MAJOR ARTICLE

Induction of Sterilizing Immunity against West Nile Virus (WNV), by Immunization with WNV-Like Particles Produced in Insect Cells

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No specific vaccine for West Nile virus (WNV) is currently available for human use. In the present study, we describe the generation of WNV-like particles (WNV-LPs) in insect cells by use of recombinant baculoviruses expressing the WNV structural proteins prME or CprME. BALB/c mice immunized with purified WNV-LPs developed WNV-specific antibodies that had potent neutralizing activities. Mice immunized with prME-like particles (prME-LPs) showed no morbidity or mortality after challenge with WNV. Immunization with prME-LPs can induce sterilizing immunity without producing any evidence of viremia or viral RNA in the spleen or brain. These results suggest that WNV-LPs hold promise as a vaccine candidate for WNV infection.

There is an urgent need for an effective prophylactic vaccine to prevent West Nile virus (WNV) transmission and infection in domestic animals and humans. A killed-virus equine vaccine is in use [1]; however, no human vaccine has been approved. Although passive immunotherapy has been shown to be effective in mouse models [2, 3], its use has been limited in humans [4]. Neither a treatment option nor a proven vaccine for the prevention of WNV infection is available at the present time. Several approaches to the development of a WNV vaccine have demonstrated immunogenicity and protective efficacy, including chimeric [5, 6], DNA [7, 8], and live attenuated vaccines [9]. Virus-like particles (VLPs) synthesized in various expression systems have been used to prevent infection with papillomaviruses [10] and rotaviruses [11]. Such an approach has also been successfully extended to other important human pathogens, such as flaviviruses [12–14]. In the present study, we report the production of WNV-like particles (WNV-LPs) containing the WNV structural proteins, prME and CprME, by use of a recombinant baculovirus in insect cells, and we evaluate the use of WNV-LPs as a vaccine in a mouse model.

MATERIALS AND METHODS

Recombinant baculovirus expressing WNV prME and CprME was generated by use of the Bac-to-Bac baculovirus expression system (Invitrogen), as described elsewhere [15]. cDNA (GenBank accession number AF202541) for prME (nt 335–2427) and CprME (nt 1–2636) was generated from WNV strain HNY1999–infected Vero cells by polymerase chase reaction (PCR) with the following 2 primer sets: for prME, 5′-CTATCAATCGGCGGAGCTC-3′ and 5′-ACCCAGTGTCAGCGTGCA-3′, and for CprME, 5′-GCGGGATCCTAATACGACTCACTATAGGGAGTAGTTCGCCTGTGTGAGCTG-3′ and 5′-GCTTCCCACATTTGRTGYTC-3′. These

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PCR-generated fragments were then cloned into the pGEM-T Easy vector (Promega). pFASTBac-prME and pFASTBac-CprME were generated by subcloning an *Eco*R1 and *Spe*I fragment into the pFASTBac-1 vector (Invitrogen). The correct recombinant baculoviruses were identified by immunofluorescence and immunoblotting with a rabbit anti-E antibody. The procedure for the production and purification of WNV-LPs was similar to that for hepatitis C virus–like particles (HCV-LPs) [15], with some modifications (see the Appendix in the electronic edition). The WNV recombinant proteins prM, E, and NS1 were produced, and rabbit antibodies against them were generated, as described in the Appendix in the electronic edition.

Four groups of 6 BALB/c mice (6–8-week-old females; Jackson Laboratories) were immunized 4 times at 3-week intervals. Mice received injections of 20 μg of WNV-LPs into each quadriceps muscle in 100 μL of PBS, on the basis of the previously described immunization protocol for HCV-LPs [14]. One group received prME-like particles (prME-LPs) alone; a second group received prME-LPs plus AS01B (50 μL); a third group received CprME-like particles (CprME-LPs) alone; and a final group received AS01B (50 μL) alone. The adjuvant AS01B, which contains monophosphoryl lipid A and QS21, was provided by GlaxoSmithKline. Serum samples were collected before immunization and 2 weeks after each immunization and were analyzed for anti-M, -E, or -NS1 antibodies by both ELISA and virus neutralization assay (see the Appendix in the electronic edition).

Mice were housed in biosafety level–3 conditions and were given food and water ad libitum. Mice were acclimatized for at least 1 week before challenge. Immunized mice and 6 age-matched, female BALB/c mice were inoculated intraperitoneally with 10^4 pfu of WNV that had been derived from an infectious clone [16]. A group of 6 age-matched, female BALB/c mice were inoculated with diluent alone (PBS, 1% fetal bovine serum). Mice were weighed and were scored daily for clinical signs of disease, including ruffled fur, hunching, and paresis. Morbidity was defined as exhibition of >10% weight loss and/or clinical signs for $\geq$2 days. Mice that exhibited severe disease were killed. Surviving mice were killed 31 days after inoculation. Mice were bled on day 3 after inoculation. Spleens and brains were harvested from mice at death or on the day of killing, and blood was also harvested from mice that were killed. Brains were divided sagitally at the midline. One-half of each brain was processed for RNA extraction, as described elsewhere [17].

The RNA from serum, spleens, and brains were analyzed for WNV in the envelope gene by real-time reverse-transcription PCR (RT-PCR) with primers, as described elsewhere [17]. RNA copies were quantified by use of a standard curve of 50–5×10^5 copies of RNA per reaction and are reported as the number of copies per milliliter of serum or per gram of tissue. The thresholds of detection for serum, spleen, and brain assays were 5×10^3 copies/mL, 1.5×10^4 copies/g, and 7.5×10^3 copies/g, respectively. Virus was titered by use of Vero cells [17]. Fixed brains were sectioned, stained with hematoxylin-eosin (HE), and blindly assessed for abnormalities by light microscopy.

RESULTS AND DISCUSSION

Recombinant baculoviruses bvWNVprME and bvWNVCprME (figure 1*A*), which contain the coding sequences for prM and E and for core, prM, and E, respectively, were shown to direct the production of WNV-LPs in insect cells. By use of a modified method described elsewhere for HCV-LPs [15], WNV-LPs were harvested and purified by iodixanol gradient centrifugation (see the Appendix in the electronic edition). WNV E protein was detected by ELISA (figure 1*B*). The peak of E reactivity corresponds to the peak total protein concentration and to buoyant densities of 1.12–1.14 g/mL. Western blot analysis (figure 1*C*) revealed that these fractions contained a 50-kDa E protein band and a 20-kDa prM protein band in both the prME-LP and CprME-LP preparations. The mature form of M protein was not detected, probably because the furin required for the proper cleavage of prM to M is not expressed efficiently in Sf9 insect cells [18]. A core protein band was also detected at 12 kDa in the CprME-LP preparation (data not shown). Examination by cryoelectron microscopy revealed that WNV-LPs are polymorphic in appearance and have a diameter of 40–60 nm (figure 1*D*). The typical yield of WNV-LPs from the procedure is ~1–2 mg/100 mL of culture, which is substantially greater than the reported yields of other flavivirus-like particles generated in mammalian cells [12, 19].

Groups of BALB/c mice ($n = 6$) were immunized with prME-LPs alone, CprME-LPs alone, prME-LPs plus AS01B, or AS01B alone, with 4 injections given at 3-week intervals. Although all of the mice immunized with prME-LPs (with or without the AS01B adjuvant) developed anti-E antibodies after the fourth immunization, AS01B enhanced the anti-E antibody response significantly, from 317 to 8128, and also enhanced the anti-M antibody response, from 50 to 142 (table 1). CprME-LPs induced weaker antibody responses to the M and E proteins. One mouse in the AS01B group died of unknown causes after the first immunization.

The pooled serum samples collected from each group at 2 weeks after the fourth immunization were assayed for titers of neutralizing antibodies (table 1). Titers were determined to be 37 in the prME-LP group and 75 in the prME-LP plus AS01B group. The CprME-LP group did not develop detectable titers of neutralizing antibody. None of the serum samples from the AS01B group had any detectable antibodies to the E and M proteins or neutralizing antibodies to WNV.

Immunized mice were challenged with 10^4 pfu of WNV. This dose is >100 times the ID_{50} identified in a previous study in 6-month-old BALB/c mice (data not shown), and it was chosen

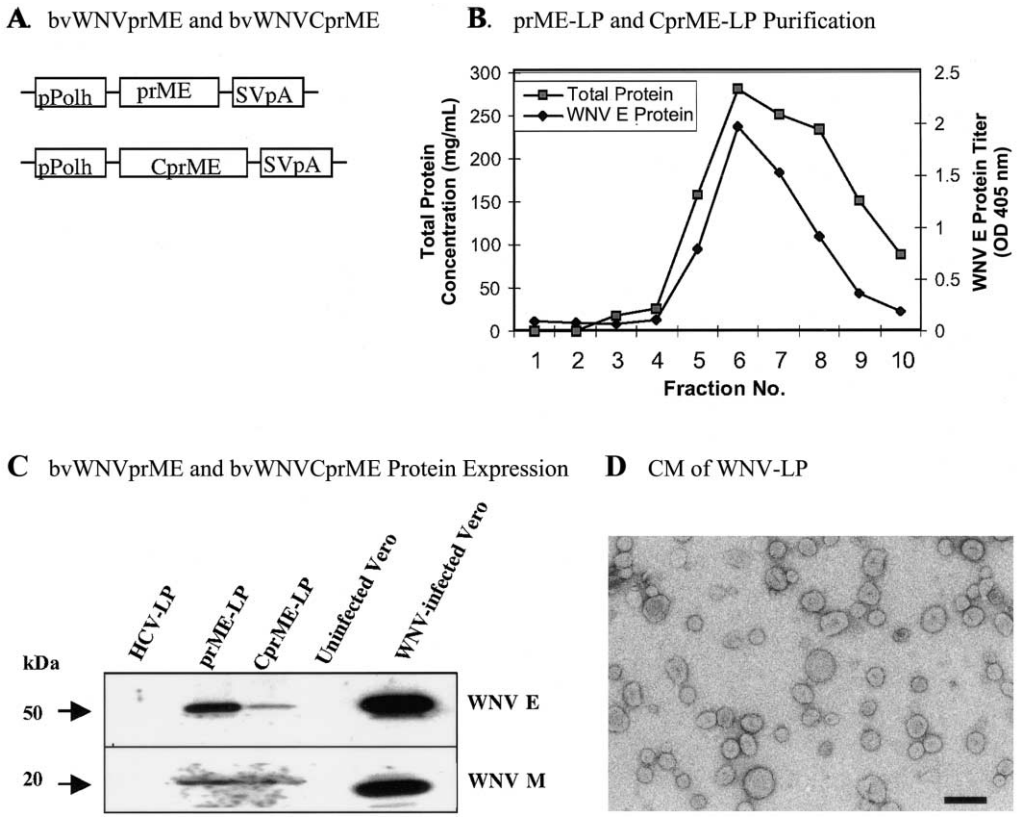

Figure 1. Construction and production of West Nile virus–like particles (WNV-LPs) in insect cells. *A,* Map depicting segments of the WNV genome in the recombinant baculovirus expression vector; the bvWNVprME construct (top) contains the coding sequences for prM and E, and the bvWNVCprME construct (bottom) contains the coding sequences for core, prM, and E. pPolh, baculovirus polyhedrin promoter; SV40pA, simian virus 40 polyadenylation sequence. *B,* Characterization of WNV-LPs. WNV-LPs were purified from Sf9 insect cells by iodixanol gradient centrifugation. Ten fractions collected from the top of the gradient were analyzed for total protein content and the titer of WNV E protein by ELISA. *C,* Western blot analysis of purified prME-like particles (prME-LPs) and CprME-like particles (CprME-LPs) with rabbit anti-E or -M antibodies. Uninfected Vero cells and hepatitis C virus–like particles (HCV-LPs) were used as negative controls, and WNV-infected Vero cells were used as a positive control. *D,* Cryoelectron micrograph (CM) of purified prME-LPs. Bar, 100 nm.

to enhance the probability of discriminating differences in morbidity among groups. Mice were challenged 2 months after the fourth immunization (table 2). Two groups of unimmunized mice (6 mice each) of similar age were included as control mice in this challenge experiment. One group was challenged with the same dose of WNV as were the immunized groups, and the other group was not challenged. Morbidity and mortality in the unimmunized/challenged group were 50% and 17%, respectively. There was no morbidity or mortality in either the prME-LP group or the prME-LP plus AS01B group. In contrast, 67% morbidity was observed in the CprME-LP group. The presence of high titers of anti-E antibodies before challenge correlated with protective immunity, and all mice had a further increase in titers of anti-E antibodies after challenge, a result consistent with the presence of an anamnestic response directed toward the VLPs, of which the E protein is the major immunogenic component. All of the surviving mice were examined for pathologic abnormalities in the brain at the time of killing on day 31 after challenge. HE-stained brain sections showed no significant neuropathologic damage.

Viral replication was analyzed after challenge, to determine whether immunization with WNV-LPs induced sterilizing protective immunity. Viremia was assayed during the peak viremic phase on day 3 after challenge (table 2 and, in the electronic edition, figure 2). Because it is possible that the immunized mice had neutralizing antibodies by day 3, viremia was measured by both plaque-forming assay and RT-PCR. Postchallenge viremia (infectious virus or viral RNA) was detected in all 6 of the mice in the unimmunized/challenged group, in 5 (83%) of the 6 mice in the CprME-LP group, and in 4 (67%) of the 6 mice in the prME-LP group; however, 0 of the 6 mice in the prME-LP plus AS01B group had circulating infectious virus or viral RNA in serum after challenge. Although 4 of the 6 mice in the prME-LP group had viral nucleic acid (as detected by RT-PCR), only 2 had infectious virus (as detected by plaque-forming assay). In addition, the geometric mean viral titer of

Table 1. Antibody response in mice immunized with West Nile virus–like particles (WNV-LPs).

	ELISA						
	Anti-WNV E protein		Anti-WNV M protein			Neutralization assay	
Mouse group	Before challenge	After challenge	Before challenge	After challenge	NS1 seroconversion, proportion of mice	Before challenge	After challenge
Unimmunized/unchallenged	ND	<50	ND	<50	0/6	ND	0
Unimmunized/challenged	ND	2432	ND	<50	4/5	ND	15
AS01B	<50	3200	<50	<50	4/4	0	37
prME-LPs	317	5689	<50	89	3/6	37	62
prME-LPs plus AS01B	8128	45,709	112	355	1/6	75	75
CprME-LPs	86	7217	<50	<50	5/6	0	57

NOTE. Serum antibody titers were determined after the last of 4 immunizations. For each group, the geometric mean of antibody titers was calculated. The titer for a mouse with a negative ELISA value at a serum dilution of 50 was arbitrarily set at 50 for the calculation of the geometric mean. The results of statistical analyses were as follows (by Mann-Whitney *U* test or Fisher's exact test). Anti-WNV E titer before challenge: $P = .018$, for prME-like particles (prME-LPs) vs. AS01B; $P = .0009$, for prME-LPs plus AS01B vs. AS01B; and $P = .026$, for prME-LPs vs. prME-LPs plus AS01B. NS1 seroconversion: $P = .024$, for prME-LPs vs. AS01B, and $P = .001$, for prME-LPs plus AS01B vs. AS01B. Neutralization titer before challenge: $P = .0007$, for prME-LPs vs. AS01B, and $P = .0003$, for prME-LPs plus AS01B vs. AS01B. CprME-LPs, CprME-like particles; ND, not done.

the prME-LP group (1.58×10^4 copies/mL) was more than an order of magnitude lower than that of the unimmunized/challenged group (2×10^5 copies/mL) ($P = .027$).

As an additional measure of postchallenge viral replication, the presence of viral RNA in the spleen and brain was determined at the time of death or at killing (day 31 after challenge). Viral RNA was detected in the brains of ~50% of the mice in the unimmunized/challenged, AS01B, and CprME-LP groups (table 2). In contrast, none of the mice that received either prME-LPs alone or prME-LPs plus AS01B had detectable viral RNA in the brain, suggesting that these mice were protected from neuroinvasion. Viral RNA was detected in the spleens of all of the mice in the unimmunized/challenged and AS01B groups, providing evidence for active replication in these control groups. Viral RNA was detected in 2 of the 6 and 5 of the 6 mice in the prME-LP and CprME-LP groups, respectively, but in 0 of the mice in the prME-LP plus AS01B group. Thus, viral replication was partially inhibited in the mice immunized with prME-LPs alone and was completely inhibited in the mice immunized with prME-LPs plus AS01B.

Seroconversion to the WNV nonstructural protein NS1 was assayed after viral challenge. Eight of the 9 mice in the unimmunized/challenged and AS01B groups and 5 of the 6 mice in the CprME-LP group developed an anti-NS1 antibody response after challenge with WNV (table 2). In contrast, only 3 of the 6 mice in the prME-LP group and 1 of the 6 mice in the prME-LP plus AS01B group seroconverted to anti-NS1 antibody, indicating that immunization with prME-LPs (espe-

Table 2. Protection of mice immunized with West Nile virus–like particles (WNV-LPs) from challenge with WNV.

				WNV detected in			
Mouse group	Virus	Morbidity	Mortality	Serum[a]	Serum[b]	Spleen[c]	Brain[c]
Unimmunized/unchallenged	Mock (diluent)	0/6	0/6	0/6	0/6	0/6	0/6
Unimmunized/challenged	WNV	3/6	1/6	5/6	6/6	6/6	3/6
AS01B	WNV	2/5	1/5	4/5	4/5	5/5	2/5
prME-LPs	WNV	0/6	0/6	2/6	4/6	2/6	0/6
prME-LPs plus AS01B	WNV	0/6	0/6	0/6	0/6	0/6	0/6
CprME-LPs	WNV	4/6	0/6	5/6	5/6	5/6	3/6

NOTE. Data are proportion of mice. Two months after the last of 4 immunizations, mice were challenged intraperitoneally with 10^4 pfu of WNV. The results of statistical analyses are as follows (by Fisher's exact test; control combines the results from the unimmunized/challenged and AS01B groups). Morbidity: $P = .03$, for prME-like particles (prME-LPs) vs. control, and $P = .03$, for prME-LPs plus AS01B vs. control. WNV detected in serum by plaque-forming assay: $P = .04$, for prME-LPs vs. control, and $P = .0016$, for prME-LPs plus AS01B vs. control. WNV detected in serum by reverse-transcription polymerase chain reaction (RT-PCR): $P = .0002$, for prME-LPs plus AS01B vs. control, and $P = .01$, for prME-LPs vs. prME-LPs plus AS01B. WNV detected in spleen: $P = .007$, for prME-LPs vs. control, and $P = .0005$, for prME-LPs plus AS01B vs. control. WNV detected in brain: $P = 0.003$, for prME-LPs vs. control, and $P = .003$, for prME-LPs plus AS01B vs. control. CprME-LPs, CprME-like particles.

[a] Positive for infectious WNV by plaque-forming assay on day 3 after challenge.

[b] Positive for viral RNA by RT-PCR on day 3 after challenge.

[c] Positive for viral RNA on day of death or killing.

cially in the presence of adjuvant) prevented productive infection and, therefore, exposure to NS1 after challenge with WNV. These results, together with the lack of detectable viremia and viral RNA in the spleens, suggest that sterilizing immunity might be achieved in mice immunized with prME-LPs.

It is not apparent why the CprME particles, differing from the prME particles only in the addition of core protein, are less immunogenic. One explanation could be that the CprME preparation is less pure, resulting in lower immunogenicity. It is also possible that the particles formed by the CprME construct are less immunogenic because of the subtle structural difference. Alternatively, the core protein may somehow diminish the immune response to the VLPs.

It is interesting to note that the neutralization titer in the mice immunized with prME-LPs plus AS01B did not increase after challenge, probably because the preexisting neutralization titer was sufficient to protect the mice from infection. It is conceivable that cell-mediated immunity induced by immunization with VLPs might contribute to the observed sterilizing immunity [14]. The relative contribution of humoral versus cellular components in the protective immunity observed here awaits future study.

Several published studies have described promising approaches to vaccine development for WNV. Chimeric or attenuated flaviviruses that are closely related to WNV have been shown to successfully protect animals from WNV infection [5, 6, 9]. DNA immunization by use of plasmid-expressing WNV proteins [7] and Kunjin virus [8] have also been applied successfully in the animal model. Despite the promise of these vaccine candidates, safety concerns will always be an issue. However, VLP-based vaccines are noninfectious and are easily controlled for quality and safety. The recent successful development of a human papillomavirus vaccine based on VLP technology [10] lends credence to the promise of this approach in the development of an effective WNV vaccine.

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