Complex Adenovirus-Mediated Expression of West Nile Virus C, PreM, E, and NS1 Proteins Induces both Humoral and Cellular Immune Responses

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West Nile Virus (WNV), a member of the family Flaviviridae, was first identified in Africa in 1937. In recent years, it has spread into Europe and North America. The clinical manifestations of WNV infection range from mild febrile symptoms to fatal encephalitis. Two genetic lineages (lineages I and II) are recognized; lineage II is associated with mild disease, while lineage I has been associated with severe disease, including encephalitis. WNV has now spread across North America, significantly affecting both public and veterinary health. In the efforts to develop an effective vaccine against all genetic variants of WNV, we have studied the feasibility of inducing both neutralizing and cellular immune responses by de novo synthesis of WNV antigens using a complex adenoviral vaccine (CAdVax) vector. By expressing multiple WNV proteins from a single vaccine vector, we were able to induce both humoral and cellular immune responses in vaccinated mice. Neutralization assays demonstrated that the antibodies were broadly neutralizing against both lineages of WNV, with a significant preference for the homologous lineage II virus. The results from this study show that multiple antigens synthesized de novo from a CAdVax vector are capable of inducing both humoral and cellular immune responses against WNV and that a multiantigen approach may provide broad protection against multiple genetic variants of WNV.

West Nile virus (WNV) is a mosquito-borne pathogen belonging to the genus Flavivirus, family Flaviviridae, and is a member of the Japanese encephalitis virus genetic group. This virus occurs in nature predominantly in a bird-mosquito transmission cycle, with humans, horses, and other domestic and wild animals being incidental hosts (36, 57). While the typical illness is a mild febrile illness, the most serious consequence of infection is fatal encephalitis in humans and horses and mortality in domestic and wild birds (33, 56). Since its initial isolation from a febrile woman in 1937 within the West Nile district of Uganda, the virus has become endemic in parts of Africa, the Middle East, Europe, Asia, and North America (15, 16, 23, 53, 60). The continuing spread of WNV highlights the inability of mosquito control to halt the spread of this virus and the need for effective vaccines for human and veterinary use.

Comprehensive studies on the phylogenetic relatedness of WNV strains stipulate the identification of at least two main lineages (9, 18, 46). WNV lineage I (WNV-I) strains are found worldwide and are associated with increased virulence and frequent encephalitis and death. WNV lineage II (WNV-II) strains were thought to be restricted to central and southern Africa and to cause relatively mild infections, with sporadic cases of encephalitis and death caused by WNV-I strains. However, in 2004 there was a highly lethal outbreak of WNV in goshawks in Hungary, and these strains showed the highest identity to WNV-II strains (5). The increased mortality from WNV infections and the spread of WNV to other European countries or continents raise serious concern that both lineages will evolve into more diverse and virulent subtypes and further validate the need for an effective, broadly protective vaccine (5).

WNV is an enveloped, single-stranded, positive-sense RNA virus with a genome of approximately 11 kb. The genome encodes a single polypeptide precursor in one open reading frame that is processed by cellular and viral proteases to yield the following structural proteins: the capsid (C) protein, the premembrane (preM) protein, and the envelope (E) glycoprotein (14). These proteins are followed by the nonstructural (NS) proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (17, 63). It is now well known that the majority of antibodies elicited during a flavivirus infection are directed to epitopes within the E and NS1 proteins (12, 50). However, most immunological studies have focused on the E protein because it induces neutralizing antibody responses (7, 8, 27, 28, 33). Recently, the significance of the role of the highly conserved NS proteins in inducing cell-mediated immune responses has been emphasized (19, 68). Therefore, the incorporation of both structural and NS proteins in a vaccine may trigger both arms of the immune response and provide broad protection against WNV.

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Currently, there is no approved commercial WNV vaccine or specific antiviral therapy for human use; consequently, the management of WNV infections is limited to supportive and palliative therapy. A number of approaches for the development of a WNV vaccine have been investigated, such as the use of an inactivated virus (35, 52, 61, 62), subunit particles (47, 84, 85), naked DNA (24, 35, 48, 70, 71, 91), cross-protection immunity induced by Japanese encephalitis virus vaccination (1, 77, 88), live-, attenuated virus (51, 78, 79, 89), recombinant virus (3, 25, 39, 50, 58, 74, 78, 79, 86), and virus replicons (2). Currently, only ChimeriVax-WNV, a vaccine based on a recombinant virus that expresses the preM and E proteins of WNV in an attenuated yellow fever virus backbone, has completed phase I clinical trials (58; Acambis, 2007), and others are in preclinical development (34, 70, 71). In general, safety has been the main concern of replication-competent vaccines, since attenuated viruses may revert to virulence and the recombinant viruses may adapt to the host cells and increase in pathogenicity, while a lack of efficacy has been the major limitation of protein subunit vaccines (34). In addition, most of these vaccine approaches target only the structural proteins of the virus, which may narrow the range of protection so that protection is offered against fewer variants or a particular lineage of WNV.

In this study, we report on the development and testing of a complex adenovirus (Ad)-based multiantigen WNV vaccine candidate that expresses the genes of the C, preM, E, and NS1 proteins of WNV. The complex Ad vaccine (CAdVax) is a replication-competent vector capable of expressing multiple antigen inserts at high levels de novo (37, 81–83). Therefore, it has the advantage of being both efficient and safe compared to the replication-competent viruses described above. We found that the vaccination of mice with the CAdVax-WNVII candidate led to the induction of WNV-specific humoral and cellular immune responses. Furthermore, we have shown that the antibody responses are broadly neutralizing against both lineages I and II and have higher levels of activity against the homologous lineage II WNV. Although other recombinant WNV vaccine approaches are currently being tested, our CAdVax-WNVII is the first nonreplicating, multiantigen vectored WNV vaccine candidate that is capable of inducing both neutralizing and cellular immune responses against both the E and the conserved C and NS1 antigens.

MATERIALS AND METHODS

Cell lines. The HEK293 (human embryonic kidney), Vero E6 (African green monkey kidney), BS-C-1 (African green monkey kidney), and M57G (mouse fibrosarcoma) cell lines were obtained from the American Type Culture Collection (Manassas, VA). HEK293, Vero E6, and BS-C-1 cell lines were maintained in Eagle (Eagle’s) minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Gibco). BS-C-1 cells were maintained in Eagle minimal essential medium (MEM) supplemented with 10% FCS, 2 mM L-glutamine (BioWhittaker), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Bio-whittaker). Mouse splenocytes were cultured in RPMI 1640 supplemented with 10% FCS, 10^{-5} M β-mercaptoethanol, 10 mM HEPES, 1% penicillin-streptomycin, and 0.1 mM nonessential amino acids.

Construction of CAdVax-WNVII. To create the Ad-based WNV vaccine vector, we subcloned the WNvNII antigen into our pLAd and pRAd shuttle vectors described previously (65–67). Once the pLAd and pRAd shuttle plasmids were completed, the CAdVax-based WNV vectors were constructed as described previously (65–67). The CAdVax-WNVII vector genome was based on a modified Ad5sub360 vector backbone, which contains deletions in the E1 and E3 regions that have also been deleted, except for orf6 (ITR, inverted terminal repeat; hCMVie, human cytomegalovirus intermediate-early promoter; BGH-poly(A), bovine growth hormone polyadenylation site; ψ, Ad packaging signal).

The WNV vector vaccine described herein is a complex adenovirus expressing the C, preM, E, and NS1 proteins of WNVII. A schematic representation of the structure of a single positive-stranded RNA genome of WNV. Open boxes, ORFs of WNV proteins that are synthesized as a polypeptide precursor, which is processed into each individual protein, as labeled (NC, noncoding region). (B) Genome structure of CAdVax-WNVII that expresses the C, preM, E, and NS1 proteins of WNVII. Large arrows, promoter regions; boxes with lines, poly(A) site, as labeled. The expression cassette containing the C and NS1 proteins is inserted at the left end of the vector within the E1 region that has been deleted from the Ad genome, and the cassette expressing the E and preM proteins is inserted in the right end of the vector into the E4 region that has also been deleted, except for orf6 (ITR, inverted terminal repeat); hCMVie, human cytomegalovirus intermediate-early promoter; BGH-poly(A), bovine growth hormone polyadenylation site; ψ, Ad packaging signal.

Complex Ad vector propagation, confirmation by sequencing analysis, and titration. CAdVax-based vectors were propagated in HEK293 cells by procedures described previously (65–67). After several rounds of single-plaque selection, candidate vaccine clones were analyzed by restriction map digestion and sequencing analysis of the vector DNA. The confirmed CAdVax vector clones were reamplified in HEK293 cells and purified by ultracentrifugation in cesium chloride gradients, as described previously (65–67). All vectors were titrated on HEK293 cells in 12-well plates by infecting triplicate wells with serial dilutions of the vectors. The resulting titers were scored as the numbers of PFU/ml. The final vector preparations were titrated and tested for the presence of replication-competent adenovirus by limiting dilutions on A459 cells (65–67). The DNA sequences of the final vaccine vector were confirmed again by restriction map digestion.

Western blotting. Vero cells were infected with the WNVII vaccine at a multiplicity of infection (MOI) of 30 for 48 h. The cell pellets were washed twice with phosphate-buffered saline (PBS) and lysed in lysis buffer (22 mM Tris-HCl, pH 7.4, with 5% Triton X-100, 2% Nonidet P-40, 20 mM NaCl, and 2 mM EDTA) on ice. The cell lysates were mixed with sample buffer (50 mM Tris, pH 6.8, 2% sodium dodecyl sulfate (SDS), 1% β-mercaptoethanol, 0.1% bromophenol blue, 10% glycerol), heated at 100°C for 10 min, and separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 4% to 15% gradient Tris-HCl gels (Bio-Rad Laboratories, Hercules, CA). The separated proteins were then transferred to an Immobilon-P polyvinylidene difluoride membrane (Millipore Corporation, Bedford, MA). The membrane was stained with Ponceau S for 5 min and washed with distilled water, and nonspecific antibody-binding sites were blocked with 5% nonfat dry milk in blocking buffer (0.05 M Tris, pH 7.5, 0.15 M NaCl, 0.1% NP-40, 0.3 mM Na2MoO4) for 1 h. The membrane was then incubated overnight at 4°C with the anti-WNV E-protein mouse monoclonal antibody from the C to the NS3 protease (90). The desired regions of the WNV genome were then amplified by high-fidelity PCR, with each primer including specific restriction sites at the 5′ and 3′ ends for subsequent cloning of the PCR fragments into the pLAd or the pRAd plasmid shuttle vector. The final products were verified by sequence analyses. The WNV preM gene sequence was modified to incorporate the ATG start codon.

Once the pLAd and pRAd shuttle plasmids were completed, the CAdVax-based WNV vectors were constructed as described previously (65–67). The CAdVax-WNVII vector genome was based on a modified Ad5sub360 vector backbone, which contains deletions in the E1, the E3, and almost all of the E4 open reading frames (ORFs), with the exception of orf6. The structure of the WNV vaccine vector used in this study is shown in Fig. 1. CAdVax-M11 (vector control) is a CAdVax-based Marburg virus vaccine. CAdVax-M11 includes the nucleoprotein of the Musoke strain of the Marburg virus. It was constructed by using the same pLAd and pRAd vectors described above.

The HEK293 vector genome that expresses the preM and E proteins of WNV. It was constructed by using the same pLAd and pRAd vectors described above.

Complex Ad vector propagation, confirmation by sequencing analysis, and titration. CAdVax-based vectors were propagated in HEK293 cells by procedures described previously (65–67). After several rounds of single-plaque selection, candidate vaccine clones were analyzed by restriction map digestion and sequencing analysis of the vector DNA. The confirmed CAdVax vector clones were reamplified in HEK293 cells and purified by ultracentrifugation in cesium chloride gradients, as described previously (65–67). All vectors were titrated on HEK293 cells in 12-well plates by infecting triplicate wells with serial dilutions of the vectors. The resulting titers were scored as the numbers of PFU/ml. The final vector preparations were titrated and tested for the presence of replication-competent adenovirus by limiting dilutions on A459 cells (65–67). The DNA sequences of the final vaccine vector were confirmed again by restriction map digestion.
Washes with PBS, the cells were probed for 1 h at room temperature with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G (IgG) antibody or an anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100 in PBS–1% bovine serum albumin. Following three washes with PBS, the cells were visualized with an Axiovert-25 microscope (Carl Zeiss, Germany) and an FITC excitation and emission filter set (Chroma Technology Corp., Rockingham, VT).

**Immunoﬂuorescence assay.** Vero cells were seeded in eight-well chamber slides. On the next day, the cells were infected at an MOI of 30 with CADVax-WNVII or CADVax-M11. At 3 days postinfection, the cells were washed once with PBS and then fixed in 3.7% formaldehyde for 5 min at room temperature. Then, the cells were blocked and permeabilized in a PBS solution with 2.5% nonfat dry milk, 0.05% Tween 20, and 0.5% Triton X-100. Where indicated, additional steps included blocking without permeabilization by using PBS and 2.5% nonfat dry milk only. The cells were probed with an anti-WNV E-protein MAb (7H2), an anti-WNV preM-protein polyonal antibody (Abcam, Cambridge, MA), an anti-WNV M-protein polyonal antibody (Abcam), or an anti-WNV NS1-protein MAb (clone 3.1112G; Chemicon). The antibodies were diluted 1:20 in PBS–1% bovine serum albumin and left overnight at 4°C. Following two washes with PBS, the cells were probed for 1 h at room temperature with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G (IgG) antibody or an anti-rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100 in PBS–1% bovine serum albumin. Following three washes with PBS, the cells were visualized with an Axiovert-25 microscope (Carl Zeiss, Germany) and an FITC excitation and emission filter set (Chroma Technology Corp., Rockingham, VT).

**Immunization of mice with CADVax-WNVII.** Outbred CD-1 mice or C57BL/6 mice (Jackson Laboratory, Charleston, SC) were immunized intraperitoneally (i.p.) on weeks 0 and 8 with 10⁷ PFU/ml. At 3 days postinfection, the cells were washed once in PBS, and then lysed for 45 min at 4°C in reticulocyte lysate buffer. A biotinylated anti-mouse capture antibody (BD Pharmingen) was added. The tetramethylbenzidine reaction was stopped with addition of 0.5 M sulfuric acid. The plates were read using an ELISA reader (BioTek Instruments, Inc., Winooski, VT). Antibody titers were determined by calculating the dilution of serum that corresponded to a signal of two times the background for that particular test. Mouse anti-WNV E-protein MAb (Bioreliance) were used as positive controls.

**Retrovirus vector design and target cell preparation.** We have generated retroviral vectors expressing either the preM and E proteins or the C and NS1 proteins of WNV by subcloning the left and the right expression cassettes described in Fig. 1 separately into plasmid pQCXIN, which is one of the Q series of retroviral expression vectors from BD Retro-X universal packaging system (BD Biosciences/Clontech). The plasmids were purified by established protocols. To generate the vectors, we followed the instructions provided by the manufacturer (BD Biosciences/Clontech) in the Retroviral Gene Transfer and Expression User Manual (6). The generated GP2-293 cells were maintained at 37°C with 5% CO₂ in Dulbecco modified Eagle medium (Gibco BRL) supplemented with 100 µg/ml penicillin G sodium, 100 µg/ml streptomycin, 1 mM l-glutamine, and 10% fetal bovine serum (HyClone). To generate retroviral vectors, the cells were seeded at 3 × 10⁵ cells per 100-mm dish. On the next day, when the cells reached confluence levels of 70 to 80%, they were cotransfected with the retroviral expression vector of choice and pSV-V-G (a plasmid expressing the vesicular stomatitis virus glycoprotein under the control of the cytomeglovirus promoter) in a 1:1 molar ratio. We used transfectin (Bio-Rad Laboratories) in a 2:1 to 1 ratio with total DNA, a set of conditions which we have found optimizes gene transfer and cell viability. Twenty-four hours after transfection, the culture medium of the transfected cells was replaced with EMEM supplemented with 10% CCS, 2 mM l-glutamine (Biowhittaker), 100 U/ml penicillin, and 100 µg/ml streptomycin (Biowhittaker). The cells were maintained for 3 days, with viral particle–containing medium collected every 24 h, aliquoted, and frozen at −80°C. MC57g cells were cultured in six-well plates in the EMEM supplemented with 10% CCS, 2 mM l-glutamine (Biowhittaker), 100 U/ml penicillin, and 100 µg/ml streptomycin (Biowhittaker). The cells were cultured at moderate confluence levels (30 to 45%) by replacing the culture medium with retrovirus-vector-containing medium collected from GP2-293 cells. The transduction efficiency was monitored by the ability of the cells to remain viable in excess of 3 weeks of culture.

**Mouse splenocyte isolation and murine IFN-γ ELISPOT assay.** Ten weeks after primary vaccinations, the C57BL/6 mice were euthanized and splenocytes were isolated by pressing through a 70-μm cell strainer under slight vacuum. The splenocytes were isolated after dissection with 70-μm cell strainers (Falcon; BD, Franklin Lakes, NJ). Red blood cells were removed with ammonium chloride-potassium lysis buffer (BioSource International, Camarillo, CA).

The splenocytes were then added at a final concentration of 2 × 10⁶ cells/well in duplicate wells of Multiscreen 96-well filter plates (Millipore, Billerica, MA) precoated with rat anti-mouse gamma interferon (IFN-γ) capture antibody (BD Pharmingen). Where indicated, retrovirus-infectected target cells (expressing the WNV preM and E antigens or the C and NS1 antigens) were added to the wells at a 1:5 ratio with the splenocytes. Concanavalin A (Sigma) was used as a positive control at a concentration of 5 µg/ml. The cells were incubated for 18 h at 37°C, followed by lysis with distilled water. The plates were then washed three times with PBS to remove the cellular debris. A biotinylated anti-mouse IFN-γ antibody (BD Biosciences) was added, and the plates were incubated for 1 h. Following washing, the plates were incubated with ExtrAvidin alkaline phosphatase (Sigma) for 1 h and then washed again. Finally, 5-bromo-4-chloro-3-indolyphosphate-nitroblue tetrazolium substrate solution (Pierce, Woburn, MA) was added, and the spots were quantified with an AID enzyme-linked immunospot (ELISPOT) assay reader (Autoimmun Diagnostika GmbH, Strassberg, Germany).

**WNV plaque reduction neutralization assay.** For each neutralization assay, 50 µl of serum from each mouse was added to 200 µl minimal essential medium (MEM) supplemented with 2% bovine growth serum (BGS; HyClone). The samples were diluted twofold by transferring 125 µl of the serum dilution to 125 µl MEM, serially resulting in working concentrations of serum ranging from 1:10 to 1:102,400. Full dilution series were run with CADVax-WNVII samples, while CAdVax-M11 and PBS control samples were tested at dilutions of 1:10 and 1:40. A positive mouse anti-WNV immune ascitic fluid control was tested at dilutions of 1:320, 1:1,280, and 1:5,120. Dilutions of the Uganda37 lineage II and the NY99 lineage I viruses were prepared so that 125 µl of virus sample contained approximately 60 PFU virus (125 µl). The virus sample was added to each dilution of serum and allowed to incubate at room temperature for 30 min. Each virus-serum combination was then assayed for plaque formation in Vero cells. For the plaque assay, Vero cells were allowed to grow to approximately 90% confluence in six-well plates. Growth medium (MEM with Earle's salts and L-glutamine) supplemented with 10% BCS, 100 U/ml penicillin (Gibco), and 100 µg/ml streptomycin (Gibco) was removed; and the cells were rinsed with PBS. Dilutions of each virus (200 µl per well) were adsorbed for 30 min before the cell monolayer was overlaid with a 50:50 mixture of 2% agar (Sigma) and 2% MEM (Gibco) containing 4% BGS. Two days following the first agar overlay, 2 ml of a
mixture of 2% agar and 4% BGS–2× MEM containing 2% neutral red was added to each well. The plaques were visualized, and the viral plaques were counted on the following day.

Statistical analysis. To analyze the data, an analysis of variance (ANOVA) model was run by using GraphPad Prism software. Once a significant interaction was found, a post-hoc analysis was performed by using a Bonferroni adjustment.

RESULTS

Construction of CAdVax-WNVII vector. Our hypothesis is that the naturally formed E glycoproteins (E and preM proteins) are essential in presenting neutralizing epitopes in the correct conformation to induce efficient neutralizing antibody production and that the highly conserved capsid and NS1 proteins are efficient in inducing broadly reactive cellular responses. The natural conformation of the glycoprotein complex can be formed only by synthesis of the proteins in cells (antigen synthesis de novo), just as in a natural viral infection. Recombinant viral proteins synthesized in bacteria are not properly glycosylated and often assume different tertiary structures. Even proteins synthesized in eukaryotic cells may be denatured during the purification process. We have decided to express the preM and E antigens and the C and NS1 antigens using vector-mediated transfer of these genes into cells. To achieve high levels of antigen synthesis from a single vector, we have developed the CAdVax vector that allows insertion of multiple transgene cassettes into a single vector (37, 65–67, 81–83). The vector has multiple deletions within the adenoviral E1, E3, and E4 genes and is replication defective, with undetectable levels of Ad protein expression (due to a lack of E1 proteins).

The preM and E antigens were inserted into the left arm of the vector by using the pLAd shuttle vector, while the C and NS1 genes were inserted into the right arm of the vector by using the pRad shuttle vector. This process of vector construction has been described previously (37, 65–67, 81–83). Both sets of antigens are flanked by a human cytomegalovirus intermediate-early promoter–BGH–poly(A) cassette to achieve high levels of expression. The resulting construct, CAdVax-WNVII, is shown in Fig. 1B. The integrity of the vector was confirmed by restriction digest mapping and by sequence analysis of the antigen inserts. CAdVax-WNVII was designed to induce cellular expression of the preM, E, C, and NS1 antigens of WNV in a single-step transduction of the cells.

Expression of WNVII preM, E, and NS1 proteins by CAdVax-WNVII. CAdVax-WNVII induces immune responses by transferring the WNV genes into cells, which results in the expression of the viral antigens in a way that mimics viral antigen expression during natural WNV infection. To confirm the expression of the correct antigen from CAdVax-WNVII, we analyzed vaccine-transduced cells by both Western blotting and immunochromatography using antibodies specific to WNV antigens. As a negative vector control, cells were transduced with CAdVax-M11, a similar vaccine vector that expresses Marburg virus proteins. In the Western blot analysis, we used MAb 7H2 to probe for the expression of E protein in Vero cells transduced with CAdVax-WNVII or CAdVax-M11. The blot membrane was separated into two strips, according to the predicted molecular weights of the target proteins, and was processed with specific antibodies. The top strip of the membrane was probed with MAb 7H2, which detected a band corresponding to the molecular mass of the E protein (~52 kDa) in CAdVax-WNVII-infected cells. In contrast, the same protein band was not detected in mock-infected or CAdVax-M11-transduced cells (Fig. 2). The bottom strip was probed with an antiactin antibody as an internal control for the amount of total protein loaded.

For immunochromatography, we transduced Vero cells with the vaccine at an MOI of 30. Two days after infection, the cells were fixed and stained with MAbs for the WNV preM, M, E, and NS1 proteins and were visualized with an FITC-conjugated secondary antibody. We found that the CAdVax-WNVII-transduced cells expressed both the preM and the E proteins efficiently with what appeared to be a more intense expression pattern on the cell surface (Fig. 3). A similar staining pattern
was observed in cells stained with an antibody specific to the M protein, the mature product cleaved from preM (Fig. 3). Control cells transduced with CAdVax-M11 showed no fluorescence, indicating that the antibody stains were specific to the membrane glycoprotein (preM- and E-protein) antigens. In contrast to the glycoprotein staining patterns, NS1 immunostaining appeared to be relatively uniform throughout the cells in CAdVax-WNVII infections. Similarly, the NS1-protein antibody did not stain the control vaccine-infected cells, showing the specificity of the immunochemical staining. These results show that CAdVax-WNVII is capable of mediating high levels of preM-, E-, and NS1-protein expression upon a single transduction of Vero cells. Unfortunately, C-protein expression was not analyzed due to a lack of antibodies specific for this protein.

Vaccination with CAdVax-WNVII induces WNVII-specific humoral responses. We believe that vector-mediated expression of the WNVII proteins will mimic that in WNVII-infected cells and therefore induce an immune response against the naturally formed viral membrane proteins that are identical to those produced in a natural WNV infection. In order to evaluate the immune response induced by our vaccine vector, CD-1 mice were injected i.p. with $1 \times 10^8$ PFU of CAdVax-WNVII or CAdVax-M11. An additional group of mice was injected with PBS as a vehicle control. Eight weeks after the primary immunizations, the mice were boosted i.p. with $1 \times 10^9$ PFU of the same vaccines. Serum samples were collected biweekly, and WNVII-reactive antibodies were detected by using indirect ELISAs with C-, preM-, E-, and NS1-protein-containing (CAdVax-WNVII-transduced) cell lysates as the immune targets.

All animals vaccinated with CAdVax-WNVII produced significant humoral antibody responses to WNV ($P < 0.05$), and these were detectable as early as 4 weeks after the primary immunization (Fig. 4). These titers remained elevated for 8 weeks, until the second booster vaccination was given to all animals at week 8. After the booster immunizations, the WNV-specific antibody titers increased sharply, demonstrating that repeated administration is feasible in a mouse model of vaccination. Conversely, the CAdVax-M11- and the PBS-vaccinated groups showed relatively no antibodies reactive to the WNV protein lysates.

Humoral immune responses induced by CAdVax-WNVII neutralize both WNVI and WNVII. The induction of neutralizing antibodies is considered more important than the induction of broadly reactive nonneutralizing antibody responses in offering protection against WNV infection. Cross-reactive but nonneutralizing immune responses against the E glycoprotein may not provide protection and have even been shown to enhance viral entry and disease symptoms for flaviviruses (75). To analyze the neutralizing antibody responses in CAdVax-WNVII-vaccinated mice, sera were collected from all mice 2 weeks after the second immunization and were used to perform plaque reduction neutralization assays against two different strains of WNV. All experimental samples and controls were tested for neutralization against both lineage I (NY99 strain) and lineage II (Uganda37) viruses. As shown in Table 1 and Fig. 5, the CAdVax-WNVII-vaccinated serum samples significantly neutralized both viruses, with 50% plaque reduction titers (PRNT<sub>50</sub>s) of 2,816 against the lineage I virus and 4,480 against the lineage II virus ($P < 0.05$). The results for sera from mice vaccinated with CAdVax-M11 were comparable to the results for sera from the saline-treated group. The results for the full series of serum dilutions are shown in Fig. 5, demonstrating the significant neutralization of both strains of WNV compared with that of the control serum samples. In addition to the broad neutralization induced by CAdVax-WNVII, these results show an approximately twofold difference in neutralizing activities in favor of the lineage II virus, which is homologous to the lineage of WNV used in the vaccine. This may indicate the need for a WNV vaccine that targets viruses from both lineages in order for the vaccine to be fully protective.

CAdVax-WNVII vaccination induces cellular immune responses against WNV antigens. Cytotoxic T lymphocytes (CTLs) play critical roles in destroying virus-infected cells, preventing virus replication, and promoting viral clearance during an infection. In addition, CTL responses tend to recognize conserved epitopes and are typically more broadly reactive than antibody responses. CTL activation typically requires the de novo synthesis of foreign proteins within antigen-
presenting cells and presentation of the processed antigen in the context of major histocompatibility complex class I molecules (11). For the detection of T-cell immune responses induced by CAdVax-WNVII vaccination, C57BL/6 mice were immunized i.p. with \( 1 \times 10^8 \) PFU of CAdVax-WNVII on weeks 0 and 8. As negative controls, groups of mice were immunized CAdVax-M11 or PBS by use of the same dose and schedule. Two weeks after the second immunization, splenocytes were collected from all animals and were used in a murine IFN-\( \gamma \)-ELISPOT assay. Splenocytes were incubated with syngeneic target cells expressing either the preM and E proteins or the C and NS1 proteins. As negative and positive controls, splenocytes were incubated with media only or concanavalin A, respectively. Following incubation, the numbers of IFN-\( \gamma \)-SFU were detected by ELISPOT assay and counted on an AID ELISPOT assay plate reader. The results are presented as the number of IFN-\( \gamma \)-SFU per \( 2 \times 10^6 \) cells. Statistical analysis was performed by using an ANOVA model (\( **, P < 0.01; *, P < 0.05 \)); bars, standard deviations; ConA, concanavalin A; RV, retrovirus.

**FIG. 6.** Vaccination with CAdVax-WNVII induces cell-mediated immune responses against multiple WNV antigens. Four C57BL/6 mice from each vaccine group were euthanized 10 weeks after the primary vaccinations, and splenocytes from each animal were collected and analyzed individually for WNV-specific CTL activity by using a murine IFN-\( \gamma \)-ELISPot assay. Splenocytes were incubated with syngeneic target cells expressing either the preM and E proteins or the C and NS1 proteins. As negative and positive controls, splenocytes were incubated with media only or concanavalin A, respectively. Following incubation, the numbers of IFN-\( \gamma \)-SFU were detected by ELISPOT assay and counted on an AID ELISPot assay plate reader. The results are presented as the number of IFN-\( \gamma \)-SFU per \( 2 \times 10^6 \) cells. Statistical analysis was performed by using an ANOVA model (\( **, P < 0.01; *, P < 0.05 \)); bars, standard deviations; ConA, concanavalin A; RV, retrovirus.

**DISCUSSION**

Three disturbing epidemiological trends for WNV have emerged during the last 10 years: (i) more frequent human and equine outbreaks; (ii) increases in the number of severe cases of human disease, with higher incidences of encephalitis and polio-like diseases; and (iii) higher avian death rates accompanying the human outbreaks (36). WNV spread across North America in only 4 years (1999 to 2003) and has had detrimental effects on both public and veterinary health as well as the economy.
To curb the epidemics, a WNV vaccine needs to be broadly protective against the different lineages of WNV. We developed a novel WNV vaccine with the hypothesis that the synthesis of multiple WNV antigens de novo will mimic a natural infection and induce both neutralizing and cell-mediated immune responses. These two arms of the adaptive immune system are crucial, in that the antibody response prevents infection and the cell-mediated immune response is more broadly reactive, playing a critical role in the prevention of viral replication and recovery from infection (26–29, 72).

Our complex Ad vaccine vector (CAdVax) is capable of inducing both neutralizing immune responses against the envelope glycoproteins preM and E and cell-mediated immune responses against the structural proteins and an NS protein, NS1. In addition to its multitransgene capability, we chose our Ad-based platform as the antigen carrier for a number of its favorable characteristics: (i) Ad5 is a relatively benign virus that causes a self-limiting common cold; (ii) the vector is replication defective and therefore provides a favorable safety profile; (iii) Ad5 is a nonenveloped DNA virus, and thus, there is no chance that the virus will incorporate or gain novel functions from the WNV genes that it carries as antigens; (iv) Ad vectors have been shown to transduce a variety of human cells and produce very high levels of transgene expression; and (v) different from other viral vectors, such as alpha virus or poxviruses, this vector expresses only the transgenes (WNV antigens), with little to undetectable levels of expression of the Ad antigens (10, 20, 32). The de novo synthesis of WNV antigens is a more efficient way to present antigens to the immune system, and the lack of Ad vector protein synthesis will avoid a competitive inhibitory effect that could detract from the immune responses directed toward the target WNV proteins.

The rationale of our antigen selection is that the flavivirus E protein is responsible for receptor binding and viral entry into cells and has been shown to be the major target of neutralizing antibodies (7, 33). The preM protein is part of the envelope-glycoprotein complex and is important in maintaining the glycoprotein conformation and stability; therefore, it enhances the presentation of the E protein to the immune system in a more natural conformation (30, 40–44, 54, 55, 64, 76, 92). The importance of incorporating the C and NS1 proteins as CTL antigens is that the sequences of these proteins are more conserved among WNV strains than are the sequences of the other glycoproteins and, therefore, should aid in the induction of broadly reactive CTL responses (13, 74). The NS1 protein is also reported to induce an antibody-dependent cellular cytotoxicity and complement fixation activity (19, 50, 69). Antibody complement-mediated cytolysis is believed to further enhance the broad CTL responses and provide broad protection against infections by different variants of WNV (19).

We have demonstrated that upon transduction of cells, CAdVax-WNVII expressed high levels of the WNV preM and E glycoproteins in the cell membrane and the NS1 protein in the cytoplasm, as shown by immunochemical staining with specific antibodies. Vaccination of mice with CAdVax-WNVII induced potent and significant WNV-specific antibody responses, as assayed by ELISA, and cell-mediated immune responses, as shown by ELISPOT assays. Neutralization assays further showed that the antibodies induced after vaccination significantly neutralized both lineage I and lineage II viruses, with a preference toward the homologous lineage II virus over the heterologous lineage I virus. Our results also show that CAdVax-WNVII vaccination did induce T-cell responses against the E and preM glycoproteins, but the responses against the capsid and NS1 antigens were indeed even higher than those against the E and preM antigens. A distinction between CD4+ and CD8+ T-cell responses was not made in these experiments. However, stimulated CD8+ cells typically release Th1-based cytokines, such as interleukin-2 and IFN-γ. Therefore, the cell-mediated responses generated by CAdVax-WNVII may be indicative of a Th1 response, which is considered crucial for the maturation of CTLs and the further elimination of virus-infected cells (38, 59). It is generally believed in the field of WNV research that vaccination against either lineage would be sufficient for protection against infections by both lineages of WNV. We selected our antigens from lineage II mainly because at the time that we started this research lineage II had been favored as the vaccine candidate because lineage II viruses appeared to be less pathogenic in humans and would be safer for use in vaccine development than the use of live, attenuated WNV. However, we were intrigued by the current results that there is clearly a preferential neutralizing activity toward the homologous lineage of WNV. These observations have been reported previously (49) and are similar to what we have observed for experimental dengue virus vaccines (37). Because of our observation of the lineage-specific neutralization of WNV strains, a future topic of our research is to determine if a vaccine should be specific to the lineage of WNV that is endemic or if a bivalent vaccine against both lineages is needed to prevent WNV epidemics.

A common concern with the use of Ad vectors as vaccine platforms is the issue of preexisting immunity to the Ad vector. It is suggested that preexisting Ad immunity would substantially reduce the efficacy of the vaccine due to the immune clearance of the Ad vaccine vector. However, recent data have shown that any reduction in infectivity upon a second administration of the vaccine can be overcome by simply increasing the vaccine dose, by using a DNA priming scheme, or by changing the routes of immunization (4, 21, 73, 80). Alternatively, preexisting vector immunity can be overcome by using alternate Ad serotypes as a vaccine backbone or coating the Ad vector particle with polyethylene glycol, which may mask the neutralizing epitopes on the capsid surface (22, 31, 45, 87). However, independent studies performed by the U.S. Army Medical Research Institute of Infectious Diseases investigating CAdVax-based Marburg virus vaccines showed the efficacies of the vaccines in repeated vaccinations, indicating that these extra measures may not be necessary (81, 83).

In conclusion, we have shown the feasibility of creating a multiantigen WNV vaccine using a unique, complex, replication-defective Ad vector. As WNV continues to spread globally, its impact on public and veterinary health as well as the economy will increase without the development of a vaccine effective against this virus. The results from this study indicate that the use of a multiantigen platform will likely be necessary.
in order to effectively immunize against the antigenically distinct lineages of WNV and that the CADvax platform is a suitable means for this development process.

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