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West Nile premembrane-envelope genetic vaccine encoded as a chimera containing the transmembrane and cytoplasmic domains of a lysosome-associated membrane protein: increased cellular concentration of the transgene product, targeting to the MHC II compartment, and enhanced neutralizing antibody response

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Abstract

A genetic vaccine for West Nile virus (WN) has been synthesized with the WN premembrane-envelope (WN preM-E) gene sequences encoded as a chimera with the transmembrane and carboxyl terminal domains of the lysosome-associated membrane protein (LAMP). The LAMP sequences are used to direct the antigen protein to the major histocompatibility class II (MHC II) vesicular compartment of transfected professional antigen-presenting cells (APCs). Vaccine constructs encoding the native WN preM-E and WN preM-E/LAMP chimera were synthesized in pVAX1 and pITR plasmid backbones. Extracts of human fibroblast 293 and monkey kidney COS-7 cells transfected with the WN preM-E/LAMP chimera constructs contained much greater amounts of E than did the cells transfected with constructs encoding the native WN preM-E. This difference in the concentration of native E and the E/LAMP chimera in transfected cells is attributed to the secretion of native E. The amount of preM protein in cell extracts, in contrast to the E protein, and the levels of DNA and RNA transcripts, did not differ between WN preM-E- and WN preM-E/LAMP-transfected cells. Additionally, confocal and immunoelectron microscopic analyses of transfected B cells showed localization of the WN preM-E/LAMP chimera in vesicular compartments containing endogenous LAMP, MHC II, and H2-M, whereas native viral preM-E lacking the LAMP sequences was distributed within the cellular vesicular network with little LAMP or MHC II association. Mice immunized with a DNA construct expressing the WN preM-E/LAMP antigen induced significant antibody and long-term neutralization titers in contrast to the minimal and short-lived neutralization titer of mice vaccinated with a plasmid expressing the untargeted antigen. These results underscore the utility of LAMP targeting of the WN envelope to the MHC II compartments in the design of a genetic WN vaccine.
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Keywords: West Nile virus DNA vaccine; LAMP; MIICs; MHC II; H-2M; Neutralizing antibody; Electron microscopy

Abbreviations: preM-E, premembrane-envelope; LAMP, lysosome-associated membrane protein; MHC, major histocompatibility complex; MIICs, MHC II-containing compartments; H-2M, peptide editor; WN, West Nile virus; JE, Japanese encephalitis virus; pVax-WN, pVAX WN preM-E DNA construct; pVax-WNL, pVAX WN preM-E/LAMP chimera DNA construct.

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Introduction

The *Flaviviridae* family of viruses includes several important pathogens that cause diseases of major public health importance, including yellow fever, dengue, Japanese encephalitis (JE), and West Nile (WN) viruses. As there are no antiviral drugs active against flaviviruses, vaccination remains the most effective means of disease prevention, with the development and application of inactivated and live attenuated virus vaccines for use in humans against yellow fever, tick-borne encephalitis, and Japanese encephalitis viruses (Barrett, 2001; Pugachev et al., 2003). These vaccines have proven efficacy; however, adverse reactions have been reported and there is a potential risk of gene reversion or recombination to a virulent virus (Berkhout et al., 1999; Gundlach et al., 2000; Liu et al., 2003). Thus, the search for the development of safer forms of vaccines continues, including genetic vaccines encoding specific viral sequences that offer many possible advantages (Donnelly et al., 2003; Gurunathan et al., 2000; Hall et al., 2003; Liu, 2003; Putnak et al., 2003).

Among flaviviruses, West Nile (WN) has recently assumed increasing importance because of its global spread. WN is an important human pathogen that is maintained in nature as a mosquito-borne viral disease of birds with humans and other mammals as occasional hosts (Petersen et al., 2003). The virus is widely distributed in Africa and Europe, and the rapid spread since 1999 in the United States is attributed to viremic migratory birds as well as enhanced mosquito distribution (Hubalek et al., 2000; Nedry and Mahon, 2003; Rappole and Hubalek, 2003). Research in the development of a variety of WN vaccines has been reported from several laboratories. Attenuated, live virus vaccines include a replicon vaccine vector encoding the infectious full-length RNA genome of Kunjin (Hall et al., 2003) and chimera viruses bearing the membrane precursor and envelope protein genes of WN on backbones of dengue (Pletnev et al., 2003) and yellow fever viruses (Arroyo et al., 2001). DNA vaccines encoding WN virus proteins that induced protection in animal challenge systems have also been described (Davis et al., 2001; Turell et al., 2003).

In this report, we describe the development of a West Nile virus genetic vaccine based upon a molecular adjuvant methodology designed to target DNA-encoded antigens to compartments of antigen-presenting cells (APCs) that contain the major histocompatibility class II (MHC II) protein known to function in antigen processing and presentation to $CD4^+$ T cells (Drake et al., 1999; Geuze, 1998; Kleijmeer et al., 1997, 2001; Turley et al., 2000). DNA constructs are synthesized with the antigen sequences linked to those of the lysosomal-associated membrane protein (LAMP) (Chen et al., 1985) containing the LAMP targeting signals (Guarnieri et al., 1993) that direct the LAMP/antigen chimera to specialized endosomal–lysosomal compartments that contain MHC II. LAMP targeting of the WN premembrane-envelope (preM-E) protein is shown to elicit a significant and long-lasting neutralizing antibody response in mice as compared to DNA encoding the wild-type WN preM-E. Several previous studies have also demonstrated the enhanced antigen-specific immune responses of such antigen/LAMP chimeras of vaccinia virus vector and DNA plasmid vaccine preparations (Marques et al., 2003; Ruff et al., 1997; Wu et al., 1995), including studies of a dengue virus vaccine (Lu et al., 2003; Raviprakash et al., 2001). The function of LAMP targeting sequences to increase the trafficking of genetic vaccines to the MHC II compartment can be viewed as particularly important for the activation of $CD4^+$ T cells and the development of memory B and $CD8^+$ T cells, an essential criterion for an effective prophylactic vaccine. An exceptional additional finding in these studies was the increased cellular concentration of the preM-E/LAMP chimera protein in transfected cells as compared to the native preM-E. Unlike the native WN E that is secreted from the cell, the WN preM-E/LAMP chimera antigen is present in the MHC II compartment of transfected B cells as shown by confocal and immunoelectron microscopy (IEM) using double and triple labeling for the chimeric viral/LAMP, MHC II, and H-2M proteins, the latter of which is closely related to antigen presentation (Neefjes, 1999). These results suggest the application of the WN preM-E/LAMP chimera genetic vaccine as a candidate for higher animal challenge studies in the development of a WN DNA vaccine.

Results

Genetic vaccine plasmid constructs and expression of the WN preM-E and preM-E/LAMP proteins

Expression plasmids of pVAX-1 and pITR containing the native WN preM-E sequences and the chimera containing the LAMP transmembrane and cytoplasmic domains replacing the corresponding WN E sequences (Fig. 1A) were constructed as described in Materials and methods. Validation of plasmid protein expression was conducted by transfection of human fibroblast 293 and monkey kidney COS-7 cells followed by SDS-PAGE of total cell extracts and Western blotting (Fig. 1B). Transfected cells showed protein bands corresponding to the processed forms of the preM and E proteins. Additional bands stained with the polyclonal antiserum were nonspecific and present in extracts of cells transfected with the empty vector. An important finding was the enhanced cellular concentration of E as an E/LAMP chimera. The amount of steady-state E protein was markedly lower in cells transfected with the native preM-E construct as compared to that present in cells transfected with the preM-E/LAMP chimera construct. Overexposed Western blot analysis showed only a slight band of the E protein in lanes containing lysates from cells

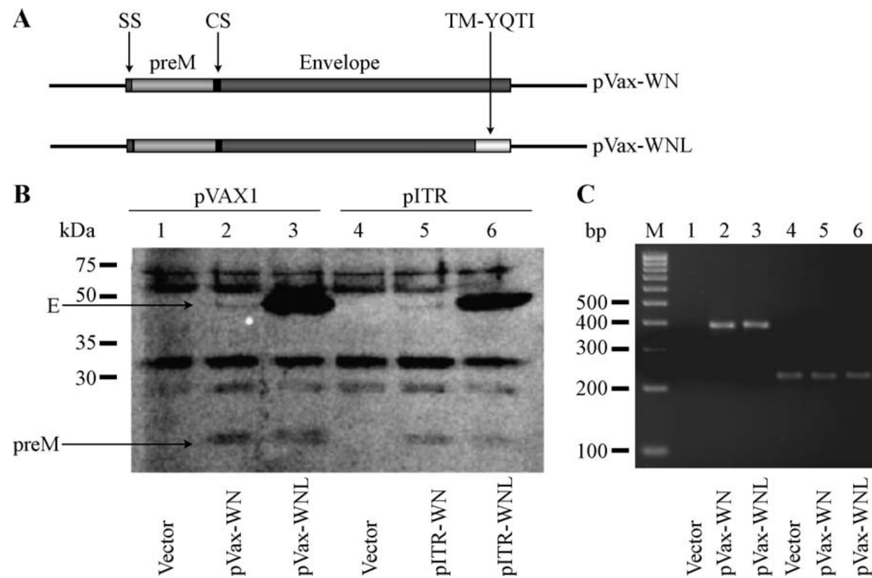
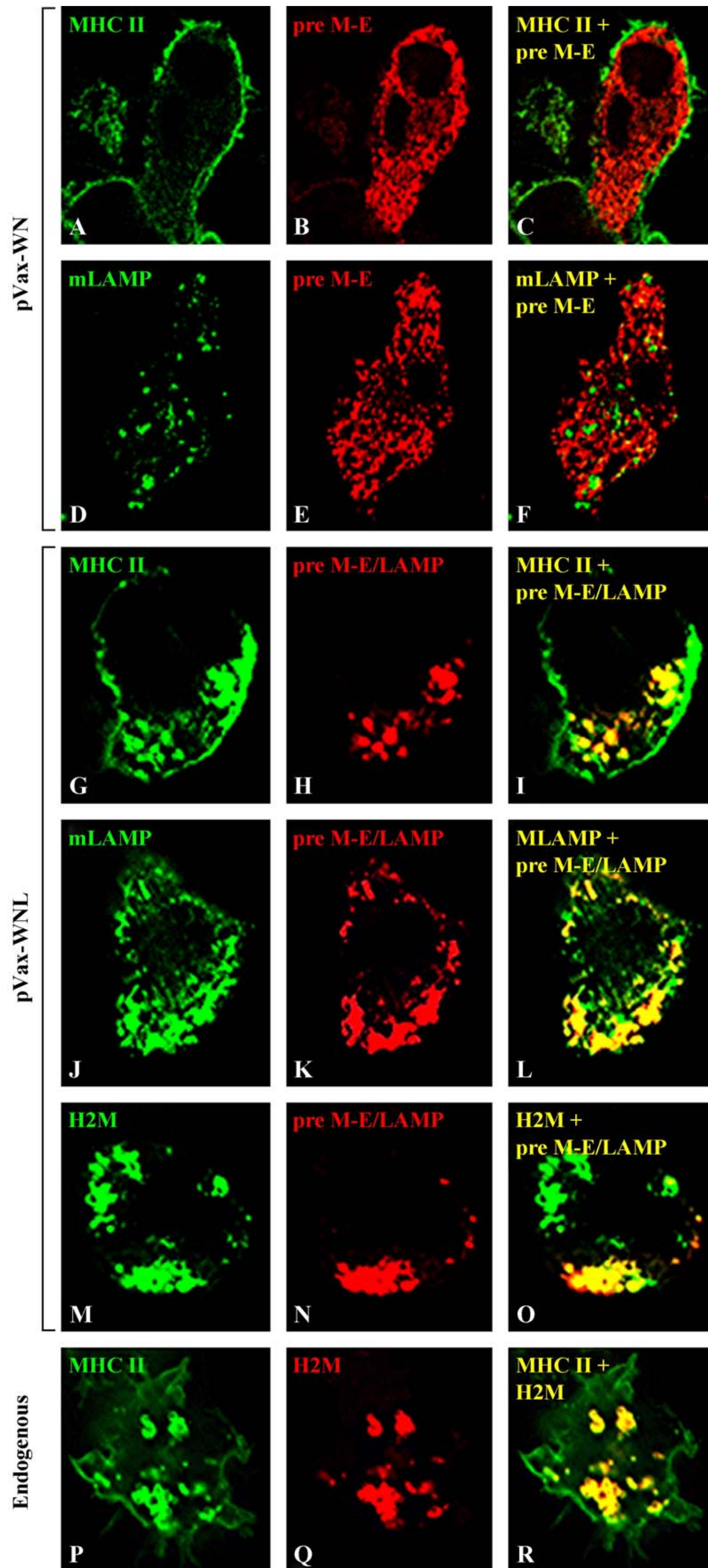


Fig. 1. Construction and expression of WN preM-E and preM-E/LAMP plasmids. (A) Schematic representation of the plasmids used in this study. The preM-E and preM-E/LAMP DNAs were constructed in the pVAX1 vector as described in Materials and methods. The pVax-WN plasmid encodes the unmodified WN preM-E, and the preM-E chimera pVax-WNL modified to include the LAMP transmembrane and carboxyl terminal signal sequences. Boxed areas represent the open reading frames as indicated. The arrows indicate the positions of the various sequences: signal sequence for endoplasmic reticulum translocation (SS), signal peptidase cleavage site between preM-E (CS), and the LAMP transmembrane and carboxyl terminal signal sequences (TM-YQTI). (B) Western blot analysis. Human 293 cells were transfected with the vector, wild-type WN preM-E, and preM-E/LAMP chimera DNA plasmids constructed in pVAX1 and pITR expression vectors as indicated, and probed with anti-WN hyperimmune ascitic fluid. The molecular weight markers are shown on the left. E and preM denote the positions of expressed WN envelope and premembrane proteins. Similar results were obtained from monkey kidney COS-7 cells transfected with the above plasmids. (C) Specific detection of WN RNA transcripts. Total RNA and DNA of transfected cells were analyzed by real-time PCR. The WN E fragments were obtained only from total RNA of cells transfected with pVax-WN (lane 2) and pVax-WNL (lane 3), and not from cells transfected with the vector (lane 1). The cDNA amplifications were not due to contaminating transfected plasmids. Lanes 4–6 indicate amplification of the pVAX1 vector backbone from total DNA of cells transfected with the vector, pVax-WN, and pVax-WNL, respectively. All of the above results are representative of three independent experiments. M, 100 bp DNA marker.

transfected with preM-E constructs. This is in contrast to the steady-state level of preM protein, which was not significantly different between the two preM-E and preM-E/LAMP cell lysates.

Further analysis to determine if differences in the amounts of E protein could be attributed to the amount of plasmid or mRNA transcription of the expression of native preM-E and preM-E/LAMP was conducted by quantitative real-time PCR analysis of cellular DNA and RNA. Total RNA and DNA were isolated simultaneously from the same COS-7 cells transfected with either pVax-WN or pVax-WNL. Specific primers were used to determine the amount of intracellular plasmid in the transfected cells. When normalized to the total amount of genomic DNA recovered, there was no significant difference in the amounts of plasmid DNA in cells transfected with pVax-WN or pVax-WNL constructs. The expression levels of WN RNA transcribed from pVax-WN and pVax-WNL plasmids in transfected COS-7 cells were also compared and no significant differences were observed (data not shown). Validation that the amplifications from reversed-transcribed RNA samples were not due to contaminating plasmids was conducted by performing reverse transcription (RT) in the presence or absence of random primers, followed by PCR using WN E-specific primers that would yield a product of 384 bp. Amplifications were observed only with samples reverse transcribed with random primers, indicative of a lack of contamination from plasmids in the total RNA samples. Reverse-transcribed WN RNA by PCR was detectable only from cells transfected with pVax-WN and pVax-WNL, and not from cells transfected with a control vector (Fig. 1C) or from mock-transfected cells (data not shown).

Fig. 2. Confocal microscopy of endogenous LAMP, MHC II, and H-2M, and WN antigens in B cells. Mouse LB27.4 B cells, transfected with either pVax-WN or pVax-WNL plasmids, were stained for the WN (red) and endogenous LAMP, MHC II, or H-2M (green) molecules. Colocalization of the viral antigens was visualized by superimposing images of the WN antigens with the endogenous proteins and shown as yellow in the merged images. The wild-type WN preM-E antigen showed a reticular distribution and did not colocalize with the endogenous MHC II (A–C) or LAMP (D–F) molecules. Note that as the steady-state expression of wild-type WN antigens was low, pixel distribution was adjusted with respect to endogenous LAMP and MHC II to enable merging. In contrast, the chimera WN preM-E/LAMP antigen showed a punctuated and vesicular profile and colocalized extensively with endogenous MHC II (G–I) and LAMP (J–L). The endogenous MHC II and H-2M showed colocalization internal of the B cells (P–R). The preM-E/LAMP viral antigen was observed to colocalize with the endogenous H-2M proteins (M–O). Image processing to adjust equal pixel density distribution was carried out on Adobe Photoshop 6.0.


pVax-WN
MHC II
pre M-E
MHC II + pre M-E
A
B
C
mLAMP
pre M-E
mLAMP + pre M-E
D
E
F
pVax-WNL
MHC II
pre M-E/LAMP
MHC II + pre M-E/LAMP
G
H
I
mLAMP
pre M-E/LAMP
MLAMP + pre M-E/LAMP
J
K
L
H2M
pre M-E/LAMP
H2M + pre M-E/LAMP
M
N
O
Endogenous
MHC II
H2M
MHC II + H2M
P
Q
R

WN preM-E/LAMP is colocalized with endogenous LAMP, MHC II, and H-2M in B cells

The cellular trafficking of the unmodified and LAMP-targeted WN preM-E protein was analyzed by comparing the cellular steady-state localization of the transgene-expressed viral proteins to the localization of endogenous LAMP, MHC II, and H-2M molecules by confocal microscopy and immunoelectron microscopy (IEM). The mouse B lymphoma cell line (LB 27.4) used in this study exhibits the phenotype of B cells similar to A20 and IIA1.6 B cells (Lankar et al., 2002). Mouse LB27.4 B cells were transfected with either the unmodified pVax-WN (preM-E) or pVax-WNL (preM-E/LAMP). Confocal microscopy showed the reticular, cytoplasmic distribution of the transgene wild-type WN preM-E protein with no significant colocalization with endogenous MHC II or LAMP molecules (Figs. 2A–F). In contrast, the WN preM-E/LAMP protein was extensively colocalized with endogenous LAMP and MHC II molecules in a typical lysosomal distribution (Figs. 2G–L). The localization of the preM-E/LAMP was also studied in relation to H-2M, an essential mediator for MHC II peptide loading and found predominantly in the late MHC II-containing compartments (MIICs; Geuze, 1998; Neefjes, 1999). Endogenous H-2M molecules in B cells are colocalized with the endogenous MHC II molecules (Figs. 2P–R), consistent with the findings in A20 and IIA1.6B cells (Lankar et al., 2002). The WN preM-E/LAMP protein was found to colocalize with the endogenous H-2M (Figs. 2M–O) as well as the MHC II, further demonstrating the altered targeting of the preM-E/LAMP protein to antigen-presenting compartments. These findings are representative of several independent experiments.

Immunoelectron microscopy (IEM) demonstration of WN preM-E/LAMP in MHC II compartments

The ability of the LAMP carboxyl terminal targeting signal to direct trafficking of the viral antigen to the MHC II compartment was further studied in detail by IEM on ultrathin sections of transfected B cells (Fig. 3). Previous studies by others have shown MHC II present in B cells in classical multilaminar (MLC) and multivesicular (MVC) vesicles and are involved in antigen processing and MHC II presentation (MIICs) (Kleijmeer et al., 1997). The transgene WN preM-E/LAMP chimera antigen was also present in these MIIC vesicles and colocalized with endogenous LAMP and MHC II molecules (Figs. 3D–F). In contrast, unmodified wild-type WN preM-E antigen was distributed throughout the cytoplasm as well as at the peripheral plasma membrane

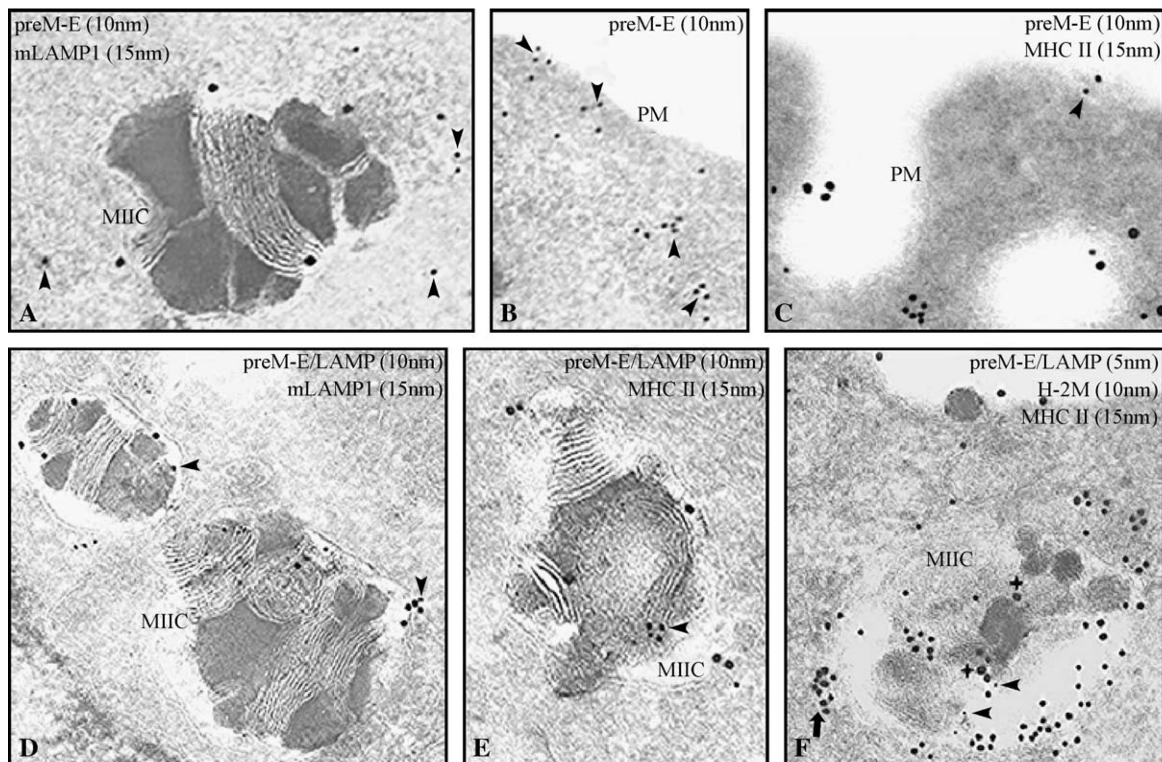


Fig. 3. Immunoelectron microscopic images (IEM) of B cells transfected with pVax WN and pVax-WNL. (A–C) Ultrathin sections of B cells transfected with pVax-WN and immunolabeled with ID4B antibody (15 nm, anti-mLAMP), M5/114 antibody (15 nm, anti-MHC II), and 6B6C-1 (10 nm, anti-E). (D–F) IEM images of B cells transfected with pVax-WNL and immunolabeled with ID4B antibody (15 nm), M5/114 antibody (15 nm), K553 (10 nm, anti-H2M), and 6B6C-1 (5 or 10 nm) showing the WN protein in the endogenous MIIC. The arrowhead, notched arrow, and star indicate representative WN E, H-2M, and MHC II proteins, respectively. PM, plasma membrane; MIIC, MHC II containing compartment.

where surface MHC II was also located and was neither colocalized with endogenous LAMP nor located within the MIICs of B cells (Figs. 3A–C). Triple immunolabeling also showed the presence of the chimera WN preM-E/LAMP antigen with endogenous H-2M molecules as well as with MHC II (Fig. 3F).

Enhanced immune response of mice to a genetic vaccine with the WN viral preM-E sequence fused to the LAMP targeting motif

The immune response to LAMP-modified WN preM-E protein was analyzed with mice and followed for 2 years after immunization. Four groups of five animals each were injected subcutaneous at the base of the tail with 50 μg of endotoxin-free plasmid DNA on days 0, 21, 42, and 63 (Fig. 4A) with pVAX vector expression constructs encoding: (a) the negative control pVax1 vector alone; (b) unmodified preM-E sequences (pVax-WN); and (c) preM-E fused to the LAMP transmembrane and cytoplasmic sequences (pVax-WNL). As a positive control, animals were injected with 10 μg total WN virion protein emulsified with complete Freund's adjuvant and subsequently boosted with the same preparation with incomplete Freund's adjuvant. The antibody endpoint titers of individual mouse in each group were measured at days 55, 62, 90, and 125 by ELISA (Fig. 4B). At day 55, two of the five unmodified preM-E vaccinated mice showed appreciable endpoint titers, which increased to three mice at day 62. However, the antibody responses of these mice were not sustained, and by day 90 onwards, these mice no longer showed significant WN-specific endpoint antibody titers. In contrast, all mice immunized with pVax-WNL showed antibody responses at day 55, with increasing endpoint titers from days 55 to 125.

Neutralizing antibodies, determined by a standard plaque reduction neutralization test (PRNT), were assayed with pooled sera of each group of mice starting with sera dilutions from 1/20 to 1/1280 in 2-fold serial increments. None of the sera samples obtained from the WN preM-E group, collected at days 62, 90, and 125, showed a significant difference in neutralizing antibody responses compared to the negative control group immunized with pVAX1 (Table 1). In contrast, there was a significant neutralization titer response in sera from mice immunized with the WN preM-E/LAMP construct at days 90 and 125. This neutralization titer response from mice immunized with WN preM-E/LAMP construct was comparable to the positive control group of mice immunized with the WN virion proteins in Fruend's adjuvant. No significant neutralization titer was detected in the pooled sera obtained at day 62 despite high WN-specific antibody titers in two of the five immunized mice. The presence of memory B cell response was assessed with the groups of mice immunized at day 716, 19 months after the last boost, and the blood collected 2 weeks later at day 730. While there was no significant neutralization with sera from the WN preM-E group, there was appreciable neutralization with sera from

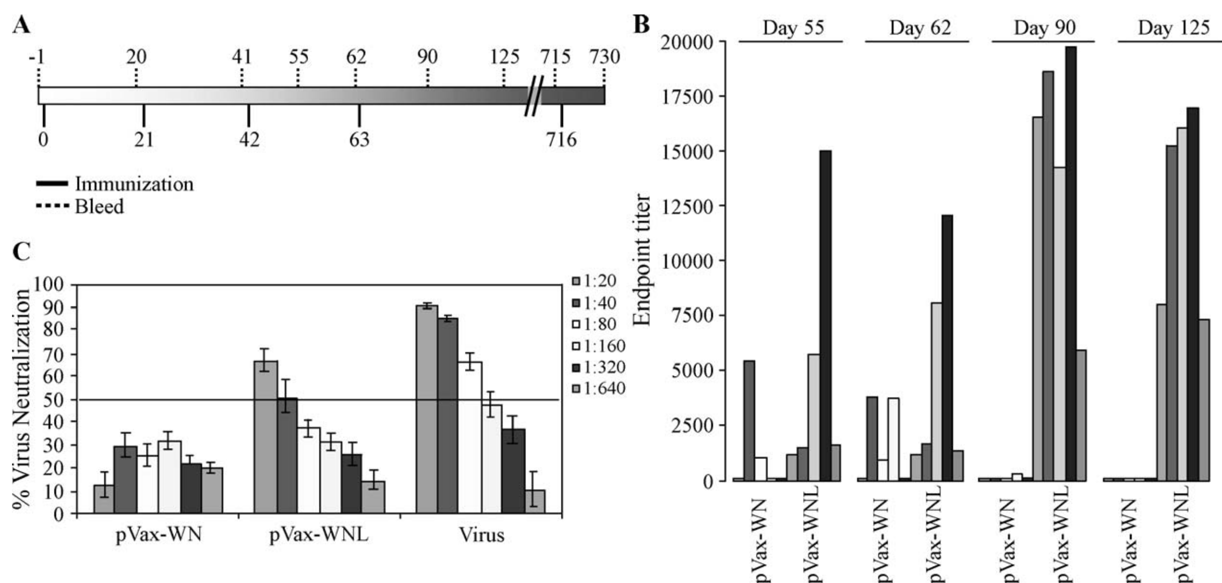


Fig. 4. Immunization protocol and antibody responses of individual mouse immunized with pVax-WN and pVax-WNL plasmids. (A) Immunization schedule. Mice ($n = 5$) were immunized on days 0, 21, 42, and 63 with the WN plasmids and virion proteins as described in Materials and methods, and blood collected from days −1 to 730 as indicated. (B) The endpoint titers of WN-specific IgG responses obtained from sera collected on days 55, 62, 90, and 125. Each bar represents the endpoint titer of serum obtained from individual mice in their respective groups. (C) Plaque reduction neutralization assay at day 730. Sera samples collected from groups of mice immunized with pVax-WN, pVax-WNL, and virion proteins were pooled and used in the PRNT assay, starting at 1:20 sera dilution. The percentage of virus neutralization was determined for each group at various dilutions and shown as a mean percentage with one standard deviation.

Table 1
50% virus neutralization titers ($PRNT_{50}$)

Groups	Day 62	Day 90	Day 125	Day 730
pVAX1	<20	<20	<20	ND
pVax-WN	<20	<20	<20	<20
pVax-WNL	<20	66	72	46
WN virus	<20	74	75	145

Sera samples from each group, collected on days 62, 90, 125, and 730, were analyzed by plaque reduction neutralization (PRNT) assay. Serum from individual mice in each group was pooled, irrespective of the seroconversion status of the sera, to obtain a representative average titer within the experiment. The serum titer that was able to reduce the plaque number formed by 50% ($PRNT_{50}$) is indicated.

the WN preM-E/LAMP group (Fig. 4C). This titer was however significantly lower than the positive control group (Table 1).

Discussion

This report expands on our previous work describing genetic vaccines based upon DNA constructs encoding the antigen as a protein chimera containing sequences of the LAMP lysosomal membrane proteins (Chikhlikar et al., 2004; de Arruda et al., 2004; Lin et al., 1996; Lu et al., 2003; Marques et al., 2003; Raviprakash et al., 2001; Rowell et al., 1995; Ruff et al., 1997; Wu et al., 1995). These LAMP sequences target the antigen chimera to cellular endosomal/lysosomal compartments that in APCs (B, macrophage, and dendritic cells) contain both LAMP and MHC II molecules and function in proteolytic processing of antigen proteins to peptide epitopes and in epitope presentation to $CD4^+$ T cells (Amigorena et al., 1994; Drake et al., 1999; Geuze, 1998; Kleijmeer et al., 1997, 2001; Turley et al., 2000). Delivery of MHC II molecules to these compartments is directed by an associated invariant chain (I-chain) that is degraded in endosomes to a peptide sequence (clip) that remains bound to the class II peptide loading groove until replaced by antigen protein epitopes in a process facilitated by mouse H2-M or human HLA-DM proteins (Busch et al., 2000; Hiltbold and Roche, 2002; Vogt and Kropshofer, 1999; Vogt et al., 1999). LAMP molecules are directed to lysosomes by a tyrosine-based carboxyl terminal targeting sequence that functions in the context of the cytoplasmic domain of the molecules (Guarnieri et al., 1993). Conventionally, antigens directed to the LAMP/MHC II compartment(s) are “foreign” molecules delivered by binding to specialized plasma membrane endocytic receptors of dendritic and other phagocytic cells (Mahnke et al., 2000; Mellman and Steinman, 2001; Parkin and Cohen, 2001). This is to be distinguished from antigen/LAMP chimera proteins that are recombinant endogenous molecules, with no natural counterpart, that enter the cell vesicular compartment by the endoplasmic reticulum translocation signal sequence, pass through the Golgi apparatus, and are directed to lysosomes by specific adaptor protein association with the LAMP carboxyl terminal targeting sequence (Bonifacino and Dell’Angelica, 1999; Bonifacino and Traub, 2003). Thus, the MHC II, dendritic cell antigen receptors, and LAMP each use different vesicular trafficking mechanisms to reach the compartment where antigen peptide epitopes bind to MHC II molecules. In this study, we have analyzed the localization of endogenous LAMP, MHC II, H2-M, and the transgene WN E/LAMP of transfected B cells by combined confocal light microscopy and immunoelectron microscopy. The data show remarkable confocal colocalization of the preM-E/LAMP transgene product with B cell MHC II, LAMP-1, and H2-M proteins. Detection of the transgene WN E protein was based on binding of monoclonal antibody 6B6C-1 that reacts with flaviviral E protein (Roehrig, 2003; Roehrig et al., 1983). It is highly likely that this antibody binds only to E/LAMP chimera or intact or perhaps partially degraded E and not to epitope peptides associated with the MHC II. It would therefore appear that it is the unprocessed or perhaps only slightly processed transgene E protein that is detected colocalized with endogenous LAMP, MHC II, and H2-M. Thus, the distribution of transgene E/LAMP correlated with that of the endogenous proteins in internal endocytic/lysosomal compartments, and not with the MHC II molecules present on the cell surface as a result of the transport of peptide-loaded molecules to the plasma membrane. At the electron microscopy level, the site of colocalization of the preM-E/LAMP with the endogenous MHC II, LAMP-1, and H2-M molecules was predominantly in unique electron dense multilaminar structures corresponding to late MIICs and, in some cases, late endosomes and exososome-like multivesicular compartments (Geuze, 1998; Kleijmeer et al., 1997). This distribution of the preM-E/LAMP was markedly different from that of the unmodified preM-E protein that was instead distributed mainly within the vesicular structures of the ER, Golgi complex, trans-Golgi network, and secretory vesicles, consistent with earlier reports of WN structural proteins being transported from the perinuclear region, along the endoplasmic reticulum (ER), to the plasma membrane during its morphogenesis (Chu and Ng, 2002; Mackenzie and Westaway, 2001). There was little apparent colocalization with endogenous LAMP or MHC II, except possibly with MHC II at the plasma membrane and infrequently with LAMP, attesting to the separate vesicular pathways of these molecules in post-Golgi trafficking. On this basis, we speculate that access of native preM-E encoded by DNA vaccines to the MHC II would entail a mechanism not observed in these transfected B cells, such as uptake of secreted protein or alternative processing pathways in other transfected APCs.

The rationale for targeting antigens to the MIICs of APCs is to increase the amount of epitopes loaded onto the MHC II molecules and thus more readily reaching the $CD4^+$ T cell receptor triggering threshold and antigen-specific $CD4^+$ T

cell responses essential for the generation and maintenance of memory B cell and $CD8^+$ T cell responses (Chan et al., 2001; Maecker et al., 1998; Tanchot and Rocha, 2003). This hypothesis is supported by evidence that LAMP-antigen chimera vaccines result in enhanced $CD4^+$ T cell responses (Marques et al., 2003) and an increased number of antigen MHC II epitopes (Fernandes et al., 2000) as compared to the corresponding native antigens. When evaluated for the effects on the humoral immune response in repeated immunization studies, mice immunized with the pVax-WNL DNA construct showed significantly higher and more sustained antibody titers. While the total WN-specific IgG titers of all mice immunized with pVax-WNL DNA increased progressively after the second and third DNA boosts, peaking at day 90 postvaccination, the antibody response from mice immunized with the pVax-WN DNA peaked earlier after the second DNA boost and subsequently showed a concomitant decline in sera titers. This low and unsustained immune response has been reported for several DNA vaccines encoding native antigens of other proteins (de Arruda et al., 2004; Fournillier et al., 1999; Martins et al., 1995; Vidalin et al., 2000). When compared with mice immunized with the wild-type pVax-WN DNA construct, the levels of neutralization antibodies elicited in mice immunized with pVax-WNL were comparable to the positive control mice immunized with the WN virions at days 90 and 125, declining at day 730 postimmunization. Although the level of neutralizing antibody response was lower compared to the positive control at 2 years post-immunization, pooled sera from pVax-WNL-vaccinated mice still showed an augmented neutralizing antibody titer in contrast to pooled sera from pVax-WN-vaccinated mice. This maintenance of a long-term immune response was also observed in our previous study with the dengue preM-E/LAMP DNA vaccine, where a high level of neutralizing antibody was maintained over a period of 1 year post-immunization (Lu et al., 2003). In another study of a West Nile preM-E DNA vaccine (Davis et al., 2001), the mouse neutralizing antibody response to the vaccine delivered by electrotransfer was high compared to that reported here. Although it is not possible to directly compare results between different immunization protocols, immunization by electrotransfer is reported to greatly increase DNA vaccine efficacy as compared to the direct immunization used in this study (Babiuk et al., 2002; Chang et al., 2001; Durieux et al., 2002; Liu and Huang, 2002; Mir et al., 1999).

We attribute the low humoral response of mice immunized with the native WN preM-E antigen to a diminished trafficking to the MIICs, not to the possibly low steady-state cytoplasmic level of the expressed E protein as found with transfected cells in vitro. This low level of E protein was shown not to be due to differences in the transfection or transcriptional efficiencies and is thus likely a posttranslational event. Taken together with the Western analysis data, where the preM protein levels were similar in pVax-WN- and pVax-WNL-transfected cells despite significant differences in the E protein, the data are congruent with the secretion of the E protein, confirming previously described studies by electron microscopy (Ng et al., 1994), and by isolation of the protein from the culture media of transfected COS-1 cells (Davis et al., 2001). In addition, previous reports have shown the secretion of the viral preM-E protein from cells transfected with WN and other JE antigenic group DNA constructs (Chang et al., 2000; Davis et al., 2001). In contrast, the chimera E/LAMP protein containing the carboxyl terminal transmembrane domain and targeting sequences of LAMP directed to an endosomal/lysosomal pathway and despite targeting to lysosomes has a higher steady-state cellular concentration. Secretion of the E protein could possibly increase its immunogenicity, as several studies have shown that DNA vaccines encoding secreted antigens generally induced higher immune responses than the same antigens retained within the cell (Andersson et al., 2000; Grode et al., 2002; Ma et al., 2002; Riedl et al., 2002). In this case of the WN E, our findings indicate that the secreted protein is not as effective in eliciting an immune response as that targeted directly to the MHC II compartment.

Materials and methods

Cell lines and virus

The cell lines Vero, BHK-21, 293, COS-7, and LB27.4 were obtained from ATCC (Rockville, MD) and maintained according to the supplier's protocols. The West Nile virus strain Wengler (obtained from Prof. Ng ML, National University of Singapore) was propagated in Vero cells at 37 °C in 5% CO_2 and the viral titer evaluated in BHK-21 cells by plaque assay as described (Gould and Clegg, 1985).

Construction of plasmid vaccines

Genomic RNA was extracted with guanidinium isothiocyanate, followed by delipidation and differential partitioning as described (Too and Maggio, 1995), and used as template for the synthesis of WN cDNA by reverse transcription (RT) using random hexamer primers and MMuLV reverse transcriptase (Promega Corporation, Madison, WI) (Sambrook and Russell, 2001). The wild-type plasmid containing the WN premembrane (preM) and envelope (E) regions, inclusive of a 34 amino acids endoplasmic reticulum translocation sequence present at the carboxyl end of the capsid, was constructed by PCR using WN-specific primers. The WN sequences were amplified using primers that incorporated an ATG start site in the context of the Kozak sequence and a translational stop codon. PCR amplification was performed using the Expand High Fidelity Taq polymerase (Roche Diagnostics GmbH, Germany) and 50 nM of each primer. The amplified viral

DNA fragment, extending from nucleotides 361 to 2457 (GenBank accession M12294), was cloned into pVAX1 (Invitrogen Life Technologies, Carlsbad, CA) to construct the wild-type WN preM-E plasmid (pVax-WN). The chimera WN preM-E/LAMP plasmid (pVax-WNL) was constructed by replacing the transmembrane and carboxyl terminal sequences of the E gene with the transmembrane and carboxyl terminal domains of the mouse LAMP-1 (Guarnieri et al., 1993). All clones were verified by sequencing. The viral sequences were also cloned into the pITR vector (Kessler et al., 1996) to construct the pITR-WN and pITR-WNL plasmids.

Cell transfection and immunochemical staining

The cellular localization of the expressed viral proteins in the mouse B lymphoma cell line LB-27.4 was studied by fluorescent confocal and immunoelectron microscopy. Cells were transfected with the pVax-WN and pVax-WNL plasmids using Lipofectamine PLUS (Invitrogen Life Technologies) for 48 h according to the manufacturer's protocol. The expression of the recombinant viral proteins was visualized by use of anti-WN hyperimmune ascitic fluid (HIAF; ATCC) or 6B6C-1 (anti-Saint Louis Encephalitis antibody cross-reactive to WN E protein; Chemicon International Inc., Temecula, CA; Roehrig et al., 1983). Localization of the viral proteins in relation to the MHC II-containing compartments and lysosomes was studied by use of the monoclonal antibodies M5/114.15.2 (anti-MHC II; BD Biosciences, San Diego, CA), K553 (anti H-2M; gift from Dr. L. Karlsson, R.W. Johnson Pharmaceutical Research Institute, CA), and ID4B (IgG2a, anti-mouse LAMP-1; (Chen et al., 1985; Hughes and August, 1982).

Confocal and immunoelectron microscopy

Confocal microscopy was carried out using an Olympus IX70-Fluoview 300 system. Image processing to adjust pixel density distribution was carried out on Adobe Photoshop 6.0 (Adobe System Corp., San Jose, CA). Immunoelectron microscopy (IEM) was performed with transfected cells fixed in a mixture of 4% paraformaldehyde and 0.2% glutaraldehyde in PBS and incubated for 2 h at room temperature. Fixed cells were processed for ultrathin cryosectioning and immunogold labeling as described previously (Kleijmeer et al., 1997). Briefly, cells were washed with PBS and PBS containing 50 mM glycine, pelleted, and embedded in 10% gelatin. Small blocks were infiltrated with 2.3 M sucrose at 4 °C for at least 2 h and then frozen in liquid nitrogen. The ultrathin cryosections were prepared with a Leica Ultracut FCS Cryomicrotome and retrieved with a 2:3 (vol/vol) mixture of 2% methylcellulose and 2.3 M sucrose. Ultrastructural analysis was performed with the same repertoire of antibodies used in confocal microscopy. The sections were then double immunolabeled with 10- and 15-nm gold particles or triple immunolabeled with 5-, 10-, and 15-nm gold particles (SPI Supplies, PA, USA). The sections were contrast-stained, embedded in a mixture of methylcellulose and uranyl acetate, and viewed using a CM120 Twin Phillips electron microscope.

RT-PCR and real-time quantitative PCR of transfected nucleic acids

The total RNA and DNA of COS-7 cells transiently transfected with the pVax-WN and pVax-WNL plasmids were isolated simultaneously by use of TRIzol Reagent (Invitrogen Life Technologies) according to manufacturer's instruction. The RNA was quantified by spectrophotometry and gel electrophoresis. The RT reaction was performed at 42 °C with 2–5 μg of total RNA, Improm-II reverse transcriptase (Promega Corporation), and random hexamers (Promega Corporation) in a total volume of 10 μl (Sambrook and Russell, 2001). The primer sets WN-f/r (WN-f, 5′ TGAAGTGTCGGGTGAAG ATGG 3′, position 1811–1831; WN-r, 5′ CTCTGAGTGTGGTGGTAAAGG 3′, position 2174–2194; GenBank accession no. M12294), Vax-f/r (Vax-f, 5′ TTCGCCACCTCTGACTT GAGC 3′, position 2870–2890; Vax-r, 5′ ATGGGCTATGAACTAAT-GACC 3′, position 78–98; Invitrogen), and Actin-f/r (Actin-f, 5′ ACAACGGCTCCGGCATGTGC 3′, position 32–51; Actin-r, 5′ GGTCATCTTTTCACGGTTGG 3′, position 341–360; GenBank accession no. AJ312092) were used in real-time PCR studies to amplify the transcribed West Nile E gene, intracellular plasmids, and the host actin gene, respectively. All real-time quantitative PCR was performed on the iCycler iQ Multi-Color Real Time PCR Detection System (Bio-Rad, Hercules, CA) with the SYBR Green PCR Master Mix containing AmpliTaq Gold DNA polymerase (Applied Biosystem, Foster City, CA). The threshold cycles (Ct) were calculated by use of the Optical interface v3.0a (Bio-Rad). All real-time quantitative PCR was performed under the following conditions: 95 °C for 10 min to activate the AmpliTaq Gold DNA polymerase, followed by 50 cycles of 1 min denaturation at 95 °C, 30-s annealing at 55 °C, and 30-s extension at 72 °C. Fluorescent detection of SYBR Green I was carried out at the extension phase. Linearized pVax-WN and an actin plasmid were used as standards in the real-time PCR.

Western blot analysis of protein expression

The expression of WN viral proteins from pVax-WN, pVax-WNL, pITR-WN, and pITR-WNL was analyzed by Western blot of the transfected human fibroblast 293 and monkey kidney COS-7 cell lines. Cells were seeded onto 60-mm plates and transfected with WN plasmids using Polyfect Transfection reagent (Qiagen GmbH, Germany) according to the manufacturer's protocol. The proteins were harvested 48 h posttransfection, resolved on a 12% SDS-PAGE, and transferred onto a nitrocellulose membrane. The

WN proteins were detected by labeling with anti-WN HIAF and SuperSignal West Pico Chemiluminescent Kit (Pierce, Rockford, IL).

Mouse immunization

Endotoxin-free WN pVax plasmids used for immunization experiments were prepared with the Qiagen Endofree Plasmid Kit (Qiagen GmbH). Groups of five 6- to 8-week female BALB/c mice were immunized by subcutaneous injection at the base of the tail with 50 μg of DNA in PBS. Retro-orbital blood required for ELISA and neutralizing antibody determinations was collected from individual mouse prior to each immunization at days 55, 90, 125, and 730 postimmunization. As a positive control, mice were injected intraperitoneally (ip) with 10 μg of WN virion protein in complete or incomplete Freund's adjuvant.

ELISA and neutralization assays

Anti-WN end-point titers (Crowther, 1995) of antibody in the serum of each mouse were determined at 3-fold serial dilutions by ELISA assay (Ansari et al., 1993) of antibody binding to WN virion antigens purified by ultracentrifugation (Gould and Clegg, 1985). Virus neutralization titers were measured by plaque reduction neutralization titer (PRNT) assay in BHK-21 cells (Russell et al., 1967). All statistical analyses were performed using GraphPad Prism 2.01 Software (GraphPad Software, San Diego, CA).

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