

## 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  $\mu\text{g}/\text{kg}$  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 (TCID<sub>50</sub>) assay and plaque assay on Vero cells. The MAbs 7H2, 5H10, and 3A3 (Bioreliance Corp., Rockville, Md.) (1); 6B6C-1 (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 the virus was purified over a 30% glycerol cushion at 25,000 × g for 2 h at 4°C. The virus was exposed to 45 kGy of gamma irradiation from a <sup>60</sup>Co 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 VLP were produced by transient transfection of prM/E-encoding constructs. The prM/E sequence of WNV strain USA99b was synthesized and cloned into the expression vector pcDNA3.1 in frame with the HA-VT20 signal peptide sequence. The constructs were transfected into HEK293T cells via lipid-mediated transfection and cultured in ultraCHO 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 prM/E construct. The construct was made as described above using a prM/E 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.

SDS-PAGE and immunoreactivity analyses using a panel of defined E protein-specific MAbs confirmed that the envelope protein was intact and antigenically conserved in all three preparations of antigen.

**Immune phage antibody libraries.** An immune library was constructed from peripheral blood donated by three WNV-infected individuals with laboratory-confirmed immunoglobulin M (IgM)-positive WNV infection. All three individuals were infected in Ontario, Canada, during the 2003 outbreak and hospitalized with neurological symptoms ranging from tremors to flaccid paralysis. All three individuals fully recovered from illness. Peripheral-blood samples were drawn 1, 2, and 3 months after recovery from acute illness, and total RNA, prepared from whole blood from each sample, was converted into cDNA using random hexamers. The library was constructed as previously described (22). PCR-amplified light chain (LC) genes were pooled and cloned into the phagemid vector PDV-C06 using SalI and NotI; PCR-amplified heavy-chain genes were then pooled and ligated into the LC library using SfiI and XhoI. The quality of the library was analyzed by sequence analysis of 71 randomly picked clones.

**Phage display selection of WNV-specific scFv.** 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 XL1-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 in the nine 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 6B6C-1, 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 *Escherichia 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.

Reformatting 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 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 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.

**Microtiter-based virus neutralization assay (VNA).** For high-throughput screening, twofold serial dilutions of MAbs were made in triplicate and mixed with 100 TCID<sub>50</sub> 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).

**PRNT.** The plaque reduction neutralization test (PRNT) was used to quantify the neutralizing potencies of selected MAbs. Briefly, Vero-E6 cells were trypsinized and counted; 2.5 × 10<sup>5</sup> cells were added to each well of a 12-well plate and incubated overnight at 37°C in a humidified CO<sub>2</sub> 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 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 the plates had been incubated 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 per well, and the plates were placed in a humidified CO<sub>2</sub> incubator for 3 days at 37°C. To facilitate counting, the plaques were stained with an overlay of CMC-PBS containing 8.25 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 CO<sub>2</sub> incubator, after which the number of visible plaques was determined.

Antibody concentrations yielding 50% and 90% neutralization (PRNT<sub>50</sub> and PRNT<sub>90</sub>, 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.

**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 procedure. Therefore, the antibody CR4283 was first 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 (K<sub>D</sub>), 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. BIAcore BIAevaluation version 3.2 (July 2001) software was used for data analysis. The bivalent analyte 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 ⇌ AB<sub>2</sub>, with on rates (k<sub>on</sub>) and off rates (k<sub>off</sub>) characterized by constants ka1 and kd1 and ka2 and kd2, respectively. The

affinity constant  $K_D$  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 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 on 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*-hydroxy-succinimide-LC-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 WNV 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<sub>50</sub> units using probit ordinal-regression analysis. For challenge experiments, 4-week-old mice ( $n = 5$ ) were inoculated i.p. with  $20 \times 50\%$  lethal dose of USA99b. Passive transfer of MAbs was carried out essentially as described previously (2). IgG1 (450  $\mu$ l) 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  $\sim 50\%$  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 DQ490721 to DQ490744.

## RESULTS

**Generation of human MAbs against WNV.** Antibody phage display libraries were constructed from peripheral blood leu-

kocytes 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  $4 \times 10^7$  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 ( $\sim 25\%$ ) (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 TCID<sub>50</sub> 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

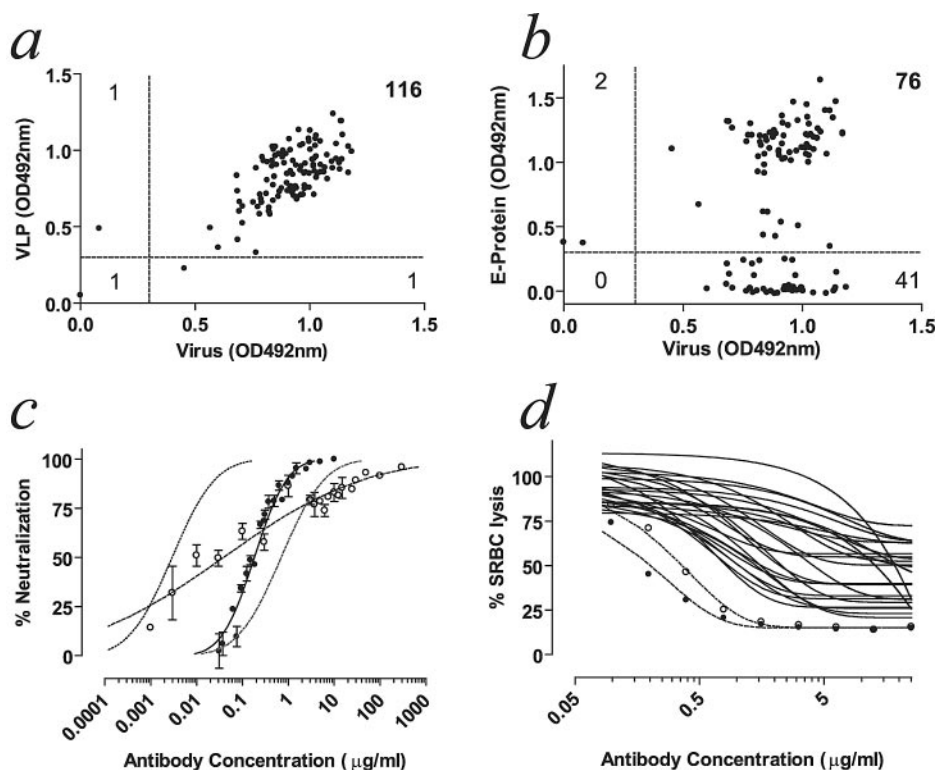


FIG. 1. Anti-WNV monoclonal antibody panel binding and functional characteristics. (a) ELISA immunoreactivities of selected monoclonal scFv phages for purified inactivated WNV along the *x* axis and purified VLP along the *y* axis and (b) purified inactivated WNV along the *x* axis and purified soluble E protein along the *y* axis. OD, optical density. (c) PRNT for the determination of *in vitro* neutralizing activities of monoclonal antibodies. Data from two independent experiments carried out in duplicate are plotted as percentage mean  $\pm$  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,  $\bullet$ ; CR4353,  $\circ$ ; 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 ( $\bullet$ ) and CR4381 ( $\circ$ ).

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. The 50% and 90% neutralization concentrations were calculated after titration of purified IgG1 MAbs using the ordinal-regression model probit. As expected all 12 MAbs exhibited neutralizing activity—CR4271 and CR4283, however, only at the highest concentrations (300 and 100  $\mu\text{g/ml}$ ), so that their PRNT<sub>50</sub> and PRNT<sub>90</sub> could not be calculated (Table 1). 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  $<10 \mu\text{g/ml}$  and were further titrated (Fig. 1d; see Table S1 in the supplemental material). The most potent antibody, CR4268, showed 50% fixation (CF<sub>50</sub>) at a concentration of 0.19 (0.15 to 0.27)  $\mu\text{g/ml}$ . Titration of purified IgG<sub>1</sub> 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  $\text{ng/ml}$  (24.7 to 35.7; 95% CI) for CR4283 to 856  $\text{ng/ml}$  (572 to 1702; 95% CI) for CR4311.

To examine the role of antibody binding strength on func-

TABLE 1. In vitro functional activities of anti-WNV antibodies

Antibody	Neutralization potency ( $\mu\text{g/ml}$ )		CF activity ( $\mu\text{g/ml}$ )
	PRNT <sub>50</sub> (95% CI)	PRNT <sub>90</sub> (95% CI)	CF <sub>50</sub> (95% CI)
CR4268	ND <sup>a</sup>	ND	0.19 (0.15–0.27)
CR4271	>100	NA <sup>b</sup>	1.12 (0.77–2.05)
CR4274	>100	NA	1.05 (0.79–1.55)
CR4283	>100	NA	0.46 (0.37–0.58)
CR4294	ND	ND	3.77 (1.1–67.8)
CR4289	2.62 (1.16–6.10)	37.4 (13.7–241)	>10
CR4299	0.78 (0.28–1.82)	10.3 (3.92–67.7)	>10
CR4311	2.91 (2.26–3.74)	39.6 (27.3–62.3)	>10
CR4325	1.45 (0.66–3.05)	15.8 (6.58–75.6)	>10
CR4353	0.026 (0.012–0.045)	36.4 (19.1–82.6)	1.20 (0.87–1.95)
CR4361	2.03 (0.90–4.34)	>100	1.54 (1.21–2.13)
CR4368	2.05 (1.07–3.76)	>100	>10
CR4374	0.18 (0.17–0.20)	0.95 (0.82–1.12)	4.18 (3.01–6.83)
CR4375	0.17 (0.12–0.23)	2.29 (1.59–3.67)	5.19 (2.69–78.3)
7H2	0.003 (0.002–0.004)	0.026 (0.020–0.037)	ND
6B6C-1	0.70 (0.37–1.55)	6.32 (2.42–106)	ND

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

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

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, CR4369, and CR4377; CDR group 2, CR274, CR4275, and CR4361; CDR group 3, CR4283, CR4284, CR4285, and CR4352; CDR group 4, 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  $k_{on}$ ,  $k_{off}$ , 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 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-

TABLE 2. Variable-gene usage in anti-WNV specific antibodies<sup>a</sup>

Light chain	No. of genes identified for indicated heavy chain						VL Total
	VH1	VH2	VH3	VH4	VH5	VH6	
VKI	8	0	2	2	5	0	17
VKIII	3	0	5	8	3	0	19
VKIV	2	0	1	1	5	0	8
VL1	16	3	12	5	0	0	36
VL2	5	0	8	0	3	0	16
VL3	8	0	21	4	4	0	37
VL6	0	0	1	0	0	0	1
VL7	0	0	2	0	0	1	3
VL10	1	0	0	0	0	0	1
VH Total	43	3	51	20	20	1	138

<sup>a</sup> No antibodies were selected with light chains from the VKII, VL4, VL5, VL8, and VL9 families.

VH:	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	VL
1-69	QVQLVQSGAEVKKPGSSVKVSKASGGTFS	S--YAIS	WVRQAPGQGLEWMG	GIIPFGTANYAQKFGQ	RVITITADESTSTAYMELSSLRSEDTAVYYCAR			
SC4325	VH	.....S.....	.....S.....	.....S.....	.....S.....	GGMATTPGLDLY	WGQGLTVTVSS	VKIV B3
2-05	QITLKESGPTLVKPTQTLTCTCFSGFSL	TSGVGVG	WIRQPPGKALEWLA	LIYWNDDKRYSPSLKS	RLTITKDTSKNQVLTMTNMDPVDATATYYCAHR			
SC4368	VH	.V.....A.....	.....V.....	.....L.R.....	.....K.....	HRYYDISGYRLFSDAFDI	WGQGLTVTVSS	VL1 1e
SC4374	VH	.....V.....	.....V.....	.....L.R.....	.....K.....	HRYYDISGYRLFSDAFDI	WGQGLTVTVSS	VL1 1e
SC4375	VH	.....V.....	.....V.....	.....L.R.....	.....K.....	HRYYDISGYRLFSDAFDI	WGQGLTVTVSS	VL1 1e
3-30	QVQLVESGGGVQPGSRSLRSCAASGFTFS	S--YGMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNKNTLYLQMNLSRAEDTAVYYCAK			
SC4299	VH	.....G..A.....	.....D.....	.....E..S.....	.....S.....	ESGGPINYYKYGVVDV	WGQGLTVTVSS	VL1 1a
SC4311	VH	.....V.....	.....K..DA.....	.....D..H.....	.....V.....RK.....R.D.....L.....R	GYNSGGHYFDY	WGQGLTVTVSS	VL1 1b
SC4353	VH	.....N..A.....	.....N..A.....	.....G.....	.....M.....	DFWSGYSMVDSEYIYMDV	WGQGLTVTVSS	VKIII A27
5-51	EVQLVQSGAEVKKPGESLKISCKSGYSYFT	S--YWIG	WVRQMPGKGLEWMG	IIPYDSDTSTRYSFPGQ	QVTISADKSISTAYLQWSSLSKASDAMYYCAR			
SC4283	VH	.....S.....T.N.T.....	.....I.....	.....S.....	.....S.....	LTFRRYSGSDSFLPPGDFDY	WGQGLTVTVSS	VKI L12
SC4271	VH	.....R.....T.N.N.....	.....V.....	.....H.....	.....N.....	RPYDYGPFYFDY	WGQGLTVTVSS	VL2 2a2
SC4289	VH	.....R.....	.....N..T.....	.....R.D.S..Y.N.....	.....H.....	DVVGWGSADYIYMDV	WGQGLTVTVSS	VKIII L2
SC4274	VH	.....L...G...L...T.....	.....T...N...S...Y.G.....	.....D.....	.....D.....	LRGPYDFWNGYRETHDAFNV	WGQGLTVTVSS	VKIV B3
SC4361	VH	.....A..F..S.T.....	.....A.....	.....A.....	.....A.....	LRGPYDFWNGYRETHDAFNV	WGQGLTVTVSS	VKIV B3
VL:	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	VH
VKI L12	DIQMTQSPSTLSASVGRVITTC	RASQSISS-----WLA	WYQQKPGKAPKLLIY	DASSLES	GVPSRFSGSGSGTEFTLTISSLQPDFATYYC	QQYNSYS		
SC4283	VL	.....A.....Q.....	.....D.....	.....S.....	.....S.....	.....T.P.....	LTFGTRVEIK	VH5 5-51
VKIII L2	EIVMTQSPATLSVSPGERATLSC	RASQSVSS-----NLA	WYQQKPGQAPRLLIY	GASTRAT	GIPARFSGSGSGTEFTLTISLQSEDFAVYYC	QQYNWNP-		
SC4289	VL	.....L...G...L...T.....	.....T...N...S...Y.G.....	.....D.....	.....D.....	.....P.....	ITFGQTRLEIK	VH5 5-51
VKIII A27	EIVLTQSPFTLSLSPGERATLSC	RASQSVSS-----SYLA	WYQQKPGQAPRLLIY	GASSRAT	GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC	QQYGSPP		
SC4353	VL	.....T.T.....	.....N.....	.....T.F.....	.....A.....	.....-	LTFPGTRKVDIK	VH3 3-30
VKIV B3	DIVMTQSPDLSAVSLGERATINC	KSSQSVLYSSNNKNYLA	WYQQKPGQPKLLIY	WASTRES	GVPSRFSGSGSGTDFTLTISLQAEDEVAVYYC	QQYYSPT		
SC4325	VL	.....S.....	.....Y.G.....	.....G.....	.....G.....	.....TL.SAW.....	FGGQTKLTVL	VH3 3-30
SC4274	VL	.....S.....	.....Y.G.....	.....G.....	.....G.....	.....TL.SAW.....	FGGQTKLTVL	VH3 3-30
SC4361	VL	.....V.....	.....NI..DN..D..F.....	.....A.....	.....L..C.....	.....N.....	ITFGQTRLEIK	VH5 5-51
VL1 1a	QSVLTQPPSVEAPRQRTVITC	SGSSSNIGNN-AVN	WYQQLPGKAPKLLIY	YDDLPS	GVSDRFSGSGSGTASLAISGLQSEDAEADYYC	AAWDDSLG--		
SC4299	VL	.....S.....Y.....	.....L..A.....	.....Y.G.....	.....G.....	.....TL.SAW.....	FGGQTKLTVL	VH3 3-30
VL1 1b	QSVLTQPPSVAAPGQKVTISC	SGSSSNIGNN-YVS	WYQQLPGTAPKLLIY	DNNKRP	GIPDRFSGSGSGTASLTIGLQTEDEADYYC	GTWDSLSA--		
SC4311	VL	.....S.....	.....T...D.....	.....H.....	.....V.....	.....T.VV.....	FGGQTKLTVL	VH3 3-30
VL1 1e	QSVLTQPPSVGAPGQRTVITC	TGSSSNIGAGYDVH	WYQQLPGTAPKLLIY	GNSNRPS	GVPSRFSGSGSGTASLAITGLQAEDEADYYC	QSYDSSLG----		
SC4374	VL	.....S.....	.....V.....	.....V.....	.....SM.....	.....G.....	FGGQTKLTVL	VH2 2-05
SC4375	VL	.....S.....	.....V.....	.....V.....	.....SM.....	.....G.....	FGGQTKLTVL	VH2 2-05
SC4368	VL	.....S.....	.....V.....	.....V.....	.....SM.....	.....G.....	FGGQTKLTVL	VH2 2-05
VL2 2a2	QSALTQPASVSGSPQSIITISC	TCTSSDVGGYNYVS	WYQQHPPGKAPKLLIY	EVSNRPS	GVSNRFSGSGSGNTASLTISGLQAEDEADYYC	SSYTSSTL-		
SC4271	VL	.....R.....	.....V.....	.....H.....	.....D.....	.....L.....	FGGQTKLTVL	VH5 5-51

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.

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 ~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

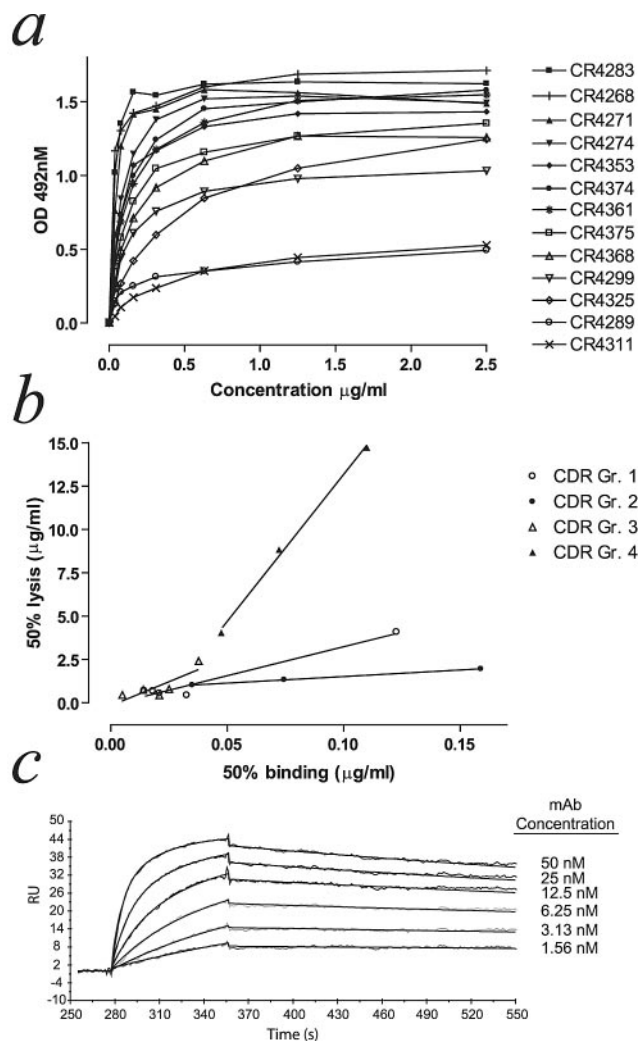


FIG. 3. Binding activities of functional anti-WNV antibodies. (a) Purified IgG1s were titrated by ELISA on directly coated WNV antigen, and the antibody concentration required for 50% saturated binding was calculated by nonlinear regression. The antibody names are ranked from lowest to highest concentration 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.

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

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

Antibody	$k_{\text{on}}$ ( $\text{ka1}; \text{M}^{-1} \text{s}^{-1}$ )	$k_{\text{off}}$ ( $\text{kd1}; \text{s}^{-1}$ )	$K_D$ (nM)
CR4374	$2.34 \times 10^4 \pm 0.04 \times 10^4$	$1.30 \times 10^{-3} \pm 0.05 \times 10^{-3}$	$56 \pm 4$
CR4353	$2.07 \times 10^5 \pm 0.05 \times 10^5$	$1.33 \times 10^{-3} \pm 0.04 \times 10^{-3}$	$6.5 \pm 0.4$
CR4283	$1.23 \times 10^6 \pm 0.43 \times 10^6$	$8.26 \times 10^{-4} \pm 3.74 \times 10^{-4}$	$0.8 \pm 0.6$

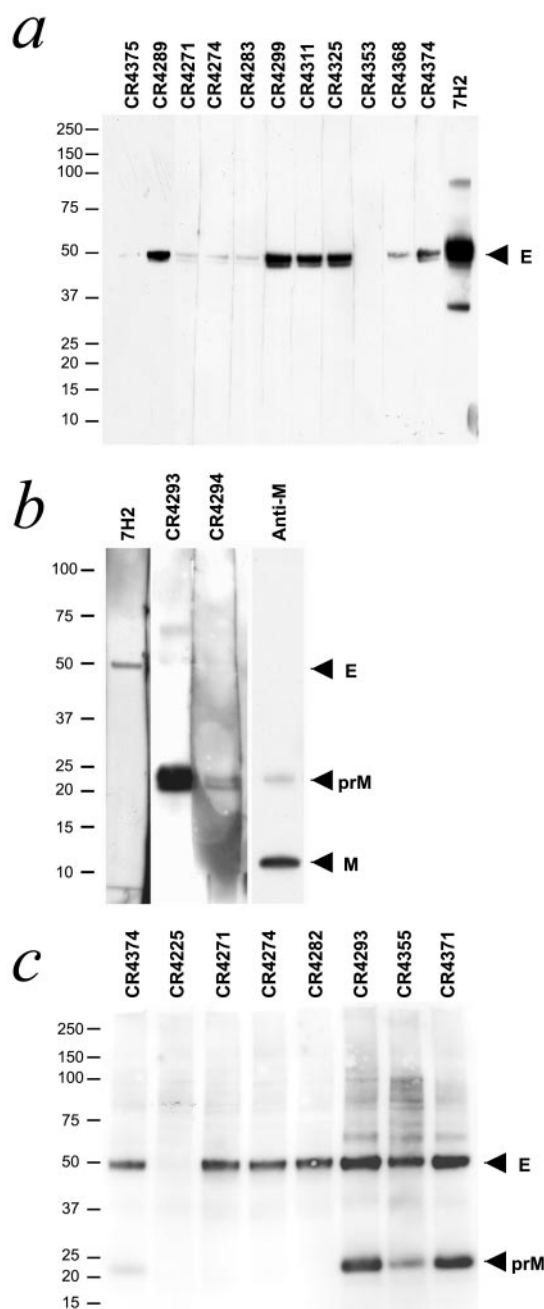


FIG. 4. Identification of anti-WNV IgG1 binding targets that are nonreactive in the E protein ELISA. (a and b) Purified IgG1s were incubated with membranes blotted with inactivated WNV separated by 4 to 12% SDS-PAGE under nonreducing conditions. The anti-E protein murine MAB 7H2 and rabbit polyclonal 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.

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

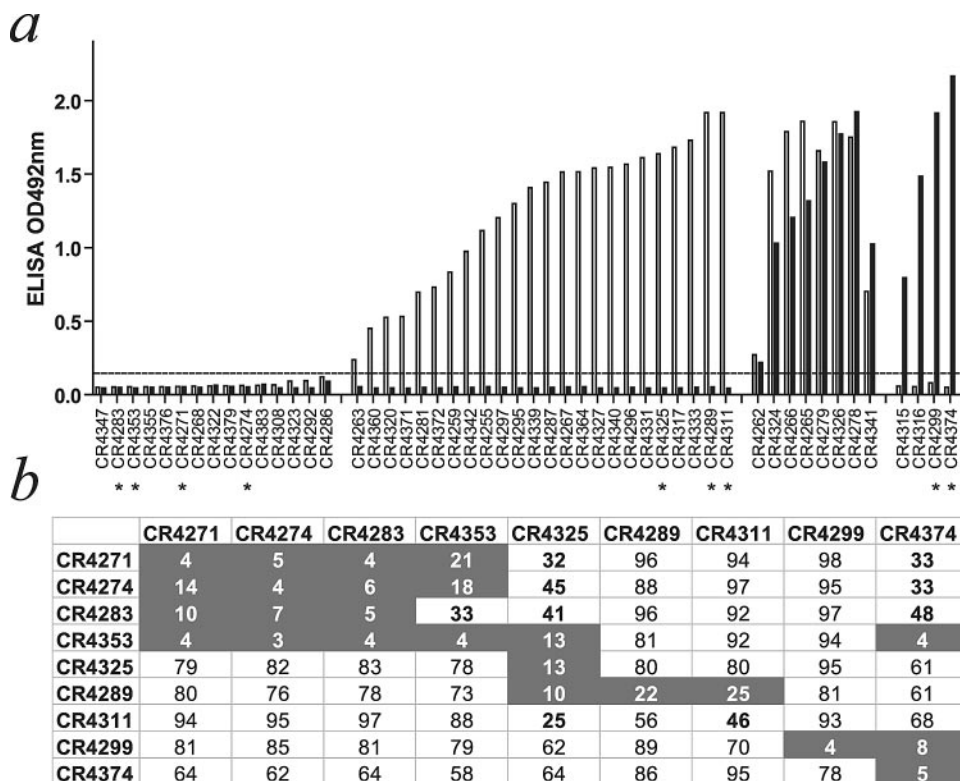


FIG. 5. West Nile virus (WNV) domain mapping by competition binding ELISA. (a) E protein was captured in microtiter plates either by immobilized 6B6C-1 MAb (filled bars) or 7H2 (open bars). The wells were then incubated with a panel of anti-WNV IgG1 at saturating concentrations. Binding was detected after anti-human IgG-HRP incubation and O-phenylenediamine (OPD) deposition. A representative experiment is shown (\* denotes neutralizing activity). (b) WNV antigen was immobilized overnight in microtiter plates and then incubated with saturating concentrations of IgG1 (columns), followed by biotinylated IgG1 (rows) at subsaturating concentrations for 5 min. Binding was detected after streptavidin-HRP incubation and OPD deposition. The tabulated data represent the mean percentage of binding compared to negative control IgG1 for three independent experiments. For clarity, percentage binding lower than or equal to 25 is shaded gray, and percentage binding of less than 50 is in boldface.

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  $\mu\text{g}/\text{kg}$  and 0.04 to 74.8 [mean and 95% CI]) was significantly different from that of CR4353 (357  $\mu\text{g}/\text{kg}$  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



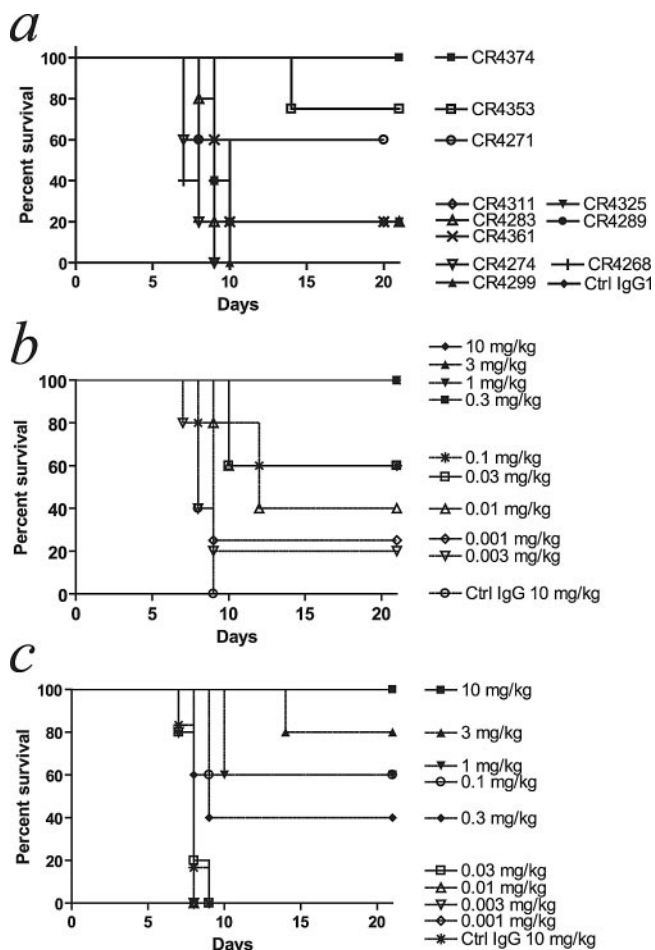


FIG. 6. Demonstration of the protective activity of anti-West Nile virus (WNV) IgG1 in a lethal WNV challenge model. BALB/c mice ( $n = 5$ ) were injected i.p. with (a) 15 mg/kg or (b and c) the indicated dose of purified IgG1 in PBS and 24 h later infected i.p. with WNV strain USA99b. The animals were 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.

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<sup>a</sup>

Binding group	Domain	No. (%) with characteristic indicated <sup>d</sup>				
		WNV binding ELISA/IP	CF 50% lysis <10 $\mu$ g/ml	Neutralization VNA <sub>66</sub> <50 $\mu$ g/ml	Affinity 50% binding <50 ng/ml	In vivo protection PD <sub>100</sub> 15 mg/kg
A	NA	15	6 (40)	4 (27)	4 (27)	1 (7)
B	II	24	2 (8)	3 (13)	0	0
C	NA	8	0	0	0	0
D	III	4	1 (25)	2 (50)	1 (25)	1 (25)
Total		51	9	9	5	2

<sup>a</sup> IP, immunoprecipitation; VNA<sub>66</sub>, antibody concentration yielding 66% neutralization; PD<sub>100</sub>, 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 to 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 potentially 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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