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Antibody Recognition and Neutralization Determinants on Domains I and II of West Nile Virus Envelope Protein▽†

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Previous studies have demonstrated that monoclonal antibodies (MAbs) against an epitope on the lateral surface of domain III (DIII) of the West Nile virus (WNV) envelope (E) strongly protect against infection in animals. Herein, we observed significantly less efficient neutralization by 89 MAbs that recognized domain I (DI) or II (DII) of WNV E protein. Moreover, in cells expressing Fc γ receptors, many of the DI- and DII-specific MAbs enhanced infection over a broad range of concentrations. Using yeast surface display of E protein variants, we identified 25 E protein residues to be critical for recognition by DI- or DII-specific neutralizing MAbs. These residues cluster into six novel and one previously characterized epitope located on the lateral ridge of DI, the linker region between DI and DIII, the hinge interface between DI and DII, and the lateral ridge, central interface, dimer interface, and fusion loop of DII. Approximately 45% of DI-DII-specific MAbs showed reduced binding with mutations in the highly conserved fusion loop in DII: 85% of these (34 of 40) cross-reacted with the distantly related dengue virus (DENV). In contrast, MAbs that bound the other neutralizing epitopes in DI and DII showed no apparent cross-reactivity with DENV E protein. Surprisingly, several of the neutralizing epitopes were located in solvent-inaccessible positions in the context of the available pseudoatomic model of WNV. Nonetheless, DI and DII MAbs protect against WNV infection in mice, albeit with lower efficiency than DIII-specific neutralizing MAbs.

West Nile virus (WNV), a positive-sense RNA virus and a member of the *Flaviviridae* family, recently became endemic in North America, with annual outbreaks of severe encephalitis occurring mostly in immunocompromised or elderly individuals. There is currently no vaccine approved for human use, and treatment is primarily supportive. The WNV genome encodes three structural proteins (C, prM/M, and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). During the course of WNV infection, antibodies are raised against prM/M and E as well as NS1, NS3, and NS5, with a majority of the protective antibody response against the E protein (12, 63).

The crystal structure of the ectodomain of the E protein has been determined for dengue virus (DENV), tick-borne encephalitis virus (TBEV), and WNV (43, 45, 48, 56, 65). Flavivirus E proteins have three separate domains and form head-to-tail homodimers on the surface of the virion. Domain I (DI) is the central structural domain and consists of a 10-stranded β-barrel. DII is formed from two extended loops that project from DI. At the end of DII is a highly conserved loop, amino acid residues 98 to 110, that has been implicated in the acid-catalyzed type II fusion event (1, 7, 44). In the E dimer, the fusion loop lies in a pocket at the DI-DIII interface of the adjacent E protein. DIII, located on the opposite side of DI, forms a seven-stranded immunoglobulin-like fold and has been implicated in receptor binding (5, 10, 14). Short, flexible linker regions connect the domains and allow for the conformational changes associated with virus maturation and fusion (65).

The structure of the WNV virion has been defined by cryoelectron microscopy (36, 47). The mature WNV is ~500 Å in diameter and has a relatively smooth surface with no apparent spikes or large projections. The 180 E monomers lay flat along the virion surface as sets of three parallel dimers. The arrangement of the 180 E monomers has quasi-icosahedral symmetry such that there are three E monomers in the asymmetric unit and three distinct chemical environments available for antibody or ligand binding (47). The reduced pH in the endosome causes the E protein to convert from a homodimer to a homotrimer and exposes the fusion loop (44).

Antibodies are critical for the control of flavivirus infection in vivo (4, 17, 18, 20, 23, 50, 59), and this protection has been correlated with neutralizing activity in vitro (32, 53, 58). However, there have been reports of strong and weak in vivo protection with nonneutralizing (6, 11, 29, 31, 34, 58) and neutralizing (30, 32, 41) monoclonal antibodies (MAbs), respectively. Several recent studies suggest that specific epitopes elicit flavivirus-reactive MAbs with particular functional activities (3, 37, 38, 50, 57, 60). Most type-specific neutralizing antibodies map to DIII of the E protein. Cross-reactive, neutralizing MAbs bind to regions outside DIII and have been mapped to

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the putative fusion loop in DII (13, 22). We recently reported a high-throughput method for identifying contact residues of DIII-specific neutralizing and nonneutralizing MAbs by using random mutagenesis and yeast surface display epitope mapping (50). This method was validated by X-ray crystallographic analysis of a neutralizing Fab fragment with DIII of WNV E protein (49). Herein, using functional, biophysical, and molecular approaches, we have defined seven distinct epitopes in DI and DII that elicit antibodies that are inherently less protective against WNV infection than previously described DIII-specific antibodies.

MATERIALS AND METHODS

Cells and viruses. BHK21, Vero, C6/36 *Aedes albopictus*, and K562 cells were cultured as previously described (16, 54). Raji cells that stably express the c-type lectin DC-SIGNR were produced by transduction with a retroviral vector and will be described elsewhere (T. C. Pierson et al., submitted for publication). The North American WNV strain 3000.0259 (passage 2) that was isolated in 2000 was used for all in vivo experiments (19). WNV reporter virus particles (RVPs) were produced in BHK21 cells using a previously described complementation strategy (54).

Generation and purification of MAbs. MAbs were generated as previously described (50) after we performed several independent splenocyte-myeloma fusions. To generate anti-E MAbs (with the exception of E1), BALB/c mice were immunized alternately with 25 μg purified soluble WNV E protein or 10^2 PFU infectious WNV at 3-week intervals. For the E1 MAb, mice were immunized only with purified WNV E protein. Splenocytes were fused to P3X63Ag8.53 myeloma cells using a previously described procedure (26). MAbs were subcloned by limiting dilution, isotyped, and purified using either protein A or protein G affinity chromatography (Invitrogen, Carlsbad, CA).

Mouse experiments. Mouse studies were approved and performed according to the guidelines of the Washington University School of Medicine Animal Safety Committee. Five-week-old wild-type C57BL/6 mice and 8-week-old FcγRI-, FcγRIII-, and FcγRIV-deficient C57BL/6 mice were purchased commercially (Jackson Laboratories, Bar Harbor, ME, and Taconic, Germantown, NY, respectively). All mice were inoculated with 10^2 PFU of WNV subcutaneously via the footpad after anesthetization with xylazine and ketamine. Purified MAbs were diluted in phosphate-buffered saline (PBS) and administered by intraperitoneal injection either 1 day before or 2 days after infection.

In vitro neutralization and enhancement activity. Plaque reduction neutralization titer (PRNT) assays were performed as described previously (17). Neutralization or enhancement of RVPs on Vero, Raji DC-SIGNR, and K562 cells was evaluated according to a previously described protocol (54).

Yeast mapping. The generation of yeast cells that express the WNV ectodomain (amino acid residues 1 to 415) or DIII (residues 296 to 415) has been described previously (50). Yeast cells that express amino acid residues 1 to 295 (DI-DII) of WNV E protein were made by engineering BamHI and XhoI restriction enzyme sites at the 5′ and 3′ ends of the ectodomain construct using PCR amplification. This fragment was cloned into the BamHI and XhoI sites of the pYD1 vector (Invitrogen) and expressed in the *S. cerevisiae* strain EBY100. DI-DII, residues 1 to 294, and DIII, residues 295 to 417, of the DENV-2 strain 16681 were also cloned into the pYD1 vector by using the BamHI and XhoI sites. Yeast cells that expressed WNV E or DENV E protein or domains were stained with 50 μl of MAb supernatant on ice for 30 min. The yeast cells were washed three times with PBS supplemented with 1 mg/ml bovine serum albumin, incubated with a 1/500 dilution of a goat anti-mouse immunoglobulin G conjugated to Alexa Fluor 647 (Invitrogen), and analyzed using a Becton Dickinson FACSCalibur flow cytometer.

Yeast library construction and screening. A mutant library of WNV E DI-DII was made using error-prone PCR (50) and had an observed mutation frequency of ~0.3%. The library was ligated into the pYD1 vector and transformed into XL2-Blue ultracompetent bacteria (Stratagene, La Jolla, CA). The resultant ~10^5 colonies were pooled, and plasmid DNA was harvested using a HiSpeed Maxi kit (QIAGEN, Palo Alto, CA) and transformed into competent EBY100 yeast. The library was screened with MAbs to identify loss-of-binding mutants as described previously (50). Single MAbs were labeled with Alexa Fluor 647. An oligoclonal pool of DI-DII-specific MAbs (E53, E60, E121, E18, and E31) was labeled with Alexa Fluor 488. The yeast library was initially stained with the Alexa Fluor 647-labeled single MAb for 30 min and then with the Alexa Fluor 488-labeled oligoclonal pool of MAbs for 30 min. Yeast cells were sorted on the single antibody-negative, oligoclonal pool-positive population. This population was enriched through three rounds of sorting, and individual colonies were tested for loss of binding by flow cytometry. Plasmid was recovered using a Zymoprep yeast miniprep kit (Zymo Research, Orange, CA), transformed into DH5α competent cells (Stratagene), and sequenced. Mutation phenotypes were confirmed using both purified MAb and hybridoma supernatant using flow cytometry. To normalize for variation in expression of some of the DI-DII mutants, the total fluorescence product (percent positive population × mean linear fluorescence intensity) of a mutant for an individual MAb was divided by the total fluorescence product for the strongest binding MAb for that mutant.

TABLE 1. Characteristics of DI- and DII-specific MAbs used in functional studies

Antibody	Isotype	WNV yeast reactivity[a]	DENV-2 yeast reactivity	Epitope(s)[c]
E18	G2a	DI-DII	DI-DII	DII-fl
E31	G2a	DI-DII	DI-DII	DII-fl
E53	G2a	DI-DII	NR[b]	DII-fl, DI-lr
E60	G2a	DI-DII	DI-DII	DII-fl
E65	G2a	DI-DII	DI-DII	DII-fl
E113	G2a	DI-DII	NR	DII-hi
E100	G2a	DI-DII	NR	DII-di
E101	G2a	DI-DII	NR	DI-lr
E48	G2a	DI-DII	NR	DII-ci
E121	G2a	DI-DII	NR	DI-lr
7H7	G2a	DI-DII	NR	DII-lr, DI-lr
7G5	G2a	DI-DII	NR	DI-li
E16	G2b	DIII	NR	DIII-lr
E34	G1	DIII	NR	DIII-lr

[a] DI-DII indicates binding to yeast expressing WNV or DENV-2 E DI-DII, with no binding to WNV or DENV-2 E DIII.
[b] NR, no reactivity to any of the DENV-2 yeast constructs.
[c] DII-fl, DII fusion loop; DI-lr, DI lateral ridge; DII-hi, hinge interface between DI and DII; DII-di, dimer interface; DII-ci, DII central interface; DII-lr, DII lateral ridge; DI-li, linker region between DI and DIII; DIII-lr, DIII lateral ridge.

RESULTS

Generation of MAbs. A total of 163 MAbs against WNV E protein were generated after immunization of mice with either purified soluble protein or infectious virus: 46 of these were partially characterized in a prior study (50). All MAbs were screened for domain localization by using a yeast surface display assay, with WNV E ectodomain, DIII, or DI-DII expressed on the yeast surface (Table 1; see Table S1 in the supplemental material). Of the 163 MAbs that recognized purified soluble E protein, 152 bound to yeast expressing WNV E ectodomain, and 89 bound to yeast expressing DI-DII but not DIII. The remaining 63 MAbs bound to DIII and not DI-DII. All MAbs were also tested for cross-reactivity using yeast cells expressing DENV-2 DI-DII or DIII alone. Thirty-four of the 90 DI-DII-specific MAbs and 2 of the 63 DIII-specific MAbs cross-reacted with DENV-2.

Neutralizing activity. Previous reports have shown that cross-reactive MAbs that localize to sites within DII often neutralize WNV and related flaviviruses (13, 22, 23, 55, 57). Historically, neutralization has been measured using a standard PRNT assay. While the PRNT assay has successfully classified many neutralizing MAbs, it may fail to identify neutralizing MAbs that interfere with virus-receptor attachment interactions not present on BHK cells. As cellular factors also impact the sensitivity of viruses to antibody neutralization (64), we tested several different cellular contexts, including cells

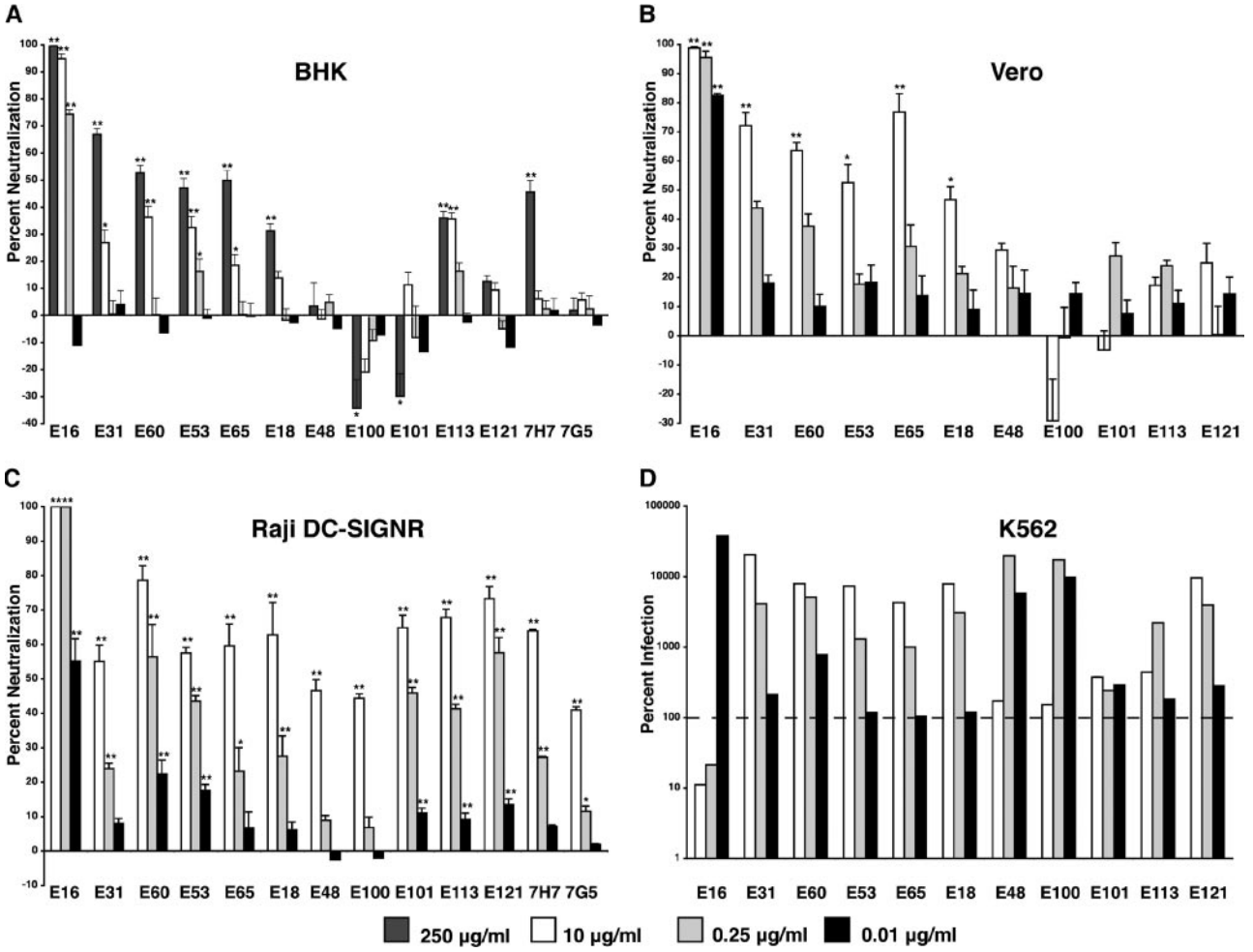

FIG. 1. In vitro neutralization and enhancement activity of DI- or DII-specific MAbs. (A) Neutralization of WNV using a PRNT assay on BHK cells. (B) Neutralization of RVPs on Vero cells. (C) Neutralization of RVPs on Raji DC-SIGNR cells. The data are expressed as percentages of the no-MAb control. The data shown are the means from at least three independent experiments. Error bars indicate the standard errors of the means, and statistical significance was determined using an unpaired, two-tailed *t* test compared to the no-MAb control (*, $P \leq 0.05$; **, $P \leq 0.01$). (D) Enhancement of RVP infection on K562 cells. The data shown are the enhancement over baseline infection without MAb. All MAbs were studied in a single experiment to allow for comparisons of the power of enhancement. Values of less than 100 indicate neutralization, whereas values greater than 100 indicate enhancement.

expressing lectins (e.g., DC-SIGNR), which promote attachment via carbohydrates rather than protein-protein interactions (15).

To evaluate the in vitro neutralization potential of the DI-DII-specific MAbs in several cell types, we used both a standard PRNT assay in BHK cells and a recently developed high-throughput flow cytometry assay that uses RVPs that express luciferase or green fluorescent protein genes (54). Using the standard PRNT assay and a range (0.01 to 250 μg/ml) of concentrations, several MAbs (7H7, E18, E31, E53, E60, E65, and E113) had significant, albeit weak, neutralizing activity (Fig. 1A). At the highest concentration of 250 μg/ml, neutralization by these DI- or DII-specific MAbs ranged from 26 to 68% ($P \leq 0.01$ compared to the no-MAb condition). E18 and E31 had been described as nonneutralizing (50); however, previous experiments were performed with hybridoma supernatant (~10 μg/ml) instead of higher concentrations of purified MAb. Interestingly, none of the DI- or DII-specific MAbs completely neutralized WNV infection. In contrast, E16 and other DIII-specific neutralizing MAbs completely inhibited infection at all but the lowest concentration of 0.01 μg/ml (Fig. 1A and data not shown). Surprisingly, MAbs E100 and E101 consistently enhanced infection by ~30% ($P \leq 0.05$) at the highest concentration tested, even though BHK cells do not express Fc γ receptors. While the mechanism for antibody enhancement in the absence of Fc γ receptors is uncertain, these MAbs could promote the formation of virus aggregates that are more readily endocytosed by BHK cells. Alternatively, these MAbs could cross-react with a surface antigen, leading to dual binding of the WNV virion and target cells, as was suggested by a recent study (28).

The purified MAbs were also screened for inhibition of RVP infection on Vero cells. The results correlated well with those of the standard PRNT assay, with the exception that E113 had no significant neutralizing activity on Vero cells (Fig. 1B). In contrast, marked differences in neutralizing activity were ob-

TABLE 2. Effect of MAb pretreatment on survival of wild-type mice

Antibody	Dose (μg)	No. of mice that survived/ total no. of mice	% Survival	*P* value[a]
PBS		5/40	13	
E60	400	17/20	85	≤0.0001
E53	400	15/20	75	≤0.0001
E18	400	16/20	80	≤0.0001
E121	400	17/20	85	≤0.0001
E113	400	19/20	95	≤0.0001
E31	400	20/20	100	≤0.0001
E48	400	18/20	90	≤0.0001
E100	400	6/20	30	≤0.01

[a] *P* values were determined using the log rank test.

TABLE 3. Effect of MAb therapy at day 2 after infection on survival of wild-type mice

Antibody	Dose (μg)	No. of mice that survived/ total no. of mice	% Survival	*P* value[a]
PBS		8/66	12	
E60	500	12/20	60	≤0.0001
E60	20	10/20	50	≤0.01
E53	500	12/31	39	≤0.0001
E53	20	6/21	29	≤0.01
E18	500	4/22	18	0.07
E18	20	3/21	14	0.65
E121	500	7/20	35	≤0.01
E121	20	4/20	20	≤0.01
E113	500	13/30	43	≤0.0001
E113	20	8/20	40	≤0.01
E31	500	18/20	85	≤0.0001
E31	20	13/20	65	≤0.0001
7H7	500	4/10	40	≤0.01
7G5	500	1/10	10	0.21
E34	500	10/10	100	≤0.0001
E34	20	8/10	80	≤0.0001
E16	500	18/19	95	≤0.0001
E16	20	15/18	83	≤0.0001

[a] *P* values were determined using the log rank test.

served when the MAbs were screened against RVP infection of Raji DC-SIGNR cells. Additional MAbs such as E100, E101, E48, 7G5, and E121 exhibited significant neutralizing activity ($P \leq 0.01$) (Fig. 1C).

The DI- and DII-specific MAbs showed less neutralizing activity than many of our DIII-specific MAbs, such as E16. To rule out the possibility that the differences in neutralization were due to decreased affinity of the DI-DII-specific MAbs for WNV E protein, binding affinities were investigated. We previously reported the affinity of E16 for purified WNV DIII to be 3.4 nM (50). In comparison, several DI- and DII-specific MAbs that neutralized infection in different cell types, including E60, E121, and E100, all had relatively similar monovalent binding affinities for WNV E protein between 0.5 nM and 6.7 nM (data not shown). Thus, the disparity in neutralizing potential between DIII- and DI-DII-specific MAbs cannot be explained by differences in binding strength alone.

Enhancing activity in Fc γ receptor-expressing cells. Many MAbs efficiently enhance flavivirus infection in cells bearing Fc γ receptors (40, 51, 52). Although not known to contribute to WNV disease, this phenomenon, also known as antibody-dependent enhancement (ADE), has been implicated in the pathogenesis of severe DENV infection (25, 35). Previous studies demonstrated that E53 and E60 enhanced WNV infection in vitro in the J774 murine macrophage cell line at a concentration of 50 μg/ml, whereas E16, a DIII-specific MAb, completely neutralized infection at this concentration (49). We tested the neutralizing and enhancing properties of DI-DII-specific MAbs in K562 cells that express FcγRII (CD32), a cell line previously used by others to study ADE (39, 54). The DIII-specific MAb E16 strongly neutralized at the two highest concentrations of MAb but enhanced infection at the low concentration of 0.01 μg/ml, consistent with previous data (Fig. 1D). In comparison, none of the DI- or DII-specific MAbs neutralized infection in K562 cells at any of the concentrations tested. Different patterns of enhancement were observed with the DI- or DII-specific MAbs of the γ_{2A} isotype, as several (E18, E31, E53, E60, E65, and E121) augmented infection at higher MAb concentrations. In contrast, two MAbs, E48 and E100, enhanced infection at the lower antibody concentrations but had infection levels near baseline at the highest dose. One MAb, E101, showed little enhancement at any concentration, and another, E113, had infection levels near baseline at the lowest concentration, a 10-fold enhancement at the intermediate concentration, and less enhancement at the highest concentration.

In vivo protection of DI- and DII-specific MAbs. While the ability to protect mice from lethal flavivirus challenge has been associated with the in vitro neutralization potential of MAbs (58), the correlation is not perfect, as other factors, including antibody effector function, may modulate protection. Previous studies have shown that MAbs that recognize a specific epitope within DIII can protect mice for up to 5 days after WNV infection (50) and that human single-chain variable-region antibody fragments that bind to regions outside DIII also protect mice for several days after infection (23). To determine whether DI- and DII-specific MAbs with different neutralization profiles had unique protective activities, passive antibody transfer studies were performed with C57BL/6 mice. Five-week-old wild-type mice were administered a single intraperitoneal dose (400 μg) of MAb 1 day prior to infection with 10^2 PFU of WNV via footpad inoculation. All neutralizing MAbs tested significantly protected against lethal infection ($P \leq 0.01$) compared to the saline control, which had a baseline survival rate of 13%, and all MAbs, except E100, protected more than 75% of the mice (Table 2). Notably, this included MAbs such as E48 and E121, which lacked appreciable neutralizing activity on BHK or Vero cells but did inhibit RVP infection in Raji DC-SIGNR cells.

As a more stringent test of protection, we tested the MAbs for their ability to control an established infection by administering them 2 days after WNV inoculation. E53, E60, E18, E113, E31, E121, 7H7, and 7G5 were given at doses of 20 μg or 500 μg. In most cases, there was significant improvement in survival compared to what was observed with the saline control (Table 3). However, differences in the level of protection were observed: the five MAbs with the greatest neutralizing activity in the PRNT assay (E53, E60, E31, E113, and 7H7) all protected at levels of ≥39% at the 500-μg dose, with E31 having the greatest effect (85% survival, $P \leq 0.0001$). In contrast, E18

TABLE 4. Effect of MAb pretreatment on survival of Fc γ receptor-deficient mice

Antibody	Dose (μg)	No. of mice that survived/ total no. of mice	% Survival	*P* value[a]
PBS		13/42	31	
E60	400	13/18	72	≤0.01
E53	400	17/28	61	≤0.01
E121	400	26/37	70	≤0.01

[a] *P* values were determined using the log rank test.

and 7G5, MAbs that neutralized less effectively on BHK cells, had no significant protective effect at the 500-μg dose (10 to 18% survival, $P \geq 0.07$), and E121 had a weak therapeutic effect at the highest dose (35% survival, $P \leq 0.0001$). In general, the DI- and DII-specific MAbs were less effective at protecting mice than E16 and E34, two DIII-specific neutralizing MAbs that protected between 95 and 100% of mice at the 500-μg dose and between 80 and 83% of mice at the 20-μg dose ($P \leq 0.0001$) (Table 3) (50).

Although DIII-specific MAbs protected in vivo primarily because of their neutralizing activity, a small part was dependent on their interaction with Fc γ receptors and, to a lesser extent, complement (42, 50). To assess whether the protection by DI- and DII-specific neutralizing MAbs was independent of Fc γ receptor function, passive transfer studies using E121, E60, and E53 were repeated with congenic C57BL/6 mice deficient in expression of the activating Fc γ receptors I, III, and IV. Because 5-week-old Fc γ receptor-deficient mice show uniform lethality after WNV infection (data not shown), older mice (8 weeks old) were used. All three DI- and DII-specific MAbs tested protected the 8-week-old Fc γ receptor-deficient mice from WNV challenge (Table 4) with an efficacy similar to that for the 5-week-old wild-type mice. Thus, much of the antibody-mediated protection appeared independent of Fc γ receptor effector function.

Epitope mapping. As differences in binding affinity did not correlate with neutralizing activity, we hypothesized that the location of MAb binding more significantly affected inhibitory activity. We mapped the amino acid residues required for MAb binding using random mutagenesis and a yeast surface display assay (8, 50) by generating a mutant library of $\sim 10^5$ DI-DII variants. Independent screens were performed with 14 MAbs, yielding 30 mutations that encompassed 25 distinct amino acid residues. The entire DI-DII-specific MAb panel was then tested for binding against these mutations (Fig. 2; see Table S2 in the supplemental material).

Seven of the mutations that abolished binding of DI- or DII-specific MAbs localized within the highly conserved fusion loop of DII (DII-fl). Several MAbs with neutralizing activity in the PRNT and RVP assays (E18, E31, E53, E60, and E65) lost binding with at least one of these mutations (Fig. 3; see Table S2 in the supplemental material). Surprisingly, 35 MAbs that lacked apparent neutralizing activity by the PRNT assay when tested with hybridoma supernatant (MAb concentration of ~10 μg/ml) also mapped to this region. As E18 and E65 neutralized poorly (<20%) at concentrations of 10 μg/ml (Fig. 1A), it is probable that some of these MAbs also neutralize at higher concentrations. Strikingly, all 34 DI- or DII-specific, cross-reactive MAbs mapped to sites within the DII-fl, and all completely lost binding with the mutation W101R. Although the residues G104, G106, and L107 were previously identified to be part of an epitope recognized by flavivirus cross-reactive MAbs (13, 22), W101 has not been described to contribute to MAb binding. Despite their overall similarity, the cross-reactive MAbs had slightly different specificities for the identified mutations. As an example, E18 lost binding with the mutations W101R, G104D, and G106E (Fig. 3A), whereas E60 lost binding with the mutations P75L, W101R, G106R, L107P, and L107R (see Table S2 in the supplemental material). A recent report indicated that the residue W233 may be involved in cross-reactive epitopes (13). However, when the mutation W233F was introduced by reverse genetics in the WNV DI-DII yeast construct, none of the 34 cross-reactive MAbs were affected. There were, however, 10 WNV-specific MAbs that exhibited reduced binding with this mutation (see Table S2 in the supplemental material).

E53, a WNV-specific MAb with neutralizing activity in BHK, Vero, and Raji DC-SIGNR cells, lost binding with the mutations P75L, T76A, T76I, R99G, G106E, G106R, L107P, and L107R (Fig. 3B). E53 was the only MAb that lost binding with a mutation at T76; notably, P75 and T76 are located on a loop that structurally supports the DII-fl through a disulfide bond between residues C74 and C105. E53 and five other fusion loop MAbs did not cross-react with DENV-2 E protein expressed on yeast and were not affected by the W101R mutation. The distinct pattern of E53 binding was also reflected by the effect of additional mutations on the lateral ridge of DI (DI-lr), as the mutations S175P and E191K reduced binding, although not to the same degree as changes in fusion loop residues.

The two remaining WNV-specific MAbs with neutralizing activity in the PRNT assay, 7H7 and E113, were mapped using independent sorts of the mutant yeast library. 7H7 lost binding with the mutations H81Y, H81R, D83V, and A86V, which

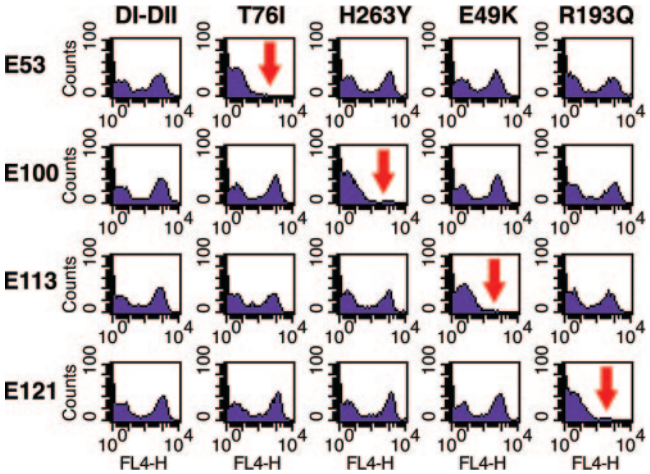

FIG. 2. Flow cytometry patterns of loss-of-function DI- or DII-specific MAb variants selected by yeast surface display. Representative histograms are shown for MAbs E53, E100, E113, and E121. Red arrows indicate mutations that result in loss of MAb binding. The data shown are representative of three independent experiments. FL4-H, log fluorescence intensity on the FL4 (660=nm) channel.

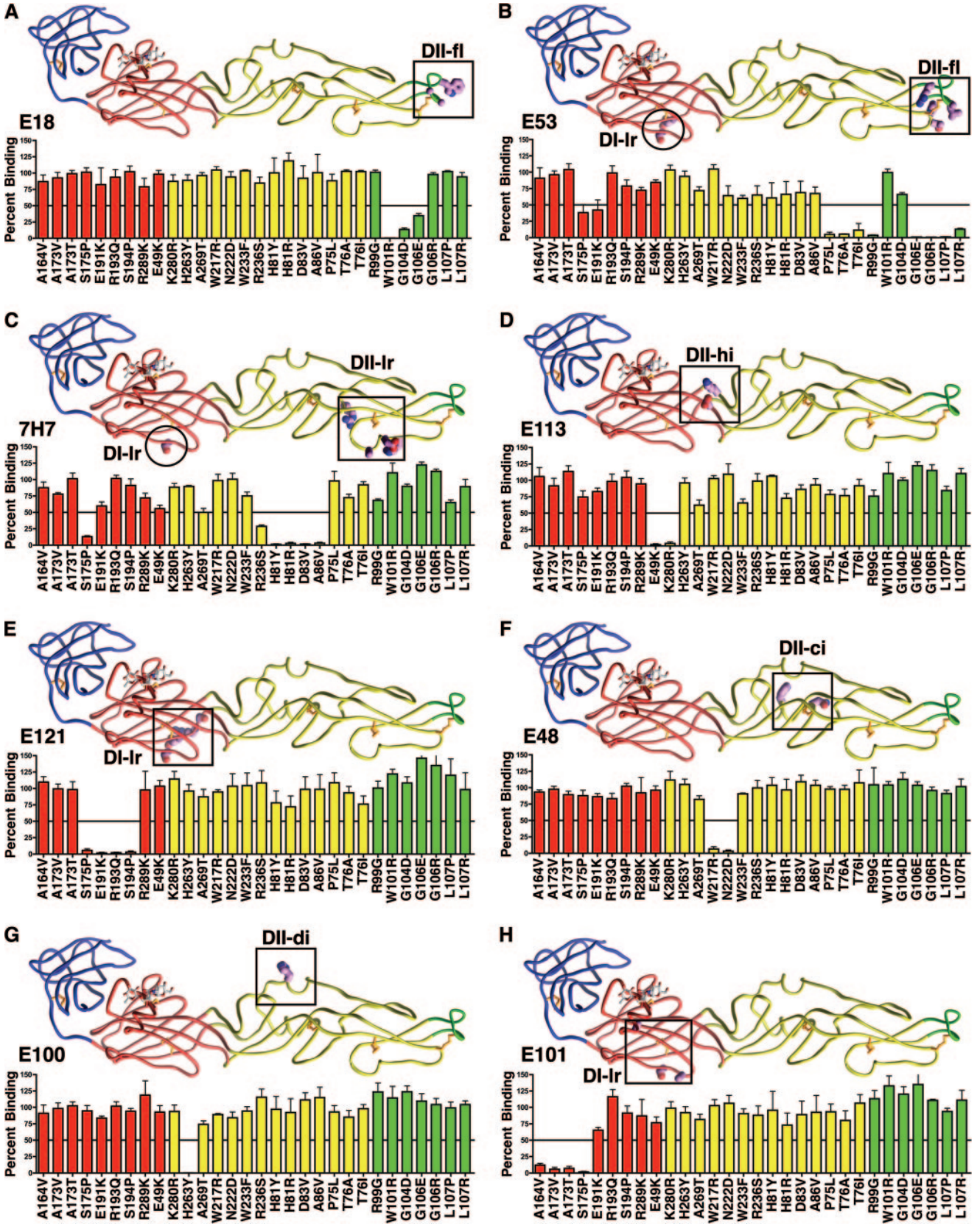

FIG. 3. Epitope mapping of DI- and DII-specific neutralizing MAbs. Binding of (A) E18, (B) E53, (C) 7H7, (D) E113, (E) E121, (F) E48, (G) E100, and (H) E101 to mutants expressed on the yeast surface. The binding of each MAb to the mutants was measured by flow cytometry, and total fluorescence was normalized to yeast expressing wild-type DI-DII. The data shown are the means from three independent experiments.

localize to the DII-lr (Fig. 3C). 7H7 binding was also reduced by the mutations R236S and S175P. R236 is located in DII and maps within ~20 Å of the residues H81, D83, and A86. In contrast, S175P, the same mutation that affected E53 binding, is located within the DI-lr and is ~60 Å from the other identified 7H7 mutations on the E monomer. E113 had a rather distinctive recognition site, as the mutations E49K and K280R eliminated binding and were located at the hinge interface between DI and DII (DII-hi) (Fig. 3D). Both of these residues localize to a region of the E protein that undergoes rotational movement between the pre- and postfusion conformations (43, 44). This site also was identified to be important for binding of a DENV-1-specific neutralizing MAb (2).

A selected group of MAbs (E121, E48, E100, E101, and 7G5) that did not neutralize in the PRNT assay but did inhibit infection on Raji DC-SIGNR cells were also mapped by independent sorts of the yeast library (Fig. 3E to H). E121 binding was eliminated with three mutations (E191K, R193Q, and S194P) that map to the DI-lr, whereas E48 binding was abolished by mutations (W217R and N222D) in the central interface of DII (DII-ci). E100 was affected only by the mutation H263Y that is located in DII near the dimer interface. E101 binding was strongly reduced or eliminated with the mutations A164V, A173T, A173V, and S175P, which are located on the DI-lr. Finally, 7G5 binding was eliminated with the mutation R289K, which maps to DI in the linker region between DI and DIII.

The 180 monomers of WNV E protein exist in three distinct chemical environments because of the quasi-icosahedral symmetry on the surface of the mature virion (36, 47). We hypothesized that the functional properties of the DI- and DII-specific MAbs could be related to their distinct recognition patterns and epitope abundance in the three symmetry environments. On the viral particle, not all E proteins have equivalently accessible epitopes: prior studies established that the DIII-specific MAb E16 was excluded from binding E proteins along the fivefold symmetry axis, resulting in a maximum binding of 120 of 180 sites (Fig. 4A) (33, 49). To understand the binding patterns of DI- and DII-specific MAbs, residues identified by mutagenesis analysis were mapped onto the E protein crystal structure and docked on the pseudoatomic model of the mature virion. Antibody epitopes that mapped to the DII-fl, such as E18 and E53, had no apparent differences in accessibility in any of the three symmetry environments (Fig. 4B and C), in contrast to what was observed with E16 (Fig. 4A). The two mutations on the DI-lr that affect E53 binding, S175P and E191K, were not contiguous with the fusion peptide mutations on the E protein monomer or dimer. However, when mapped on the mature virion, S175 and E191 at the fivefold symmetry axis cluster with the fusion loop residues at the threefold symmetry axis and are approximately 27 Å apart. Thus, some of the mutations may influence binding in a subset of symmetry environments and modulate the relative avidity of binding for a given asymmetric unit.

Mutations that affected binding of the neutralizing MAb 7H7 also had a unique distribution on the mature virion. The DII-lr residues H81, D83, and A86 were located adjacent to each other along the interface of the twofold and fivefold symmetry axes (Fig. 4D), which may create steric conflict and preclude simultaneous antibody binding. These same residues, located on the threefold symmetry axis, also appear obscured by DIII from the twofold symmetry axis. Based on this, we speculate that 7H7 binding may be limited to 60 of the 180 sites. In contrast, mutations that affect E113, a MAb with neutralizing activity in the PRNT assay, were surface accessible at the DII-hi and had no apparent differences among the three symmetry axes (Fig. 4E). Finally, E121 is typical of several of the MAbs (e.g., E48 and E101) that inhibited infection only in Raji DC-SIGNR cells: the residues, which our mutational analysis suggests as contact sites, paradoxically were poorly accessible on the mature virion (Fig. 4F). Experiments with a four-layer trap enzyme-linked immunosorbent assay and intact RVPs (data not shown) provided additional evidence of the variable surface accessibility of specific epitopes on the virion. MAbs that recognized the DIII-lr epitope exhibited increased binding to intact RVPs compared to DI-DII-specific MAbs. Furthermore, DI-DII-specific neutralizing MAbs recognized the RVPs variably, although there was no direct correlation between binding and neutralizing activity.

DISCUSSION

In this report, we characterized a panel of 89 MAbs that bound to DI or DII of the WNV E protein. Neutralization profiles were determined for several MAbs and were found to be both cell-type-specific and less potent than DIII-specific neutralizing MAbs previously described (3, 50, 60). In addition, ADE occurred across a wider range of concentrations with DI- or DII-specific MAbs than with neutralizing, DIII-specific MAbs. Postexposure in vivo protection correlated with PRNT activity, whereas preexposure protection correlated with neutralization of RVPs on Raji DC-SIGNR cells. In all cases, protection with the DI- or DII-specific MAbs was less than that previously observed with DIII-specific neutralizing MAbs. The DI-DII-specific MAbs with the strongest neutralizing activity mapped to the putative fusion loop, although in total, six distinct neutralizing epitopes were identified throughout DI and DII.

Flavivirus neutralization has been routinely assessed by a PRNT assay on either BHK or Vero cells, and this assay has successfully identified several MAbs that efficiently neutralize WNV (3, 21, 23, 50, 55, 60). The most potent neutralizing MAbs bind a specific epitope on the lateral surface of DIII (3, 50, 60). However, the neutralizing and protective potential of MAbs that bind E protein outside DIII has been less clear. Although we identified several DI- and DII-specific MAbs with neutralizing activity, none completely neutralized WNV in the

Error bars indicate the standard errors of the means. The colors red, yellow, blue, and green indicate domains I, II, and III and the fusion loop, respectively. Mutations that resulted in ≥50% reduction of MAb binding were mapped (shown in magenta and boxed) onto the WNV E protein crystal structure (Protein Data Bank accession code 2HG0). For MAbs E53 and 7H7, residues that compose the primary binding site within DII are boxed and secondary sites in DI are circled. Epitopes are labeled using the same nomenclature defined in Table 1.

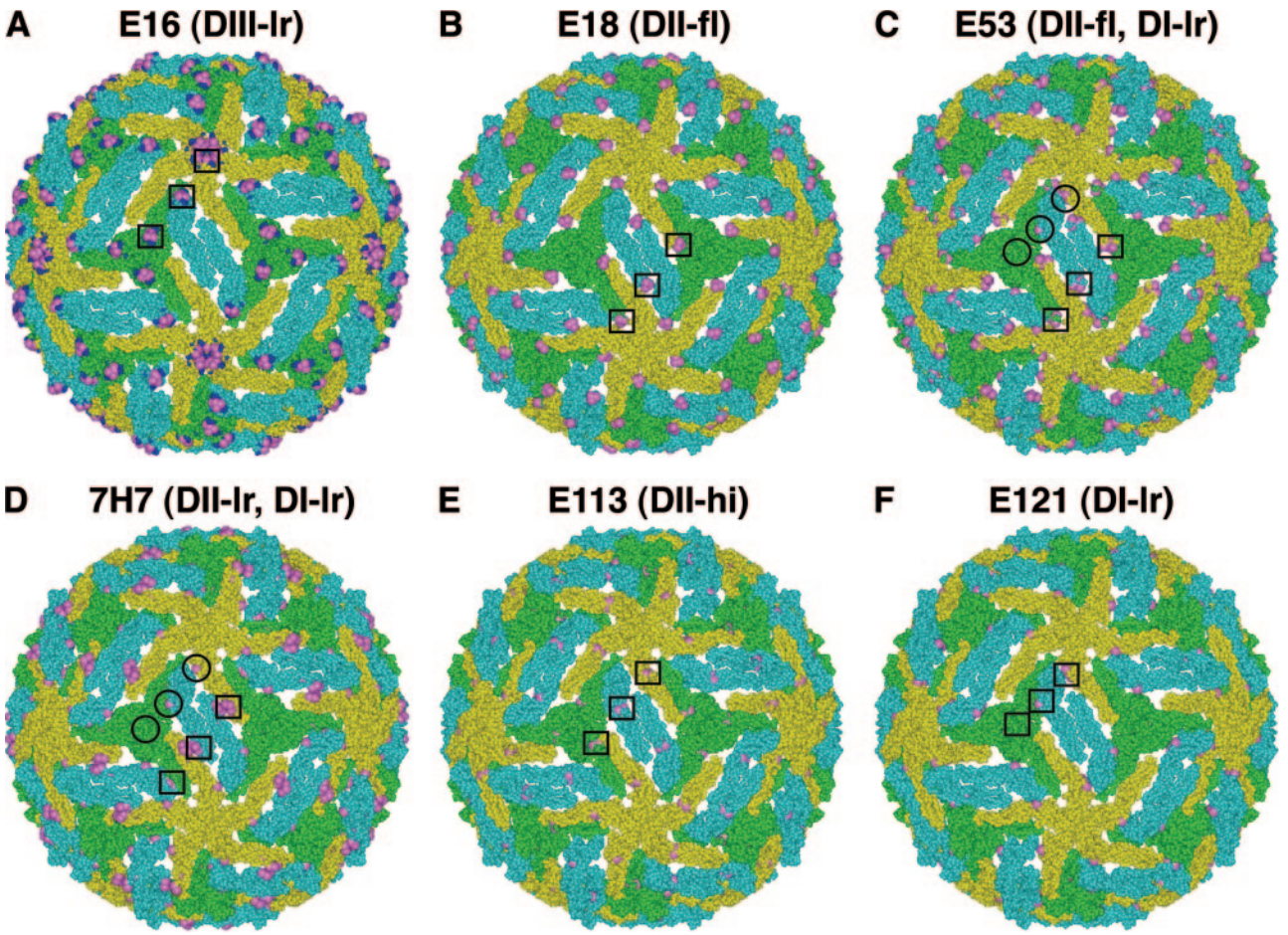

FIG. 4. Epitope expression on the WNV virion. Yeast display epitope residues (magenta) for (A) E16, (B) E18, (C) E53, (D) 7H7, (E) E113, and (F) E121 were mapped onto the pseudoatomic model of the mature WNV virion. For E16, the blue indicates additional contact residues as determined by X-ray crystallography. Virions are depicted as 2.0-Å-radius Cα atoms and are colored according to their symmetry axes, twofold (cyan), threefold (green), and fivefold (yellow). Epitopes are boxed on one E protein in each symmetry axis. Secondary binding sites in DI for E53 and 7H7 are circled in each symmetry axis.

PRNT assay, even at very high concentrations (e.g., 250 μg/ml), and all were far less potent than the DIII-specific MAbs E16 and E34. We did, however, observe significant differences in the neutralizing potential of DI-DII-specific MAbs depending on the cell type and assay. Several DI- or DII-specific MAbs that had no neutralizing activity in the PRNT assay significantly neutralized RVP infection of Raji DC-SIGNR cells. These differences were due to the cell type used and not the form of infectious particle, as neutralization of RVPs on Vero cells closely matched neutralization of infectious WNV on BHK cells. Although a complete understanding of the basis for cell-type-dependent neutralization warrants further study, it could reflect differences in the mechanism of neutralization. DIII-specific neutralizing MAbs primarily block WNV infection at a postattachment step, possibly by inhibiting viral fusion in the endosome (49). DI- and DII-specific MAbs could neutralize infection through distinct mechanisms, such as blocking receptor attachment. As several candidate WNV attachment factors have been identified (e.g., $\alpha_v\beta_3$ integrin [9] and DC-SIGNR [15]), DI-DII-specific MAbs could have cell-specific neutralizing potential by blocking some, but not all, WNV-receptor interactions.

DI- and DII-specific neutralizing MAbs behaved distinctly from DIII-specific MAbs in infection assays with a cell line expressing Fc γ receptors; in general, they showed ADE at higher concentrations of MAb. Unlike the DIII-specific neutralizing MAb E16, none of the DI-DII-specific MAbs neutralized infection of these cells at any of the tested concentrations. The enhancing pattern of DI-DII-specific MAbs could reflect their inherent inability to completely neutralize infection even at high concentrations of MAb: populations of unneutralized virus may infect cells bearing Fc γ receptors. Alternatively, differences in the mechanism of neutralization (attachment versus postattachment) could explain the distinct enhancement patterns. Neutralizing MAbs, such as E16, that block infection at a postattachment step (49) should inhibit regardless of the entry receptor. In contrast, if DI-DII-specific MAbs neutralized WNV by blocking attachment, then on Fc γ receptor-expressing cells, virus binding could proceed through an alternate ligand, the Fc moiety of the antibody. While ADE has not

been reported to contribute to WNV pathogenesis, it is believed to influence DENV disease expression during secondary infection (25, 35). Given the decreased protective nature of DI-DII-specific MAbs in mice, it is possible that antibodies that localize to this region could be protective against infection in some cell types and possibly pathological in others.

Although DI- or DII-specific MAbs against WNV E protein protected mice when administered prior to infection, their efficacy was reduced when added 2 days after infection. In contrast, neutralizing MAbs that map to DIII protected mice and hamsters when administered 5 days after WNV infection (46, 50) and 4 days after infection with the closely related flavivirus Saint Louis encephalitis virus (41, 58). The finding of decreased in vivo activity of neutralizing antibodies that map to DII is consistent with those of a study with human single-chain antibodies against WNV (23). In terms of the relative efficacy among the DI-DII-specific MAbs in vivo, the two neutralizing MAbs (E31 and E60) that conferred the most protection at 2 days after infection mapped to sites within the DII-fl. Indeed, the single-chain human antibody with the greatest in vivo potency also mapped at least partially to residues within this region (T. Oliphant, L. H. Gould, E. Fikrig, and M. S. Diamond, unpublished data). However, mapping to the fusion loop was not always associated with therapeutic activity, as E18 offered little protection in the postinfection treatment model. Similarly, 4G2, a flavivirus cross-reactive MAb that maps to the fusion loop (13), offered various levels of protection in vivo depending on the model used (30, 32).

Approximately 45% (40 of 89) of the DI- or DII-specific MAbs showed markedly reduced binding to WNV E protein, with mutations in the DII-fl. This was the largest percentage of any of the identified epitopes and included all but two of the DENV-2 cross-reactive MAbs. This finding agrees with those of recent studies that have mapped flavivirus cross-reactive neutralizing MAbs to the fusion loop (13, 22). In contrast, DI- and DII-specific neutralizing MAbs that mapped outside the fusion peptide were poorly cross-reactive. However, our MAbs were generated, in part, after immunization with soluble recombinant E protein. Preliminary studies suggest that the human antibody repertoire against infectious WNV may be directed away from DIII neutralizing epitopes and toward the inherently less neutralizing immunodominant epitopes on DI and DII. Only 8% (4 of 51) and 0% (0 of 11) of human single-chain antibodies that reacted with DIII were isolated from phage display libraries of WNV-infected and naïve patients, respectively (23, 62). Our own preliminary data with human MAbs isolated from transformed B cells from secondarily DENV-infected patients indicate that the majority of MAbs are weakly neutralizing in BHK cells and map to the DII-fl (C. Simmons, M. Beltramello, F. Sallusto, A. DaSilva, M. S. Diamond, and A. Lanzavecchia, unpublished data). Although more-definitive studies that examine the human antibody repertoire are required, the cross-reactive DII epitope may be immunodominant and the DIII-specific neutralizing epitope less dominant. It is intriguing to consider that flaviviruses, in some way, manipulate the humoral response to direct antibody specificity away from highly protective epitopes.

Surprisingly, many of the DI-DII-specific MAbs with neutralizing activity appear to bind to epitopes on the E protein that are poorly accessible on the surface of the mature virion, results that are consistent with those of a recent study on DII-specific flavivirus cross-reactive antibodies (61). Based on the crystal structures of the TBEV and DENV E proteins, the DII-fl appears to be buried at the DIII interface of the adjacent protein that forms the E homodimer (43, 45, 56). This should apparently preclude antibody binding to the fusion loop. The observation that several MAbs bind to and neutralize WNV suggests that viral particles may not be as static as the cryo-electron microscopic pseudoatomic model suggests. Indeed, the ectodomain of WNV E protein was crystallized as a monomer, suggesting weaker dimer interactions than with DENV or TBEV (48). Lower dimer affinity within the virion could allow MAb access to buried residues near the fusion loop. Three MAbs (E121, E48, and E101) with in vitro neutralizing activity and preexposure protective capacity also appeared to bind distinct regions (DII-ci and DI-lr) that were poorly accessible on the mature virion. Taken together, we speculate that the mature virion structure may be more fluid than previously hypothesized. Alternatively, infection in vivo produces heterogeneous virus populations containing partially mature virions that express various amounts of prM and are more accessible to binding of these antibodies. In support of this, a recent study demonstrated that at least some infectious particles contain uncleaved prM (15). Moreover, the level of uncleaved prM on the virion markedly affects antibody binding and neutralizing activity of a subset of antibodies (24, 27; S. Nelson, M. S. Diamond, T. C. Pierson, unpublished data).

In summary, we have defined seven distinct epitopes on DI and DII of WNV that elicit MAbs with neutralizing activity against infection of several cell types. Our experiments suggest that DI- and DII-specific antibodies are inherently less protective against WNV infection in vitro and in vivo than previously described DIII-specific antibodies. Indeed, the enhancing profiles of DI-DII-specific MAbs are concerning, especially for flavivirus vaccines (e.g., DENV) where ADE is suspected to contribute to pathogenesis. Based on this, we suggest that eliminating or altering specific DI-DII epitopes may be a way to redirect antibody responses toward the more protective neutralizing epitope in DIII. Flavivirus vaccines that target epitopes in DIII that neutralize infection regardless of the mode of cellular entry may be more effective and possibly safer.

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