Induction of Epitope-Specific Neutralizing Antibodies against West Nile Virus[▽]

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Previous studies have established that an epitope on the lateral ridge of domain III (DIII-Ir) of West Nile virus (WNV) envelope (E) protein is recognized by strongly neutralizing type-specific antibodies. In contrast, an epitope against the fusion loop in domain II (DII-fl) is recognized by flavivirus cross-reactive antibodies with less neutralizing potential. Using gain- and loss-of-function E proteins and wild-type and variant WNV reporter virus particles, we evaluated the expression pattern and activity of antibodies against the DIII-Ir and DII-fl epitopes in mouse and human serum after WNV infection. In mice, immunoglobulin M (IgM) antibodies to the DIII-Ir epitope were detected at low levels at day 6 after infection. However, compared to IgG responses against other epitopes in DI and DII, which were readily detected at day 8, the development of IgG against DIII-Ir epitope was delayed and did not appear consistently until day 15. This late time point is notable since almost all death after WNV infection in mice occurs by day 12. Nonetheless, at later time points, DIII-Ir antibodies accumulated and comprised a significant fraction of the DIII-specific IgG response. In sera from infected humans, DIII-Ir antibodies were detected at low levels and did not correlate with clinical outcome. In contrast, antibodies to the DII-fl were detected in all human serum samples and encompassed a significant percentage of the anti-E protein response. Our experiments suggest that the highly neutralizing DIII-Ir IgG antibodies have little significant role in primary infection and that the antibody response of humans may be skewed toward the induction of cross-reactive, less-neutralizing antibodies.

West Nile virus (WNV) is a neurotropic, positive-sense RNA virus that has become endemic to North America (28). WNV is a member of the *Flaviviridae* family of viruses, along with other important human pathogens such as dengue virus (DENV), Japanese encephalitis virus, tick-borne encephalitis virus, and yellow fever virus. Although most cases of WNV infection are asymptomatic, it can cause severe encephalitis and death in immunocompromised or elderly individuals (39). At present, treatment is supportive, with no specific therapy or vaccine available for human use.

The WNV genome encodes three structural (capsid [C], premembrane/membrane [prM/M], and envelope [E]) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. The E proteins of flaviviruses, including WNV, have three domains and form head-to-tail homodimers on the surface of the mature virion (27, 38). Domain I (DI) is the central structural domain and consists of a 10-stranded β -barrel. Domain II (DII) is formed from two extended loops that project from DI. At the end of DII is a highly conserved loop, amino acid residues 98 to 110, that has been implicated

in the acid catalyzed type II fusion event (1, 9, 35). DIII, located on the opposite side of DI, adopts a seven-stranded immunoglobulin-like fold and has been implicated in cellular attachment (8, 12, 14). Short, flexible linker regions connect the domains and allow for the conformational changes associated with virus maturation and fusion (60).

Neutralizing antibodies are essential for the control of WNV infection in vivo (6, 16–18, 20, 44, 45). Specific amino acid residues have been defined that are critical for the binding of DII- and DIII-specific neutralizing monoclonal antibodies (MAbs) against WNV (4, 10, 44, 50). Using X-ray crystallography, the structure of a strongly neutralizing DIII-specific MAb, E16, was determined in complex with DIII (42). The binding epitope consisted of four discontinuous loops along the lateral ridge of DIII (DIII-Ir). Introduction of mutations at the core residues of the DIII-Ir epitope (residues S306, K307, T330, and T332) reduced or abrogated binding of not only E16 but also all other DIII-specific, strongly neutralizing MAbs (4, 44, 50). The fusion loop within DII elicits cross-reactive antibodies with relatively weak inhibitory activity; recent mapping studies have defined the core residues of this epitope as W101, G106, and L107 (13, 19, 45, 51). Whereas DIII-Ir MAbs neutralize potently in all cells tested, DII-fl MAbs inhibit to a lesser degree and not on all cell types. Accordingly, MAbs against the DII-fl were less effective than DIII-Ir MAbs at preventing or treating WNV infection in vivo (20, 45).

Study of the epitope specificity of the humoral response

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during the course of flavivirus infection has begun to explain the protective capacity of antibodies *in vivo*. In serum from convalescent horses, the levels of DIII-Ir antibodies were low, but in some cases correlated with neutralizing activity (49). However, a recent report that evaluated 138 human MABs derived from three convalescent WNV-infected patients showed that 92% of the E-specific MABs were non-neutralizing and reacted with regions outside of DIII (52). Consistent with this, immune serum from DENV-infected patients showed reduced binding to tick-borne encephalitis virus subviral particles that contained mutations in DII at position L107 in the fusion loop (51). These latter experiments suggest that the human immune response may be directed away from generating antibodies to the highly protective DIII-Ir epitope. In this report, using gain- and loss-of-function E protein variants, we evaluated the kinetics of development DIII-Ir and DII-fl antibodies in mice and compared this to serum from humans after WNV infection. Our experiments suggest that induction of the highly neutralizing DIII-Ir immunoglobulin G (IgG) antibodies is delayed in mice and variable in humans after WNV infection. Moreover, in humans, antibody responses appear to be skewed toward the induction of less-neutralizing DII-fl-specific antibodies.

MATERIALS AND METHODS

Cloning and protein expression. The WNV E ectodomain (residues 1 to 404) and DIII (residues 296 to 404) of the New York 1999 strain were amplified from an infectious cDNA clone (5) by high-fidelity Platinum *Taq* PCR (Invitrogen). The PCR product was cloned into the pET21a bacterial expression plasmid (EMD Biosciences, San Diego, CA) using flanking *Nde*I and *Xho*I restriction sites. The mutations K307E and T330I were introduced into the DIII construct iteratively, and the mutation W101R was introduced into the E ectodomain by using a QuikChange mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. All mutations were confirmed by bidirectional sequencing.

The generation of a WNV-DENV2 DIII chimera was accomplished by transplanting the WNV DIII-Ir epitope onto the DENV2 DIII backbone. Amino acid exchange was informed by X-ray crystal structures of WNV-E (41), DENV-2 E (34), and the E16-WNV DIII complex (42). To construct the WNV-DENV2 DIII chimeric protein, we swapped all four segments of WNV DIII that are directly contacted by the E16 Fab, as defined by interactions within a 4-Å distance (42). Specifically, we transposed residues that compose the N-terminal linker (WNV positions 303 to 308), BC loop (positions 330 to 334), DE loop (positions 363 to 370), and the FG loop (positions 388 to 391). Two of the sixteen directly contacted DIII residues are conserved between WNV and DENV2 (Tyr 302 and Gly 331). We also included 8 WNV residues (303, 304, 334, 363, 364, 369, 370, and 388) that flank the E16 epitope to increase the chances of mimicking the wild-type loop conformations and provide chemical similarity to the neighboring regions. To transplant the WNV DIII-Ir epitope, we used *in vitro* oligonucleotide assembly PCR (21). A series of overlapping primers (Table 1) that encoded for a WNV-DENV2 DIII chimera with flanking *Nde*I and *Xho*I restriction sites was designed by using the program DNAWorks (<http://helixweb.nih.gov/dnaworks>). The resultant PCR fragment was digested and cloned into the pET21a vector.

All constructs were expressed in *Escherichia coli* and purified by using an oxidative refolding protocol as described previously (44). Briefly, the cDNA plasmids were transformed into BL21 Codon Plus *E. coli* cells (Stratagene), grown in Luria broth, and protein expression was induced for 4 h with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 37°C. Inclusion bodies containing insoluble aggregates were denatured in the presence of 6 M guanidine hydrochloride and 20 mM β-mercaptoethanol and refolded in the presence of 400 mM L-arginine, 100 mM Tris-base (pH 8.0), 2 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 5 and 0.5 mM reduced and oxidized glutathione, respectively. Refolded protein was separated from aggregates on a Superdex 75 or 200, 16/60 size-exclusion column using fast-protein liquid chromatography (GE Healthcare, Piscataway, NJ).

Protein characterization. Recombinant proteins (DIII, WNV-DENV2 DIII chimera, E ectodomain, and E ectodomain W101R) were confirmed as approx-

TABLE 1. Primers used to generate the WNV-DENV2 DIII-Ir chimeric protein

Primer	Sequence (5' to 3')
1.....	TACATCATATGCAACTGAAAGGCATGCTTACGGC
2.....	TGAACGCCTTGCTGCACACGCCGTGAAGACATGCGCTTTCAG
3.....	TGTGCAGCAAGGCGTTCAAAGTTGTGAAAGAGATCGCGGA
4.....	GATGGTGCCATGCTGCGTTTCCGCGATCTCTTTCACAACT
5.....	CGCAGCATGGCACCATCGTTATTCGCGTGCAATACACCGG
6.....	TACACGGGCGGTCGGTACCGGTGATTGCACGCGAATAAC
7.....	TACCGACGGCCGTGTAAGATCCCGTTTGAGATCATGGAC
8.....	ACGTGGCGCTTCTCCAGGTCCATGATCTCAAACGGGATCT
9.....	CTGGAGAAGCGCCACGTCCTGGGCGGTTTAATCACGGTGA
10.....	GGCGACGCTAACGATCGGATTCACCGTGATTAACACGGCCC
11.....	CCGATCGTTAGCGTCGCCACGCCAATGCAAGGTTAATA
12.....	ACGGCGGTTTCGGCTCGATATTAACCTTGGCATTGGCGGT
13.....	GGCCGAACCGCGTTCGGCGACTCTTACATCATCATCGGT
14.....	AATTGCTGCTCGCCGCGATGATGATGTAAGATGTAAGTGT
15.....	GCGGCGAGCAGCAATTAAGCTGAAGTGGTTCAAGAAGGG
16.....	CTGGCCGATGCTGCTGCCCTTCTTGAACAGTTCAGCTTT
17.....	CAGCAGATCGGCCACGATGTCGAGACGACCATGCGTGGT
18.....	CTCGAGTTCAGGCCACCGCATGGTTCGTCTC

imately folded and disulfide bonded in the following manner: (i) elution as a monodispersed peak at the appropriate size on a gel filtration column (data not shown); (ii) immunoreactivity with all expected MABs based on parallel yeast surface display studies with DIII and E protein mutants and chimera (Fig. 1B and 4A; also data not shown); (iii) mass spectrometry at the W. M. Keck Facility (Yale University, New Haven, CT)—for the wild-type DIII and WNV-DENV2 DIII chimera average molecular weights of 11,723 and 13,258, respectively, were measured, a finding consistent with disulfide bond formation; (iv) circular dichroism spectroscopy; and (v) crystallographic analysis.

The circular dichroism spectroscopy was recorded on a Jasco J-720 spectropolarimeter after all proteins were buffer exchanged into a solution with 20 mM NaF and 10 mM sodium phosphate (pH 7.0). Measurements were taken in the far-UV region (180 to 260 nm) in a quartz cell with a path length of 0.01 cm. Protein secondary structure was calculated by using the CONTINLL or CDSSTR software (available at <http://lamar.colostate.edu/~sreeram/CDPro>). The loss-of-function (K307E/T330I) and gain-of-function (WNV-DV2 DIII chimera) proteins were within 2.9% of β-strand and 0.4% α-helix compositions of wild-type WNV DIII or DV2 DIII. Variation for all DIII proteins was less than 5% for β-strand and 4% for α-helix from the DSSP (for definition of secondary structure of proteins given a set of three-dimensional coordinates) values calculated from the X-ray crystal structures. Wild-type and W101R E proteins had the same calculated circular dichroism values for both α-helix and β-strand structure content. The variation for these was less than 5% for β-strand and 6.4% for α-helix from the DSSP values calculated from the X-ray crystal structure. The crystallographic analysis was performed for *E. coli*-derived wild-type WNV DIII and E proteins (42; data not shown).

SPR. Surface plasmon resonance (SPR) experiments were performed on a BiAcCore2000 (Biacore) at 25°C with 150 mM NaCl, 3 mM EDTA, 15 mM HEPES (pH 7.5), and 0.005% (vol/vol) Tween 20 as the flow buffer. Antibody was coupled to Biacore CM5 chips after sequential treatment with 0.2 M N-ethyl-N'-(dimethylaminopropyl)carbodi-imide (EDC) and 50 N-hydroxysuccinimide ester for 7 min, E16 or E9 anti-WNV MABs (44) dissolved in 20 mM sodium citrate (pH 4.5), and 1 M ethanolamine-HCl (pH 8.0). Baselines were stabilized after three injections of 0.1% sodium dodecyl sulfate in flow buffer for 1 min each. Flow rates of 80 μl/min with WNV DIII or WNV-DENV2 DIII chimera were used during analysis. Injection of 20 mM glycine (pH 3.0) was used to regenerate the chip after each injection. Each experiment was performed in triplicate and the k_{off} and k_{on} values were obtained by global curve fitting of the Langmuir model in the program Clamp (<http://www.cores.utah.edu/interaction/clamp.html>).

Mouse experiments. Mouse studies were approved and performed according to the guidelines of the Washington University School of Medicine Animal Safety Committee. Eight-week-old wild-type C57BL/6 mice were purchased commercially (Jackson Laboratories, Bar Harbor, ME) and inoculated with 10² PFU of WNV subcutaneously via the footpad after anesthetization with xylazine and ketamine. Sera were collected from groups of mice at different times after infection, heat inactivated at 56°C for 30 min, and stored as aliquots at -80°C.

Human serum collection and human MABs. Serum was obtained from convalescent patients who were participating in a longitudinal study of WNV infection 4 to 6 months after the onset of symptoms (ranging from fever to neuroin-

FIG. 1. Expression of loss- and gain-of-function of DIII variant proteins. (A) Sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis showing the recombinant E proteins after purification. (B) ELISA comparing binding of MAb to either the wild-type or K307E/T330I mutant protein. (C) Ribbon diagram of the WNV E DIII protein. The 23 residues that were transferred to the DENV2 DIII backbone are shown in blue, and the two residues mutated, K307 and T330, are shown in magenta.

vasive disease). The Research Ethics Board at McMaster University approved this human trial. The human MAb against WNV were obtained from three convalescent patients by using phage display screening and were previously described (52).

Serologic analysis. Endpoint titers for mouse and human serum were determined by using a WNV protein enzyme-linked immunosorbent assay (ELISA) as described previously (32), with the following modifications. To measure DIII-Ir-specific antibodies, two separate ELISAs with a loss-of-function (DIII-K307E/T330I) or gain-of-function (WNV-DENV2 DIII chimera) protein were performed.

For the ELISA with a loss-of-function DIII, wild-type DIII or DIII-K307E/T330I was diluted to 5 $\mu\text{g}/\text{ml}$ in 0.1 M sodium carbonate buffer (pH 9.3) and adsorbed on 96-well Maxi-Sorp microtiter plates (Nalge Nunc, Rochester, NY) overnight at 4°C. After blocking with phosphate-buffered saline (PBS), 2% bovine serum albumin (BSA), and 0.05% Tween 20 (PBS-BT) for 1 h at 37°C, fourfold serial dilutions of serum in PBS-BT were incubated for 1 h at room temperature. Plates were washed with PBS plus 0.05% Tween 20 and incubated with either biotin-conjugated goat anti-mouse IgG or IgM (1 $\mu\text{g}/\text{ml}$; Sigma-Aldrich) or biotin-conjugated goat anti-human IgG (0.3 $\mu\text{g}/\text{ml}$; Sigma-Aldrich) for 1 h at room temperature. After being washed, all plates were incubated with streptavidin-horseradish peroxidase (2 $\mu\text{g}/\text{ml}$; Zymed) for 1 h at room temperature and developed with tetramethylbenzidine substrate (Dako, Carpinteria, CA). After the addition of 1 N H_2SO_4 , the optical density at 450 nm was measured and adjusted for background by subtracting the optical density at 450 nm of blocked control wells. Best-fit lines were calculated, and endpoint titers were determined as three standard deviation units above the background signal by using GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA). The level of DII-Fl MAb was measured in an identical manner using the wild-type E protein and a W101R mutant E. In all cases, a MAb, E9, or a previously defined serum sample was used to control for site density of the proteins as well as plate-to-plate variation.

For the ELISA with a gain-of-function WNV-DENV2 DIII chimera, E111, a DIII-specific flavivirus cross-reactive mouse IgG2a (45) was diluted to 10 $\mu\text{g}/\text{ml}$ in sodium carbonate buffer and adsorbed overnight at 4°C. After blocking, DENV2 DIII, WNV-DENV2 DIII chimera, or BSA (10 $\mu\text{g}/\text{ml}$) was incubated for 1 h at room temperature. Subsequently, plates were incubated with fourfold serial dilutions of human serum, biotin-conjugated goat anti-human IgG, and streptavidin-horseradish peroxidase. Plates were developed, and titers were determined as described above.

WNV RVP and neutralization assays. Neutralization assays were performed according to a previously described protocol (46) using wild-type and mutant WNV reporter virus particles (RVP). Briefly, WNV RVP were produced by transfection of BHK21 cells that stably propagate a green fluorescent protein-expressing lineage II WNV replicon (46) with DNA plasmids encoding the wild-type or mutant (T332K) structural genes of WNV New York 1999 as described previously (46). RVP were harvested at 48 h after transfection, filtered through a 0.22- μm -pore-size filter, and frozen in aliquots at -80°C .

Neutralization assays were performed with the B-lymphoblastoid cell line Raji that stably expresses the C-type lectin and the WNV attachment ligand, DC-SIGNR (Raji-R) (47). To measure neutralization potential of the mouse and

human serum samples, WNV RVP stocks were diluted and incubated with nine threefold dilutions in duplicate of heat-inactivated serum for 60 min at room temperature (in a 200- μl volume). For the human MAb dose-response experiment, 19 twofold dilutions of antibody in triplicate were performed. Antibody-RVP complexes were then added to preplated cells (5×10^4 cells per well of 96-well plate in a 300- μl total volume). Infection was measured by flow cytometry at 48 h postinfection. At least 5×10^4 events were collected at each point. The 50% effective concentration (EC_{50}) of each serum preparation was predicted by nonlinear regression analysis using a variable slope (GraphPad Prism 4).

Yeast expression and staining. Yeast expressing wild-type DIII or variants were generated and stained as described previously (44). Briefly, human MAb were diluted to 50 $\mu\text{g}/\text{ml}$ in PBS plus 1 mg of BSA/ml (PBS-BSA) and then incubated on ice for 30 min. The cells were washed three times with PBS-BSA and incubated with a 1/500 dilution of goat anti-human IgG conjugated to Alexa Fluor 647 (Invitrogen) for 30 min on ice. Yeast cells were analyzed with a Becton Dickinson FACSCalibur flow cytometer.

RESULTS

Mouse antibody response against DIII-Ir epitope. Previous studies have defined epitopes on the DIII-Ir and the DII-Fl of WNV E protein that elicit type-specific and cross-reactive neutralizing MAb, respectively (4, 10, 13, 44, 45, 50, 59). Although MAb mapping to the DIII-Ir have potent inhibitory capacity in passive transfer prophylaxis and therapeutic models in vivo (37, 44), their function during the course of natural infection remains uncertain. Indeed, a recent study demonstrated that the antibody response to this epitope in single serum samples from acutely infected horses was variable, with some animals having little antibody that recognized the DIII-Ir epitope (49). To determine the kinetics of the antibody response against the DIII-Ir neutralizing epitope in mice, we developed an ELISA using loss-of-function and gain-of-function DIII variants.

Initially, a loss-of-function variant was generated with WNV DIII incorporating mutations at K307E and T330I (Fig. 1). These residues were identified as critical amino acid contacts for DIII-Ir neutralizing MAb using yeast surface display mapping (44), X-ray crystallography (42), sequencing of neutralization escape mutants (4, 10), and site-specific substitutions (50). Mutation of these two residues in tandem eliminated binding of 10 potentially neutralizing DIII-specific MAb without reducing the binding of 10 other non-neutralizing DIII-specific MAb (Fig. 1B). As an independent test, we engineered a gain-of-function WNV-DENV2 DIII chimera in which the E16

TABLE 2. Binding affinities of WNV MAbs to wild-type and WNV-DENV2 DIII-Ir proteins

MAb	Mean binding affinity ^a ± SD					
	WNV DIII			WNV-DENV2 DIII-Ir		
	<i>k_a</i> (M ⁻¹ s ⁻¹ × 10 ⁴)	<i>k_d</i> (s ⁻¹ × 10 ⁻³)	<i>K_D</i> (nM)	<i>k_a</i> (M ⁻¹ s ⁻¹ × 10 ⁴)	<i>k_d</i> (s ⁻¹ × 10 ⁻³)	<i>K_D</i> (nM)
E16	5.3 ± 2.8	0.5 ± 0.03	9.8 ± 3.2	11 ± 1.6	0.5 ± 0.002	4.4 ± 0.6
E9	4.5 ± 2.1	10.6 ± 0.04	240 ± 50	0	0	0

^a Affinities were measured by SPR, and the results are the average of three independent experiments.

MAb neutralizing epitope (corresponding to amino acid residues in the N-terminal linker, BC, DE, and FG loops of DIII as defined by X-ray crystallography) (42) was transplanted in its entirety onto the DENV2 DIII (Fig. 1C). Among the 23 amino acids that were swapped, 8 residues (303, 304, 334, 363, 364, 369, 370, and 388) that are not directly involved in E16 binding were also transferred to maintain conformational stability. The loss- and gain-of-function DIII proteins folded as expected and had wild-type disulfide binding and secondary structure elements as judged by circular dichroism, mass spectrometry, and size exclusion chromatography (see Materials and Methods). To establish the immunoreactivity of the chimeric protein, we compared binding of a WNV-specific neutralizing DIII-Ir MAb (E16) and WNV-specific poorly neutralizing DIII MAb (E9) that maps outside of the DIII-Ir epitope near residue H396 (44). An SPR assay demonstrated that E16 bound both wild-type and chimeric DIII with comparable affinity (4.4 nM versus 9.8 nM). As predicted, E9 bound the wild-type DIII (240 nM) but not the WNV-DENV2 DIII chimera (Table 2). Neither E16 nor E9 demonstrated appreciable binding to wild-type DENV2 DIII when assayed in parallel (data not shown).

Studies with mice lacking soluble IgM have established that the early WNV-specific IgM response is critical for limiting virus dissemination to the central nervous system (17). WNV-specific IgM is routinely detected beginning at day 4, whereas WNV-specific IgG does not appear until day 8 after infection

(16, 32). To examine the contribution of the DIII-Ir epitope to the neutralizing response, we analyzed by ELISA the WNV-specific antibody reactivity over time (days 6, 8, 10, 15, 20, 30, and 90 after mouse infection) with the wild-type, loss-of-function (DIII-K307E/T330I), and gain-of-function (WNV-DENV2 DIII chimera) proteins. In addition, at day 30 a group of mice was boosted with 10⁴ PFU WNV, and sera were analyzed 7 days later to assess an early memory response to the DIII-Ir epitope. To maintain proper conformation of the WNV-DENV2 chimera in the solid phase, it was necessary to capture the protein with a mouse IgG MAb, E111; thus, analysis of mouse antibodies with this variant was limited to WNV-specific IgM. In each case, antibody reactivity was compared to an internal control protein for the particular assay (loss-of-function assay, wild-type WNV DIII; gain-of-function assay, wild-type DENV2 DIII).

Consistent with previous findings (16, 32), IgM that recognized WNV E ectodomain peaked 8 days after infection and then declined rapidly (Fig. 2A). In contrast, the DIII-specific IgM response was relatively constant and significantly lower than the E titer throughout the time course and comprised 10 to 25% of the overall WNV-specific IgM response. Interestingly, low levels (endpoint titers of ~1/40) of IgM antibody bound to DIII in naive serum, likely reflecting the reactivity of natural IgM to WNV, analogous to results observed for vesicular stomatitis, lymphocytic choriomeningitis, and influenza viruses (3, 43). When the levels of IgM were compared be-

FIG. 2. Detection of IgM against DIII-Ir in mouse serum. (A) Levels of IgM antibody against purified WNV E and DIII as determined by ELISA. *P* values were determined by using an unpaired, two-tailed *t* test (*, *P* ≤ 0.05; **, *P* ≤ 0.01). (B) Levels of IgM against the wild-type and K307E/T330I mutant WNV DIII proteins. *P* values were determined by using an unpaired, two-tailed *t* test (NS, difference was not statistically significant; *, *P* ≤ 0.05; **, *P* ≤ 0.01). (C) Levels of IgM that react with WNV-DENV2 DIII chimera. The means and standard deviations of at least five mice per time point are shown. Dashed lines indicate the limit of detection for each assay. Due to limiting quantities of serum to perform assays in independent replicates, the limit of sensitivity in panel C is slightly lower (threefold) than in panels A and B.

FIG. 3. Detection of IgG against DIII-Ir in mouse serum. (A) Levels of IgG against purified WNV E and DIII as determined by ELISA. Mice that were challenged with 10^4 PFU of WNV on day 30 and harvested 7 days later are labeled as boost. (B) Levels of IgG against the wild-type and K307E/T330I mutant WNV DIII proteins. *P* values were determined by using an unpaired, two-tailed *t* test (NS, difference was not statistically significant; **, $P \leq 0.01$). The means and standard deviations of at least five mice per time point are shown. Dashed lines indicate limit of detection for each assay. (C) Endpoint titer concentrations of an MAb, E9, against the wild-type and K307E/T330I proteins. The means and standard deviations of 12 independent experiments are shown.

tween WNV DIII and DIII-K307E/T330I, a significant difference of ~65% ($P \leq 0.05$) existed between the two proteins at all time points except with the naive serum (Fig. 2B). As an independent confirmation, the IgM-specific ELISA was repeated with the WNV-DENV2 DIII chimera and DENV2 DIII. A consistent and comparable IgM titer was measured against the WNV-DENV2 DIII chimera but not DENV2 DIII (Fig. 2C). These experiments suggest that in mice WNV-specific IgM recognizes the DIII-Ir epitope at early time points after infection.

As observed previously in mice (16, 32), IgG that recognized WNV E ectodomain was initially detected at 8 days after infection and then increased rapidly over time. Somewhat surprisingly, DIII-specific IgG was not detected consistently until day 10, since only 20% (one of five) day 8 samples were positive (Fig. 3A). The E-specific IgG titer peaked at day 20 after infection, whereas the DIII-specific titer continued to rise through day 90. At days 15 and 20, the DIII-specific antibodies made up ~10% of the WNV E-specific IgG response, whereas by day 30 the fraction rose to ~25% of the total. When the levels of IgG that reacted with WNV DIII and DIII-K307E/T330I were compared, a statistically significant difference was observed beginning at day 20 ($P < 0.01$), although a noticeable trend was present at day 15 ($P = 0.06$) (Fig. 3B). At day 10, there was no significant difference in IgG reactivity with WNV DIII and DIII-K307E/T330I ($P = 0.6$), suggesting that the development of DIII-Ir IgG was delayed. This difference was not due to variable adsorption of DIII and DIII-K307E/T330I since the E9 MAb, which maps outside of the DIII-Ir epitope, recognized both proteins equivalently ($P = 0.7$) (Fig. 3C).

Antibody response against DII-fl epitope. The fusion loop on DII of flavivirus E proteins elicits flavivirus cross-reactive antibodies that have less neutralizing activity than antibodies that recognize the DIII-Ir epitope (13, 45, 51). This is likely because this epitope is buried or at least partially hidden on the mature virion (51). To determine the kinetics of the antibody response against the DII-fl epitope in mice, we again utilized a loss-of-function protein ELISA. Using a yeast surface display

assay, prior mutational analysis revealed a complete loss of binding 34 of 40 DII-fusion loop specific MAbs with the single mutation, W101R (45). A loss-of-function W101R WNV E protein was generated recombinantly (Fig. 1A). This loss-of-function variant folded correctly and had wild-type disulfide binding and secondary structure elements as judged by circular dichroism and size exclusion chromatography (see Materials and Methods). Initial analysis with wild-type and W101R E proteins confirmed the loss-of-binding phenotype of DII-fl MAbs (Fig. 4A). Subsequently, mouse serum was tested for reactivity with these proteins. Notably, there was no significant difference in the IgM titers at days 6 and 8 ($P > 0.6$) and only a slight, albeit significant, difference at day 10 ($P < 0.05$) (Fig. 4B). In contrast to what was observed with DIII-Ir antibodies, no significant differences in the IgG response were observed with wild-type and W101R E proteins, although a trend was observed at day 30 and after boosting ($P = 0.07$) (Fig. 4C). Again, these differences were not due to adsorption variability between wild-type and W101R E proteins ($P > 0.2$) (Fig. 4D).

Epitope-specific antibody responses in human patient serum. The kinetic experiments suggested that DII-fl antibodies comprised a much smaller fraction of the WNV-specific antibody response in C57BL/6 mice than the DIII-Ir antibodies. Moreover, in the early phases of infection, DIII-Ir antibodies were present in the IgM but not the IgG fraction. We next assessed the epitope specificity of WNV-antibody response from sera from convalescent human patients that had experienced distinct clinical phenotypes after WNV infection: subclinical (cases identified by blood donation), mild febrile illness (West Nile fever), meningitis, or encephalitis. Earlier studies with seven subclinical human samples that used a competitive ELISA with a Fab fragment of a DIII-Ir antibody suggested that at least some individuals developed DIII-Ir-specific antibodies (44). A total of 35 human serum samples, including 5 of the earlier samples, were tested by ELISA for reactivity with the wild-type DIII, K307E/T330I DIII, WNV-DENV2 DIII chimera, wild-type E ectodomain, and the W101R variant (Table 3). A total of 30 of 35 samples were taken between 4 and

FIG. 4. Detection of DII-fl antibodies in mouse serum. (A) ELISA comparing MAb binding to wild-type and W101R WNV E proteins. (B) Levels of serum IgM against the wild-type and W101R WNV E proteins. (C) Levels of serum IgG against the wild-type and W101R WNV E proteins. *P* values were determined by using an unpaired, two-tailed *t* test (NS, difference was not statistically significant; *, *P* ≤ 0.05). The means and standard deviations of at least five mice per time point are shown. Dashed lines indicate limit of detection for each assay. (D) Endpoint titer concentrations of an MAb, E9, against the wild-type WNV E and W101R proteins. The means and standard deviations of 12 independent experiments are shown.

7 months after infection, whereas 3 of 5 of the subclinical samples were obtained within 1 month of the presumed infection. As expected, given the convalescent status of the patients, the majority of samples contained low titers of IgM to E and DIII (data not shown), which precluded IgM epitope analysis.

In contrast to inbred mice, which had similar titers between subjects at each time point, there was substantial variability (42-fold difference among patient sera) in the E-specific antibody titers. DIII-specific antibodies comprised an average of 7.3% (range, 0.6 to 50.5%) of the total IgG antibody response in convalescent samples, in contrast to the ~25% observed 30 days after primary infection in the mouse. An even more profound difference was observed in the DIII-Ir antibody response. In human convalescent samples the DIII-Ir antibodies, on average, accounted for only 1.6% of the E-specific IgG response compared to ~18% of the response in mice at day 30 after infection. This pattern was observed with both the loss- and gain-of-function DIII proteins. As an independent confirmation of the low frequency of DIII-Ir antibodies in serum, we screened human MAbs that were generated previously from convalescent WNV patients (52). Only 4 of 51 human MAbs against WNV E protein were previously shown to bind DIII. Notably, only 1 of these, CR4374, showed loss-of-binding to a K307E mutant DIII expressed on the surface of yeast or to the K307E/T330I recombinant DIII (Fig. 5A and data not shown). Importantly, this MAb demonstrated the strongest neutralizing activity of any of the human MAbs. In comparison, CR4299, a

DIII-specific MAb that retained binding to the K307E mutant, had much weaker inhibitory activity (Fig. 5B). In contrast to what we observed with mouse serum and in support of the idea that the DII-fl epitope may be immunodominant in humans generally (20, 52), a marked reduction in serum antibody binding was observed with the W101R mutant (*P* < 0.001). Nonetheless, substantial variability was observed with human samples: the level of DII-fl antibody varied from 8.8 to 91.0% of the total E ectodomain-specific response with an overall median and mean of 65 and 61%, respectively (Table 3).

Antibodies that recognize the DIII-Ir and DII-fl epitopes protect mice and hamsters from lethal WNV infection to various degrees (20, 37, 44, 45). However, the in vitro correlates of in vivo protection or severe disease in humans are less clear. To evaluate whether the epitope specificity and titer of human serum antibodies correlated with outcome, we compared the titers of DIII-Ir and DII-fl antibodies in convalescent human serum with the neuroinvasive or non-neuroinvasive clinical phenotype (Fig. 6). In all cases, including the overall levels of E, DIII, DIII-Ir, and DII-fl specific antibodies, no correlation between the immunoreactivity of a given E protein and clinical phenotype was apparent (*P* > 0.4). However, this analysis was limited by the existence of only a single convalescent-phase serum sample. It remains possible that correlations could be made between epitope specificity and clinical outcome with prospective samples obtained at different stages of the acute

TABLE 3. Antibody titers in WNV-infected humans

Patient identification no.	Diagnosis ^a	Time of collection	Antibody endpoint titer		Difference between DIII and K307E/T330I antibody endpoint titers ^b	WNV-DENV2 DIII chimeric protein antibody endpoint titer	Difference between E and W101R antibody endpoint titers ^c	% of total E antibodies ^d		
			E	DIII				DIII	DIII-Ir	DIII-fl
77329	MF	5 mo	1,044	157	18	53	302	15.0	1.7	28.9
77326	MF	4 mo	19,103	1,040	271	80	10,553	5.4	1.4	55.2
77316	MF	5 mo	15,042	1,328	45	37	7,364	8.8	0.3	49.0
77310	MF	6 mo	8,921	402	140	133	4,751	4.5	1.6	53.3
77325	M	4 mo	10,792	273	61	<20	8,485	2.5	0.6	78.6
77323	MF	4 mo	2,345	200	37	<20	1,415	8.5	1.6	60.3
77324	MF	4 mo	6,933	339	256	139	4,455	4.9	3.7	64.3
55301	MF	7 mo	35,983	18,180	25	<20	6,936	50.5	0.1	19.3
55307	MF	6 mo	14,259	237	50	43	9,823	1.7	0.4	68.9
77304	E	6 mo	25,968	193	24	<20	22,422	0.7	0.1	86.3
77307	ME	6 mo	1,856	384	106	72	1,055	20.7	5.7	56.8
55308	ME	7 mo	12,879	1,561	942	1,280	4,268	12.1	7.3	33.1
77306	MF	6 mo	34,557	562	430	72	25,676	1.6	1.2	74.3
77332	ME	4 mo	1,008	44	4	<20	350	4.4	0.4	34.7
55315	MF	5 mo	6,064	167	42	108	3,691	2.8	0.7	60.9
77309	MF	6 mo	4,827	229	248	<20	3,265	4.7	5.1	67.6
77303	ME	6 mo	5,690	246	0	23	4,268	4.3	0.0	75.0
44302	ME	5 mo	6,502	1,019	255	320	2,910	15.7	3.9	44.8
55314	MF	5 mo	2,703	123	0	<20	1,799	4.6	0.0	66.6
77321	MF	5 mo	26,191	267	150	36	2,314	1.0	0.6	8.8
77308	ME	6 mo	3,830	296	5	54	2,255	7.7	0.1	58.9
77315	ME	6 mo	3,187	254	216	56	2,231	8.0	6.8	70.0
77322	MF	5 mo	4,689	219	101	80	3,455	4.7	2.2	73.7
77311	ME	6 mo	21,073	789	531	292	11,890	3.7	2.5	56.4
66301	ME	6 mo	22,534	839	0	33	7,663	3.7	0.0	34.0
77327	MF	4 mo	9,543	572	0	<20	6,926	6.0	0.0	72.6
99301	MF	5 mo	6,322	267	0	51	4,834	4.2	0.0	76.5
77330	MF	5 mo	28,295	169	32	<20	25,761	0.6	0.1	91.0
77320	E	5 mo	6,511	317	0	<20	4,901	4.9	0.0	75.3
77331	MF	5 mo	4,962	117	10	52	3,924	2.4	0.2	79.1
9321234	SC	27 days	16,360	155	16	<20	12,513	0.9	0.1	76.5
9321509	SC	32 days	23,214	185	21	<20	19,388	0.8	0.1	83.5
9321446	SC	34 days	30,559	1,004	427	<20	19,095	3.3	1.4	62.5
9321217	SC	14 days	6,745	249	68	<20	5,053	3.7	1.0	74.9
9321812	SC	152 days	9,598	571	19	<20	5,660	5.9	0.2	59.0

^a MF, mild febrile illness; M, meningitis; E, encephalitis; ME, meningoencephalitis; SC, subclinical illness.

^b The DIII K307E/T330I antibody endpoint titer was subtracted from the DIII antibody endpoint titer. All comparisons were made using data from the same ELISA plate.

^c The E W101R antibody endpoint titer was subtracted from the E endpoint antibody titer.

^d For DIII, the percentage was calculated by dividing the DIII titer by the E titer and multiplying that value by 100. For DIII-Ir, the percent DIII-Ir (of total anti-E response) was calculated by dividing the DIII-K307E/T330I titer by the DIII titer and multiplying that value by the percent DIII antibodies. For DIII-fl, the percentage was calculated by dividing the E-W101R titer by the E titer and multiplying that value by 100.

infection. Unfortunately, as of yet no such clinical trials have been performed with WNV-infected patients.

Contribution of DIII-Ir epitope specific responses to neutralizing activity of serum. Antibodies to the DIII-Ir epitope have potent neutralizing activity in vitro and in vivo (4, 44, 50) and appear to comprise a larger percentage of the antibody response in mice than in humans. To evaluate their contribution to the neutralization potential of serum, we utilized a previously established high-throughput, flow cytometric neutralization assay with WNV RVP (45–47). Because K307E/T330I RVP showed significantly decreased infectivity (data not shown), we generated a distinct mutant WNV RVP that contained a single mutation, T332K: these RVP eliminated binding and neutralization of virtually all DIII-Ir-specific MAbs and yet maintained virus infectivity (4, 50). We compared the neutralization curves and 50% inhibition points (i.e., EC₅₀ values) of serial dilutions of mouse and human serum on wild-type and T332K containing RVP. Mouse serum neutralization profiles

showed consistent differences between wild-type and mutant RVP (Fig. 7A). In contrast, the human serum showed little or no difference in neutralization of wild-type or T332K RVP (Fig. 7B and data not shown), even in serum samples that contained antibodies to the DIII-Ir epitope. The fold differences in the EC₅₀ values (wild type versus mutant) were compared, with significant increases observed at all time points in the mouse serum compared to the convalescent human serum ($P < 0.0001$) (Fig. 7C). This included day 6, when WNV-specific IgG is absent and WNV-specific IgM mediates neutralization (17). We also compared the wild-type RVP EC₅₀ values between human and mouse serum (Fig. 7D). Despite similar E-specific antibody titers as determined by ELISA between day 30 mouse serum and the human samples (Fig. 3A and Table 3), the mouse serum, at both days 6 and 30, had superior neutralizing activity compared to the convalescent human samples ($P < 0.0001$). Taken together, these data suggest that DIII-Ir antibodies contribute more significantly to the

FIG. 5. Human MAbs recognize DIII-ir epitope. (A) Flow cytometry histograms of human MAbs binding to yeast expressing wild-type and mutant DIII. Arrow indicates eliminated or greatly reduced MAb binding. The data are representative of three independent experiments. (B) Human MAb neutralization curves of WNV RVP on Raji DC-SIGNR cells. Representative curves from three independent experiments are shown.

neutralizing potential of serum in mice than in humans and that the human antibody response is directed toward less-neutralizing epitopes.

DISCUSSION

An intact humoral response is critical for the control of neuroinvasive WNV infection (16, 17, 32, 33). In this report, we evaluated the kinetics and magnitude of the antibody response against two distinct epitopes on the WNV E protein with discrete functional characteristics. One epitope, DIII-ir, is recognized by type-specific antibodies with potent neutralizing and therapeutic activity (4, 42, 44, 50). The other, DII-fl, is detected by flavivirus cross-reactive antibodies with less neutralizing and protective activity (13, 45). Given the differences in B-cell V-D-J repertoire and the variation in class II major histocompatibility complex alleles among different species, it was not unexpected to see a species-related difference in the antibody response against the two epitopes. However, several interesting observations can be made, which may have implications for targeted vaccine development. In mice, DIII-ir IgM antibodies were detected soon after infection and contributed to the early neutralizing potential of serum. For unclear reasons, there was a delay in isotype switching of

DIII-ir antibodies to IgG, since these were not reliably measured until day 15 after infection. Given that the vast majority of mortality in C57BL/6 mice after WNV infection occurs between days 8 and 12 (16, 55, 56), DIII-ir IgG antibodies likely do not contribute to protection against primary infection. Moreover, in mice, DII-fl antibodies comprised a smaller percentage of the total antibody response compared to DIII-ir antibodies. In humans, only a subset of individuals generated a significant IgG antibody response against the DIII-ir epitope. In almost all human cases, a large fraction of the E-specific antibodies was directed against the less protective DII-fl epitope.

DIII-ir IgM in mice was observed soon after WNV infection in mice. Sequence analysis of the V-D-J regions of ~10 different DIII-ir strongly neutralizing MAbs suggest that some mice (e.g., BALB/c and C57BL/6) have germ line gene configurations that can produce antibodies recognizing the DIII-ir epitope (S. Johnson and M. Diamond, unpublished observations). This could in part explain why neutralizing IgM were detected soon after infection. At present, it remains unclear which subset of B cells produce IgM that recognizes the DIII-ir epitope. CD5⁺ B-1 cells generate natural IgM, are unresponsive to B-cell receptor-mediated growth signals, and instead

FIG. 6. Comparison of non-neuroinvasive and neuroinvasive antibody levels for specific E variants. Titers of antibodies against E (A), DIII (B), DIII-Ir (C), and DII-II (D) were compared among patients with non-neuroinvasive and neuroinvasive WNV disease. Panels B, C, and D show the percentage of total E antibody titers. *P* values were determined by using an unpaired, two-tailed *t* test (NS, difference was not statistically significant).

undergo apoptosis upon B-cell receptor cross-linking, whereas conventional CD5⁺ B-2 cells expand in an antigen-dependent fashion after B-cell receptor cross-linking and costimulation (2). Of note, we did not detect DIII-Ir antibodies in naive serum, although we cannot rule out that their presence was below our limit of detection. More definitive subset depletion or epitope-based ELISPOT studies are needed to define the B-cell population that produces the earliest DIII-Ir antibody response in mice.

Despite an early IgM response to the DIII-Ir epitope there was a delay in the isotype switch to IgG of antibodies against this epitope in mice. The lag was considerable such that DIII-Ir IgG was not consistently measured until between days 10 and 15, a time after which the majority of mortality had occurred in mice after WNV infection. In contrast, there was no global delay in IgG responses, since significant WNV E protein antibody titers were measured in all infected mice by day 8. Although we cannot yet explain the epitope specific delay in isotype switching, it could be due to a requirement for germinal center formation in lymphoid tissues. Whereas some IgG antibodies can be produced in parafollicular zones, others require T-cell help, specific cytokines, or somatic hypermutation and are generated exclusively in germinal centers (7, 23). Experiments with signaling lymphocyte activation molecule-associated protein-deficient mice, which have impaired germinal center formation, production of class-switched IgG, and development of memory B-cell germinal center formation (15, 26, 58), are in progress to directly address this question.

Although both BALB/c and C57BL/6 mice generate mono-

clonal (44, 50) and polyclonal antibodies that recognized the DIII-Ir epitope, other species, including humans, showed significant variability. Accordingly, neutralization profiles with WNV RVP and mouse serum were affected more significantly by a mutation at T332K, which abolishes binding and neutralization of DIII-Ir antibodies (47). Our evaluation of convalescent human serum suggests that only a subset of infected individuals generate IgG against this DIII-Ir epitope, and this accounted for a relatively small fraction of the neutralizing antibody response. One possible criticism of our analysis is that the human serum samples were obtained from individuals infected with heterogeneous WNV isolates, which might not bind to recombinant proteins derived from the New York 1999 strain of WNV. However, an alignment of all 83 published WNV isolates in North America between 1999 and 2006 showed virtually no change in amino acid sequences in the fusion loop or DIII of the E protein (G. Ebel, unpublished data): only four amino acid changes were seen in any of the residues of DIII, and each change was observed as a single mutation in a single strain and occurred at residues Q296, V364, N394, and H395, which do not engage strongly neutralizing MAbs as defined by crystallography (42). Thus, to date, the type-specific epitope in DIII and cross-reactive epitope in DII are completely conserved in all North American WNV isolates.

The apparent lack of immunodominance of the highly protective DIII-Ir epitope in humans is also consistent with two recent studies that generated human MAbs: only 2% (1 of 51) and 0% (0 of 5) of unique human single chain antibodies

FIG. 7. Contribution of DIII-Ir antibodies to neutralizing activity in mouse and human serum. (A) Representative neutralization curves for a day 30 mouse serum sample against the wild-type and T332K RVP. (B) Representative neutralization curve for a convalescent human serum sample (patient 55308) against the wild-type and T332K RVP. (C) Fold differences in EC_{50} titers (wild type over T332K mutant) for mouse and human serum samples. Means and standard deviations are shown for human samples ($n = 15$) and mouse samples ($n = 5$ for each time point). P values were determined by using an unpaired, two-tailed t test (***, $P \leq 0.0001$). (D) EC_{50} titers against wild-type RVP for mouse and human serum samples. Means and standard deviations are shown. P values were determined as in panel C.

(scFv) generated from immune or nonimmune patient B cells by phage display reacted with the DIII-Ir epitope (20, 52). In our sample collection, some human sera contained DIII-Ir IgG, whereas others did not. Unfortunately, we could not establish a correlation between the development of an antibody response against the DIII-Ir epitope and clinical outcome. Although studies with a larger patient cohort are required for confirmation, our preliminary data suggest that IgG against this epitope does not contribute significantly to protection against primary WNV infection. This is similar to what has been observed in human immunodeficiency virus infections, where the level or type of antibodies does not predict disease progression (53, 61). Alternatively, serum sampling 4 to 6 months after infection may give an incomplete picture of the function of particular antibodies at a given stage of disease. Because the majority of human samples in our study were acquired at a single time point and not as part of a multiple-time-point prospective study, we cannot ascertain when in the course of the infections the DIII-Ir-specific IgG developed. Finally, our results with human serum are also consistent with results obtained with sera from WNV-infected horses: the equine IgG response to the DIII-Ir epitope was also variable and comprised only a small fraction of the antibodies directed against DIII (49).

The fusion loop is highly conserved among flaviviruses, and

antibodies against this epitope are in general highly cross-reactive (13, 19, 45, 51). Earlier studies established that DII-fl IgG MAbs behaved distinctly from DIII-Ir MAbs in infection assays in cells. In general, they neutralized infection less efficiently in cells lacking $Fc\gamma$ receptors and enhanced infection across a wide range of antibody concentrations in cells expressing $Fc\gamma$ receptors (42, 45). Accordingly, they showed decreased efficacy in preventing or treating WNV or DENV infection in vivo (24, 25, 45). The experiments in the present study are particularly intriguing since they suggest that DII-fl antibodies are produced by all humans against WNV and comprise a significant fraction of the anti-E protein response. Moreover, preliminary data with human MAbs isolated from B cells from secondarily DENV-infected patients indicate that the majority of E-specific antibodies also map to the DII-fl (C. Simmons, M. Beltramello, F. Sallusto, M. Diamond, and A. Lanzavecchia, unpublished results). Based on this, we speculate that the cross-reactive DII-fl epitope is immunodominant in humans.

The experiments with human and horse sera (49) suggest that some animals do not make significant responses against the highly protective DIII-Ir epitope. Based on passive transfer studies in rodents, it would be desirable to design vaccines that elicit high-titer DIII-Ir antibodies, which block at a postattachment stage (42). To date, WNV vaccine design has focused on

eliciting a strong neutralizing response against the E protein, but the epitope specificities of the generated antibodies are largely unknown (11, 22, 29–31, 36, 40, 48, 54). Complicating the issue, many of the initial immunization studies have been performed in mice. Because our studies show that mice generate distinct antibody responses against specific epitopes, some caution may be required in applying mouse vaccination results to humans. Although administration of live attenuated WNV vaccines to humans and nonhuman primates has induced relatively low-titer WNV-specific neutralizing antibodies that control viremia (36, 48, 57), the epitope specificity of the response remains unclear. Further study into the naturally occurring and vaccine-induced antibody repertoire should enhance our understanding of the immune correlates of protection from WNV disease and promote the development of novel vaccine strategies, which focus on eliciting highly protective DIII-Ir antibodies.

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REFERENCES

- Allison, S. L., J. Schlich, K. Stiasny, C. W. Mandl, and F. X. Heinz. 2001. Mutational evidence for an internal fusion peptide in flavivirus envelope protein E. *J. Virol.* **75**:4268–4275.
- Baumgarth, N., O. C. Herman, G. C. Jager, L. Brown, and L. A. Herzenberg. 1999. Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system. *Proc. Natl. Acad. Sci. USA* **96**:2250–2255.
- Baumgarth, N., O. C. Herman, G. C. Jager, L. E. Brown, L. A. Herzenberg, and J. Chen. 2000. B-1 and B-2 cell-derived immunoglobulin M antibodies are nonredundant components of the protective response to influenza virus infection. *J. Exp. Med.* **192**:271–280.
- Beasley, D. W., and A. D. Barrett. 2002. Identification of neutralizing epitopes within structural domain III of the West Nile virus envelope protein. *J. Virol.* **76**:13097–13100.
- Beasley, D. W., M. C. Whiteman, S. Zhang, C. Y. Huang, B. S. Schneider, D. R. Smith, G. D. Gromowski, S. Higgs, R. M. Kinney, and A. D. Barrett. 2005. Envelope protein glycosylation status influences mouse neuroinvasion phenotype of genetic lineage 1 West Nile virus strains. *J. Virol.* **79**:8339–8347.
- Ben-Nathan, D., S. Lustig, G. Tam, S. Robinson, S. Segal, and B. Rager-Zisman. 2003. Prophylactic and therapeutic efficacy of human intravenous immunoglobulin in treating West Nile virus infection in mice. *J. Infect. Dis.* **188**:5–12.
- Berek, C., A. Berger, and M. Apel. 1991. Maturation of the immune response in germinal centers. *Cell* **67**:1121–1129.
- Bhardwaj, S., M. Holbrook, R. E. Shope, A. D. Barrett, and S. J. Watowich. 2001. Biophysical characterization and vector-specific antagonist activity of domain III of the tick-borne flavivirus envelope protein. *J. Virol.* **75**:4002–4007.
- Bressanelli, S., K. Stiasny, S. L. Allison, E. A. Stura, S. Duquerroy, J. Lescar, F. X. Heinz, and F. A. Rey. 2004. Structure of a flavivirus envelope glycoprotein in its low-pH-induced membrane fusion conformation. *EMBO J.* **23**:728–738.
- Chambers, T. J., M. Halevy, A. Nestorowicz, C. M. Rice, and S. Lustig. 1998. West Nile virus envelope proteins: nucleotide sequence analysis of strains differing in mouse neuroinvasiveness. *J. Gen. Virol.* **79**(Pt. 10):2375–2380.
- Chu, J. H., C. C. Chiang, and M. L. Ng. 2007. Immunization of flavivirus West Nile recombinant envelope domain III protein induced specific immune response and protection against West Nile virus infection. *J. Immunol.* **178**:2699–2705.
- Chu, J. J., R. Rajamanonmani, J. Li, R. Bhuvanathan, J. Lescar, and M. L. Ng. 2005. Inhibition of West Nile virus entry by using a recombinant domain III from the envelope glycoprotein. *J. Gen. Virol.* **86**:405–412.
- Crill, W. D., and G. J. Chang. 2004. Localization and characterization of flavivirus envelope glycoprotein cross-reactive epitopes. *J. Virol.* **78**:13975–13986.
- Crill, W. D., and J. T. Roehrig. 2001. Monoclonal antibodies that bind to domain III of dengue virus E glycoprotein are the most efficient blockers of virus adsorption to Vero cells. *J. Virol.* **75**:7769–7773.
- Czar, M. J., E. N. Kersh, L. A. Mijares, G. Lanier, J. Lewis, G. Yap, A. Chen, A. Sher, C. S. Duckett, R. Ahmed, and P. L. Schwartzberg. 2001. Altered lymphocyte responses and cytokine production in mice deficient in the X-linked lymphoproliferative disease gene SH2D1A/DSHP/SAP. *Proc. Natl. Acad. Sci. USA* **98**:7449–7454.
- Diamond, M. S., B. Shrestha, A. Marri, D. Mahan, and M. Engle. 2003. B cells and antibody play critical roles in the immediate defense of disseminated infection by West Nile encephalitis virus. *J. Virol.* **77**:2578–2586.
- Diamond, M. S., E. M. Sitati, L. D. Friend, S. Higgs, B. Shrestha, and M. Engle. 2003. A critical role for induced IgM in the protection against West Nile virus infection. *J. Exp. Med.* **198**:1853–1862.
- Engle, M. J., and M. S. Diamond. 2003. Antibody prophylaxis and therapy against West Nile virus infection in wild-type and immunodeficient mice. *J. Virol.* **77**:12941–12949.
- Goncalves, A. P., R. H. Purcell, and C. J. Lai. 2004. Epitope determinants of a chimpanzee Fab antibody that efficiently cross-neutralizes dengue type 1 and type 2 viruses map to inside and in close proximity to fusion loop of the dengue type 2 virus envelope glycoprotein. *J. Virol.* **78**:12919–12928.
- Gould, L. H., J. Sui, H. Foellmer, T. Oliphant, T. Wang, M. Ledizet, A. Murakami, K. Noonan, C. Lambeth, K. Kar, J. F. Anderson, A. M. de Silva, M. S. Diamond, R. A. Koski, W. A. Marasco, and E. Fikrig. 2005. Protective and therapeutic capacity of human single-chain Fv-Fc fusion proteins against West Nile virus. *J. Virol.* **79**:14606–14613.
- Hoover, D. M., and J. Lubkowski. 2002. DNAWorks: an automated method for designing oligonucleotides for PCR-based gene synthesis. *Nucleic Acids Res.* **30**:e43.
- Iglesias, M. C., M. P. Frenkiel, K. Mollier, P. Souque, P. Despres, and P. Charneau. 2006. A single immunization with a minute dose of a lentiviral vector-based vaccine is highly effective at eliciting protective humoral immunity against West Nile virus. *J. Gene Med.* **8**:265–274.
- Jacob, J., G. Kelsoe, K. Rajewsky, and U. Weiss. 1991. Intracloonal generation of antibody mutants in germinal centres. *Nature* **354**:389–392.
- Johnson, A. J., and J. T. Roehrig. 1999. New mouse model for dengue virus vaccine testing. *J. Virol.* **73**:783–786.
- Kaufman, B. M., P. L. Summers, D. R. Dubois, and K. H. Eckels. 1987. Monoclonal antibodies against dengue 2 virus E-glycoprotein protect mice against lethal dengue infection. *Am. J. Trop. Med. Hyg.* **36**:427–434.
- Kim, I. J., C. E. Burkum, T. Cookenham, P. L. Schwartzberg, D. L. Woodland, and M. A. Blackman. 2007. Perturbation of B cell activation in SLAMF-associated protein-deficient mice is associated with changes in gammaherpesvirus latency reservoirs. *J. Immunol.* **178**:1692–1701.
- Kuhn, R. J., W. Zhang, M. G. Rossmann, S. V. Pletnev, J. Corver, E. Lencches, C. T. Jones, S. Mukhopadhyay, P. R. Chipman, E. G. Strauss, T. S. Baker, and J. H. Strauss. 2002. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. *Cell* **108**:717–725.
- Lanciotti, R. S., J. T. Roehrig, V. Deubel, J. Smith, M. Parker, K. Steele, B. Crise, K. E. Volpe, M. B. Crabtree, J. H. Scherret, R. A. Hall, J. S. MacKenzie, C. B. Cropp, B. Panigrahy, E. Ostlund, B. Schmitt, M. Malkinson, C. Banet, J. Weissman, N. Komar, H. M. Savage, W. Stone, T. McNamara, and D. J. Gubler. 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. *Science* **286**:2333–2337.
- Ledizet, M., K. Kar, H. G. Foellmer, T. Wang, S. L. Bushmich, J. F. Anderson, E. Fikrig, and R. A. Koski. 2005. A recombinant envelope protein vaccine against West Nile virus. *Vaccine* **23**:3915–3924.
- Lieberman, M. M., D. E. Clements, S. Ogata, G. Wang, G. Corpuz, T. Wong, T. Martyak, L. Gilson, B. A. Collier, J. Leung, D. M. Watts, R. B. Tesh, M. Siirin, A. Travassos da Rosa, T. Humphreys, and C. Weeks-Levy. 2006. Preparation and immunogenic properties of a recombinant West Nile subunit vaccine. *Vaccine*.
- Mason, P. W., A. V. Shustov, and I. Frolov. 2006. Production and characterization of vaccines based on flaviviruses defective in replication. *Virology* **351**:432–443.
- Mehlhop, E., and M. S. Diamond. 2006. Protective immune responses against West Nile virus are primed by distinct complement activation pathways. *J. Exp. Med.* **203**:1371–1381.
- Mehlhop, E., K. Whitby, T. Oliphant, A. Marri, M. Engle, and M. S. Diamond. 2005. Complement activation is required for induction of a protective antibody response against West Nile virus infection. *J. Virol.* **79**:7466–7477.
- Modis, Y., S. Ogata, D. Clements, and S. C. Harrison. 2003. A ligand-binding pocket in the dengue virus envelope glycoprotein. *Proc. Natl. Acad. Sci. USA* **100**:6986–6991.
- Modis, Y., S. Ogata, D. Clements, and S. C. Harrison. 2004. Structure of the dengue virus envelope protein after membrane fusion. *Nature* **427**:313–319.
- Monath, T. P., J. Liu, N. Kanasa-Thanas, G. A. Myers, R. Nichols, A. Deary,

- K. McCarthy, C. Johnson, T. Ermak, S. Shin, J. Arroyo, F. Guirakhoo, J. S. Kennedy, F. A. Ennis, S. Green, and P. Bedford. 2006. A live, attenuated recombinant West Nile virus vaccine. *Proc. Natl. Acad. Sci. USA* **103**:6694–6699.
37. Morrey, J. D., V. Siddharthan, A. L. Olsen, G. Y. Roper, H. Wang, T. J. Baldwin, S. Koenig, S. Johnson, J. L. Nordstrom, and M. S. Diamond. 2006. Humanized monoclonal antibody against West Nile virus envelope protein administered after neuronal infection protects against lethal encephalitis in hamsters. *J. Infect. Dis.* **194**:1300–1308.
38. Mukhopadhyay, S., B. S. Kim, P. R. Chipman, M. G. Rossmann, and R. J. Kuhn. 2003. Structure of West Nile virus. *Science* **302**:248.
39. Nash, D., F. Mostashari, A. Fine, J. Miller, D. O'Leary, K. Murray, A. Huang, A. Rosenberg, A. Greenberg, M. Sherman, S. Wong, and M. Layton. 2001. The outbreak of West Nile virus infection in the New York City area in 1999. *N. Engl. J. Med.* **344**:1807–1814.
40. Ng, T., D. Hathaway, N. Jennings, D. Champ, Y. W. Chiang, and H. J. Chu. 2003. Equine vaccine for West Nile virus. *Dev. Biol.* **114**:221–227.
41. Nybakken, G. E., C. A. Nelson, B. R. Chen, M. S. Diamond, and D. H. Fremont. 2006. Crystal structure of the West Nile virus envelope glycoprotein. *J. Virol.* **80**:11467–11474.
42. Nybakken, G. E., T. Oliphant, S. Johnson, S. Burke, M. S. Diamond, and D. H. Fremont. 2005. Structural basis of West Nile virus neutralization by a therapeutic antibody. *Nature* **437**:764–769.
43. Ochsenbein, A. F., T. Fehr, C. Lutz, M. Suter, F. Brombacher, H. Hengartner, and R. M. Zinkernagel. 1999. Control of early viral and bacterial distribution and disease by natural antibodies. *Science* **286**:2156–2159.
44. Oliphant, T., M. Engle, G. E. Nybakken, C. Doane, S. Johnson, L. Huang, S. Gorlatov, E. Mehlhop, A. Marri, K. M. Chung, G. D. Ebel, L. D. Kramer, D. H. Fremont, and M. S. Diamond. 2005. Development of a humanized monoclonal antibody with therapeutic potential against West Nile virus. *Nat. Med.* **11**:522–530.
45. Oliphant, T., G. E. Nybakken, M. Engle, Q. Xu, C. A. Nelson, S. Sukupolvi-Petty, A. Marri, B. E. Lachmi, U. Olshevsky, D. H. Fremont, T. C. Pierson, and M. S. Diamond. 2006. Antibody recognition and neutralization determinants on domains I and II of West Nile virus envelope protein. *J. Virol.* **80**:12149–12159.
46. Pierson, T. C., M. D. Sanchez, B. A. Puffer, A. A. Ahmed, B. J. Geiss, L. E. Valentine, L. A. Altamura, M. S. Diamond, and R. W. Doms. 2006. A rapid and quantitative assay for measuring antibody-mediated neutralization of West Nile virus infection. *Virology* **346**:53–65.
47. Pierson, T. C., Q. Xu, S. Nelson, T. Oliphant, G. E. Nybakken, D. H. Fremont, and M. S. Diamond. 2007. The stoichiometry of antibody-mediated neutralization and enhancement of West Nile virus infection. *Cell Host Microbe* **1**:135–145.
48. Pletnev, A. G., M. S. Claire, R. Elkins, J. Speicher, B. R. Murphy, and R. M. Chanock. 2003. Molecularly engineered live-attenuated chimeric West Nile/dengue virus vaccines protect rhesus monkeys from West Nile virus. *Virology* **314**:190–195.
49. Sanchez, M. D., T. C. Pierson, M. M. Degrace, L. M. Mattei, S. L. Hanna, F. Del Piero, and R. W. Doms. 2007. The neutralizing antibody response against West Nile virus in naturally infected horses. *Virology* **359**:336–348.
50. Sanchez, M. D., T. C. Pierson, D. McAllister, S. L. Hanna, B. A. Puffer, L. E. Valentine, M. M. Murtadha, J. A. Hoxie, and R. W. Doms. 2005. Characterization of neutralizing antibodies to West Nile virus. *Virology* **336**:70–82.
51. Stiasny, K., S. Kiermayr, H. Holzmann, and F. X. Heinz. 2006. Cryptic properties of a cluster of dominant flavivirus cross-reactive antigenic sites. *J. Virol.* **80**:9557–9568.
52. Throsby, M., C. Geuijen, J. Goudsmit, A. Q. Bakker, J. Korimbocus, R. A. Kramer, M. Clijsters-van der Horst, M. de Jong, M. Jongeneelen, S. Thijssen, R. Smit, T. J. Visser, N. Bijl, W. E. Marissen, M. Loeb, D. J. Kelvin, W. Preiser, J. ter Meulen, and J. de Kruijff. 2006. Isolation and characterization of human monoclonal antibodies from individuals infected with West Nile Virus. *J. Virol.* **80**:6982–6992.
53. Turbica, I., F. Simon, J. M. Besnier, B. LeJeune, P. Choutet, A. Goudeau, and F. Barin. 1997. Temporal development and prognostic value of antibody response to the major neutralizing epitopes of gp120 during HIV-1 infection. *J. Med. Virol.* **52**:309–315.
54. Wang, T., J. F. Anderson, L. A. Magnarelli, S. J. Wong, R. A. Koski, and E. Fikrig. 2001. Immunization of mice against West Nile virus with recombinant envelope protein. *J. Immunol.* **167**:5273–5277.
55. Wang, T., T. Town, L. Alexopoulou, J. F. Anderson, E. Fikrig, and R. A. Flavell. 2004. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat. Med.* **10**:1366–1373.
56. Wang, Y., M. Lobigs, E. Lee, and A. Mullbacher. 2003. CD8⁺ T cells mediate recovery and immunopathology in West Nile virus encephalitis. *J. Virol.* **77**:13323–13334.
57. Wolf, R. F., J. F. Papin, R. Hines-Boykin, M. Chavez-Suarez, G. L. White, M. Sakalian, and D. P. Dittmer. 2006. Baboon model for West Nile virus infection and vaccine evaluation. *Virology* **355**:44–51.
58. Wu, C., K. B. Nguyen, G. C. Pien, N. Wang, C. Gullo, D. Howie, M. R. Sosa, M. J. Edwards, P. Borrow, A. R. Satoskar, A. H. Sharpe, C. A. Biron, and C. Terhorst. 2001. SAP controls T-cell responses to virus and terminal differentiation of TH2 cells. *Nat. Immunol.* **2**:410–414.
59. Zhang, S., L. Li, S. E. Woodson, C. Y. Huang, R. M. Kinney, A. D. Barrett, and D. W. Beasley. 2006. A mutation in the envelope protein fusion loop attenuates mouse neuroinvasiveness of the NY99 strain of West Nile virus. *Virology* **353**:35–40.
60. Zhang, Y., W. Zhang, S. Ogata, D. Clements, J. H. Strauss, T. S. Baker, R. J. Kuhn, and M. G. Rossmann. 2004. Conformational changes of the flavivirus E glycoprotein. *Structure* **12**:1607–1618.
61. Zwart, G., L. van der Hoek, M. Valk, M. T. Cornelissen, E. Baan, J. Dekker, M. Koot, C. L. Kuiken, and J. Goudsmit. 1994. Antibody responses to HIV-1 envelope and gag epitopes in HIV-1 seroconverters with rapid versus slow disease progression. *Virology* **201**:285–293.