 293T cells transfected with mock (M), wild-type (WT; pCB-D1), and mutant E constructs were subjected to Western blot analysis by using mixed sera (shown in Fig. 5A): sera from 10 patients with primary DENV2 infection, 7 at early (A) and 3 at later time points (C), and from 11 patients with secondary DENV2 infection, 7 at early (B) and 4 at later time points (D). Primary or secondary infection was determined as described previously (53, 59). Day 1 was defined as the day of onset of fever. The data are presented as described for Fig. 5. One representative experiment of more than two was shown. d7, day 7. (E and F) The intensities of E protein bands of the wild type and mutants as well as recognition indices were analyzed as described for Fig. 5. Data are mean recognition indices ± standard errors for patients with primary (E) and secondary (F) infections to each mutant E protein.

gions of E protein in human sera were reported based on peptide ELISA (28, 46). Using Western blot analysis under both reducing and nonreducing conditions, we found that the majority of antibodies to E as well as to PrM and NS1 proteins were conformation sensitive. Mutational study has shown that the six disulfide bridges formed by 12 strictly conserved cysteine residues of DENV2 E protein were critical for the epitope expression of E protein and therefore would contribute to the sensitivity of the majority of anti-E antibodies to β-mercaptoethanol (47). It is likely that the disulfide bridges formed by the highly conserved cysteine residues in the PrM and NS1 proteins also contribute to the sensitivity of anti-PrM and anti-NS1 antibodies to β-mercaptoethanol. Consistent with these results, previous studies of mouse MAbs against E and NS1 proteins revealed that 61% and 80%, respectively, were sensitive to β-mercaptoethanol treatment (10, 45).

While antibodies against DENV E protein are known to play a critical role in both protection and enhancement of disease, especially after primary infection (21, 22, 39), the extent of cross-reactivity to different serotypes, the relative amounts of homologous and heterologous anti-E antibodies, and the relationship to neutralization remain unclear. Using a blocking experiment on sera at the early convalescent period, we demonstrated for the first time that more than 90% of anti-E antibodies in most cases after primary infection were cross-reactive and only a minor proportion were type specific (Table 2). Moreover, the majority of the cross-reactive antibodies recognized epitopes involving the highly conserved residues at the fusion loop of domain II. Further studies with a larger sample size at a later time point would substantiate these observations and provide important information for our understanding of the pathogenesis of DHF/DSS and for future design of subunit vaccines against DENV as well.

An analysis of the E-binding activity for different serotypes in patients with primary DENV2 infection, though the activity varied among different patients, revealed that the cross-reactivities to DENV1 and DENV3 (95.5% to 100%) were generally higher than that to DENV4 (78.5% to 99.8%) (Table 2). This could be due to the lower degree of amino acid similarity between DENV2 and DENV4 E proteins than between E proteins of DENV2 and other serotypes (63% versus 69%) (34, 54). In agreement with previous reports, a primarily monotypic neutralization pattern for DENV2 was found in all seven cases with primary DENV2 infection (1, 8, 29). This finding, together with the results for E-binding activity, suggested that the majority of anti-E antibodies generated after primary infection were cross-reactive and nonneutralizing against heterologous serotypes. Whether the large amounts of cross-reactive nonneutralizing antibodies could persist for a long time remains to be investigated. If these antibodies persist, it is likely that they may contribute to antibody-dependent enhancement and an increase in disease severity during secondary infection years or even decades later, as has been demonstrated in several cohort studies (13, 18, 19, 20). The observation that only a minor proportion (0% to 8.6% in most cases) of anti-E antibodies generated after primary infection were type specific was surprising. Although the possibility that the DENV2-specific E-binding activity measured by our blocking experiment contained some subcomplex-specific binding activity cannot be completely ruled out, such a possibility would not affect the main finding that only a minor proportion of E-binding activity was DENV2 specific. It is likely that these minor type-specific antibodies account for the monotypic neutralization pattern and the life-long protection against that serotype. Examining how these type-specific neutralizing antibodies, compared to those cross-reactive nonneutralizing antibodies, persist over time would further our understanding of the pathogenesis of dengue greatly. In this regard, it is worth noting that a recent study of sera collected 4 to 8 or 20 to 22 years after primary DENV infection reported that the titers of homologous neutralizing antibodies increased over time and those of heterologous neutralizing antibodies decreased over time (16).

Our finding that the binding of polyclonal anti-E antibodies in patients with primary infection was almost completely abolished by single point mutations 101WA and 108FA at the fusion loop of domain II was unexpected and interesting. A previous study of a panel of mouse flavivirus group-reactive anti-E MAbs has identified three distinct yet overlapping epitopes containing conserved fusion loop residues plus residues outside of the fusion loop, including 104G, 106G, and 107L plus 231W; 104G and 106G plus 231W; and 104G plus 231W and 126E (6). Another study reported that the binding of 12 broadly flavivirus cross-reactive anti-E MAbs was affected to different degrees by mutations of fusion loop residue 107L (54). Similarly, a fusion loop residue (106G) and a residue outside the fusion loop (317H) were found to be the epitope of broadly reactive Fab antibody 1A5 from chimpanzees (12). It is conceivable that the conserved fusion loop residues identified here, 101W, 108F, and 107L, were the common residues of several different but overlapping epitopes recognized by the predominantly cross-reactive anti-E antibodies present in the serum. Although primarily based on data at early convalescent period, our findings raised the possibility that the spectrum of anti-E antibodies generated in patients with primary infection might be more restricted than previously appreciated. Of note was that polyclonal sera from patients with secondary infection, compared with those with primary infection, were less affected by the mutations at the fusion loop (Fig. 6E and F), suggesting a process of broadening the spectrum of anti-E antibody responses during secondary infection. This is also in agreement with a previous report that about 30% of the cross-reactivity in sera from six dengue patients, mostly secondary infection cases, was reduced by mutation of fusion loop residue 107L of E protein of the tick-borne encephalitis virus (54).

Studies of a large panel of mouse MAbs against domain III of WNV E protein have identified an important neutralizing epitope at the lateral ridge of domain III (2, 41, 49). Another study of 12 MAbs against domain III of DENV2 E protein revealed a similar epitope at the lateral ridge recognized by type-specific neutralizing MAbs, as well as an adjacent epitope at the A strand of domain III recognized by subcomplex-specific neutralizing MAbs (55). A recent study of 89 mouse MAbs against domain I or II of WNV E protein has reported seven distinct epitopes, including the epitope at the fusion loop of domain II (residues 101W, 104G, 106G, and 107L), recognized by flavivirus group-reactive MAbs (42). Among the 89 MAbs, the majority (more than 94%, 34/36) of those cross-reactive to DENV2, presumably the flavivirus group-reactive MAbs, were

mapped to the fusion loop of domain II (42). Similarly, a study of the human antibody repertoire using the single-chain phage display library from WNV-infected individuals revealed that 47% (24/51) of anti-E human MAbs bound to domain II and 8% (4/51) bound to domain III (57). These observations resonate with our findings that the majority of anti-E antibodies after primary infection were cross-reactive and nonneutralizing and recognized fusion loop residues of domain II, whereas only a minor proportion were type specific and neutralizing against the homologous serotype. Probably due to the cryptic nature and poor accessibility of the fusion loop epitope on the surface of the mature virion, flavivirus group-reactive MAbs against the fusion loop had no neutralizing activity or less-potent neutralizing activity than those type-specific neutralizing MAbs against domain III (41, 42, 54). Therefore, our findings supported the hypothesis that the human antibody repertoire against DENV may be directed toward the less neutralizing but immunodominant epitope involving the fusion loop residues at domain II and away from the more potent type-specific neutralizing epitope at domain III, as was recently proposed for WNV infection (42, 57). Further studies to delineate the epitopes recognized by type-specific neutralizing antibodies and those recognized by cross-reactive nonneutralizing antibodies generated during the natural course of infection would provide new insight into humoral immune responses against DENV and critical information for a rational design of subunit DENV vaccine as well.

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