Human Monoclonal Antibodies against West Nile Virus Induced by Natural Infection Neutralize at a Postattachment Step


Departments of Pathology and Immunology, Molecular Microbiology, and Medicine, Washington University School of Medicine, St. Louis, Missouri 63110; Department of Medical Microbiology, University Medical Center Groningen, University of Groningen, 9700 RB Groningen, The Netherlands; Crucell Holland B.V., 2301 CA Leiden, The Netherlands; and Viral Pathogenesis Section, Laboratory of Viral Diseases, National Institutes of Health, Bethesda, Maryland 20892

Received 9 February 2009/Accepted 16 April 2009

West Nile virus (WNV) is a neurotropic flavivirus that is now a primary cause of epidemic encephalitis in North America. Studies of mice have demonstrated that the humoral immune response against WNV limits primary infection and protects against a secondary challenge. The most-potent neutralizing mouse monoclonal antibodies (MAbs) recognize an epitope on the lateral ridge of domain III (DIII-lr) of the envelope (E) protein. However, studies with serum from human patients show that antibodies against the DIII-lr epitope comprise, at best, a minor component of the human anti-WNV antibody response. Herein, we characterize in detail two WNV-specific human MAbs, CR4348 and CR4354, that were isolated from B-cell populations of convalescent patients. These MAbs strongly neutralize WNV infection of cultured cells, protect mice against lethal infection in vivo, and yet poorly recognize recombinant forms of the E protein. Instead, CR4348 and CR4354 bind determinants on intact WNV virions and subviral particles in a pH-sensitive manner, and neutralization is altered by mutations at the dimer interface in domain II and the hinge between domains I and II, respectively. CR4348 and CR4354 human MAbs neutralize infection at a postattachment step in the viral life cycle, likely by inhibiting acid-induced fusion within the endosome.

West Nile encephalitis virus (WNV) is a positive-polarity, single-stranded RNA virus of the genus Flavivirus within the family Flaviviridae. Other members of this genus that cause significant human disease include dengue virus (DENV), St. Louis encephalitis virus, Japanese encephalitis virus (JEV), yellow fever virus, and tick-borne encephalitis virus (TBEV). Flaviviruses are translated as a single polypeptide, which is then cleaved by host and viral proteases into three structural (capsid [C], premembrane [prM], and envelope [E]) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins (reviewed in references 42 and 43).

WNV cycles in nature between several species of birds and Culex mosquitoes, with humans and other mammals as dead-end hosts (25, 62). Infection causes syndromes ranging from a mild febrile illness to severe encephalitis and death (13, 72). WNV has spread globally and causes outbreaks with thousands of severe human cases annually in the United States. An age of mild febrile illness to severe encephalitis and death (13, 72). WNV has spread globally and causes outbreaks with thousands of severe human cases annually in the United States. An age of WNV infection.

The mature WNV virion has a ~500-Å diameter and consists of a single RNA genome surrounded by the capsid protein, a lipid bilayer, and a shell of the prM/M and E proteins (31, 55). X-ray crystallography studies have elucidated the three-domain structure of the flavivirus E protein (30, 48, 50, 58, 67). Domain I (DI) is a central, eight-stranded β-barrel, which contains the only N-linked glycosylation site in WNV E. Domain II (DII) is a long, finger-like protrusion from DI and contains the highly conserved fusion peptide at its distal end. Domain III (DIII) adopts an immunoglobulin-like fold at the opposite end of DI and is believed to contain a site for receptor attachment (6, 8, 40).

Within an infected cell, progeny WNV are assembled initially as immature particles. In immature virions, three pairs of E and prM interact as trimers and form 60 spiked projections withicosahedral symmetry (85, 86). Exposure to mildly acidic conditions in the trans-Golgi secretory pathway promotes virus maturation through a structural rearrangement of the E proteins and cleavage of prM to M by a furin-like protease (41, 83). Mature WNV virions are covered by 90 antiparallel E protein homodimers, which are arranged flat along the surface in a herringbone pattern with quasi-icosahedral symmetry (55).

Upon binding to poorly characterized cell surface receptors, internalization of WNV is believed to occur through receptor-mediated, clathrin-dependent endocytosis (1, 79, 80). After trafficking to Rab5- and/or Rab7-positive endosomes (38, 79), the mildly acidic pH within the lumen of the endosome induces structural alterations in the flavivirus E protein (7, 49), which includes changes in its oligomeric state (7, 49, 77). During this process, also known as type II fusion, the hydrophobic peptide on the fusion loop of DII of the E protein inserts into the
endosomal membrane, thus physically joining the host and viral membranes, which allows the infectious RNA genome to enter the cytoplasm (32, 33).

Humoral immunity is an essential component of the protective host response against flaviviruses including WNV (reviewed in references 64 and 68). Studies by several groups have shown that the neutralization of WNV can occur after antibodies bind to a series of discrete epitopes on all three domains of the E protein (3, 12, 22, 59, 61, 71). To date, the most potently neutralizing monoclonal antibodies (MAbS) localize to an epitope on the lateral ridge of DIII (DIII-Ir). One well-characterized strongly neutralizing mouse MAb, E16, blocks infection primarily at a postattachment step (57) and requires the engagement of only a fraction of its epitopes on the surface of the virion (66). Studies of the human antibody response to WNV infection reveal that, in contrast to mice, antibodies that bind the DIII-Ir epitope comprise a minor component of the neutralizing humoral response in most individuals (60).

In this study, we characterized two strongly neutralizing novel human MAbs (CR4348 and CR4354) that were selected from an antibody phage display library constructed from B cells of subjects that survived WNV infection (78). We demonstrated that both MAbs are WNV specific, bind weakly to recombinant or yeast surface-displayed E proteins, exhibit pH-sensitive binding to viral particles, and protect against lethal infection in mice. Our experiments suggest that these human MAbs map to distinct epitopes and neutralize infection at a postattachment stage, likely by inhibiting the acid-catalyzed viral fusion step.

MATERIALS AND METHODS

Preparation of virus, subviral particles, and pyrene-labeled virus. WNV strain New York 99 (NY99c) infectious clone (5) are detailed below. WNV subviral particles (SVP) were generated after transfection of BHK21-15 cells with a pcDNA3.1 plasmid expressing premembrane (prM) and envelope (E) genes (36) from the NY99 WNV strain using FuGENE HD (Roche) according to manufacturer’s instructions. Supernatants containing SVP were collected 48 h after transfection, filtered through a 0.2-μm filter, and stored aliquoted at −80°C. Labeling of WNV with the fluorescent probe pyrene was performed essentially as described previously for alphaviruses (73, 81). Briefly, BHK21-15 cells cultured in the presence of 15 μg/ml of 16-(1-pyrenyl)hexadecanoid acid (Invitrogen) were infected with WNV at an multiplicity of infection of 4. At 24 h postinfection, the medium was harvested and clarified by low-speed centrifugation, and pyrene-labeled WNV particles were pelleted by ultracentrifugation in a Beckman type 19 rotor for 15 h at 48,500 g at 4°C. The virus particles were further purified on an Optiprep (Axis-Shield) density (15 to 55%, wt/vol) gradient by ultracentrifugation in a Beckman SW41 rotor for 18 h at 100,000 × g at 4°C. The infectious titer was determined by 50% tissue culture infective dose (TCID50) assay. Briefly, serially diluted WNV were mixed with Vero cells in duplicate or triplicate with either Vero or BHK21-15 cells. Viral adsorption proceeded for one hour at 37°C, followed by an overlay with 1% low-melt agarose (SeaPlaque) in α-modified Eagle medium and 4% FBS. After solidification, plaques were visualized 3 to 4 days later following fixation with 2 ml of 10% formaldehyde, removal of agarose plugs, and staining with 1% (wt/vol) crystal violet in 20% (vol/vol) ethanol. Plaques were counted and then normalized to the average of data from six control wells in which WNV was mixed with DME containing 10% FBS and no antibody.

Neutralization assays. (i) PRNT. In many experiments, the neutralizing activity of MAbs was determined using a 50% plaque reduction neutralization test (PRNT50) analysis. Briefly, serially diluted MAbs were mixed 1:1 with 106 PFU of WNV in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) (Omega Scientific) and incubated for 1 h at 37°C. The WNV-MAb mixture was then added to individual wells of a six-well tissue culture plate in duplicate or triplicate with either Vero or BHK21-15 cells. Viral adsorption proceeded for one hour at 37°C, followed by an overlay with 1% low-melt agarose (SeaPlaque) in α-modified Eagle medium and 4% FBS. After solidification, plaques were visualized 3 to 4 days later following fixation with 2 ml of 10% formaldehyde, removal of agarose plugs, and staining with 1% (wt/vol) crystal violet in 20% (vol/vol) ethanol. Plaques were counted and then normalized to the average of data from six control wells in which WNV was mixed with DMEM containing 10% FBS and no antibody.

(ii) Pre- and postattachment neutralization assays. To assay for postattachment neutralization, a PRNT assay was completed essentially as described above with the following modifications. All solutions and Vero cells were prechilled to 4°C, and 106 PFU of WNV were then added to each well of cells, and viral adsorption was allowed for 1 h at 4°C. Wells were then washed three times with medium, and MAb at the specified concentrations was added. Virosion-antibody complexes were allowed to form for 1 h at 4°C, followed by three washes with chilled medium. Cells were then warmed to 37°C, and the PRNT was completed as described above. In parallel, a PRNT with all cells and solutions at 4°C was performed in which MAb and WNV were mixed for 1 h at 4°C prior to addition to cells (preattachment assay).

(iii) RVP assay. WNV and mutant reporter virus particles (RVP) were generated as described previously (65, 66). Separate plasmids expressing the wild-type E and prM genes and the cap gene of WNV wild type were transfected into a yeast cell that stably propagates a WNV replication expressing green fluorescent protein. In some experiments, the pr-M-E plasmid was mutated using the QuikChange site-directed mutagenesis kit (Stratagene) to introduce specific amino acid substitutions. In other experiments, RVP were produced from 293T cells at various stages of maturation (immature, partially mature, or fully mature) according to previously published protocols (56). Supernatants containing RVP were harvested 48 h after transfection, filtered through a 0.2-μm filter, and aliquoted at −80°C. RVP were incubated with serial dilutions of MAb under conditions of antibody excess at room temperature (RT) for 1 h. Subsequently, MAb-RVP mixtures were added to Raji-DCSIGNR cells, which stably express the DC-SIGNR attachment factor (14), and were incubated at 37°C for 48 h. Infected cells were assayed for green fluorescent protein expression using a BD FACSArray flow cytometer. Alternatively, human C32DA (Fc-γRIIA)-expressing K562 cells were used to assay for antibody-dependent enhancement of infection.

Yeast surface display of WNV E proteins. The generation of Saccharomyces cerevisiae cells that express the WNV E protein ectodomain (amino acid residues 1 to 415) or DIII (residues 296 to 415) was described previously (59). Yeast cells expressing WNV E or DIII were washed in a solution containing phosphate-buffered saline (PBS), 2% bovine serum albumin, and 0.02% gelatin. Supernatants containing SVP were collected 48 h after transfection, filtered through a 0.2-μm filter, and stored aliquoted at −80°C. RVP were incubated with serial dilutions of MAb under conditions of antibody excess at room temperature (RT) for 1 h. Subsequently, MAb-RVP mixtures were added to Raji-DCSIGNR cells, which stably express the DC-SIGNR attachment factor (14), and were incubated at 37°C for 48 h. Infected cells were assayed for green fluorescent protein expression using a BD FACSArray flow cytometer.

Antigen capture and solid-phase ELISA. Nunc MaxiSorp polystyrene 96-well plates were coated either overnight at 4°C with murine DIII-Ir MAb (10 μg/ml) or for 1 h at 37°C with the soluble recombinant WNV E protein ectodomain (10 μg/ml) generated as described previously (57), in a pH 9.3 carbonate buffer. Plates were washed three times in enzyme-linked immunosorbent assay (ELISA) wash buffer (PBS with 0.02% Tween 20) and blocked for 1 h at 37°C with ELISA block buffer (PBS, 2% bovine serum albumin, and 0.02% Tween 20). SVP or WNV infectious virions (wild type or mutant) were captured on plates coated with murine DIII-Ir MAb for 1 h at RT. Subsequently, plates were rinsed five times in wash buffer and then incubated with anti-WNV or control human IgG1 (10 μg/ml in block buffer) in triplicate for 1 h at RT. Plates were washed five times and then incubated with biotinylated rabbit anti-human IgG antibody (1:1250 dilution; Southern Biotech) 1 h at RT in blocking buffer. Plates were washed again five times and then sequentially incubated with 2 μg/ml of horse-radish peroxidase-conjugated streptavidin (Vector Laboratories) and tetramethylbenzidine-substrate (Dako). The reaction was stopped with the addition of 2 N H2SO4 to the medium, and absorption (450 nm) was read using an ELISPOT microplate reader (Bio-Rad). In the pH-dependent ELISA, plates were washed four times with wash buffer and once with the indicated pH buffer (150 mM NaCl, 0.05% Tween 20, and 50 mM MES [morpholinolinesulfonic acid] [pH 6.0]) or wash buffer (pH 7.4) after trapping of SVP. The plate was incubated for 30 min

Downloaded from http://jvi.asm.org/ by guest on November 1, 2017.
at RT with the indicated pH buffer and then washed five times in wash buffer followed by normal completion of the capture ELISA protocol.

**Western blots.** The recombinant WNV E protein, SVP, or infectious WNV was diluted in 2× sodium dodecyl sulfate loading buffer with or without β-mercaptoethanol (5%, vol/vol) and incubated at RT or 95°C for 10 min as indicated. Samples were loaded into wells of a NuPage (Invitrogen) 4 to 12% Bis-Tris-gradient gel and electrophoresed. Protein was transferred onto a polyvinylidene difluoride transfer membrane using the iBlot system (Invitrogen). Membranes were rinsed in PBS-0.05% Tween 20 (wash buffer) with gentle shaking for 10 min at RT and then blocked overnight with block buffer (5% dry milk in wash buffer) with shaking at RT. After five 10-min washes, membranes were stained with primary human MAB (1 µg/ml diluted in block buffer with normal goat serum added at a 1:250 dilution) for 1 h at RT. After five additional washes, membranes were incubated with horseradish peroxidase-conjugated goat anti-human antibody (diluted 1:5000 in block buffer; Sigma) for 1 h. Membranes were then washed five times for 10 min in wash buffer and developed using ECL reagent (Amersham).

**Generation of neutralization escape mutants.** WNV was incubated with 25 µg/ml of CR4348 or CR4354 for 1 h at RT in DMEM. The mixture was added to Vero cells in a six-well plate at a multiplicity of infection of 1. After infection for 2 h at 37°C, wells were washed three times with DMEM, and fresh medium containing 25 µg/ml of MAb was added. Virus growth under antibody selection was allowed for 48 h at 37°C. At each passage, half of the supernatant was mixed 1:1 with 50 µg/ml of MAb for 1 h. The remaining half of the supernatant was aliquoted and stored at −80°C. After three passages under MAb selection, virus-containing supernatants were tested by PRNT for escape from neutralization by CR4348 or CR4354. After confirmation of the escape phenotype, an aliquot of the supernatant was used in a Vero cell plaque assay under MAb selection. Plaques were visualized by overlaying with neutral red, and sterile glass Pasteur pipettes were used to isolate resistant virus from single plaques. Plaque-purified virus was amplified under MAb selection (25 µg/ml overnight at 37°C. Vero cells were scrapped from wells, and total cellular RNA was isolated using an RNeasy kit (Qiagen). cDNA was amplified using a reverse primer (5′- TGCCGGCTGATGTCTATGG-3′) in the WNV NS1 gene and served as a template for PCR amplification of the prM and E genes using forward (454F [5′-AGCGTAGGAGCAGTTACCC-3′]) and reverse (2501R) primers. The prM and E genes were then directly sequenced from gel-purified PCR products, and the neutralization escape mutant sequence was compared to the sequence of the laboratory stock WNV that was passaged and plaque purified in parallel in the absence of MAb selection.

**WNV infectious cDNA clone and mutant generation.** The two-plasmid WNV-NY99 cDNA clone (36) was used to create wild-type and mutant infectious WNV. Single amino acid substitutions were introduced into plasmid pWNAB by site-directed mutagenesis, Wild-type and mutant plasmid pWNAB (encoding nucleotides 1 to 2495 of the WNV genome) and wild-type plasmid pWNCG (encoding nucleotides 2495 to 11029) were grown in SURE+2 supercompetent Escherichia coli cells (Stratagene) at RT. Each plasmid was digested with XbaI and EcoRI, treated with WNV restriction endonucleases, and the prM/E fragment of pWNAB and pWNCG, respectively, were gel purified and ligated with T4 DNA ligase (Invitrogen) at 4°C overnight. The reaction mixtures were then heat inactivated, digested with XbaI to linearize the DNA, treated with proteinase K, extracted twice with phenol and chloroform, and precipitated with ethanol at −20°C overnight. All DNA was used as a template for in vitro DNA-dependent RNA transcription with the AmpliScribe T7 kit (Epitope) with the addition of an mG(5′)-ppp(5′)-sCAp analog (New England Biolabs). Transcription reactions were run at 37°C for 5 h, and the reaction mixture was then electro- porated (three pulses at 850 V, 25 µF, and +310) into BHK21-15 cells. Cells were added to a T75 tissue culture flask in DMEM with 10% FBS and observed for the onset of cytopathic effects. Once cytopathic effects were observed, the virus-containing supernatant was collected, cellular debris was pelleted, and supernatant aliquots were frozen at −80°C. The cells remaining in the T75 flask were harvested and used as a source of viral RNA to confirm that the desired mutant sequence was retained.

**Structural analysis.** The coordinates for the WNV E protein (RCSB accession number 2HGO) were divided by domains and fit onto the cryo-electron microscopy structure for mature DENV (RCSB accession number 1K4R) using CCP4MG to create a model of the WNV E protein dimer. Distances were calculated and figures were prepared using PyMol (http://www.pymol.org).

**Mouse experiments.** Mouse studies were approved and performed according to the guidelines of the Washington University School of Medicine Animal Safety Committee. Three- to four-week-old outbred NIH Swiss mice (Harlan) were infected by intraperitoneal injection with WNV-NY99c dilute in Hanks’ balanced salt solution containing 1% heat-inactivated FBS. For antibody pro-uction studies, 1 day prior to infection, mice were treated by intraperitoneal injection with 50 µg of the indicated MAb or vehicle control diluted in 100 µl PBS. Mice were monitored daily for 21 days for mortality and were euthanized when moribund. In some experiments, passive transfer of MAbs was performed with 5-week-old C57BL/6 mice (Jackson Laboratories) as described previously (59).

**Fusion assay.** The fusion of pyrene-labeled WNV with liposomes was monitored continuously in a Fluorolog-3-22 fluorometer (BFI Optics) at excitation and emission wavelengths of 345 nm and 475 nm, respectively. Liposomes (large unilamellar vesicles with a diameter of ∼200 nm) were prepared by a freeze-thaw extrusion procedure as described previously (73). Liposomes consisted of a mixture of phosphatidylcholine from egg yolk, phosphatidylethanolamine prepared by the transphosphatidylolation of egg phosphatidylcholine, and cholesterol in a molar ratio of 1:1:2. The lipids were obtained from Avanti Polar Lipids. The concentration of phospholipids was determined by phosphate analysis.

Pyrene-labeled WNV (1.24 µg of total viral protein) and liposomes (final concentration, 200 µM phospholipid) were mixed in a final volume of 665 µl in a solution containing 5 mM HEPES, 150 mM NaCl, and 0.1 mM EDTA (pH 7.4) with continuous stirring in a temperature-controlled cuvette at 37°C. At 0 s, the medium was acidified by the addition of 35 µl of a solution containing 0.1 MES and 0.2 M acetic acid, which was pretreated with NaOH to achieve the final desired pH. The fusion scale was calibrated such that 0% fusion corresponded to the initial excimer fluorescence value. The 100% value was obtained through the addition of 35 µl of a 0.4 M octaethylenynglycol monooctadecyl ether (Fluka Chemie AG) to achieve an infinite dilution of the probe. The extent of fusion was determined 60 s after acidification. To determine the influence of the MAbs on membrane fusion, pyrene-labeled WNV was incubated with different concentrations of MAbs for 1 h at RT prior to mixing with liposomes.

**Statistical analysis.** All data were analyzed using Prism software (GraphPad, San Diego, CA). Kaplan-Meier survival curves were analyzed by the log-rank test. For neutralization assays, an unpaired Student’s t test was used to determine significance. For ELISA data, a paired Student’s t test was used to determine significance.

**RESULTS**

**Characterization of strongly neutralizing human MAbs.** Previous studies that mapped the epitope specificity of inhibitory antibodies in convalescent-phase human serum samples suggested that only a fraction of WNV-infected patients developed antibodies against the strongly neutralizing epitope on DIII-Ir of the E protein (60). Consistent with this, few DIII-specific human MAbs were isolated from phase display scFv libraries from infected patients (78), and none were selected from very large libraries of pooled sera from uninfected donors (22). As human convalescent-phase serum retains strong neutralizing activity, we hypothesized that antibodies with distinct specificity must contribute to the inhibitory activity.

Antibody-phase display libraries constructed from peripheral blood lymphocytes of three convalescent patients after WNV infection were screened for reactivity on WNV antigen as described previously (78). All selected monoclonal phages specifically bound a preparation of inactivated WNV and immobilized prM-E-containing SVP, SVP, in addition to containing prM/M proteins, display 60 E protein homodimers in a lipid bilayer (18), whereas virions have 90 E protein homodimers in a distinct icosahedral arrangement (39, 55). Two MAbs, CR4348 and CR4354, which demonstrated strong neutralizing activity in pilot functional assays, tested negative for binding to the recombinant E protein by ELISA (data not shown); in contrast, other neutralizing human MAbs that were identified in the screen and previously characterized (e.g., CR4374) (60, 78) readily bound the recombinant E protein.

In both the PRNT and WNV RVP assay, CR4348 (PRNT50 = 536 ng/ml; RVP 50% effective concentration [EC50] = 146 ng/ml) and CR4354 (PRNT50 = 88 ng/ml; RVP EC50 = 26...
ng/ml) inhibited infection strongly albeit slightly less than that achieved with a humanized version of a therapeutic WNV type-specific mouse MAb, E16 (Hu-E16), that maps to the DIII-lr epitope (59) (Fig. 1A and B). CR4348 and CR4354 appeared to be distinct from Hu-E16, as they did not efficiently recognize the ectodomain of the E protein when expressed as a purified protein or displayed on the surface of yeast cells (Fig. 2A and B). These neutralizing human MAbs also efficiently neutralized variant WNV RVP composed of E proteins encoding a T332K mutation that abrogates the neutralization of virtually all DIII-lr MAbs (Fig. 1C) (3, 59). Additionally, both human MAbs likely do not recognize linear epitopes, as they did not identify prM, M, or E by Western blotting under reducing or nonreducing conditions (Fig. 2C).

Because CR4348 and CR4354 neutralized WNV but did not appear to recognize the recombinant E protein, we speculated that they might inhibit infection by binding to an epitope on prM that was not be detected by Western blotting. To test this, we took advantage of a series of WNV RVP preparations that differ with respect to the efficiencies of the maturation process; similar preparations were used previously to define the differential neutralizing activity of anti-WNV MAbs generated in mice (56). WNV RVP were produced as fully mature (in cells overexpressing the furin protease), partially and heterogeneously mature (standard conditions), or largely immature (in the presence of NH₄Cl, which inhibits furin cleavage) particles. These three types of RVP differ in their content of prM, ranging from virtually none (mature) to >95% (immature). CR4348 and CR4354 neutralized prM-containing or -absent particles equivalently (Fig. 3), eliminating the pr peptide as part of the epitope for either MAb.

An alternative possibility was that CR4348 and CR4354 do not bind a viral protein but instead inhibit WNV infection by binding a host cell surface receptor and blocking attachment and/or entry. However, if the neutralizing MAbs CR4348 and CR4354 recognized a structural protein on the virion, they should enhance infection in Fc-γRIIA-expressing cells when the stoichiometry of binding falls below the neutralization threshold (51, 66). The addition of subneutralizing concentrations of CR4348 and CR4354 to K562 cells that display human CD32 (Fc-γRIIA) enhanced infection in a manner that was analogous to that observed with Hu-E16 (Fig. 1D). This experiment suggests that CR4348 and CR4354 recognized a protein on the surface of the infectious virion.

To corroborate these findings, we used a capture ELISA to measure the direct binding of CR4348 and CR4354 to SVP and infectious WNV virions. Notably, CR4348 and CR4354 bound to both SVP and infectious virus (P = 0.002) (Fig. 2D). However, CR4348 and CR4354 did not bind to DENV particles, consistent with their inability to neutralize these viruses in a plaque reduction assay (data not shown). Thus, biochemical and functional analyses suggested that CR4348 and CR4354 neutralize WNV by virtue of their ability to bind an epitope on one of the viral structural proteins (M or E) that requires a specific oligomeric arrangement present on virions or SVP.

Neutralization by CR4348 and CR4354 occurs primarily at a postattachment step. Antibody neutralization of enveloped viruses may occur by inhibiting receptor attachment, internal-
FIG. 2. Binding of human MAbs to recombinant protein, SVP, and virions. (A) Yeast display assay. The WNV E protein ectodomain (Ecto-E) (amino acids 1 to 415) (left) or DIII alone (amino acids 296 to 415) (right) was expressed on the surface of yeast cells; stained with CR4348 (dotted lines), CR4354 (dashed lines), Hu-E16 (solid lines), or irrelevant human IgG1 (filled area) MAbs; and detected by flow cytometry. Data from one representative experiment of three are shown. (B) ELISA. The ectodomain (amino acids 1 to 415) of the E protein was adsorbed onto microtiter plates for 1 h at 37°C or overnight at 4°C. After blocking, wells were incubated with the indicated MAbs, and ELISA was performed as detailed in Materials and Methods. Data shown are representative of data from three experiments performed in triplicate, with error bars representing standard deviations. Abs, absorbance. (C) Western blot. The recombinant E protein ectodomain (~39 kDa), SVP (glycosylated full-length E protein) (~44 kDa), or WNV virus (unglycosylated full-length E protein) (~43 kDa) was assessed for binding by the indicated human MAbs to the E or prM (~15-kDa) protein. Samples were prepared in sodium dodecyl sulfate sample buffer with or without heating to 95°C and with or without 5% (vol/vol) β-mercaptoethanol (β-ME). (D) A capture ELISA was used to detect binding of MAbs to WNV SVP (left) and virions (right). Microtiter plates were coated with murine E16, incubated with SVP or virus, and detected with the indicated human IgG1 MAbs. Dashed lines indicate the background of the assay with an isotype control MAb.
ization, and/or endosomal fusion (63, 88). To determine the stage of the viral entry pathway at which CR4348 and CR4354 inhibit infection, we performed pre- and postattachment neutralization assays (11, 29, 57). CR4348 and CR4354 were incubated with WNV before or after virus binding to a monolayer of Vero cells, and infection was measured by a plaque reduction assay. As expected, CR4348 and CR4354 efficiently neutralized infection when premixed with virus (Fig. 4A). Both MAbs also inhibited WNV infection when added after virus adsorption to the cell surface, indicating that at least part of their neutralizing activity was at a postattachment step of the viral life cycle. When added after attachment, CR4354 was

FIG. 3. Effect of virus maturation state on neutralization by CR4348 and CR4354. CR4348 (left) and CR4354 (right) were incubated with MAbs prior to infection of Raji-DCSIGNR cells. RVP were prepared normally (mixture of mature, immature, and partially mature particles), in the presence of NH₄Cl (immature), or in cells overexpressing furin protease (mature) to create virions of different maturation states. These virions were incubated with MAbs prior to infection of Raji-DCSIGNR cells. Data shown are combined results from three independent experiments performed in duplicate.

FIG. 4. Mechanism of WNV neutralization by CR4348 and CR4354. (A) Pre- and postattachment inhibition assays. To determine whether the MAbs neutralize WNV infection after cellular attachment, Vero cells were prechilled to 4°C, and 10⁷ PFU of WNV were added to each well for 1 h at 4°C. After extensive washing at 4°C, the MAbs were added for 1 h at 4°C, and the PRNT protocol was then completed (dashed lines) (Post). In comparison, a standard preincubation PRNT with all steps performed at 4°C is shown for reference. In this case, virus and MAb were incubated together for 1 h at 4°C, prior to addition to cells (solid lines) (Pre). Data shown are representative of data from three experiments performed in duplicate, with error bars representing standard deviations. (B) pH sensitivity of MAb binding to SVP. The capture ELISA protocol was modified such that the pH was changed (pH 6.0 or 7.4) for 30 min immediately before captured SVP were detected by the indicated human IgG1 MAbs. Data from one representative experiment of three performed in triplicate are shown, with error bars representing standard deviations. Dashed lines indicate the background of the assay with an isotype control MAb. Abs, absorbance. (C and D) Fusion of pyrene-labeled WNV with liposomes. (C) Pyrene-labeled WNV was incubated with or without 50 nM of the indicated MAbs for 1 h at room temperature prior to mixing with liposomes and acidification at pH 6.3. Fusion was measured online, as described in Materials and Methods. Representative fusion data for at least three independent experiments are shown. Curve a, no antibody; curve b, 50 nM of the indicated MAbs; curve c, 50 nM CR4348; curve d, 50 nM CR4354. (D) Extent of WNV fusion with increasing concentrations of antibody. The extent of fusion was determined at 60 s upon acidification and is shown as a percentage of the control (no antibody) (pH 6.3). White bars, isotype-matched control MAb; dark gray bars, CR4348; light gray bars, CR4354. Representative fusion data for at least three independent experiments are shown.
more potent than CR4348 in neutralizing infection. Importantly, no pre- or postattachment neutralization was detected with a nonbinding isotype control, and a largely postattachment pattern of inhibition was seen with Hu-E16 (Fig. 4A), as observed previously (57).

**pH sensitivity of CR4348 and CR4354 binding.** The exposure of flavivirus virions or SVP to acidic pH in solution or in the endosome prompts a rapid structural rearrangement (7, 19, 49, 77). Given that CR4348 and CR4354 preferentially recognize epitopes present on virions and SVP but not on recombinant E proteins (Fig. 2), we hypothesized that changes in the oligomeric arrangement of E that are associated with shifts in pH might alter immunoreactivity. To test this, we used our SVP capture ELISA, exposing particles to different pH (pH 6.0 or 7.4) conditions for 30 min prior to pH normalization (pH 7.4) and incubation with MAbs. Notably, our control neutralizing MAb, Hu-E16, did not show pH-sensitive binding (Fig. 4B). In contrast, the exposure of SVP to pH 6.0, which should promote E protein rearrangement and irreversible trimer formation, significantly reduced the levels of binding of both CR4348 and CR4354 ($P \leq 0.003$).

**CR4348 and CR4354 inhibit WNV fusion.** Since both CR4348 and CR4354 have the capacity to neutralize WNV infection at a postattachment step, we investigated whether these MAbs would also affect WNV fusion. To evaluate this, we utilized a model liposome fusion assay with pyrene-labeled virus that was previously used to monitor the fusion dynamics of TBEV (9, 19, 75–77). Briefly, pyrene-labeled WNV was preincubated with different concentrations of MAbs for 1 h at RT and then mixed with liposomes at 37°C. Fusion was triggered by the acidification of the mixture to pH 6.3, which is the optimal pH for WNV fusion (B. Moesker, J. Wilschut, and J. Smit, unpublished observations). In the absence of antibodies or in the presence of 50 nM (7.5 μg/ml) of a nonbinding control MAb, fusion was essentially complete within seconds upon acidification. In contrast, preincubation of the virus with CR4348 or CR4354 potently inhibited fusion activity (Fig. 4C). Both MAbs reduced the extent of fusion in a dose-dependent manner (Fig. 4D). CR4354 inhibited fusion at concentrations of 1 nM (0.15 μg/ml) and above, whereas for CR4348, a minimal concentration of 10 nM (1.5 μg/ml) was required. This difference in potency was also reflected in the maximum level of fusion inhibition at saturating antibody concentrations. CR4354 and CR4348 neutralized ~85% and ~65% of the virus particles, respectively (Fig. 4D). Thus, in the model liposome assay, CR4348 and CR4354 can block the low-pH-catalyzed fusion of the majority of WNV virions; this supports the idea that these MAbs act at a postattachment step. Nonetheless, even under conditions of saturating antibody concentrations, a fraction of the virus particles resists complete inhibition by these MAbs, although the rate of fusion of the residual fractions is substantially lower than that in the absence of antibody.

**Generation of neutralization escape mutants.** Because CR4348 and CR4354 recognized a determinant on the WNV virion or SVP, which is not readily apparent on recombinant or yeast-displayed forms of E, we generated neutralization escape mutants to further define their epitopes. After three sequential virus passages on Vero cells under CR4348 or CR4354 selection (25 μg/ml), WNV was no longer neutralized by these MAbs in plaque reduction assays (Fig. 5A). To determine the mutations that conferred the escape phenotype, RNA sequences obtained from plaque-purified escape variants were compared to the wild-type WNV sequence derived from virus passaged in parallel in the absence of antibody selection. All (five of five) sequences from CR4348 escape variants contained the same single-nucleotide change encoding a T208I mutation in the E protein; in addition, two of the five sequences also had an independent H246Y mutation in the E protein. In contrast, all (15 of 15) CR4354 escape variants contained a single-nucleotide mutation, encoding a K136E mutation in the E protein; no other nucleotide changes were observed in any of the CR4354 variants.

To establish that these amino acid substitutions conferred the neutralization escape phenotypes observed, we utilized two reverse-genetic systems. First, RVP with single amino acid mutations were generated and analyzed for MAb neutralization. Whereas Hu-E16 neutralized all mutant and wild-type RVP equivalently, the T208I and K136E RVP were not efficiently neutralized by CR4348 and CR4354, respectively, even at concentrations of 15 μg/ml of MAb (Fig. 5B). H246Y RVP were neutralized by CR4348 but only at the highest doses of antibody tested; the concentration at which 50% inhibition (EC50) occurred was increased by >31-fold ($P < 0.02$) compared to that of wild-type RVP (Fig. 1B).

As an independent confirmation, we introduced these substitutions into a wild-type, infectious New York 1999 WNV cDNA clone (NY99ic) (5) to generate mutant viruses. Analogous results were obtained with all genetically engineered mutant WNV and MAbs using a plaque reduction assay on BHK21-15 cells (Fig. 5C). Thus, results of experiments with RVP and infectious cDNA clones confirmed the sequencing results and suggest that CR4348 and CR4354 likely bind distinct epitopes on the WNV E protein. To directly determine the contributions of these amino acids to MAb binding, we used mutated NY99ic WNV in a capture ELISA (Fig. 5D). As expected, Hu-E16 recognized all three variant viruses. CR4354 failed to bind to WNV with a K136E mutation but did recognize the other viruses, establishing that a change in K136 confers a loss-of-binding phenotype. In contrast, no decrease in the level of CR4348 binding was observed for the single T208I or H246Y variant or a double T208I H246Y variant (data not shown). Thus, these two individual mutations, which strongly impact CR4348 neutralization, do not alter MAb binding in the capture ELISA.

To gain a better understanding of why CR4348 and CR4354 recognized intact virions or subviral particles but not recombinant E proteins, we mapped the residues that conferred escape from MAb neutralization onto the existing WNV E protein crystal structures (30, 58). K136, T208, and H246 are all solvent-accessible residues (Fig. 6A). K136 is located at the end of DI adjacent to the hinge between DI and DII. This hinge rotates ~20° to 30° during structural rearrangements of the E protein associated with low-pH-induced viral fusion in late endosomes (7, 49). The flexibility of the DI-DII hinge may be less dynamic on an icosahedral virion or SVP than the soluble recombinant protein, possibly explaining the differential reactivity of the CR4354 epitope. In contrast, the conformationally sensitive nature of CR4348 binding may be due to a preferential reactivity with WNV E protein dimers. T208 and H246 are present in DII and, although relatively distant (~43 Å) within an individual E protein monomer, are
separated by ~19 Å across the dimer interface, which is within the spatial limits of an antibody footprint (Fig. 6B). It is important that in contrast to the TBEV and DENV E proteins, which readily form dimers in solution, the soluble WNV E protein is largely monomeric (30, 58). Consistent with the mapping and binding data that suggest that the two human MAbs recognize distinct epitopes, CR4348 efficiently neutralized the K136E WNV variant, and CR4354 inhibited infection of the T208I and H246P variants (data not shown).

Given these mapping data, we hypothesized that CR4348 might recognize oligomeric forms of the E protein that are not prevalent in our purified recombinant preparation. As mentioned above, ELISA plates coated with the WNV E protein overnight at 4°C showed no immunoreactivity with either CR4348 or CR4354 (Fig. 2B). However, coating plates with the E protein at 37°C resulted in a modest yet significant signal by CR4348, possibly due to oligomeric interactions between E proteins that occur more favorably at a higher temperature, prior to adsorption (Fig. 2B).

Phenotype of WNV variants in cell culture. Comparisons of E protein amino acid sequences of WNV isolates that varied geographically and temporally showed that T208 and H246 were completely conserved. K136 was completely conserved among lineage 1 WNV isolates but varied in the less-virulent lineage 2 and 3 strains, with alanine and serine substitutions, respectively (data not shown). To evaluate whether the mutations associated with the neutralization escape of CR4348 and CR4354 were functionally important for virus replication and
affected viral virulence, growth analyses using BHK21-15 cells was performed. The plaque morphologies of the three mutant viruses (K136E, T208I, and H246Y) in BHK21-15 cells was compared to that of wild-type WNV strain New York 1999. The wild type and K136E and T208I variants had similar large-plaque morphologies, whereas the H246Y mutant had a small-plaque phenotype (data not shown).

**In vivo protection studies.** Passive transfer of neutralizing MAbs against WNV confers protection against disease in mice (22, 59, 61, 69, 78) and hamsters (53, 54). To evaluate the potency of human MAbs, protection studies were performed using wild-type C57BL/6 mice after infection with WNV-NY99ic. Analogous to results with Hu-E16 (59), prophylaxis with low (1.4 and 0.4 μg, respectively) doses of CR4348 and

---

**FIG. 6.** Structural mapping of MAb epitopes. (A) WNV E protein dimer, with K136 (CR4354) in blue and T208 and H246 (CR4348) in magenta. (B) Close-up view of the WNV E DII dimer interface, with the CR4348 epitope highlighted in magenta. (C) Surface display model of the WNV E DI-DII hinge region with epitope recognition sites of CR4354 and the corresponding WNV residues of other antiflavivirus MAbs (E113, 503, NARMA3, B2, and 4B6C-2) listed in Table 4. Note that residue 136 is labeled CR4354 but is also part of the 503 epitope, and residue 126 is labeled B2 but is also part of the 503 and 4B6C-2 epitopes.
CR4354 completely protected mice against lethal WNV encephalitis (Table 1). No protective effect was observed in these or other previously reported studies (17, 59, 78) using isotype control antibodies.

To characterize the protective activity of the more potent of the two neutralizing MAbs, CR4345, in greater detail, infection studies with wild-type and K136E escape variant WNV were repeated using highly susceptible 3- to 4-week-old NIH Swiss mice (Table 2); this strain was selected because WNV morbidity and mortality show a linear virus dose dependence (4). Consistent with the cell culture plaque morphology data, the lethals of wild-type and K136E WNV-NY99ic were not different, with 50% lethal dose values of 0.28 and 0.24 PFU, respectively (P > 0.8). Sequencing of viral RNA from brains of mice infected with wild-type or K136E variant WNV-NY99ic revealed no amino acid changes: WNV in the brain after infection with wild-type and mutant viruses contained K and E residues at position 136, respectively (data not shown). To confirm the neutralization escape phenotype, mice were pretreated with 50 µg (~100 times the minimum protective dose in C57BL/6 mice) of CR4354 MAb 1 day prior to infection with ~3 × 10³ PFU of wild-type or K136E variant WNV-NY99ic. CR4354 protected 94% (15 of 16) of mice from wild-type NY99ic challenge, whereas only 19% (3 of 16) of mice challenged with K136E-NY99ic survived despite CR4354 prophylaxis. In contrast, Hu-E16 protected nearly all mice after infection with either the wild type or K136E-NY99ic. Collectively, these data confirm the highly protective activity of CR4354 in vivo and the CR4354 escape phenotype of the K136E mutation.

**DISCUSSION**

Previous studies suggested that antibodies that map to the DI-DII-ir epitope do not account for the majority of neutralizing activity in serum from human patients or horses (60, 70). In this study, we characterized the functional properties of two neutralizing human MAbs, CR4348 and CR4354, that recognize distinct epitopes. Both MAbs inhibit WNV infection in vitro and in vivo but did not recognize closely or distantly related flaviviruses, including St. Louis encephalitis virus or DENV (M. Throsby, M. Vogt, and M. Diamond, unpublished results). Biochemical studies demonstrate that these MAbs have similar but not identical profiles: they bind to conformationally sensitive epitopes on E proteins displayed on virions or SVP in a pH-sensitive manner but recognize recombinant E proteins in solution or displayed on the surface of yeast poorly, if at all. Functional experiments suggest that both human MAbs neutralize infection primarily at a postattachment stage in the viral life cycle, specifically through the inhibition of viral fusion with the endosomal membrane.

The CR4354 recognition site was established by neutralization escape and reverse-genetic experiments and localized to the DI-DII hinge interface at residue K136. This hinge is highly flexible, as the angle between DI and DII on DENV E rotates 27° during the transition from the immature to the mature state after the furin-mediated cleavage of prM (87). The hinge then rotates back 30° during the prefusion-to-postfusion domain rearrangement (7, 49). Two X-ray crystal structures of similarly prepared WNV E proteins also have distinct DI-DII hinge angles, differing by 5° (30, 58). This inherent flexibility of the DI-DII hinge could explain the lack of CR4354 binding to soluble and yeast surface-displayed E, as these recombinant E proteins may not display the native hinge that is found on virions or SVP. Indeed, the exposure of SVP to acidic conditions that alter the DI-DII hinge angle significantly reduced the level of binding of CR4354.

Although the characteristics of CR4354 appear relatively unique among anti-WNV MAbs, several other antiflavivirus neutralizing MAbs that localize to this region have been described (Table 3). The anti-WNV MAb that maps closest to this region is mouse MAb E113, which binds a determinant along the DI-DII hinge interface at residues E49 and K208 (Fig. 6C). E113 is protective in vitro (EC₅₀ of ~0.25 µg/ml) and in vivo, but unlike CR4354, E113 binds recombinant and yeast-displayed E proteins efficiently (61). The importance of this epitope for antibody neutralization is reflected by the characterization of several DI-DII-inhibitory antiflavivirus MAbs. Notably, MAbs (503, NARMA3, and B2) that recognize the DI-DII hinge are strongly inhibitory against the closely

### Table 1. Effect of MAb pretreatment on survival of C57BL/6 mice

<table>
<thead>
<tr>
<th>MAb</th>
<th>Dose (µg)</th>
<th>No. of survivors/total no. of mice</th>
<th>% Survival</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td></td>
<td>4/20</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>CR4348</td>
<td>14</td>
<td>4/4</td>
<td>100</td>
<td>≤0.01</td>
</tr>
<tr>
<td>CR4348</td>
<td>4.2</td>
<td>5/5</td>
<td>100</td>
<td>≤0.01</td>
</tr>
<tr>
<td>CR4348</td>
<td>1.4</td>
<td>5/5</td>
<td>100</td>
<td>≤0.01</td>
</tr>
<tr>
<td>CR4348</td>
<td>0.42</td>
<td>3/5</td>
<td>60</td>
<td>0.13</td>
</tr>
<tr>
<td>CR4348</td>
<td>0.14</td>
<td>3/5</td>
<td>60</td>
<td>0.10</td>
</tr>
<tr>
<td>CR4348</td>
<td>0.042</td>
<td>2/5</td>
<td>40</td>
<td>0.31</td>
</tr>
<tr>
<td>CR4348</td>
<td>0.014</td>
<td>1/5</td>
<td>20</td>
<td>0.70</td>
</tr>
<tr>
<td>CR4354</td>
<td>14</td>
<td>5/5</td>
<td>100</td>
<td>≤0.01</td>
</tr>
<tr>
<td>CR4354</td>
<td>4.2</td>
<td>5/5</td>
<td>100</td>
<td>≤0.01</td>
</tr>
<tr>
<td>CR4354</td>
<td>1.4</td>
<td>5/5</td>
<td>100</td>
<td>≤0.01</td>
</tr>
<tr>
<td>CR4354</td>
<td>0.42</td>
<td>5/5</td>
<td>100</td>
<td>≤0.01</td>
</tr>
<tr>
<td>CR4354</td>
<td>0.14</td>
<td>5/5</td>
<td>80</td>
<td>0.02</td>
</tr>
<tr>
<td>CR4354</td>
<td>0.042</td>
<td>2/5</td>
<td>40</td>
<td>0.21</td>
</tr>
<tr>
<td>CR4354</td>
<td>0.014</td>
<td>1/5</td>
<td>20</td>
<td>0.57</td>
</tr>
</tbody>
</table>

*P values were determined using the log-rank test and compared to the PBS controls.

### Table 2. Effect of the K136E substitution on MAb protection of NIH Swiss mice

<table>
<thead>
<tr>
<th>MAb</th>
<th>NY99ic WNV</th>
<th>No. of survivors/total no. of mice</th>
<th>% Survival</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>Wild type</td>
<td>4/16</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Hu-E16</td>
<td>Wild type</td>
<td>15/16</td>
<td>100</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CR4354</td>
<td>Wild type</td>
<td>15/16</td>
<td>94</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PBS</td>
<td>K136E</td>
<td>1/16</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Hu-E16</td>
<td>K136E</td>
<td>15/16</td>
<td>94</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CR4354</td>
<td>K136E</td>
<td>3/16</td>
<td>19</td>
<td>0.37</td>
</tr>
</tbody>
</table>

*P values were determined using the log-rank test and compared to the PBS controls.

b NIH Swiss mice were pretreated with 50 µg of human MAb (Hu-E16 or CR4354) at day −1. On day 0, mice were infected with 3 × 10³ PFU/mouse of wild-type NY99ic or K136E-NY99ic virus and monitored for survival.
TABLE 3. Flavivirus MAbs that localize to the DI-DII hinge interface

<table>
<thead>
<tr>
<th>MAb</th>
<th>Virus</th>
<th>Epitope(s)</th>
<th>Immunogen(s)</th>
<th>Source of MAb</th>
<th>Neutralization*</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR4354</td>
<td>WNV</td>
<td>K136</td>
<td>Natural infection</td>
<td>Human</td>
<td>++</td>
<td>78, this paper</td>
</tr>
<tr>
<td>E113</td>
<td>WNV</td>
<td>E49, K280</td>
<td>Infectious virus + recombinant E protein</td>
<td>Mouse</td>
<td>+</td>
<td>61</td>
</tr>
<tr>
<td>503</td>
<td>JEV</td>
<td>S275, K136, I126</td>
<td>Infectious virus</td>
<td>Mouse</td>
<td>++</td>
<td>34, 35, 52</td>
</tr>
<tr>
<td>NARMA3</td>
<td>JEV</td>
<td>Q52</td>
<td>Infectious virus</td>
<td>Mouse</td>
<td>++</td>
<td>27, 37</td>
</tr>
<tr>
<td>B2</td>
<td>JEV</td>
<td>I126</td>
<td>Vaccine + infectious virus</td>
<td>Chimpanzee</td>
<td>++</td>
<td>21</td>
</tr>
<tr>
<td>4B6C-2</td>
<td>Murray Valley encephalitis virus</td>
<td>A126, R128, F274, S276, S277</td>
<td>Purified virus</td>
<td>Mouse</td>
<td>++</td>
<td>28, 47</td>
</tr>
</tbody>
</table>

* ++, greater than 90% inhibition via any in vitro assay or capable of 90% protection in any lethal in vivo model; +, any significant in vitro neutralization or in vivo protective capacity; ND, not determined.

related JEV, which is a member of the same serogroup as WNV (21, 27, 35, 52).

Neutralization escape experiments suggested that T208 and H246 are recognition sites near the DII dimer interface for MAb CR4348. The importance of these residues for neutralization was confirmed using reverse-genetic approaches by introducing these mutations into WNV RVP and the infectious cDNA clone. Although the mutation of these two residues abolished neutralization, they did not prevent MAb CR4348 binding in capture ELISAs, suggesting that other amino acids that were not revealed in our selection experiments contribute to the epitope. While T208 and H246 lie spatially far apart (−43 Å) within the E monomer, likely beyond the footprint of the antibody paratope, they reside significantly closer (−19 Å) across the dimer interface. Thus, CR4348 may recognize an epitope that is sensitive to the oligomeric state of the E protein. Consistent with this, CR4348 does not stably bind WNV E on yeast cells or in the solid phase when adsorbed at a low temperature (4°C). However, when WNV E was adsorbed onto microtiter plates at 37°C, we reproducibly observed modest levels of binding. Furthermore, CR4348 binds poorly to SVP that have been exposed to mildly acidic solutions; a decrease in pH induces a structural rearrangement in flavivirus E proteins, resulting in dimer dissociation and trimer formation (7, 49).

The CR4348 MAb epitope is structurally and functionally unique among characterized anti-WNV MAbs. Although CR4348 recognizes residues at the DII dimer interface that are proximal to mouse anti-WNV E MAb E100 (61), the two differ in several respects: E100 maps more distal from DI at residue H263, only modestly neutralizes WNV in cell culture (EC50 ~ 10 μg/ml), recognizes both soluble and yeast forms of E, and shows a quite limited protective efficacy against lethal WNV infection in mice. However, a DII dimer interface MAb with more-similar properties for the distantly related flavivirus TBEV has been described (Table 4). MAb A5 (26) maps to residue E207 along the dimer interface (45), is strongly neutralizing in culture (26), and partially blocks TBEV fusion in a pyrene excimer liposome fusion assay (74). Moreover, the binding of at least some cross-reactive neutralizing flavivirus MAbs (e.g., 4G2 and 6B6C-1) that map to the fusion peptide in DI are also affected by mutations of residues (E231) along the dimer interface (10). One speculation as to why so few MAbs with these functional properties have been described is that they may not be identified with screens or immunization protocols using recombinant E proteins.

One limitation to mapping analyses by neutralization escape, which may be important for MAbs that show a loss of function with mutations in a flexible hinge, is the inability to exclude a distal binding site that is modulated allosterically by the hinge. A lack of binding to yeast or soluble E could reflect that these recombinant forms of E are truncated at amino acid 415 and lack the C-terminal stem-anchor and transmembrane regions. The lack of an identification of an escape mutant in the highly conserved C-terminal regions could be due to a poor viability of these variants. Unfortunately, because these MAbs bind poorly to recombinant E proteins, co-crystallography (44, 57), nuclear magnetic resonance (82), or saturation mutagenesis (23, 24, 46) approaches to identify the structural epitope are not possible. Instead, cryo-electron microscopy studies with Fab-virion complexes (31, 44) are planned to confirm the location of the epitope on the virion. Such experiments will be especially important for MAbs like CR4348, which shows a loss of neutralization but not binding with mutations at the DII dimer interface.

We previously described E16, a strongly neutralizing WNV-specific mouse MAb that maps to the DIII-Ir of the E protein (59) and inhibits infection by blocking viral membrane fusion in endosomes (B. Thompson, B. Moesker, J. Wilschut, J. Smit,

<table>
<thead>
<tr>
<th>MAb</th>
<th>Virus</th>
<th>Epitope(s)</th>
<th>Source of MAb</th>
<th>Neutralization*</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR4348</td>
<td>WNV</td>
<td>T208, H246</td>
<td>Human</td>
<td>++</td>
<td>78, this paper</td>
</tr>
<tr>
<td>E100</td>
<td>WNV</td>
<td>H263</td>
<td>Mouse</td>
<td>+</td>
<td>61</td>
</tr>
<tr>
<td>A5</td>
<td>TBEV</td>
<td>E207</td>
<td>Mouse</td>
<td>+</td>
<td>26, 45</td>
</tr>
</tbody>
</table>

* ++, greater than 90% inhibition via any in vitro assay or capable of 90% protection in any lethal in vivo model; +, any significant in vitro neutralization or in vivo protective capacity; ND, not determined.
M. Diamond, and D. Fremont, unpublished data). Although CR4348 and CR4354 also strongly neutralize infection and affect a postattachment step in the viral life cycle, they likely inhibit WNV by a mechanism distinct from that of E16. CR4354 appeared to inhibit infection almost equivalently when it was added before or after attachment. In contrast, CR4348 and E16 showed somewhat enhanced neutralizing activity when added prior to attachment. Moreover, in the liposomal model system, E16 completely blocked fusion at different pH values ranging from pH 6.3 to 5.0, consistent with ELISA and surface plasmon resonance data showing pH-independent binding of E16 to SVP (Thompson et al., unpublished). In contrast, CR4354 and CR4348 do not completely block low-pH-induced fusion of the virus with liposomes, with some residual fusogenic activity when added prior to attachment. Moreover, in the liposomal model system, E16 completely blocked fusion at different pH values ranging from pH 6.3 to 5.0, consistent with ELISA and surface plasmon resonance data showing pH-independent binding of E16 to SVP (Thompson et al., unpublished).

Epitopes may be advantageous, as was demonstrated previously for a therapeutic antibody cocktail against rabies virus (2). The combination of neutralizing MAbs that recognize distinct epitopes may be advantageous, as was demonstrated previously for a therapeutic antibody cocktail against rabies virus (2). The characterization of potently neutralizing MAbs like CR4348 and CR4354 that map to distinct regions and inhibit virus by different mechanisms suggests that this may be feasible.

ACKNOWLEDGMENTS

We thank members of our laboratories for helpful discussions, James Brien for experimental assistance, Jolanda Smit for critical comments and suggestions, and Syd Johnson for preparation of MAb Hu-E16. This work was supported by grants from the NIH (grant U01 AI061373 [M.S.D.]) and the Midwest Regional Center of Excellence for Biodefense and Emerging Infectious Diseases Research (grant U54 AI057160).

REFERENCES


