Isolation and Characterization of Human Monoclonal Antibodies from Individuals Infected with West Nile Virus

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Monoclonal antibodies (MAbs) neutralizing West Nile Virus (WNV) have been shown to protect against infection in animal models and have been identified as a correlate of protection in WNV vaccine studies. In the present study, antibody repertoires from three convalescent WNV-infected patients were cloned into an scFv phage library, and 138 human MAbs binding to WNV were identified. One hundred twenty-one MAbs specifically bound to the viral envelope (E) protein and four MAbs to the premembrane (prM) protein. Enzyme-linked immunosorbent assay-based competitive-binding assays with representative E protein-specific MAbs demonstrated that 24/51 (47%) bound to domain II while only 4/51 (8%) targeted domain III. In vitro neutralizing activity was demonstrated for 12 MAbs, and two of these, CR4374 and CR4353, protected mice from lethal WNV challenge at 50% protective doses of 12.9 and 357 µg/kg of body weight, respectively. Our data analyzing three infected individuals suggest that the human anti-WNV repertoire after natural infection is dominated by nonneutralizing or weakly neutralizing MAbs binding to domain II of the E protein, while domain III-binding MAbs able to potently neutralize WNV in vitro and in vivo are rare.

West Nile virus (WNV) is a member of the Japanese encephalitis virus serocomplex of flaviviruses, is transmitted by mosquitoes, and infects birds and horses, as well as humans (17). Genomic analysis has revealed two genetic lineages of WNV: lineage 1 viruses, circulating in the United States, Europe, the Middle East, Africa, India, and Australia, and lineage 2 viruses, isolated from sub-Saharan Africa and Madagascar (23). Alarming recent epidemics of lineage 1 WNV have been associated with significant rates of morbidity and mortality in humans (12, 14, 19); however, neither a specific treatment for individuals infected with the virus nor a preventive vaccine is available. The recognition of WNV as an agent of neurological disease with long-term sequelae, in combination with its continuing geographical expansion, has increased the urgency with which such treatment options are being sought.

The positive-stranded RNA of the flavivirus genome encodes a single polyprotein that, when processed, produces three structural proteins—capsid (C), precursor membrane (prM), and envelope (E)—and seven nonstructural (NS) proteins. Experiments in murine models and extrapolation from clinical data from related flaviviruses suggest that a prompt humoral response is required to control viremia and to prevent viral dissemination into the central nervous system and, consequently, severe disease (8–10, 34). The target of most in vivo protective monoclonal antibodies (MAbs) generated by murine hybridoma technology is the E protein, although protective MAbs have been reported that bind to the M and NS1 proteins (32). With the exception of the last target, which is not associated with the virion, protective activity is strongly correlated with in vitro neutralizing activity (34). Structural analysis of flavivirus E protein has identified three domains (26–28, 31). The finger-like domain II harbors the fusion peptide that, in the endosomal trimeric form of E protein, mediates cellular fusion, and the immunoglobulin-like domain III exposes peptide loops with a putative role in cellular receptor binding. These two regions are flexibly connected by domain I, which forms a hinge region important in the pH-dependent shift from the prefusion antiparallel homodimeric form of E protein to the trimeric form. Neutralizing epitopes have been described within all three domains of the E protein; however, the most potent neutralizing MAbs have been mapped to domain III (30, 32).

Characterization of the binding specificities and functional activities of MAbs generated during natural WNV infection of humans has not been carefully carried out. In this study, a cloned antibody repertoire, constructed from three patients infected with WNV, was generated as a source of human MAbs with neutralizing activities against WNV. A large panel of unique MAbs that bound specifically to WNV was isolated, although only a small fraction demonstrated in vitro neutralizing activity against the virus, and only two of those MAbs

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were found to be protective in a murine WNV challenge model.

**MATERIALS AND METHODS**

**Virus strains and murine MAbs.** WNV designation USA99b (strain 385-99), isolated from a snowy owl at the Bronx zoo in New York City during the 1999 epidemic, was obtained after one passage from the University of Texas Medical Branch, Galveston, Texas. Virus working stocks were grown and titrated by 50% tissue culture infective dose (TCID50 assay and plaque assay on Vero cells. The MAbs 7H2, 5H10, and 3A3 (Bioreliance Corp., Rockville, Md.) (1); 6B6C1 (Cheminor International, Temecula, Calif.) (33, 34); and 4G2 (38) have been previously described. Polyclonal antibody against WNV M protein was purchased from Immunex, Sorrento Valley, Calif.

**Antigen preparation.** To maximize the chance that all potential antibody binding epitopes present on WNV would be accessible during the library selection procedure, three different preparations of viral antigens were produced: an inactivated whole-virus preparation, soluble recombinant E protein, and recombinant virus-like particles (VLP) that display on their surfaces the M and E proteins in native configuration. To produce viral antigen for selection, Vero cells were infected with USA99b and cultured for 6 days. The supernatant was harvested, and the virus was purified over a 25% sucrose cushion at 25,000 × g for 2 h at 4°C. The virus was exposed to 45 kGy of gamma irradiation from a 60Co source. Gamma irradiation was chosen for inactivation because its disruptive action on nucleic acid was judged unlikely to affect potential epitopes on the viral antigen to the same degree as cross-linking fixation methods, such as with formaldehyde or β-mercaptoethanol, commonly used for viral inactivation.

Recombinant E protein was produced by transient transfection of prM/E-expressing cells. The prM/E sequence was amplified by PCR amplification of phage supernatant after 72 h, purified through a 30% glycerol cushion as described above, and resuspended in phosphate-buffered saline (PBS).

Soluble recombinant E protein was expressed by transient transfection of a transmembrane-truncated prM/E construct. The construct was made as described above using a prM/E gene product truncated by 95 amino acids at its cytoplasmic domain. The virus was further purified over a 20% sucrose cushion at 40,000 × g for 2 h at 4°C. The resulting fractions were cloned into eukaryotic expression vectors containing constant antibody domains. IgG1 was expressed by transient transfection of mammalian cells in serum-free medium. The IgG concentrations in harvested supernatants were measured by a modified high-performance liquid chromatography method (11) and used for further experimentation or, alternatively, purified over protein A affinity columns. The concentrations of purified MAbs were measured by optical absorbance at 280 nm, and the purity and integrity of the MAbs were analyzed by reducing and nonreducing SDS-PAGE. Titration of IgG1 binding by ELISA was performed as described above with horse-radish peroxidase (HRP)-coupled mouse anti-human secondary antibody (Jackson Immunoresearch). Data were analyzed by nonlinear regression using Prism software (version 4.02) to calculate the antibody concentration required for 50% binding activity.

**Virus-neutralized virus neutralization assay (VNA).** For high-throughput screening, twofold serial dilutions of MAbs were made in triplicate and mixed with 100 TCID50 units of virus in a volume of 50 μl for 60 min at 37°C. Trypsinized Vero cells (50 μl) were then added to each microwell plate well and incubated for 7 days. Wells containing evidence of cytopathic viral activity were scored. The end point antibody titer was calculated as the lowest dilution at which two or more wells were free from cytopathic activity (60% neutralization).

**Screening of bacterial MAbs.** Screening was carried out using the domain-mapping ELISA, as described below. Screening was carried out using the domain-mapping ELISA, as described below. Screening was carried out using the domain-mapping ELISA, as described below. Screening was carried out using the domain-mapping ELISA, as described below. The neutralizing potencies of selected MAbs were scored. Vero-E6 cells were trypsinized and counted; 2.5 × 104 cells were added to each well of a 12-well plate and incubated overnight at 37°C in a humidified CO2 incubator. Serial dilutions (10-fold) of a titrated stock of WNV USA99b were made in complete medium. Equal-volume (250-μl) mixtures of virus (100 PFU) and serial dilutions of purified IgG1 MAbs were incubated in duplicate at 37°C for 1 h. Dillutions of both virus and MAbs were done in Dulbecco's modified Eagle's medium. The mixture (400 μl) was then added to the 12-well plates containing Vero cell monolayers after careful aspiration of the overnight medium. After incubating at 37°C for 1 h, a 1.5-ml overlay of carboxymethyl-cellulose (CMC) medium with 10% (vol/vol) fetal bovine serum was added well per well, and the plates were placed in a humidified CO2 incubator for 3 days at 37°C. For scoring, the plates were stained with the appropriate antibody (with antibody staining count) containing 0.825 mg/ml of neutral red (2 ml neutral red at 33 mg/liter in 80 ml CMC-PBS) added to each well on top of the previous overlay. The plates were incubated for another day at 37°C in a humidified CO2 incubator, after which the number of visible plaques was determined.

Antibody concentrations yielding 50% and 90% neutralization (PRNT50 and PRNT90, respectively) were determined for each MAb by linear regression using the Bliss combination model (13.0). Antibodies were compared directly in the regression model, with significant level alpha set at 0.05. Antibody concentrations yielding 50% and 90% neutralization were estimated from the model, together with 95% confidence interval (CI) values. SPR analysis of IgG1. Surface plasmon resonance (SPR) analysis was performed on a BIAcore3000. Direct immobilization of inactivated WNV to the sensor chip proved impossible due to the pH sensitivity of WNV to the coupling buffer. Therefore, the antibody (C4A2g3 was immobilized to a CM5 sensor chip by amine coupling and then used to capture inactivated WNV. Analysis of the antibodies was performed at 25°C at a constant flow rate of 30 μl/min using HBS-EP (10 mM HEPES [pH 7.4], 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20) as a running buffer. To determine the association rate, dissociation rate, and affinity (Kd), a concentration series from 0.4 to 1,000 nM of each antibody (40 μl) was injected at a constant flow rate of 30 μl/min. BIAevaluation version 3.2 (July 2001) software was used for data analysis. The bivalent analytic model was applied to account for avidity effects due to the bivalency of IgG antibodies. The rate equations in this model are as follows: (A) B + AB with rate constants (koff) off rates (koff) characterized by constants k1 and k-1 and k2 and k-2, respectively. The
affinity constant $K_a$, was determined as $k_{off}/k_{on}$ (kd1/ka1), using data from two independent experiments.

**Domain mapping and antibody binding competition.** An ELISA-based assay was used to map antibody binding to the E protein domain. Microtiter plates were coated with the murine MAbs 7H2, binding to domain III of E protein (1, and 6B6C-1, binding to domain II of E protein (34), and incubated for 60 min with purified E protein. After being washed, anti-E protein MAbs were added to the wells, and binding was detected by incubation with anti-human IgG antibody conjugated to HRP.

The assay was also carried out with anti-E protein scFv instead of reformatted IgG1 to avoid the possibility that adjacent epitopes would be blocked by steric hindrance from the larger IgG1 molecules. The assay was the same as described above, except that scFv containing a vesicular stomatitis virus tag were detected by incubation with anti-vesicular stomatitis virus antibody conjugated to HRP.

Direct competition of anti-WNV IgG1 molecules was measured by coating microtiter plates with inactivated virus or VLP and then incubating them with unlabeled IgG1 for 1 h, followed by biotin-labeled IgG1 for 5 min; the plates were washed, and biotinylated IgG1 was detected with streptavidin-HRP. To verify the results, the experiment was repeated using domain II MAbs 4G2 and domain III MAbs 3A3 and SH10.

**Immunoprecipitation and Western blotting of VLP transfectants.** Immunoprecipitation was carried out as previously described (13). IgG1 was coupled to protein A beads. 293T cells, transiently transfected with the VLP expression construct, were harvested and lysed at a concentration of $3 \times 10^7$ cells/ml and then incubated on ice in the presence of Triton X-100 for 1 h. The lysates were centrifuged at 20,000 × g and then biotinylated by addition of sulfo-N-hydroxysuccinimide-activated LC-1-LC-biotin. Lysates were precleared with empty protein A beads and then incubated for 2 h with antibody-conjugated beads at 4°C. The beads were washed extensively, and bound immunocomplexes were eluted by the addition of Laemmli buffer. The eluates were separated by SDS-PAGE under both non-reducing and reducing conditions. After being blotted on polyvinylidene difluoride membranes, the biotinylated proteins were detected with streptavidin-HRP and enhanced chemiluminescence. For Western blotting, recombinant E protein or VLP was loaded onto a 4 to 12% gradient gel. After being blotted, individual IgG1s were incubated on separate strips and detected with anti-human IgG-HRP and chemiluminescence.

**CF assay.** The complement fixation (CF) activities of the anti-WNV MAbs were measured as described previously (20). Briefly, MAbs were incubated overnight at 4°C in microtiter plates coated with inactivated WNV in the presence of human serum (Sigma) as a complement source. Sheep red blood cells (SRBC) (5% [vol/vol]) and anti-SRBC polyclonal serum were preincubated on ice for 60 min and then added to the wells for a further 60 min at 37°C with intermittent shaking. The degree of SRBC lysis, which is inversely correlated with complement fixation, was measured as optical density at 540 nm. Data were analyzed by nonlinear regression using Prism software (version 4.02) to calculate the antibody concentration required for 50% complement fixation and the 95% confidence interval.

**Murine challenge model.** Female BALB/c mice were purchased from Harlan at 21 days and used at 28 days. The mice were kept in isolator cages under pathogen-free conditions. All experiments were approved and performed according to the Dutch law on animal experiments in an AAALAC internationally accredited facility. The intraperitoneal 50% lethal dose in 4-week-old BALB/c mice, determined after titration of the WNV USA99b at four dilutions in two independent experiments, was calculated as 9.5 TCID_{50} units using probit ordination analysis. For challenge experiments, 4-week-old mice ($n = 5$) were inoculated i.p. with 20× 50% lethal dose of USA99b. Passive transfer of MAbs was carried out essentially as described previously (2). IgG1 (450 µg) was injected i.p. 24 h before virus injection. To control antibody injection, human MAb serum levels were monitored, and animals without measurable IgG1 in the serum were excluded from the group for analysis. The average antibody concentration in sera taken from mice immediately prior to virus injection was ~50% of the injected dose. The animals were monitored twice daily for clinical signs of infection, including fur ruffling, hunchback posture, and hind-limb paralysis. When signs of illness were detected, the mice were immediately sacrificed.

**Nucleotide sequence accession numbers.** scFv sequences from the neutralizing MAb panel are available in the GenBank database under the accession numbers DQ407212 to DQ40744.

RESULTS

**Generation of human MAbs against WNV.** Antibody phage display libraries were constructed from peripheral blood leucocytes collected from three patients with confirmed WNV infections. In total, nine serum samples, obtained at three time points from each of the donors, were screened for neutralization activity against WNV (USA99b; 385-99) in a microtiter-based VNA. All sera exhibited neutralization activity ranging from 1:400 to 1:1,600; no correlation was observed between the neutralization titer and time following infection. VH (immunoglobulin gamma) and VL regions from lymphocytes of all nine samples were amplified and cloned into the phagemid PDV-C06 to generate an scFv-expressing phage library. The final library size was $4 \times 10^6$ individual clones. DNA sequence analysis of 71 randomly picked scFv showed that 79% contained complete open reading frames composed of 24 different VH germ line and 30 different VL germ line genes, indicating a degree of diversity comparable to those of natural human repertoires (7).

Antigen from WNV was prepared in three different forms for the selection of anti-WNV MAbs from immune libraries. The immune antibody phage display library was incubated with inactivated WNV or VLP or soluble E protein immobilized on immunotubes. A second round of selection was again carried out with the three antigens so that all nine possible selection combinations were performed, a strategy that generally results in a diverse panel of MAbs (22). A total of 480 monoclonal scFv phages were screened for binding activity to inactivated WNV preparations. Alignment of DNA sequences from the binding scFv phages identified 117 unique scFv (~25%) (see Table S1 in the supplemental material). All monoclonal phages were demonstrated to specifically bind to WNV, but not to rabies virus included as a viral-antigen negative control or to fetal bovine serum-coated plates. Although almost all phage MAbs also specifically bound immobilized recombinant VLP and there was a direct correlation in terms of binding intensity (Fig. 1a), 41 of them did not bind to immobilized recombinant E protein (Fig. 1b). This may be because their binding site is not present on the truncated recombinant form of E protein. Alternatively, the binding epitope may span the homodimeric or heterodimeric form of E protein or the binding epitope is found on another surface protein. Binding to an NS protein of the virus was excluded, as VLP do not express the NS proteins.

A second set of selection experiments was conducted to focus selection on domain III of the E protein, the target of the most potent WNV-neutralizing MAbs. E protein was captured by the domain II binding antibody 6B6C-1 to block this region and effectively present domain III. After 300 colonies were screened, a further 21 scFv that contained unique sequences were isolated.

**Neutralizing activities of anti-WNV MAbs.** Key functional activities of the MAbs in the panel were investigated. The generally accepted in vitro correlate of protection from flavivirus infection is virus neutralization (34). To screen for this activity, the VNA was used. All 138 phage antibodies selected from the immune library were reformatted as IgG1 molecules and transiently expressed (3); 119 MAbs were produced in serum-free medium at concentrations sufficient for further testing (64 ± 34 µg/ml [mean ± standard error of the mean]). IgG1-containing supernatants were assayed for neutralizing activity against 100 TCID_{50} units of WNV strain USA99b. Twelve of the 119 MAbs tested reached the end point of 66% neutralization at a concentration of <50 µg/ml (see Table S1 in
A PRNT was performed on Vero cells to accurately determine the in vitro potencies of the neutralizing MAbs identified in the VNA. Data from two independent experiments carried out in duplicate are plotted as percentage mean ± standard error of the mean of the test IgG1 plaque number compared to negative control IgG1. A nonlinear-regression line was fitted using the ordinal-regression model probit. CR4374, CR4353, CR4347, 7H2, dotted line; 6B6C-1, solid line. (d) The CF activity of anti-WNV IgG1 was measured by the degree of SRBC lysis by free complement after preincubation of complement with WNV and IgG1. The results of two independent experiments were calculated as the mean percentage of SRBC lysis compared to negative control IgG1 wells. Nonlinear-regression lines fitted to the data are shown, and data were plotted for CR4268 (●) and CR4381 (○).
tional activity, the 50% binding activities of MAbs were plotted against the CF50 (Fig. 3b). No direct correlation was found between these two characteristics; however, an effect was observed when MAb specificity was taken into account by grouping MAbs containing the same CDR3H (CDR group 1, CR4271, CR4272, CR4273, CR4369, and CR4377; CDR group 2, CR274, CR4275, and CR4361; CDR group 3, CR4283, CR4284, CR4285, CR4272, CR4273, CR4369, and CR4377; CDR group 2, CR274, CR4374, CR4375; and CR4353, CR4354, and CR4355). For example, in CDR group 2, there was little effect of affinity on CF activity, in contrast to CDR group 4, where affinity had a pronounced effect on CF activity. This suggests that both affinity and specificity influence the CF activities of anti-WNV MAbs.

Affinity determination was performed for three MAbs by SPR; a representative tracing for antibody CR4283 is shown in Fig. 3c. The $k_\text{on}$, $k_\text{off}$, and absolute $K_D$ values were calculated for the two MAbs with the highest neutralization potencies, CR4374 and CR4353, and the best antibody in the ELISA titration, CR4283 (Table 3).

**Determination of MAb binding target on WNV.** To facilitate analysis, 64 MAbs representing unique CDR3H sequences (and thus potentially unique specificities) were chosen based on VLP ELISA binding; the other 8 specificities could not be expressed as IgG1. Of these, 51 bound specifically to E protein by ELISA (data not shown), and the binding specificity was consistent with the results of the phage antibody ELISA. Twenty-one of the 51 were immunoreactive with E protein, separated by SDS-PAGE under nonreducing conditions, and detected by Western blotting (data not shown). The other 30 MAbs thus either bind conformational epitopes not preserved under these conditions, or their affinities are too low to detect by this method (13). All of the MAbs in the neutralizing panel that were reactive to E protein in ELISA detected E protein after Western blotting (Fig. 4a), with the exception of CR4353. The reactivity of this antibody to E protein was confirmed by immunoprecipitation as described below (data not shown). Only one IgG1 from the panel, CR4374, was immunoreactive with E protein under reducing conditions, indicating that it recognizes a continuous linear epitope (data not shown). Specific binding of the reference antibody 7H2 to a 50-kDa band was pulled down with 8 out of the remaining 15 specificities could not be expressed as IgG1. Of these, 51 bound specifically to E protein by ELISA (data not shown), and the binding specificity was consistent with the results of the phage antibody ELISA. Twenty-one of the 51 were immunoreactive with E protein, separated by SDS-PAGE under nonreducing conditions, and detected by Western blotting (data not shown). The other 30 MAbs thus either bind conformational epitopes not preserved under these conditions, or their affinities are too low to detect by this method (13). All of the MAbs in the neutralizing panel that were reactive to E protein in ELISA detected E protein after Western blotting (Fig. 4a), with the exception of CR4353. The reactivity of this antibody to E protein was confirmed by immunoprecipitation as described below (data not shown). Only one IgG1 from the panel, CR4374, was immunoreactive with E protein under reducing conditions, indicating that it recognizes a continuous linear epitope (data not shown). Specific binding of the reference antibody 7H2 to a 50-kDa band confirmed the identity of E protein under both nonreducing and reducing conditions.

**Immunoprecipitation of VLP-transfected lysates** was performed to establish the binding targets of the MAbs not reactive to recombinant E protein in ELISA or Western blotting. A 50-kDa band was pulled down with 8 out of the remaining 15 MAbs that were negative in the E protein ELISA. Stripping of blots and staining with the reference antibody 7H2 confirmed the identity of E protein. Interestingly, two patterns were observed: in more than half the cases, a band of ~20 kDa was coprecipitated with the 50-kDa E protein band, which corresponds to the molecular weight of prM (confirmed on stripped blots by a specific polyclonal serum), while in the other population, this band was absent (Fig. 4c). This difference may be related to the accessibility of epitopes on the endoplasmic reticulum-restricted heterodimeric prM-E protein complex compared to the surface-exposed homodimeric form of E pro-
tein. If this is the case, CR4271, CR4274, and CR4282 are only capable of binding the latter form, while the epitope is present in both forms for the other MAbs shown (CR4355, CR4371, and CR4374). The antibody CR4293 consistently pulled down the /H11011 20-kDa band more intensely than the 50-kDa band. Western blotting confirmed that this antibody, and CR4294 with the same CDR3H region, recognized prM but not M protein (Fig. 4b).

Of the seven remaining unidentified MAbs, three were not able to immunoprecipitate any band, and thus their binding targets could not be identified. We have previously correlated failure in this experimental system with poor antibody affinity (13). Four MAbs, CR4258, CR4321, CR4348, and CR4354, were consistently found to immunoprecipitate bands that did not correspond to the molecular weights of viral proteins (data not shown).

**Mapping of MAb binding to WNV-E domains.** E protein ELISA-positive MAbs from the first set of selections (without domain III bias) were assayed for binding to E protein in a capture assay designed to differentiate between E protein domains. Monomeric soluble E protein was captured either by the domain III binding monoclonal 7H2 or the domain II binding monoclonal 6B6C-1. Of the 51 E protein binding MAbs that were tested, binding of 4 was completely blocked when E protein was captured by 7H2, but not when captured by 6B6C-1 (group D), and thus they were predicted to recognize an epitope in domain III of the E protein (Fig. 5a). In direct contrast, 24 MAbs were blocked with 6B6C-1 but not when captured by 7H2 (group B) and thus were predicted to bind at or close to the 6B6C-1 epitope mapped to the tip of domain II (34). Eight MAbs were able to bind WNV E protein captured by either antibody (group C) and thus could potentially bind in any of the three domains. Finally, 15 E protein binding scFv were not able to bind when captured in either of the orientations (group A). To rule out the possibility that steric hindrance was interfering with the binding of some MAbs, the assay was repeated with scFv expressed in soluble form. Although scFv are five times smaller than the reformatted IgG1, no difference in the binding pattern was observed (data not shown). To confirm these results, the domain III binding MAbs 3A3 and 5H10 or the domain II binding antibody 4G2 was substituted for 7H2 or 6B6C-1, respectively; no difference in binding pattern was observed (data not shown).

All unique MAbs isolated in the second group of selections, where 6B6C-1-captured E protein was the selection target, bound to E protein in both orientations (data not shown). Thus, no new domain III binding MAbs were isolated in this assay.

The binding characteristics of the neutralizing antibody panel were further investigated in cross-blocking assays on WNV. All MAbs blocked their own binding, as expected (Fig. 5b). Four of the MAbs, CR4271, CR4283, CR4353, and CR4274, all inhibited binding to one another either when preincubated with the inactivated WNV preparation or when

![FIG. 2. West Nile virus-neutralizing MAb sequences. VH sequences and VL sequences of scFv were aligned with germ line sequences. Framework (FR), CDR, sequence identities (.) and deletions (-) outside the CDR3 sequence, and VL germ line usage are indicated.](http://www.jvi.org/issue/2006/07/07/6987.html)
added as the biotinylated detection antibody. Consistent with the domain-mapping data, the domain III binding MAbs CR4299 and CR4374 also competed with each other, but only when CR4374 was incubated first with WNV antigen and not when CR4299 was the first antibody, perhaps reflecting the lower affinity of CR4299 (Fig. 3a). Similarly, the domain II binding MAbs CR4289, CR4311, and CR4325 inhibited each

TABLE 3. Affinity constants of three anti-WNV antibodies as determined by SPR

<table>
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<tr>
<th>Antibody</th>
<th>$k_{on}$ (ka1; M$^{-1}$ s$^{-1}$)</th>
<th>$k_{off}$ (kd1; s$^{-1}$)</th>
<th>$K_D$ (nM)</th>
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<tr>
<td>CR4374</td>
<td>$2.34 \times 10^7 \pm 0.04 \times 10^6$</td>
<td>$1.30 \times 10^{-3} \pm 0.05 \times 10^{-3}$</td>
<td>$56 \pm 4$</td>
</tr>
<tr>
<td>CR4353</td>
<td>$2.07 \times 10^5 \pm 0.05 \times 10^5$</td>
<td>$1.33 \times 10^{-3} \pm 0.04 \times 10^{-3}$</td>
<td>$6.5 \pm 0.4$</td>
</tr>
<tr>
<td>CR4283</td>
<td>$1.23 \times 10^6 \pm 0.43 \times 10^6$</td>
<td>$8.26 \times 10^{-4} \pm 3.74 \times 10^{-4}$</td>
<td>$0.8 \pm 0.6$</td>
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other’s binding to WNV, but not in all orientations, again likely a result of differences in affinity.

**Protective capacity of anti-WNV MAbs in WNV-infected mice.** To assess the protective activities of the neutralizing panel of MAbs, a challenge model was developed in 4-week-old BALB/c mice similar to a previously described model (2). Antibodies with neutralization potency in vitro were first screened for in vivo activity at a concentration of 15 mg/kg (Fig. 6a). Although three of the tested MAbs protected more than half of the animals, CR4271 was not significantly different from the isotype IgG1 control MAb as measured by the log rank test \((P = 0.156)\), in contrast to CR4374 \((P = 0.003)\) and CR4353 \((P = 0.007)\). The two MAbs demonstrating protection in the first experiment were titrated in a half-log concentration range from 10 mg/kg to 0.001 mg/kg. Titration of CR4374 (Fig. 6b) and CR4353 (Fig. 6c) showed a dose response both in terms of the number of surviving animals per group and also in terms of survival time. The difference between the two antibodies was highly significant (log rank test; \(P < 0.001\)). The 50% protective dose was calculated for each antibody after titration using the ordinal-regression model probit. The 50% protective dose of CR4374 (12.9 \(\mu\)g/kg and 0.04 to 74.8 [mean and 95% CI]) was significantly different from that of CR4353 (357 \(\mu\)g/kg and 4.3 to 1,166 [mean and 95% CI]; \(P < 0.01\)).

**DISCUSSION**

Immune repertoires generated in response to acute WNV infection or any flavivirus infection have not been well characterized in humans or primates. Although antibody phage display repertoires from dengue virus-infected chimpanzees (25) and yellow fever virus-infected patients (5) have been reported and nonimmune human repertoires have been screened for reactivity against WNV (16), only small numbers of MAbs were isolated and analyzed. Here, scFv phage display was used to clone the antibody repertoires from three patients with mild self-limiting WNV-induced disease, and 138 WNV-specific MAbs were identified with a large degree of VH and VL gene diversity. Screening for in vitro functional activity identified a subset of MAbs with neutralizing and/or CF activity that varied over a 2-log-unit concentration range; however, the majority exhibited activity only at higher concentrations (see Table S1 in the supplemental material). These observations are in contrast to a previous antibody repertoire cloned against rabies virus, where 44% of the repertoire was focused on only one VH3 gene (22) and 25% had potent or very potent neutralizing activities. These observations could stem from differences in natural infection versus immunization. However, it has been shown that in mice infected with vesicular stomatitis virus, a rhabdovirus closely related to rabies virus, high-affinity...
MAbs with potent neutralizing activity are rapidly induced (35). Thus, these observations may also reflect inherent differences in the way the immune system responds to these different virus families and indicate that the immunodominant epitopes on WNV are not associated with potent antiviral activity.

In total, 76 out of 117 phage MAbs (isolated from the first set of selections on the WNV immune libraries) bound E protein as measured by specific ELISA. E protein binding MAbs could be further segregated into four groups based on domain-mapping experiments (Table 4). Group A comprised MAbs that were unable to bind in an ELISA format to E protein when it was captured with either the domain II binding MAb 6B6C-1 or the domain III binding MAb 7H2. Four of these MAbs, CR4274, CR4283, CR4271, and CR4353, demonstrated neutralizing activity and were analyzed in more detail. They were among the best-binding MAbs in the neutralizing panel, based on binding activity measured by ELISA, but they bound only to viral antigen or VLP, not to monomeric secreted E protein. Cross-blocking experiments indicated that they bound the same or overlapping epitopes, and immunoprecipitation experiments demonstrated that this epitope is not present on the prM/E protein heterodimer. Taken together the data are consistent with binding to a conformational epitope on the E protein homodimer possibly formed by residues of domain I (5). In addition, MAbs in this group were frequently isolated during selections. In functional terms, MAbs from group A were weakly neutralizing but contained the best complement-fixing MAbs. CR4353 was an exception; it neutralized the virus in vitro over a surprisingly wide concentration range, but it failed to completely neutralize the virus even at high antibody concentrations. The in vitro neutralization activity of CR4353 contrasts with both CR4374 and the murine MAbs tested and may be due to unusual binding properties or a different mechanism of neutralization.

Group B consisted of 24 MAbs, almost half the MAbs tested, that competed for binding with the domain II binding antibody 6B6C-1. The epitope of 6B6C-1 has been mapped to the highly conserved fusion peptide of E protein, and the MAb is cross-reactive with all flaviviruses; neutralization activity is due to blocking of virus fusion with the cell membrane (32). Only three MAbs in this group were shown to have moderate in vitro neutralization activity, while the majority did not neutralize.

In contrast, only four MAbs competed for binding with the domain III murine neutralizing MAb 7H2. Two of the four MAbs in group D showed neutralizing activity in vitro, and CR4374 was a potent protective MAb in vivo; however, unlike in group A or B, MAbs in this group were not commonly isolated. Of note, CR4374 also had only a moderate affinity of 10^8 M. In addition, alternative selection strategies that block

### Table 4. Summary of anti-E protein MAb characteristics indicated

<table>
<thead>
<tr>
<th>Binding group</th>
<th>Domain</th>
<th>WNV binding ELISA/IP</th>
<th>CF 50% lysis &lt;10 μg/ml</th>
<th>Neutralization VNA_{66} &lt;50 μg/ml</th>
<th>Affinity 50% binding &lt;50 ng/ml</th>
<th>In vivo protection PD_{100} 15 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NA</td>
<td>15</td>
<td>6 (40)</td>
<td>4 (27)</td>
<td>4 (27)</td>
<td>1 (7)</td>
</tr>
<tr>
<td>B</td>
<td>II</td>
<td>24</td>
<td>2 (8)</td>
<td>3 (13)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>NA</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>III</td>
<td>4</td>
<td>1 (25)</td>
<td>2 (50)</td>
<td>1 (25)</td>
<td>1 (25)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>51</td>
<td>9</td>
<td>9</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

*IP, immunoprecipitation; VNA_{66}, antibody concentration yielding 66% neutralization; PD_{100}, antibody concentration giving 100% protection; NA, not applicable.
selection of domain II binders did not result in isolation of more MAbs binding to domain III.

Our finding of a relative paucity of domain III binders in the repertoire of WNV-infected patients is supported by recent studies involving primates and humans (5, 16, 25). In contrast, the immunization protocols used to generate murine hybridomas have yielded panels containing domain III binding MAbs with potent neutralization activities, although extensive screening was required for their isolation (30, 36). Taken together, this analysis of three WNV-infected individuals indicates that domain III binding MAbs form a minority of the specificities in the human immune repertoire after WNV infection and that an immunodominant epitope appears to be located around the fusion peptide in domain II of the E protein.

In addition to MAbs binding to E protein, we identified four MAbs against prM protein. M reactivity of these MAbs was not detectable by Western blotting. CF activity was observed for CR4294 on intact virus (Table 1), which may indicate binding to surface-displayed M protein; however, it could equally well be due to the presence of uncleaved prM in the virus preparation. None of the anti-prM binding MAbs had neutralizing activity, and therefore, they are unlikely to contribute substantially to the protective immune response.

To explore the roles of the binding target and binding affinity in MAb functional activity, we compared binding activity as measured by ELISA to both neutralizing and CF activities of MAb groups containing the same CDR3H. In the case of CR4368, CR4374, and CR4375 (CDR group 4), which bind to domain III of the E protein, there was a direct correlation between binding activity and both CF and neutralizing activities. However, for MAbs in CDR group 1, CDR group 2, and CDR group 3, which all bind an epitope on the homodimeric E protein (group A in Fig. 5a), the influence of affinity was not as important, suggesting that this region of the E protein is particularly permissive to complement deposition. Together, the data illustrate that the epitope of a MAb can have an important influence on the role affinity plays with respect to its functional activity.

In the present study, we provide the first description of fully human IgG1 MAbs that neutralize WNV. Consistent with historical data from flavivirus E protein-reactive antibody panels, we found a correlation between in vitro neutralization potency and in vivo protection (34); however, there was no association between potent CF activity and in vivo protection. CR4268, the most potent complement-fixing MAb in vitro, provided no significant protection in mice against lethal WNV challenge, nor did any of the MAbs that demonstrated 50% CF activity at concentrations of <3 μg/ml (CR4271, CR4274, and CR4361). In contrast, CR4374 was the most potent neutralizing antibody in terms of PRNT<sub>50</sub> and PRNT<sub>90</sub> and was 100% protective in a challenge model at a dose of 300 μg/kg. The MAbs described here are in the same range of in vitro and in vivo potencies as other recently reported MAbs (16, 30).

To summarize, of the 51 E protein binding MAbs analyzed in detail, we showed that 24 MAbs bind to domain II and 15 MAbs to an unidentified region formed only on the homodimeric form of E protein. In both groups, a small number of MAbs were shown to be weakly neutralizing (the exception being CR4353), while the region targeted by the latter group appeared to be permissive for complement fixation. The focus of the antibody repertoire on poorly neutralizing or nonneutralizing epitopes of the E protein may explain the comparatively low neutralizing activity found in human serum after WNV infection and could even have a role in exacerbating the disease process. Nonneutralizing MAbs have been associated with an enhancement of the infectious process through Fc receptor or complement-mediated uptake in macrophages (4, 15, 18), and such an activity has been recently demonstrated in vitro for MAbs binding to domain II of WNV (29). Taken together, our data from three acutely infected individuals suggest that WNV infection induces a diverse antibody repertoire containing only a small number of MAbs potently neutralizing WNV and that these MAbs target domain III of the WNV E protein. Thus, the potencies of WNV and other flavivirus vaccines may be enhanced by strategies that focus the immune response away from immunodominant epitopes and toward the critical neutralization epitope on domain III of the envelope protein.

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