JOURNAL OF VIROLOGY, May 2001, p. 4040–4047 Vol. 75, No. 9
0022-538X/01/$04.00+0 DOI: 10.1128/JVI.75.9.4040–4047.2001

West Nile Virus Recombinant DNA Vaccine Protects Mouse and Horse from Virus Challenge and Expresses In Vitro a Noninfectious Recombinant Antigen That Can Be Used in Enzyme-Linked Immunosorbent Assays

BRENT S. DAVIS,[1] GWONG-JEN J. CHANG,[1]* BRUCE CROPP,[1] JOHN T. ROEHRIG,[1] DENISE A. MARTIN,[1] CARL J. MITCHELL,[1] RICHARD BOWEN,[2] AND MICHEL L. BUNNING[1]

Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Public Health Service, U.S. Department of Health and Human Services,[1] and Colorado State University,[2] Fort Collins, Colorado 80522

Received 15 November 2000/Accepted 29 January 2001

Introduction of West Nile (WN) virus into the United States in 1999 created major human and animal health concerns. Currently, no human or veterinary vaccine is available to prevent WN viral infection, and mosquito control is the only practical strategy to combat the spread of disease. Starting with a previously designed eukaryotic expression vector, we constructed a recombinant plasmid (pCBWN) that expressed the WN virus prM and E proteins. A single intramuscular injection of pCBWN DNA induced protective immunity, preventing WN virus infection in mice and horses. Recombinant plasmid-transformed COS-1 cells expressed and secreted high levels of WN virus prM and E proteins into the culture medium. The medium was treated with polyethylene glycol to concentrate proteins. The resultant, containing high-titered recombinant WN virus antigen, proved to be an excellent alternative to the more traditional suckling-mouse brain WN virus antigen used in the immunoglobulin M (IgM) antibody-capture and indirect IgG enzyme-linked immunosorbent assays. This recombinant antigen has great potential to become the antigen of choice and will facilitate the standardization of reagents and implementation of WN virus surveillance in the United States and elsewhere.

Between late August and early September 1999, New York City and surrounding areas experienced an outbreak of viral encephalitis that caused seven deaths with 62 confirmed cases. Concurrent with this outbreak, local health officials observed increased mortalities among birds (especially crows) and horses. The outbreak was subsequently shown to be caused by West Nile (WN) virus, based on monoclonal antibody (MAb) mapping and gemonic sequences detected in human, avian, and mosquito specimens (4, 17, 22). Virus activity detected during the ensuing winter months (5, 6, 13) indicated that the virus had established itself in North America. In 2000, surveillance data reported from the northeastern and mid-Atlantic states confirmed an intensified epizootic-epidemic transmission and a geographic expansion of the virus. Numerous cases of infection in birds, mosquitoes, and horses as well as cases in humans were documented (6).

WN fever is a mosquito-borne flavivirus infection that is transmitted to vertebrates primarily by various species of *Culex* mosquitoes. Like other members of the Japanese encephalitis (JE) antigenic complex of viruses, including JE, St. Louis encephalitis (SLE), and Murray Valley encephalitis viruses, WN virus is maintained in a natural cycle between arthropod vectors and birds. The virus was first isolated from a febrile human in the West Nile district of Uganda in 1937 (38). It was soon recognized as one of the most widely distributed flaviviruses, with its geographic range including Africa, the Middle East, western Asia, Europe, and Australia (18). Clinically, WN fever in humans is a self-limited acute febrile illness accompanied by headache, myalgia, polyarthropathy, rash, and lymphadenopathy (28). Rarely, though, acute hepatitis or pancreatitis has been reported, and cases in elderly patients are sometimes complicated by encephalitis or meningitis (7).

Currently, no human or veterinary vaccine is available to prevent WN virus infection, and mosquito control is the only practical strategy to combat the spread of disease. Recently, we reported the development of a highly immunogenic recombinant DNA vaccine for JE virus that induced protective immunity in mice following a single intramuscular (i.m.) injection (8). COS-1 cells transformed with this plasmid secreted the premembrane (prM) and envelope (E) proteins in the form of extracellular subviral particles (EPs) into culture medium. We also demonstrated that partially purified EPs not only induced a protective immune response in mice, but more importantly could serve as a noninfectious recombinant antigen (NRA) in both immunoglobulin M (IgM)-antibody capture (MAC) and indirect IgG enzyme-linked immunosorbent assays (ELISAs) (A. R. Hunt and G. J. Chang, submitted for publication). Because WN virus is closely related to JE virus, a recombinant WN virus plasmid was constructed in this study to investigate its potential as a source of NRA for diagnosis and as a candidate DNA vaccine to prevent WN virus infection.

MATERIALS AND METHODS

Cell culture and virus strain. COS-1 cells (American Type Culture Collection, Manassas, Va.; 1650-CRL) were grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Inc.,

* Corresponding author. Mailing address: P.O. Box 2087, Fort Collins, CO 80522-2087. Phone: (970) 221-6497. Fax: (970) 221-6476. E-mail: gxc7@cdc.gov.

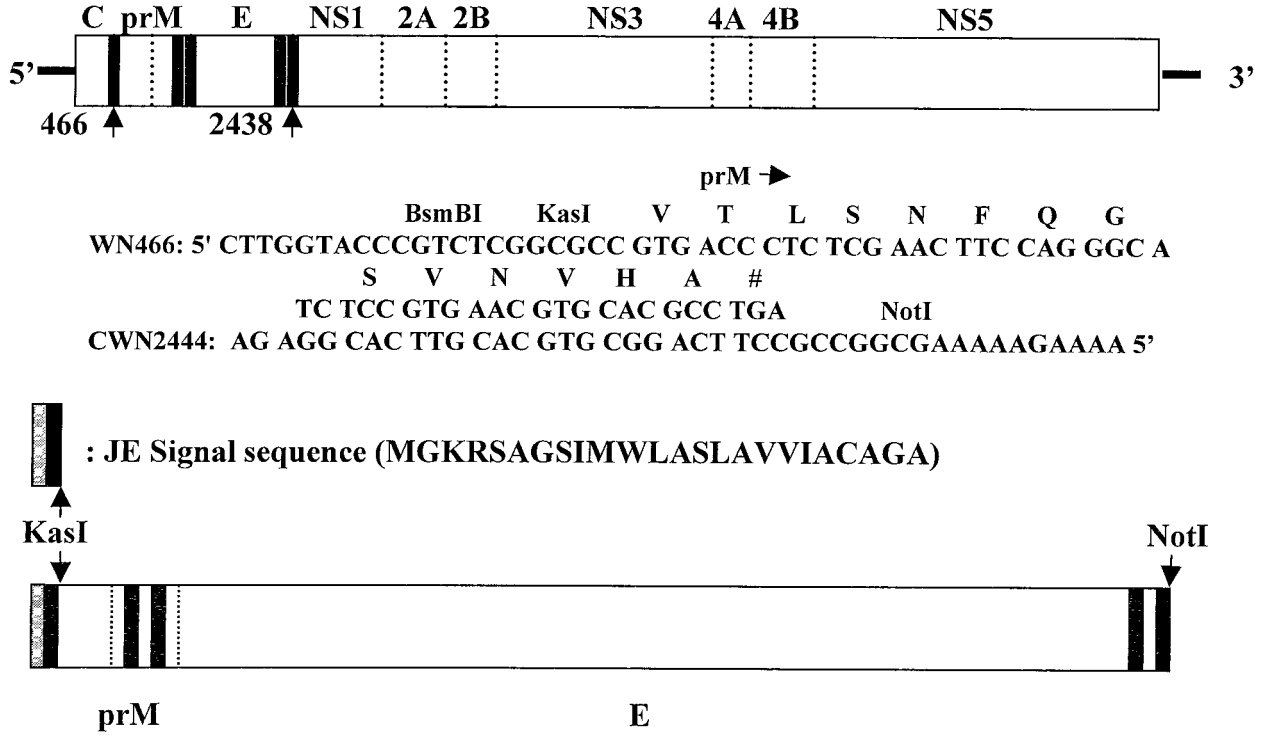

FIG. 1. Map of the WN virus genomic region (top) and oligonucleotides used in RT-PCR to construct the transcription unit for the expression of WN virus prM and E coding regions (bottom). Potential transmembrane helices of viral proteins are indicated by black boxes.

Logan, Utah), 1 mM sodium pyruvate, 1 mM nonessential amino acids, 17 ml per liter of 7.5% $NaHCO_3$, 5 ml per liter of 1 M HEPES (Bio Whittaker, Walkersville, Md.), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Vero cells were grown under the same conditions used for COS-1 cells. Two WN virus strains (NY99-6480, a mosquito isolate, and BC787, a bird isolate), isolated from the outbreak in New York in 1999, were used for challenge experiment or mosquito inoculation. The NY99-6480 strain was propagated in the Vero cell culture. The BC787 strain was passed once in suckling-mouse brain. The NY99-6480 strain used for immunological or biochemical studies was gradient purified by precipitating in 7% polyethylene glycol 8000 (PEG-8000; Fisher Scientific, Fair Lawn, N.J.) followed by ultracentrifugation on 30% glycerol–45% potassium tartrate gradients (30).

Construction of plasmid expressing WN virus prM and E proteins. Genomic RNA was extracted from 150 μl of Vero cell culture medium infected with strain NY99-6480 using a QIAamp viral RNA kit (Qiagen, Santa Clarita, Calif.). Extracted RNA, resuspended in 80 μl of diethyl pyrocarbonate-treated water (Sigma, St. Louis, Mo.), was used as a template in a reverse transcriptase-PCR (RT-PCR) for the amplification of WN virus prM and E genes. Primer sequences (Fig. 1) were designed based on the published sequence (22). Restriction enzyme sites for *Bsm*BI and *Kas*I were incorporated at the 5′ terminus of the cDNA amplicon. An in-frame termination codon followed by a *Not*I restriction site was introduced at the 3′ terminus of the cDNA amplicon. The RT-PCR and molecular cloning protocols used are essentially identical to those reported previously (8).

The WN virus cDNA amplicon was digested with *Kas*I and *Not*I enzymes, and the resulting 998-bp (nucleotides −1470 to 2468) fragment of the cDNA was inserted into the *Kas*I and *Not*I sites of a pCBJESS vector to form an intermediate plasmid, pCBINT. pCBJESS was derived from the pCBamp plasmid, which contained the cytomegalovirus early gene promoter and translational control element and engineered JE virus signal sequence element (8) (Fig. 1). The cDNA amplicon was subsequently digested with *Bsm*BI and *Kas*I enzymes, and the remaining 1,003-bp fragment (nt −466 to 1469) was inserted into the *Kas*I site of pCBINT vector to form pCBWN (Fig. 2). Automated DNA sequencing was performed on an ABI Prism 377 sequencer (Applied Biosystems/Perkin Elmer, Foster City, Calif.) according to the manufacturer's recommended procedures. The recombinant plasmid which had a correct prM and E sequence was identified (22).

Immunochemical characterization of the recombinant WN virus antigen. COS-1 cells were electroporated with plasmid pCBWN using the protocol described in a previous publication (8). Electroporated cells were seeded onto 75-cm^2 culture flasks or in a 12-well tissue culture dish containing one sterile coverslip per well. All flasks and 12-well plates were kept at 37°C in a 5% CO_2 incubator. Forty hours following electroporation, coverslips containing adherent cells were removed from the wells, washed briefly with phosphate-buffered saline (PBS), fixed with acetone for 2 min at room temperature, and allowed to air dry. The flavivirus E protein-specific MAb 4G2 (17), WN virus mouse hyperimmune ascitic fluid (HIAF), and normal mouse serum (NMS) at a 1:200 dilution in PBS were used as the primary antibodies to detect protein expression by an indirect immunofluorescence antibody assay (IFA), as described previously (8).

Tissue culture medium was harvested 40 and 80 h following electroporation. Antigen capture (Ag-capture) ELISA was used to detect secreted WN virus antigen in the culture medium of transiently transformed COS-1 cells. MAb 4G2 and horseradish peroxidase-conjugated MAb 6B6C-1 were used to capture the WN virus antigens and detect captured antigen, respectively (8, 15, 34).

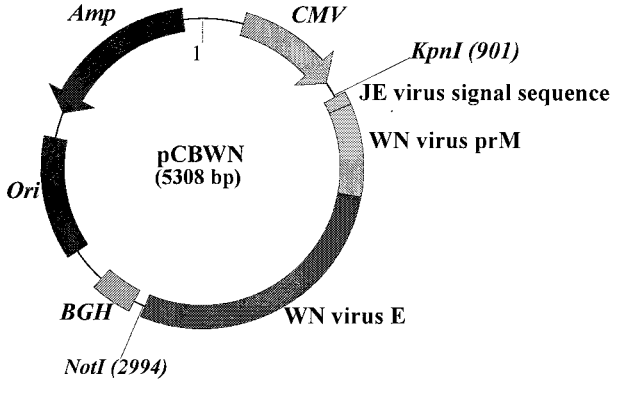

FIG. 2. Map of the recombinant WN virus plasmid pCBWN. The transcription unit contains the human cytomegalovirus early gene promoter (CMV), JE virus signal sequence, WN virus prM and E gene region, and bovine growth hormone poly(A) signal (BGH).

WN virus antigen in the medium was concentrated by precipitation with 10% PEG-8000. The precipitant was resuspended in TNE buffer (50 mM Tris, 100 mM NaCl, 10 mM EDTA [pH 7.5]), clarified by centrifugation, and stored at 4°C. Alternatively, the precipitant was resuspended in lyophilization buffer (0.1 M Trizma and 0.4% bovine serum albumin in borate-saline buffer [pH 9.0]), lyophilized, and stored at 4°C. Lyophilized preparations were used as the antigen for the evaluation in MAC- and indirect IgG ELISAs.

WN virus antigen concentrated by PEG precipitation and resuspended in TNE buffer was extracted with 7.0% ethanol to remove residual PEG (2). Ethanol-extracted antigens and gradient-purified WN virions were analyzed on a NuPAGE 4 to 12% gradient bis-Tris gel in an Excel Plus electrophoresis apparatus (Invitrogen Corp., Carlsbad, Calif.) and monitored by electroblotting onto nitrocellulose membranes using an Excel Plus blot unit (Invitrogen Corp.). WN virus-specific protein was detected by Western blot using WN virus-specific mouse HIAF and MAb 4G2, and NMS was used as a negative serum control (8).

MAC- and indirect IgG ELISAs. The qualities of lyophilized WN virus NRA produced by the pCBWN-transformed COS-1 cells described in the previous section were evaluated by ELISAs. One vial of lyophilized NRA, representing antigen harvested from 40 ml of tissue culture fluid, was reconstituted in 1.0 ml of distilled water and compared with the reconstituted WN virus-infected suckling mouse brain (SMB) antigen provided as lyophilized β-propiolactone-inactivated sucrose-acetone extracts in our facility (9). Coded human specimens were tested concurrently with antigens in the same test at the developmental stage. The MAC- and IgG ELISA protocols employed were identical to the published methods (18, 24). Human serum specimens were obtained from the serum bank in our facility, which consists of specimens sent to the division for WN virus confirmation testing during the 1999 outbreak. In these tests, screening MAC- and IgG ELISAs were performed on a 1:400 specimen dilution. Specimens yielding a positive/negative (P/N) optical density (OD) ratio of between 2 and 3 are considered suspected positives. Suspect serum specimens were tested further in two other tests, ELISA end-point titration and plaque reduction neutralization test (PRNT), for confirmation. Specimens registering P/N ratios of ≥3.0 are considered positive.

Animal vaccination and protection studies. Groups of 10 3-week-old female ICR outbred mice were used in the study. Mice were injected i.m. with a single dose of pCBWN or green flurorescent protein (GFP)-expressing plasmid (pEGFP) DNA (Clonetech, San Francisco, Calif.). The plasmid DNA was purified from *Escherichia coli* XL-1Blue cells with EndoFree Plasmid Giga kits (Qiagen) and resuspended in PBS (pH 7.5) at 1.0 μg/μl. Mice that received 100 μg of pEGFP were used as unvaccinated controls. Mice were injected with the pCBWN plasmid at a dose of 100, 10, 1.0, or 0.1 μg in a volume of 100 μl. Groups that received 10, 1.0, or 0.1 μg of pCBWN were vaccinated by the electrotransfer-mediated in vivo gene delivery protocol using the EMC-830 square-wave electroporator (Genetronics Inc., San Diego, Calif.). The electrotransfer protocol was based on the method reported by Mir et al. (25). Immediately following DNA injection, transcutaneous electric pulses were applied by two stainless steel plate electrodes, placed 4.5 to 5.5 mm apart, at each side of the leg. Electrical contact with the leg skin was ensured by completely wetting the leg with PBS. Two sets of four pulses of 40 V/mm 25 ms in duration with a 200-ms interval between pulses were applied. The polarity of the electrode was reversed between the sets of pulses to enhance electrotransfer efficiency.

Mice were bled every 3 weeks following injection, and the WN virus-specific antibody response was evaluated by Ag-capture ELISA and PRNT. Challenge experiments were performed by two methods. Half of the mouse groups were challenged intraperitoneally (i.p.) at 6 weeks postvaccination with 1,000 50% lethal doses (LD_{50}) (1,025 PFU/100 μl) of NY99-6480 virus. The LD_{50} was determined previously by i.p. inoculation of 10-week-old adult ICR mice (data not shown). The remaining mice were each exposed to the bites of three *Culex tritaeniorhynchus* mosquitoes that had been infected with NY99-6480 virus 7 days prior to the challenge experiment. Mosquitoes were allowed to feed on mice until they were fully engorged. Mice were observed twice daily for 3 weeks after challenge.

Mixed-bred mares and geldings of various ages used in this study were shown to be WN virus and SLE virus antibody negative by ELISA and PRNT. Four horses were injected i.m. with a single dose (1,000 μg/1,000 μl in PBS [pH 7.5]) of pCBWN plasmid. Serum specimens were collected every other day for 38 days prior to virus challenge, and the WN virus-specific antibody response was evaluated by MAC- or IgG ELISA and PRNT.

Two days prior to virus challenge, 12 horses (4 vaccinated and 8 control) were relocated into a biosafety level 3 containment building at Colorado State University. The eight unvaccinated control horses were the subset of a study that was designed to investigate WN virus-induced pathogenesis in horses and the potential of horses to serve as amplifying hosts (M. L. Bunning, R. A. Bowen, B. Davis, N. Kumar, M. Godsey, D. Baker, D. Hettler, and C. J. Mitchell, submitted for publication). Horses were each challenged by the bite of 14 or 15 *Aedes albopictus* mosquitoes that had been infected by NY99-6425 or BC787 virus 12 days prior to horse challenge. Mosquitoes were allowed to feed on horses for 10 min. Horses were examined for signs of disease twice daily. Body temperature was recorded, and serum specimens were collected twice daily from days 0 (day of infection) to 10 and then once daily through day 14. Pulse and respiration were recorded daily after challenge. The collected serum samples were tested by plaque titration for detection of viremia and by MAC- or IgG ELISA and PRNT for antibody response. Vaccinated horses were euthanized by pentobarbital overdose at 14 days after virus challenge and necropsied for gross pathological and histopathological examination, and their carcasses were incinerated within the containment facility.

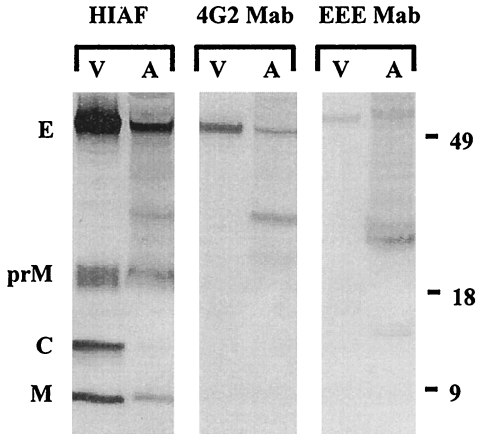

FIG. 3. Comparison of Western blot reactivity between NRA produced by pCBWN-transformed COS-1 cells (A) and gradient-purified WN virion proteins (V). WN virus-specific mouse HIAF, flavivirus E-specific cross-reactive MAb 4G2, and eastern equine encephalitis virus monoclonal antibody (EEE MAb) were used at a 1:200 dilution in the assay. Sizes are shown in kilodaltons.

Serological tests. Pre- and postvaccination as well as postchallenge serum specimens were tested for antibody-binding ability to purified WN virion or recombinant antigen by ELISA, for neutralizing (Nt) antibody by PRNT, and for antibodies that recognize purified WN virus proteins by Western blotting (8, 27). PRNT was performed with Vero cells, as previously described (8), using NY99-6480 virus. Endpoints were determined at a 90% plaque-reduction level.

RESULTS

Plasmid construct and transient expression of WN virus antigen. Expression of the prM and E genes of WN virus was assayed by transfection of plasmid pCBWN into COS-1 cells. This plasmid was constructed by inserting the WN virus cDNA that encoded the sequence between the prM and E genes into the pCBJESS vector to obtain pCBWN (Fig. 2). WN virus-specific protein was detected by IFA on transiently transformed COS-1 cells (data not shown). These cells also secreted E, prM, and M proteins into the culture medium, that were detected by WN virus HIAF or flavivirus E protein-reactive MAb 4G2 in a Western blot analysis. All three proteins had a similar reactivity and were identical in size to the gradient-purified virion E, prM, and M proteins (Fig. 3).

NRA as an antigen for diagnostic ELISA. An Ag-capture ELISA employing flavivirus group-reactive anti-E MAb 4G2 and 6B6C-1 was used to detect NRA secreted into the culture fluid of pCBWN-transformed COS-1 cells. The antigen could be detected in the medium 1 day following transformation, and the maximum ELISA titer (1:32 to 1:64) in the culture fluid without further concentration was observed between days 2

TABLE 1. IgM and IgG reactivity to NRA and SMB antigen in a panel of coded human serum specimens[a]

Specimen no. (day postonset)	ELISA titer								Nt antibody titer
	IgM				IgG				
	NRA	SMB			NRA	SMB			
		NY99 virus	Eg-101 virus	SLE virus		NY99 virus	Eg-101 virus	SLE virus	
Positive control	4.19	4.46	5.16		4.54	5.00	4.82		ND
1 (20)	7.42	7.15	6.59	3.74	7.22	6.30	5.12	7.93	1:1,280
2 (14)	4.26	3.12	4.34	1.14	1.14	1.31	1.40	1.8	ND
3 (16)	6.00	5.39	6.18	2.08	1.61	1.85	1.95	2.53	<1:10
4 (30)	3.04	2.23	2.47	1.21	8.48	7.83	6.34	ND	1:160
5 (68)	5.01	5.85	6.28	1.44	1.10	1.14	1.19	1.02	ND
6 (?)	7.30	4.11	3.51	1.11	8.57	8.84	7.76	11.54	1:320
7 (43)	1.90	1.76	2.69	0.79	7.82	7.66	6.40	3.61	ND
8 (20)	1.12	0.98	0.95	0.91	1.53	1.64	1.70	ND	ND
9 (3)	1.53	1.37	2.17	1.13	6.88	7.12	6.07	ND	1:5120
10 (3)	6.43	4.69	6.04	1.87	3.94	4.23	4.38	2.31	<1:10
11 (7)	6.71	4.59	5.78	1.86	3.74	4.30	3.48	2.74	1:20
12 (10)	1.14	1.06	1.17	0.96	6.30	6.59	5.11	7.28	ND
13 (?)	1.13	0.90	1.13	1.33	1.20	1.38	1.41	ND	ND
14 (6)	7.43	6.64	8.03	1.71	7.90	7.05	5.44	4.69	1:80
15 (3)	7.44	6.98	8.32	2.89	2.96	3.04	2.43	2.44	1:160
16 (13)	2.88	2.30	4.17	1.01	1.07	1.37	1.47	1.80	<1:10
17 (4)	1.20	0.85	0.90	1.07	0.94	1.34	1.34	1.49	ND
18 (8)	1.09	0.97	1.01	ND	0.67	0.77	0.75	ND	ND
19 (73)	1.04	0.96	0.95	ND	0.71	0.80	0.79	ND	ND
20 (8)	1.10	1.14	1.01	ND	0.72	0.82	0.82	ND	ND
21 (0)	1.06	0.92	0.88	0.91	0.79	0.88	1.11	0.90	ND
Total no. positive	11	11	13		10	10	10		

[a] SLE virus MAC and IgG ELISAs were conducted as a separate test. Nt antibody titer was assayed in a 90% plaque reduction neutralization test against WN virus. ND, not done.

and 4. NRA was concentrated by PEG precipitation, resuspended in lyophilization buffer, and lyophilized for preservation. For diagnostic test development, one vial of lyophilized NRA was reconstituted with 1.0 ml of distilled water and titrated in the MAC- or indirect IgG ELISA using WN virus-positive and -negative reference human sera (18, 24). Dilutions to 1:320 and 1:160 of the NRA were found to be the optimal concentrations for use in MAC- and IgG ELISA, respectively. These dilutions resulted in a P/N OD_{450} ratio of 4.19 and 4.54 for MAC and IgG tests, respectively (Table 1). The WN virus SMB antigens produced by the NY99-6480 and Eg101 strains were used at a 1:320 and 1:640 dilution for MAC-ELISA and a 1:120 and 1:320 dilution for IgG ELISA. The negative control antigens, PEG precipitates of the culture medium of normal COS-1 cells and normal SMB antigen, were used at the same dilutions as the respective NRA and SMB antigens. Human serum specimens, diluted to 1:400, were tested concurrently in triplicate with virus-specific and negative control antigens. For the positive test result to be valid, the OD_{450} for the test serum reacted with viral antigen (P) had to be at least twofold greater than the corresponding OD value of the same serum reacted with negative control antigen (N).

The reactivities of NRA and NY99-0648, Eg101, and SLE virus SMBs were compared by MAC- and IgG ELISAs using 21 coded human serum specimens (Table 1). Of the 21 specimens, 19 had similar results on all three antigens (8 negative and 11 suspect or positive). Eighteen specimens were also tested separately using SLE virus SMB antigen. Only 3 of 13 Eg-101 SMB-positive specimens were suspect or positive in the SLE virus MAC-ELISA (Table 1). None of the WN antigen-negative specimens was positive by SLE virus MAC-ELISA. This result provided further evidence that anti-WN virus IgM did not cross-react significantly with other flavivirus antigens (39) and was specific to diagnose acute WN virus infection regardless of the antigen (NRA or SMB) used in the test. All of the specimens were also tested concurrently by indirect IgG ELISA, and 10 of 21 specimens were positive with all three antigens.

The two discrepant serum specimens (7 and 9), both from the same patient, collected on day −3 and day −43 after onset of disease, respectively, were IgM negative with NRA and NY99-6480 SMB antigen and suspect for IgM positive to Eg-101 SMB antigen in the screening test (Table 1). To investigate these two discordant specimens further, six sequentially collected specimens from this patient were retested by end-point MAC- and IgG ELISAs. A greater than 32-fold serial increase in the MAC-ELISA titer between days 3 and 15 could be demonstrated with all antigens (Table 2). Cerebrospinal fluid collected on day 9 after onset of disease also confirmed that this patient indeed was recently infected by WN virus. The cerebrospinal fluid had an IgM P/N reading of 13.71 and 2.04 against Eg-101 and SLE virus SMB antigens, respectively (data not shown). All other specimens had an end-point IgM titer of 1:1,600 or less with all three antigens when using the absolute P/N cutoff of 3.0. Compatible IgG titers were observed with all three antigens used in the test.

Immunogenicity and protective efficacy of candidate DNA vaccine in ICR mice. ICR mice were immunized by i.m. injection of pCBWN or pEGPF. The mice were bled 3 and 6 weeks after immunization. Individual sera were tested by IgG ELISA,

TABLE 2. Comparison of ELISA titers[a] obtained with three antigens on sequential serum specimens from one patient

Day postonset	IgM titer			IgG titer		
	NRA	SMB		NRA	SMB	
		NY99	Eg-101		NY99	Eg-101
3[b]	<1:400	<1:400	<1:400	1:1,600	1:3,200	1:3,200
15	1:6,400	>1:12,800	>1:12,800	1:1,600	1:1,600	1:3,200
30	<1:400	<1:400	1:1,600	1:3,200	1:3,200	1:3,200
43[c]	<1:400	<1:400	<1:400	1:3,200	1:3,200	1:3,200
204	<1:400	<1:400	<1:400	1:800	1:1,600	1:1,600
344	<1:400	<1:400	<1:400	1:800	1:1,600	1:800

[a] Titers were calculated using an absolute P/N ratio cutoff of 3.0.
[b] Random sample 9 in Table 1.
[c] Random sample 7 in Table 1.

and pooled sera from 10 mice in each group were assayed by PRNT. All the mice vaccinated with pCBWN had IgG ELISA titers ranging from 1:640 to 1:1,280 3 weeks after vaccination (data not shown). The pooled sera collected at 3 and 6 weeks in the test I group had an Nt antibody titer of 1:80 (Table 3). None of the serum specimens from pEGFP control mice displayed any ELISA or Nt antibody to WN virus.

To determine if the single i.m. vaccination of pCBWN could protect mice from WN virus infection, we challenged mice with NY99-6480 virus either by i.p. injection or by exposure to the bite of virus-infected *Culex* mosquitoes. It was evident that the presence of WN virus antibody correlated with protective immunity, since all mice immunized with WN virus DNA remained healthy after virus challenge (Table 3). All control mice developed symptoms of central nervous system infection 4 to 6 days later and died an average of 6.9 and 7.4 days after i.p. or infected-mosquito challenge, respectively. In the vaccinated group, the pooled sera collected 3 weeks after virus challenge (9 weeks postimmunization) had Nt antibody titers

TABLE 3. Evaluation of protective immunity conferred by DNA vaccine for WN virus in mice

Test	Plasmid and dose[a] (μg)	Titer[b] on wk p.v.:			Challenge method[c]	% Survival	Avg survival time (days)
		3	6	9			
I	pCBWN						
	100.0	1:80	1:80	1:640	i.p.	100	
	100.0	1:80	1:80	1:320	Mosq.	100	
	pEGFP (control)						
	100.0	<1:10	<1:10		i.p.	0	6.9
	100.0	<1:10	<1:10		Mosq.	0	7.4
II	pCBWN						
	100.0		1:160		i.p.	100	
	10.0, E		1:160		i.p.	100	
	1.0, E		1:80		i.p.	100	
	0.1, E		1:40		i.p.	100	
	pEGFP (control)						
	100.0		<1:10		i.p.	0	7.0

[a] Groups of 10 ICR mice were immunized via a single i.m. injection or i.m. followed by electrotransfer (E).
[b] Serum neutralization titers of postvaccination (p.v.) pooled sera were determined as described in Materials and Methods. Week 9 sera were collected from the surviving mice 3 weeks after virus challenge.
[c] Mice were challenged by i.p. injection of 1,000 LD_{50} of virus or by exposure to the bite of three virus-infected mosquitoes (Mosq.).

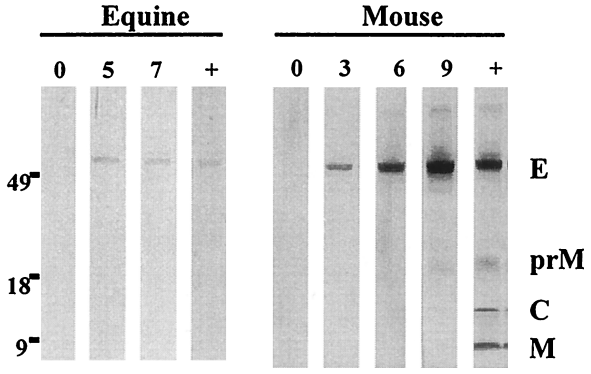

FIG. 4. WN virus-specific reactivity of pre- and postchallenge serum specimens obtained from mice and horses immunized with WN virus DNA vaccine. Pooled serum specimens from the mice and horses used in the experiments were tested at a 1:25 dilution by Western blot analysis using purified WN virion as the antigen. Western blot results obtained with pooled horse sera collected before DNA vaccination (week 0), 5 weeks postvaccination or before virus challenge (week 5), and 2 weeks postchallenge (week 7). Positive horse serum (lane +) was from control horse 16, which had PRNT titer of >1,280 on week 4 after virus challenge. Western blot results obtained with pooled mouse sera collected before DNA vaccination (week 0), 3 and 6 weeks postvaccination or before virus challenge (weeks 3 and 6), and 3 weeks postchallenge (week 9). Mouse HIAF at a 1:250 dilution was used as the positive control (lane +). Sizes are shown in kilodaltons.

of 1:320 or 1:640 (Table 3). Pooled vaccinated mouse sera reacted only with E protein in the Western blot analysis (Fig. 4).

Enhancing immunogenicity of the candidate vaccine by electrotransfer. Mir et al. reported that the local application of electric pulses increases the efficiency and reproducibility of in vivo plasmid transfer to muscle fibers after i.m. injection (25). To determine if this unique electrotransfer protocol increases the immunogenicity of the candidate vaccine, we immunized groups of 10 mice by this technique with pCBWN (10.0 to 0.1 μg per animal). At 6 weeks after immunization, all electrotransfer-immunized groups had a pooled Nt titer equal to or greater than 1:40 and were completely protected from virus challenge (test II in Table 3). The group immunized by electrotransfer with 0.1 μg of DNA, the lowest dose tested in this study, had an Nt titer of 1:40, representing only a fourfold difference compared with animals receiving 100 μg of pCBWN by i.m. injection. This result suggested that electrotransfer could be an alternative immunization protocol to enhance the immunogenicity and protective efficacy of our DNA vaccine.

Immunogenicity and protective efficacy of the candidate vaccine in horses. Four horses were vaccinated i.m. with a single dose of pCBWN and bled every other day prior to infected-mosquito challenge on day 39. No systemic or local reaction was observed in any vaccinated horse. Individual horse sera were tested by PRNT. Vaccinated horses developed Nt antibody titers of ≥1:5 between days 14 and 31 (Table 4). Endpoint titers for vaccinated horses 5, 6, 7, and 8 on day 37 (2 days prior to mosquito challenge) were 1:40, 1:5, 1:20, and 1:20, respectively. To determine if the DNA vaccine could protect horses from WN virus infection, we challenged vaccinated and unvaccinated control horses by allowing each horse to be bitten by approximately 15 virus-infected mosquitoes. Horses vaccinated with the pCBWN plasmid remained healthy after virus challenge. None of them developed detectable viremia or fever

TABLE 4. Serum Nt antibody titers and protective immunity elicited by a single i.m. injection of WN virus DNA vaccine in horses

Sample (day)	Titer							
	Vaccinated horse no.				Unvaccinated control horse no.			
	5	6	7	8	9	10	14	15
Postvaccination								
12	<1:10	<1:10	<1:10	<1:10				
14	1:10	<1:10	<1:10	<1:10				
16	1:10	<1:10	<1:10	<1:10				
18	1:40	<1:10	<1:10	<1:10				
20	1:40	<1:10	<1:10	<1:10				
22	1:40	<1:10	1:10	<1:10				
28	1:40	<1:10	1:20	1:10				
31	1:40	1:5	1:20	1:10				
37	1:40	1:5	1:20	1:20				
Postchallenge								
2	1:40	1:5	1:20	1:20	<1:2	<1:2	<1:2	<1:2
4	1:40	1:10	1:20	1:40	<1:2	<1:2	<1:2	<1:2
6	1:40	1:10	1:40	1:40	<1:2	<1:2	<1:2	<1:2
8	1:80	1:20	1:40	1:40	1:80	<1:10	<1:10	<1:10
10	1:80	1:20	1:40	1:80	>1:320	<1:10	<1:10	1:160
12	1:160	1:40	1:80	1:160	>1:320	1:20	1:20	>1:320
14	>1:320	1:40	1:160	1:160	>1:320	1:160	1:10	1:160
Viremia titer[a] range (day)					1.3–2.4 (4)	1.0–1.6 (6)		1.3–2.2 (2)

[a] Viremia titer was expressed as $\log_{10}$ Vero cell PFU/ml of serum.

from days 1 to 14. All unvaccinated control horses became infected with WN virus after exposure to infected-mosquito bites. Seven of the eight unvaccinated horses developed viremia that appeared during the first 6 days after virus challenge. Viremic horses developed Nt antibody between days 7 and 9 after virus challenge. The only horse from the entire study to display clinical signs of disease was horse 11, which became febrile and showed neurologic signs beginning 8 days after infection. This horse progressed to severe clinical disease within 24 h and was euthanized on day 9 (Bunning et al., submitted). Four horses, 9, 10, 14, and 15, presenting viremia for 0, 2, 4, or 6 days were selected and used as examples in this study (Table 4). Virus titers ranged from $10^{1.0}$ PFU/ml of serum in horse 10, the lowest level detectable in our assay, to $10^{2.4}$ PFU/ml in horse 9. Horse 14 did not develop detectable viremia during the test period. However, this horse was infected by the virus, as evidenced by Nt antibody detected after day 12.

Anamnestic Nt antibody response was not observed in vaccinated horses, as evidenced by the gradual increase in Nt titer during the experiment. Existing Nt antibody in the vaccinated horse prior to mosquito challenge could suppress initial virus infection and replication. Without virus replication, the challenge virus antigen provided by infected mosquitoes may not contain sufficient antigen mass to stimulate an anamnestic immune response in the vaccinated horse. All vaccinated horses were euthanized 14 days after virus challenge. Gross pathological and histopathological lesions indicative of WN virus infection were not observed (data not shown).

DISCUSSION

We previously designed a recombinant eukaryotic expression plasmid that contained an optimal genetic constellation to enhance the expression and secretion of JE virus prM and E proteins into the culture medium of a stably transformed cell line. A single i.m. injection of recombinant plasmid DNA induced a long-lasting protective immunity and prevented JE in mice (8). The recombinant antigens, formed as EPs that were produced by this stably transformed cell line, also elicited high anti-JE virus Nt antibody (Hunt and Chang, submitted). Our PEG-concentrated recombinant JE virus antigen has also proven to be an excellent alternative to traditional SMB antigen used in the diagnostic MAC- and indirect IgG ELISAs (Hunt and Chang, submitted).

The same strategy was applied to construct a recombinant WN virus plasmid, pCBWN, in the present study. We theorized that an effective JE virus transmembrane signal peptide (TSP) is one of the most important factors that influence downstream protein translocation and topology, thus dictating correct processing of JE virus prM and E proteins by the host-encoded signalase and endopeptidase (8). In the present study, the WN virus-encoded TSP was replaced by JE virus TSP (Fig. 1). As expected, transiently transformed COS-1 cells expressed and secreted prM/M and E proteins into the culture medium, which could be detected by Ag-capture ELISA. Western blot analysis confirmed the presence of three major proteins identical in size to the E, prM, and M of gradient-purified WN virions (Fig. 3). More importantly, prM-to-M processing was very similar between the recombinant antigen and virion protein.

The SignalP computer program was used to calculate the cleavage site (C), signal peptide (S), and combined cleavage site (Y) scores of JE virus and WN virus TSP by the method of Nielsen et al. (29). As predicted, SignalP indicated that replacement of WN virus TSP for JE virus TSP did not adversely effect the proper signalase cleavage site. Replacement for JE virus TSP did increase raw cleavage site (C), signal peptide (S), and combined cleavage site (Y) scores significantly (data not shown). More importantly, the most significant predictor of the

TSP (Y) was increased from 0.375 to 0.617. The physical properties of the recombinant antigen expressed by plasmid pCBWN were not characterized in this study. Physical structure and secretion of the antigen expressed by a recombinant plasmid can influence the vaccine potential of flavivirus DNA vaccines. The recombinant antigen expressed by plasmid pCBWN could assemble and form a secreted EP that is very similar to the well-characterized JE and tick-borne encephalitis virus antigens (3, 21; Hunt and Chang, submitted). It has been demonstrated that the plasmid construct encoding an EP of tick-borne encephalitis virus prM and E protein is the most potent vaccine construct of a series of plasmids expressing different forms of the E protein (1). This observation shows that our WN virus plasmid has excellent vaccine potential in mice and horses and supports the notion that a portion of WN virus prM and E proteins is likely expressed and secreted as EPs.

The use of the prM-E gene cassette as the flavivirus DNA vaccine has been reported for different strains of JE virus (8, 19, 23) and other flaviviruses, such as SLE virus (31), dengue serotype 1 and 2 viruses (20, 32, 33), Murray Valley encephalitis virus (11), and Russian spring-summer encephalitis and tick-borne Central European encephalitis viruses (36). In general, vaccine potential, measured by induction of Nt antibody and protective efficacy by virus challenge, can be improved by multiple i.m., intradermal, or gene gun-mediated intradermal deliveries of DNA vaccine. The gene gun immunization enhances the uptake of DNA by the professional antigen-presenting cells in the dermis and the opportunity for intracellular processing of in vivo-synthesized antigen directly in these cells (10). However, gene gun immunization requires that plasmid DNA be applied to the surface of gold beads (12), a step that could become expensive if multiple vaccine administrations are needed. In addition, the amount of DNA on gold particles that can be administered in a single application is usually limited to 2.5 μg per mg of gold beads. Studies conducted in monkeys with Russian spring-summer and tick-borne Central European encephalitis virus DNA vaccines have indicated that between 3 and 12 applications per monkey may be require to achieve the effective vaccination dosage (35).

Electroporation is commonly used to introduce foreign DNA into prokaryotic and eukaryotic cells ex vivo. Recently, Mir et al. described a similar procedure that uses electric pulses to enhance foreign DNA uptake by muscle fiber (25). They demonstrated that this i.m. electrotransfer method increases reporter and therapeutic gene expression by several orders of magnitude in various muscles in mouse, rat, rabbit, and monkey models. Furthermore, its clinical application, electrochemotherapy, is being pursued in oncology. Using WN virus DNA, we demonstrated that the i.m. electrotransfer method has the potential to improve the immunogenicity of the DNA vaccine. The lowest dose tested (0.1 μg of DNA per animal) is sufficient to induce complete protective immunity by our vaccine in mice (Table 3). The short, intense electric pulse applied in the current protocol is safe and well tolerated in humans (14, 26, 37). However, an attempt to apply this method to equines was not successful due to intolerance of this host to the electric current (data not shown).

As a safe alternative to the traditional SMB antigen preparation and to streamline production efforts, we have cloned and derived a stably transformed COS-1 cell line, C2, that constitutively expresses and secretes recombinant WN virus antigen into the culture medium (G. J. Chang and D. Holmes, unpublished data). The antigen produced by C2 cells has been used to replace the transiently expressed recombinant antigen. Thus far, 250 vials of recombinant WN virus antigen have been produced and more than 100 vials have been shipped to various states and local health departments for their surveillance activities. Each vial contains a sufficient amount of antigen to test at least 250 specimens concurrently and in triplicate by MAC- and IgG ELISAs. Based on our estimation, each vial of NRA contains an equivalent amount of antigen produced by four-fifths of a suckling mouse brain. Thus, it has great potential to become the antigen of choice, with a major impact on the standardization of flavivirus reagents and implementation of WN virus surveillance in the United States and elsewhere.

We believe that NRA prepared by our technique could also be used as a biosynthetic subunit vaccine, since feasibility has been demonstrated previously for the antigenically related JE virus (21; Hunt and Chang, submitted). Currently, we have directed our efforts to using the pCBWN plasmid as a candidate DNA vaccine to prevent WN virus encephalitis in horses. Studies to define an optimal vaccine regimen, long-term immunogenicity and efficacy, and field safety are in progress. As an alternative approach, vaccination could be achieved by priming the host with DNA, followed by a booster injection with NRA.

ACKNOWLEDGMENTS

We thank Genetronics Inc., San Diego, Calif., for use of the EMC-830 square-wave electroporator and G. Kuno and B. Miller for useful discussion and advice. We are grateful to D. Hettler and D. Holmes for superb technical assistance.

ADDENDUM IN PROOF

Recently, Konishi et al. (E. Konishi, A. Fujii, and P. W. Mason, J. Virol. **75:**2204–2212, 2001) reported the isolation of a CHO cell line constitutively producing subviral extracellular particles of JEV only after elimination of the prM processing site. In the present study, a line of COS cells producing WNV subviral particles with an intact processing site was obtained.

REFERENCES

1. **Aberle, J. H., S. W. Aberle, S. L. Allison, K. Stiasny, M. Ecker, C. W. Mandl, R. Berger, and F. X. Heinz.** 1999. A DNA immunization model study with constructs expressing the tick- borne encephalitis virus envelope protein E in different physical forms. J. Immunol. **163:**6756–6761.
2. **Aizawa, C., S. Hasegawa, C. Chih-Yuan, and I. Yoshioka.** 1980. Large-scale purification of Japanese encephalitis virus from infected mouse brain for preparation of vaccine. Appl. Environ. Microbiol. **39:**54–57.
3. **Allison, S. L., K. Stadler, C. W. Mandl, C. Kunz, and F. X. Heinz.** 1995. Synthesis and secretion of recombinant tick-borne encephalitis virus protein E in soluble and particulate form. J. Virol. **69:**5816–5820.
4. **Anderson, J. F., T. G. Andreadis, C. R. Vossbrinck, S. Tirrell, E. M. Wakem, R. A. French, A. E. Garmendia, and H. J. Van Kruiningen.** 1999. Isolation of West Nile virus from mosquitoes, crows, and a Cooper's hawk in Connecticut. Science **286:**2331–2333.
5. **Anonymous.** 2000. Update: surveillance for West Nile virus in overwintering mosquitoes —New York, 2000. Morb. Mortal. Wkly. Rep. **49:**178–179.
6. **Anonymous.** 2000. Update: West Nile virus activity—Northeastern United States, 2000. Morb. Mortal. Wkly. Rep. **49:**820–822.
7. **Asnis, D. S., R. Conetta, A. A. Teixeira, G. Waldman, and B. A. Sampson.** 2000. The West Nile virus outbreak of 1999 in New York: the Flushing Hospital experience. Clin. Infect. Dis. **30:**413–418.
8. **Chang, G. J., A. R. Hunt, and B. Davis.** 2000. A single intramuscular injection of recombinant plasmid DNA induces protective immunity and prevents

Japanese encephalitis in mice. J. Virol. **74:**4244–4252.
9. **Clarke, D. H., and J. Casals.** 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am. J. Trop. Med. Hyg. **7:**561–573.
10. **Cohen, A. D., J. D. Boyer, and D. B. Weiner.** 1998. Modulating the immune response to genetic immunization. FASEB J. **12:**1611–1626.
11. **Colombage, G., R. Hall, M. Pavy, and M. Lobigs.** 1998. DNA-based and alphavirus-vectored immunisation with prM and E proteins elicits long-lived and protective immunity against the flavivirus, Murray Valley encephalitis virus. Virology **250:**151–163.
12. **Eisenbraun, M. D., D. H. Fuller, and J. R. Haynes.** 1993. Examination of parameters affecting the elicitation of humoral immune responses by particle bombardment-mediated genetic immunization. DNA Cell Biol. **12:**791–797.
13. **Garmendia, A. E., H. J. Van Kruiningen, R. A. French, J. F. Anderson, T. G. Andreadis, A. Kumar, and A. B. West.** 2000. Recovery and identification of West Nile virus from a hawk in winter. J. Clin. Microbiol. **38:**3110–3111.
14. **Heller, R., M. J. Jaroszeski, D. S. Reintgen, C. A. Puleo, R. C. DeConti, R. A. Gilbert, and L. F. Glass.** 1998. Treatment of cutaneous and subcutaneous tumors with electrochemotherapy using intralesional bleomycin. Cancer **83:** 148–157.
15. **Henchal, E. A., M. K. Gentry, J. M. McCown, and W. E. Brandt.** 1982. Dengue virus-specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. Am. J. Trop. Med. Hyg. **31:**830–836.
16. **Hubalek, Z., and J. Halouzka.** 1999. West Nile fever—a reemerging mosquito-borne viral disease in Europe. Emerg. Infect. Dis. **5:**643–650.
17. **Jia, X. Y., T. Briese, I. Jordan, A. Rambaut, H. C. Chi, J. S. Mackenzie, R. A. Hall, J. Scherret, and W. I. Lipkin.** 1999. Genetic analysis of West Nile New York 1999 encephalitis virus. Lancet **354:**1971–1972.
18. **Johnson, A. J., D. A. Martin, N. Karabatsos, and J. T. Roehrig.** 2000. Detection of antiarboviral immunoglobulin G by using a monoclonal antibody-based capture enzyme-linked immunosorbent assay. J. Clin. Microbiol. **38:**1827–1831.
19. **Konishi, E., M. Yamaoka, Khin-Sane-Win, I. Kurane, K. Takada, and P. W. Mason.** 1999. The anamnestic neutralizing antibody response is critical for protection of mice from challenge following vaccination with a plasmid encoding the Japanese encephalitis virus premembrane and envelope genes. J. Virol. **73:**5527–5534.
20. **Konishi, E., M. Yamaoka, I. Kurane, and P. W. Mason.** 2000. A DNA vaccine expressing dengue type 2 virus premembrane and envelope genes induces neutralizing antibody and memory B cells in mice. Vaccine **18:**1133–1139.
21. **Konishi, E., S. Pincus, E. Paoletti, R. E. Shope, T. Burrage, and P. W. Mason.** 1992. Mice immunized with a subviral particle containing the Japanese encephalitis virus prM/M and E proteins are protected from lethal JEV infection. Virology **188:**714–720.
22. **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.
23. **Lin, Y. L., K. L. Chen, C. L. Liao, C. T. Yeh, S. H. Ma, J. L. Chen, Y. L. Huang, S. S. Chen, and H. Y. Chiang.** 1998. DNA immunization with Japanese encephalitis virus nonstructural protein NS1 elicits protective immunity in mice. J. Virol. **72:**191–200.
24. **Martin, D. A., D. A. Muth, T. Brown, A. J. Johnson, N. Karabatsos, and J. T. Roehrig.** 2000. Standardization of immunoglobulin M capture enzyme-linked immunosorbent assays for routine diagnosis of arboviral infections. J. Clin. Microbiol. **38:**1823–1826.
25. **Mir, L. M., M. F. Bureau, J. Gehl, R. Rangara, D. Rouy, J. M. Caillaud, P. Delaere, D. Branellec, B. Schwartz, and D. Scherman.** 1999. High-efficiency gene transfer into skeletal muscle mediated by electric pulses. Proc. Natl. Acad. Sci. USA **96:**4262–4267.
26. **Mir, L. M., L. F. Glass, G. Sersa, J. Teissie, C. Domenge, D. Miklavcic, M. J. Jaroszeski, S. Orlowski, D. S. Reintgen, Z. Rudolf, M. Belehradek, R. Gilbert, M. P. Rols, J. Belehradek, Jr, J. M. Bachaud, R. DeConti, B. Stabuc, M. Cemazar, P. Coninx, and R. Heller.** 1998. Effective treatment of cutaneous and subcutaneous malignant tumours by electrochemotherapy. Br. J. Cancer **77:**2336–2342.
27. **Monath, T. P., and R. R. Nystrom.** 1984. Detection of yellow fever virus in serum by enzyme immunoassay. Am. J. Trop. Med. Hyg. **33:**151–157.
28. **Monath, T. P., and T. F. Tsai.** 1997. Flaviviruses, p. 1133–1186. *In* D. D. Richman, R. J. Whitley, and F. G. Hayden (ed.), Clinical virology. Churchill-Livingtone, New York, N.Y.
29. **Nielsen, H., S. Brunak, and G. von Heijne.** 1999. Machine learning approaches for the prediction of signal peptides and other protein sorting signals. Protein Eng. **12:**3–9.
30. **Obijeski, J. F., A. T. Marchenko, D. H. Bishop, B. W. Cann, and F. A. Murphy.** 1974. Comparative electrophoretic analysis of the virus proteins of four rhabdoviruses. J. Gen. Virol. **22:**21–33.
31. **Phillpotts, R. J., K. Venugopal, and T. Brooks.** 1996. Immunisation with DNA polynucleotides protects mice against lethal challenge with St. Louis encephalitis virus. Arch. Virol. **141:**743–749.
32. **Porter, K. R., T. J. Kochel, S. J. Wu, K. Raviprakash, I. Phillips, and C. G. Hayes.** 1998. Protective efficacy of a dengue 2 DNA vaccine in mice and the effect of CpG immuno-stimulatory motifs on antibody responses. Arch. Virol. **143:**997–1003.
33. **Raviprakash, K., T. J. Kochel, D. Ewing, M. Simmons, I. Phillips, C. G. Hayes, and K. R. Porter.** 2000. Immunogenicity of dengue virus type 1 DNA vaccines expressing truncated and full length envelope protein. Vaccine **18:** 2426–2434.
34. **Roehrig, J. T., J. H. Mathews, and D. W. Trent.** 1983. Identification of epitopes on the E glycoprotein of Saint Louis encephalitis virus using monoclonal antibodies. Virology **128:**118–126.
35. **Schmaljohn, C., D. Custer, L. VanderZanden, K. Spik, C. Rossi, and M. Bray.** 1999. Evaluation of tick-borne encephalitis DNA vaccines in monkeys. Virology **263:**166–174.
36. **Schmaljohn, C., L. Vanderzanden, M. Bray, D. Custer, B. Meyer, D. Li, C. Rossi, D. Fuller, J. Fuller, J. Haynes, and J. Huggins.** 1997. Naked DNA vaccines expressing the prM and E genes of Russian spring summer encephalitis virus and Central European encephalitis virus protect mice from homologous and heterologous challenge. J. Virol. **71:**9563–9569.
37. **Sersa, G., B. Stabuc, M. Cemazar, B. Jancar, D. Miklavcic, and Z. Rudolf.** 1998. Electrochemotherapy with cisplatin: potentiation of local cisplatin antitumour effectiveness by application of electric pulses in cancer patients. Eur. J. Cancer **34:**1213–1218.
38. **Smithburn, K. C., T. P. Hughes, A. W. Burke, and J. H. Paul.** 1940. A neurotropic virus isoalted from the blood of anative of Uganda. Am. J. Trop. Med. Hyg. **20:**471–492.
39. **Tardei, G., S. Ruta, V. Chitu, C. Rossi, T. F. Tsai, and C. Cernescu.** 2000. Evaluation of immunoglobulin M (IgM) and IgG enzyme immunoassays in serologic diagnosis of West Nile virus infection. J. Clin. Microbiol. **38:**2232–2239.