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 preventative 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.

West Nile virus (WNV) is an arbovirus and 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.

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...
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 titered by 50% tissue culture infective dose (TCID_{50}) assay and plaque assay on Vero cells. The MAbs 7H2, 5H10, and 3A3 (Bioreliance Corp., Rockville, Md.) (1); 6B6C1 (Chemicon International, Temecula, Calif.) (33, 34); and 4G2 (38) have been previously described. Polyclonal antibody against WNV M protein was purchased from Imgenex, 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 concentrated by ultracentrifugation 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-propiolactone, commonly used for viral inactivation.

Recombinant E protein was produced by transient transfection of prME-encoding plasmids. The prE/EM/E construct from WNV strain USA99b was synthesized and cloned into the expression vector pDNA3.1 in frame with the HAVT20 signal peptide sequence. The constructs were transfected into HEK293T cells via lipid-mediated transfection and cultured in ultrCHO medium. The VLP were harvested 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 prME construct. The construct was made as described above using a prME gene product truncated by 95 amino acids at its carboxyl terminus and fused with a myc and six-histidine tag. Protein was expressed by transient transfection of HEK293T cells as described above, and the supernatant was harvested after 5 days. E protein was affinity purified over His-trap columns and further purified by gel filtration on G-200 columns. The final product was greater than 99% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

**Selections were carried out as described previously (6, 22), CT helper phage was used to rescue phage libraries (21). Briefly, MaxiSorp Nunc-Immuno Tubes (Nunc) were coated with purified WNV E protein, VLP, or inactivated viral antigen in PBS. The tubes were incubated with the blocked library for 2 h at room temperature and washed with PBS containing 0.1% Tween 20 and then with PBS, followed by elution of bound phage with 50 mM glycine-HCl, pH 2.2; neutralization with 1 M Tris-HCl, pH 7.5; and infection of XLI-Blue. After growth, colonies were scraped from the plates for rescue and a second round of selection. Two rounds of selection were carried out on the three different antigens to maximize the possible combinations. Individual scFv phage preparations were prepared from single colonies and screened for WNV-specific binding in a phage enzyme-linked immunosorbent assay (ELISA) (6) against VLP antigen, inactivated virus antigen, or E protein diluted in PBS, and used to coat Nunc-Immuno MaxiSorp plates (Nunc). Positive clones were sequenced to identify unique antibody sequences. An alternative selection procedure was also performed to increase the number of scFv phage binding to domain III of the E protein. Second-round selections were carried out as described above with first-round libraries from each of the WNV antigen formats on purified E protein captured by the antibody 6B6C1 as described below. Screening was carried out using the domain-mapping ELISA, as described below.

**Production of soluble scFv and IgG1 MAbs.** Soluble scFv were produced in nonsuppressing Excherichia coli bacteria (strain SF110F) essentially as described previously (37). Following periplasmic extraction, scFv were dialyzed against PBS and expression levels were analyzed by SDS-PAGE/Western blotting.

Recombinant of scFv phage into full-length IgG1 molecules has been described (3). Briefly, variable heavy (VH) and light (VL) genes were PCR amplified using primers to restore the human framework and append restriction sites. The resulting fragments 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 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 horseradish 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.

**High-throughput screening.** Twofold serial dilutions of MAbs were made in triplicate and mixed with 100 TCID_{50} 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 microtiter 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 (66% neutralization).

**Microtiter-based virus neutralization assay (VNA).** For high-throughput screening, twofold serial dilutions of MAbs were made in triplicate and mixed with 100 TCID_{50} 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 microtiter 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 (66% neutralization). The plaque reduction neutralization test (PRNT) was a complementation test used to determine the neutralizing potencies of selected MAbs. Briefly, Vero-E6 cells were trypsinized and counted; 2.5 × 10^5 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. Dilutions of both virus and MAbs were made 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 the plates had been incubated at 37°C for 1 h, a 1.5-m1 overlay of carboxymethylcellulose (CMC) medium with 10% (vol/vol) fetal bovine serum was added per well, and the plates were placed in a humidified CO2 incubator for 3 days at 37°C. To facilitate counting, the plaques were stained with a mixture (400 μl) of 0.5% crystal violet in 40% formaldehyde or 0.005% surfactant P20) as a running buffer. To determine the association rate, k_{a1} and k_{a2}, and the dissociation rate, k_{d1} and k_{d2}, a concentration series from 0.4 to 1,000 nM antibody were prepared using the ordinal-regression model probit run on SPSS software (version 13.0). Antibodies were compared directly in the regression model, with significance level alpha set at 0.05. Antibody concentrations yielding 50% and 90% neutralization (PRNT_{50} and PRNT_{90}, respectively), together with 95% confidence intervals (CI), were derived using the ordinal-regression model probit run on SPSS software (version 13.0). Antibodies were compared directly in the regression model, with significance level alpha set at 0.05. Antibody concentrations yielding 50% and 90% neutralization were estimated from the model, together with 95% CI.

**Sensitivity of scFv and IgG1 MAbs.** 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 procedure. Therefore, the antibody CTRA239 was first immobilized to a CMS 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 (K_{d}), 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 and AB + B ⇌ AB2, with on rates (k_{on}) and off rates (k_{off}) characterized by constants k_{a1} and k_{d1} and k_{a2} and k_{d2}, respectively. The
affinity constant $K_D$ was determined as $k_{on}/k_{off}$ (kd1/k1a1), 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 $H2E$, 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 MAb 4G2 and domain III MAbs 3A3 and 5H10.

**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 in ice in the presence of Triton X-100 for 1 h. The lysates were centrifuged at 20,000 $\times g$ and then biotinylated by addition of sulfo-N-hydroxysuccinimide-activated LS-CLC-biotin. Lysates were preincubated 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.

**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 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 conditions 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 ($64 \pm 34 \mu g/ml$ [mean $\pm$ standard error of the mean]). IgG1-containing supernatants were assayed for neutralizing activity against $100 \text{TCID}_{50}$ units of WNV strain USA99b. Twelve of the 119 MAbs tested reached the end point of 66% neutralization at a concentration of $<50 \mu g/ml$ (see Table S1 in the supplemental material). All monoclonal phages were demonstrated to specifically bind to WNV, but not to rabies vaccine 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 ($64 \pm 34 \mu g/ml$ [mean $\pm$ standard error of the mean]). IgG1-containing supernatants were assayed for neutralizing activity against $100 \text{TCID}_{50}$ units of WNV strain USA99b. Twelve of the 119 MAbs tested reached the end point of 66% neutralization at a concentration of $<50 \mu g/ml$ (see Table S1 in the supplemental material).
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, 7H2, dotted line; 6B6C-1, solid line. The level of neutralizing activity correlated well between the two assays. The PRNT titration data of CR4374 and CR4353, the two most potent neutralizing MAbs, are shown, together with the regression curves of two murine neutralizing MAbs, 7H2 and 6B6C-1, for comparison (Fig. 1c). Note the different pattern of neutralization for CR4353 compared to CR4374 and the mouse MAbs.

**Complement fixation of anti-WNV MAbs.** In addition to neutralizing activity, complement-mediated lysis of WNV has been proposed as a potential clearance mechanism for anti-WNV MAbs (24). Of the 119 IgG1s produced, 28 showed CF activity above background at concentrations of 0.19 (0.15 to 0.27) ng/ml and were further titrated (Fig. 1d; see Table S1 in the supplemental material). The most potent antibody, CR4268, showed 50% fixation (CF50) at a concentration of 0.19 (0.15 to 0.27) ng/ml. Titration of purified IgG1 from the panel of neutralizing MAbs identified seven MAbs with measurable activity, although none as potent as CR4268 (Table 1).

**Sequence analysis.** More detailed sequence analysis of the 138 WNV binding scFv established that 72 had unique VH complementarity determining region 3 (CDR3H) sequences—the region shown to have the most influence over antibody binding specificity (39). The other 66 sequences differed by point mutations in the framework or CDR regions of the heavy and light chains. Alignment with VH and VL germ line genes revealed a large degree of diversity in the germ line families selected (Table 2; see Table S1 in the supplemental material). In Fig. 2, the sequences of the 12 neutralizing MAbs are shown aligned with their closest VH (Fig. 2a) or VL (Fig. 2b) genes. Note that CR4274 and CR4361, as well as CR4368, CR4374, and CR4375, share the same CDR3H.

**Binding activity and affinity determination of functionally active anti-WNV MAbs.** The binding activities of the functionally active MAb panel were determined first by ELISA titration on WNV antigen (Fig. 3a). The MAbs have been ranked in the figure on the basis of the concentration required to reach 50% of saturated binding calculated by nonlinear regression. The values ranged from 29.3 ng/ml (24.7 to 35.7; 95% CI) for CR4283 to 856 ng/ml (572 to 1702; 95% CI) for CR4311. To examine the role of antibody binding strength on func-
tional activity, the 50% binding activities of MAbs were plotted against the CF<sub>50</sub> (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, CR4269, and CR4377; CDR group 2, CR274, CR4274, CR4361, and CR4377; CDR group 3, CR4283, CR4284, CR4285, CR4272, CR4273, CR4369, and CR4377; CDR group 2, CR274, CR4368, CR4374, and CR4375).

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 <i>k<sub>off</sub></i>, <i>k<sub>on</sub></i>, and absolute <i>KD</i> 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 confirmed by Western blotting (data not shown). The other 30 specificities could not be detected by this method (13).

### Table 1. In vitro functional activities of anti-WNV antibodies

<table>
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<tr>
<th>Antibody</th>
<th>PRNT&lt;sub&gt;50&lt;/sub&gt; (95% CI)</th>
<th>PRNT&lt;sub&gt;90&lt;/sub&gt; (95% CI)</th>
<th>CF&lt;sub&gt;50&lt;/sub&gt; (95% CI)</th>
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<tr>
<td>CR4268</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.19 (0.15–0.27)</td>
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<td>CR4271</td>
<td>&gt;100</td>
<td>NA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.12 (0.77–2.05)</td>
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<td>CR4274</td>
<td>&gt;100</td>
<td>NA</td>
<td>1.05 (0.79–1.55)</td>
</tr>
<tr>
<td>CR4283</td>
<td>&gt;100</td>
<td>NA</td>
<td>0.46 (0.37–0.58)</td>
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<tr>
<td>CR4294</td>
<td>ND</td>
<td>ND</td>
<td>3.77 (1.1–67.8)</td>
</tr>
<tr>
<td>CR4289</td>
<td>2.62 (1.16–6.10)</td>
<td>37.4 (13.7–241)</td>
<td>&gt;10</td>
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<td>CR4299</td>
<td>0.78 (0.28–1.82)</td>
<td>10.3 (3.92–67.7)</td>
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<td>CR4311</td>
<td>2.91 (2.26–3.74)</td>
<td>39.6 (27.3–62.3)</td>
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<td>CR4325</td>
<td>1.45 (0.66–3.05)</td>
<td>15.8 (6.58–75.6)</td>
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<td>CR4353</td>
<td>0.026 (0.012–0.045)</td>
<td>36.4 (19.1–82.6)</td>
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<td>CR4361</td>
<td>2.03 (0.90–4.34)</td>
<td>&gt;100</td>
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<td>CR4368</td>
<td>2.05 (1.07–3.76)</td>
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<td>&gt;10</td>
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<tr>
<td>CR4374</td>
<td>0.18 (0.17–0.20)</td>
<td>0.95 (0.82–1.12)</td>
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<td>CR4375</td>
<td>0.17 (0.12–0.23)</td>
<td>2.29 (1.59–3.67)</td>
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<td>7H2</td>
<td>ND</td>
<td>0.026 (0.020–0.037)</td>
<td>ND</td>
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<tr>
<td>6B6C-1</td>
<td>0.70 (0.37–1.55)</td>
<td>6.32 (2.42–106)</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> ND, not determined.

<sup>b</sup> NA, not applicable.

### Table 2. Variable-gene usage in anti-WNV specific antibodies

<table>
<thead>
<tr>
<th>Light chain</th>
<th>No. of genes identified for indicated heavy chain</th>
<th>VL Total</th>
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<tr>
<td>VH1</td>
<td>8</td>
<td>17</td>
</tr>
<tr>
<td>VH2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>VH3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>VH4</td>
<td>5</td>
<td>19</td>
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<td>VH5</td>
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<td></td>
</tr>
<tr>
<td>VH6</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> No antibodies were selected with light chains from the VKII, VL4, VL5, VL8, and VL9 families.
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 it was 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
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 incubated first with WNV antigen and not

\[ \text{TABLE 3. Affinity constants of three anti-WNV antibodies as determined by SPR} \]

<table>
<thead>
<tr>
<th>Antibody</th>
<th>( k_{\text{on}} ) ( \text{(ka1; M}^{-1}\text{s}\text{)} )</th>
<th>( k_{\text{off}} ) ( \text{(kd1; s}\text{)} )</th>
<th>( K_D ) ( \text{(nM)} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR4374</td>
<td>( 2.34 \times 10^4 \pm 0.04 \times 10^4 )</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 10^9 \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>
</tr>
</tbody>
</table>

FIG. 3. Binding activities of functional anti-WNV antibodies. (a) Purified IgG1s were titrated by ELISA on directly coated WNV antigen, and the antibody concentration required for 50% saturated binding was calculated by nonlinear regression. The antibody names are ranked from lowest to highest concentration required for 50% binding activity. OD, optical density. (b) To correlate CF activity with binding activity, the MAb concentration required for 50% CF activity was plotted against the concentration required for 50% binding activity. Antibodies were grouped based on the VH CDR3H sequence, and linear-regression lines were plotted to determine if the binding target specificity influenced the interaction between MAb affinity and CF activity. (c) Representative sensorgram of MAb CR4283 analyzed by SPR, showing raw data and curves fitted using a bivalent analyte model.

FIG. 4. Identification of anti-WNV IgG1 binding targets that are nonreactive in the E protein ELISA. (a and b) Purified IgG1s were incubated with membranes blotted with inactivated WNV separated by 4 to 12% SDS-PAGE under nonreducing conditions. The anti-E protein murine MAb 7H2 and rabbit polyclonal anti-M serum were included as positive controls. The migration of a molecular weight marker is indicated. (c) Immunoprecipitation of biotinylated VLP-transfected mammalian cell lysates with anti-WNV IgG1 expressed in serum-free medium was analyzed under nonreducing conditions by SDS-PAGE separation, membrane transfer, and probing with streptavidin-HRP. The migration of a molecular weight marker is indicated.
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 \((P = 0.001)\). 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-infected 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

![FIG. 6. Demonstration of the protective activity of anti-West Nile virus (WNV) IgG1 in a lethal WNV challenge model. BALB/c mice (n = 5) were injected i.p. with (a) 15 mg/kg or (b and c) the indicated dose of purified IgG1 in PBS and 24 h later infected i.p. with WNV strain USA99b. The animals were monitored twice daily and euthanized when clinical signs of infection appeared. Kaplan-Meier survival curves are shown for (a) a combined neutralizing MAb panel, (b) CR4374, and (c) CR4353.](http://jvi.asm.org/)

**TABLE 4. Summary of anti-E protein MAb characteristics indicated a**

<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>

a IP, immunoprecipitation; VNA_{66}, antibody concentration yielding 66% neutralization; PD_{100}, antibody concentration giving 100% protection; NA, not applicable.
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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REFERENCES