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TITLE: Dengue Virus-Like Particle Vaccine

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14. ABSTRACT

The aim of the project was to develop and validate Dengue virus (DENV) VLP based vaccines for the elicitation of broadly reactive immune responses. This approach built on expertise and tools already developed by the members of the University of Pittsburgh's Center for Vaccine Research for Emerging Diseases and Biodefense. Synthetic genes were generated by GeneArt and cloned into the Center's expression plasmid. Gene cassettes were generated for all 4 subtypes of dengue virus. Expression of these genes was verified by direct transfection of 293T cells. Cell lysates and supernatants were harvested and SDS-PAGE performed followed by immunoblotting. While vaccines were effective at eliciting high titer IgG to homologous virus, virus-like particles for dengue virus were more efficient for eliciting neutralizing antibodies. A tetravalent VLP vaccine was efficient at eliciting antibodies against all 4 subtypes of dengue virus. Data obtained from these mouse models can be used as the basis for future non-human primate and human vaccine and challenge studies.

15. SUBJECT TERMS

dengue virus, virus-like particles, neutralizing antibodies, cell-mediated immunity, vaccine, baculovirus, DNA vaccine

Table of Contents

Introduction

A major problem in the development of effective dengue virus (DENV) vaccines is the diverse strains of virus in circulation that do not readily offer cross-protective immunity due to variant viral envelope (E) proteins.

Our hypothesis is that Consensus DENV E proteins, presented to the immune system on a virus-like particle (VLP), will elicit broader immunity against diverse primary E proteins and provide broader neutralization activity against heterologous DENV isolates compared to a mixture of primary E constructs.

This project proposed to construct and compare the immune responses generated with several dengue VLP vaccine strategies: (1) a mixture of four VLPs, each with a primary envelope sequence; (2) a mixture of four VLPs, each with a serotype consensus envelope sequence; and (3) a chimeric VLP with all four envelope sequences on a single particle (primary or consensus envelope sequences). For each VLP construct strategy, two different delivery systems were employed: an *in vivo* "genetic VLP" DNA/particle prime-boost vaccine delivery system and an *in vitro* baculovirus-expressed "purified VLP" vaccine delivery system.

Body

The approved Statement of Work is as follows:

STATEMENT OF WORK: Independently, and not as an agent of the Government, the Contractor shall furnish all necessary services, qualified professional, technical, and administrative personnel, material, equipment and facilities, not otherwise provided by the Government under the terms of this contract, as needed to perform the tasks set forth below.

Specifically the Contractor shall provide:

1. **Dengue virus-like particles.** The Contractor shall develop and validate Dengue virus (DENV) VLP based vaccines for the elicitation of broadly reactive immune responses. This approach will build on expertise and tools already developed by the members of the University of Pittsburgh's Center for Vaccine Research for Emerging Diseases and

Biodefense. Data obtained from these mouse models will be used as the basis for future non-human primate and human vaccine and challenge studies.

1 (a) DNA plasmids expressing VLPs from mammalian cells. The Contractor shall construct vaccines that elicit immune responses against DENV isolates representing each serotype. Two vaccine approaches are described in the proposal. The first vaccine strategy uses DNA plasmids expressing the Capsid (C), pre-membrane (prM), and envelope (E) gene segments from DENV. DNA plasmids will be constructed in Year 1 using gene segments representing each of the 4 DENV serotypes (wild type or consensus). Plasmids will be transfected mammalian cells *in vitro* resulting in VLP secretion followed by ultracentrifugation purification. Mice will be primed with DNA plasmids expressing each vaccine intradermally by gene gun and then boosted with the same purified VLPs intranasally. Serum and broncheoalveolar lavage (BAL) will be assayed for antibody responses and spleens and lymph nodes (LN) for cellular responses. Mice will be vaccinated with a single vaccine expressing VLPs representing a single serotype or all four vaccines representing each serotype. Immune responses elicited in mice vaccinated with a single vaccine will be compared to the tetravalent VLP vaccination. Anti-DENV antibodies will be assayed for specificity to DENV proteins by ELISA, the affinity to DENV protein by surface plasmon resonance (SPR), and the ability to inhibit DENV infection (neutralization). Cellular responses will be assayed against C, prM/M, and E proteins from cells collected in the spleen and LNs. The elicited immune responses will be assayed for the ability to recognize viral proteins representing each of the four serotypes of DENV. The data generated by these experiments will be deposited in the Center Database where they will be accessible by all members of the Center for Vaccine Research. These *in vivo* experiments should be completed within the first 36 of the start of the contract.

1 (b) Purification of VLPs from baculovirus vectors. The Contractor shall construct vaccines that elicit immune responses against DENV isolates representing each serotype. The second vaccine strategy uses bacmids expressing the C, prM, and E from wild type or consensus gene segments for packaging in baculovirus. Each baculovirus vector will be used to infect Sf9 insect cells and the expressed DENV genes will secrete VLPs into the supernatant, which will be purified by ultracentifugation as vaccine immunogens. VLPs will contain either a single DENV E protein representing a single DENV serotype or a single VLP will contain all four DENV E proteins representing all four DENV serotypes. Mice will be

vaccinated with purified VLPs intranasally and the same immune analysis will be performed as described in 1(a) and the data will be deposited in the Center Database These experiments should be completed in Years 3 and 4 of the contract.

Table 1: SOW Milestones with Due Dates

Milestone	Baseline Plan Date	Rev Plan Date	Act Comp Date	Remark
Gene Synthesis	16-Jan-08		16-Jan-08	Aim 1
Plasmid Construction	16-Jan-08	31-May-08	31-May-08	Aim 1
VLP Purifications	16-Jul-08	30-Sep-08	30-Sep-08	Aim 1
VLP Characterization	16-Jul-08	30-Sep-08	21-Nov-08	Aim 1
Vaccinations	16-Jan-09	15-Mar-09	15-Mar-09	Aim 1
Bacmid Construction	16-Jul-09		10-Apr-09	Aim 2
Baculovirus Infection	16-Jul-09	15-May-09	20-May-09	Aim 2
Vaccinations	16-Oct-09		16-Oct-09	Aim 1
Determine Ab reactivity & maturity	16-Jan-10		16-Jan-10	Aim 1
VLP Purification	16-Jan-10		16-Jan-10	Aim 2
VLP Characterization	16-Jan-10		16-Jan-10	Aim 2
Determine the breadth of NAbs	16-Apr-10		16-Apr-10	Aim 1
Vaccinations	16-Apr-10		16-Apr-10	Aim 2
Determine the breadth of T cell response	16-Jul-10		16-Jul-10	Aim 1
Vaccinations	16-Jan-11		16-Jan-11	Aim 2
Determine Ab reactivity & maturity	16-Apr-11		16-Apr-11	Aim 2
Determine the breadth of NAbs	16-Apr-11		16-Apr-11	Aim 2
Determine the breadth of T cell response	16-Jul-11		16-Apr-11	Aim 2

Summary of Work Completed During the 4-Year Contract

The following individuals worked on experiments, as outlined in the Statement of Work, during the 4 year period of performance: Dr. Ted M. Ross, Dr. Nikolaos Vasilakis, Dr. Shannan Rossi, Dr. Jared Evans, Dr. Xian-Chun Tang, Mr. Matthew Dunn, Ms. Nicole Beatty, and Mrs. Hai-Rong Lu. There were several areas of progress in the development of a Dengue virus-like particle vaccine including the design of consensus E sequences for each serotype of Dengue, the generation of virus-like particle vaccines, and determination of broadly reactive immunity associated with these vaccines. These consensus sequences were designed using over 200 sylvatic and contemporary Dengue E amino acid sequences and the most common amino acid was engineered at each location. These sequences were combined into one opening reading frame to generate a C, prM, E gene cassette to express the virus-like particle. These synthetic genes were generated by GeneArt and cloned into our expression plasmid. Gene cassettes were generated for all 4 subtypes of dengue virus. Expression of these genes was verified by

direct transfection of 293T cells. Cell lysates and supernatants were harvested and SDS-PAGE performed followed by immunoblotting.

In addition to mammalian gene expression systems, each gene cassette was cloned into a bacmid expression vector from Invitrogen and protein expression was verified. The bacuolvirus system for protein and dengue particle expression was establishment by Dr. Xianchun Tang. Dr. Tang effectively grew Sf9 insect cells in spinner flasks and amplified baculovirus in these cell cultures. He generated bacmids for efficient production of dengue virus-like particles from infected insect cells. However, the efficiency of particle purification and inactivating the baculovirus by beta-propriolactone inactivation methods proved quite difficult. Dr. Tang moved to working with a more efficient system, influenza virus, and he productively produced various types of VLPs for influenza. These accomplishments led to 2 first author publications (see appendix). However, after 18 months of experimentation and discussion with colleagues, flavivirus VLPs for dengue virus were not expressed or efficiently purified and the approach was abandoned in favor of an alternative method using yeast, described below.

Initially, the team started with the DENV-2 isolate as a prototype for the additional vaccines. The plasmids were constructed and the protein expressed as detected by western blot. However, the efficiency of secretion was poor. Therefore, the construct was modified to express the VLP gene segments in two ways: 1) the addition of NS3B/NS4 to efficiently cleavage the capsid and 2) the construction of the prM/E construct with the C proteins provided in *trans*. It was determined that the VLPs could not express without viral proteases, which were not part of the original vaccines. The team decided to work out the conditions of VLP expression using a related flavivirus, West Nile virus. Particles could be released as subvirion particles (SVPs) from mammalian cells using the prM-E cassette only. SVPs were generated for West Nile virus and all 4 dengue virus subtypes. In order to retain the capsid in our VLPs, we invited Dr. Jared Evans and his lab to join the team to express out C-prM-E gene cassettes in a yeast-based expression system, *Pichia pastoris*, that allows for efficient proteolytic cleavage of the capsid protein from the prM-E gene product. Once again, the system was first used with West Nile virus to work out conditions, and then it was moved to dengue virus. All four subtypes of dengue virus were produced as SVPs from mammalian cells and VLPs from yeast.

All four dengue virus prototype strains were produced in Vero cells and plaque tittered, as well as immunohistochemically stained for expression. All viruses efficiently expressed and virus

could be detected for 16 days post-infection. These viruses will be necessary to determine vaccine efficacy in neutralization assays.

For immunogenicity studies, female BALB/c mice (n=10/group; age 6-8 weeks) were purchased from Harlan Sprague Dawley, (Indianapolis, IN, USA), immunized with each DNA vaccine plasmid by gene gun (particle bombardment with 2 μg DNA coated on gold bullets) or with purified SVPs and then boosted with the same dose on weeks 3 and 6. In addition, a tetravalent combination of all 4 dengue vaccines was also used for vaccination. Blood was collected from anesthetized mice via the retro-orbital route on weeks 5 and 8 post vaccination and then centrifuged at 6000 rpm for 10 min to separate the serum. Sera were transferred to new vials and frozen at -20°C.

A quantitative ELISA was performed to assess anti-DIII specific IgG in serum of vaccinated mice. Individual wells of a 96 microtiter plate were coated overnight at 4°C with dengue DIII proteins produced from transfected 293T cells and then blocked (25°C for 2 hr) with PBS supplemented with Tween-20 (0.05%) and nonfat dry milk (5%). Each serum sample was serially diluted and incubated (25°C for 2 hr). Following serial washes with PBS Tween-20 (0.05%), samples were incubated (25°C for 1 hr) with HRP conjugated goat anti-mouse IgG (1:5000) or one of four IgG subclasses (IgG_1, IgG_{2a}, IgG_{2b}, or IgG_3) (Southern Biotechnology, Birmingham, AL) diluted in PBS Tween-20 (0.05%) and nonfat dry milk (5%). Unbound antibody was removed and after additional washes samples were incubated with TMB substrate, and the colorimetric change was measured as the optical density at 405 nm using a plate reader (Biotek Powerwave XS, Winooski, VT USA). The O.D. value of the age-matched naïve sera was subtracted from the OD values of the antisera from the vaccinated mice. Results were recorded as the geometric mean titer (GMT) ± the standard error of the mean (SEM).

Vaccinations with the dengue SVP vaccines produced high titer anti-E DENV antibodies specific to each subtype and across subtypes. Purified VLPs elicited high titer antibody to the individual Denv-1 and Denv-3 E proteins. Mice vaccinated Denv-2 and Denv-4 were more likely to have high titers to themselves, but there were across reactive IgG titers against all 4 dengue viruses.

Neutralization titers showed similar results. The yeast-based VLPs were not tested in immunogenicity studies due to lack of funds. The tetravalent vaccine has been produced and

the VLPs were used to vaccinate mice showed broadly reactive IgG titers and neutralization titers against all 4 subtypes of dengue.

We also compared our SVP vaccines against two alternative strategies produced in the Ross Laboratory, DIII and Ecto E (E80) based dengue vaccines. The SVP strategy appears more efficient in producing serum IgG and neutralizing antibodies to dengue virus than DIII or Ecto E versions. No T cell responses were ever detected using any of the dengue vaccines produced in the Ross or Evans laboratories.

Table 2: Neutralizing Titers elicited by Dengue Vaccines in Mice

		Neutralizing Titers against Serotype (*PRNT50*)			
		DENV-1	DENV-2	DENV-3	DENV-4
Sample	Notation				
SVP-DNA					
DENV-1	A	X	>1:160	<1:10	X
DENV-2	B	X	>1:320	<1:10	X
DENV-3	C	X	1:40	1:320	X
DENV-4	D	X	<1:10	<1:10	X
Tetravalent	E	X	>1:160	1:20	X
Mock	F	X	<1:10	<1:10	X
Purified SVP					
DENV-1	G	X	1:80	<1:10	X
DENV-2	H	X	>1:320	<1:10	X
DENV-3	I	X	1:20	>1:160	X
DENV-4	J	X	<1:10	<1:10	X
Tetravalent	K	>1:320	>1:320	>1:160	1:40
Ecto E-C3d					
DENV-1	L	1:20	1:20	1:10	1:20
DENV-2	M	1:20	X	1:10	1:40
DENV-3	N	<1:10	<1:10	1:10	<1:10
DENV-4	O	1:20	X	1:10	1:160
Tetravalent	P	1:40	X	1:10	1:10
DIII-C3d					
Tetravalent DII+SVP boost	R	<1:10	X	<1:10	<1:20
Tetravalent	S	<1:10	X	<1:10	1:10
Tetravalent DII+SVP boost	T	1:10	X	<1:10	<1:10

X = not completed

Key Research Accomplishments

- Establishment of Dengue virus program in the Ross and Evans laboratories in Center for Vaccine at the University of Pittsburgh.
- Generation of subvirion particle using consensus E proteins.
- Establishment of a yeast-based expression system at the University of Pittsburgh.
- Establishment of a baculovirus expression system in the Ross Laboratory at the University of Pittsburgh.
- Elicitation of high titer anti-dengue antibodies, with neutralization activity, across subtypes of dengue with a single consensus E gene product.

Reportable Outcomes/Project Bibliography

No manuscripts were submitted that are related to the Statement of Work.

Published manuscripts related to establishing expression systems funded by this work include:

- *Dunn MD, Rossi SL, Carter DM, Vogt MR, Mehlhop E, Diamond MS, Ross TM. Enhancement of Anti-DIII Antibodies by the C3d Derivative P28 Results in Lower Viral Titers and Augments Protection in Mice. 2010. Virology J. 7:95-106.*
- *Tang X-C, Lu H-R, Ross TM. Hemagglutinin Displayed Baculovirus Protects Against Highly Pathogenic Influenza. 2010. Vaccine. 28:6821-6831.*
- *Tang X-C, Lu H-R, Ross TM. Baculovirus-produced Influenza Virus-like Particles in Mammalian Cells Protect Mice from Lethal Influenza Challenge. 2011. Viral Immunol. 24:1-9.*

Two book chapters were derived based upon the Dengue and West Nile virus projects inspired including:

- *Ross TM. Dengue Virus. 2010. Clin Lab Med. 30(1):149-160.*
- *Rossi SL, Ross TM, Evans JD. West Nile Virus. 2010. Clin Lab Med. 30(1):47-65.*

One manuscript is in preparation that is related to the Statement of Work:

- *Ross TM, Tang X-C, Lu H-R, Strong LS, Evans JD. Elicitation of broadly reactive anti-dengue neutralizing antibodies by virus-like particle based consensus E vaccine. 2011. In preparation.*

Invention Disclosures

- *Title: Universal Dengue Vaccine; Internal Reference Number 02226; Status: Marketing-Active*

Funding Applied for: None

Personnel Receiving Pay from the Research Effort

- UPMC Personnel: Charles R. Rinaldo, PhD, Principal Investigator
  No other UPMC personnel received direct pay from this award.
- A subcontract with the University of Pittsburgh covered all other personnel directly involved with the execution of this contract.

Employment Opportunities Received for University of Pittsburgh Personnel:

- Dr. Nikoloas Vasilakis: Assistant Professor, University of Texas Medical Branch-Galveston.
- Dr. Shannan Rossi: Post-Doctoral Fellow, University of Texas Medical Branch-Galveston.
- Dr. Xian-Chun Tang: Post-Doctoral Fellow, Harvard University Medical Center-Boston.

Conclusions

While all vaccines were effective at eliciting high titer IgG to homologous virus, virus-like particles for dengue virus are more efficient to elicit neutralizing antibodies. A tetravalent VLP vaccine was efficient at eliciting antibodies against all 4 subtypes of dengue virus.

References

None

Appendices

APPENDIX 1

Enhancement of anti-DIII antibodies by the C3d derivative P28 results in lower viral titiers and augments protection in mice

2010. Virology J. 7:95-106

Dunn, MD, Rossi, SL, Carter, DM, Vogt, MR, Mehlhop, E, Diamond, MS, Ross, TM

Dunn et al. Virology Journal 2010, 7:95
http://www.virologyj.com/content/7/1/95

VIROLOGY JOURNAL

RESEARCH Open Access

Enhancement of anti-DIII antibodies by the C3d derivative P28 results in lower viral titers and augments protection in mice

Matthew D Dunn1, Shannan L Rossi[1,2], Donald M Carter[1], Matthew R Vogt[3], Erin Mehlhop[3], Michael S Diamond[3] and Ted M Ross*[1,2]

Abstract

Antibodies generated against West Nile virus (WNV) during infection are essential for controlling dissemination. Recent studies have demonstrated that epitopes in all three domains of the flavivirus envelope protein (E) are targets for neutralizing antibodies, with determinants in domain III (DIII) eliciting antibodies with strong inhibitory properties. In order to increase the magnitude and quality of the antibody response against the WNV E protein, DNA vaccines with derivatives of the WNV E gene (full length E, truncated E, or DIII region, some in the context of the pre-membrane [prM] gene) were conjugated to the molecular adjuvant P28. The P28 region of the complement protein C3d is the minimum CR2-binding domain necessary for the adjuvant activity of C3d. Delivery of DNA-based vaccines by gene gun and intramuscular routes stimulated production of IgG antibodies against the WNV DIII region of the E protein. With the exception of the vaccine expressing prM/E given intramuscularly, only mice that received DNA vaccines by gene gun produced protective neutralizing antibody titers (FRNT80 titer >1/40). Correspondingly, mice vaccinated by the gene gun route were protected to a greater level from lethal WNV challenge. In general, mice vaccinated with P28-adjuvated vaccines produced higher IgG titers than mice vaccinated with non-adjuvanted vaccines.

Introduction

West Nile virus (WNV) is a single-stranded positive polarity enveloped RNA virus and member of the Flavivirus genus of the *Flaviviridae* family. The genome (11 kb) encodes for three structural proteins (Capsid [C] [1], pre-membrane [prM] that is cleaved to form a mature membrane [M] [2] and Envelope [E] [1]) and seven nonstructural gene products (NS1, 2A, 2B, 3, 4A, 4B and 5). WNV is transmitted by mosquitoes and causes morbidity and mortality in birds, horses, and humans. Since 1999, there have been over 29,000 cases that reached clinical attention and resulted in greater than a thousand deaths http://www.cdc.gov/ncidod/dvbid/westnile/surv&control.htm within the United States as reported to the Centers for Disease Control and Prevention. As the geographic distribution of this virus continues to expand, naïve human populations are put at greater risk, making the need for a licensed vaccine and/or antiviral treatment pressing [3].

The host immune response is critical for limiting virus spread and disease. Results from genetically engineered mice indicate that both the innate (*e.g.*, interferon) and the adaptive (B and T cells) immune responses control WNV infection [4]. The production of antibodies is essential to protection against WNV infection [5], and passive antibody transfer of anti-WNV neutralizing antibodies can prevent or treat lethal infection [6]. The primary target of the neutralizing antibody response is the E protein, which is the most accessible structural glycoprotein on the surface of the virion [7]. Structural analysis of the soluble ectodomain of flavivirus E proteins reveals three domains [8,9]. Domain I is an 8-stranded β-barrel that participates in the conformational changes associated with the acidification of the endosome. Domain II, which contains 12 β-strands, has important roles in dimerization, trimerization, and virus-mediated fusion [10-12]. Domain III adopts an immunoglobulin-like fold that contains the most distal projecting loops on the

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mature virion [13,14], and has been hypothesized to contain a binding site for cell attachment [15]. Even though neutralizing antibodies are generated against epitopes in all three domains, many highly neutralizing antibodies cluster to epitopes in DIII [16].

Our laboratory and others have demonstrated that the fusion of C3d to an antigen results in enhanced immunogenicity of the fused antigen [5,16,17,19,21,24,31,32,35,36]. C3d is the final degradation product of the third component of complement (C3). The most commonly proposed mechanisms for C3d adjuvanticity involves C3d binding to the complement receptor 2 (CR2) that is located on the surface of follicular dendritic cells (FDC), B cells, and T cells in many species (for review, see [17]). C3d stimulates antigen presentation by FDCs and helps to maintain immunological B cell memory. On B cells, C3d interacts with CR2, CD19 and CD81 surface molecules. CD19 has a long intracellular tail that triggers a signaling cascade that results in cell activation and proliferation. Simultaneous ligation of CR2 by C3d and surface immunoglobulin by antigen activates two signaling pathways that synergize to activate B cells, thereby leading to enhanced antibody secretion against the fused antigen. Multimers of a 28 amino acid peptide of C3d (P28), which contains the predicted minimum CR2 binding domain, have been demonstrated to have similar adjuvant properties as the entire C3d molecule [18]. The P28 molecule is ~9% the size of the entire C3d molecule and therefore, is an attractive adjuvant to elicit enhanced B cell responses to a vaccine antigen.

Currently, there are no effective anti-WNV treatments and there are no Food and Drug Administration (FDA)-licensed vaccines for humans. The FDA has approved a WNV vaccine for horses and other exotic animals, based upon a formalin-inactivated killed virus (WNV Innovator™, Fort Dodge Animal Health), but these require annual boosting. Several experimental vaccines for humans based upon live-attenuated virus, purified protein, viral vectors, or DNA plasmids are under development (see reviews [19-21]) although none has advanced beyond phase II. In theory, WNV E DIII protein is an attractive target for vaccine development because many strongly protective MAbs (in vitro and in vivo) against flaviviruses, including WNV have been localized to this region DIII (reviewed in [22]). Moreover, previous studies have demonstrated the recombinant WNV DIII is a plausible vaccine candidate when administered as a recombinant protein [23], but less effective when expressed from a DNA plasmid [24]. In this study, we developed candidate WNV DNA vaccines with greater immunogenicity and protection using DIII or truncated E proteins conjugated to the molecular adjuvant P28.

Materials and methods

Virus and cell lines

WNV (TX114 strain), isolated from a blue jay in Texas in 2002 was used for all studies except for the passive antibody transfer experiment. The virus was propagated once in Vero cells, aliquotted, and then frozen at -80°C. For the passive antibody transfer experiments, the lineage 1 New York WNV strain 3000.0259 that was isolated in 2000 was passaged once in C6/36 *Aedes albopictus* cells to generate an experimental stock. 293T (human embryonic kidney) and Vero (African green monkey kidney) cells were maintained in DMEM with 10% FBS and 1% penicillin/streptomycin (P/S). Raji cells stably expressing DC-SIGNR were maintained as described [25].

Construction and expression of DNA vaccine plasmids

The WNV prM/E eukaryotic expression vector, pCBWN, has been previously described [26] and encodes the prM and E gene segments (accession number DQ211652) from the strain NY99-6480 strain [26]. To generate an Ecto E DNA vaccine, the glycine residue at position 706 was converted by mutagenesis to a TAG stop codon (Fig. 1A) [27]. The DIII of E (amino acids 296-415) was cloned in frame with the tPA leader sequence in pTR600 (Fig. 1A). A second set of plasmids were constructed to express Ecto E or DIII fused in frame with P28 [18,27]. Each gene sequence encoding for two functional copies of P28 was cloned at the 3' end of Ecto E or DIII using unique restriction endonuclease sites. A BamHI restriction endonuclease site was introduced using site directed mutagenesis immediately 5' to the TAG stop site. A $(Gly_4\text{-}Ser)_2$ linker was cloned in between each P28 gene. All DNA vaccine plasmids were amplified in *Escherichia coli*, purified using anion-exchange resin columns (Qiagen, Valencia, CA) and stored at 20°C in dH_2O. Plasmids were verified by appropriate restriction enzyme digestion and sequencing.

293T cells were transfected with 30 μg of DNA using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Cell culture supernatants were collected 48 hrs post-transfection. Approximately 1.5% of sample volume was loaded onto a 10% polyacrylamide/SDS gel. The resolved proteins were transferred onto a Immobilon PVDF membrane (Millipore, Temecula, CA) and incubated with a 1:5000 dilution of the WNV specific monoclonal antibody (mAb 8150, Chemicon, Temecula, CA) in PBS containing 0.05% Tween-20 and 5% nonfat dry milk. After extensive washing, bound antibodies were detected using a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-mouse antiserum, and visualized by chemiluminescence (Western Lightning™, Perkin Elmer, Waltham, MA).

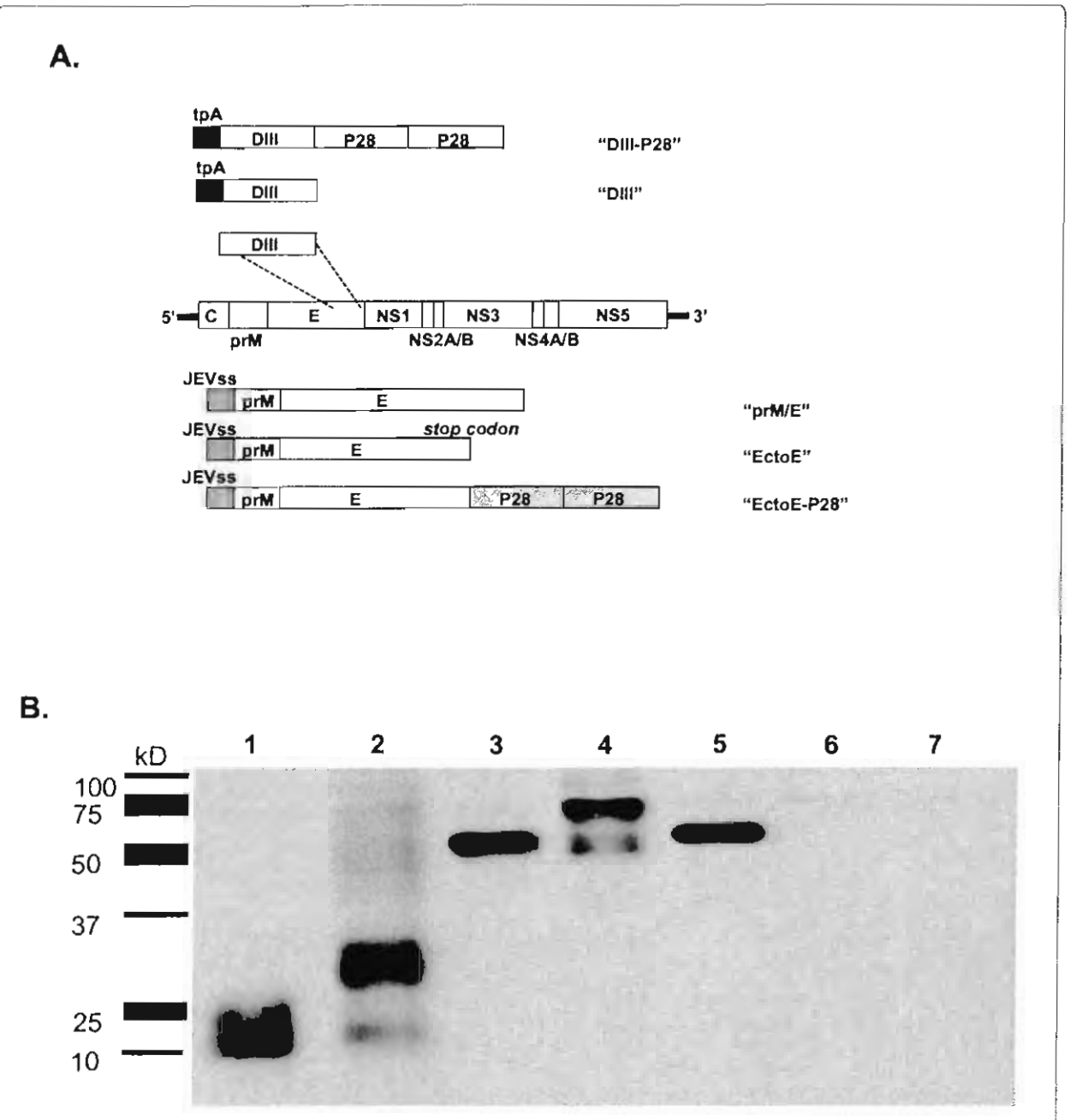

Figure 1 Schematic diagram of constructs and expression of vaccine plasmids. A. A diagram of the WNV genome is shown in the center, and the segments of the genome used in the vaccine constructs are shown above (DIII-modified) and below (prM/E-modified). The construct expressing prM/E was previously described [26]. The DIII region of the E gene (amino acids 586-705) was cloned downstream of the tpA leader sequence, and in some cases, P28 was also cloned in frame and directly after the 3' end of the DIII gene. An artificial BamHI site and stop codon was engineered at position 705 in the E gene to create the truncated Ecto E gene, and P28 was cloned into the Ecto E construct using the BamHI site to create the Ecto E-P28 construct. **B.** Supernatants from 293T cells transiently transfected with plasmid DNA were assessed by SDS-PAGE and Western blot. The membrane was probed by with the DIII-specific monoclonal antibody, 7H2. Lane 1: DIII-DNA; Lane 2: DIII-P28-DNA; Lane 3: prM/Ecto E-DNA; Lane 4: prM/Ecto E-P28-DNA; Lane 5: prM/E-DNA; Lane 6: P28-DNA only; Lane 7: Vector DNA only.

Virus titrations and immunohistochemistry

All virus titrations were performed on Vero cell monolayers. Briefly, cells were incubated with indicated serial dilutions of virus or mouse serum for 1 hour at 37°C. Subsequently, the virus/serum inocula were removed and replaced with a semi-solid overlay of carboxy-methylcellulose in OptiMEM or 1% low melting point agarose (SeaPlaque) in α-MEM (Invitrogen) supplemented with 3 or 4% FBS. Cultures were incubated for 24-72 hours prior to fixation with a 50:50 v/v mixture of methanol and acetone or 10% formadehyde. For plaque assays, staining was performed with 1% (w/v) crystal violet in 20% ethanol and scored visually on a light box. For focus formation assays, foci were visualized by immunohistochemistry.

Immunohistochemistry was performed on fixed and dried cell monolayers by first rehydrating with 3% FBS in PBS (blocking buffer) for 1 hour, then replacing the media with a monoclonal antibody specific to WNV E protein (7H2; BioReliance Corporation, Rockville, MD) diluted in blocking buffer and incubated for at least 1 h. The primary antibody solution was removed and monolayers were washed thrice in PBS prior to adding the goat anti-mouse secondary antibody conjugated to peroxidase diluted in blocking buffer. After 1 h, monolayers were washed thrice in PBS again. WNV-infected cells were visualized by adding the peroxidase substrate (Enzo Diagnostics, Farmingdale, NY).

Vaccination and Viral Challenge

Female C57BL/6 mice (n = 5-8 mice per group; aged 6-8 weeks) were purchased from Harlan Sprague Dawley, (Indianapolis, IN, USA), immunized with each DNA vaccine plasmid intramuscularly (IM, 50 μg DNA injection into thigh) or by gene gun (particle bombardment with 2 μg DNA coated on gold bullets) and then boosted with the same dose on weeks 3 and 6. In some cases 0.2 μg or 0.02 μg of vaccine plasmid as a dose response was administered in a mixture of vector plasmid to keep a total of 2 μg total DNA vaccine. Blood was collected from anesthetized mice via the retro-orbital route on weeks 5 and 8 post vaccination, then centrifuged at 6000 rpm for 10 min to separate the serum. Sera were transferred to new vials and frozen at -20°C.

For challenge, naïve or vaccinated mice administered 1000 focus forming units (FFU) of WNV (TX114 strain) in a volume of 0.1 ml by the intraperitoneal route. WNV was diluted in a filtered solution of 10% fetal bovine serum (FBS) in phosphate buffered saline (PBS) prior to the mice infections (diluents). Mice were weighed daily to determine percent weight loss, and monitored to determine the severity of sickness. Moribund mice (severe lethargy, hunched posture and ruffled fur) were euthanized by CO_2 asphyxiation and recorded as dead for the next day. All mouse experiments were performed in accordance and with approval of the Washington University or University of Pittsburgh Animal Studies guidelines under BSL-3 conditions.

Passive transfer of antiserum to naïve mice

Five week old C57BL/6 mice (Jackson Laboratories) were infected by subcutaneous route with 10^2 PFU of WNV 3000.0259 diluted in Hank's Balanced Salt Solution containing 1% heat-inactivated FBS. For antibody protection studies, one day prior to infection mice were treated by IP injection with indicated amounts of immune (gene gun vaccinated mice) or naïve serum diluted in 100 μl PBS. Mice were monitored daily for 21 days for morbidity and mortality.

Enzyme-Linked Immunoabsorbant Assay (ELISA)

A quantitative ELISA was performed to assess anti-DIII specific IgG in serum of vaccinated mice. Individual wells of a 96 microtiter plate were coated overnight at 4°C with WNV DIII proteins produced from transfected 293T cells and then blocked (25°C for 2 hr) with PBS supplemented with Tween-20 (0.05%) and nonfat dry milk (5%). Each serum sample was serially diluted and incubated (25°C for 2 hr). Following serial washes with PBS Tween-20 (0.05%), samples were incubated (25°C for 1 hr) with HRP conjugated goat anti-mouse IgG (1:5000) or one of four IgG subclasses (IgG_1, IgG_{2a}, IgG_{2b}, or IgG_3) (Southern Biotechnology, Birmingham, AL) diluted in PBS Tween-20 (0.05%) and nonfat dry milk (5%). Unbound antibody was removed and after additional washes samples were incubated with TMB substrate, and the colorimetric change was measured as the optical density at 405 nm using a plate reader (Biotek Powerwave XS, Winooski, VT USA). The O.D. value of the age-matched naïve sera was subtracted from the OD values of the antisera from the vaccinated mice. Results were recorded as the geometric mean titer (GMT) ± the standard error of the mean (SEM).

Focus Neutralization Reduction Assay (FRNT)

Sera from individual mice were heat inactivated at 56°C for 30 min. In some cases, sera from moribund or surviving mice following WNV infection were pooled. Pooled sera were diluted 1:10 in DMEM supplemented with 1% FBS, P/S and HEPES, and serially diluted 2-fold thereafter. Pooled naïve sera from uninfected, unvaccinated C57Bl/6 mice were used as a negative control. The 7H2 antibody, which neutralizes WNV in tissue culture [16], was diluted in naïve mouse sera and used as a positive control. Each dilution was incubated in an equal volume of media containing WNV for 1 h at 37°C. The virus-antibody solutions were then placed in duplicate wells in a 24-well plate containing a confluent Vero monolayer and incubated at 37°C for 1 h. Monolayers were rinsed free of

unbound virus-antibody solution, rinsed an additional time with PBS, and then covered with the CMC overlay. After 48 hours, monolayers were fixed with a 50:50 v/v methanol and acetone solution. WNV foci were detected by immunohistochemistry as described above. Titer was determined as the dilution in which there was 50% ($FNRT_{50}$) or 80% ($FRNT_{80}$) or greater reduction in the number of WNV foci by immunohistochemical staining.

Results

Construction of WNV Vaccine Plasmids

DNA plasmids were constructed that contained either the complete E gene (in context with the precursor viral gene prM "prM/E") or portions of the E gene (ectodomain (Ecto) or domain III (DIII)) (Fig 1A). A second set of plasmids was generated with these same gene sequences conjugated to two copies of the molecular adjuvant P28 to enhance antibody responses to the conjugated antigen (Fig. 1A). All of these gene cassettes were cloned directly downstream of a cytomegalovirus promoter to drive efficient transcription. Each plasmid efficiently expressed the E gene insert in transiently transfected 293T cells as determined by Western blot of clarified cell supernatant with a WNV-specific anti-E MAbs (Fig 1B). DNA plasmids expressing DIII only produced a protein ~10-20 kD in size. The addition of P28 resulted in an expressed protein of ~30 kD (Fig 1B; lanes 1 and 2). Ecto E (~65 kD) and Ecto E-P28 (~70 kD) were efficiently secreted into the supernatants of transiently transfected cells (Fig 1B; lanes 3 and 4). In addition, a 65 kD protein representing E was detected in supernatants from cells transiently transfected with DNA expressing the prM/E gene cassette, which produces subviral particles (SVPs) (Fig 1B; lane 5). As expected, mock-transfected or vector-only transfected cell supernatants showed no reactivity with WNV anti-E MAbs.

The molecular adjuvant P28 enhances the anti-WNV antibody response

Mice were vaccinated with the panel of DNA vaccines via one of two routes: gene gun (GG) or intramuscular (IM) at weeks 0, 3, and 6. On week 8, serum samples were collected and the anti-WNV DIII antibody levels were tested by ELISA from individual clarified sera samples (Fig. 2). C57BL/6 mice immunized with all of the DNA plasmids via the gene gun route developed high titers of anti-WNV DIII antibodies. In contrast, mice vaccinated by an intramuscular route with DIII-DNA had significantly lower total IgG titers that were significantly enhanced by conjugation of P28 (Fig. 2A). The enhancement effect was observed following gene gun administration of plasmids only at lower doses of vaccine (Fig 2B). Mice vaccinated IM with Ecto E, Ecto E-P28 or prM/E developed similar titers (Fig 2A).

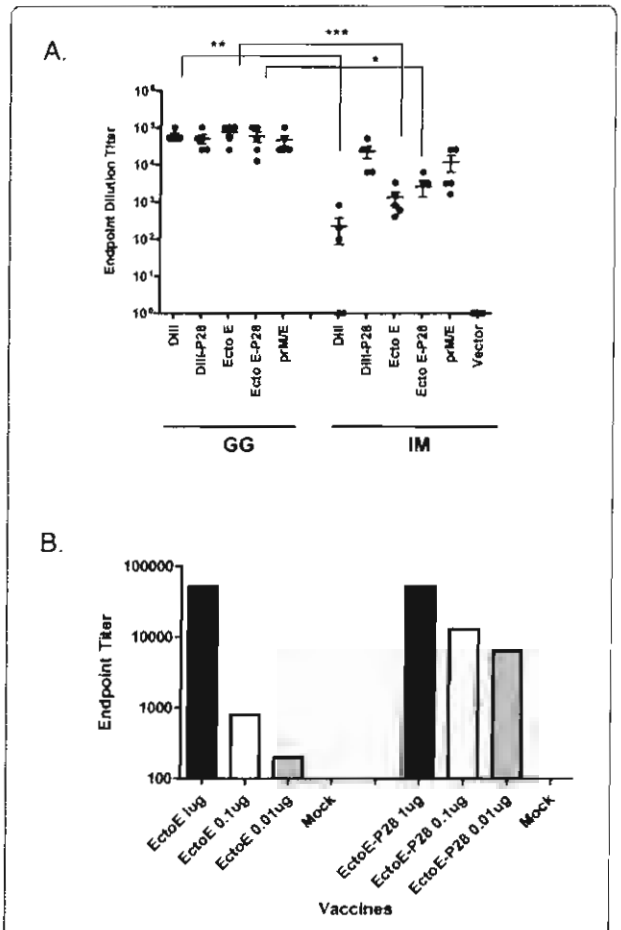

Figure 2 Vaccine elicited anti-DIII antibodies. Total IgG titers were measured by ELISA on WNV DIII-coated plates from mice vaccinated ID or IM with DNA plasmids encoding sections of the WNV E gene, with or without molecular adjutant P28 on week 8. Each dot represents an individual mouse. Undetectable antibody titers were arbitrarily assigned a titer of 1. Error bars denote the standard error within the samples with a measurable titer. Representative data from 1 of 2 experiments shown. A 2-way unmatched ANOVA with a Bonferroni post-test was used to determine the significance of the data between groups, which is denoted by asterisks; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

To characterize further the immune response elicited by these vaccines, the IgG subtypes of the elicited anti-DIII antibodies were determined (Table 1). Gene gun DNA vaccination elicited primarily a T-helper (Th)-2 (characterized by IgG1 isotype), whereas DNA plasmids administered intramuscularly elicited more of a Th-1 response (characterized by IgG2 isotype). C57BL/6 mice immunized by gene gun with DIII- or DIII-P28-DNA elicited predominately IgG_1 and IgG_{2b}. Similar antibody isotypes were elicited with Ecto E and Ecto E-P28 expressing plasmids. Interestingly, the prM/E plasmid elicited a broader IgG isotype profile via both ID and IM

Table 1: Anti-DIII Antibody Isotypes

C57BL/6 Gene Gun	IgG1	IgG2a*	IgG2b	IgG2c
DIII	0.44	N.A.	0.18	0.00
DIII-P28	0.43	N.A.	0.24	0.00
Ecto E	0.41	N.A.	0.19	0.00
Ecto E-P28	0.38	N.A.	0.12	0.01
prM/E	0.44	N.A.	0.36	0.18
C57BL/6 Intramuscular	IgG1	IgG2a	IgG2b	IgG2c
DIII	0.13	N.A.**	0.01	0.04
DIII-P28	0.41	N.A.	0.14	0.02
Ecto E	0.23	N.A.	0.08	0.00
Ecto E-P28	0.23	N.A.	0.04	0.01
prM/E	0.25	N.A.	0.20	0.11

*IgG2a isotype not expressed in C57BL/6 mice.
**N.A. = Not applicable since the IgG2a subtype is not expressed in C57BL/6 mice.

routes and included anti-DIII antibodies of the IgG_{2c} subclass.

Protection of mice against lethal WNV challenge

At week 10 (4 weeks from the final vaccination), C57BL/6 mice were challenged with a lethal dose (10^3 FFU) of WNV. All unvaccinated and 80% of vector-only vaccinated mice (IM) infected with WNV died by day 11 post-challenge (Fig. 3). The vector-only vaccinated mouse that survived challenge nonetheless showed signs of morbidity (hunchback posture and fur ruffling) and lost ~15% of body weight, but recovered by 1 month post infection (Fig. 4). All mice vaccinated with the prM/E plasmid construct survived lethal challenge (Fig. 3) and did not lose

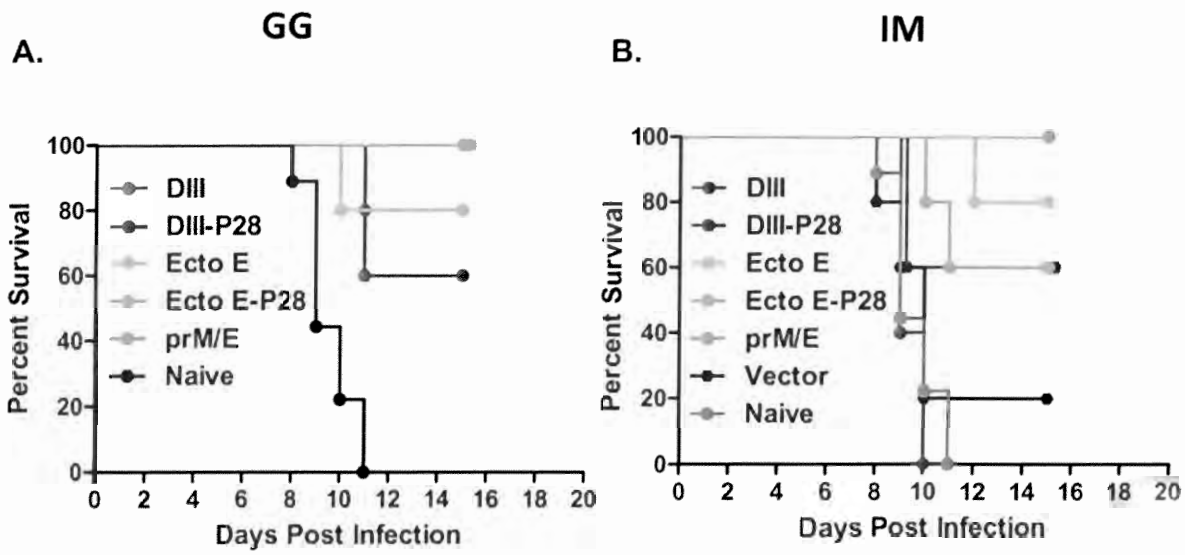

Figure 3 WNV challenge of vaccinated mice Mice that were vaccinated with indicated plasmids were challenged with 10^3 FFU of WNV and assessed daily for survival. Mice that were moribund were euthanized and counted as dead the following day. MOCK-challenged mice, which were injected with diluent only, did not die or show signs of illness, and are not shown on the survival curves. The results are pooled from at 5 mice.

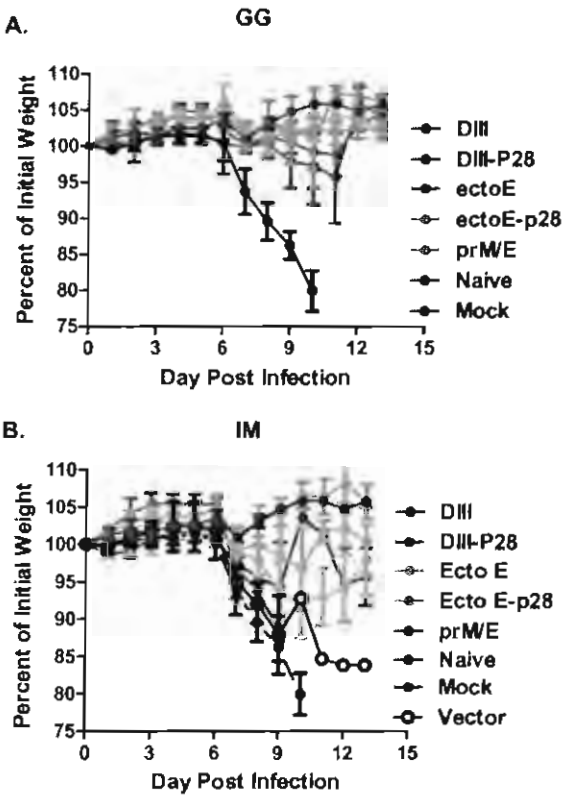

Figure 4 Weight loss curves of WNV-infected vaccinated mice. The weights of mice vaccinated by the GG (**A**) and IM (**B**) routes and challenged with 10^3 FFU of WNV were recorded daily. Dead and moribund mice were included in the weight loss curves on the day of death, but not after. The daily weight of each mouse was compared to her weight the day of challenge, and data are shown as the average percentage of initial weight for each cohort. The color scheme is identical for both panels. Error bars represent the standard error for all samples available at that time point.

weight, regardless of the vaccination route. In general, mice vaccinated by gene gun had higher rates of survival and less weight loss compared to mice vaccinated intramuscularly. Eighty percent of mice vaccinated ID with DIII-DNA survived challenge with little weight loss (Fig. 3A and 4) whereas, no mice vaccinated IM with DIII-DNA survived challenge (Fig. 3B, $p < 0.004$). All of these mice lost weight prior to succumbing to infection (Fig. 4C). Conjugation of P28 to DIII enhanced the survival rate to 60% when the DNA was administered IM (Fig. 3B, $p < 0.046$). Interestingly, the disparity in survival between ID and IM vaccination routes was also apparent in mice vaccinated with Ecto E DNA (Fig. 3). Eighty percent of mice vaccinated ID or IM with Ecto E-DNA survived challenge, and conjugation of P28 to Ecto E increased the survival rate (100%) of ID-vaccinated animals, but slightly decreased rate in IM-vaccinated mice (60%, Fig 3). Although these survival results were not statistically different between these groups, survival did correlated with weight loss, as mice that lost more weight had the highest morbidity and mortality (Fig. 4).

To determine if protection against WNV challenge prospectively correlated with a reduction in viremia, the sera at day 2 post-infection in each vaccine group was analyzed for infectious virus by a focus forming assay. Vaccinated C57BL/6 mice that survived infection (Fig. 5) had viremia that was at or below the limit of detection at day 2 post-challenge. With the exception of one mouse vaccinated with the Ecto E vaccine, all mice that died had a viremia at day 3 of greater than 4×10^2 FFU/ml (Fig 5).

Neutralization titers

Sera from mice immunized with the DNA vaccines were assayed for the ability to neutralize WNV infection in cell culture (Table 2). Serum samples collected at week 8 and again at 1 month post challenge (week 14 of study) were divided into two groups: (1) mice that survived subsequent lethal challenge and (2) mice that did not survive challenge (obtained from terminally moribund mice immediately prior to euthanasia). Mice vaccinated with any of the DNA vaccines by gene gun that survived challenge had high neutralizing titers at eight weeks (1/80-1/320; $FRNT_{80}$), whereas those that died from challenge had lower titers <1/20 (Table 2). In contrast to gene gun vaccination, only mice vaccinated with prM-E DNA intramuscularly had high neutralizing titers, which again correlated with survival. Mice vaccinated via gene gun with DNA plasmids expressing DIII, Ecto E, or these immunogens conjugated to P28 had titers <1/20 ($FRNT_{80}$) did not survive infection. Similar results were observed using a $FRNT_{50}$, albeit the titers were higher than $FRNT_{80}$. Regardless of the route of vaccination, mice that survived challenge exhibited an immunological boost by 14 weeks since neutralizing titers rose following infection ($FRNT_{80}$; 1/320-1/1280).

Passive sera transfer protects mice from virus infection

Although several mice immunized with DIII plasmids survived infection, it remained unclear mechanistically whether this was due to antibodies or possibly, memory $CD4^+$ and $CD8^+$ T cell responses To determine if anti-DIII antibodies alone could afford protection against WNV infection, pooled antiserum from each gene gun vaccinated group was transferred intraperitoneally into naïve 5 week-old C57BL/6 mice, which were then challenged with 10^2 PFU of WNV (Fig. 6). Fifty to seventy percent of mice administered sera from mice vaccinated with DIII, Ecto E or Ecto E-P28 survived challenge 21 days post-infection. Sera from mice vaccinated with DIII-P28 or prM-E showed greater (80-90%) survival compared to DIII alone, although this did not attain statistical significance. As expected, nearly all mice (10% survival))

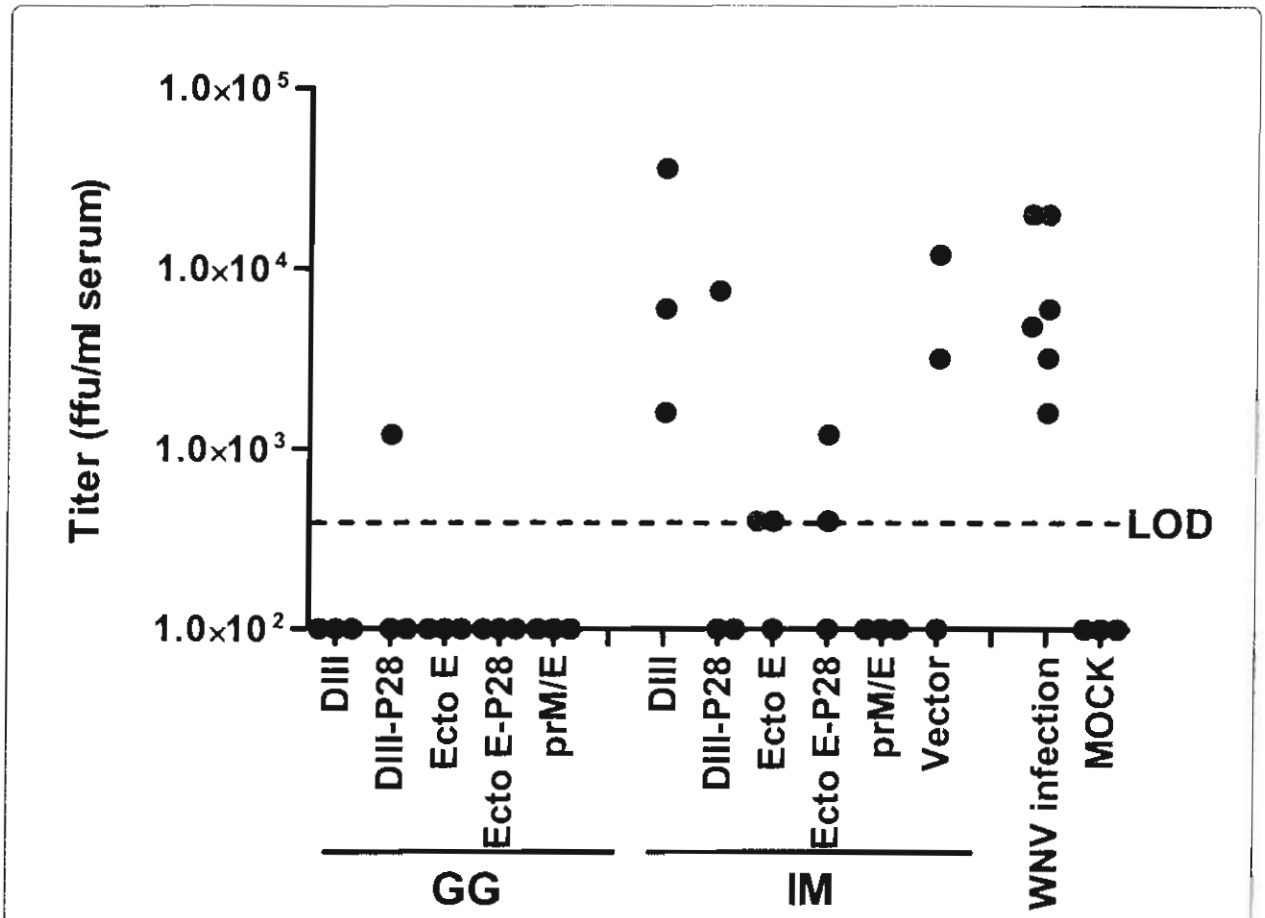

Figure 5 Viremia of protected and unprotected mice following WNV challenge. Sera taken from the blood of three mice from each group 2 days after infection were titrated on Vero cells to determine levels of virenia. Each point represents the titer from one mouse; black and red dots denote animals that survived and died, respectively. The dashed line represents the limit of detection (LOD) for this assay, which was 4 × 10^2 FFU/ml of serum. Mice with a viremia less than the LOD were arbitrarily shown as dots at the bottom of the graph.

administered naïve sera succumbed to virulent WNV infection.

Discussion

Although it has been a decade since the emergence of WNV in North America, there remains no effective, licensed vaccine to combat WNV induced disease in humans. Although candidate vaccines have not advanced beyond phase I and II clinical trials for humans [19,28], there are currently approved inactivated and DNA vaccines licensed for use in horses and geese. Since neutralizing antibodies may serve as a primary protective function against challenge [5], recent vaccine strategies have focused on using the ectodomain of E or different domains within E to elicit neutralizing anti-WNV antibodies [23,24,26,29-33]. Recent attention has been focused on DIII as a potential immunogen because structural and functional studies suggest that many protective antibodies against WNV recognize this highly conserved epitopes within this region. Some DIII-specific neutralizing antibodies are particularly potent in blocking viral fusion and escape from the endosome [34,35].

In this study, a series of DNA-based vaccines expressing the full length E, Ecto E or the DIII domain of E were fused to the molecular adjuvant P28 to enhance antibody titers. The addition of P28 to DIII or Ecto E increased the anti-DIII IgG antibody titer in C57BL/6 mice. However, a high anti-DIII antibody titer was not sufficient to completely protect against WNV infection. Mice vaccinated with nearly all gene gun delivered vaccines elicited similar high-titer anti-DIII antibodies, however, only the prM/E and Ecto E-P28 vaccinated mice were completely protected from lethal challenge.

Table 2: FRNT and Mouse Survival

Sample	Group	Survival [# pooled/# in vaccinated group]	FRNT50*	FRNT80	FRNT50	FRNT80
GG			Pre-infection		Post-infection	
1	DIII	Yes [4/5]	640	80	2560	320
2	DIII	No [1/5]	<20	<20		
3	DIII-P28	Yes [3/5]	320	80	1280	320
4	DIII-P28	No [2/5]	40	20		
5	ectoE	Yes [4/5]	320	320	2560	640
6	ectoE	No [1/5]	40	<20		
7	ectoE-28	Yes [5/5]	640	320	2560	640
8	prM/E	Yes [5/5]	320	160	1280	640
9	naive	No [5/5]	20	<20		
IM			Pre-infection		Post-infection	
10	DIII	No [5/5]	<20	<20		
11	DIII-p28	Yes [3/5]	20	<20	1280	640
12	DIII-p28	No [2/5]	20	<20		
13	ectoE	Yes [4/5]	40	20	2560	1280
14	ectoE	No [1/5]	20	<20		
15	ectoE-p28	Yes [3/5]	40	20	2560	640
16	ectoE-p28	No [2/5]	<20	<20		
17	prM/E	Yes [5/5]	320	160	2560	640
18	vector	Yes [1/5]	40	<20	640	80
19	vector	No [4/5]	<20	<20		

* Titers shown as the reciprocal titer

Several studies have previously demonstrated the immunogenicity of the E protein DIII domain [23,29,36-40]. However, immunization of purified recombinant DIII has not consistently elicited high-titer neutralizing antibodies, thereby indicating that the neutralizing epitope may be poorly immunogenic in the context of a soluble protein, or that a dominant non-neutralizing epitope is present on the soluble DIII but is not exposed in the context of the virion. Expression of DIII from a DNA vaccine plasmid also has been less than optimal in eliciting neutralizing antibodies. The results from our study suggest that DIII may be less efficiently secreted from transfected cells (data not shown), which may in part explain the lowered immune responses seen during vaccination with DIII alone. Conjugation of P28 may assist DIII protein secretion from transfected cells, helping to explain why the P28-conjugated vaccines elicited higher DIII-specific antibody titers (by ELISA) and higher protection against lethal challenge than non-conjugated vaccines in some cases. Although the use of molecular adjuvants, such as P28, did not skew the antibody repertoire, they did increase the efficacy of the response of DIII-based DNA vaccines. An analogous increase in overall titer was observed when JEV or WNV DIII was linked to IL-15 [24].

Prospective studies have shown a direct correlation between the level of neutralizing antibody prior to challenge, the magnitude of viremia, and survival rates in mice [5]. Nonetheless, some mice vaccinated IM with DIII-P28, Ecto E, and Ecto E-P28 were protected from challenge despite the absence of high-titer pre-challenge neutralizing antibodies ($FRNT_{80} \leq 20$). Although further mechanistic studies are required, we suggest three possible explanations: (a) non-neutralizing antibodies are protective through complement and/or Fc?R-dependent functions. Indeed, we have previously seen this phenotype with mAbs against WNV NS1, which is absent from the virion [41]; (b) the *in vitro* neutralization assay does

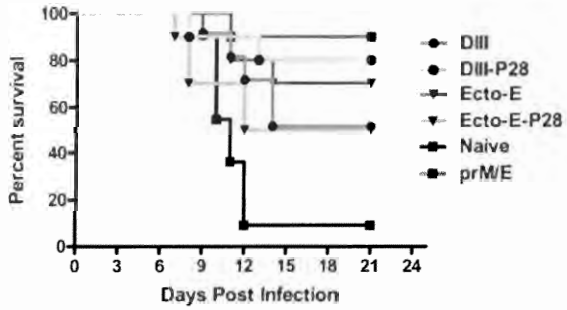

Figure 6 Passive transfer of antibody from vaccinated mice protects against lethal WNV challenge. Five week-old C57BL/6 mice were pre-treated with 25 μl of serum from the indicated vaccinated mice or naive animals at day -1 by intraperitoneal injection. On day 0, mice were infected with 10^2 PFU of WNV by subcutaneous route and monitored for survival. All vaccinated mice showed statistically significant protection ($P < 0.05$, log rank test) compared to mice receiving naive sera. Data reflect two independent experiments with a total of $n = 10$ mice per arm.

not accurately reflect possible neutralization of virus *in vivo*. Antibodies that block virus attachment of one cell type (*e.g.*, Vero cells) may not function effectively against a second more physiologically relevant cell type (*e.g.*, dendritic cells). Of note, differences in neutralization potency among cell types were observed with mAbs against epitopes in DI and DII of WNV E protein [42]; and/or (c) T cell responses to peptide epitopes in the E protein independently contribute to protection. The isotype of the polyclonal antibody in part determines the effector functions of the anti-WNV antibodies and identifies the T helper cell bias (required for antibody class switching). Antibodies of the IgG2a/c and IgG2b subclass fix complement proteins C1q and C3 and can opsoznize and inhibit flavivirus infection [43-45]. IgG2a/c bind FcγRI with high avidity facilitating enhanced uptake of virus-antibody complexes by macrophages. The predominant IgG isotype detected was IgG1 indicating a Th2 bias. However, IgG2b was detected in almost all vaccine groups, with a strong level of this isotype and IgG2c detected in prM/E vaccinated mice (Table 1), which may help to explain the effectiveness of these vaccines. C57BL/6 mice do not express IgG2a, if this isotype was associated with protection, could have been an explanation for the inconsistent protection with these vaccines.

C3d and P28 have been used as effective molecular adjuvants to elicit high titer antibodies against other pathogens [18,27,46-53]. This study extends this platform to WNV, and likely other flaviviruses due to the similarity in E protein structure and function within the genus. Interestingly, gene gun administration of DNA plasmids elicited higher titer antibody responses and broader protection against WNV infection than through the IM route. For mice that were vaccinated gene gun, there was a clear correlation between viral neutralization titers and survival. This correlate was less apparent in IM-vaccinated mice, with most mice surviving infection having low neutralization titers. This discrepancy in survival may be explained, at least in part, by the types of cells that internalize the DNA plasmids and express and/or present these antigens. Muscle cells and infiltrating appear to internalize plasmid DNA following IM administration [54]; these cell types may not efficiently secrete these viral proteins. Gene gun differs from intramuscular or intradermal injection of DNA with a needle and syringe in that it results in direct delivery of the vaccine into the intracellular environment [55]. Gene gun delivery of DNA plasmids is complex and can involve both non-professional antigen presenting cells (APC), such as keratinocytes and professional APCs, such as Langerhans cells [56] and [57]. Compared to other routes of delivery, gene gun inoculations can induce both antibody and $CD8^+$ T cell responses with substantially lower doses of DNA. The effectiveness of this system is likely related to the use of a delivery technology that deposits DNA directly into cells [55]and [57] as well as the immune competence of the epidermis as a delivery site [58] and [59]. Skin cells likely traffic to the draining lymph nodes where the expressed proteins are processed and presented to immune cells [60,61].

Passive transfer studies with serum from vaccinated mice to naïve mice established that antibody generated after immunization was sufficient for protection. The percentage of mice surviving challenge after passive transfer appeared similar to the percentage of vaccinated mice that survived after direct challenge. Although further studies with depleting anti-CD4 and CD8 mAbs are required to precisely evaluate the contribution of T cells to protection in these vaccinated mice, it is noteworthy that for C57BL/6 mice, the immunodominant H-2b T cell epitopes for WNV fall outside of DIII [62-64].

In general, immunogens based upon Ecto E elicited better protective responses than those based upon DIII. Likely, WNV E proteins contain multiple neutralizing epitopes in separate domains and therefore, a broader panel of neutralizing antibodies can be generated. The prM/E plasmid produces SVPs, which are effective immunogens since they contain conformationally relevant prM and E protein [14,65,66]. However, Ecto E-P28 elicited comparable immune responses and protection as prM/E when delivered ID, indicating that DNA vaccination can be as effective as the prM/E vaccines currently used for animal vaccines.

The results from the WNV vaccines described in this report indicate that DIII can be an effective immunogen when expressed from a DNA plasmid, when conjugated to a molecular adjuvant like P28 and delivered as a gene

gun based DNA vaccine. The mechanism of delivery could account for the induction of protective responses. Gene gun elicits a T cell helper type 2 (Th2) bias, as indicated by the predominance of elicited IgG_1, which may be just as an important factor in eliciting neutralizing antibodies as the immunogen epitopes. Nonetheless, our studies have not yet shown that DIII elicits superior neutralizing responses when conjugated to a C3d molecular adjuvant. Although further studies are warranted, we speculate that this is due to the presence of immunodominant non-neutralizing epitopes on the A-B loop that is normally solvent inaccessible [67]. Reverse genetic studies are underway to create DIII variants that lack this immunodominant epitope and thus, focus the immune response on the lateral ridge epitope, which is recognized by highly neutralizing antibodies. By combining this molecular approach with the addition of P28, alone or with other vaccine modalities, we believe it will be possible to create a catalogue of safe immunogens that elicit high-titer neutralizing antibody responses against all flaviviruses.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MDD and DMC constructed and characterized the vaccines, MDD and SLR performed animal vaccinations, SLR performed virological analysis, and MDD, SLR, MRV, EM immune analysis. MDD, SLR, MSD, and TMR wrote the manuscript. MSD and TMR conceived the studies and participated in experimental design and coordination. All authors read and approved the manuscript.

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APPENDIX 2

Hemagglutinin Displayed Baculovirus Protects Against Highly Pathogenic Influenza

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Hemagglutinin Displayed Baculovirus Protects Against Highly Pathogenic Influenza

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ABSTRACT

Baculovirus (BV) replicating in insect cells can express a foreign gene product as part of its genome. The influenza hemagglutinin (HA) can be expressed from BV and displayed on the surface of baculovirus (HA-DBV). In this study we first generated six recombinant baculoviruses that expressed chimeric HAs with segments of the BV glycoprotein (gp64). The signal peptide (SP) and cytoplasmic tail (CT) domains of gp64 can enhance the display of HA from A/PR8/34 on BV surface, while the transmembrane (TM) domain of gp64 impairs HA display. Different doses of either live or β-propiolactone (BPL)-inactivated HA-DBV were administered to BALB/c mice. Live HA-D8V elicited higher hemagglutination-inhibition (HAI) titers than BPL-inactivated HA-DBV, and provided sterilizing protection. A second generation recombinant BV simultaneously displaying four HAs derived from four subclades of H5N1 influenza viruses was constructed. This tetravalent H5N1 HA-DBV vaccine elicited HAI titers against all four homologous H5N1 viruses, significantly decreasing viral lung titers of challenged mice and providing 100% protection against lethal doses of homologous H5N1 viruses. Moreover, mice vaccinated with HA-DBV had high levels of IFN-γ-secreting and HA-specific CD8+ T cells. Taken together, this study demonstrates that HA-DBV can stimulate strong humoral, as well as cellular immune responses, and is an effective vaccine candidate for influenza.

1. Introduction

Each year, seasonal influenza causes over 300,000 hospitalizations and 36,000 deaths in the US alone [1]. The emergence of the novel H1N1 influenza virus in 2009 demonstrated how quickly a new influenza pandemic can sweep across the world. The spread of highly pathogenic H5N1 viruses in birds and coincident infections in humans have raised the concerns that H5N1 viruses may cause a new pandemic in humans. Vaccination is an effective method to prevent influenza infection. There are two influenza vaccine approaches licensed in the US; the inactivated, split vaccine and the live-attenuated virus vaccine. Inactivated vaccines can efficiently induce humoral immune responses but generally only poor cellular immune responses.

Baculoviruses are a family of large rod-shaped enveloped viruses with a large circular double-stranded DNA genome (80–200 kb). Baculoviruses infect some insects, but not mammals [2]. *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the most well studied baculovirus and most extensively used for protein expression because the polyhedron (PH) and p10 promoters are efficient promoters [3]. Compared to other protein expression approaches, the baculovirus expression vector system (BEVS) produces abundant protein yields with appropriate eukaryotic glycosylation and other modifications. Recently, this system has also been used for virus-like particle (VLP) production for vaccines against HIV, HPV and influenza [4–6]. However, the BV-derived VLPs are always accompanied with BV contamination. Therefore, separating VLPs from contaminating BV is one of the obstacles that need to be overcome.

Due to its low cytotoxicity and absence of pre-existing antibodies [7,8], AcMNPV has emerged as a potent vaccine vector [9–13]. Foreign immunogens or peptides can be displayed on the envelope of AcMNPV by fusion with the baculovirus major envelope protein gp64 [14,15]. Based on the baculovirus display system, some efficient vaccines have been studied not only for viral diseases, but also for parasitic disease, such as, classical swine fever virus [16], influenza virus [12,17–19], avian reovirus [11], bovine herpesvirus [20], *Plasmodium berghei* [13,14], and *Plasmodium fal-*

Abbreviations: AcMNPV, *Autographa californica* multiple nucleopolyhedrovirus; BEVS, baculovirus expression vector system; BPL, β-propiolactone; CT, cytoplasmic tail; CTL, cytotoxic T lymphocytes; DBV, displayed baculovirus; HA, hemagglutinin or hemagglutination assay; HAI, hemagglutination inhibition; IFU, infectious unit; PFU, plaque form unit; SP, signal peptide; TM, transmembrane; VLP, virus-like particle.

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Table 1
Primers used for PR8-HA displaying constructs

Primer	Primer sequence (5'-3')	Primer annotation
A	CGCTGATCAGCCACC**ATG**CTACTGGTAAATCAGTCACAC	Forward primer for gp64 Signal peptide with *Bcl* I site
B	CGAGCTCGTCGACAGGCCTGAATTCGGATCCCGCAAAGGCAGAATGCGCC	Reverse primer for gp64 Signal peptide with multiple cloning sites
C	CAGGCCTGTCGACGAGCTCGCGGCCGCGTTCATGTTTGGTCATGTAG	Forward primer for gp64 TM-CT with multiple cloning sites
D	AAGCGGCCG**TTA**ATATTGTCTATTACGGTTTCTAATC	Reverse primer for gp64 TM-CT with *Eag* I site
E	CAAGTCGACGCCACC**ATG**AAGGCAAACCTACTGGTCC	Forward primer for HA of PR8 virus with *Sal* I site
F	CTCGCGGCCGC**TCA**GATGCATATTCTGCACTGC	Reverse primer for HA of PR8 virus with *Not* I site
G	GCGGGATCCGCAGACACAATATGTATAGGC	Forward primer for PR8 HA without SP (with *BamH* I)
H	AACGCGGCCGCAATCTGATAGATCCCCATTGATTC	Reverse primer for PR8 HA without TM, CT (with *Not* I)
I	GGCTCTAGA**TTA***ATATTGTCTATTACGGTTTC*TACACATCCAGAAACTGATTGC	Reverse primer for PR8 HA with CT of gp64 (with Xba I)

Underlined sequences are restriction enzyme sites. Bolded sequences are start or stop codons.

ciparum [8]. Most BV display strategies rely on gp64 protein which is the major envelope protein of baculovirus. Both influenza HA and baculovirus gp64 are type I transmembrane glycoproteins comprised of an amino-terminal signal peptide domain, carboxy-proximal transmembrane domain and cytoplasmic tail domain. Both proteins mediate viral entry into the host cells and efficient virion budding [21,22]. HA and gp64 proteins get incorporated into the infected host cell membrane. During the budding process, the budding virons pick up the protein as the constituent viral envelope [19,23]. Therefore, influenza HA can be displayed on the surface of baculovirus [12,17,19,24]. In this study, we investigated how the SP, TM, CT domains of gp64 influenced HA incorporation into BV. HA-DBVs protected mice against virus challenge even with low doses of vaccine. Moreover, we constructed a recombinant baculovirus simultaneously displaying four HAs derived from four subclades of H5N1 influenza. Vaccination with the tetravalent HA-DBVs stimulated strong humoral and cellular immune responses and protected mice against lethal H5N1 influenza virus challenges.

2. Materials and methods

2.1. Cells and viruses

Spodoptera frugiperda (Sf9) cells were propagated at 28 °C in Sf-900II serum free medium. Cells were infected by each recombinant baculoviruses at a multiplicity of infection (MOI) of 0.1–1.0 and virus supernatants were collected 4 days post-infection. Influenza viruses used in this study included the subtype H1N1, A/Puerto Rico/8/1934 (PR8), or the PR8-reassortant H5N1 viruses representing A/Vietnam/1203/2004 (VN/04), A/Indonesia/5/05 (IN/05), A/Whooper Swan/244/Mongolia/05 (WS/05), and A/Anhui/1/05 HA (AH/05). Each reassortant virus expressed the HA and NA derived from H5N1 viruses and the internal protein genes came from A/PR/8/1934 donor virus. Each virus was used to infect mice as previously described [25].

2.2. Construction of plasmids and recombinant baculoviruses

The SP, TM, and CT domains of the gp64 gene were amplified from Bacmid DNA by PCR. The full-length or ectodomain of HA genes from mouse-adapted PR8 were amplified by PCR from one plasmid containing full-length HA of the PR8 virus. A series of plasmids encoding the SP, TM, CT regions of gp64 and various portions of HA were generated using the following strategy. Nine primers (A-I as shown in Table 1) were used to generate the chimeric HA-gp64 genes. Primers A and B were used to amplify the gene fragment encoding the gp64 SP. Primers C and D were used to amplify the gene fragment encoding gp64 TM and CT. Primers E and F were used to amplify full-length HA of PR8. Primers G and H were used to amplify the ectodomain of PR8 HA (without SP, TM and CT). Primers E and I were used to amplify PR8 HA, but the CT derived from gp64. Primers E and H were used to amplify PR8 HA without TM and CT. Primers G and F were used to amplify PR8 HA without SP. Primers G and I were used to amplify PR8 HA without SP, and CT deriving from gp64. Appropriate fragments were serially inserted into pFastBac transfer vector (Invitrogen, Carlsbad, CA) in frame. Thus, each construct expresses chimeric PR8 HA proteins (Fig. 1A and B). All recombinant baculoviruses were generated using the Bac-to-Bac system (Invitrogen, Carlsbad, CA) and designated as Bac-HA, Bac-spHA, Bac-spHAct, Bac-HAct, Bac-HAtmct, Bac-spHAtmct.

The recombinant virus selection and amplification were performed following standard protocols. The infectious titers of recombinant baculoviruses were determined by the BacPAK Baculovirus Rapid Titer Kit (Clontech, Mountain View, CA) and are expressed as infectious units per milliliter (ifu/ml).

2.3. Purification of HA-displayed baculovirus

The recombinant baculoviruses were produced by infecting Sf9 cells at an MOI of 0.1. Supernatants were collected 4 days after

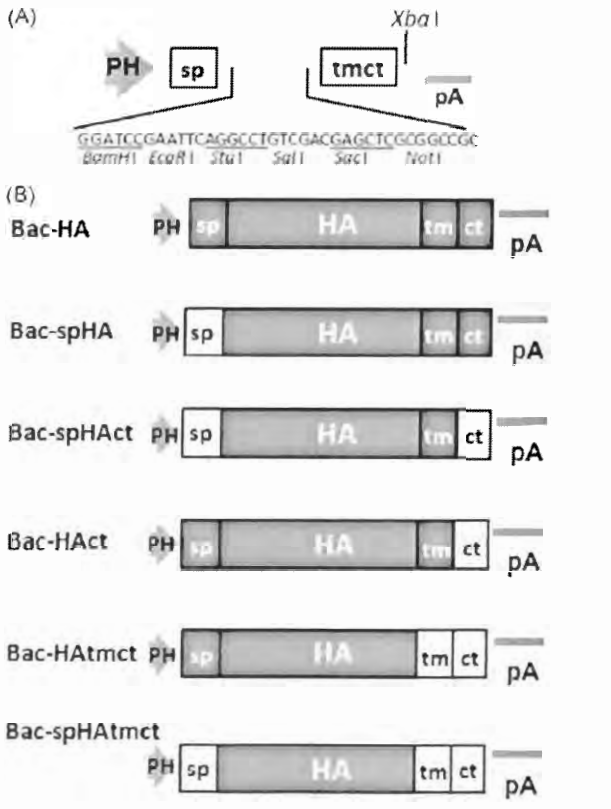

Fig. 1. Schematic illustration of the HA-pseudotyped baculovirus. (A) Modified transfer vector with SP, TM, CT domain sequences of gp64 and multiple cloning sites. (B) Schematic diagram of chimeric HA constructs. All components derived from HA are shown in dark gray while those from gp64 are shown in shadow.

infection and were clarified by centrifugation at 3000 g for 10 min at 4 °C to remove cell debris. Viral particles were precipitated via ultracentrifugation (100,000xg through 20% glycerol, w/v) for 4 h at 4 °C. The pellets were subsequently resuspended in PBS and stored at 4 °C. The viral titer was determined using the BacPAK Baculovirus Rapid Titer Kit.

2.4. Hemagglutination assay for HA-displayed baculoviruses

A series of 2-fold dilutions of HA- displayed baculovirus in PBS were prepared and incubated at 25 °C for 30 min with 50 μl of 1% turkey red blood cells (tRBCs), or 1 h with 50 μl of 1% horse red blood cells (hRBCs) (Lampire Biologicals, Pipersville, PA, USA). The extent of hemagglutination was inspected visually, and the highest dilution capable of agglutinating red blood cells was determined.

2.5. Hemadsorption assays

Insect Sf9 cells (infected or uninfected with recombinant baculovirus containing HA genes or no HA genes) were diluted to a concentration of $1x10^6$ cell/ml in PBS. 100 μl of cells were mixed with 10 μl of 1% red blood cells and shaken gently for 10 min at room temperature. Then 10 μl of the suspension was pipetted on a glass plate and observed under a microscopy [26].

2.6. Western blot analysis

The supernatants from rBV infected Sf9 cells or purified baculoviruses were subjected to Western blot analysis. Mouse Anti-PR8 HA polyclonal antibody (produced in our lab) and mouse anti-vp39 monoclonal antibody (kindly provided by Professor Matt Welch from University of California, Berkeley) was used to detect proteins. The primary antibodies were detected with goat anti-mouse monoclonal antibodies conjugated with horseradish peroxidase (1:5000, SouthernBiotech, Birmingham, Alabama).

2.7. Vaccinations

Female BALB/c mice (*Mus musculis*, females, 6–8 weeks) were purchased from Harlan Sprague Dawley (Indianapolis, IN, USA). Mice were housed in microisolator units and allowed free access to food and water and were cared for under USDA guidelines for laboratory animals. Mice (10 groups, 15 mice per group) were vaccinated with live or BPL inactivated Bac-spHAct at 3 different doses (1×10^8, 2×10^7, and 4×10^6 ifu/mouse) or with wild-type (wt) baculovirus (1×10^8 ifu), mammalian cell derived VLPs (6 μg) and PBS as control via intramuscular injection at week 0 and boosted with the same doses at week 3 (Table 2). The mammalian cell derived VLPs were produced by simultaneously transfecting 293T cells with three plasmids encoding HA, NA, and M1 genes of PR8 virus respectively as previously described [27]. The choice of dose is based on previous studies in our laboratory (unpublished data).

2.8. Hemagglutination inhibition (HAI) assays

Blood samples were collected from anesthetized mice via retro-orbital plexus puncture before immunization and at 2 weeks after each immunization (week 2, 5). After the blood samples were clotted and centrifuged, serum samples were collected. The HAI assay was used to assess functional antibodies to HA able to inhibit agglutination of erythrocytes. To inactivate non-specific inhibitors, aliquots of each serum sample were treated with receptor-destroying enzyme (RDE; Denka Seiken Co., Japan) overnight at 37 °C, heat-inactivated at 56 °C for 30 min, diluted to 1:5 with PBS [28]. RDE-treated sera (25 μl) were diluted serially two-fold in v-bottom 96-well microtiter plates. An equal volume of influenza virus, adjusted to approximately 8 HA units/50 μl was added to each well. The plates were covered and incubated at room temperature for 30 min followed by the addition of 50 μl freshly prepared 1% tRBCs or hRBCs in PBS. The plates were mixed by agitation, covered, and allowed to set for 30 min or 1 hr at 25 °C. The HAI titer was determined by the reciprocal of the last dilution that contained non-agglutinated RBCs. Positive and negative serum controls were included on each plate.

2.9. Challenge and viral load

Challenge infections were performed as previously described [25]. At 3 weeks after the final immunization, ketamine-anesthetized mice were intranasally infected with 1,500 plaque forming units (pfu) of A/PR/8/1934 virus (equivalent to 10 × the 50% lethal dose [LD_{50}]) in 50 μl of PBS. Mice were weighed daily and analyzed for disease (*i.e.* weight loss, ruffling fur, inactivity). Mice that lost greater than 20% of body weight were humanely euthanized. One day 3 and 6 post-challenge, five mice from each group were sacrificed and the lungs were harvested. The tissues were homogenized (the lung from one mouse was homogenized in 1 ml PBS), and viral load was determined by plaque assay on Madin-Darby canine kidney (MDCK) cells as previously described [25].

2.10. Construction of multiple-HA-displayed baculovirus

In order to introduce four expression cassettes into baculovirus, pFastBac Dual plasmid was firstly modified to contain two PH promoters and two multiple cloning sites (p2PH). The SP region of gp64 with Flag or His tag was inserted into p2PH to make the transfer vector p2PHsp. Two pairs of compatible restriction sites (*Asc* I -*Mlu* I, *Spe* I –*Avr* II) were introduced into p2PHsp as shown in Figure 2A. Appropriate ectodomain of HAs from four H5N1 influenza viruses were PCR amplified from the following virus strains: A/Vietnam/1203/2004 (clade 1), A/Indonesia/5/05 (clade 2.1), A/Whooper Swan/244/Mongolia/ 05 (clade 2.2), A/Anhui/1/05 HA (clade 2.3). The four-HA plasmid pHA1.0/2.1/2.2/2.3 was constructed in two stages (Fig. 2B). (i) We first constructed two dual-HA plasmids. HA fragments of VN/04 and IN/05 were cloned into one p2PHsp to obtain plasmid pHA1.0/2.1. HA fragments of WS/05 and AH/05 were cloned into another p2PHsp to obtain plasmid pHA2.2/2.3. (ii) The fragment containing the HA2.2 and HA2.3 cassettes, along with their promoter-terminator, was excised with *Asc* I and *Avr* II from pHA2.2/2.3 and cloned in between the *Mlu* I and *Spe* I sites in the pHA1.0/2.1 to obtain the four-HA plasmid pHA1.0/2.1/2.2/2.3 (p4HA). Recombinant baculoviruses were generated using the Bac-to-Bac system and designated as Bac-HA1.0/2.1, Bac-HA2.2/2.3 and Bac-HA1.0/2.1/2.2/2.3 (Bac-4HA). Multiple-HA-displayed baculoviruses were propagated and purified as above. Protein expression was checked by Western-blot, hemagglutination assay and hemadsorption assay.

2.11. Evaluation for the tetravalent H5N1 vaccine candidate in mouse model

Mice (9 groups, 12 mice per group) were intramuscularly vaccinated with Bac-HA2.2, Bac-4HA, or *wt* BV (1×10^7 ifu/mouse) at week 0 and 3 (Table 2). Serum was collected at weeks 2 and 5 to determine anti-HA-specific antibody titer. For virus challenge, anesthetized mice were infected intranasally with 5000 pfu of A/VN/04, A/IN/05, or A/WS/05 viruses in 50 μl of PBS per mouse at 3 weeks after the final immunization. Five mice from each group were sacrificed on day 3 post-challenge for examining virus replication in lungs. Five mice in each group were monitored daily for survival and morbidity post infection. Mice that lost greater than

Table 2
Mouse study groups and protective efficacy

Vaccines used in each mouse group	Immunization dose (ifu)	Virus and dose used for challenge (pfu)	Clinical signs**	Protection (%)
PR8 HA-displayed BV				
G1: Live Bac-spHAct	$1x10^8$	A/PR8 ($1.5x10^3$)	Healthy	100
G2: Live Bac-spHAct	$2x10^7$	A/PR8 ($1.5x10^3$)	Healthy	100
G3: Live Bac-spHAct	$4x10^6$	A/PR8 ($1.5x10^3$)	Healthy	100
G4: Live *wt* baculovirus	$1x10^9$	A/PR8 ($1.5x10^3$)	Sick (+++)	0
G5: Inactivated Bac-spHAct	$1x10^8$	A/PR8 ($1.5x10^3$)	Healthy	100
G6: Inactivated Bac-spHAct	$2x10^7$	A/PR8 ($1.5x10^3$)	Healthy	100
G7: Inactivated Bac-spHAct	$4x10^6$	A/PR8 ($1.5x10^3$)	Sick (+)	100
G8: Inactivated *wt* baculovirus	$1x10^8$	A/PR8 ($1.5x10^3$)	Sick (+++)	0
G9: PR8 VLP*	6 μg	A/PR8 ($1.5x10^3$)	Healthy	100
G10: PBS		A/PR8 ($1.5x10^3$)	Sick (+++)	0
H5N1 HA-displayed BV				
G1: Live Bac-HA2.2	$1x10^7$	A/VN/04 ($5x10^3$)	Sick (++)	60
G2: Live Bac-HA2.2	$1x10^7$	A/IN/05 ($5x10^3$)	Sick (++)	40
G3: Live Bac-HA2.2	$1x10^7$	A/WS/05 ($5x10^3$)	Healthy	100
G4: Live Bac-HA1.0/2.1/2.2/2.3	$1x10^7$	A/VN/04 ($5x10^3$)	Healthy	100
G5: Live Bac-HA1.0/2.1/2.2/2.3	$1x10^7$	A/IN/05 ($5x10^3$)	Healthy	100
G6: Live Bac-HA1.0/2.1/2.2/2.3	$1x10^7$	A/WS/05 ($5x10^3$)	Healthy	100
G7: Live *wt* baculovirus	$1x10^7$	A/VN/04 ($5x10^3$)	Sick (+++)	0
G8: Live *wt* baculovirus	$1x10^7$	A/IN/05 ($5x10^3$)	Sick (+++)	0
G9: Live *wt* baculovirus	$1x10^7$	A/WS/05 ($5x10^3$)	Sick (+++)	0

* VLP was produced by transfecting 293T cells with three plasmids expressing HA, NA and M1 of PR8 virus.

** Mice with +++ signs showed severe illness. Clinical signs were determined by body weight losses and mouse symptoms of illness. +++, lost in body weight of over 20% and riffling fur; ++, some mice died and some had only10–20% weight loss with riffling fur; +, 10–20% decreases in body weight, with riffling fur; healthy, < 5% body weight changes and no riffling fur.

20% of body weight were euthanized. The ability of each vaccine to protect against homologous or heterologous challenge was compared to separate groups of *wt*-baculovirus vaccinated control mice that were subsequently challenged with each reassortant virus. The remaining mice in each group were used to determine the elicitation of anti-HA specific cellular responses by murine IFNγ enzyme linked immunospot (IFNγ-ELISPOT) assay (R & D Systems, Minneapolis, MN, USA) and MHC class I pentamer staining (ProImmune, Oxford, UK).

2.12. IFNγ-ELISPOT assays

Spleens were harvested from vaccinated mice at day 6 and day 9 post-challenge and splenocytes were isolated for IFNγ-ELISPOT assays as previously described [29]. Briefly, pre-coated anti-IFNγ plates were incubated (25 °C for 2 h) with cRPMI (200 ml) and then incubated with freshly isolated splenocytes (5×10^5/well). Splenocytes were stimulated with the single peptides representing the immunodominant H2-Kd $CD8^+$ T cell epitopes HA_{533} and NP_{147} or as a negative control the unspecific Ova_{257} peptide (Pepscan Presto, Leystad, Netherlands). Both HA_{533} and NP_{147} peptides were originally derived from the PR8 (H1N1) virus, but they are conserved in H5N1 influenza viruses [29]. Additional wells were stimulated with PMA (50ng)/ionomycin (500ng) or were mock stimulated. In addition, IL-2 was added to all wells (10 units/ml). After 48 h stimulation, plates were washed with PBS-Tween (3 ×) and were incubated overnight at 4 °C with anti-mIFNγ antibody. The plates were washed and then incubated (25 °C for 2 h) with strepavidin

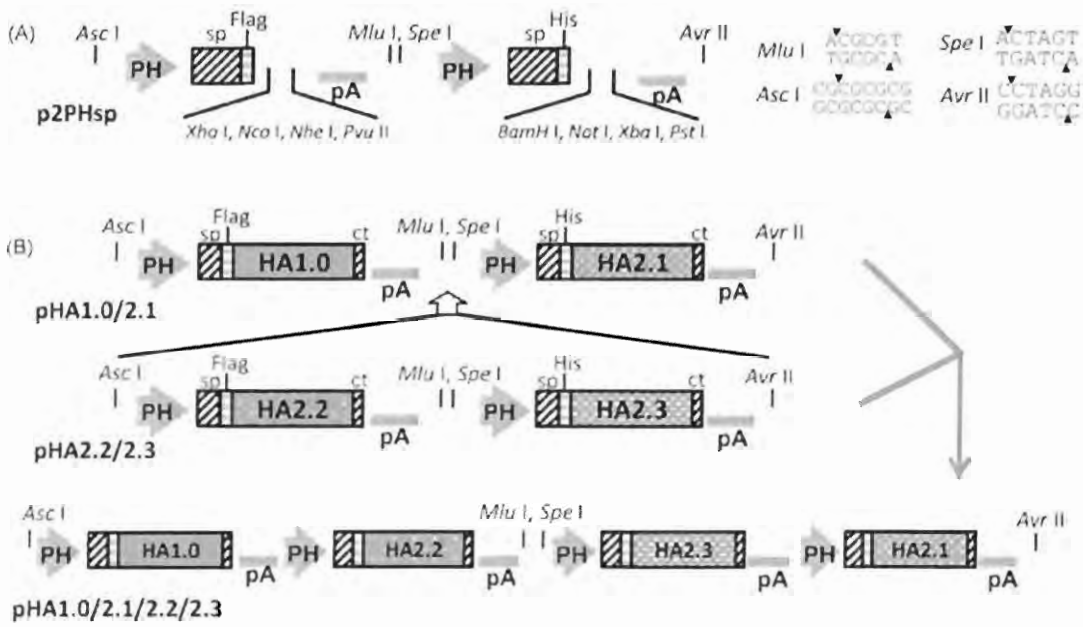

Fig. 2. Construction of four-unite transfer vector pHA1.0/2.1/2.2/2.3 (A) Dual-PH promoter transfer vector with SP of gp64 and two multiple cloning sites. (B) Stepwise construction of pHA1.0/2.1/2.2/2.3

conjugated to alkaline phosphatase. Following extensive washing, cytokine/antibody complexes were incubated at room temperature with BCIP/NBT chromagen until spots appeared. The plates were rinsed with dH_2O and air-dried at 25 °C. Spots were counted by an ImmunoSpot ELISPOT reader (Cellular Technology Ltd., Cleveland, OH, USA).

2.13. Flow cytometry

In order to detect influenza-specific $CD8^+$ T cells, MHC class I pentamer staining was employed. The $CD8^+$ T cell responses to NP_{147} are dominant followed by HA_{533} responses in influenza virus infected BALB/c mice. Lung lymphocytes were isolated from infected mice at day 6 and 9 post-challenge as previously described [29]. The cells were washed with FACS buffer (PBS, 1% FBS, 0.1% sodium azide) and then blocked with anti-CD16/CD32 mouse Fc receptor block (BD Biosciences, San Jose, CA, USA), followed by staining with a murine MHC-I encoded allele Kd-specific pentamer for the HA_{533} epitope or NP_{147} epitope conjugated to phycoerythrin (PE). Lymphocytes were subsequently stained with anti-CD8 antibodies conjugated to Pacific Blue, anti-CD3 antibodies conjugated to PerCP and anti-CD19 antibodies conjugated with APC-Cy7 (BD Biosciences, San Jose, CA, USA). The cells are then incubated with a viability dye (Molecular Probes, Invitrogen, Eugene, OR, USA). Once the surface staining was complete the cells were washed with FACS buffer, then fixed in 1% formalin/PBS and the cells were acquired using a LSRII flow cytometer (BD Biosciences, San Jose, CA, USA).

3. Results

3.1. Construction of recombinant baculoviruses

In order to investigate the gp64 components that may influence incorporation of HA on to baculovirus, six novel chimeric genes were constructed. The coding sequences for the signal peptide, transmembrane and cytoplasmic tail domains of HA were replaced with those of gp64 (Fig. 1B): Bac-HA, expressing full length HA; Bac-spHA, expressing ectodomain of HA with SP derived from gp64; Bac-spHAct, expressing ectodomain of HA with SP and CT derived from gp64; Bac-HAct, expressing HA with CT derived from gp64; Bac-HAtmct, expressing ectodomain of HA with TM and CT derived from gp64; Bac-spHAtmct, expressing ectodomain of HA with SP, TM and CT derived from gp64. All constructs were derived from the mouse adapted influenza virus A/PR/8/34 (H1N1). The hypothesis was that the SP of the gp64 would facilitate the translocation of the chimeric HA to the insect cell plasma membrane and the TM and CT domains of gp64 will stabilize the chimeric HA incorporated into virus envelope.

3.2. Confirmation of HA expression and incorporation into baculovirus

To determine whether the HA expressed by BV is properly translocated to the insect cell surface, BV infected and uninfected insect cells were incubated with tRBCs for agglutination. Approximately 80% RBCs were absorbed on the insect cells infected with baculoviruses containing HA genes. In contrast, no RBC absorption was observed for the uninfected insect cells or cells infected with baculovirus without HA gene (supplemental Figure 1). Therefore, the HA proteins expressed in insect cells were translocated to the cell surface, were properly folded maintaining their hemagglutination activity.

To confirm the expression of each chimeric HA, Sf9 cells were infected with these recombinant baculoviruses at a MOI ~1.0, and harvested at 4 days post-infection and the expressed HAs were quantified by scanning densitometry (Fig. 3A). Equivalent concentrations of recombinant baculoviruses were loaded in each well and the amount of incorporated HA was normalized to vp39 (the major baculovirus capsid protein). HA proteins were expressed at similar levels by all the six constructs (Fig 3A).

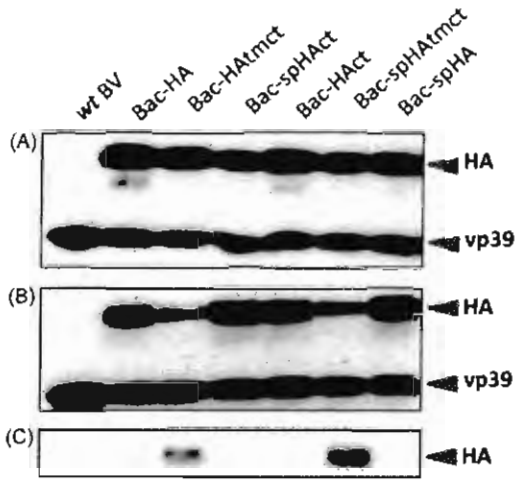

Fig. 3. Western-blot assay of HA-displayed baculovirus. (A) Supernatants from infected Sf9 cells probed with HA and vp39 antibodies. (B) Pelleted rBV by ultracentrifugation probed with HA and vp39 antibodies. (C) Supernatants after ultracentrifugation of rBV probed with HA antibody.

To confirm that each HA was incorporated on the envelope of baculoviruses, supernatants from infected Sf9 cells were used to perform hemagglutination assay. All recombinant baculovirus containing HA gene bound tRBCs, but baculoviruses without an HA gene did not agglutinate tRBCs. Furthermore, at the same titer of baculovirus (5x 10^7 ifu/ml), Bac-spHAct had the highest HA titer (1:64) while Bac-spHAtmct and Bac-HAtmct had the lowest HA titer (1:2), indicating that the different domains of gp64 (SP, TM and CT) affected the efficiency of HA incorporation into baculovirus. In order to verify whether all expressed HAs are incorporated into baculovirus, HA-DBVs from infected Sf9 cells were pelleted by ultracentrifugation and the supernatants and pelleted fractions were analyzed. Four DBV pellets from cells infected with Bac-HA, Bac-spHA, Bac-spHAct, and Bac-HAct incorporated similar amounts of each chimeric HA, while two DBV pellets (Bac-HAtmct and Bac-spHAtmct) incorporated about 50% less HAs (Fig. 3B). Supernatants from Bac-spHAtmct and Bac-HAtmct had some unbound HAs while the other four constructs did not have detectible HAs after ultracentrifugation (Fig. 3C). Therefore, Bac-spHAct was chosen as the template for further vaccine studies.

3.3. HA-DBVs elicit hemagglutination-inhibition activity

Mice (BALB/c, n=15/group) were vaccinated with either 1) live HA-DBV (Bac-spHAct) with the HA derived from the A/PR/8/34, 2) the same BV inactivated with BPL, 3) *wt* BV, 4) purified PR8 VLPs produced in mammalian cells or 5) mock vaccinated with PBS. Serum samples were evaluated for the ability to inhibit PR8 influenza virus induced hemagglutination of tRBCs. All Bac-spHAct vaccinated mice had detectable HAI titer against PR8 vitus from serum collected at week 2 and 5 (Fig. 4). Two weeks after the first vaccination, the average HAI titers for live Bac-spHAct groups (1×10^8, 2×10^7, and 4×10^6 ifu/mouse) were between 118 and 373, while the average HAI titers for BPL-inactivated Bac-spHAct groups (1×10^8, 2×10^7, and 4×10^6 ifu/mouse, same doses but inactivated) were between 38 and 56. Following the second vaccination, HAI titers increased from the first dose (~10 fold) in mice vaccinated with live Bac-spHAct vaccine, while the HAI titers

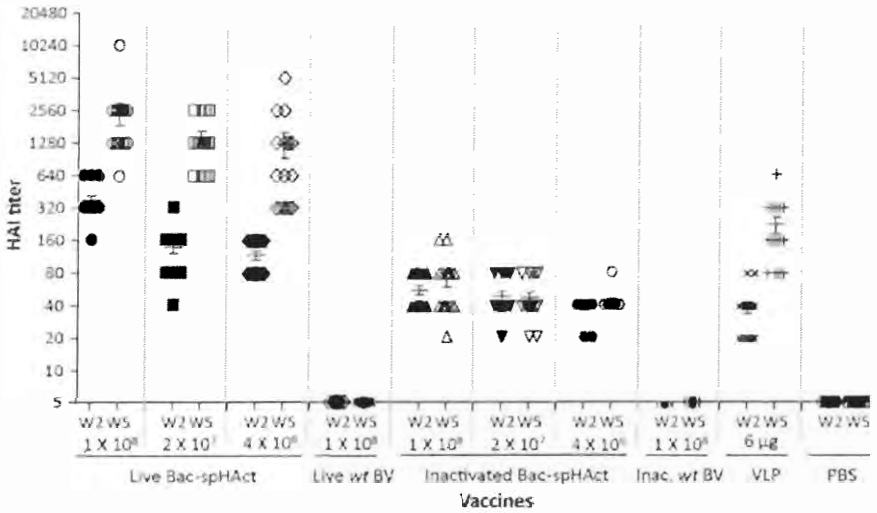

Fig. 4. Hemaggutination-inhibition (HAI) titers. Mice (n = 15/group) immunized intramuscularly with live/inactivated Bac-spHAct, *wt* BV, VLPs or mock vaccination. Week 2 (W2), and week 5 (W5) serum HAI antibody responses were assessed against PR8 virus. Bars indicate geometric mean titer (GMT) +/- SEM.

from mice vaccinated with BPL-inactivated Bac-spHAct vaccines did not increase significantly. Mice that were immunized with the live Bac-spHAct vaccinated groups had significant higher HAI titers than the mice vaccinated with the BPL-inactivated Bac-spHAct. Remarkably, the live Bac-spHAct vaccinated mice had higher HAI titers compared to VLP-vaccinated mice after the primary and boost immunization. As expected, there were no HA inhibiting antibody responses elicited in mice that were immunized with *wt* BV.

3.4. Viral titers in lungs of vaccinated mice post challenge

Lung viral titers were determined at days 3 and 6 post-challenge (Fig. 5). Unvaccinated mice and mice vaccinated with live or inactivated *wt* BV had high viral titers in their lungs (~1×10^6 pfu/ml). Mice which were immunized with a mammalian cell-derived VLP vaccine showed a 1000-fold reduction of viral titer. However, mice vaccinated with live Bac-spHAct, irrespective of the dose, did not have detectable virus (<10 pfu/ml) in their lungs. In contrast, mice vaccinated with inactivated Bac-spHAct had virus titers that ranged from 1×10^3 to 1×10^5 pfu/ml at day 3 (Fig. 5A). By day 6, mice vaccinated with inactivated Bac-spHAct with 1×10^8 ifu showed a reduction in lung viral titer, whereas mice vaccinated with a lower dose of inactivated Bac-spHAct maintained similar viral titers as day 3 (Fig. 5B). Since mice vaccinated with the live HA-DBV elicited immune response that blocked PR8 virus infection, even if immunized with a very low dose (4×10^6 ifu/mouse). Therefore, additional studies were performed using a live HA-DBV regimen.

3.5. Immunization with Bac-spHAct confers protection from lethal PR8 virus challenge

To evaluate the protective efficacy of different vaccine strategies of Bac-spHAct, mice were challenged intranasally with a lethal dose of PR8 virus. All mice vaccinated with either live or BPL inactivated *wt* BV or non-vaccinated mice lost greater than 20% of their original body weight and died from complications associated with infection by day 5-8 post challenge (Fig. 6). All mice vaccinated with live Bac-spHAct or VLP vaccines were protected from lethal challenge without weight loss, regardless of vaccination dose (Fig. 6A and B). In contrast, mice vaccinated with inactivated Bac-spHAct lost some weight following challenge (Fig. 6C), and one out of five mice vaccinated with the lowest dose of inactivated Bac-spHAct

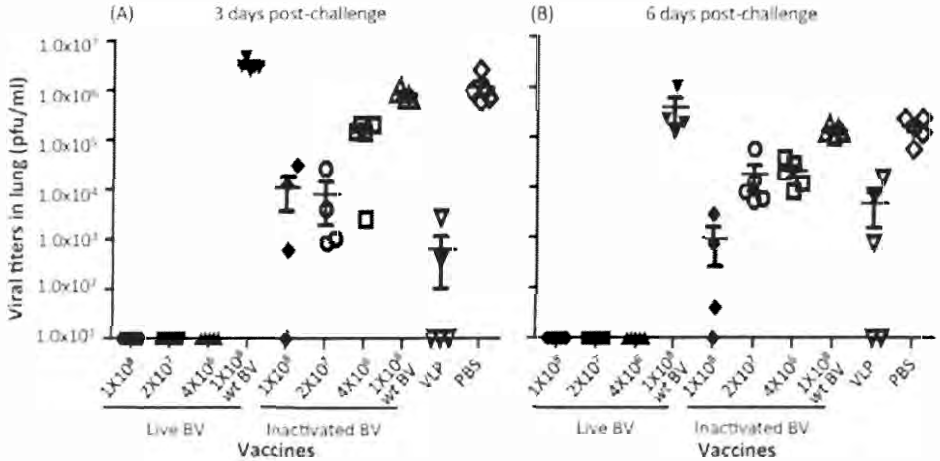

Fig. 5. Virus titers in lungs at day 3 and 6 post-challenge. Mice (n = 5/group) immunized intramuscularly with live/inactivated Bac-spHAct, *wt* BV, VLPs or mock vaccination. At week 3 after the final immunization, immunized mice were intranasally infected with a lethal dose of mouse-adapted PR8 virus (10 LD50). Mice were sacrificed on day 3 (A) and day 6 (B) post-challenge and lungs were collected for plaque assay.

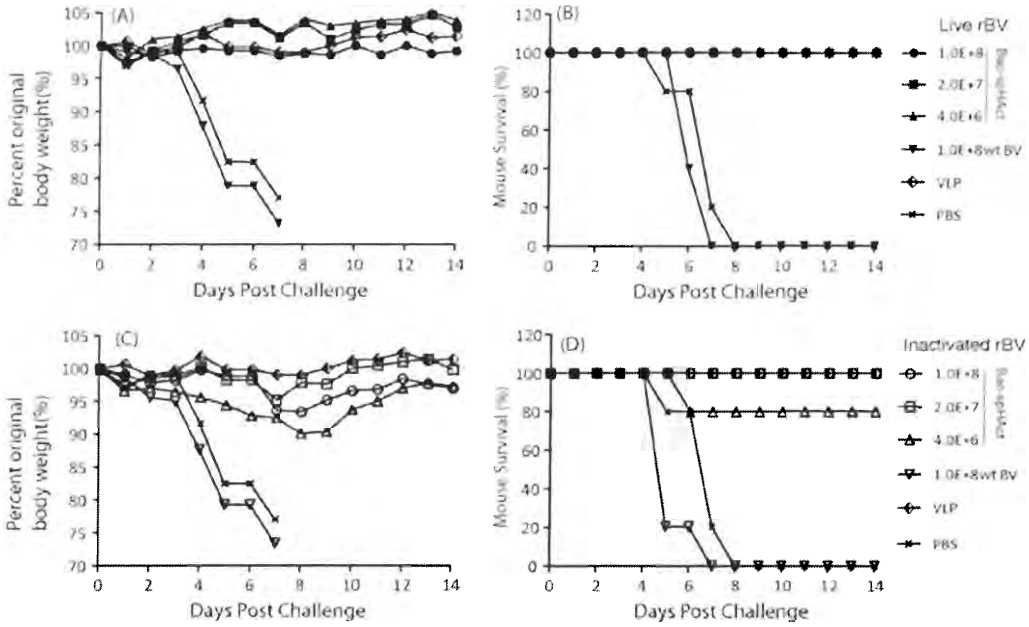

Fig. 6. Protection of mice from lethal PR8 challenge. At week 3 after the final immunization, immunized mice (n = 5/group) were intranasally infected with a lethal dose of mouse-adapted PR8 virus (10 LD_{50}). Mice were monitored daily for 14 days. (A) Body weight changes of mice immunized with live Bac-spHAct, *wt* BV, VLP, PBS. (B) Percent survival of mice immunized with live Bac-spHAct, *wt* BV, VLP, PBS. (C) Body weight changes of mice immunized with BPL-inactivated Bac-spHAct, *wt* BV, VLP, PBS. (B) Percent survival of mice immunized with BPL-inactivated Bac-spHAct, *wt* BV, VLP, PBS.

(4×10^6 ifu/mouse) died after challenge (Fig. 6D). All the other mice vaccinated with Bac-spHAct survived from challenge.

3.6. HAI antibody titers elicited by Bac-HA2.2 or Bac-4HA vaccines

Since the comparison of PR8 HA-displayed constructs indicated that SP and CT domains of gp64 can enhance the HA incorporation into baculovirus, HA-DBV were constructed to contain chimeric HAs derived from four subclades of H5N1 influenza viruses which were fused with SP and CT domains of gp64. The HA displayed on the surface of baculovirus maintain hemagglutination activity. Mice were vaccinated with live H5N1 HA-DBV (1×10^7 ifu/mouse) of either a monovalent HA-DBV (Bac-HA2.2) or a tetravalent HA-DBV (Bac-4HA). Two weeks after primary vaccination, the HAI titers to all H5N1 viruses were undetectable or low (<1:10), regardless of the vaccine administered (data not shown). At week 5 following the second vaccination, mice vaccinated with the monovalent HA-DBV (Bac-HA2.2) had an average HAI titer of 1:100 against A/WS/05, low (≤1:20) HAI titer against heterologous viruses (VN/04, IN/05, AH/05). In contrast, mice vaccinated with the tetravalent HA-DBV (Bac-4HA) had HAI titers against all four viruses (average HAI titers: 116 against VN/04, 120 against IN/05, 90 against WS/05, 80 against AH/05), with a seroconversion rate ranging from 86-94%. As expected, *wt* BV vaccinated mice had no detectable HAI titers. (Fig. 7)

3.7. Protection against heterologous or homologous H5N1 viral challenge

To test whether immunization protects mice from a lethal infection with reassortant H5N1 influenza viruses, mice that received either Bac-HA2.2, Bac-4HA, or *wt* BV vaccine were challenged intranasally with lethal doses of either VN/04, IN/05 or WS/05 viruses (Fig. 8 and Table 2). All mice vaccinated with Bac-4HA were protected from death following lethal challenge with VN/04, IN/05 or WS/05 reassortant viruses. All mice vaccinated with Bac-HA2.2 were protected from lethal challenge with homologous WS/05, whereas only 60% of the mice infected with heterologous VN/04 and 40% of mice infected with IN/05 were protected. All mice vaccinated with *wt* BV lost greater than 20% of their original weight and had to be euthanized or died from complications associated with infection by day 6- 9 post-challenge.

Lung viral titers at day 3 post-challenge were analyzed to determine virus replication in the lung (Fig. 9). The *wt* BV immunized mice groups showed high viral titer (~1×10^6 pfu/ml), regardless of the challenge virus, while significantly lower viral titers were detected in the Bac-4HA vaccinated groups. High titers of virus replication were also observed in the mice that received Bac-HA2.2, albeit lower than the titers observed in mice immunized with *wt* BV. These results indicate that Bac-4HA can induce protective immune responses that can protect from challenge with

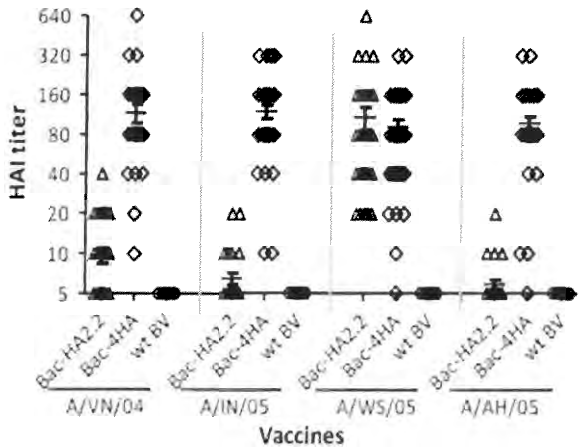

Fig. 7. Hemaggutination-inhibition (HAI) titers against H5N1 viruses. Mice (n = 36/group) were immunized intramuscularly with Bac-HA2.2, Bac-4HA, *wt* BV. Week 5 serum HAI antibody responses were assessed against VN/04, IN/05, WS/05, AH/05 viruses. Bars indicate geometric mean titer (GMT) +/- SEM.

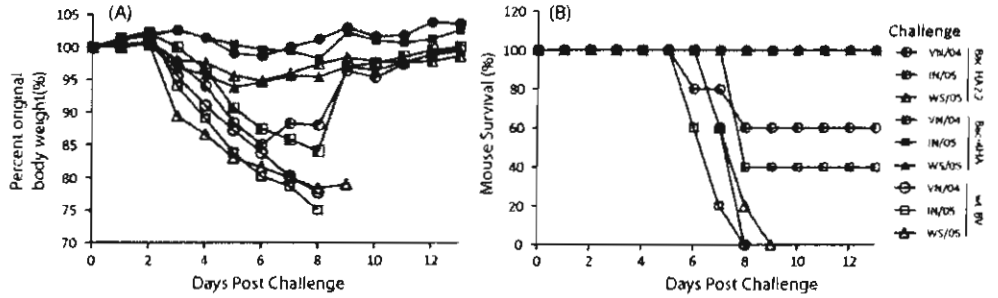

Fig. 8. Protection of mice from lethal H5N1 viruses' challenge. At week 3 after the final immunization, immunized mice (n = 5/group) were intranasally infected with a lethal dose of VN/04, IN/05, WS/05 viruses. Mice were monitored daily for 13 days. (A) Body weight changes after challenge. (B) Percent survival after challenge.

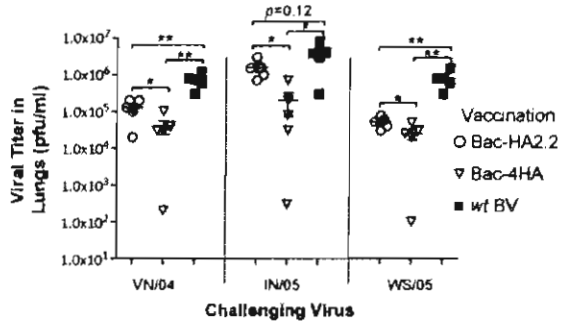

Fig. 9. Virus titers in lungs at day 3 post-challenge. Mice (n = 5/group) were immunized intramuscularly with Bac-HA2.2, Bac-4HA, *wt* BV. At week 3 after the 2nd immunization, immunized mice were intranasally infected with a lethal dose of VN/04, IN/05, WS/05. Mice were sacrificed on day 3 post-challenge and lungs were collected for plaque assay. * $p < 0.05$, ** $p < 0.01$

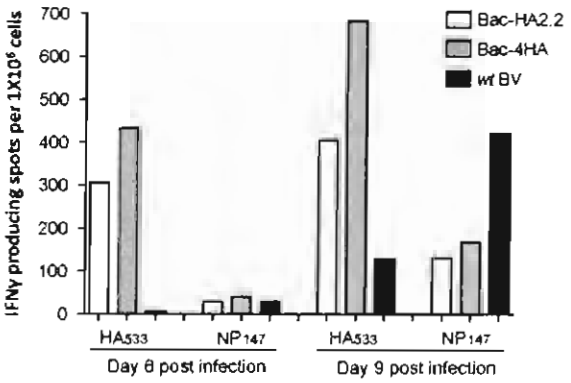

Fig. 10. IFNγ-ELISPOT assays. Splenocytes were collected on day 6 and 9 post-infection with IN/05. Each sample was stimulated with HA_{533}, NP_{147}, and Ova peptides. The spot number from Ova peptide stimulation was substracted.

VN/04, IN/05 or WS/05 influenza viruses, while Bac-HA2.2 can induce partial protective immune responses against heterologous virus (VN/04, IN/05).

3.8. Cell-mediated immunity elicited by HA-DBV

We determined the magnitude of T-cell responses induced by HA-DBV using IFNγ-ELISPOT and flow cytometry. Splenocytes were harvested at 6 and 9 days post challenge and stimulated *in vitro* with H2d-restricted $CD8^+$ T cell specific peptide HA_{533} [29]. As expected, after HA peptide stimulation, *wt* BV vaccinated mice had T cell responses similar to the negative controls (unstimulated or stimulated with irrelevant peptide) (Fig. 10). In contrast, mice vaccinated with Bac-HA2.2 or Bac-4HA vaccines had significant higher IFNγ responses (300-460 spots/$1x10^6$ cells) following HA peptide stimulation (Fig. 10). After NP_{147} peptide stimulation, IFNγ responses were detected at low levels in all vaccinated mice on day 6 post infection, which is to be expected since the NP epitope is not present in the vaccine. The ELISPOT assay was performed also on day 9 post challenge, which is the peak of the T cell response during a primary infection. With HA_{533} peptide stimulation, mice vaccinated with Bac-HA2.2 or Bac-4HA had 400–700 spots while mice vaccinated with *wt* BV had only 130 spots per $1x10^6$ cells, this is expected because the T cell response in HA-DBV vaccinated mice is a recall response which must be stronger than primary response in *wt* BV vaccinated mice. With NP_{147} peptide stimulation, mice vaccinated with Bac-HA2.2 or Bac-4HA had 150 spots while mice vaccinated with *wt* BV had 400 spots per $1x10^6$ cells. This disparity is most likely due to the fact that *wt* BV vaccinated mice were not protected from infection with the influenza virus which resulted in a robust activation of influenza specific T cell responses, whereas in the HA-DBV vaccinated mice a large proportion of the challenge virus was neutralized by antibodies or cleared by influenza specific T cells, therefore resulting in a lower frequency of NP specific T cells on day 9 post challenge.

To determine the influenza specific T cell response in lung, lung cells were collected at day 6 and 9 post-challenge with IN/05 and analyzed via staining with a pentamer specific for T cells recognizing the HA_{533} or NP_{147} epitopes [29] (supplemental Figure 2A & B). On day 6 post-challenge, the percentage of NP-pentamer$^+$/$CD8^+$ T cells in all vaccinated/infected mice was similar to unvaccinated/uninfected mice and 2.6% HA-pentamer$^+$/$CD8^+$ T cells were detected in the lungs of mice vaccinated with Bac-HA2.2 and Bac-4HA. As expected, there were no HA-pentamer$^+$/$CD8^+$ T cells in *wt* BV vaccinated mice (Table 3). On day 9 post-challenge, HA-DBV vaccinated mice had 4.6-5.4% NP-pentamer$^+$/$CD8^+$ T cells in their lungs, whereas *wt* BV vaccinated mice had 15% of their lung lymphocytes stain positive for the NP-pentamer. This revealed same

Table 3
Percentage of pentamer positive $CD8^+$ T cells in lung

Challenge after vaccination	Day 6 post infection			Day 9 post infection		
	Bac-HA2.2	Bac-4HA	*wt* BV	Bac-HA2.2	Bac-4HA	*wt* BV
HA pentamer$^+$/$CD8^+$ (%)	2.6	2.6	0.1	26.1	20.8	1.3
NP pentamer$^+$/$CD8^+$ (%)	0.2	0.5	0.6	5.4	4.6	15

phenomenon as IFNγ-ELISPOT results on day 9 post-challenge. In contrast, 26.1% of cells collected from mice vaccinated with Bac-HA2.2 and 20.8% from Bac-4HA vaccinated mice were HA-pentamer$^+$/CD8$^+$, and only 3.1% were detected HA-pentamer$^+$/CD8$^+$ in *wt* BV vaccinated mice, since recall immune responses in the former were stronger than primary immune responses in the later.

4. Discussions

In this study, we investigated the efficiency of influenza HA displayed on the surface of baculovirus and its utility as a vaccine candidate. Baculovirus surface display has been extensively used for the analysis of protein-protein interaction [30], drug screening [31], monoclonal antibody generation [32], as well as vaccine production [9,11,13,18]. Initially, vaccines were developed that fused epitopes or peptides to the envelope protein of AcMNPV, gp64, which resulted in surface display of these peptides on the baculovirus surface. Subsequently, it was found that some native viral envelope proteins can be displayed on the baculovirus surface, even without the fusion with gp64, such as HIV-1 gp120 [14], influenza HA [24], vesicular stomatitis virus glycoprotein [33]. However, so far no comprehensive studies have investigated whether fusion of native proteins results in efficient display on the baculovirus surface as a delivery vehicle for vaccines. In this study, the SP, TM, and CT domains of gp64 were examined to enhance foreign antigen display on the baculovirus surface. The signal peptide of the membrane protein plays an important role in directing protein to the endoplasmic reticulum membrane and trafficking [34]. The TM domain of baculovirus envelope is critical for protein trafficking, membrane anchoring, membrane fusion, and viral budding [35,36]. The CT domain of a viral envelope protein may influence envelope incorporation and virus budding, since the CT domains interact with the components of viral core [37,38]. The CT domain of gp64 has been shown to enhance the incorporation of influenza HA into baculovirus [19]. It is not known if SP and TM domains of gp64 have similar functions. Therefore, we constructed six recombinant baculoviruses expressing six chimeric or native HAs. All six HAs were expressed, translocated to the infected cell surface and incorporated into baculovirus envelope. Importantly, all constructs expressed HA at similar levels (Fig. 3A), suggesting the substitutions of these three domains does not significantly affect HA expression. However, not all expressed HA were incorporated into mature baculovirus with equal efficiency (Fig. 3B & C). The HA containing TM domain of gp64 resulted in unbound HA, indicating that TM domain of HA is important for HA incorporating into virions. Roth *et al.* reported that substitutions of TM domains of HA with VSV-G and herpes simplex virus glycoprotein C had minimal effect on the HA ectodomain [39], but the replacement or mutation in the TM domain of HA affected its folding, stability, and virus-cell membrane fusion [21,36,40]. Hemagglutination titer of influenza virus can reflect the abundance of properly folded hemagglutinin on a viral particle. At an equivalent virus titer, Bac-spHAct had the highest hemagglutination titer. Therefore, it was chosen for subsequent mouse study.

We investigated the efficacy of HA-DBV as an influenza vaccine. There is a direct correlation between the HAI titers and protection against influenza challenge. Yang *et al.* reported that HA displayed BV can successfully elicit functional antibodies although they did not analyze protection by challenging the immunized mice [19]. Recently, Prabakaran *et al.* reported that intranasal or gastrointestinal delivery of HA-DBV protected mice against H5N1 influenza virus infection [12,18]. All these studies indicated that HA-DBV was a promising influenza vaccine candidate. In this study, we investigated the dosage of HA-DBV as a vaccine candidate in mouse model and compared live and inactivated HA-DBV. Our results indicated that live HA-DBV elicited strong humoral immune responses as indicated by the HAI titers, even at a low dose (4×10^6 ifu/mouse), whereas, the inactivated HA-DBV induced low HAI titers. After challenge, viral titers in lungs were determined on day 3 and day 6 post-challenge. We found that all mice vaccinated with live Bac-spHAct had undetectable viral titers in their lungs on day 3 and 6 post challenge, suggesting that antibodies induced by live Bac-spHAct conferred sterilizing immunity. Most mice vaccinated with inactivated Bac-spHAct had detectable lung virus titers by day 3 post challenge. Some mice in the VLP-vaccinated group had detectable viral titers in their lungs, indicating that the efficacy of the live HA-DBV is superior to the VLP vaccine, which is most likely the result of the strong adjuvant property of baculovirus. All *wt* BV-vaccinated mice had lung viral titers similar to unvaccinated mice. Viral lung titers correlated with protection: mice with low viral titers were protected. Even though baculoviruses are unable to replicate in mammalian cells, only the live HA-DBV vaccines, not the inactivated ones, elicited high titer protective immune responses. This may be due to two reasons. First, the BPL-inactivation process may affect the conformation of displayed HA proteins. The hemagglutination titer of Bac-spHAct was reduced following BPL-inactivation. Second, the adjuvant effect of BVs can be observed with low number of virions (10^4-10^3 pfu/mouse), but totally abolished if BVs were inactivated by UV irradiation, treatment with Triton X-100, or binary ethylenimine [41].

BV contains a large genome (80–200 kb) [3]. This enables insertion of large foreign DNA fragments or construction of multivalent vaccines. Influenza viruses have many serotypes in nature. A single influenza infection may be sufficient to provide lifelong immunity to the invading strain or serotype, but cannot provide protection against emerging serotypes. H5N1 avian influenza virus has the potential to emerge as a pandemic threat in humans. So far, H5N1 influenza viruses are divisible into 10 clades on the basis of phylogenetic analysis of HA genes [42]. The cross-clade protections are very poor, so multivalent H5N1 influenza vaccines are critically important for preventing its spread. The major human infections were caused by clades 1, 2.1, 2.2 and 2.3. Therefore, we constructed an rBV that expressed four HAs derived from these four subclades of H5N1 influenza viruses. In a mouse study, we found that monovalent H5N1 vaccine induced poor cross-clade antibody responses, but multivalent H5N1 vaccine elicited broadly-reactive antibody responses against all the HA subtypes included in the DBV. These correlated with protection rates and viral titers in lung. Some mice did not have detectable HAI titers, but survived from lethal dose virus challenge, which may be result of cellular immune responses clearing some virally infected cells. Previous studies have reported that virus-specific CTL play an important role in the recovery and protection during influenza virus infection, especially when a protective antibody titer is absent [43,44].

To investigate influenza-specific T cell responses elicited by HA-DBV, IFNγ-ELISPOT and MHC-I pentamer staining were performed. On day 6 post-challenge, the recall of HA-specific IFNγ-secreting memory T cells were detected in HA-DBV vaccinated mice, but not in *wt* BV vaccinated mice. Little or no NP-specific IFNγ-secreting T cells were detected in all vaccinated mice since the NP protein was not included in the HA-DBV [45,46]. On day 9 post infection, which is close to the peak of the primary response, NP-specific IFNγ-secreting T cells can be measured. Meanwhile, there was a much higher frequency of HA-specific IFNγ-secreting T cells in HA-DBV vaccinated mice compared to *wt* BV vaccinated mice. Similarly, the frequency of HA-pentamer positive CD8$^+$ T cells was significantly higher in HA-DBV vaccinated mice compared to *wt* BV vaccinated mice on both day 6 and 9 post-challenge. These data indicated that HA-specific CD8$^+$ T cells were induced by the HA-DBV vaccine and memory T cells were present in the immunized

mice. Even though cellular immune responses cannot confer sterilizing immunity, they are able to reduce the severity of infection and lower morbidity and mortality rates [47], and antigen-specific memory T cells are able to rapidly respond to a secondary virus infection [45]. Furthermore, cellular immune responses to the conserved epitopes contained in vaccines may provide cross-protective immunity against different subtypes of influenza virus infection [48–50].

Baculoviruses have strong adjuvant activity to promote humoral and cellular immune responses against coadministered antigens, activate dendritic cells maturation, induce the production of cytokines, chemokines, and type I IFNs [41,51–53]. Viral DNA is responsible for the adjuvant properties of BVs [41]. Intranasal vaccination of *wt* BV can stimulate robust innate immune responses in the respiratory tract that protect mice against lethal doses challenge of mouse-adapted PR8 [52] and H5N1 influenza viruses (data not shown). After two intramuscular immunizations, mice vaccinated with mammalian cell-derived influenza VLPs (1 μg) coadministered with 10^5 pfu live *wt* BV had HAI titers 1:80-1:320. In contrast, mice vaccinated with the VLPs (1 μg) alone or VLPs with inactivated *wt* BV did not have detectable HAI titer (data not shown) implying that contaminating BVs may play an important role in the strong immunogenicity of baculovirus vector-derived VLP vaccines [54,55]. In influenza VLPs have been expressed from BV expression system. During the process of removing of contaminating BVs from the influenza VLP preparation may reduce the effectiveness of the influenza VLP vaccine because influenza HA molecules that are displayed on the BV surface, are eliminated from the final product [55]. This might be true for other enveloped VLPs, because envelope proteins from other viruses may be displayed on the surface of BV.

BV cannot replicate in mammalian cells and they are not cytotoxic [56], therefore, the use of BV as a potential adjuvant should be considered. However, similar to other viral vectors, antibodies against BV may prevent its widespread use due to anti-BV antibodies. It is remarkable that low doses (10^3pfu/mouse) of BVs act as an effective adjuvant [41]. Therefore, reducing BV concentration and elongating vaccination intervals may prevent memory responses to BV administration.

DBVs have several advantages as a vaccine platform. DBVs are easy to generate, grow efficiently without the addition of fetal calf serum, and they are stable under refrigeration. Displayed proteins, expressed from either insect and mammalian cells have similar protein processing and post-translational modifications and they form native structures on the BV surface. The baculovirus genome allows for insertion of large foreign DNA segments or the construction of multivalent vaccines. There is little or no observable cytopathic effect following administration of high doses of BV. Taken together, HA-DBV may be a promising vaccine platform for multiple infectious disease pathogens.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2010.08.040.

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APPENDIX 3

Baculovirus-produced Influenza Virus-like Particles in Mammalian Cells Protect Mice from Lethal Influenza Challenge

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Baculovirus-Produced Influenza Virus-like Particles in Mammalian Cells Protect Mice from Lethal Influenza Challenge

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Abstract

Influenza virus-like particles (VLPs) are effective vaccines against influenza infection, which can be produced either in insect cells by recombinant baculovirus (BV) infection or in mammalian cells by DNA plasmid transfection. However, VLPs produced from baculovirus/insect cells are difficult to purify due to baculovirus contamination; VLPs produced by plasmid transfection are limited by scale-up capability. In this study, a BacMam BV, in which three CMV-promoters drive the hemagglutinin, neuraminidase, and matrix of influenza virus was constructed. This baculovirus can deliver these genes into mammalian cells/hosts and subsequently influenza VLPs can be produced and secreted from transduced cells. Transduction conditions were optimized and influenza VLPs were purified from transduced 293T cells. Mice were vaccinated with BV transduction-produced VLPs, plasmid transfection-produced VLPs, and BacMam BV. Two vaccinations of each vaccine induced high hemagglutination-inhibition (HAI) titers and prevented influenza virus infection. In contrast, following a single vaccination, all mice vaccinated with each vaccine had significantly lower lung viral titers compared to unvaccinated mice. Remarkably, mice vaccinated with a single dose of BV transduction-produced VLPs survived challenge, whereas mice vaccinated with one dose of BacMam BV- or plasmid transfection-produced VLPs had 60–80% survival. This finding is particularly significant for producing easily purified VLPs. The BacMam system is an alternative strategy for VLP production, which is easy to scale up and purify. Besides, BacMam BV can be used as a gene delivery vector to produce VLPs *in vivo*, to stimulate immune responses.

Introduction

INFLUENZA INFECTION IS A MAJOR THREAT TO HUMAN HEALTH. Vaccination is still the best method to prevent influenza infection. Current influenza vaccines are produced in embryonated chicken eggs, but the efficiency of this method is limited. New strategies for influenza vaccines have focused on the development of cell culture-based production. Virus-like particles (VLPs) are a promising vaccine approach because they stimulate both humoral and cellular immune responses (1–3). Influenza VLPs can be readily produced in insect or mammalian cells via the simultaneous expression of hemagglutinin (HA), neuraminidase (NA), and matrix (M1) proteins (4). The baculovirus expression vector system (BEVS) has been widely used to produce VLPs for a variety of viruses due to its high expression level, eukaryotic processing feature, and scale-up capability (5). In the last decade, various influenza VLPs have been produced with the baculovirus/insect cell expression system (6–10). However, separating progeny baculovirus particles from the influenza VLPs is still the biggest challenge for this vaccine approach because baculoviruses, which replicate efficiently in insect cells ($>10^8$ pfu/mL), are similar in size to influenza VLPs (6,10). Another method of producing influenza VLPs is co-transfection of plasmids encoding HA, NA, and M1 genes of influenza virus into mammalian cells (4). This method produces VLPs that are easily purified, but this strategy is inefficient, expensive, and difficult to scale up.

In 1983, Volkman *et al.* found that baculovirus infected 35 vertebrate cell lines, but without detectable baculovirus replication (11). Subsequently, mammalian cells transduced by baculovirus could efficiently express some proteins under the transcriptional control of mammalian promoters (12–14). Many cell types can be transduced by baculovirus, including cell lines derived from human, non-human primate, rabbit, rodent, porcine, bovine, and fish hosts (15). In addition, baculovirus can transduce non-dividing cells (16) and primary cells *in vivo* (17). Recombinant baculoviruses containing

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mammalian expression cassettes are referred to as BacMam baculoviruses. Due to their high efficiency and low cytotoxicity during the gene delivery process, BacMam baculoviruses have been used as novel vectors for gene therapy and vaccine development (18–24).

In this study, we constructed a BacMam baculovirus containing HA, NA, and M1 expression cassettes that were driven by cytomegalovirus (CMV) promoters. We speculated that BacMam baculoviruses would deliver these influenza genes into mammalian cells and result in the production of influenza VLPs. Since baculoviruses cannot replicate in mammalian cells, this strategy would not produce any baculovirus particles during influenza VLP production. After transduction of mammalian cells *in vitro*, we found that influenza VLPs can be easily generated and purified. In addition, since BacMam baculoviruses can transduce primary cells *in vivo* without cytotoxicity, these baculoviruses can potentially be used as a safe gene delivery vector to produce influenza VLP *in vivo*. Mice were immunized with purified VLPs produced from baculovirus-transduced mammalian cells, or directly immunized with this BacMam baculovirus. Both induced influenza-specific immune responses and provided mice sterilizing protection against influenza challenge.

Materials and Methods

Cells and viruses

Spodoptera frugiperda (Sf9) cells were propagated at 28°C in Sf-900II serum-free medium (Invitrogen, Carlsbad, CA). Cells were infected by recombinant baculoviruses at a multiplicity of infection (MOI) of 0.1–1.0, and virus supernatants were collected 4 d post-infection. The influenza virus used in this study was a mouse-adapted influenza virus, A/Puerto Rico/8/1934 (PR8, H1N1).

Plasmids and recombinant baculovirus construction

In order to construct multiple genes expressing recombinant baculoviruses, pFastBac Dual plasmid (Invitrogen) was modified to contain two CMV promoters (p2CMV) and two multiple cloning sites based on a vector we previously modified (25). CMV promoter and HSV tk/SV40 poly A signal sequences were amplified by overlapping PCR and inserted into the pFastBac vector backbone. At the same time two compatible restriction sites (*Asc* I and *Mlu* I) were introduced into p2CMV as shown in Fig. 1A and Supplementary Fig. 1 (see online supplementary material at http://www.liebertonline.com). The M1 gene segment from PR8 was

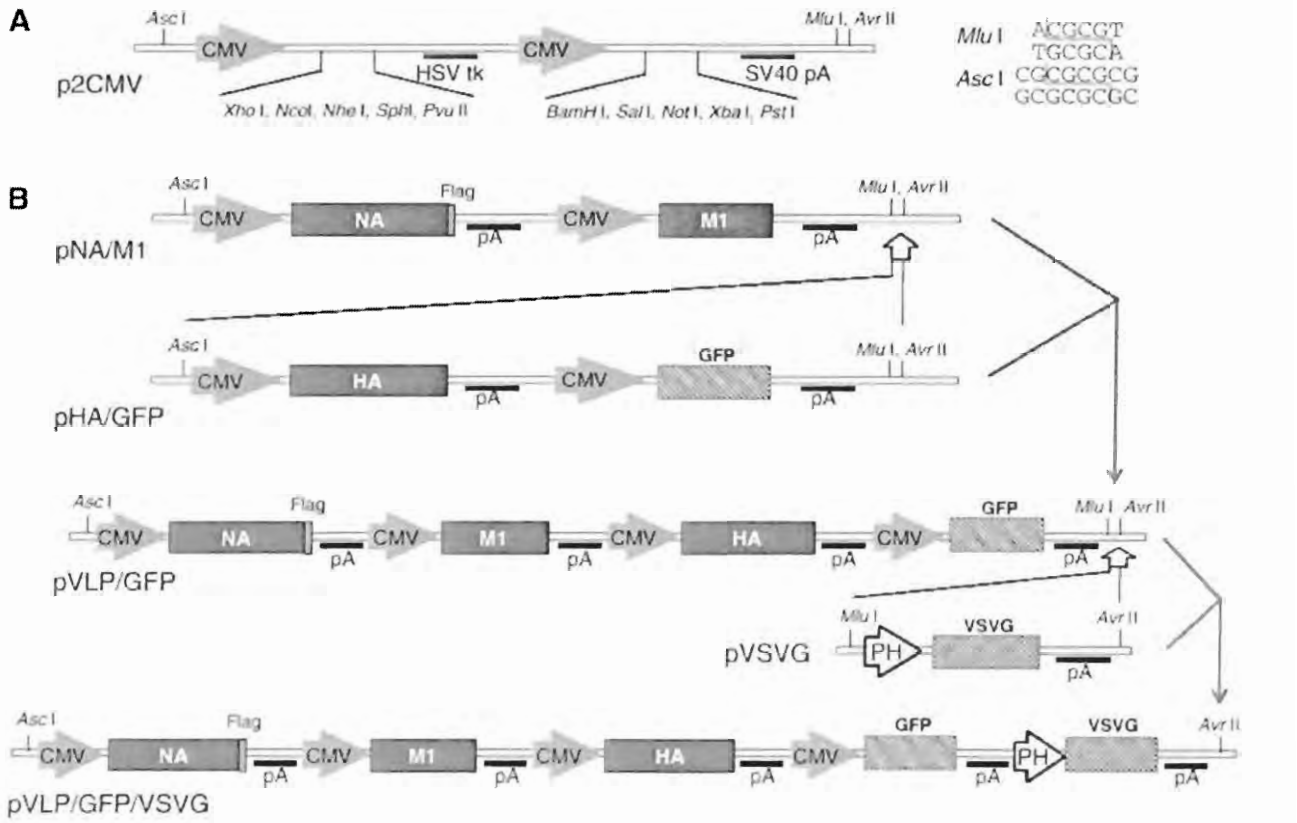

FIG. 1. Construction of five-unit transfer vector that can express influenza HA, NA, and M1, as well as GFP and VSVG. (**A**) Dual-CMV promoters transfer vector with two multiple cloning sites, two compatible restriction sites (*Asc* I and *Mlu* I), and two polyA signal sequences (HSV tk and SV40 pA). (**B**) Stepwise construction of pVLP/GFP/VSVG. The fragment containing HA and GFP was excised using *Asc* I and *Avr* II, and ligated into the compatible sites of pNA/M1 to obtain a four-unit plasmid, pVLP/GFP. VSVG was excised suing *Mlu* I and *Avr* II and ligated into pVLP/GFP to get the five-unit plasmid pVLP/GFP/VSVG.

codon optimized for mammalian cell expression and synthesized by GENEART (Regensburg, Germany). HA and NA of the PR8 virus, green fluorescent protein (GFP), and vesicular stomatitis virus glycoprotein (VSVG) genes were amplified by PCR. A flag tag was added at the C-terminal of NA. Transfer vectors were constructed as indicated in Fig. 1B. Briefly, NA and M1 were cloned into one p2CMV vector, and HA and EGFP were cloned into another p2CMV; then the fragment containing HA and GFP was excised using *Asc* I and *Avr* II, and ligated into the compatible sites of pNA/M1 to obtain the four-unit plasmid pSVP/GFP; VSVG was cloned into another vector, p2PH (25), then excised with *Mlu* I and *Avr* II and ligated into pSVP/GFP to get plasmid pSVP/GFP/VSVG. All recombinant baculoviruses (BV-HA/GFP, BV-VLP/GFP, and BV-VLP/GFP/VSVG) were generated using the Bac-to-Bac system (Invitrogen). The recombinant virus selection and amplification were performed following standard protocols.

Purification of recombinant baculoviruses

The recombinant baculoviruses were produced by infecting Sf9 cells at an MOI of 0.1. Supernatants were collected 4 d after infection and were clarified by centrifugation at 3000 g for 10 min at 4°C to remove cell debris. Viral particles were precipitated via ultracentrifugation (27,000 rpm using a Sorvall SW28 rotor) for 4 h at 4°C. The pellets were subsequently resuspended in phosphate-buffered saline (PBS) and stored at 4°C. The viral titer was determined using the BacPAK Baculovirus Rapid Titer Kit (Clontech, Mountain View, CA) and was expressed as infectious units per milliliter (ifu/mL).

Optimization of baculovirus transduction

To optimize the transduction efficiency, human embryonic kidney cells (293T) were seeded onto 6-well plates at a density of 5×10^5 cells/well in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C overnight. Before transduction, the medium was removed and the cells were washed twice with PBS. The cells were transduced with BV-VLP/GFP or BV-VLP/GFP/VSVG at different MOI (20, 40, 60, 80, and 100) in 0.5 mL PBS. The cells were incubated at 28°C for a range of periods (1, 2, 4, 6, and 8 h). Following incubation, the viral inocula were removed, followed by two washes with PBS, and then 2 mL complete DMEM (cDMEM) with or without 5 mM sodium butyrate (Sigma-Aldrich, St. Louis, MO) were added. The transduced cells were cultured at 37°C for 72 h. To compare baculovirus transduction and plasmid transfection, 293T cells were seeded onto 6-well plates at a density of 1×10^6 cells/well in cDMEM and incubated at 37°C overnight. The cells were transfected with 1 μg plasmid pVLP/GFP and 5 μL lipofectamine 2000 in 0.5 mL OPTI-MEM (Invitrogen). After 5 h incubation at 37°C, the transfection medium was replaced with 2 mL cDMEM. The cells were cultured at 37°C for 72 h. The expression of GFP was monitored daily by fluorescence microscopy.

Western blot and hemagglutination assays for VLP production

Supernatants from transduced or transfected cells were harvested after 72 h of culture and were clarified by centrifugation at 3000 g for 10 min at 4°C to remove cell debris. The supernatants were subjected to Western blot analysis. The supernatants from BV-VLP transduced or pVLP-transfected 293T cells were examined for functional VLPs by hemagglutination assay. Briefly, 50 μL of serial twofold dilutions were prepared in PBS, followed by incubation with 50 μL of 1% turkey red blood cells (tRBCs; Lampire Biologicals, Pipersville, PA) at 25°C for 30 min. The extent of hemagglutination was inspected visually, and the highest dilution capable of agglutinating red blood cells was determined.

VLP production and purification

293T cells were transduced with BV-VLP/GFP/VSVG in PBS at an MOI of 60. The transduced cells were cultured in cDMEM containing 5 mM sodium butyrate for 72 h at 37°C. The VLP produced from baculovirus transduction was represented VLP-BV. Another VLP produced following transient transfection was used as a control. Briefly, 293T cells were transiently transfected with a plasmid (pVLP) encoding M1, NA, and HA of PR8 virus, and incubated for 72 h at 37°C. Culture supernatants (200 mL) from transduced or transfected cells were collected and were clarified by centrifugation at 3000 g for 10 min at 4°C to remove cell debris. VLPs were precipitated via ultracentrifugation as described above. The pellets were resuspended in 1 mL PBS and loaded onto 20–60% sucrose gradients, and sedimented by ultracentrifugation for 18 h at 27,000 rpm at 4°C. Fractions containing influenza VLPs were collected and diluted in PBS followed by ultracentrifugation again. The pellets were resuspended in PBS and stored at 4°C. Baculovirus residues were checked with the BacPAK Baculovirus Rapid Titer Kit. Protein concentration was determined by bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL). All VLPs used in this study were based on total protein. The hemagglutination titer of VLPs was examined with an HA assay.

Mouse immunization and challenge

Female BALB/c mice (6–8 wk old) were purchased from Harlan Sprague Dawley (Indianapolis, IN). The mice (8 groups, 10 mice per group) were vaccinated: (1) once or twice with 3 μg VLP-BV representing VLPs derived from BV-VLP/GFP/VSVG transduction; (2) once or twice with 3 μg VLP-tsf representing VLP derived from pVLP transfection; (3) once or twice with 1×10^8 ifu BV-VLP representing purified BV-VLP/GFP/VSVG; (4) twice with 1×10^8 ifu BV representing purified wild-type baculovirus; and (5) with PBS. All vaccines were administered intramuscularly to the mice without any adjuvants.

For virus challenge, ketamine-anesthetized mice were intranasally infected with 1500 pfu of A/PR/8/1934 virus (equivalent to 10× the 50% lethal dose [LD_{50}]) in 50 μL of PBS at 3 wk after the final immunization. For measurement of virus replication in the lungs, five mice from each group were sacrificed on day 3 post-challenge. The other five mice were monitored daily for clinical signs of influenza infection (i.e., weight loss, ruffling fur, or inactivity) and body weight was recorded each day. Mice that lost greater than 20% of body weight were euthanized.

Hemagglutination inhibition (HAI) titer in sera and viral titer in lungs

Blood samples were collected from anesthetized mice via retro-orbital plexus puncture at 2 wk after each immunization.

After the blood samples were clotted and centrifuged, serum samples were collected. The HAI assay was used to assess the functional antibodies to HA able to inhibit agglutination of erythrocytes as previously described (3).

Three days after challenge, five mice from each group were euthanized. The lungs were harvested and were homogenized by passing them through a cell strainer (BD Biosciences, Bedford, MA) in 1 mL of PBS. The homogenates were centrifuged at 1000×g for 10 min. The lung supernatants were collected for plaque assay on Madin-Darby canine kidney (MDCK) cells as previously described (3).

Results

Optimization of transduction conditions

Previous studies reported that many factors affect the efficiency of baculovirus transduction, including cell lines, transduction medium, incubation time, MOI, VSVG, and sodium butyrate (26–28). Initially, we investigated the protein expression after baculovirus transduction in several cell lines, such as Vero, HeLa, MDCK, HEK293, and 293T, and found that 293T is the most susceptible cell line and yields the highest protein expression (data not shown). As previously reported, PBS had the highest transduction efficiency as the transduction medium (26). Two hours is long enough for baculovirus transduction into 293T cells. Therefore, transduction was conducted in PBS for 2 h followed by two washes with PBS or DMEM. Then, the effect of MOI was investigated by transducing 293T cells with BV-VLP/GFP at different MOIs. Following 72 h of incubation, supernatants were harvested and NA expression was determined by Western blot (Fig. 2A). The intensity of each band in the Western blot was analyzed with ImageJ software (http://rsb.info.nih.gov/ij/index.html) (29). NA expression increased with higher MOI, and saturated at MOI greater than 60. Therefore, we used MOI of 60 for further investigation.

Previous studies demonstrated that VSVG can affect baculovirus transduction both *in vitro* and *in vivo* (30), and sodium butyrate can enhance gene expression in baculovirus-transduced cells (28). Therefore, the effects of VSVG and butyrate on baculovirus transduction of 293T cells were examined. At MOI of 60, 293T cells were transduced with BV-VLP/GFP or BV-VLP/GFP/VSVG. After transduction, the cells were cultured with or without sodium butyrate. Under the same transduction and culture conditions, the cells transduced with BV-VLP/GFP/VSVG showed a higher level of fluorescence intensity than with BV-VLP/GFP, indicating that these cells were more efficiently transduced (Fig. 2B and C) (30). Furthermore, the addition of 5 mM sodium butyrate enhanced GFP expression (Fig. 2C and D). In addition, plasmid transfection results in a high level of GFP expression (Fig. 2E), but the cells transfected with DNA plasmid had more dead/dying cells compared to cells transduced with baculovirus (Fig. 2F and G) (31).

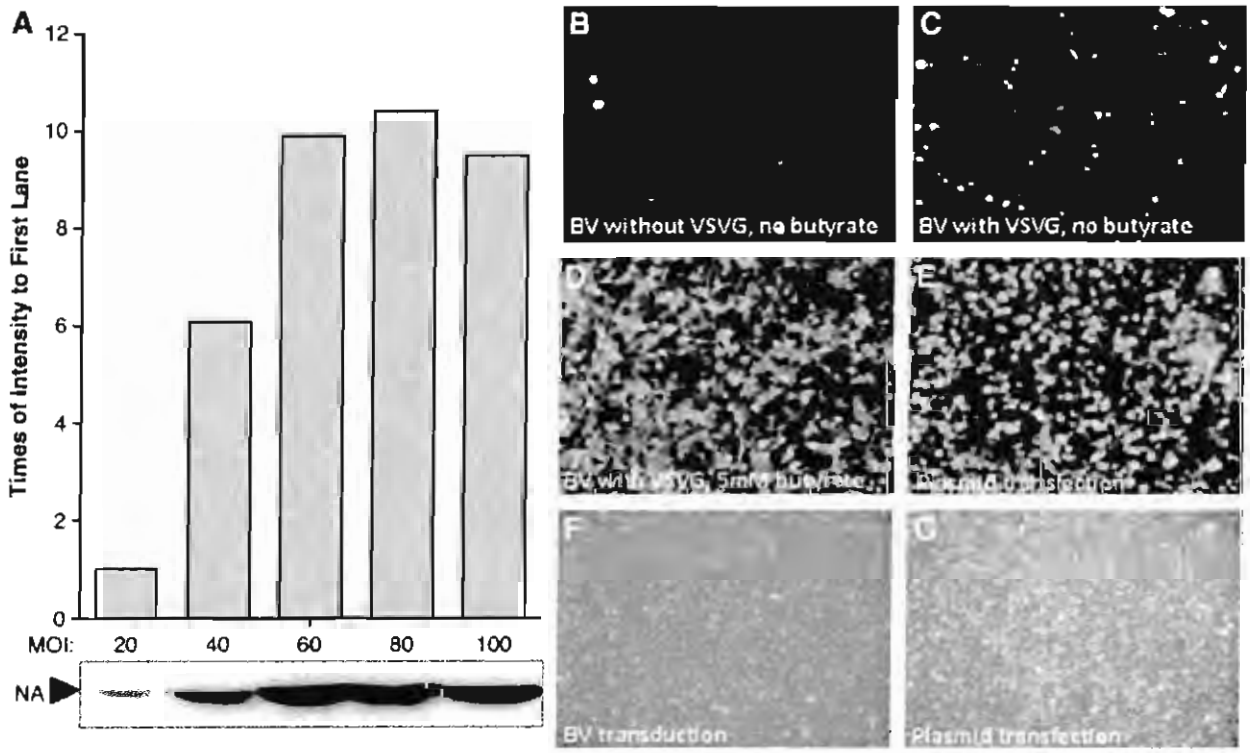

FIG. 2. Optimization of BV transduction conditions. (**A**) 293T cells were transduced with different MOIs of BV-VLP/GFP. NA expression was analyzed by Western blot probed with flag-tag antibody. The intensity of bands was analyzed with ImageJ software. The first lane (MOI = 20) was used as baseline. (**B**) 293T cells transduced with BV-VLP/GFP at MOI = 50, no sodium butyrate. (**C**) 293T cells transduced with BV-VLP/GFP/VSVG at MOI = 50, no sodium butyrate. (**D** and **F**) 293T cells transduced with BV-VLP/GFP/VSVG at MOI = 50, with 5 mM sodium butyrate. (**E** and **G**) 293T cells transfected with pVLP/GFP (1 μg DNA/10^6 cells). Round and gray cells in E and G are dead/dying cells. Color images available online at www.liebertonline.com/vim

Influenza VLP production

To investigate whether the HA, NA, and M1 can be expressed simultaneously in baculovirus-transduced or plasmid-transfected mammalian cells, 293T cells were transduced with BV-VLP/GFP/VSVG at an MOI of 60, or transfected with pVLP/GFP. As shown in Fig. 3A, three bands corresponding to the molecular weights of HA, NA, and M1 were detected in the supernatants of BV-VLP/GFP/VSVG-transduced and pVLP/GFP-transfected cells, but not in the supernatant of wild-type BV-transduced cells.

HA on viral particles or VLPs has the ability to agglutinate red blood cells, while soluble HA cannot agglutinate RBCs, and the hemagglutination titer can reflect the concentration of viral particles (32). Therefore, the functional VLPs can be examined by hemagglutination assay. The supernatants from 293T cells transduced with BV-VLP without VSVG had no detectable HA titer (Fig. 3B). However, the addition of VSVG in BacMam BV increased the HA titer to 1:8. The addition of 5 mM sodium butyrate to the culture medium increased the HA titer to 1:128, which was 4 times higher than 293T cells directly transfected with plasmid pVLP/EGFP. In addition, following sucrose gradient purification, there was no detectable baculovirus in the purified VLP products by plaque assay, although baculovirus DNA could be detected by PCR (data not shown).

Analysis of the immune response

The immunogenicity of purified VLPs and BacMam baculovirus were examined in BALB/c mice by intramuscular injection at week 0 and/or 3. Serum samples were collected 2 wk after each vaccination (weeks 2 and 5), and evaluated for the ability to inhibit influenza virus-induced hemagglutination of tRBCs (HAI), which is a main indicator for the HA-specific immune response. The seroprotection rate (defined as the percentage of subjects with an HAI titer $\geq$1:40) is a well-accepted criterion for the evaluation of influenza vaccine efficacy. A seroprotection rate >70% is one requirement for an influenza vaccine, according to the EU Committee for Human Medicinal Products (CHMP) (33).

After one immunization with VLP-BV, 80% of mice had detectable HAI titers ($\geq$1:10) against PR8 virus; 35% of mice had an HAI titer $\geq$1:40. After one immunization with VLP-tsf, 35% of mice had detectable HAI titers, while none had an HAI titer $\geq$1:40. After one immunization with BV-VLP, 50% of mice had detectable HAI titers, while 10% of mice had an HAI titer $\geq$1:40. After the second vaccination, a significant rise in HAI titers of all immunized mice could be observed. The average HAI titers of mice vaccinated with VLP-BV, VLP-tsf, and BV-VLP were 1:240, 1:100, and 1:112, respectively (Fig. 4). The seroprotection rates for all three vaccines ranged between 80 and 100%, and met the CHMP requirement for immunogenicity. Remarkably, mice

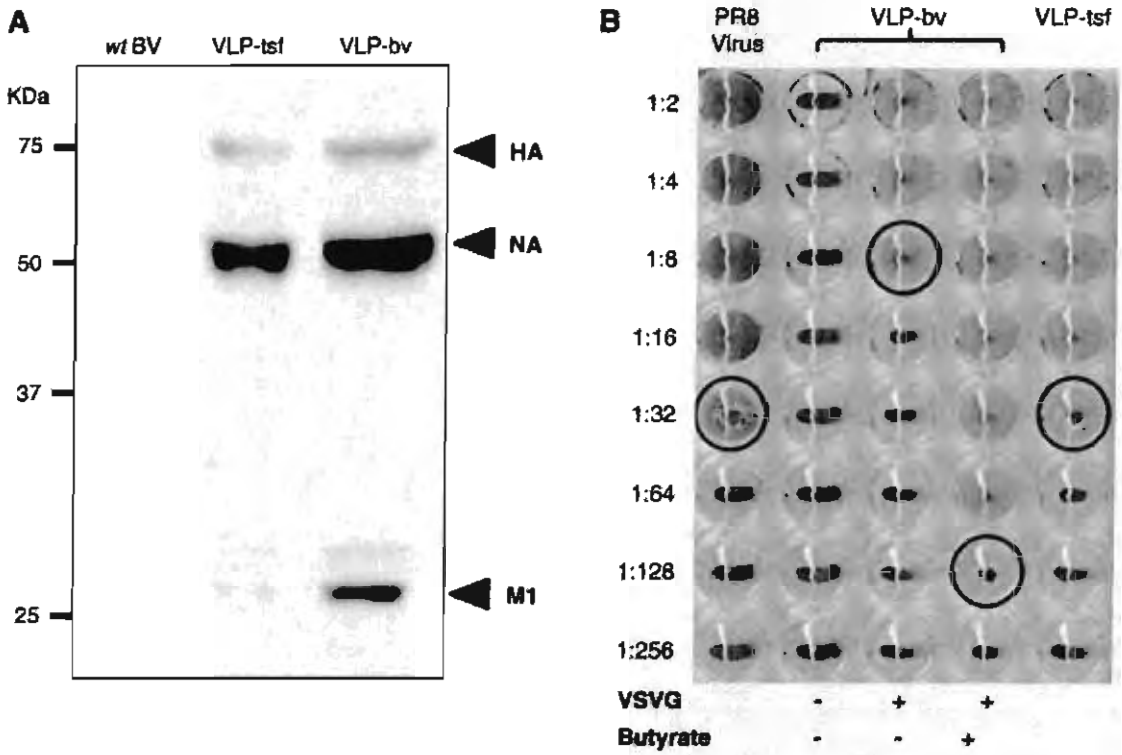

FIG. 3. VLP production from BV transduction or plasmid transfection. (A) Western blot assay of VLP produced from BV transduction (with BV-VLP/GFP/VSVG and sodium butyrate) and plasmid transfection. Mouse anti-PR8 HA polyclonal antibody (1:1000), mouse anti-flag tag monoclonal antibody (1:5000; Sigma-Aldrich), and mouse anti-M1 monoclonal antibody (1:200; AbD Serotec, Raleigh, NC) were mixed for detecting PR8 HA, NA, and M1 in one blot. (B) Hemagglutination activity of VLP produced by plasmid transfection (1 μg DNA/10^6 cells) or BV-VLP transduction (MOI = 60). Color images available online at www.liebertonline.com/vim

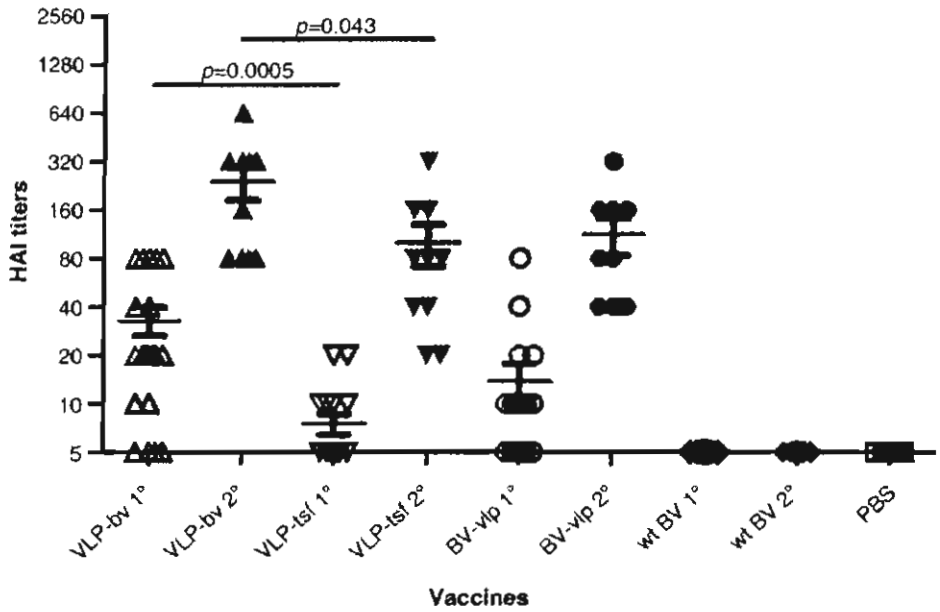

FIG. 4. Hemagglutination-inhibition (HAI) titers. Mice were immunized once (1°) or twice (2°) intramuscularly with VLP derived from BV transduction (VLP-BV), VLP derived from plasmid transfection (VLP-tsf), BV producing PR8 VLP (BV-VLP), wild-type BV (wt BV), and PBS at a 3-week interval (10 mice/group). Blood samples were taken 2 wk after each vaccination. Serum HAI antibody titers were assessed against PR8 virus. Bars indicate geometric mean titer (GMT) ± SEM.

vaccinated with VLP-BV had significantly higher HAI titers compared to mice vaccinated with VLP-tsf, after both priming and boosting immunization. As expected, all mice vaccinated with wt BV and mock vaccinated had no detectable HAI titers.

Protection from lethal viral challenge

To evaluate the protective efficacy of each vaccine, all vaccinated mice were challenged intranasally with a lethal dose of mouse-adapted PR8 virus. Lung viral titers were determined at day 3 post-challenge. Mice vaccinated with wt BV or PBS had high viral lung titers ($\sim 1\times10^6$ pfu/mL), while the mice that received only one vaccination with VLP-BV, VLP-tsf, or BV-VLP had significantly lower viral lung titers (2×10^4 to 2×10^5 pfu/mL) (Fig. 5). There were no significant differences between the three different vaccines. All mice that received two vaccinations had undetectable viral titers (<10 pfu/mL) in their lungs.

All mice vaccinated with wt BV or PBS had signs of morbidity (e.g., ruffling fur, shivering, and inactivity), and had lost greater than 20% of their original body weight by days 8–9 post-challenge (Fig. 6). All mice vaccinated once with VLP-BV were protected with an average of 7% weight loss (open triangles in Fig. 6). Eighty percent of mice vaccinated once with VLP-tsf were protected against influenza challenge, and 60% of mice vaccinated with BV-VLP were protected, even though some mice showed signs of sickness. However, all mice survived without any signs of sickness or weight loss following two vaccinations with VLP-BV, VLP-tsf, or BV-VLP.

Discussion

VLPs mimic the overall structure of parental virus particles without packaging infectious genetic material (34). It is a promising approach to the production of vaccines due to its low risk and high immunogenicity (35). VLP vaccines have been produced from yeast-, insect-, mammalian-, and plant-based systems for a variety of viral pathogens (5). VLPs produced from mammalian cells have several advantages, such as ease of purification and similar post-translational modifications and cytoplasmic trafficking processes as wild-type viruses. However, mammalian-based production systems are hampered by high costs and difficulties with production scale-up. The use of yeast and insect expression systems are inexpensive and relatively easy to scale-up, but the VLP purification is always a challenge because VLPs produced in yeast cells are usually not secreted, and VLPs produced in insect cells are accompanied by baculovirus particles (10,36). Baculoviruses are rod-shaped particles roughly 70 nm×320 nm in size (37,38), which are similar in size to influenza virions. Many methods have been developed to improve the purification of VLPs derived from baculovirus/insect-cell systems, including density-gradient ultracentrifugation, size-exclusion chromatography, and affinity chromatography. These methods are efficiently purifying some small, non-enveloped VLPs, such as norovirus (38 nm) and human papillomavirus (40–50 nm) (5,39), but they are not efficient for other larger VLPs, such as HIV (100–120 nm) or influenza VLPs (80–120 nm) (6,40–42).

In this study, we developed a new strategy to produce influenza VLPs in mammalian cells with baculovirus vector. BacMam baculoviruses can be produced using a standard method in insect cells. Gene expression in transduced cells can last over 16 days (43). Due to its rapid and facile gene expression in various mammalian cells, the BacMam system has been used as a substitute for direct plasmid transfection (44). Influenza VLPs can be produced by simultaneously expressing HA, NA, and M1 in insect/mammalian cells. To

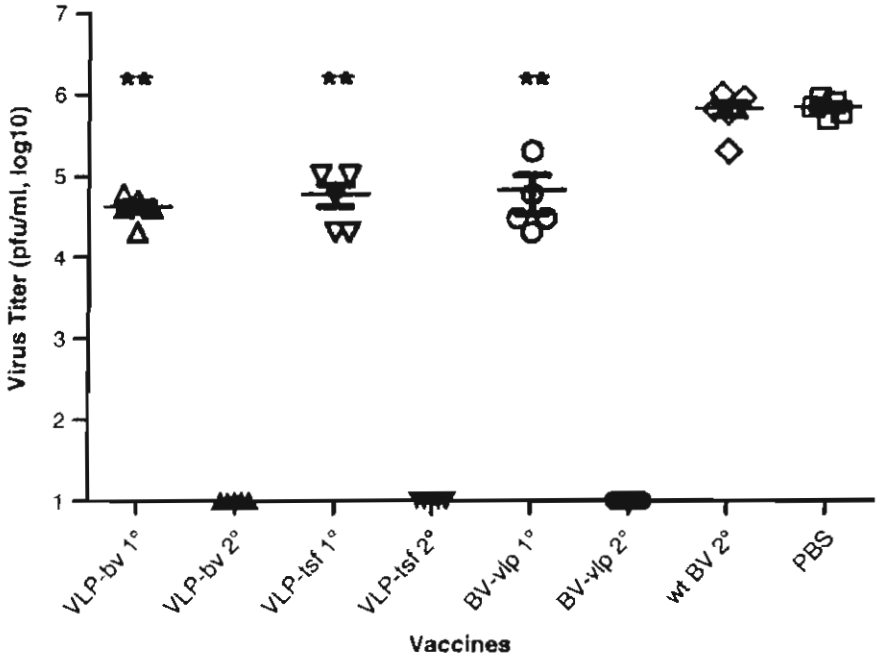

FIG. 5. Virus titers in lungs at day 3 post-challenge. Mice were immunized once (1°) or twice (2°) intramuscularly with VLP-BV, VLP-tsf, BV-VLP, wt BV, and PBS. At week 3 after the last immunization, the immunized mice were intranasally infected with a lethal dose of mouse-adapted PR8 virus (10 LD_{50}). Lung samples from individual mice in each group (five mice per group) were collected on day 3 post-challenge, and each sample was diluted in 1 mL PBS. The titers are presented as $\log_{10}$ pfu per mL (**$p < 0.01$ compared with the wt BV 2° and PBS groups).

ensure gene uptake and expression in target cells, we developed a new BacMam baculovirus carrying all information necessary to induce influenza VLP production. The resulting constructs, BV-VLP/EGFP and BV-VLP/EGFP/VSVG, allowed for the production of influenza VLPs. Transduction efficiency was optimized and VLP production was characterized. Since baculovirus cannot replicate in mammalian cells as it does in insect cells (11), the purification of VLPs produced from the BacMam system is much easier than VLPs produced from insect cells. The BacMam system is also easy to scale up by using bioreactors such as the baculovirus/insect-cell expression system (44). Therefore, the BacMam system combines the advantages of both baculovirus/insect cell expression and DNA plasmid transfection methods: it is inexpensive, rapid, and facile for experiments for gene overexpression, and is easy to scale up and purify. Of

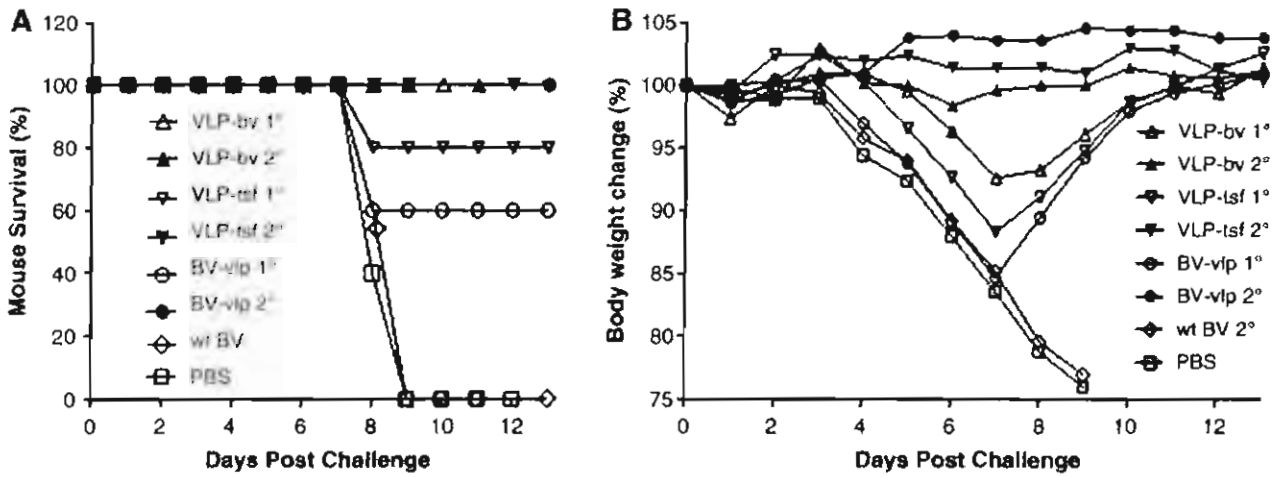

FIG. 6. Protection of mice from lethal PR8 challenge. Mice were immunized once (1°) or twice (2°) intramuscularly with VLP-BV, VLP-tsf, BV-VLP, wt BV, and PBS. At week 3 after the final immunization, the mice were intranasally infected with a lethal dose of mouse-adapted PR8 virus (10 LD_{50}) (five mice per group). The mice were monitored daily for 13 d. (**A**) Percent survival after challenge. (**B**) Body weight changes after challenge.

course, there are also some limitations, such as repetitious washing of transduced cells that would be labor-intensive for suspension culture; two-tiered banking systems for both mammalian and insect cells are needed to implement this strategy.

Vaccination of mice with *in vitro*-produced influenza VLPs or with BacMam BV induced equally high antibody responses following two vaccinations (HAI titers 1:20–1:640). Remarkably, VLPs produced via BV transduction induced significantly higher HAI titers than VLPs produced via plasmid transfection (Fig. 4). This effect may be because VLPs produced via plasmid transfection contain a higher content of cellular proteins. During direct transfection of culture cells with plasmids, the transfection reagents are potentially cytotoxic, which may result in a high degree of cell death and subsequent cell debris contamination of the VLP preparation. However, the BacMam BV transduction process is not associated with cytotoxicity, even at high MOI (>500) (31). At equal protein concentrations, the VLPs produced via BV transduction have 2–4 times higher HA titers than VLPs produced via plasmid transfection, suggesting a higher portion of influenza VLPs with functional HA on the particles compared to VLPs produced via plasmid transfection. An additional reason could be that baculovirus DNA may remain in the VLP products derived from the BacMam system, which could serve as adjuvant for this vaccine. Baculovirus has strong adjuvant effects even at low concentrations (10^3 pfu), and baculoviral DNA, but not protein, has adjuvant activity (45). We found that baculovirus DNA was detectable by PCR from the purified VLPs, although no baculovirus could be detected by plaque assay (data not shown).

To evaluate the protection induced by VLP vaccines, all vaccinated mice were challenged with a homogeneous, mouse-adapted influenza virus (A/PR/8/1934), administered at a lethal dose. The viral clearance in lungs provides a sensitive indicator for assessing protective efficacy. At 3 d post-challenge, all mice in the control groups showed high lung viral titers. Mice vaccinated two times with VLP-BV, VLP-tsf, and BV-VLP had no detectable virus post-challenge. Mice receiving one vaccination had two- to 50-fold reductions in viral lung titers compared to unvaccinated or wt BV vaccinated mice (Fig. 5). These results indicate that for all three vaccines, two vaccinations can induce strong immune responses that are sufficient to prevent infection. In contrast, one vaccination reduces viral replication, but does not prevent infection. As for the protection seen after one vaccination, the results indicated that one vaccination with VLP-BV provided 100% protection, while one vaccination with VLP-tsf or BV-VLP conferred 80% and 60% protection, respectively. Although BV-VLP is a little less effective in protection compared to other VLPs, it is still a promising vaccine strategy because of its ease of scale-up. Since BV cannot infect mammals and does not cause cytopathic effects (11,18), safety issues are greatly reduced. Of course, more studies are needed to address its safety. Better protection should be achievable by giving higher doses. Recently, it was demonstrated that high doses of BV delivering influenza HA protected chickens against avian influenza virus challenge, while a low dose did not elicit protection (21). Therefore, protection with a single vaccination would be expected if a higher dose of VLP-tsf or BV-VLP is administered.

When considered together with previous data, the results of our current studies suggest that BacMam system-derived influenza VLPs represent a promising and safe vaccine candidate for protection against influenza infections. Due to its low cytotoxicity and high gene delivery efficiency, the BacMam baculovirus represents a significant advance, and it is a promising alternative vaccine strategy. The major drawback of directly using BV as a gene delivery vector is that it can induce immune responses against the BV vector, which will limit the repeat use of this vector. Future studies to evaluate the potential benefits of using this strategy in controlling other infectious diseases are needed.

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Author Disclosure Statement

No competing financial interests exist.

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APPENDIX 4

Dengue Virus

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Dengue virus

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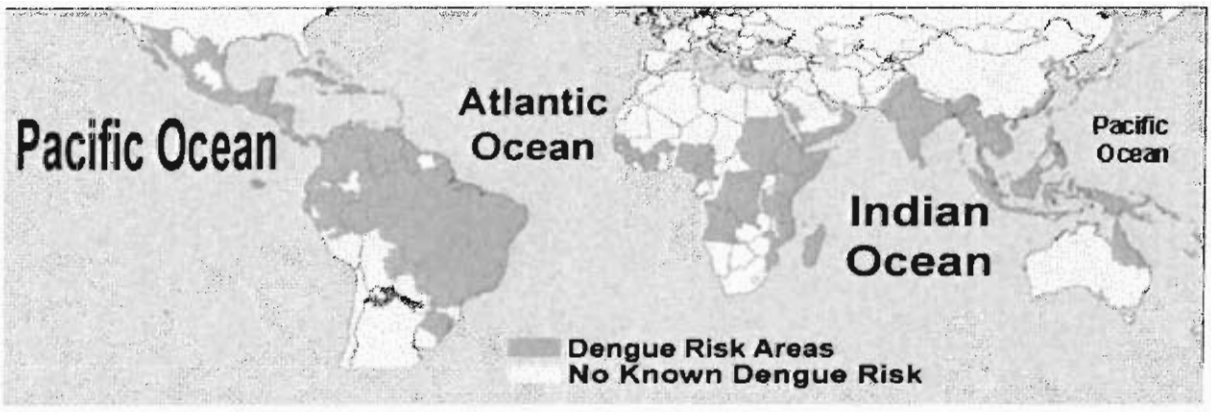
Abstract

Dengue viruses cause a spectrum of diseases by the four serotypes. Dengue is the most prevalent arthropod-borne virus affecting humans today. Due in part to population growth and uncontrolled urbanization in tropical and subtropical countries, breeding sites for the mosquitoes that transmit dengue virus have proliferated. In turn, successful vector control programs have been eliminated, often due to lack of governmental funding. Dengue viruses have evolved rapidly, as they have spread worldwide, and genotypes associated with increased virulence has spread across Asia and the Americas. This chapter will describe the virus, clinical and epidemiological, and treatments/vaccines associated with dengue infection.

Overview

Dengue fever (DF), the most prevalent arthropod-borne viral illness in humans, is caused by the dengue virus (DENV). The four serotypes of DENV (DENV 1-4) are transmitted to humans primarily by the *Aedes aegypti* mosquito. DENV, is a member of the *Flaviviridae* family and is related to the viruses that cause yellow fever and the Japanese, St. Louis, and West Nile encephalitides [1]. Infection by DENV causes a spectrum of clinical diseases that range from an acute debilitating, self-limited febrile illness, known as dengue fever (DF) to a life-threatening hemorrhagic and capillary leak syndrome of dengue hemorraghic fever/dengue shock syndrome (DHF/DSS). DENV causes an estimated 25 to 100 million cases of DF and 250,000 cases of DHF per year worldwide, with 2.5 billion people at risk for infection [2, 3]. At present, no approved antiviral treatment or vaccine is in use, and therapy is supportive in nature.



Epidemic DHF first appeared in the 1950s in Southeast Asia, and by 1975 it had become a leading cause of hospitalization and death among children in many countries in that region. In the 1980s, DHF began a second expansion into Asia, and in countries where DHF is endemic, the epidemics have become progressively larger over the last 15 years (CDC, http://www.cdc.gov/ncidod/dvbid/dengue).

The Americas have seen the most dramatic rises in the emergence of dengue cases. The mosquito vector for dengue was eradicated in most of the region as part of the Pan American Health Organization's (PAHO) yellow fever eradication campaign in the 1950s and 1960s. The *Aedes aegypti* eradication program was officially discontinued in the United States and other Western Hemisphere regions, leading to re-infestation of the mosquito vector in most countries during the 1980s and 1990s. By 1997, the geographic distribution of *Aedes aegypti* was wider than its distribution before the eradication program (Figure 1). Dengue is now endemic in

much of the Western Hemisphere (Figure 2). Hyperendemicity, the presence of multiple circulating serotypes, is wide-spread in most countries and epidemics caused by multiple serotypes are more frequent.

Recent Dengue in the U.S.A.

- Dengue epidemics occurred in the USA in the 1800s and the first half of the 1900s
- Recent indigenous transmission
  - Texas:
    - 1980: 23 cases, first locally acquired since 1945
    - 1986: 9 cases, 1995: 7 cases, 1997: 3 cases, 1998: 1 case, 1999: 18 cases, 2005: 25 cases
  - Hawaii:
    - 2001-2002: 122 cases (first since 1944)

- The first indigenous transmission of dengue in the United States since the 1940s occurred in Texas in 1980.
- Other recent clusters in the United States also occurred in Texas, where 25 locally acquired cases occurred in 2005.
- A dengue outbreak occurred in Hawaii during 2001-2002. In this case Aedes albopictus mosquitoes were responsible for virus transmission. in homes.

There are four grades of DHF. For all grades the four criteria for DHF must be met.

1. In Grade 1, fever and nonspecific constitutional symptoms are present and the only hemorrhagic manifestation is provoked, that is, a positive tourniquet test (which will soon be described).
2. In Grade 2, in addition to the Grade I manifestations, there is spontaneous bleeding.
3. Grades 3 and 4 are Dengue Shock Syndrome. Grade 3 is incipient shock with signs of circulatory failure.
4. In Grade 4, the patient has profound shock, with undetectable pulse and blood pressure.

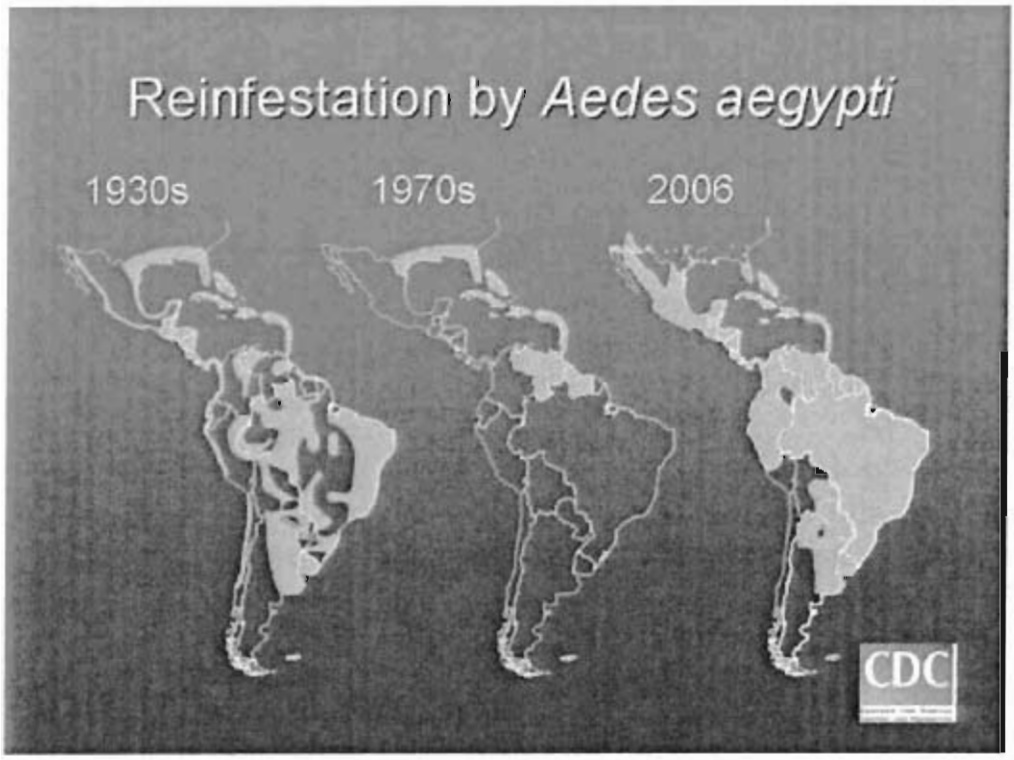


Figure 1: Reinfestation of *Aedes aegypti* in the Americas. Unfortunately, the success of the eradication campaign was not sustained. Beginning in the early 1970s, it began to be disbanded, and many countries channeled their limited resources into other areas. Consequently, *Aedes aegypti* began to reinfest the countries from which it had been eradicated. Comparing the 1970 with the 2006 map, we see the mosquito reestablishing itself throughout Central America and most of South America. As the mosquito has spread, the number and frequency of dengue epidemics have increased, as has dengue hemorrhagic fever activity in the Americas.

Source: PAHO, 2001

Virology

DENV is an enveloped virus with a single-stranded, positive-sense 10.7 kilobase RNA genome [4], which is translated as a single polyprotein and then cleaved into three structural proteins (C, prM/M, E) and seven non-structural (NS) proteins by virus- and host-encoded proteases. In primary DENV infection, virus enters target cells after the envelope (E) protein adheres to cell surface receptors, such as DC-SIGN on dendritic cells [5]. Viral uptake occurs by receptor-mediated endocytosis. Endosomal acidification induces a conformational change in the E protein resulting in fusion of the viral and endosomal membranes and nucleocapsid release into the

cytoplasm [6, 7]. Virus assembly occurs at the endoplasmic reticulum (ER), and virions are exocytosed via Golgi-derived secretory vesicles [8].

Epidemiology

Following the bite of a mosquito, usually *Aedes aegypti* or *Aedes albopictus* [2], DENV can cause a range of illnesses from mild to severe. There are four distinct serotypes of DENV. Primary infection with one DENV serotype provides lifelong immunity to that specific serotype. However, when an individual is infected with a different serotype of DENV, there is an increased risk of severe dengue disease [9]. This can occur with all four serotypes, therefore, in regions with multiple endemic serotypes, the risk of severe disease is higher.

Pathogenesis

The pathogenesis of DHF/DSS, the most severe form of DENV infection, reflects a complex interplay of the host immune response and the viral determinants of virulence [2, 10, 11]. Epidemiological studies have suggested an immune system linkage as there is an increased risk of DHF with secondary infection and in children within the first year of life born to DENV-immune mothers [12-15]. From these observations, the hypothesis of antibody-dependent immune enhancement of infection (ADE) emerged. In support of the ADE pathogenesis concept, antibody enhancement of DENV infection in monocytes *in vitro* correlated with increased risk for DHF [15, 16], and peak viremia was increased in patients with severe secondary DENV infection [17, 18]. Differences in specific genetic determinants among viral isolates [19-21] may also affect virulence as some DENV strains fail to cause severe disease [22, 23]. Finally, a pathologic cytokine response that occurs after extensive T cell activation may contribute to the capillary leak syndrome associated with DHF [11]. Elevated levels of cytokines, including IFN-gamma, TNF-alpha, and IL-10, to some extent correlate with severe disease [24-28]; and disease severity has been associated with activation of $CD8^+$ T cells and the expansion of serotype-reactive low-affinity DENV-specific T cells that produce high levels of vasoactive cytokines [29-33].

Clinical Presentations

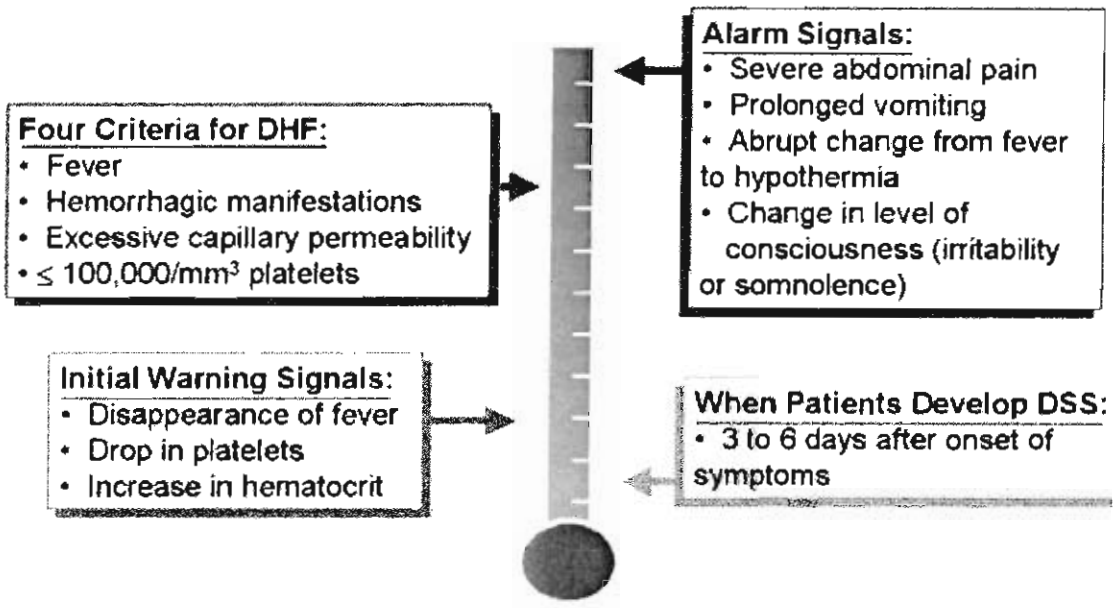
Dengue fever may present in many forms; as an undifferentiated febrile illness with a maculopapular rash, particularly in children, flu-like symptoms, or as classic Dengue with two or more symptoms, such as fever, headache, bone or joint pain, muscular pain, rash, pain behind the eyes, petechial hemorrhaging. Often, there is prolonged fatigue and depression. During dengue epidemics, hemorrhagic complications may also appear, such as bleeding from the gums, nosebleeds, and bruising. Case fatalities due to DF are low, but case fatality due to

DHF can be high. There is no specific treatment for dengue fever except for symptomatic treatment, rest, and rehydration.

DHF is characterized by spontaneous bleeding, plasma leakage, fever, and thrombocytopenia. Four clinical manifestations need to be observed to be classified as DHF. These include: 1) fever, 2) hemorrhagic episodes with the presence of at least one of the following: a positive tourniquet test; petechiae, ecchymoses, or purpura; or bleeding from mucosa, gastrointestinal tract, injection sites, or others), 3) plasma leakage due to increased capillary permeability, and 4) thrombocytopenia (100,000 mm3 or less). Moderate to marked thrombocytopenia with concurrent hemoconcentration is a distinctive clinical laboratory finding of DHF. However, in order to distinguish DHF from DF, an observation of plasma leakage manifested by a rising hematocrit value (*i.e.,* hemoconcentration) must be observed.

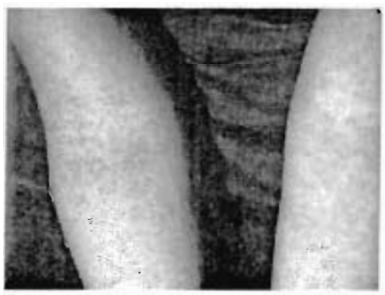
The normal course of DHF lasts between 7-10 days, and with appropriate intensive maintenance of the circulating fluid volume, mortality may be reduced to less than 1%. Only severe DF and DHF cases should be hospitalized. Serological tests are necessary to confirm cases of dengue. However, these tests may take several days [34, 35]. Developing countries may not have the resources to perform these expensive confirmatory assays and therefore, many suspected cases of dengue are not fully diagnosed. In severe cases of DHF, the patient's condition may suddenly deteriorate after a few days of fever; the temperature will drop, followed by signs of circulatory failure; and the patient may rapidly go into a critical state of shock (dengue shock syndrome) and die within 12-24 hours, or quickly recover following appropriate volume replacement therapy.

Dengue shock syndrome (DSS) is the most severe form of DHF, and is characterized by the presence of all four DHF clinical manifestations, as well as circulatory failure. All three manifestations of circulatory failure must be present: rapid and weak pulse, narrow pulse pressure or hypotension for age of patient, and cold, clammy skin and altered mental state.



Diagnosis

Establishing a laboratory diagnosis of dengue infection is critical for diagnosis of dengue. A major challenge for disease surveillance and case diagnosis is that the dengue viruses produce asymptomatic infections and a spectrum of clinical illness ranging from a mild, nonspecific viral syndrome to fatal hemorrhagic disease. Important risk factors for DHF include the strain and serotype of the infecting virus, as well as the age, immune status, and genetic predisposition of the patient. The most common method to detect the virus is culture or detection of anti-dengue antibodies by serology. Virus can be cultured *in vitro* or by detection of viral RNA and specific dengue virus antigens. Countries that do not have access to sophisticated laboratory tests rely on identification of early clinical and/or simple laboratory indicators that can provide a reliable diagnosis of dengue prior to hospitalization. Early distinction between dengue and other febrile illnesses could help identify patients that should be monitored for signs of DHF.



Differential Diagnosis

Febrile illnesses such as measles, typhoid fever, leptospirosis, and severe acute respiratory syndrome (SARS) can produce symptoms similar to DF [36-41]. At presentation, these illnesses may share similar clinical features, including headache, myalgia, and rash.

The differential diagnosis of dengue includes:

- Influenza
- Measles
- Rubella
- Malaria
- Typhoid fever
- Leptospirosis
- Meningococcemia
- Rickettsial infections
- Bacterial sepsis and
- Other viral hemorrhagic fevers.

Treatment and long-term outcomes.

There are no specific anti-virals that can kill the virus. However supportive care and treatment can go a long way to treat DF. Paracetamol and other anti-pyretics can be used to treat fever. Bone pain should be treated by analgesics or pain killing tablets. During episodes of DHF/DSS, the mortality rate in the absence of hospitalization can be as high as 50%. With proper treatment, such as intravenous fluid replacement, the mortality rate is greatly reduced.

Vaccines and Immunity

Multiple correlates of protection have been described for dengue. However, the primary correlate appears to be long-term homotypic protection [42, 43] or short-term heterotypic protection [42, 44]. The majority of protective antibodies are directed at the surface E glycoprotein [45, 46], however, antibodies to the M and NS1 proteins show some protective efficacy [47]. Passively transferring antibodies from a seroconverted animals results in decreased infection and disease following challenge [45, 47]. In addition, maternal antibodies decrease disease in infants [15, 48]. Using *in vitro* neutralization assays, antibodies directed against the E protein prevent virus infection [49]. Antibodies that block viral attachment or prevent fusion to target cells neutralize virus infection [50, 51]. In addition to neutralization, antibodies that

mediate cell-mediated cytotoxicity reduce virus infection in both complement independent [52, 53] and complement dependent mechanisms [54]. Cellular immune responses are generally weakly protective [55]. However, these responses are critical for viral clearance [56, 57]. Innate immune responses directed against non-structural proteins, such as NS4B (a putative IFN antagonist), appears to mediate viral escape [58].

Currently, no DENV vaccine is approved by the U.S. Food and Drug Administration. Four related, but serologically distinct DENV can cause disease. Non-neutralizing, cross-reactive antibodies may contribute to DHF pathogenesis via antibody dependent enhancement, an effective vaccine must induce high-titer neutralizing antibodies against all four strains [59, 60]; failure to do so could increase the risk of severe disease upon natural challenge. To circumvent this problem, tetravalent live-attenuated candidate vaccines are in varying stages of development [61-65]. In clinical trials, tetravalent serologic responses were observed in some individuals, but many developed do not develop high titer neutralizing antibodies despite multiple immunizations [66, 67]. Subunit based vaccines, as purified proteins or DNA plasmid, are alternative vaccine strategies. Repeated immunization of purified recombinant DENV DIII or DIII-encoding plasmids induced protective antibody in mice, albeit at relatively low neutralizing titers [68-72].

Both live attenuated vaccines and non-replicating vaccines, such as inactivated virus vaccines, virus-like particles or DNA vaccines have been developed for dengue (Table X). These vaccines elicit protective neutralizing antibodies. These vaccines can elicit long-lasting immunity against the specific serotype of DENV. However, they are poorly cross-reactive against infection with another subtype of DENV.

Table 1 | A partial list of dengue vaccine candidates that are under development

Vaccine type	**Vaccine developer(s)**	**Clinical testing status**	**Refs**
Live attenuated	WRAIR/GSK	Biologicals Tetravalent, Phase II	57,116
Live attenuated	Mahidol University/sanofi pasteur	No current testing	77
Live attenuated, chimeric	NIAID, NIH	Monovalent (DENV-1 – 4), Phase I/II	73,89
Live attenuated, chimeric	Acambis/sanofi pasteur	Tetravalent, Phase I	74,92
Live attenuated, chimeric	CDC/InViragen	Preclinical	93
Inactivated virus	WRAIR	Preclinical	97
Subunit	Hawaii Biotech	Begins 2007	97
DNA	Navy Medical Research Center	Monovalent (DENV-1), Phase I	99

GSK, GlaxoSmithKline; NIAID, National Institute of Allergy and Infectious Diseases; NIH, National Institutes of Health; WRAIR, Walter Reed Army Institute of Research

Type	Sponsor	Stage of Development
Live Attenuated		
tetravalent	Mahidol Univ/Sanofi/Pasteur	Phase I
tetravalent	WRAIR/GSK	Phase II
Chimeric		
Chimerivax (17D YF)	Acambis/Sanofi-Pasteur	Phase I
DENV-2 (16681, PDK53)	CDC/InViragen	Preclinical
DENV-2/4d30 (all serotypes)	NIAID, NIH	Phase I/II
DENV-1	US FDA	Phase I
DNA		
Several approaches	Various	
(*i.e.* Domain III, prM/E, NS1)	NMRC/Univ Pittsburgh	Phase I/Preclinical
Inactivated		
Several approaches	WRAIR	Preclinical
SVP/VLPs		
Subunit/Recombinant		
Baculovirus (E, NS1)	Various	
Replication-defective AV (E)	Replivax-UTMB/Acambis	Preclinical
Yeast (C/prM/E, E-IIBsAg)	various	Preclinical
E. coli (E, E-NS1)	Various	Preclinical
Drosophila cells	Hawaii Biotech	Phase I
DNA	Univ Pittsburgh	Preclinical

Figure Legends

Figure SRa. A world map denoting the areas that had WNV human disease prior to 2003. Kunjin, the lineage Ib WNV, is also shown. *Image reprinted with permission [Solomon et al., 2003]. GET COPYRIGHT PERMISSIONS*

Figure SR1. The number of confirmed human cases of WN disease in the United States in 2008.

http://www.cdc.gov/ncidod/dvbid/westnile/Mapsactivity/surv&control08Maps_PrinterFriendly.htm

Figure SR2. A simplified diagram of the WNV transmission cycle. The maintenance of WNV in nature depends upon many avian and mosquito species. Humans and other incidental hosts (like horses) become infected when WNV-infected mosquito takes a bloodmeal from them.

Figure SR3. Timecourse of WNV infection and antibody response in humans as estimated from blood donations. WNV genomes are detected by commercially available kit by TMA assay. IgM and IgG are measured by ELISA (enzyme-linked immunosorbant assay. ID-TMA: individual donation transcription-mediated activation performed once (1x) or 6 times (6x). ID-NAT: individual donation nucleic acid test. MP-NAT: minipooled nucleic acid test. *Figure reprinted with permission [Busch et al., 2008].* *(NEED TO GET PERMISSIONS)*

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APPENDIX 5

West Nile Virus

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West Nile Virus

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KEYWORDS

- West Nile virus • Flavivirus • Infection
- Pathogenesis • Diagnosis

OVERVIEW

Since its isolation in Uganda in 1937, West Nile virus (WNV) has been responsible for thousands of cases of morbidity and mortality in birds, horses, and humans. Historically, epidemics were localized to Europe, Africa, the Middle East, and parts of Asia and primarily caused a mild febrile illness in humans. However, in the late 1990s, the virus became more virulent and spread to North America. In humans, the clinical presentation ranges from asymptomatic (approximately 80% of infections) to encephalitis/paralysis and death (less than 1% of infections). There is no FDA (Food and Drug Administration)-licensed vaccine for human use, and the only recommended treatment is supportive care. Individuals that survive infection often have a long recovery period. This article reviews the current literature summarizing the molecular virology, epidemiology, clinical manifestations, pathogenesis, diagnosis, treatment, immunology, and protective measures against WNV and WNV infections in humans.

VIROLOGY AND MOLECULAR BIOLOGY OF WNV

West Nile virus is a positive-stranded RNA virus in the family Flaviviridae (genus *Flavivirus*), that includes other human pathogens, such as dengue, yellow fever, and Japanese encephalitis viruses.[1,2] The virion consists of an envelope surrounding an icosahedral capsid approximately 50 nm in size. The approximately11-kilobase genome encodes a single open reading frame, which is flanked by 5′ and 3′ untranslated regions. The polyprotein of approximately 3000 amino acids is cleaved into 10 proteins by cellular and viral proteases (**Fig. 1**). Three of these proteins are the structural components required for virion formation (capsid protein [C]) and assembly into viral particles (premembrane [prM] and envelope [E] proteins). The other 7 viral proteins are nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) and are all necessary for genome replication. NS3 contains an ATP-dependent

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Structural

C: Capsid
(pr)M: (pre)Membrane
E: Envelope

Non-Structural

NS1: secreted glycoprotein
NS2A: inhibits IFN-β promoter activity
NS2B: virus protease cofactor
NS3: virus protease, helicase
NS4A: membrane alteration
NS4B: membrane alteration, IFN inhibition
NS5: methyltransferase, RdRP

Fig. 1. WNV genome. A representation of the WNV genome including the 3 structural proteins that make up virion particle and the 7 nonstructural proteins necessary for virus replication and immune evasion.

helicase and in conjunction with the NS2B protein, a serine protease, which is required for virus polyprotein processing. NS5 is a methyltransferase and RNA-dependent RNA polymerase. The other NS proteins are small, generally hydrophobic proteins of disparate functions. NS1 is a secreted glycoprotein implicated in immune evasion.[3] NS2A plays a role in virus assembly and inhibiting interferon (IFN)-β promoter activation.[4,5] NS4A is responsible for a rapid expansion and modification of the endoplasmic reticulum (ER) that helps establish replication domains.[5–8] NS4B blocks the IFN response.[9–12] All the NS proteins seem to be necessary for efficient replication.[13]

The flavivirus life cycle consists of 4 principal stages: attachment/entry, translation, replication, and assembly/egress (reviewed in {Clyde, 2006 #103,[2]}).WNV enters cells via receptor-mediated endocytosis, and is transported into endosomes. The WNV receptor is unknown. Several cell-surface proteins are potential WNV receptors (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin [DC-SIGN], integrin $\alpha_v\beta_3$),[14–16] and the receptor required for WNV binding and entry may vary by cell type. Acidification of the endosomal compartment causes a conformational change in the E protein, resulting in fusion of the viral and endosomal membranes and release of the virus nucleocapsid into the cytoplasm.[17,18] The viral RNA is translated and the polyprotein is processed. Genome replication is carried out in specific domains established by the viral proteins.[19,20] As stated earlier, viral proteins cause massive expansion and modification of the ER. Two domains are important in replication and virus protein processing: vesicle packets and convoluted membranes, respectively (**Fig. 2**).[20–25] Following replication and translation, genomes are packaged into virions, which mature through the ER-Golgi secretion pathway.[19,20,26,27] Progeny viruses are released by exocytosis.

PHYLOGENY

The most current phylogenetic studies based on complete or partial genome sequences indicate 5 lineages of WNV.[28] The virus that entered North America belongs to lineage I (clade Ia). This lineage also contains viruses found in Europe,

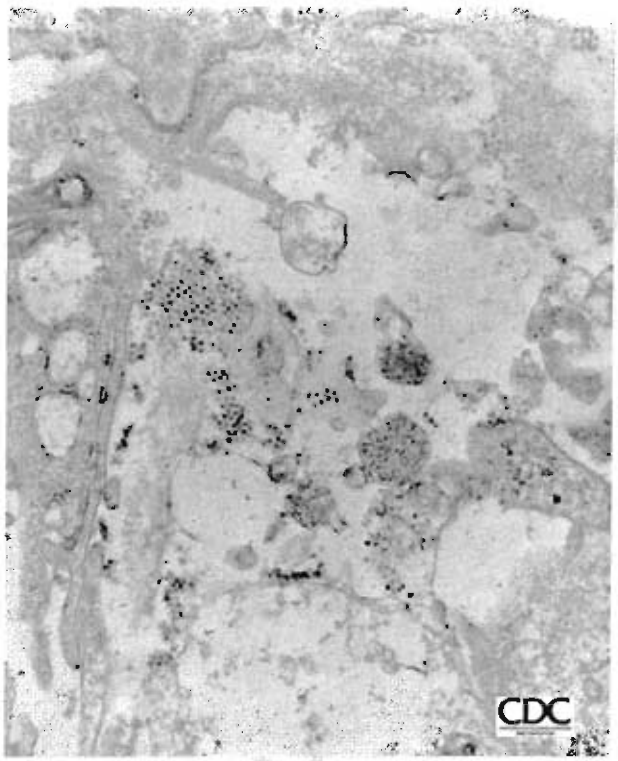

Fig. 2. WNV isolated from brain tissue from an infected crow. The tissue was cultured in a Vero cell for a 3-day incubation period. The Vero cells were fixed in glutaraldehyde, dehydrated, placed in an Epon resin, thin sectioned, placed on a copper grid, and stained with uranyl acetate and lead citrate. The grids were then placed in the electron microscope and viewed. Total magnification, image 65,625x. (*Courtesy of* Bruce Cropp, microbiologist, Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention [CDC].)

the Middle East, and Africa. The genome of Kunjin virus, the Australian strain of WNV, is also in the lineage I group (clade Ib). Lineage II contains WNV mainly of African origin. Although there are exceptions, in general, lineage I (clade Ia) viruses can cause severe human neurologic disease, whereas lineage I (clade Ib) and lineage II viruses generally cause a mild, self-limiting disease. Not much is known about the viruses that comprise lineage III, IV, and V.

EPIDEMIOLOGY

WNV is maintained in nature in a cycle between birds and mosquitoes (**Fig. 3**). Although many different species of mosquito are capable of maintaining this cycle, the *Culex* species play the largest role in natural transmission (**Fig. 4**). Not all infected mosquitoes feed preferentially on birds, which can lead to other animals, including humans, becoming infected. Humans (and horses) are incidental or "dead-end" hosts in this cycle, because the concentration of virus within the blood (viremia) is insufficient to infect a feeding naïve mosquito. Other natural modes of WNV transmission have been documented but occur rarely. WNV transmission can occur between infected

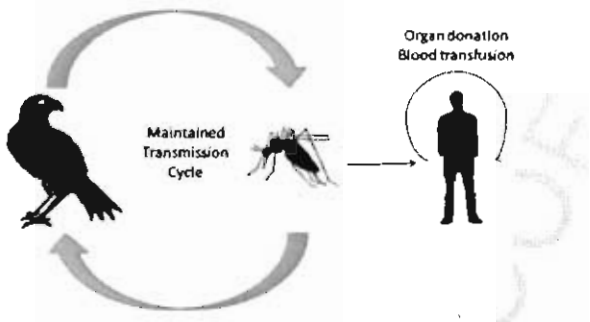

Fig. 3. The WNV transmission cycle. The maintenance of WNV in nature depends on many avian and mosquito species. Humans and other incidental hosts (like horses) become infected when WNV-infected mosquito takes a bloodmeal from them.

mother and newborn via the intrauterine route[29–31] or possibly by breast-feeding.[32] A recent study of pregnant women who became infected with WNV during the 2003 to 4 transmission in the United States suggested that adverse side effects in the newborn infant due to WNV infection of the mother are rare, and those cases with infant illness/infection/mortality may be associated with WNV infection that occurred while the mother was infected within 1 month prepartem.[33]

Within the human population, the virus can spread between individuals by more artificial means. In the early 2000s, patients that received tainted blood or organs from viremic donors became infected.[34–37] These events highlighted the need to safeguard blood and organ donations from potentially viremic, yet healthy, donors, and relatively few infections via this route of transmission have been reported since 2004.

The epidemiology of WNV is continuously changing. The virus was initially isolated from a febrile woman in Uganda in 1937.[38] Since then, few outbreaks of WNV in human or horse populations have been recorded until the beginning of the 1990s. When disease was observed in humans, symptoms were typically mild and neurologic complications were rare.[39,40] The exceptions were outbreaks in Israel in the early 1950s and France in the 1960s, which were characterized by encephalitis in humans and horses. A series of outbreaks in the 1990s brought WNV into the spotlight; epidemics in Algeria, Morocco, Tunisia, Italy, France, Romania, Israel, and Russia were associated with uncharacteristically severe human disease, including neurologic

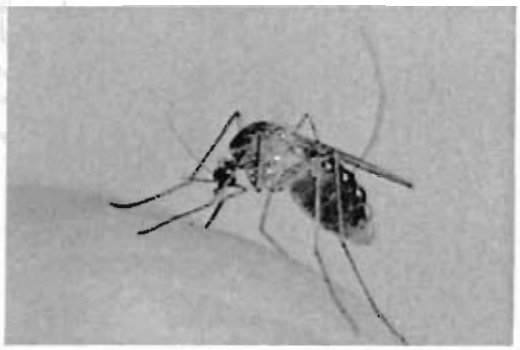

Fig. 4. *Culex* mosquito. The *Culex* species of mosquito, the most common vector of WNV, [Q16] feeding. (*Courtesy of* US Geological Survey.)

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complications and death.[39,41–43] In the summer of 1999, a cluster of patients with encephalitis in New York City signaled the entry of WNV into North America. The sequence of the 1999 New York strain of WNV is closest in identity to a viral isolate from Israel,[44] but how the virus traversed the Atlantic Ocean is still a mystery. In the past decade, there have been thousands of reported human cases of WNV disease (WN fever and WN encephalitis) accompanied by more than 1000 deaths (**Table 1**). The geographic range of the virus currently extends north into Canada, west across all 48 contiguous states, and south into Mexico, the Caribbean, and Central and South America (**Figs. 5** and **6**) {Blitvich, 2008 #876}. Since 2007, in addition to ongoing circulation of WNV in the Western Hemisphere, there have been outbreaks or isolations of WNV in Volograd (Russia) {Platonov, 2008 #877}, South Africa {Venter, 2009 #878}, Hungary {Krisztalovics, 2008 #879}, Romania {Popovici, 2008 #880}, and Italy {Rossini, 2008 #881}(see **Fig. 5**). In 2008 alone, there were 1338 cases of WNV disease reported to the Centers for Disease Control and Prevention (CDC) and resulted in 43 deaths within the United States (http://www.cdc.gov/ncidod/dvbid/westnile/surv&controlCaseCount08_detailed.htm).

CLINICAL PRESENTATION

It is difficult to accurately predict the incubation period of WNV in humans (time from mosquito bite/infection to the presentation of symptoms), but it is approximately 2 to 15 days.[34,45] The majority (>80%) of WNV infections are asymptomatic. Symptomatic infections manifest primarily as a mild, self-limiting febrile illness. However, approximately 1% of infected people develop neurologic infections and disease. Most symptomatic patients exhibit mild illness with fever, sometimes associated with headache, myalgias, nausea and vomiting, and chills.[34,46–49] Further, some patients briefly present with papular rash on the arms, legs, or trunk. These symptoms follow a fairly predictable pattern, with illness generally lasting less than 7 days. However, several patients experience severe fatigue and malaise during convalescence.

Approximately 5% of patients with symptomatic WNV infection develop neurologic disease. WNV neurologic symptoms include meningitis, encephalitis, and poliomyelitislike disease, presented as acute flaccid paralysis.[50] WNV encephalitis and meningitis are characterized by rapid onset of headache, photophobia, back pain, confusion, and continuous fever. The WNV poliomyelitislike syndrome is characterized by acute onset of asymmetric weakness and absent reflexes without pain. Patients presenting with flaccid paralysis require further testing to determine nature and degree of disease. Diagnostic tests, including cerebrospinal fluid (CSF) examination, should be performed to differentiate WNV infection from stroke, myopathy, and Guillan-Barré syndrome. Other clinical symptoms may include tremor, myoclonus, postural instability, bradykinesia, and signs of parkinsonism.

PATHOGENESIS

Understanding the full range of WNV pathogenesis in humans has been difficult, mainly because of the difference in virulence between WNV strains, the high prevalence of asymptomatic or subclinical infections, and the relative infrequency of laboratory-confirmed human infections. Little has been published about human infections with WNV of limited virulence. Most of our current knowledge regarding WNV pathogenesis resulted from animal models (mostly rodent) infected under controlled conditions with a known amount of needle-inoculated virus, which may not accurately reflect the course of a natural infection in humans. Nevertheless, many documented

Table 1
Summary of confirmed human cases of WN disease in the United States, 1999–2008[a]

Year	No. States Reporting[b]	Total Cases	Deaths	CFR[c]	Neurologic Involvement[d]	WN Fever[e]	Other Symptoms
1999	1	62	7	11.29%	59	3	0
2000	3	21	2	9.52%	19	2	0
2001	10	66	10	15.15%	64	2	0
2002	39 + DC	4156	284	6.83%	2946	1160	50
2003	45 + DC	9862	264	2.68%	2866	6830	166
2004	40 + DC	2539	100	3.94%	1142	1269	128
2005	43 + DC	3000	119	3.97%	1294	1607	99
2006	43 + DC	4269	177	4.15%	1459	2616	194
2007	43	3630	124	3.42%	1217	2350	63
2008	45 + DC	1356	44	3.24%	687	624	45

Abbreviations: CDC, Centers for Disease Control and Prevention; CFR, case fatality rate; DC, District of Columbia.

[a] Data obtained from the CDC, accessed on May 13, 2009.

[b] The number of states reporting CDC-confirmed cases of WNV infections in humans.

[c] CFR determined as percentage of deaths from total CDC-confirmed reported cases.

[d] Neurologic involvement is comprised of encephalitis, meningitis.

[e] WN fever; febrile illness with no neurologic involvement.

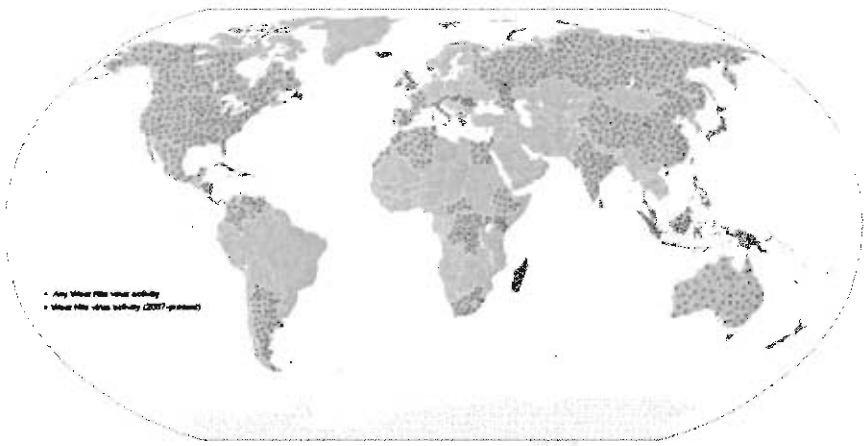

Fig. 5. Distribution of WNV. Countries with historic or recent (2007 to present) WNV activity (isolations from mosquitoes, birds, horses, or humans) are highlighted in red and blue, respectively.

accounts follow the course of infection in humans suffering from WN fever and WN encephalitis resulting from a virulent lineage I WNV infection.

WNV-infected mosquitoes transmit the virus to humans following a bloodmeal from the host. During this process, mosquito saliva contaminated with WNV is deposited in the blood and skin tissue. Virus contained within the skin is presumed to infect resident dendritic cells, such as Langerhans cells (MHCII+/NLDC145+/E-cadherin+ cells), which then traffic to the draining lymph node.[51,52] Shortly thereafter, virus amplifies in the tissues and results in a transient, low-level viremia lasting a few days, and it typically wanes with the production of anti-WNV IgM antibodies.[53] Following viremia, the virus infects multiple organs in the body of the host, including the spleen, liver, and kidneys. Eight days after onset of symptoms, WNV was detected in the urine (viruria)

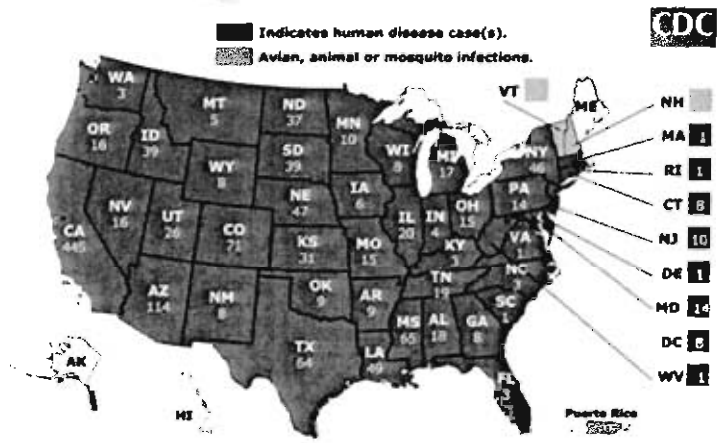

Fig. 6. The number of confirmed human cases of WNV disease in the United States in 2008. (*Courtesy of* CDC.)

of a patient with encephalitis,[54] which is consistent with animal (hamster) experiments demonstrating viruria[54] and the presence of viral infection in the kidneys.[55,56]

On entering the central nervous system (CNS), WNV causes severe neurological disease. WNV may enter the brain through a combination of mechanisms that facilitates viral neuroinvasion, such as direct infection with or without a breakdown of the blood-brain barrier (BBB) or virus transport along peripheral neurons. High viremia may easily lead to an infection of the brain if the BBB is disrupted, and it is correlated with severity of infection in experimentally infected mice.[57] Viremia and high viral titers in the periphery alone do not predict neuroinvasion. Host proteins, such as death-associated protein kinase-related 2 (Drak2), intercellular adhesion molecule 1 (ICAM-1), macrophage migration inhibitory factor (MIF), and matrix metalloproteinase 9 (MMP-9), have all been implicated in altering BBB permeability during WNV infection.[58–61] The virus may pass into the CNS without disrupting the BBB.[62] The host's response to infection may also contribute to WNV pathogenesis. Studies from experimentally infected mice suggest that the innate immune sensing molecule toll-like receptor 3 (TLR3) may play a role in WNV invasion of CNS,[63] possibly by mediating the upregulation of tumor necrosis factor (TNF)-α, thereby resulting in capillary leakage and increased BBB permeability.[64] The proinflammatory chemokines/cytokines, monocyte chemoattractant protein 5 (MCP-5), MIF, IFN-γ–inducible protein 10 (IP-10), monokine induced by IFN-γ (MIG), IFN-γ, and TNF-α, were all upregulated in the brains of experimentally infected mice, suggesting that the host immune response may be at least partially responsible for neurologic symptoms of the disease.[58,65] However, an increase in BBB leakage does not accurately predict WNV-induced mortality in hamsters, nor does lethal infection increase BBB permeability in all strains of mice.[66] WNV may enter the brain by direct infection and retrograde spreading along neurons in the periphery.[67] Entering the brain via infected peripheral neurons is a likely route, because the level of viremia is low and leakage into the CNS by a breakdown of the BBB is less likely compared with animals with a high titer of circulating WNV in the blood. The discrepancies observed regarding BBB compromise suggest that further research is required to determine the exact mechanism through which WNV enters the CNS.

DIAGNOSIS

Diagnosis of WNV infection depends on several factors, including environmental conditions, behaviors, and clinical symptoms. Patient history provides crucial clues to diagnosis. For example, if a patient presents with clinical symptoms, including fever and headache, one must consider the distribution of WNV and its mosquito vector. WNV infection must be considered in endemic areas, especially during the summer months. Furthermore, the patient history should suggest exposure to mosquitoes through outdoor activities. An initial physical examination should confirm clinical symptoms of fever, headache, myalgia, or the more severe meningitis and flaccid paralysis. The presence of mosquito bites on the skin also helps diagnosis.

To confirm the initial diagnosis, specific laboratory tests must be ordered (**Table 2**). To date, the most consistent way to verify WNV infection is serology.[47,49] WNV antigen-specific enzyme-linked immunosorbent assay (ELISA) confirms infection. Serological tests include acute or convalescent samples of serum or CSF to determine the WNV-specific antibody profile by ELISA. The best test involves IgM-specific ELISA (MAC-ELISA) in which serum is collected within 8 to 21 days after the appearance of clinical symptoms. This test is commercially available and relatively inexpensive.[34] Also, serology can be performed to analyze immune responses. The presence of

Table 2
Laboratory tests and diagnosis of WNV infection

Test	Positive Results
CBC	Anemia, lymphopenia, thrombocytopenia
IgM-specific ELISA	WNV-specific IgM antibodies detected
PRNT	Known virus stock growth inhibited in tissue culture by serum, indicating neutralizing antibodies
NAT	PCR amplification shows presence of WNV genome RNA
Virus isolation/plaque assay	Serum or CSF contain virus as seen in plaque assay
CSF analysis	Antibodies and/or virus present in ELISA or plaque assay, Elevated protein and increased polymorphonuclear cells, negative gram stain
EMG/NCS	Severe effects on anterior horn cells

Abbreviations: CBC, complete blood count; ELISA, enzyme-linked immunosorbent assay; EMG, electromyogram; NAT, nucleic acid testing; NCS, nerve conduction studies; PCR, polymerase chain reaction; PRNT, plaque reduction neutralization test.

reactive lymphocytes or monocytes in CSF samples indicates WNV neurologic infection. More dramatically, a massive influx of polymorphonuclear cells occurs. In patients with WNV neuroinvasion, more than 40% of cells in the CSF are neutrophils.[68] Plaque reduction and neutralization tests allow for identification of virus specificity. A virology test can directly confirm the presence of virus. Serum or CSF is collected, and virus is amplified within permissive cells and sequenced. This test is time-consuming and expensive. Finally, molecular biological tools can be used to confirm the presence of virus. The nucleic acid test is a powerful tool for detecting WNV genomes. Serum or CSF collected during the initial phases of virus infection can be directly amplified or used to detect viral RNA by quantitative reverse transcription polymerase chain reaction with virus-specific primers.

Magnetic resonance imaging suggests abnormalities in the brain and meninges of WNV-infected patients presenting with CNS disease (**Fig. 7**).[46,69,70] The regions of the CNS most commonly affected are basal ganglia, thalami, brain stem, ventral horns, and spinal cord. However, most of these studies were performed retrospectively. Thus, the results do not provide predictive capabilities to WNV infection.

DIFFERENTIAL DIAGNOSIS

Several diseases manifest as symptoms similar to WNV, including bacterial meningitis and those caused by the encephalitides viruses, such as the Japanese and Murray Valley encephalitis virus. Therefore, differential diagnosis is crucial to determining WNV infection. A differential diagnosis is required when a patient presents with unexplained febrile illness, encephalitis or extreme headache, or meningitis. Thus far, the only manner to differentiate between causes of encephalitis/meningitis is diagnostic and serological laboratory tests to identify the specific pathogen causing the symptoms.

TREATMENT AND LONG-TERM OUTCOMES

Currently, patients infected with WNV have limited treatment options. The primary course of action is supportive care. There is no FDA-licensed vaccine to combat

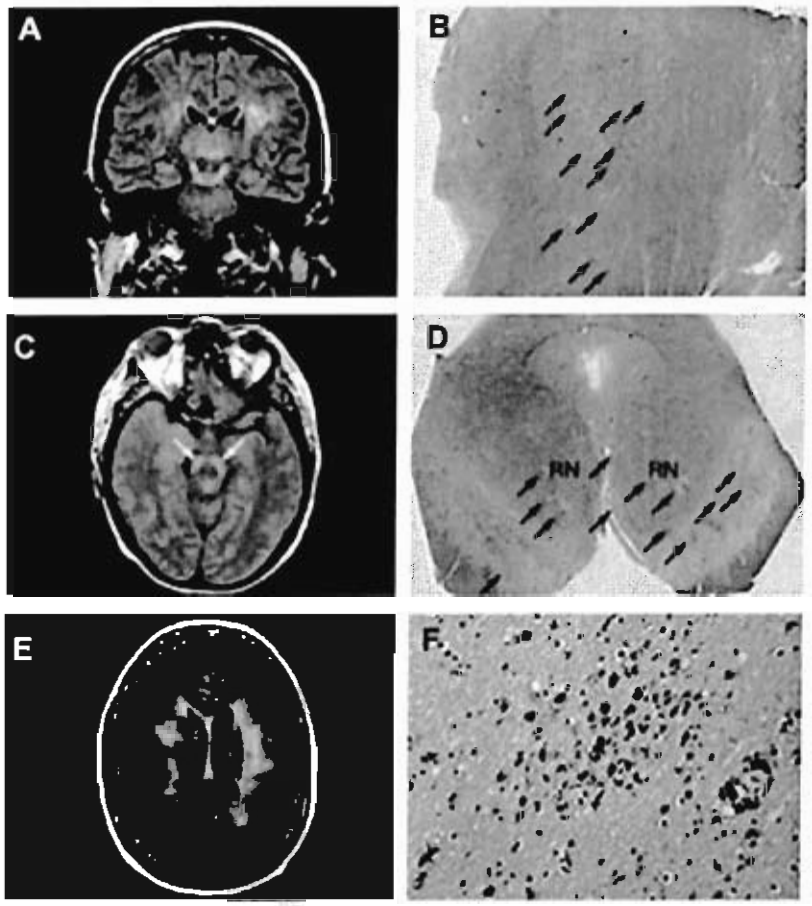

Fig. 7. Radiographic and neuropathologic findings in WNV encephalitis. (*A*) Coronal fluid-attenuated inversion recovery (FLAIR) magnetic resonance image shows an area of abnormally increased signal in the thalami, substantia nigra (extending superiorly toward the subthalamic nuclei), and white matter. (*B*) Corresponding tissue section from the same patient at autopsy 15 days later stained with Luxol fast blue-periodic acid Schiff for myelin shows numerous ovoid foci of necrosis and pallor throughout the thalamus and subthalamic nucleus (*arrows*). (*C*) Axial proton density image at the level of the midbrain shows a bilaterally increased signal in the substantia nigra (*arrows*). (*D*) Corresponding tissue section at autopsy stained with Luxol fast blue-periodic acid Schiff illustrates multifocal involvement of the substantia nigra (*arrows*), with nearly 50% of the area destroyed; the red nuclei are clearly affected. (*E*) Axial FLAIR image at the level of the lateral ventricle bodies shows a bilaterally increased signal within the white matter. A scan performed approximately 5 months earlier demonstrated an abnormal signal in the left periventricular white matter. This signal increased once WNV encephalitis developed, and the lesions in the right cerebral white matter (left side of photograph) were new. (*F*) Photomicrograph taken from the right periventricular white matter immunostained with the HAM56 antibody shows numerous macrophages in perivascular areas (*lower right*) and diffusely throughout the white matter (*center*). (*From* Kleinschmidt-DeMaster BK, Marder BA, Levi ME, et al. Naturally acquired West Nile virus encephalomyelitis in transplant recipients: clinical, laboratory, diagnostic, and neuropathological features. Arch Neurol 2004;61:1210–20; with permission.)

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WN disease in humans, despite the research of many laboratories and institutions and the vaccines available for use in horses.

Furthermore, there is no effective antiviral to combat WNV infection. Two classical antiviral compounds, IFN and ribavirin, showed promising results in vitro,[71,72] but it is unclear if these compounds are effective in patients.[73–77] Passively transferring anti-WNV immunoglobulin has been shown to be effective in mouse and hamster models[78] and may be helpful in patients.[79,80]

Long-term complications (1 year or more postinfection) are common in patients recovering from WNV infection. The most common self-reported symptom is fatigue and weakness, although myalgia, arthralgia, headaches, and neurologic complications, such as altered mental depression, tremors, and loss of memory and concentration, are not uncommon.[81] There is also evidence from animal models[55,82,83] and human autopsies[84,85] that the virus may persist in some individuals, as measured by isolation of virus or viral genomes or antigen months after infection or symptom presentation. Experimentally infected hamsters show long-term neurological sequelae, which seems to coincide with the presence of viral antigen and genome within areas of the brain showing neuropathology.[83] Although the direct evidence of persistence in humans is limited at this time, many patients have long-lasting WNV-specific IgM titers in the serum and CNS, suggesting that persistent infections may be more common than previously indicated.[86–88]

Table 3
WNV vaccines. A partial list of licensed and preclinical vaccines against WNV

Type	Antigen	Sponsor	Stage of Development
Chimeric (vector)			
Recombitek (canarypox)	WNV-prM-E	Merial	Licensed for horses
ChimeriVax (yellow fever virus)	WNV-prM-E	Acambis	Phase II
WNV-DENV4 (dengue virus 4)	WNV-prM-E	NIAID/NIH	Phase II
DNA			
WNV-DIII	WNV-DIII	Multiple laboratories	Preclinical
WNV-E	WNV-E	Multiple laboratories	Preclinical
WNV-prM-E	WNV-prM-E	Multiple laboratories	Preclinical
Inactivated/killed			
Innovator	Whole virus	Fort Dodge Animal Health	Licensed for horses
Subvirion particles/viruslike particles			
WNV-prM-E	WNV-prM-E	Multiple laboratories	Preclinical

Abbreviations: NIAID, National Institute of Allergy and Infectious Diseases; NIH, National Institutes of Health.

Data from http://www.fortdodgelivestock.com, http://www.merial.com, http://www.intervetusa.com, http://www.clinicaltrials.gov.

IMMUNITY

The innate and adaptive immune responses mounted against WNV are critically important for controlling infection. Type I IFNs (-α and -β) are important for limiting virus levels, reducing neuronal death, and increasing survival.[57] The amount of IFN made by the host in response to infection seems to be at least partly dependent on the strain or virulence of the virus; mice infected with lineage I WNV with attenuating mutations produce less type I IFN than mice infected with virulent lineage I WNV.[89] Furthermore, WNV strains that are more resistant to the affects of IFN (like some virulent lineage I viruses) are more virulent than IFN-sensitive strains (like lineage II strains).[90]

The adaptive immune response also plays a role in controlling infection. Studies using WNV-infected genetically engineered knockout mice indicate that T-[91–96]and B-[97]cells are critical for controlling infection. CD8+ T-cell recruitment to the brain by neurons expressing the chemokine CXCL10 and by CD40-CD40 ligand interactions help reduce the viral burden in the brain and increase survival in experimentally infected mice.[94,98] B cells are activated within the lymph nodes of WNV-infected mice 48 to 72 hours after infection in an IFN-α/-β–signaling dependent manner, and B cells secreting WNV-specific IgM were detected on day 7 postinfection.[99] IgM is critically important for the control of early WNV infection, and passive transfer of WNV-specific IgM could protect IgM-deficient mice from lethal WNV infection.[100] Approximately 3 to 4 days after WNV-specific IgM is detectable, anti-WNV IgG titers are measurable in patients.[53] IgG is the predominant antibody, most probably conferring long-term immunity against WNV re-infection. Although not enough data exist, immunity against WNV in convalescent patients is presumed to be lifelong.

VACCINATION

Although no FDA-approved vaccine exists for human use, there are effective, licensed vaccines for the treatment of horses. This success has encouraged others to develop these and other strategies for human vaccines. Currently, there are several ongoing clinical trials.

There are several strategies being pursued for WNV vaccine development (**Table 3**). The first strategy is inoculation of multiple doses of inactivated virus.[101,102] Fort Dodge Animal Health developed this strategy by formalin-inactivating whole virus. This formulation has been approved for horses. The second strategy involves the production of WNV antigens from a heterologous virus backbone. The vectors being used are canarypox (Recombitek), yellow fever virus (ChimeriVax), and dengue 4 (WNV-DEN4).[103–106] The Recombitek vaccine has been licensed for use in horses. The third approach is DNA vaccination. WNV structural antigens (prM-E) are expressed from DNA plasmids.[107] The final strategy is inoculation with purified viral proteins.[108–111] These proteins can be produced in mammalian cell culture, bacteria, or yeast. A recent study by Seino and colleagues[112] compared the efficacy of 3 available vaccines. Their study showed that horses vaccinated with the live, chimeric virus in the yellow fever or canarypox vectors had fewer clinical signs of WNV disease than animals receiving inactivated virus.

SUMMARY

In summary, WNV infection is a serious threat to public health, especially to the immunocompromised and the elderly. The virus is maintained in an enzootic cycle between mosquitoes and birds, with humans and other mammals as incidental hosts. Since its

introduction to the Western hemisphere in 1999, WNV has spread across North and South America in fewer than 10 years. Most human infections are asymptomatic. However, clinical manifestations range from fairly mild febrile illness to very severe neurological sequelae, including acute flaccid paralysis and encephalitis. Currently, the virus is the most significant cause of viral encephalitis in the United States. Efficient diagnosis of WNV infection requires a detailed history, including potential exposure to contaminated mosquitoes, and sensitive serological and virology assays. Recent studies have explained virus-host interactions, including pathogenesis and immune evasion. Lastly, there are no prophylactic or therapeutic measures that exist to combat the diseases caused by WNV infection, which warrants future research.

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