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Identification of Neutralizing Epitopes within Structural Domain III of the West Nile Virus Envelope Protein

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Using a panel of neutralizing monoclonal antibodies, we have mapped epitopes in domain III of the envelope protein of the New York strain of West Nile virus. The ability of monoclonal antibodies that recognize these epitopes to neutralize virus appeared to differ between lineage I and II West Nile virus strains, and epitopes were located on the upper surface of domain III at residues E307, E330, and E332.

West Nile (WN) virus is a member of the Japanese encephalitis (JE) serocomplex of the family *Flaviviridae*, genus *Flavivirus*. WN virus is a mosquito-borne virus, primarily transmitted by *Culex* mosquitoes to a number of vertebrates including humans. Human infections with WN virus may result in presentations ranging from subclinical infection to a mild undifferentiated fever to potentially fatal encephalitis. More severe disease presentation generally occurs in older individuals (16). Phylogenetic analysis of WN virus strains has defined two major lineages, I and II (9, 10). With very few exceptions, lineage II WN virus strains have been isolated only in Africa and Madagascar, while lineage I strains have a wide distribution including Africa, Europe, and North America.

The flavivirus envelope (E) protein is a major determinant of tropism and the primary target of virus-neutralizing antibody. In particular, structural domain III of E has been proposed elsewhere as a putative receptor-binding domain (13). Studies with several flaviviruses—including JE, yellow fever, dengue, and Murray Valley encephalitis viruses—have identified epitopes recognized by neutralizing antibodies within this domain (4, 14, 15, 17). Although neutralizing epitopes have been identified in other domains of the E protein, antibodies binding to domain III are reported elsewhere to be the most efficient at blocking virus attachment to cells, supporting the proposed role of this domain in receptor binding (6). Specific mutation of residues within domain III has also been shown elsewhere to affect virulence and tropism of flaviviruses (11, 12).

We used a panel of domain III-reactive monoclonal antibodies (MAbs) and a mouse brain tissue membrane receptor preparation (MRP) to select variants of representative lineage I (strain 385-99) and II (strain H-442) WN virus strains to identify residues that are potentially involved in neutralizing epitopes and receptor binding interactions. In addition, we investigated the effects of these mutations on the virulence of these viruses in a mouse neuroinvasion model.

Expression and purification of recombinant WN virus E protein domain III. Recombinant E protein structural domain III (r-EIII, comprising amino acids 296 to 415) from neuroinvasive lineage I WN virus strain 385-99 was expressed from *Escherichia coli* DH5α as a glutathione *S*-transferase fusion by using the pGEX-2T vector (Amersham Pharmacia Biotech, Piscataway, N.J.) by protocols similar to those described by Bhardwaj et al. (3). Homogeneity of cleaved, purified r-EIII was confirmed by mass spectroscopy (data not shown).

Neutralization of WN virus by domain III-reactive MAbs. Origins and propagation of WN strains 385-99 and H-442 have been described previously (2). Five commercially available WN virus E protein-specific MAbs (BioReliance Corp., Rockville, Md.) were tested for reactivity with r-EIII in a nonreducing Western blot assay (Fig. 1). Four MAbs—5H10, 3A3, 7H2, and 5C5—bound to r-EIII. Product literature reported that none of these four MAbs bound to St. Louis encephalitis virus antigens, and only MAb 5C5 bound weakly ("+/−") to JE virus. These r-EIII-reactive MAbs strongly neutralized lineage I strain 385-99 in plaque reduction neutralization titer assays in Vero cells (Table 1). For these assays, 10-fold dilutions of virus were mixed with MAb (250 ng of MAb in a 200-μl total reaction volume) or tissue culture medium only and incubated at room temperature for 60 min. Virus titers in MAb and control reaction mixtures were then determined by plaque assay in Vero cells. Lineage II strain H-442 was not neutralized by these antibodies (Table 1). The fifth MAb (3D9; cross-reactive with JE virus but not St. Louis encephalitis virus) did not bind r-EIII and did not have neutralizing activity.

Selection of neutralization-resistant variants of strain 385-99. In order to identify residues that might define epitopes recognized by these neutralizing antibodies, potential neutralization-resistant escape variants of strain 385-99 were selected and amplified in Vero cells. At least three plaques per MAb were picked from wells of plaque reduction neutralization titer plates (i.e., virus grown in the presence of each MAb). These plaque-purified viruses were then tested for escape from neutralization by their selecting antibody (evidenced by less than 1 $\log_{10}$ reduction in virus titer in the presence of the selecting MAb). One neutralization-resistant variant was identified for each of MAbs 5H10 and 5C5, and these were designated MAb^R-5H10 and -5C5, respectively (Table 1). Viral RNA was extracted from wild-type 385-99 and MAb^R-5H10 and -5C5

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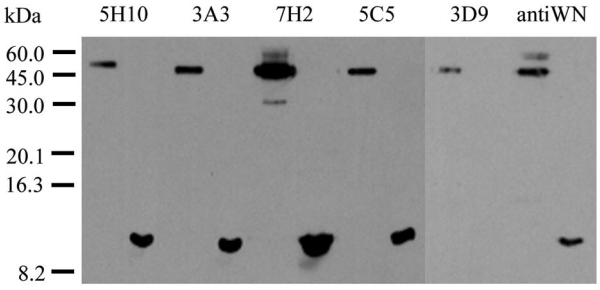

FIG. 1. Reactions of anti-WN virus E protein MAbs and polyclonal mouse hyperimmune ascitic fluid ("antiWN") with viral E protein in a WN virus-infected Vero cell lysate (left lane of each set) or purified, recombinant WN virus E protein domain III (right lane of each set) in a nonreducing Western blot.

variants with the QiaAmp viral RNA extraction kit (Qiagen Inc., Valencia, Calif.) according to the manufacturer's protocol. Regions of viral genome corresponding to genes for structural proteins prM and E were reverse transcription-PCR amplified as described previously (2). The sequence of strain 385-99 in this region did not differ from that of the related strain 382-99 (GenBank accession no. AF196835). Single-nucleotide mutations were identified in the E protein genes of MAb^R-5H10 and -5C5 variants at residues 989 (AC$\underline{T}$→A$\underline{T}$T) and 920 (A$\underline{A}$G→A$\underline{G}$G) encoding amino acid changes at E330 (Thr→Ile) and E307 (Lys→Arg), respectively. These residues lie in proximity to each other on the outside upper face of domain III, a region which would likely play a role in receptor binding by the intact WN virion (Fig. 2).

Cross-neutralization assays with all four MAbs showed that each variant did not escape neutralization by the MAbs equally (Table 1). In particular, MAb 5C5 retained strong neutralizing activity against the MAb^R-5H10 variant, while 5H10 was unable to neutralize either variant, suggesting that these antibodies recognized spatially related but distinct epitope structures. This observation was consistent with the supplier's descriptions of differences in the specificities of these two MAbs as outlined above. In addition, MAb 7H2 still neutralized both mutants, although not as strongly as it did wild-type strain 385-99. The relatively small surface of domain III would dictate that multiple epitopes may be formed by overlapping groups of residues.

Selection of a mouse brain MRP binding escape variant. WN virus strains have previously been reported to differ in their abilities to bind to mouse brain MRP, although these differences were not correlated with mouse virulence phenotypes (1). Binding indices ($\log_{10}$ reduction in virus titer following incubation with MRP) ranged from approximately 1.0 to 3.5. An MRP binding escape variant of strain H-442 (MRP binding index, 3.3) was selected as described previously (8) and designated H-442-MRP^R (Table 1). Strain 385-99 had a much lower MRP binding index (1.0) and was not suitable for selecting MRP binding variants. Nucleotide sequencing of E protein genes of wild-type H-442 (GenBank accession no. AF459403) and H-442-MRP^R viruses identified a single-nucleotide substitution at position 995 (A$\underline{A}$G→A$\underline{G}$G) encoding a Lys→Thr substitution at amino acid E332. This residue lies in the same region of domain III as the 385-99-MAb^R mutations at E307 and E330 (Fig. 2).

Mutations at residue E332 affect neutralization and MRP binding phenotypes of strain H-442. A comparison of domain III sequences revealed that E332 was one of three residues that differed between strains 385-99 and H-442: E312 (Leu or Arg, respectively), E332 (Lys or Thr, respectively), and E369 (Ala or Ser, respectively). The E332 (Lys→Thr) mutation identified in H-442-MRP^R was a reversion to the strain 385-99 residue. Given the location of this residue in the region of the putative neutralizing epitopes described above, we tested the ability of anti-WN virus MAbs to neutralize this variant (Table 1). H-442-MRP^R was efficiently neutralized by all four MAbs, although not as strongly as was lineage I strain 385-99, suggesting that the Thr at E332 is important to the antigenic structure of this region in strain 385-99 but that the two additional variable residues (E312 and E369) also contribute to the antigenic differences between strains 385-99 and H-442. All three variable residues were located on the upper surface of the domain III predicted structure (Fig. 2).

Two neutralization-resistant variants of H-442-MRP^R were selected with MAb 5H10 (designated H-442-MRP^R/MAb^R-1 and -2). These variants contained substitutions at nucleotide 920 (A$\underline{A}$A→A$\underline{G}$A) or 921 (AA$\underline{A}$→AA$\underline{T}$) of the E gene that encoded a Lys→Arg or Lys→Asn substitution at E307, respec-

TABLE 1. Neutralization, MRP binding, and mouse neuroinvasion characteristics of wild-type and MAb^R and/or MRP^R variant WN viruses

Virus	Mutation(s)	Neutralization index for MAbs[a]:				MRP binding index[b]	i.p. LD_{50} (PFU)[c]
		5H10	5C5	3A3	7H2		
385-99 wild type		2.3	2.5	2.9	3.6	1.0	0.5
385-99 MAb^R-5C5	E307 Lys→Arg	0.3	−0.2	0.5	1.7	1.0	1.3
385-99 MAb^R-5H10	E330 Thr→Ile	0.4	2.3	0.6	2.3	1.1	0.5
H-442 wild type		0.2	−0.1	0.1	0.1	3.3	3.2
H-442 MRP^R	E332 Lys→Thr	1.8	2.0	2.4	2.9	−0.4	0.3
H-442 MRP^R/MAb^R-5H10-1	E332 Lys→Thr E307 Lys→Arg	0.1	−0.2	0.1	1.3	2.9	1.3
H-442 MRP^R/MAb^R-5H10-2	E332 Lys→Thr E307 Lys→Asn	0.2	0.3	0.0	1.1	1.2	5.0

[a] Neutralization index is $\log_{10}$ reduction in virus titer in the presence of MAb (250 ng/reaction) compared with a culture medium-only control.
[b] MRP binding index is $\log_{10}$ reduction in virus titer in the presence of mouse brain MRP compared with a buffer-only control.
[c] i.p., intraperitoneal; LD_{50}, 50% lethal dose. Values are for 3- to 4-week-old female NIH Swiss mice. All animal experimentation was conducted in accordance with National Institutes of Health and institutional guidelines.

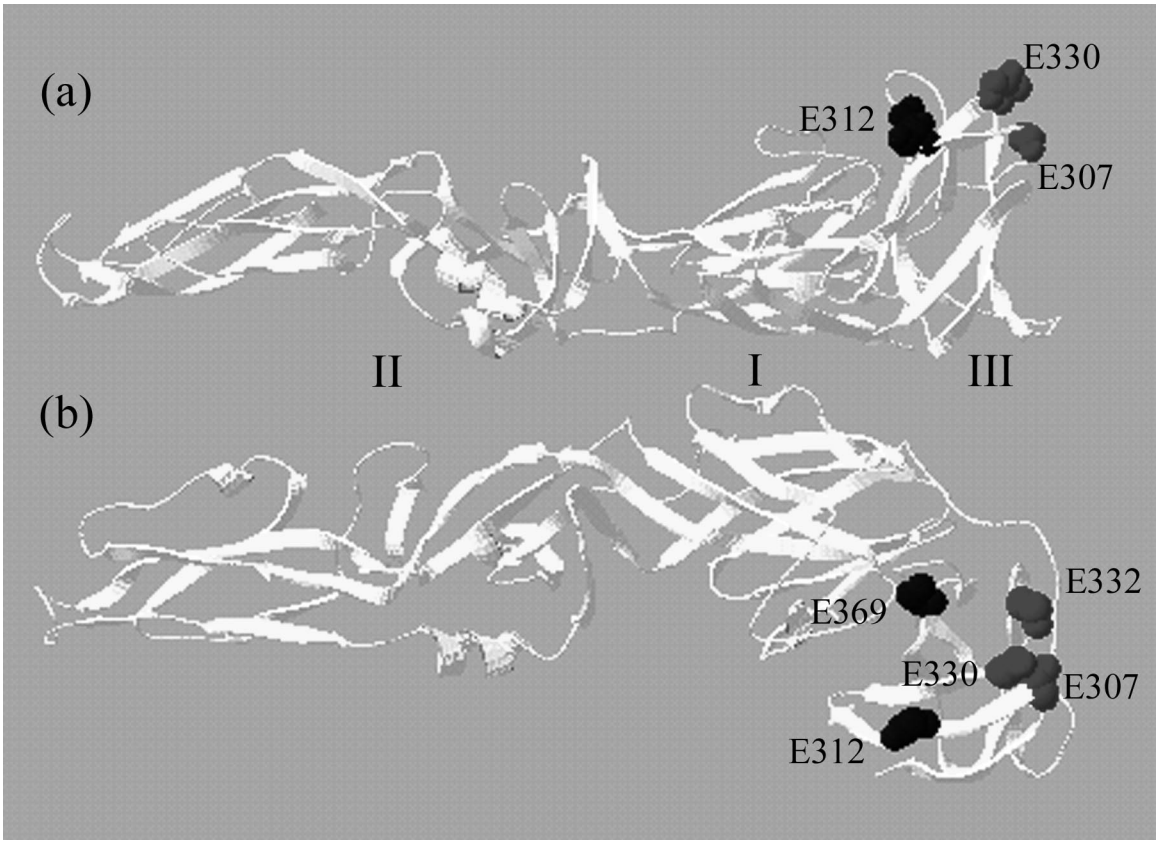

FIG. 2. Locations of residues (E307, E330, and E332, shown in dark gray) associated with escape from neutralization by MAbs and/or binding to mouse brain tissue MRP in side-on (a) and overhead (b) views of a predicted structure of the WN virus E protein based on that of the tick-borne encephalitis virus E protein crystal structure (13). Other domain III residues that differed between strains 385-99 and H-442 (E312 and E369) are shown in black. The WN virus structure was derived with the Swiss-Model structure prediction server via Expasy (www.expasy.ch) and drawn with Swiss-PDB Viewer (7).

tively. These changes were consistent with the observed escape from neutralization by MAb 5H10 of the 385-99 MAb^R-5C5 (E307 Lys→Arg) variant and further confirmed that both E307 and E330 contribute to the MAb 5H10 epitope. Both MRP^R/MAb^R variants escaped neutralization by all four MAbs although, as was observed with the MAb^R variants of strain 385-99, MAb 7H2 retained some neutralizing activity against these variant viruses (Table 1).

Mouse virulence of MAb^R/MRP^R mutants. Previously we reported that both wild-type strains, 385-99 and H-442, were highly virulent in a mouse neuroinvasion model (2). To assess the effects of mutations in domain III on virulence phenotype, the lethality of each variant virus was determined by intraperitoneal inoculation of virus in groups of 3- to 4-week-old female NIH Swiss mice (Table 1). Up to 10-fold differences were observed in 50% lethal doses for variant viruses compared with their parent strains, suggesting that certain combinations of residues in domain III can influence neuroinvasion, perhaps by altering receptor binding affinity or some other undefined mechanism. In particular, the virulence of the strain H-442 MRP^R variant was more comparable to that of wild-type 385-99, suggesting that the mutation at E332 might have enhanced the infectivity of the variant compared to wild-type H-442. Mutations at E307 in MAb^R variants of either virus were slightly attenuating. However, no mutation(s) resulted in complete attenuation of the neuroinvasion phenotype.

Conclusions. This is the first study of reactivity of MAbs with the New York strain of WN virus and highlights the importance of domain III as a target for neutralizing antibodies and as a likely receptor-binding domain. Similar to our findings, Chambers et al. (5) reported a Lys→Glu mutation at E307 of a WN virus strain from Israel that was associated with neutralization escape but had no effect on mouse virulence of the virus. The mutations in domain III associated with escape from neutralization described here had little effect on neuroinvasion by these viruses, suggesting that the neuroinvasive phenotype may be primarily driven by other virus and/or host factors. The identification of multiple mutations at residue E307 (Lys→Arg/Asn/Glu) under MAb selective pressure points to this region as a hot spot for antigenic variation and might suggest that this residue is less critical to the functional architecture of domain III than are other surrounding residues.

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