

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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Solicitud de Patente de Invención titulada: "Vacunas de ácido nucleico para
prevención de la infección de flavivirus"Solicitante: The Government of the United States of America as represented by the
Secretary of the Department of Health and Human Services, Centers for
disease control and prevention**RESPUESTA AL AUTO NOTIFICADO POR FIJACIÓN EN LISTA NO. 332****DEL 24 DE SEPTIEMBRE DE 2010, OFICIO NO. 12569 (EXAMEN DE PATENTABILIDAD)**

ÁLVARO CORREA ORDÓÑEZ, mayor de edad y vecino de Bogotá D.C., identificado tal como aparece al pie de mi firma, en mi carácter de apoderado de la sociedad en referencia, doy respuesta al auto emanado por ese Despacho dentro del término de legal:

1. Objeciones del Examinador**1.1 Reivindicaciones:**

El Examinador menciona que la reivindicación 2, que es dependiente de la 1, menciona ácidos nucleicos que comprenden una longitud mayor a la caracterizada en la reivindicación 1 puesto que ahora se caracteriza la longitud total de las secuencias, lo cual hace que esta reivindicación dependiente no especifique o limite la invención sino que por el contrario la amplíe.

Con relación a la reivindicación 7, su Despacho menciona que dicha reivindicación no aporta características técnicas de la invención, sino que trata es de un uso que se le puede dar a la misma.

Por último, con relación a la reivindicación 8, su Despacho establece que no está definido por sus características técnicas, esto es por su estructura, secuencias CDRs, lo cual no permite limitarlo. Adicionalmente, la reclamación propuesta implica un proceso biológico natural.

1.2 Excepciones a la patentabilidad

Su Despacho asevera que las reivindicaciones 5 y 6 tratan de un método de tratamiento aplicado a animales o humanos y que según la norma comunitaria los métodos de diagnóstico y tratamiento están excluidos de patentabilidad, la materia reclamada no podrá ser patentada.

1.3 Análisis de patentabilidad

Su Despacho establece que los documentos **D1** (Chang, et.al, 2000. Journal of Virology, vol 74, No.9:4244-4252), y **D2** (WO 98/37911) y **D3** (WO 99/63095) afectan el requisito de nivel inventivo de la presente invención debido principalmente a lo siguiente:

*“El documento **D3** divulga moléculas de ácido nucleico que contienen unidades de transcripción las cuales codifican antígenos de proteínas M y E de Flavivirus. Entre los flavivirus pueden estar el virus de la encefalitis Japonesa, dengue, fiebre amarilla y virus de encefalitis de St. Louis.*

*Las diferencias entre **D3** y la solicitud son dos:*

- 1. Los posibles flavivirus que pueden hacer parte del antígeno que compone la molécula de DNA, puesto que **D3** no menciona los flavivirus: virus Powassan y virus West Nile.*

Sin embargo, dada la indicación en la solicitud que el segundo flavivirus puede ser seleccionado de un grupo determinado de Flavivirus, es decir, que no siempre contiene el virus Powassan o el virus del Oeste del Nilo, entonces esta diferencia no siempre está presente.

2. *La secuencia señal utilizada, puesto que en D3 se emplea la secuencia señal es el promotor temprano del citomegalovirus, mientras que en la solicitud se emplea la secuencia señal de una proteína estructural del virus de la encefalitis Japonesa.*

Sin embargo, esta diferencia resulta derivada del conocimiento que tenía el experto en la materia a partir del estado del arte. D1 ya había realizado preparaciones de vacuna en las cuales se utilice segmentos de DNA derivado del virus de la encefalitis japonesa.

Por lo tanto, un experto en la materia podría haber desarrollado la invención tal como es descrita en la solicitud, a partir de las enseñanzas de D1 y D3, puesto que la combinación de los dos tipos de vacunas allí divulgadas da lugar a una construcción como la de la solicitud.

Adicionalmente, en el estado del arte existían otras divulgaciones que le habrían servido al experto en la materia para haber desarrollado la invención tal como es planteada, este es el caso de D2, dicho documento divulga vacunas quiméricas de flavivirus, es decir, producidas a partir de segmentos de varios flavivirus.

La diferencia entre D2 y la solicitud en estudio es que esta última tiene una secuencia señal derivada del virus de la encefalitis japonesa y no del virus de fiebre amarilla. Sin embargo, D1 ya había realizado preparaciones de vacuna en las cuales se utilice segmentos de DNA derivado del virus de la encefalitis japonesa.

En dicho documento se compara las secuencias entre el plásmido fabricado a partir de los genes M y E de este virus contra los mismos segmentos de otros flavivirus, y sugiere la mayor efectividad del virus JEV en lograr títulos anticuerpos inmunizantes.

Concluye su Despacho que la materia reivindicada no puede considerarse inventiva frente a las divulgaciones de D1 y D3 o D1 y D2.

2. Argumentos a favor de la patentabilidad de la solicitud

Con el objeto de atender a las observaciones del examinador y de conformidad con el artículo 34 de la Decisión 486 que permite al solicitante en cualquier momento del trámite efectuar modificaciones a la solicitud sin que se amplíe la invención originalmente presentada, me permito presentar un nuevo capítulo reivindicatorio conformado por 4 reivindicaciones.

En particular, se cancelaron las reivindicaciones 5 a 8 y se modificó la reivindicación 1 en la cual se especifica que el antígeno quimérico comprende la secuencia del virus de encefalitis japonesa y un segundo flavivirus.

Con relación a la observación de su Despacho en cuanto a que la reivindicación 2, que es dependiente de la 1, menciona ácidos nucleicos que comprenden una longitud mayor a la caracterizada en la reivindicación 1 puesto que ahora se caracteriza la longitud total de las secuencias, lo cual hace que esta reivindicación dependiente no especifique o limite la invención sino que por el contrario la amplíe, mi representada aclara que la reivindicación 1 es más amplia que la reivindicación 2 ya que la reivindicación 1 requiere solamente que el constructo de ácido nucleico incluya porciones de las secuencias mencionadas (específicamente las regiones codificadoras del constructo).

Por el contrario, la reivindicación 2, requiere que la molécula del ácido nucleico específicamente incluya la secuencia completa de nucleótidos de la indicada SEQ ID NOs.

Sin embargo, para superar la anterior observación, se presenta la reivindicación 2 como una reivindicación independiente.

2.1 Nivel inventivo

El nivel inventivo de una solicitud se encuentra definido en el Artículo 18 de la Decisión 486 y se establece cuando *“si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica”*.

De conformidad con el anterior artículo, mi representada considera respetuosamente que los documentos citados por su Despacho **D1** (Chang, et.al, 2000. Journal of Virology, vol 74, No.9:4244-4252), y **D2** (US 6,696,281) no afectan el nivel inventivo de la solicitud en estudio por las siguientes razones:

Se hace notar a su Despacho que las reivindicaciones 5 a 8 se cancelaron del pliego reivindicatorio.

Por otro lado, la reivindicación 1 se modificó de tal forma que se especifica que el antígeno quimérico comprende la secuencia del virus de encefalitis Japonesa y de un segundo

flavivirus.

2.1.1 Con relación a la combinación de D1 y D3

Respetuosamente mi representada considera que su Despacho está confundido acerca de las enseñanzas de los documentos D1 y D3. Ambos documentos describen el trabajo del inventor y revelan los constructos idénticos del ácido nucleico del virus de encefalitis Japonesa (JEV)-Pedje2-7, pCEJE, pREJE y pRCJE (Por favor, ver el ejemplo 2 del documento D3 y la página 4246, columna 2 de D1). Todos los constructos del ácido nucleico del JEV incluyen una secuencia señal del JEV (desde la proteína prM) y codifica las proteínas M y E del JEV (Sin embargo, los constructos varían en el tipo de promotor y en la señal polyA).

Aunque, D3 también describe la construcción de vectores similares del ácido nucleico para otros flavivirus, incluyendo el virus de fiebre amarilla, el virus de encefalitis St Louis y el virus del dengue, ninguno de los documentos D1 o D3 enseñan o sugieren combinar una secuencia señal del JEV con un antígeno desde un segundo flavivirus o un antígeno quimérico que tenga la secuencia de aminoácidos del JEV y de un segundo flavivirus.

Las enseñanzas de los documentos D1 y D3 se limitan a los constructos del ácido nucleico que tienen secuencia de un único flavivirus.

Por el contrario, las reivindicaciones modificadas que ahora se presentan como respuesta a este examen de patentabilidad requieren que el ácido nucleico codifique una secuencia señal y un antígeno desde un segundo flavivirus (que no es JEV) o un antígeno quimérico que contiene la secuencia del JEV y un segundo flavivirus.

Adicionalmente, la reivindicación 1 especifica que la secuencia señal es una secuencia señal del JEV modificada con ingeniería de la SEQ ID NO: 14 o SEQ ID NO:27, secuencias de las cuales no se enseñan ni se sugieren en ninguno de los documentos D1 y D3.

Por lo tanto, a partir de las enseñanzas de D1 y D3 no hubiera sido obvio combinar una secuencia señal de un primer flavivirus (tal como el JEV) con un antígeno de un segundo flavivirus o un antígeno quimérico con una secuencia de dos diferentes flavivirus. En particular, no habría sido obvio que tal combinación resultara en un constructo de ácido nucleico que una vez se introduce en la célula, fuera capaz de expresar un antígeno

inmunogenico de flavivirus (tal como partículas subvirales de flavivirus) tal como el inventor lo demostrado en la presente solicitud de patente.

Adicionalmente, no habría sido obvio o predecible que el constructo de ácido nucleico que codifica una secuencia señal del JEV en combinación con un antígeno de un segundo flavivirus, o en combinación con un antígeno de flavivirus quimérico, sería capaz de expresar el antígeno de flavivirus en cantidades suficientes para permitir el uso del antígeno de flavivirus en ensayos de diagnóstico de flavivirus, o permitir el uso del ácido nucleico como una vacuna del DNA del flavivirus.

2.1.2 Con relación a la combinación de D1 y D2

Como se mencionó anteriormente, **D1** no enseña ni tampoco sugiere combinar una secuencia señal del JEV, particularmente una secuencia señal del JEV manipulada/modificada de SEQ ID NO: 14 o SEQ ID NO: 27, con un antígeno de un segundo flavivirus o un antígeno quimérico con la secuencia de JEV y un segundo flavivirus. De igual forma, **D2** no enseña ni tampoco sugiere una secuencia señal del JEV en combinación con un antígeno de un segundo flavivirus, o en combinación con un antígeno quimérico que tiene secuencias de aminoácidos del JEV y un segundo flavivirus.

Más bien lo que enseña **D2** es el virus de fiebre amarilla en el cual la secuencia nucleotida para prM/E ha sido retirada o truncada tal que se produce la proteína prM/E no funcional del virus de la fiebre amarilla. La secuencia prM/E del virus de la fiebre amarilla se reemplaza con una secuencia de nucleótidos que codifica la proteína prM/E de un segundo flavivirus.

A partir de las enseñanzas de **D3** no se tiene claridad si la secuencia señal prM del virus quimérico se deriva del virus de fiebre amarilla, o si la secuencia prM/E del segundo flavivirus incluye la secuencia señal prM. Sin embargo, ninguna posibilidad conduce a la enseñanza de una *secuencia señal del JEV* en combinación con un antígeno a partir de un segundo flavivirus, o en combinación con un antígeno quimérico que comprende la secuencia del JEV y un segundo flavivirus tal como se reivindica en la presente invención.

3. Argumentos adicionales a favor del nivel inventivo:

Se hace notar a su Despacho que el inventor de la presente solicitud ha publicado un

número de manuscritos que claramente demuestran las propiedades superiores de los constructos del ácido nucleico del flavivirus reclamado.

Adicionalmente, la solicitud por sí misma describe propiedades inmunogénicas superiores de las vacunas del flavivirus reclamadas.

Por lo tanto, mi representada desea presentar los siguientes argumentos y copias de publicaciones del inventor así como otros documentos que se relacionan a continuación. Las copias adjuntas se presentan como evidencia del nivel inventivo de las composiciones reclamadas.

Como se describe en el ejemplo 11 de la presente solicitud, una inyección intramuscular única del constructo pCBWN del virus West Nile (que codifica la secuencia señal prM del JEV y las proteínas M y E del virus West Nile) provocó anticuerpos neutralizantes en caballos y suministró una completa protección contra la inoculación del virus del tipo silvestre. Por el contrario, todos los caballos sin vacunar se infectan con el virus West Nile tipo silvestre, y 7 de los 8 animales sin vacunar desarrollan viremia. Este trabajo se describe además en Davis et al (J. Virol 75:4040-4047, 2001).

El éxito de los métodos reivindicados se evidencia además por el departamento de agricultura de los Estados Unidos de América (USDA) aprobado (Julio, 2005) del pCBWN como una vacuna veterinaria para la prevención de la infección del virus West Nile en caballos. La licencia USDA del pCBWN es la primera licencia otorgada para una vacuna DNA para uso en animales. Adjunto se encuentra una copia de la gaceta de prensa de la USDA que reporta el otorgamiento de dicha licencia. Esta vacuna también muestra que protege cóndores de California en peligro de extinción por la infección del virus del West Nile (Chang et al., Vaccine 25:2325-2330, 2007). La vacuna contra el virus de West Nile provocó inmunidad protectora no solamente en pájaros adultos, sino también en polluelos y en pollitos recién eclosionados.

Adicionalmente, los cóndores cautivos de California fueron protegidos contra el virus West Nile que circula naturalmente durante el periodo de estudio. Por favor, note que la vacunación de los cóndores de California, pCBWN se modificó para reemplazar el gen resistente a ampicilina con un gen resistente a kanamicina. (Por favor, ver páginas 2326 de Chang et al. 2007) pero es por lo demás idéntico al constructo revelado en la presente invención.

Además, Martin et al. (J. Infect. Dis. 196:1732-1740, 2007) describe un ensayo clínico para humano en fase I de la vacuna DNA contra el virus West Nile que es análoga al pCBWN. Como el pCBWN, la vacuna humana (VRC-WNVDNA017-00VP) contiene una secuencia de señal modificada del JEV (SEQ ID NO:14) y el antígeno prM/E del virus West Nile (Por favor, ver la columna izquierda, página 1734 de Martin et al.). La vacuna humana fue segura y bien tolerada, y provocó células T y respuestas del anticuerpo en sujetos vacunados. En gran medida, la vacuna humana provocó anticuerpos neutralizantes en todos los sujetos que recibieron un régimen de 3 dosis, a los niveles mostrados para ser protegido contra la infección del virus de West Nile tipo-silvestre en caballos (ver resumen y figura 2).

Con relación al virus de encefalitis St. Louis, el ejemplo 12 enseña que la dosis única del plásmido pCDSLE4- 3, que contiene una unidad transcripcional codifica la secuencia prM del JEV y una proteína M y una proteína E del virus de encefalitis St. Louis, resulta en seroconversión del 100% de los animales inoculados. Adicionalmente, una publicación de fecha de prioridad posterior del inventor y sus colegas (Purdy et al., J. Clin. Microbiol. 42 (10):4709-4717,2004) describe el constructo de flavivirus (pCB8SJ2) que incluye la secuencia señal del JEV, de la proteína prM del virus de encefalitis St. Louis (SLEV) y una proteína E quimérica con 80% de la secuencia del aminoácido SLEV y un 20% de la secuencia de aminoácido JEV (ver figura 1 la secuencia de señal JEV se abrevia "JESS". La expresión transitoria de pCBSJ2 resulta en "una secreción mejorada dramáticamente de la SLEV VLPs" comparada con pCDSLE4MU4, un plásmido codifica la longitud completa del prM SLEV y de las proteínas E (ver página 4712). La expresión eficiente del flavivirus VLPs es importante para la producción de un antígeno recombinante para ensayos de diagnostico del flavivirus.

Adicionalmente, la capacidad de un constructo DNA del flavivirus para producir eficientemente VLP es crítica cuando se utiliza como una vacuna DNA.

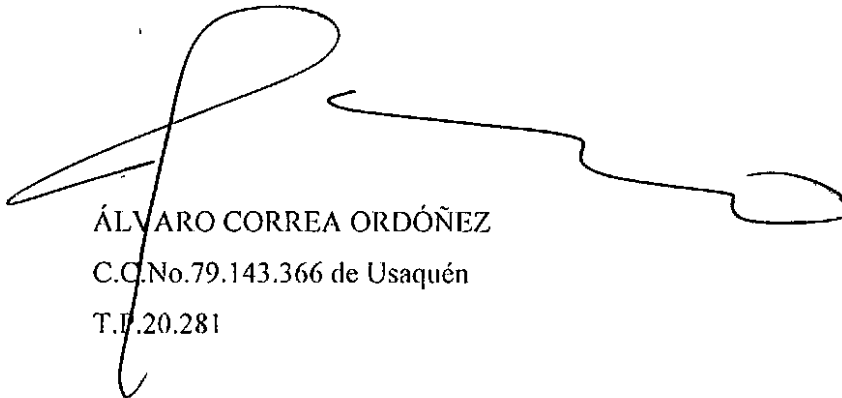
Además, el inventor ha demostrado que una unidad transcripcional que codifica una secuencia señal del JEV prM, la proteína M del virus del dengue y una proteína E quimérica que comprende una secuencia de aminoácidos del virus del dengue y JEV que es capaz de provocar inmunidad protectora contra la infección del virus del dengue en ratones vacunados. Estos estudios se describen en el ejemplo 20 de la actual solicitud y se describe además en una publicación de Chang et al (Virology 306:170-180, 2003). Los estudios hechos por Chang et al, demuestran que el constructo de la vacuna del virus del dengue (pCB8D2-2J-2-9-1)

provoca anticuerpos neutralizantes en ratones y confiere protección pasiva para cachorros lactantes de hembras vacunadas cuando se inoculan con el virus de dengue tipo silvestre.

De conformidad con lo anteriormente expuesto, existe más que una amplia evidencia para demostrar que las moléculas del ácido nucleico del flavivirus reivindicado muestran ventajas significantes con respecto al arte previo y por lo tanto, la presente invención es inventiva.

En virtud de lo anterior, solicito respetuosamente que se estudie el nuevo capítulo reivindicatorio y se acepten los argumentos arriba expuestos y en consecuencia, se otorgue el privilegio de patente de invención para el invento que se tramita en este expediente.

Atentamente,



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Anexos: Capítulo reivindicatorio modificado
 Publicaciones (6) que sustentan el nivel inventivo.

REIVINDICACIONES

1. Un ácido nucleico aislado que comprende una unidad transcripcional que codifica una secuencia de señal de una proteína estructural del virus de la encefalitis Japonesa y un antígeno flavivirus inmunogénico, en donde el antígeno es de un segundo flavivirus seleccionado del grupo que consiste de virus de fiebre amarilla, virus dengue serotipo 1, virus dengue serotipo 2, virus dengue serotipo 3, virus dengue serotipo 4, virus Powassan, virus de encefalitis San Louis y virus West Nile, o el antígeno es un antígeno quimérico que comprende la secuencia de aminoácido del virus de encefalitis Japonesa y un segundo flavivirus seleccionado del grupo que consiste de virus de fiebre amarilla, virus dengue serotipo 1, virus dengue serotipo 2, virus dengue serotipo 3, virus dengue serotipo 4, virus Powassan, virus de encefalitis San Louis y virus West Nile, en donde la unidad transcripcional dirige la síntesis del antígeno, en donde el ácido nucleico aislado es ADN, la secuencia de señal es una secuencia de señal de virus de encefalitis Japonesa construido mediante ingeniería que tiene la secuencia de la SEQ ID No: 14 o SEQ ID No:27, y el antígeno se selecciona del grupo que consiste de una proteína M, una proteína E, tanto una proteína M como una proteína E, una porción de una proteína M, una porción de una proteína E, y tanto una porción de una proteína M como una porción de una proteína E, o cualquier combinación de estos, que comprende una secuencia de

- nucleótido seleccionada del grupo que consiste de las posiciones 911-2987 de la SEQ ID No: 15, posiciones 910-2965 de la SEQ ID No:19, posiciones 910-2986 de la SEQ ID No: 21, posiciones 910-2953 de la SEQ ID No: 23, posiciones 910-2964 de la SEQ ID No: 44 y las posiciones 910-2964 de la SEQ ID No: 46.
2. El ácido nucleico que comprende una secuencia de nucleótido seleccionada del grupo que consiste de la SEQ ID No: 15, SEQ ID No: 19, SEQ ID NO: 21, SEQ ID No: 23, SEQ ID NO:44, y SEQ ID NO: 46.
 3. Una célula que comprende el ácido nucleico de la reivindicación 1 y que expresa y que secreta el antígeno flavivirus inmunogénico de la reivindicación 1.
 4. Una composición para la prevención o tratamiento de infección mediante un flavivirus que comprende el ácido nucleico de la reivindicación 1 y un portador farmacéuticamente aceptable para inmunizar un sujeto contra la infección mediante un flavivirus.

Prospective immunization of the endangered California condors (*Gymnogyps californianus*) protects this species from lethal West Nile virus infection

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Abstract

West Nile virus (WNV) has caused significant morbidity and mortality in humans, mammals, and both native and exotic birds in North America since its emergence in New York City in 1999 and its subsequent spread westward. Prior to the arrival of WNV to the western United States, prospective vaccination was conducted for the entire population of endangered California condors, both in captivity and in the wild. Here we show that this vaccine is safe for condors, stimulates protective immunity in adults, nestlings, and newly hatched chicks. Most importantly, we demonstrate protection of captive birds exposed to naturally circulating WNV during the 2004 transmission season. The prospective vaccination of the entire population of California condors before the arrival of WNV has thus potentially saved this endangered species from subsequent lethal WNV encephalitis, and possible extinction.

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Keywords: West Nile virus; DNA vaccine; California condors

1. Introduction

The California condor (*Gymnogyps californianus*) once soared over much of the North American continent, but the condor population was reduced to only 22 individuals by 1982 due to a variety of factors predominately associated with human activities. The species was deemed endangered in 1974. Soon after that, the California Condor Recovery Program was founded in order to reverse this decline and to establish two separate, sustainable wild populations. By 2005, the condor population had increased steadily to 273, including 135 birds in captive breeding programs, 127 field-released birds, and 11 birds in field pens awaiting release (http://www.peregrinefund.org/condor_factsheet.asp).

West Nile virus (WNV) has spread throughout most of North America, the Caribbean islands, and portions of Central America since its emergence in New York City in 1999 [1,2]. Phylogenetic analysis indicated that the NY-99 virus was most closely related to a WNV isolated from a dead goose in Israel in 1998 [3]. This virus strain causes significant morbidity and mortality in humans, mammals, and both native and exotic birds. As of 28 September 2005, 18,520 human cases were confirmed in the United States, including 716 fatalities. WNV is known to infect at least 35 species of mammals and 334 species of birds (http://www.nwhc.usgs.gov/disease_information/west_nile_virus/affected_species.jsp). Birds in the Corvidae family are particularly vulnerable to WNV infection; 100% mortality has been observed in several experiments [4,5]. *Falconiformes* (raptors), including the vulture and condor family *Cathartidae*, are particularly vulnerable to WNV infection [6]. Although primarily mosquito-borne, certain bird species

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may become infected with WNV after ingesting dead animals that were carrying the virus [6]. California condors are opportunistic scavengers, feeding only on the carcasses of animals. In addition to the risk posed by virus-infected mosquitoes, this scavenger behavior may increase the risk of WNV infection significantly for field-released birds. Thus, prospective immunization of the entire condor population before the spread of WNV to the western United States may deem essential to the survival of this species.

In the summer of 2002, researchers from the California Condor Recovery Team (CCRT) learned that experimental inoculation of captive birds with the formalin-inactivated WNV vaccine licensed for horses (Fort Dodge Animal Health, Fort Dodge, IA) failed to stimulate detectable neutralizing antibodies [7]. An experimental DNA vaccine, proven to protect against WNV infection in several mammal and bird species [8,9], was therefore considered to be the only viable alternative for protecting the condor population. Here we show that: (1) this vaccine is safe for California condors; (2) the vaccine stimulates protective neutralizing antibodies in adults, nestlings, and newly hatched chicks; (3) this vaccine protects vaccinated captive birds from naturally circulating WNV during the 2004 transmission season.

2. Materials and methods

2.1. Condors and vaccine

This vaccination was conducted based on the approved animal use protocol joined developed by the Centers for Disease Control and Prevention (CDC) and the CCRT. Four Andean condors (*Vultur gryphus*, close relative of California condors and not in the endangered species list) and six California condors (not essential for breeding program) in Los Angeles Zoo (LAZ) were selected for the pilot experiment (Table 1) to address the risk of acute adverse reactions caused by undetectable levels of residual antibiotics, *Escherichia coli*-derived endotoxin or Alu-gel in the vaccine. Remain-

ing population of California condors were then recruited to the follow-up study.

The vaccine DNA (pVWN) used in this study was modified by replacing the ampicillin resistance gene in the published pCBWN construct [8] with a kanamycin resistance gene derived from the pVAX plasmid (Invitrogen, Carlsbad, CA). COS-1 cells transformed with pVWN expressed WNV premembrane/membrane (prM/M) and envelope (E) proteins in the form of extracellular virus-like particles (data not shown). The pVWN plasmid DNA produced at CDC, National Center for Infectious Diseases, Division of Vector-Borne Infectious Diseases (DVBID), Fort Collins, Colorado, was used to vaccinate 4 Andean condors and 34 California condors maintained in LAZ. To produce this plasmid DNA, *E. coli* strain XL-1 Blue cells (Stratagene, La Jolla, CA) were transformed with the pVWN plasmid and propagated for 14–16 h in 1 × LB broth containing 100 µg/ml of kanamycin. The plasmid DNA was extracted, purified with an EndoFree plasmid Giga kit (Qiagen Inc., Valencia, CA), and resuspended in an elution buffer (0.5 mM Tris, pH 8.5). For each batch of purified plasmid, automated DNA sequencing was performed with a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA) according to the manufacturer's recommended procedure. Purified plasmid DNA containing the correct sequence was used to formulate the experimental vaccine. The phosphate-buffered saline (PBS)–DNA solution was combined with 1% aluminum phosphate gel (Alu-gel; Superfos Biosector, Frederikssund, Denmark) to obtain a final concentration of 500 µg DNA/ml/dose. The vaccine for the remaining condors was produced and donated by Aldevron, LLC (Fargo, ND).

2.2. Vaccination, serum collection and the plaque-reduction neutralization test (PRNT)

Two-dose regimen (500 µg/1.0 ml/dose, 3-week apart) was followed if the situation permits. The vaccine was given by intramuscular injection on the leg muscle. Captive birds were kept under constant observation for the first 8 h after

Table 1
Pilot study to determine the safety and immunogenicity of WNV DNA vaccine in endangered California condors

Species and ID	Age (Y and M)	90% plaque-reduction neutralization antibody titer (PRNT ₉₀)							
		Pre-vaccinated serum		Day postvaccination (DPV) serum					
		SLEV ^a	WNV ^a	DPV	WNV ^a	DPV	WNV ^a	DPV	WNV ^a
Andean C-1	37Y10M	64	4	21	64	60	64	416	16
Andean C-2	37Y10M	nd ^a	<4	21	64	60	256	416	32
Andean C-3	5M	nd	<4	21	128	60	128	281	32
Andean C-4	9Y6M	4	4	21	128	60	128	403	32
Calif. C-1	6Y6M	nd	<4	21	8	60	16	386	16
Calif. C-2	6Y6M	nd	<4	21	4	60	32	386	16
Calif. C-3	4Y7M	nd	nd	nd	nd	60	32	385	16
Calif. C-4	4Y8M	4	4	21	128	60	64	386	32
Calif. C-5	1Y5M	nd	<4	21	128	60	64	386	64
Calif. C-6	18Y6M	nd	<4	21	<4	60	128	nd	nd

nd, not done; year and month (Y and M); St. Louis encephalitis virus (SLEV); West Nile virus (WNV).

^a Virus used in PRNT.

13

each vaccination, and then followed by daily observation for 10 days. One to 2 ml of serum was collected from birds in the pilot study before each vaccination and 60-day postvaccination (60DPV). However, vaccination and serum collection of captive condors in the breeding program was carried out in a manner that would minimize disturbing the birds' normal breeding activities. Field-released birds in Northern Arizona, Southern California and Baja California were also vaccinated and serum collected when the birds were captured for medical or other reasons. Thus, a large numbers of field-released birds received only one dose of vaccine and no postvaccination serum specimens were available for some wild birds. Consequently, the timing of the second vaccination and the follow-up serum collections varied across individuals and deviated from the standardized regimens in the pilot experiment.

All serum specimens were assayed for the presence of protective antibodies by plaque-reduction neutralization tests at the CDC laboratory. WNV-specific antibody titers were measured in serum samples using a PRNT, as previously described [8]. Briefly, 100 μ l of the 10-fold serial dilution of each serum sample was added to in a 96-well microtiter plate. A stock solution of WNV-NY-99-4132 (four Vero passages) with a viral titer of approximately 200 plaque forming unit/100 μ l was added to each serum dilution in equal volume (100 μ l). The serum/virus mixtures were incubated at 37 °C and 5% CO₂ for 1 h and then 100 μ l was inoculated in duplicate onto 6-well Vero cell plates. The plates were treated with the double overlay method as previously described and the average number of plaques for each sample was compared to the average number of plaques produced by a virus back titration to determine the end-point of the 90% of mean neutralization. For the St. Louis encephalitis virus (SLEV) PRNT, strain MSI-7 was used.

2.3. Expression of WNV nonstructural protein-1 (NS-1) and NS-1-capture enzyme-linked immunosorbent assay (ELISA)

The recombinant WNV-NS-1 protein to identify natural exposure of WNV in vaccinated birds was produced from a stably transformed cell line (COS-WNV-NS-1) that has been selected for constitutive expression and secretion of NS-1 protein into culture media. The NS-1 expression plasmid was constructed by inserting the entire coding region of the WNV-NS-1 gene, amplified from the NY-99 strain, into a plasmid containing an engineered Japanese encephalitis virus signal sequence [8]. The authenticity of the NS-1 protein was confirmed by using two methods: an indirect fluorescent antibody assay (IFA) using a WNV-specific mouse hyperimmune ascitic fluid or the NS-1-specific mouse monoclonal antibody T17, and an antigen-capture enzyme-linked immunosorbent assay using hyperimmune rabbit anti-WNV-NS-1 antiserum as the capture antibody and T17 as the indirect detector (data not shown).

For the direct NS-1-capture ELISA, the ELISA plate was coated overnight at 4 °C with 1:100 dilutions of the con-

dor serum specimens, washed with washing buffer (PBS with 0.05% Tween-20) five times, then incubated for 1 h at 37 °C with an optimal concentration of recombinant NS-1 protein or media derived from normal COS-1 cell culture. The NS-1 protein had been titrated by antigen-capture ELISA to determine the concentration that consistently generated an OD₄₅₀ of ~1.0. The unbound NS-1 was removed by washing the plate five times with washing buffer. The bound NS-1 was detected by incubation with a 1:5000 dilution of antibody T17, followed by a peroxidase-conjugated goat anti-mouse IgG. The ELISA results were expressed as positive-to-negative ratio (P/N) values. Positive (P) values were calculated by averaging the A₄₅₀ values obtained from the experimental serum samples incubated with positive viral antigen. Negative (N) values were determined as the average A₄₅₀ for the experimental sera incubated with the normal COS-1 cell antigen.

2.4. Statistical analysis

One-way analysis of variance (ANOVA) and Student–Newman–Keuls test were used to determining the influence of the postvaccination serum collection intervals on the neutralizing antibody titers using MedCalc statistical package (Berkley, CA).

3. Results and discussions

3.1. Safety and protective antibodies response in vaccinated condors

The first concern in this study, as with any new vaccine or with vaccination in a previously untested species, was the risk of acute adverse reactions. Additionally, because the condor courtship starts at the beginning of the year; the vaccine would ideally need to be administered no later than early December in order to minimize any impact on the captive breeding program caused by capture and restraint. In the pilot study, three captive Andean condors in LAZ were vaccinated to address vaccine safety concerns. These birds were kept under constant observation for the first 8 h after each vaccination, and then followed by daily observation for 10 days; acute adverse reactions or other vaccine-related incidences were not observed. The research team therefore expanded the pilot study and selected one additional Andean condor and six California condors of low breeding value to receive the first dose of vaccine.

All condors in the pilot experiments showed excellent neutralizing antibody responses by 60DPV. Calif. C-6, the only bird without a detectable PRNT titer by 21DPV, showed a significant increase in PRNT titer after the second vaccination, consistent with the strong priming response of this vaccine (Table 1). Its PRNT₉₀ titer reached 128 at 60DPV. Pre-existing antibodies against SLEV (an endemic American flavivirus in the same antigenic complex) that cross-reacted

with WNV in 3 of the 10 experimental birds (Table 1) had no affect on the birds' ability to efficiently raise WNV neutralizing antibody after vaccination. The age of the experimental birds [ranging from 5 months (5M) to 37 years and 9 months (37Y9M)] did not influence the antibody response. In later follow-ups, the neutralizing antibody titers remained between 16 and 64 in serum specimens collected from 281 to 416DPV (Table 1). No unusual behaviors, health changes, or side effects were observed for any of the four Andean or six California condors in the pilot experiment. The experimental vaccine, therefore, appeared to be safe and efficacious in stimulating protective immunity in vaccinated birds.

Due to the urgent nature of this study and the encouraging safety and immunogenicity results, researchers in the CCRT expanded the vaccination regimen to include all condors in the three captive breeding facilities. The entire population of California condors, captive and field-released, received at least one dose of the vaccine by the end of 2004. No health, behavior or breeding changes have occurred that can be attributed to the vaccine. Pre-vaccine serum specimens were collected from 193 condors and at least 1 postvaccination specimen was collected for 167 of these birds. Many of these birds had multiple postvaccination sera collections up to 724DPV, thus providing a detailed pre-postvaccination data set for detailed examination of the immunogenicity of this WNV DNA vaccine. Thirteen pre-vaccine specimens contained neutralizing antibodies ($\text{PRNT}_{90} \geq 4$) against St. Louis encephalitis virus (data not shown). Overall, almost 90% of birds with postvaccination sera collections (PVC) seroconverted ($\text{PRNT}_{90} \geq 4$) and neutralizing antibody titers continued to increase until about 1 year postvaccination (excluding the single bird with a sixth collection, one-way ANOVA: $F\text{-ratio} = 45.58$, $p < 0.001$). Furthermore, the Student–Newman–Keuls test indicated a statistically significant difference between PVC1, PVC2, and PVC3. The

neutralizing antibody titer was highest in PVC4; however, there was no statistical difference between the third, fourth or fifth collections.

3.2. Vaccination protected condors from natural WNV infection

WNV was first isolated in California in July 2003 from a pool of *Culex tarsalis* mosquitoes collected from Imperial County in Southern California. Soon after, the virus had dispersed to the city of Riverside and into Los Angeles County [10]. Los Angeles County conducts intense mosquito surveillance in the areas surrounding LAZ. The first WNV-positive mosquito pools were detected on 7 July 2004 including one WNV-positive pool sampled near the condor holding area. All captive condors in the Southern California breeding facilities had been vaccinated by summer 2003. Condor serum specimens collected before and after 7 July indicated that, some of these vaccinated condors might be exposed to naturally circulating WNV in 2004.

The first indications of natural infection with WNV were increases in PRNT titers of greater than 4-fold in 8 of 12 available paired serum specimens collected before and after 7 July 2004 from LAZ (Table 2). The PRNT titers ranged from <4 to 64 before, and increased to 256 to >4096 after July 7 (Table 2). These large magnitude PRNT increases are indicative of exposure to WNV. Amongst the condors, similar increases (though lower magnitude) were only observed for some individuals following secondary vaccination, and these eight birds had all received their final vaccinations by summer 2003. Moreover, very similar large magnitude paired sera PRNT titer increases were observed in WNV DNA vaccinated and experimentally challenged mice and horses [8].

Limited virus replication in the vaccinated host from natural exposure is expected to generate antibodies to the

Table 2

Serological evidences supporting the potential natural exposure of West Nile virus in the DNA vaccine immunized California condors

ID	Serum collected before July 07, 2004				Serum collected after July 07, 2004			
	Collection day	DPV	Serological assay		Collection day	DPV	Serological assay	
			PRNT 90%	ELISA NS-1			PRNT 90%	ELISA NS-1
1	11/13/2003	336	64	<2	07/22/2004	587	256	21.9
95	08/07/2003	233	8	<2	08/04/2004	595	4096	13.7
120	11/13/2003	352	8	<2	07/22/2004	603	2048	2.62
139	08/15/2003	206	<4	<2	10/27/2004	643	512	2.44
320	11/13/2003	76	<4	<2	07/27/2004	332	2048	14.6
180	11/14/2003	385	16	<2	08/09/2004	653	>4096	<2
189	11/13/2003	76	8	<2	07/31/2004	336	2048	<2
290	01/15/2004	135	8	<2	08/04/2004	336	2048	<2
216	06/15/2004	369	4	<2	12/01/2004	543	4	<2
295	11/27/2003	90	32	<2	07/31/2004	337	8	<2
315	11/13/2003	76	8	<2	07/26/2004	332	4	<2
319	11/13/2003	76	4	<2	07/26/2004	332	16	<2

The enzyme-linked immunosorbent assay (ELISA) results are expressed as positive/negative (P/N) values. Only $\text{P/N} \geq 2$ is interpreted as a positive result against NS-1 protein. PRNT, plaque-reduction neutralization test.

Table 3

Persistence of maternally transferred antibodies and influence of persisting antibodies on immunological responses of vaccinated newly hatched condor chicks

Female			Chick											
ID	Collection	PRNT	ID	Hatch date	Posthatched and pre-vaccination collection						Postvaccination collection day			
					DPH (d)	PRNT	DPH (d)	PRNT	DPH (d)	PRNT	DPV (d)	PRNT	DPV (d)	PRNT
Unvaccinated group														
11	07/31/2003	128	340	05/09/2004	32	2								
84	09/22/2003	32	341	05/09/2004	5	16								
111	11/18/2003	<4	333	04/22/2004	64	<4								
31	05/25/2004	32	338	05/04/2004	31	8	91	4	124	<4				
69	09/21/2003	32	344	05/15/2004	31	16	91	<4	119	4				
Vaccinated group														
39	07/31/2003	128	332	04/21/2004	31	16	91	4	121	4	18	32		
192	11/19/2003	4	328	04/11/2004	92	<4					?	16		
11	07/31/2003	128	355	06/24/2004	31	4	91	<4			29	32		
43	03/12/2003	32	351	05/28/2004	29	8	54	<4			38	>128	71	>128

?, the day of collection is unknown; DPH, day-posthatch; PRNT, 90% plaque-reduction neutralization test; DPV, day-postvaccinations.

nonstructural proteins, including the antigenic NS-1 in particular. Because the vaccine only contains the structural prM and E proteins, vaccinated condors with detectable anti-NS-1 antibodies provide serological evidence of natural infection. Thus, a direct NS-1-capture ELISA was developed for detecting anti-NS-1 antibodies in serum specimens (Table 2). All 12 available serum pairs collected before July 7, 2004, were ELISA-negative for anti-NS-1 antibodies (P/N < 2, Table 2). Four birds were negative for anti-NS-1 antibodies, who maintained similar PRNT after 7 July. However, five of eight suspected WNV-exposed condor serum specimens collected after 7 July 2004 were anti-NS-1 antibody-positive (P/N ≥ 2, Table 2). We were not able to conduct a virus isolation experiment, the definitive demonstration of WNV infection, with any of these serum specimens as they had been heat-inactivated.

Because of the general susceptibility of raptors to, and the documented deaths of native black vultures (*Coragyps atratus*) and turkey vulture (*Cathartes aura*) from lethal WNV encephalitis [5,11], WNV infection is probably lethal to California condors. In fact, two California condor deaths due to WNV infection have been documented recently. A 3-month old wild-hatched chick that died 25 August 2005, tested positive for WNV (Press release, US Fish and Wildlife Service 09-13-2005). Another 9-month old immature condor, which had not been vaccinated and was in a holding pen awaiting field release, died in February 2005. The suspected cause of death was WNV since viral RNA was detected in multiple internal organs collected during autopsy (Rideout, personal communication). A separate study of maternal antibody prevalence and chick vaccination demonstrated that PRNT titers of all unvaccinated chicks dropped to <4 by 91 days posthatching (Table 3). We speculate that maternal antibodies had diminished to below protective levels and resulted in the deaths of these two birds upon exposure to WNV. These cases support the lethality of WNV infection for condors and demonstrate a potentially devastating loss of California con-

dors had this endangered species been left unprotected against WNV.

Four chicks with (titer ≥ 4) or without (titer < 4) maternal antibodies were vaccinated with one dose of vaccine between 54 and 121 days after hatching. Vaccinated chicks showed rise in neutralizing antibody titers (ranging from 16 to >128) at 18–38 days postvaccination, regardless of their maternally transferred antibody status and age (Table 3). Based upon these results and observations the CCRT currently recommends vaccinating newly hatched captive chicks at the age of 1–2 months, and attempting to vaccinate wild-hatched chicks in their nests providing that they are accessible.

4. Conclusion

Introduction of WNV into the United States in 1999 has created major concerns for human and animal health and for wildlife conservation. In addition to the normal transmission to humans involving the bite of an infected mosquito, transfusion-related WNV infection has seriously jeopardized the safety of blood supply [12]. Currently, there is no human vaccine available to reduce the risk of WNV infection. Active surveillance in conjunction with costly mosquito control and individual protection are the only available strategies to combat the potentially devastating effects of this disease in humans. The predecessor of this experimental vaccine, pCBWN, was the first fully licensed DNA vaccine, which was licensed by the US Department of Agriculture for use in horses (Press release APHIS 07-18-2005). Additionally, a human WNV DNA vaccine trial utilizing the transcription unit derived from the vaccine in this work is currently being conducted through the National Institutes of Health (Press release NIH-NIAID 04-18-2005). Since the entire population of California condors is monitored closely by CCRT, this study provides a unique opportunity to investigate and understand the causal correlations between PRNT titer, primary

vaccine failure and immune protection; and to address the potential long-term effects on DNA vaccine related adverse events. This detailed and long-term vaccine study has therefore not only protected a highly endangered species from lethal WNV infection but it has and will continue to produce important insights relevant to ongoing human WNV DNA vaccine development and trials.

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A West Nile Virus DNA Vaccine Induces Neutralizing Antibody in Healthy Adults during a Phase 1 Clinical Trial

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(See the article by Ledizet et al., on pages 1741–8, and the editorial commentary by Petersen and Roehrig, on pages 1721–3.)

Background. West Nile virus (WNV) is a mosquito-borne flavivirus that can cause severe meningitis and encephalitis in infected individuals. We report the safety and immunogenicity of a WNV DNA vaccine in its first phase 1 human study.

Methods. A single-plasmid DNA vaccine encoding the premembrane and the envelope glycoproteins of the NY99 strain of WNV was evaluated in an open-label study in 15 healthy adults. Twelve subjects completed the 3-dose vaccination schedule, and all subjects completed 32 weeks of evaluation for safety and immunogenicity. The development of a vaccine-induced immune response was assessed by enzyme-linked immunosorbent assay, neutralization assays, intracellular cytokine staining, and enzyme-linked immunospot assay.

Results. The vaccine was safe and well tolerated, with no significant adverse events. Vaccine-induced T cell and antibody responses were detected in the majority of subjects. Neutralizing antibody to WNV was detected in all subjects who completed the 3-dose vaccination schedule, at levels shown to be protective in studies of horses, an incidental natural host for WNV.

Conclusions. Further assessment of this DNA platform for human immunization against WNV is warranted.

Trial registration. ClinicalTrials.gov identifier: NCT00106769.

West Nile virus (WNV) is a vector-borne member of the *Flavivirus* genus, which includes several clinically and economically important human pathogens, such as yellow fever virus, 4 serotypes of dengue virus, and Japanese encephalitis virus (JEV). WNV was initially

isolated in Uganda in 1937 and was first recognized in the United States in 1999, when it caused an epidemic of encephalitis and meningitis in New York City. WNV has since spread across North America [1] and into portions of Central and South America [2, 3]. In 2005, there were 3000 cases of WNV infection in humans reported in the United States, and, as of 11 December 2006, 4052 cases had been reported for the year in 42 states [4, 5].

WNV naturally exists as an enzootic infection in mosquitoes and birds, although a large number of incidental hosts have been identified, including humans, horses, and alligators [6]. The principal form of transmission to humans is from the bite of an infected mosquito. There is no evidence of person-to-person spread, but transmission of WNV has occurred by blood transfusion, by organ transplantation, by breast-feeding, transplacentally, and in the laboratory [1, 7].

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WNV infections in humans can be severe but are often sub-clinical or may present as a mild to moderate febrile illness. Approximately 1 in 150 infected persons have a serious illness with involvement of the central nervous system [8], and, although cases of severe WNV infection (including meningitis and encephalitis) have been reported in otherwise healthy young adults [9], the risk of severe disease and death increases in elderly persons and in immunocompromised individuals [2]. The first known genetic risk factor for severe and fatal WNV infection has recently been described in patients with the defective CCR5 allele, CCR5 Δ 32, and is possibly related to a lack of CCR5 regulation of WNV-infected leukocytes [10, 11]. Although intravenous immunoglobulin has been investigated as a therapeutic intervention for severe cases of WNV [12], the standard of care for WNV infection is supportive.

Several lines of evidence suggest an important role for antibody in protection from and clearance of flavivirus infections [13]. Surface envelope (E) proteins are the primary target for the humoral response against flavivirus infection. The mature WNV virion is composed of 180 copies of the E protein, arranged with an unusual herringbone icosahedral symmetry. The E protein is thought to mediate interactions with the cell surface and promotes fusion between viral and cellular membranes. In addition, virions incorporate a second protein, the premembrane (prM) protein, which is cleaved during virion maturation into a smaller virion-associated membrane (M) peptide. Of interest, expression of the prM and E proteins in cells results in the formation and release of a virus-like subviral particle that shares many of the structural, antigenic, and functional properties of mature infectious virus.

Since its introduction into North America, WNV infection of horses has become a significant problem, with a 30%–40% mortality rate and as many as 5000 cases of sick horses per year since 1999 [14, 15]. A formalin-inactivated whole-virus WNV vaccine for the prevention of WNV infection in horses (Innovator; Fort Dodge Animal Health) has been available since 2002 and is being evaluated in other species [7, 16]. A recombinant canarypox-based vaccine (Recombitek; Merial) expressing the prM and E genes has been shown to induce neutralizing antibodies and protect horses against experimental challenge with WNV [17] and was approved for veterinary use in the United States in 2004. A yellow fever–WNV chimeric vaccine was also licensed by the US Department of Agriculture (USDA) in 2007 for use in horses [18]. The equine DNA vaccine, pCBWN (Fort Dodge Animal Health in collaboration with the Centers for Disease Control and Prevention [CDC]), also encodes for the prM and E proteins from WNV and, in WNV challenge studies in both mice and horses, elicits neutralizing antibody and confers protection against viremia [19]. In July 2005, this DNA vaccine was licensed by the USDA for the prevention of WNV infection in horses and represents the first license granted for a DNA vaccine for use in animals [14]. The DNA vaccine licensed for use in horses has

recently been shown to protect endangered California condors from WNV infection [20].

Several approaches for the development of a vaccine in humans are also being investigated. A live attenuated WNV vaccine based on chimeric flavivirus technology (ChimeriVax–West Nile; Acambis) has completed phase 1 and is currently in phase 2 trials [21]. Several other vaccine approaches have shown promise in animal models, including a recombinant canarypox vector expressing the prM and E genes [17], a recombinant subunit vaccine expressing E and nonstructural protein 1, and a live attenuated recombinant measles vaccine expressing the secreted form of the E glycoprotein of WNV [22].

Candidate DNA vaccines have been evaluated in phase 1 studies of healthy adults and have been shown to be safe and immunogenic for other pathogens, including HIV and Ebola virus [23, 24]. A promising vaccine approach for the prevention of WNV infection is a single-plasmid recombinant DNA vaccine encoding the WNV prM and E proteins described in the present report of a phase 1 clinical trial. This approach is unique among the potential WNV candidate vaccines being investigated for use in humans in that it eliminates the concern typically associated with live attenuated vaccines. It neither induces nor is susceptible to antivector immunity, which is a concern in vector-based gene-delivery vaccine candidates. In addition, it encodes for proteins that have the potential to form a multivalent antigen *in vivo*, has the ability to induce T cell and neutralizing antibody responses, and it is very similar to the DNA vaccine licensed for the prevention of WNV infection in horses [19].

SUBJECTS, MATERIALS, AND METHODS

Study design. The Vaccine Research Center (VRC) 302 protocol was a single-site, open-label, phase 1 study to examine the safety, tolerability, and immune response to an investigational recombinant DNA WNV vaccine. Healthy adult subjects 18–50 years of age who were negative for WNV IgG were recruited at the VRC, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH). The experimental guidelines of the US Department of Health and Human Services were followed in the conduct of the clinical research, and the protocol was approved by the NIAID Institutional Review Board. Fifteen subjects were enrolled between 18 April and 7 October 2005. Vaccine was administered on study days 0, 28 ($\pm$ 7 days), and 56 ($\pm$ 7 days), with at least 21 days between injections.

Vaccine (VRC-WNV DNA017-00-VP) was administered at a 4-mg dose via intramuscular injection in the lateral deltoid by use of the Biojector 2000 Needle-Free Injection Management System. The dose and route studied in this trial were based on clinical trials of VRC DNA vaccines for other pathogens [23–25]. Adverse reactions and subject safety were evaluated by laboratory and clinical evaluations at scheduled study visits. Adverse events were coded using the Medical Dictionary for Regulatory

Activities (MedDRA), and the severity of adverse events was graded using a scale of 0 to 5 (based on the NIH Division of AIDS table for grading the severity of adverse events; version 1.0; December 2004). Local and systemic solicited reactogenicity—including pain, erythema, swelling, myalgia, malaise, headache, chills, nausea, and temperature—was collected by subject self-report on 5-day diary cards after each injection. Subjects were followed for a total of 32 weeks, and the study was completed in July 2006.

Vaccine. VRC-WNV DNA017-00-VP is composed of a single, closed, circular plasmid DNA macromolecule (VCL-6428) constructed to produce the prM and E proteins of the WNV E glycoprotein. The plasmid was based on an analogous construct shown to protect mice and horses from virus challenge [19]. The plasmid encodes for a single polypeptide encompassing a modified signal sequence from JEV fused upstream of the WNV prM and E coding sequences cloned into the expression vector VR-1012 (CMV backbone). This backbone encoding the CMV promoter and 5' intron A was also used to construct a 4-plasmid HIV multiclade DNA vaccine previously shown to be safe and immunogenic in healthy adults [23]. The coding sequence for the first 6 aa was optimized for expression in humans by using commonly used codons. The WNV prM and E sequence in VCL-6428 is identical to that in the NY99 human WNV isolate. In vitro expression of this gene product results in the formation of noninfectious virus-like particles called "subviral particles" (SVPs). The plasmid in this vaccine is incapable of replication in animal cells and does not permit the generation of an infectious virion even if recombination or gene duplication were to occur.

This DNA vaccine has been produced in bacterial cell cultures containing a kanamycin-selection medium. The process involved *Escherichia coli* fermentation, purification, and formulation as a sterile, liquid, injectable dosage form for intramuscular injection. After growth of bacterial cells harboring the plasmid, the plasmid DNA was purified from cellular components.

Vaccine was prepared under current good manufacturing practice conditions by Vical. The vaccine was manufactured at a 4-mg dose in PBS and met lot-release specifications before use in the clinical trial.

Measurement of antibody responses by ELISA. Duplicate wells of serial dilutions of volunteer serum samples were incubated for 1 h at 37°C on commercially available WNV recombinant antigen-coated plates (Focus Technologies), followed by horseradish peroxidase-conjugated antibody (30 min at room temperature) and tetramethylbenzidine substrate (10 min at room temperature). Color development was stopped by the addition of 0.9 mol/L sulfuric acid, and plates were read within 30 min at 450 nm on the Molecular Devices Spectramax 384-plus ELISA plate reader. The mean optical density for each dilution was corrected for the mean optical density of the same dilution of the preimmunization sample. End-point titers for each vol-

Table 1. Summary of demographic characteristics at enrollment.

Category, parameter	All vaccinees (n = 15)
Sex	
Male	10 (67)
Female	5 (33)
Age	
18–20 years	2 (13)
21–30 years	9 (60)
31–40 years	2 (13)
41–50 years	2 (13)
Mean ± SD	28.5 ± 8.2
Range	19–44
Race	
American Indian/Alaska Native	0
Asian	0
Black or African American	2 (13)
Native Hawaiian or other Pacific Islander	0
White	13 (87)
Multiracial	0
Other/unknown	0
Ethnicity	
Non-Hispanic/Latino	14 (93)
Hispanic/Latino	1 (7)
Body mass index	
<18.5	0
18.5–24.9	6 (40)
25.0–29.9	7 (47)
≥30.0	2 (13)
Mean ± SD	26 ± 4
Range	21.0–34.8
Education	
Less than high school graduate	0
High school graduate/GED	5 (33)
College/university	9 (60)
Advanced degree	1 (7)

NOTE. Data are no. (%) of subjects, unless otherwise indicated. Age represents age at enrollment day. Height and weight (used for calculation of body mass index) were from screening evaluation before enrollment. GED, General Educational Development.

unteer were established as the last dilution with a corrected pre-immunization OD >0.2.

Measurement of neutralizing antibody responses by plaque reduction neutralization (PRNT). PRNT was performed with Vero cells, as previously described [26], using NY99-6480 virus. End-point titers were determined for 50% and 75% plaque reduction.

Measurement of antibody-mediated neutralization by use of reporter-virus particles (RVPs). WNV RVPs composed of the structural proteins of the NY99 strain of WNV and a sub-genomic replicon were produced by complementation in BHK-21 cells, as described elsewhere [27]. Antibody-mediated

neutralization was measured using a Raji B lymphoblastoid cell line that expresses the WNV attachment factor CD209L (DC-SIGNR), as described elsewhere [28]. WNV RVPs were incubated with serial 3-fold dilutions of volunteer serum at room temperature for 2 h and then added to 5×10^4 cells plated on the day of the assay. The concentration of RVPs used in each experiment was validated empirically to establish assay conditions of antibody access at all informative points of the dose-response profile for each volunteer. Infection was monitored as a function of green fluorescent protein expression (encoded by the replicon) at 48 h after infection by flow cytometry. Data were analyzed by curve fitting and nonlinear regression in order to describe the shape of the dose response and calculate an EC_{50} . Data are presented as the reciprocal dilution of serum corresponding to the EC_{50} and are adjusted to consider the final 300- μ L volume of the neutralization reaction.

Measurement of T cell responses by enzyme-linked immunospot (ELISpot) assay. ELISpot assay was performed on subject samples at baseline and at weeks 2, 6, 8, 10, 12, and 32, as described elsewhere [24]. Cells were stimulated overnight with vaccine insert-specific peptide pools (WNV-E and WNV-M) at 2×10^5 cells/well. Results are expressed as mean spot-forming cells per million peripheral blood mononuclear cells.

Measurement of T cell responses by intracellular cytokine staining (ICS). $CD4^+$ and $CD8^+$ T cell responses were measured by ICS at baseline and at weeks 2, 6, 8, 10, 12, and 32, as described elsewhere [24]. Cells were stimulated with vaccine insert-specific peptide pools (WNV-E and WNV-M). Cells were permeabilized, washed, and stained with directly conjugated anti-human CD3, CD4, CD8, interferon (IFN)- γ , and interleukin (IL)-2 antibodies and were assessed for CD3, CD8, CD4, and IFN- γ /IL-2 expression on a FACSCalibur flow cytometer (BD Immunocytometry Systems).

Statistical methods. All assays are treated as binary (responders/nonresponders). We use the usual central, exact 95% confidence intervals (CIs) for binomial rates. We are 97.5% confident that the true response rates in the antibody assays are higher than the lower limit. Calculations were done using R software (version 2.3.1; R Foundation). A positive T cell response for ICS and ELISpot data was based on composite criteria as previously described in 4 published studies of candidate vaccines [23, 24, 29]. SAS (version 9.0; SAS Institute) and S-plus (version 6.2; Insightful) were used for analyses.

RESULTS

Study population demographics. A total of 15 healthy adults were enrolled. Table 1 includes demographic data on subject sex, age, race/ethnicity, body mass index (BMI, calculated as weight in kilograms divided by the square of height in meters), and educational level at the time of enrollment. The subject population was 67% male, with a mean age of 28.5 years. Subjects were

Table 2. Summary of maximal local and systemic reactogenicity.

Symptom, intensity	All vaccines (n = 15)
Local reactogenicity	
Pain/tenderness	
None	2 (13.3)
Mild	11 (73.3)
Moderate	2 (13.3)
Swelling	
None	6 (40.0)
Mild	9 (60.0)
Moderate	0
Redness	
None	5 (33.3)
Mild	10 (66.7)
Moderate	0
Any local symptom	
None	1 (6.7)
Mild	12 (80.0)
Moderate	2 (13.3)
Systemic reactogenicity	
Malaise	
None	11 (73.3)
Mild	3 (20.0)
Moderate	1 (6.7)
Myalgia	
None	13 (86.7)
Mild	0
Moderate	2 (13.3)
Headache	
None	11 (73.3)
Mild	3 (20.0)
Moderate	1 (6.7)
Chills	
None	14 (93.3)
Mild	0
Moderate	1 (6.7)
Nausea	
None	14 (93.3)
Mild	0
Moderate	1 (6.7)
Temperature	
None	15 (100)
Mild	0
Moderate	0
Any systemic symptom	
None	9 (60.0)
Mild	4 (26.7)
Moderate	2 (13.3)

NOTE. Data are no. (%) of subjects, unless otherwise indicated. Local injection site reactions were recorded by clinicians 30–45 min after an injection and were then recorded as self-assessments at home by subjects on a 5-day diary card. Systemic reactions were recorded as self-assessments at home by subjects on a 5-day diary card after each injection. There were no reports of severe local or systemic symptoms after vaccination.

Table 3. Antibody titers.

Subject	Reciprocal ELISA titer		Reciprocal PRNT titer		
	Week 12	Week 32	50%, week 12	75%	
				Week 12	Week 32
A	480	30	32	16	16
B	Not done	90	Not done	Not done	8
C ^a	Negative	Negative	Negative	Negative	Negative
D	1920	30	64	32	8
E	480	Negative	64	32	16
F	1920	30	32	4	8
G ^b	120	30	4	Negative	8
H ^b	120	Negative	2	Negative	2
I	480	30	64	32	16
J	1920	90	128	16	8
K	480	90	64	16	32
L	1920	90	128	32	16
M	480	30	64	8	8
N	7680	810	16	4	4
O	480	90	16	16	2

NOTE. Reciprocal titers are shown for each subject as assessed by ELISA and plaque-reduction neutralization (PRNT) at weeks 12 and 32. Positive responses are reported as a reciprocal titer.

^a Received 1 dose of vaccine.

^b Received 2 doses of vaccine.

predominantly white (87%) and non-Hispanic/Latino (93%). The mean BMI was 26. All subjects had an educational level of high school or higher, with 60% having college-level degrees.

Vaccine safety. The diary cards showed that 93% (14/15) of subjects reported at least 1 local injection site symptom (mild to moderate pain/tenderness, mild induration, or mild skin erythema) after a vaccination. When systemic symptoms were recorded on diary cards, they were primarily mild and included malaise, myalgia, headache, and nausea. No subjects reported fever or severe reactogenicity (table 2). A superficial, self-limited cutaneous erosion of ~1 cm in diameter, which healed without treatment or sequelae, was noted after 1 vaccination; this was similar to superficial skin erosions observed with a candidate HIV DNA vaccine also administered by Biojector [29].

There were no serious adverse events. Twelve of 15 subjects completed the vaccination schedule. Three subjects were withdrawn from the vaccination schedule because of concomitant illness; 2 after the second vaccination and 1 after the first vaccination. One subject developed a benign grade 1 condition, aquagenic wrinkling of the palms, which occurred 12 days after the second vaccination and continued intermittently through the follow-up period. This condition has been described after administration of COX-2 inhibitors [30] and in people with cystic fibrosis (CF) gene mutations [31]. This subject had not taken concomitant medications, was healthy, and had a negative evaluation for CF gene mutations after the onset of this benign condition. One subject experienced a grade 2 methicillin-resistant

Staphylococcus aureus skin infection distant to the vaccination site, 2 weeks after the second vaccination. One subject was given a diagnosis of streptococcal pharyngitis and vaginal candidiasis, which occurred 25 days after the first vaccination. These events resolved without residual sequelae.

Safety and immunogenicity data for all subjects (*n* = 15) are included in this intent-to-treat analysis through the time points available. All subjects were followed for 32 weeks for safety and immune response; 1 subject who completed the 3-dose vaccination schedule did not present for the study visit and blood collection at week 12; therefore, that subject's week 12 immune response could not be assessed. Overall, study vaccinations were well tolerated and safe in the healthy subjects (age range, 19–44 years).

Antibody responses. Vaccine-induced humoral immune responses were assessed at weeks 8, 12, and 32. Subjects are represented by an identifier on the basis of age (designated by the letters A–O). Vaccine-induced antibody, assessed by ELISA at week 12 relative to baseline, was present in all subjects who received 2 or 3 doses of vaccine (table 3).

To determine whether vaccine-induced WNV-specific antibody could neutralize virus, 2 different methods were used to detect antibody-mediated neutralization. First, neutralizing titers for each subject at weeks 12 and 32 were estimated using PRNT on Vero cells. Vaccine-induced neutralizing antibody was detected in all vaccinees who completed the 3-dose vaccination schedule and were assessed at week 12 (11/11 [100%; 95% CI,

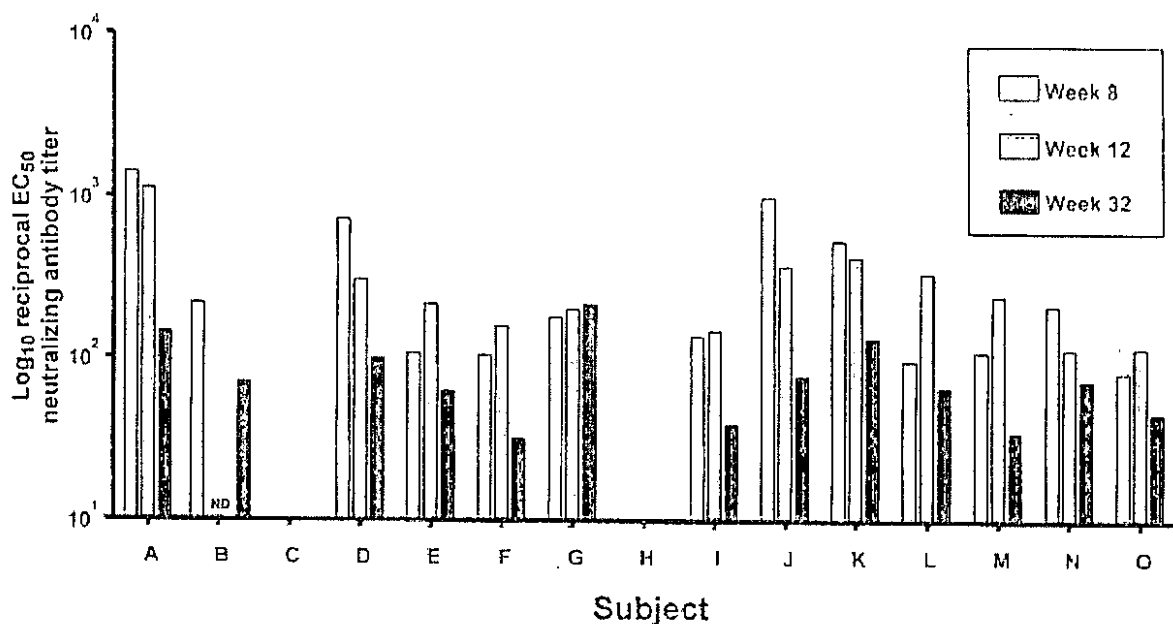


Figure 1. Serum samples from vaccinees at week 8 (before the third vaccination), week 12 (4 weeks after the third vaccination), and week 32, assessed for the presence of neutralizing antibody by a West Nile virus reporter-virus particle (RVP) neutralization assay. The X-axis shows individual vaccine clinical trial subjects, and the Y-axis shows the log₁₀ reciprocal EC₅₀ neutralizing antibody titer. Subject B was not assessed at the week 12 time point because of visit noncompliance. Subjects G and H received 2 doses of vaccine. Subject C received 1 dose of vaccine. ND, not done.

71.5%–100%}) and week 32 (12/12 [100%; 95% CI, 73.5%–100%]). A response was also seen at week 32 in both subjects who received 2 doses of vaccine (table 3).

To complement the PRNT, neutralization profiles of serum from each volunteer at weeks 8, 12, and 32 was studied using a recently described quantitative approach. WNV RVPs are

pseudoinfectious virions that are capable of a single round of infection and that allow WNV entry to be measured as a function of reporter gene expression. For each vaccinee, a profile of the neutralization activity in serum at each collection point was measured using 7 or 8 dilutions of serum. In agreement with data from the PRNT, neutralizing antibody was demonstrated in

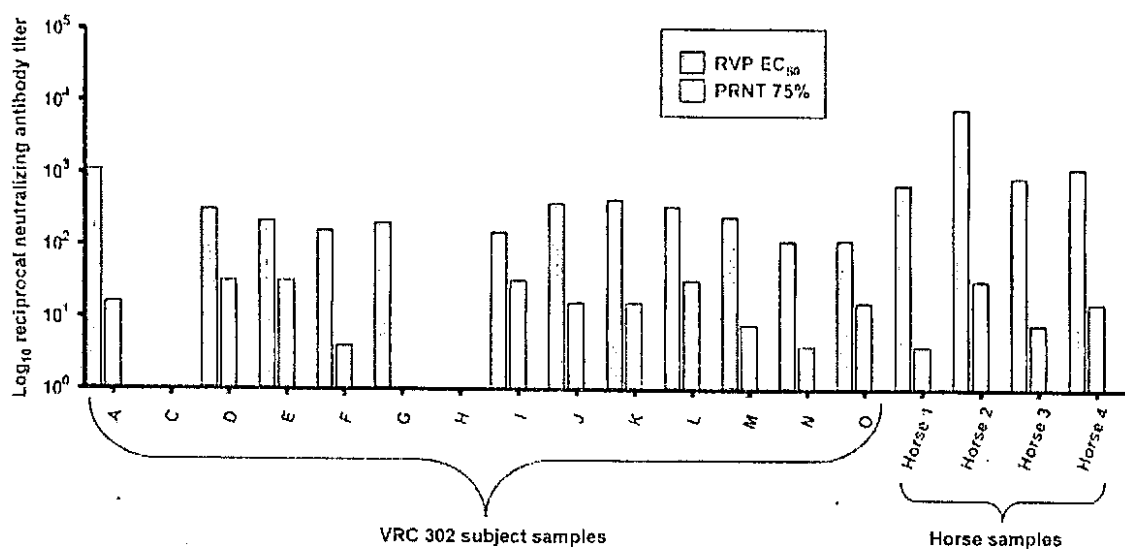


Figure 2. Serum samples from vaccinees at week 12 (4 weeks after the third vaccination) and serum samples from horses 3 weeks after receipt of a 1-mg dose of pCBWN West Nile virus (WNV) DNA vaccine, assessed by WNV reporter-virus particles (RVP) neutralization assay and by plaque reduction neutralization (PRNT). The X-axis shows individual vaccine clinical trial subjects or horse samples, and the Y-axis shows the log₁₀ reciprocal antibody titer. Subject B was not assessed at the week 12 time point because of visit noncompliance. Subjects G and H received 2 doses of vaccine. Subject C received 1 dose of vaccine. VRC, Vaccine Research Center.

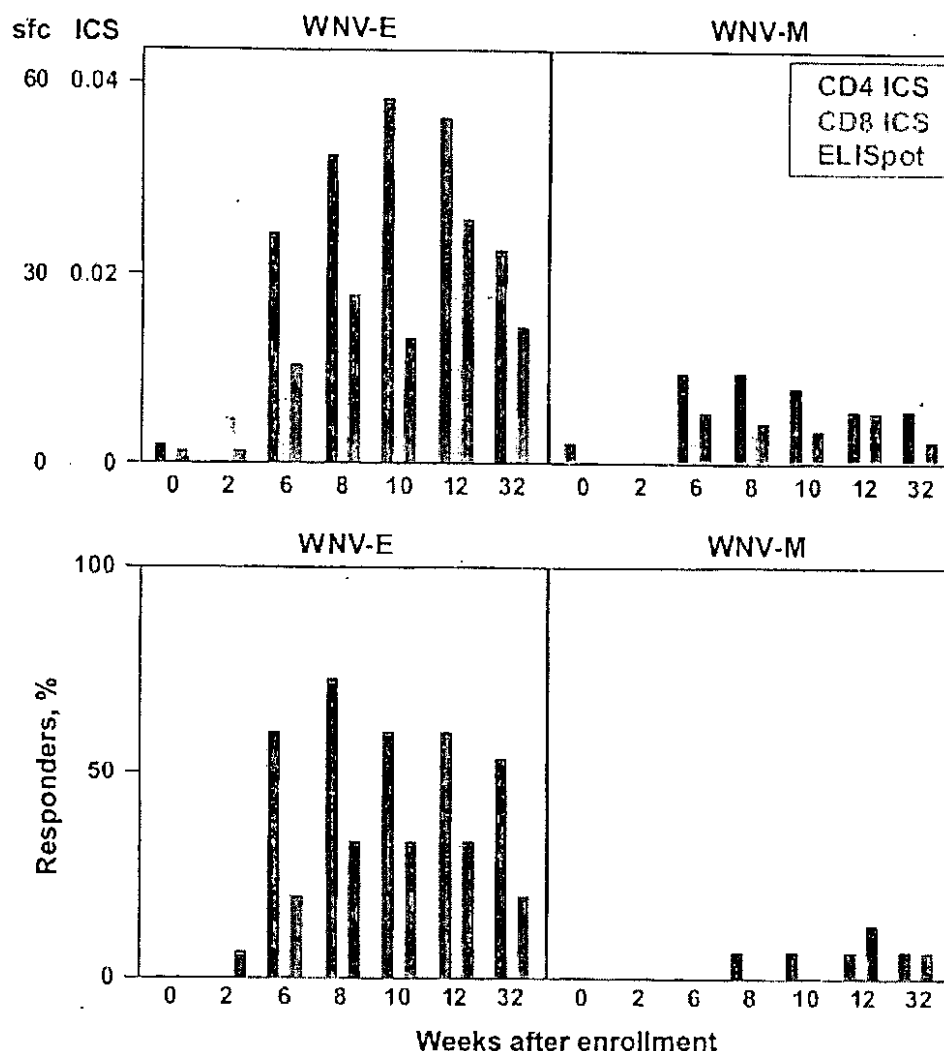


Figure 3. T cell responses specific for West Nile virus (WNV) DNA vaccine insert-specific peptide pools (WNV-E (*left*) and WNV-M (*right*)), assessed by enzyme-linked immunospot (ELISpot) assay and intracellular cytokine staining (ICS). Data are shown for the entire study (weeks 0–32). The magnitude of ELISpot and ICS responses are shown in the top graphs, and the frequency of responses among all subjects is shown in the bottom graphs. The threshold for positivity by ELISpot assay is a mean of 59 sfc per million peripheral blood mononuclear cells. For ICS responses, a positive response required that the *P* value from a Fisher's exact test of the difference between the antigen-specific response and the negative control response be $< .01$ and that the antigen-specific response be at least 0.0241% (for CD3⁺CD4⁺ responses) or at least 0.0445% (for CD3⁺CD8⁺ responses) higher than the negative control response.

all subjects who received 3 doses of vaccine and in 1 subject who received 2 doses of vaccine. Subjects developed neutralizing antibody after the second vaccination by week 8; antibody titers typically peaked at week 8 or 12 and remained positive at week 32, albeit at a lower level (figure 1).

To compare the magnitude of the neutralization response elicited by this DNA vaccine in humans to a response that confers protection to challenge by WNV, we evaluated the neutralizing titers in serum obtained from horses that received an equine WNV DNA vaccine (Fort Dodge Animal Health and the CDC) encoding for the same construct as the vaccine used in this clinical trial. That vaccine is USDA approved for use in horses and has been shown to elicit neutralizing antibody by PRNT [19]. We evaluated serum from a sample of these horses by the

RVP neutralizing antibody assay. The postvaccination titers 3 weeks after a single 1-mg dose of the WNV DNA vaccine in horses was similar to the titers elicited in humans in this clinical trial after 2 or 3 doses of vaccine (figure 2).

T cell responses. The majority of vaccine-specific T cell responses were elicited against WNV-E, and CD4⁺ T cell responses were more frequent than CD8⁺ T cell responses. The peak frequency and magnitude of the response occurred between weeks 8 and 12 (figure 3). Vaccine-specific T cell responses to WNV-E were detected by ELISpot assay in 8 (53%) of 15 subjects. CD4⁺ T cell responses to WNV-E as measured by ICS were detected in 14 (93%) of 15 subjects. A T cell response was not induced in the subject who received only 1 injection. A T cell response to WNV-M was detected in 4 (27%) of 15 subjects, and a CD8⁺ T

cell response as measured by ICS was detected in 1 subject and was present against both WNV-E and WNV-M.

DISCUSSION

WNV represents an economically and medically important re-emerging flavivirus that continues to cause morbidity and mortality in North America. The virus has spread across North and Central America and into South America since its emergence in New York in 1999. Neutralizing antibody is known to provide protection against flaviviruses in animal models and humans. A DNA vaccine encoding for WNV proteins prM and E protects horses from WNV infection in a challenge model [19] and is currently USDA approved for veterinary use.

Production of bacteria-derived plasmid DNA vaccines has many favorable features, including (1) ease and flexibility of construction, (2) scalable manufacturing capacity, (3) stability, (4) intracellular production of vaccine antigen, (5) transient expression, (6) no induction of antivector immunity, (7) induction of both T cell and antibody responses, and (8) low occurrence of systemic reactogenicity.

This WNV candidate vaccine was well tolerated in 15 healthy subjects. The rate and severity of reactogenicity reported is similar to that reported in previous studies of an Ebola virus DNA vaccine [24] and a multiclade HIV DNA vaccine [23].

DNA vaccines have been shown to be safe and to elicit vaccine-induced humoral and cellular immune responses for HIV and Ebola virus [23–25]. Although these DNA vaccines are able to elicit virus-specific antibodies detected by immunoprecipitation and Western blotting or by ELISA [23, 32], they have not been highly efficient at eliciting neutralizing antibodies, possibly because these viruses are generally less sensitive to antibody neutralization. The ability of this WNV DNA vaccine to elicit neutralizing antibody could possibly be due to the formation of virus-like particles produced by the vaccine-encoded proteins. E and prM are known targets of WNV-neutralizing antibody, and SVP formation may allow for the authentic antigenic sites constrained by presentation in the icosahedral structure to efficiently induce the relevant antibody specificity.

The T cell immune response and neutralizing antibody elicited by this WNV DNA vaccine further suggests that needle-free immunization of DNA plasmids can reproducibly elicit significant cellular and humoral immune responses and merits further clinical investigation. The consistent humoral immunity elicited by the WNV DNA vaccine described here likely reflects a combination of factors, including in vivo SVP formation, optimization of vector design, the purity of the DNA product, delivery, and sensitive immunological assays. This clinical trial represents the first report of neutralizing antibody activity elicited by a DNA vaccine in humans. Although further studies are needed, including investigation of the possibility of dose reduction, this level of neutralizing antibody activity is known to be protective

in animal models of infection [19] and suggests that DNA plasmid immunization is a promising vaccine approach for the prevention of WNV infection. Correlates of protection from animal models of infection are essential to determine the efficacy of candidate vaccines designed to prevent emerging infectious diseases. Established correlates of protection applied to the US Food and Drug Administration Animal Rule could potentially be used to license a vaccine for the prevention of WNV infection in humans and to establish efficacy for licensure of vaccines against other emerging and reemerging infectious diseases.

VACCINE RESEARCH CENTER (VRC) 302 STUDY TEAM

The VRC 302 Study Team includes Brenda Larkin, Margaret McCluskey, LaSonji Holman, Laura Novik, Pamela Edmonds, LaChonne Stanford, Woody Dubois, Tiffany Alley, Erica Eaton, Sandra Sitar, Ericka Thompson, Andrew Catanzaro, Joseph Casazza, Laurence Lemiale, and Rebecca Sheets.

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Enhancing biosynthesis and secretion of premembrane and envelope proteins by the chimeric plasmid of dengue virus type 2 and Japanese encephalitis virus

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Abstract

We have constructed a series of plasmids encoding premembrane (prM) and envelope (E) protein genes of dengue virus type 2 (DEN-2). These plasmids included an authentic DEN-2 prM-E construct (pCBD2-14-6), and two chimeric constructs, 90% DEN-2 E-10% Japanese encephalitis (JE) virus E (pCB9D2-1J-4-3) and 80% DEN-2 E-20% JE E (pCB8D2-2J-2-9-1). Monoclonal antibody (MAb) reactivity indicated that all three plasmids expressed authentic DEN-2 virus E protein epitopes representative of flavivirus domains 1, 2, and 3. However, only the pCB8D2-2J-2-9-1 construct secreted high levels of prM, M (membrane), and E proteins into the culture fluid of plasmid-transformed COS-1 cells. The major portion of the prM and E proteins expressed by COS-1 cells transformed by pCBD2-14-6 or pCB9D2-4-3 plasmids remained membrane-bound. The results supported the notion that an unidentified membrane retention sequence is located between E-397 and E-436 of DEN-2 virus E protein. Replacing the carboxyl-terminal 20% of DEN-2 E (397–450) with the corresponding JE sequence had no effect on anti-DEN-2 MAb reactivity, indicating that this region is antigenically inert, although it is required for antigen secretion. Plasmid pCBD2-2J-2-9-1, which expressed secreted forms of prM/M and E that have the potential to form subviral particles, was superior to other constructs in stimulating an antibody response. Ninety percent neutralization titers ranging from 1:40 to >1:1000 were observed in seven of nine serum specimens from pCB8D2-2J-2-9-1-immunized mice. Eleven of twelve 2-day-old neonatal mice, derived from a pCB8D2-2J-2-9-1 immunized female mouse, survived intraperitoneal challenge of DEN-2 New Guinea C virus.

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Introduction

¹Dengue (DEN) fever, an acute infection that occurs in subtropical and tropical areas, is the most important arthropod-borne viral disease of humans (Monath, 1994). DEN viruses belong to the family of *Flaviviridae*, genus *flavivirus*. There are four distinct DEN virus serotypes: DEN-1, -2, -3, and -4. Infection with any of these viruses may be

asymptomatic, or cause a self-limited febrile illness known as dengue fever (DF). In a small percentage of cases, however, infection results in a life-threatening dengue hemorrhagic fever or dengue shock syndrome (DHF/DSS). There are approximately 100 million cases of DF occurring annually worldwide and an estimated 500,000 hospitalized DHF/DSS reported. A safe and effective DEN vaccine is essential to provide protection for all four virus serotypes in DEN endemic and epidemic regions of the world. A live-attenuated DEN vaccine has shown promising results in phase 1 clinical trials (Bhamarapravati and Sutee, 2000; Kanesthasan et al., 2001). The reverse genetic technique using infectious cDNA clones derived from yellow fever (YF)

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vaccine virus (17D), attenuated DEN-2 vaccine virus (PDK-53), and genetically engineered, attenuated DEN-4 (814669) has greatly increased the potential of creating a genetically engineered, live-attenuated chimeric DEN vaccine (Blaney et al., 2001; Guirakhoo et al., 2000; Huang et al., 2000).

The use of DNA-based vaccines is a novel and promising immunization approach for the development of flavivirus vaccines (Chang et al., 2001). Few of the flavivirus DNA vaccines have been evaluated in the experimental animals other than mice. Candidate DNA vaccine for Russian spring-summer encephalitis, Central European encephalitis, and DEN-1 viruses have been evaluated in nonhuman primates (Kochel et al., 2000; Raviprakash et al., 2000; Schmaljohn et al., 1999). Japanese encephalitis (JE) virus DNA vaccine was evaluated in swine (Konishi et al. 2000b). Recently, we reported the development of highly immunogenic, recombinant DNA vaccines for JE and West Nile (WN) viruses that induced protective immunity in mice following a single intramuscular (im) injection (Chang et al., 2000; Davis et al., 2001). The recombinant WN virus construct also induced protective immunity in horses following a single im injection (Davis et al., 2001).

The protective immunity induced after DNA vaccination may correlate with the ability of plasmid DNA to direct synthesis of secreted premembrane/membrane (prM/M) and envelope (E) proteins by the host cells. COS-1 cells transformed with tick-borne encephalitis (TBE), JE or WN plasmids secreted the TBE, JE or WN prM/M and E proteins in the form of extracellular subviral particles (EPs) into the culture fluid (Allison et al., 1999; Davis et al., 2001; Hunt et al., 2001). The highly immunogenic, noninfectious EPs have potential not only as biosynthetic subunit vaccines but also as serodiagnostic antigens to replace virus-infected suckling mice brain preparations (Davis et al., 2001; Hunt et al., 2001; Konishi and Fujii, 2002; Konishi et al., 1997, 2001). In the present study, we have identified a potential membrane retention region between E-397 and E-436 of DEN-2 virus E protein. Replacing DEN-2 virus E-436 to E-450 with the corresponding E region of JE virus to construct a chimeric plasmid containing 90% DEN-2 E and 10% JE E gene had only minor effect on the prM and E secretion. However, the DEN-2-JE virus chimeric plasmid, consisting of the transmembrane signal sequence of JE virus, followed by prM and 80% E (E-1 to 397) of DEN-2, and 20% E of JE virus (corresponding to DEN-2 E-397 to E-450), secreted prM/M and E proteins efficiently in plasmid transformed COS-1 cells. The immune response in mice after im immunization of DEN-2 plasmids correlated with the efficiency of prM/M and E secretion. Only the construct efficiently secreting prM/M and E antigens had the capacity to stimulate high-titered neutralizing (Nt) antibodies in plasmid-vaccinated mice.

Results

Transient expression of DEN-2 virus recombinant antigen

Expression of the prM and E genes of DEN-2 virus or a chimeric E gene from a combination of DEN-2 and JE virus sequences (80% DEN-2/20% JE or 90% DEN-10% JE) was accomplished by separate transformations of each of the three recombinant DEN-2 DNA plasmids into COS-1 cells. The basic plasmid design was based on results from previous studies with JE virus and WN virus recombinant plasmids in which plasmid-transformed cells expressed and secreted authentic viral proteins into the cell culture fluid (Chang et al., 2000; Davis et al., 2001). Transient expression of DEN-2 recombinant proteins was initially assessed by Ag-capture ELISA of cell-culture supernatants and by immunofluorescence antibody assay (IFA) of acetone-fixed, transformed COS-1 cells (Chang et al., 2000). The optimum antigen expression was determined to be 48 h following electroporation.

Epitope mapping of the E protein expressed by transiently transformed COS-1 cells

The DEN-2 protein expressed by each of the recombinant plasmids was evaluated by IFA using a panel of murine monoclonal antibodies (MAbs) with known reactivity to DEN-2 virus (Table 1) (Henchal et al., 1985; Roehrig et al., 1998). The MAb panel included antibodies reactive with each of the three antigenic domains of the E protein of flaviviruses as well as prM and C proteins (Guirakhoo et al., 1989; Rey et al., 1995). The MAbs specific for flavivirus antigenic Domains 2 and 3 showed nearly identical qualitative reactivity with DEN-2 virus and each of the three plasmid-expressed proteins. Domain 1 specific MAb 1B4C-2 also showed a similar reactivity pattern with all expressed proteins. However, two of the Domain 1 specific MAbs, 2B3A-1 and 9A4D-1, were much less reactive with E protein expressed by plasmids pCBD2-14-6 and pCB9D2-1J-4-3 as shown by endpoint titration (values in parentheses, Table 1). Comparison of the endpoint titers revealed the apparent poor expression of epitopes C3 and C4 in constructs containing 100% DEN-2 E and 90% DEN-2 E-10% JE E. MAb 2H2, specific for prM, had the same reactivity with antigen expressed by all three plasmids. Anti-C MAb 1A2A-1 reacted well with DEN-2 virus and had low-level, nonspecific reactivity with the plasmid-expressed viral proteins, which included prM and E, but not C.

Comparison of secreted protein and membrane-bound protein produced by each of the three DEN-2 recombinant plasmids

Similar amounts of cell-culture fluid were harvested from COS-1 cells 48 h posttransformation for each recombinant DEN-2 plasmid. The DEN-2 viral antigens secreted

Table 1

Characterization of DEN-2 E glycoprotein epitopes expressed by the recombinant DEN-2 plasmids as determined by the indirect immunofluorescence antibody assay (IFA)

Antibody	Antigenic domain ^c	PRNT ^d	Controls ^a		Plasmid construct ^a		
			DEN-2-infected cells	Normal cells	pCBD2-14-6 (100D2-E)	pCB9D2-1J-4-3 (90D2-E-10JE-E)	pCB8D2-2J-2-9-1 (80D2-E-20JE-E)
4G2	2	+/-	4+	—	4+	4+	4+
6B6C-1 (A1)	2	+/-	4+	—	4+	4+	3–4+
4E5 (A2)	2	Yes	3+	—	3–4+	3–4+	2–3+
1B7 (A5)	2	Yes	3–4+	—	4+	4+	2–3+
1B4C-2(C1)	1	No	3–4+ (8000)	—	2–3+ (4000)	2–3+ (4000)	2–3+ (8000)
2B3A-1 (C3)	1	No	3–4+ (≥3200)	—	3+ (100)	2+ (100)	2–3+ (≥3200)
9A4D-1 (C4)	1	No	3–4+	—	2–3+ (400)	1–3+ (400)	3+ (≥12800)
3H5 (B2)	3	Yes	4+	—	4+	4+	4+
10A4D-2 (B3)	3	Yes	2–3+	—	3–4+	3–4+	2–3+
1A1D-2 (B4)	3	Yes	4+	—	3–4+	4+	3–4+
9D12-6	?	Yes	2–4+	—	2–3+	2–3+	3–4+
2H2	prM	No	4+	—	4+	3–4+	3–4+
1A2A-1	Capsid	No	2–3+	—	1+	2+	1–2+

^a IFA substrates were acetone-fixed COS-1 cells, infected with DEN-2 16681, uninfected controls, or transformed with a DEN-2 recombinant plasmid.

^b Monoclonal antibodies were used at a predetermined optimum dilution based on reactivity with DEN-2 16681 virus. For some MAbs, endpoint titers, shown in parentheses, are reported and for others, only qualitative values are reported based on a scale from 1+ to 4+, with 3–4+ considered positive, 2+ equivocal, and 1+ negative.

^c Antigenic domains based on the E-glycoprotein of TBE virus (12, 30).

^d Plaque-reduction neutralization activity at a 1:100 dilution of ascitic fluid, using a 90% plaque-reduction endpoint, except for 4G2 and 9D12-6, for which a 50% neutralization endpoint is reported (14, 33).

into untreated culture supernatants were first assessed by Ag-capture ELISA (Table 2). The cells transformed with 100% DEN-2 E and 90% DEN-2 E-10% JE E plasmids did not secrete DEN-2 viral antigens in replicate transformation experiments, thus, were ELISA-negative in unconcentrated culture supernatants. However, cells transformed with the 80% DEN-2 E-20% JE E plasmid secreted DEN-2 viral antigens and demonstrated ELISA titers between 1:8 and 1:32 in different transformation experiments. To increase

the antigen concentration that may be undetectable by either Ag-capture ELISA or Western blot, an equivalent volume of the culture fluid from each plasmid was concentrated 100-fold by polyethylene glycol (PEG) precipitation, followed by ethanol extraction to remove PEG, which interfered with subsequent analysis by polyacrylamide gel electrophoresis. The relative amount of secreted antigen expressed by each plasmid was determined by Ag-capture ELISA analysis of both PEG-precipitated and ethanol-extracted cell-culture

Table 2

Detection of secreted and membrane-bound DEN-2 recombinant protein by antigen-capture ELISA

Plasmid	Sample type	Endpoint ELISA titer
pCBD2-14-6 (100D2-E)	Culture fluid	<1:1
	PEG-precipitated culture fluid ^a	<1:10
	PEG-precipitated, ethanol-extracted culture fluid ^b	<1:20
	Hydrophobic membrane protein preparation ^c	1:160
pCB9D2-1J-4-3 (90D2-E-10JE-E)	Culture fluid	<1:1
	PEG-precipitated culture fluid ^a	<1:10
	PEG-precipitated, ethanol-extracted culture fluid ^b	<1:20
	Hydrophobic membrane protein preparation ^c	1:80
pCB8D2-2J-2-9-1 (80D2-E-20JE-E)	Culture fluid	1:8–1:32
	PEG-precipitated culture fluid ^a	1:640
	PEG-precipitated, ethanol-extracted culture fluid ^b	1:80
	Hydrophobic membrane protein preparation ^c	1:80
pEGFP	Culture fluid	<1:1
	PEG-precipitated culture fluid ^a	<1:10
	PEG-precipitated, ethanol-extracted culture fluid ^b	<1:10
	Hydrophobic membrane protein preparation ^c	<1:10

^a Culture supernatant from plasmid-transformed cells was precipitated with 10% polyethylene glycol (PEG) and resuspended in 1/100th of original volume.

^b PEG-precipitated culture supernatant was extracted with 4% ethanol to remove PEG and the pellet was resuspended in 1/5 of the volume extracted.

^c Hydrophobic membrane fractions were prepared as described under Materials and methods.

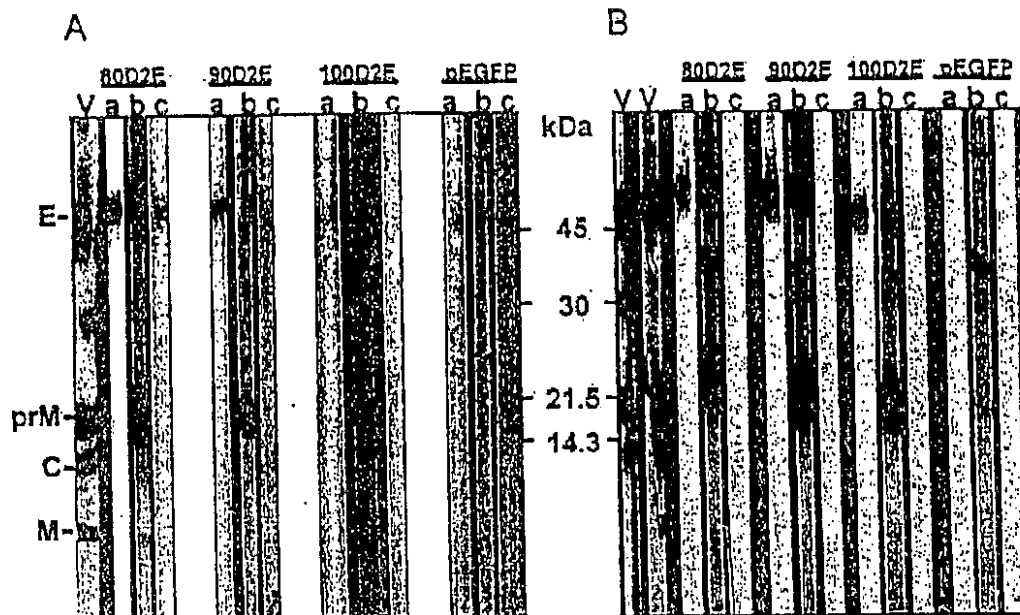


Fig. 1. Comparison of secreted and membrane-bound recombinant protein by Western blot. (A) Analysis of secreted recombinant antigen following PEG-precipitation and ethanol extraction of culture fluid for DEN-2 plasmids pCB8D2-2J-2-9-1 (80D2E), pCB9D2-1J-4-3 (90D2E), and pCBD2-14-6 (100D2E), and control plasmid pEGFP. Lane 1 (V), purified DEN-2 virus stained by Gold Blot (Owl Separation Systems, Portsmouth, NH). Reactivity of secreted, recombinant antigen from each plasmid with (a), anti-envelope (E) specific MAb 1A6A-8; (b), a mixture of MAb 1A6A-8, anticapsid (C) specific MAb 1A2A-1, antiserum specific for a DEN-2 virus membrane (M) protein peptide (M protein aa 1-34), and antiserum specific for DEN-2 virus premembrane (prM) protein; and (c), normal mouse ascites. (B) Analysis of recombinant plasmid-transformed cell hydrophobic membrane proteins. Lane 1 (V), purified DEN-2 virus stained by Gold Blot; Lane 2 (V), reactivity of purified DEN-2 virus with a mixture of MAb 1A6A-8, MAb 1A2A-1, antiserum specific for DEN-2 virus M protein, and antiserum for DEN-2 virus prM protein. Reactivity of isolated hydrophobic membrane proteins from each plasmid-transformed cell line with (a), MAb 1A6A-8; (b), a mixture of MAb 1A6A-8, MAb 1A2A-1, antiserum specific for DEN-2 virus M protein, and antiserum for DEN-2 virus prM protein; and (c), normal mouse ascites.

fluid preparations (Table 2). Secreted antigen was detected only from cells transformed with pCB8D2-2J-2-9-1, which contained 80% DEN-2 E and 20% JE E genes. The recombinant plasmids containing either 100% DEN-2 E or 90% DEN-2 E-10% JE E genes produced no ELISA-detectable antigen in the culture fluid, despite efforts to concentrate expressed protein.

Western blot analysis was also used to evaluate the production of secreted antigen by each of the DEN-2 recombinant plasmids. For comparison purposes, equivalent volumes of PEG-precipitated, ethanol-extracted cell-culture supernatant were run on NuPAGE gradient gels, electroblotted to nitrocellulose, and analyzed using MAbs or polyclonal antisera capable of reacting with all DEN-2 structural proteins (Fig. 1A). Western blot analysis showed greater sensitivity in detecting recombinant antigen than Ag-capture ELISA since DEN-2-specific proteins were detected in the culture fluid from two of the plasmids, pCB8D2-2J-2-9-1 and pCB9D2-1J-4-3. Plasmid pCB8D2-2J-2-9-1 expressed the greatest amount of secreted antigen which was shown to be composed of E, prM, and M proteins. The amount of M protein seemed to be variable for replicate transformations, perhaps due to varying efficiency of prM processing (data not shown). Relatively less secreted antigen was produced by pCB9D2-1J-4-3, and barely

detectable levels were found for the pCBD2-14-6 preparation, which appeared to contain relatively less expressed E protein, especially if the nonspecific reactivity of the E-specific MAb, 1A6A-8, on the control pEGFP was taken into consideration (Fig. 1A, lanes a, b for 14-6 and GFP).

Since E, prM, and M are membrane-associated proteins throughout their intracellular synthesis, any assessment of the expression of these proteins by the three recombinant DEN-2 plasmids should include an evaluation of cell-membrane preparations from plasmid-transformed cells. The Mem-PER Mammalian Membrane Protein Extraction Reagent Kit (Pierce, Rockford, IL) was used to isolate the integral membrane proteins from equivalent numbers of transformed cells for each recombinant plasmid. Hydrophobic proteins were separated from hydrophilic proteins by phase partitioning. Preliminary analysis by Ag-capture ELISA indicated that the hydrophilic protein fraction was nonreactive (data not shown); however, the hydrophobic protein fractions from COS-1 cells transformed with each of the recombinant DEN-2 plasmids had similar titers in ELISA tests (Table 2). These results indicated that recombinant antigen was expressed by all three plasmids following transformation, but was not always secreted.

Confirmation of the ELISA results for the hydrophobic

Table 3
Immunogenicity of three DEN-2 recombinant plasmids in ICR mice

Plasmid DNA ^b	Mouse No.	ELISA on DEN-2 virus				PRNT on DEN-2 virus ^a Endpoint titer 9 wks. p.v.	PRNT on JE virus ^a Endpoint titer 9 wks. p.v.	
		Screen 3 wks. p.v. ^c		Screen 6 wks. p.v. ^c				
		1:100	1:400	1:100	1:400			
pCB8D2-2J-2-9-1 (80D2-E-20JE-E)	Pool, 1,2,4-10	ND ^d	ND	+	+	64,000	ND	ND
	1	+	+	+	+	64,000	>1000	<2
	2	+	+	+	+	32,000	>1000	<2
	4	+	+	+	+	16,000	200	<2
	5	+	+	+	+	4000	<10	<2
	6	+	+	+	+	16,000	200	<2
	7	+	-	+	+	64,000	100	<2
	8	+	-	+	+	8000	40	<2
	9	+	+	+	+	6400	<2	<4
	10	+	+	+	+	64,000	>1000	<2
pCB9D2-1J-4-3 (90D2-E-10JE-E)	Pool, 1-10	ND	ND	+	+	1000	ND	<2 ^e
	1	-	-	+	-	400	<10	ND
	2	+	-	+	+	200	<10	ND
	3	+	+	+	+	4000	<2	≤4
	4	+	-	+	-	200	<10	ND
	5	-	-	+	+	400	<10	ND
	6	+	+	+	+	4000	<2	2
	7	-	+/-	-	-	100	<10	ND
	8	-	-	-	-	200	<10	ND
	9	+	-	+	-	4000	<2	<2
pCBD2-14-6 (100D2-E)	Pool, 1-10	ND	ND	+	-	200	<2 ^f	<2 ^g
	1	-	-	-	-	400	<10	ND
	2,3,6-9	-	-	-	-	<100	ND	ND
	4	+	+	+	+	1000	<2	<2
	5	-	-	+	-	2000	8	<2
	10	+	-	-	-	<100	ND	ND
PEGFP	Pool, 1-10	-	ND	-	ND	<100	<2	<2

^a PRNT, plaque-reduction neutralization test, 90% neutralization endpoint.

^b Mice were immunized intramuscularly with 100 µg plasmid DNA on weeks 0 and 3.

^c ELISA screens used sera diluted 1:100 and 1:400.

^d ND, not done.

^e Pool, 1, 2, 4, 5, 7, 8.

^f Pool, 2, 3, 6–10.

^g Pool, 1–3, 6–10.

protein fractions was accomplished by Western blot (Fig. 1B). Equivalent volumes of hydrophobic protein fractions from each of the plasmid-transformed cells were diluted according to the manufacturer's recommendations for SDS–polyacrylamide gel electrophoresis to reduce band and lane distortion. Immunoblotting with E-, prM-, C-, and M-specific MAbs or polyclonal antisera showed that all three recombinant DEN-2 plasmids produced similar amounts of recombinant antigen composed of E and prM. No M protein was detected, either because it was not processed from prM or because the levels were too low to be detected. Despite efforts to reduce band distortion, high levels of detergent in the hydrophobic protein samples apparently caused E and prM to run in a slightly aberrant manner (slower migration) compared to samples without such high concentrations of detergents (compare E and prM migration in Figs. 1A and 1B).

Comparison of the immune response in mice vaccinated with three different DEN-2 recombinant DNA plasmids

To compare the extent and functionality of antibody responses stimulated by DNA vaccination, 3-week-old ICR mice were immunized by im injection with 100 µg of either pCB8D2-2J-2-9-1, pCB9D2-1J-4-3, pCBD2-14-6, or pEGFP on weeks 0 and 3. Outbred mice were selected for this study to provide the genetic heterogeneity expected in nonhuman primates planned for future study. Mice were bled 3, 6, and 9 weeks after the primary immunization. Individual and pooled sera were tested by indirect ELISA, using screening dilutions of 1:100 and 1:400 at 3 and 6 weeks postvaccination and endpoint titrations at 9 weeks postvaccination. Nine-week sera were also tested by plaque reduction neutralization test (PRNT) with both DEN-2 and JE viruses. The ELISA results showed that after one immu-

nization (3-week sera) all mice given pCB8D2-2J-2-9-1 had seroconverted, whereas only 50% of pCB9D2-1J-4-3- and 20% of pCBD2-14-6-vaccinated mice reacted with DEN-2 virus (Table 3). By 9 weeks postvaccination, all mice vaccinated with either pCB8D2-2J-2-9-1 or pCB9D2-1J-4-3 demonstrated anti-DEN-2 ELISA reactivity; however, the geometric mean titers were significantly different, 1:20,000 vs 1:708, respectively. Only 40% of pCBD2-14-6-immunized mice had anti-DEN-2 ELISA titers greater than 1:100. A Western blot of pooled 9-week sera from pCB8D2-2J-2-9-1-immunized mice on purified DEN-2 virus showed that the immunodominant response was to the E glycoprotein, with only slight reactivity detected to prM and M (data not shown).

An even more significant result in terms of evaluating the vaccine potential of the three DEN-2 plasmids was the induction of virus-Nt antibody in seven of nine mice immunized with pCB8D2-2J-2-9-1. The titers reported are based on a 90% plaque-reduction endpoint (Table 3); however, if a 70 or 50% neutralization endpoint is used, nine of nine sera have PRNT titers of $\geq 1:2$ or $\geq 1:40$, respectively (data not shown). Ninety percent neutralization titers ranged from 1:40 to $>1:1000$ for the seven sera with Nt activity. None of the mice immunized with pCB9D2-1J-4-3 produced Nt antibody, and only 1 of 10 sera from pCBD2-14-6-vaccinated mice neutralized virus, but at a titer of only 1:8. We did not challenge these vaccinated mice with DEN-2 virus since 12-week-old ICR mice are resistant to DEN-2 virus challenge by either intraperitoneal (ip) or intracranial route. For the purpose of comparison, mice challenged with DEN-2 New Guinea C strain by the ip route had a mean neutralization titer of 1:10 (Konishi et al., 2000a).

Since two of the recombinant plasmids contained JE virus E-gene sequences, all sera were evaluated for JE virus Nt activity; however, no activity was detected at the 90% neutralization endpoint for mice in any of the immunization groups. Mice immunized with the control plasmid pEGFP showed no reactivity to either DEN-2 or JE viruses.

Passive protection of neonatal mice derived from pCB8D2-2J-2-9-1-vaccinated female

Female ICR mice were vaccinated with one dose of 100 $\mu\text{g}/100 \mu\text{l}$ of pCB8D2-2J-2-9-1 plasmid DNA at the age of 3 weeks. For evaluation of passive protection by maternal antibody, pups were obtained from a female that had Nt titer of 1:160 at 9-weeks postvaccination. Pups were challenged ip at 2 days after birth with 50,000 PFU/50 μl of NGC virus. Pups from an unvaccinated female, challenged at 1 day after birth with 50,000 PFU/50 μl of NGC virus, were used as unvaccinated controls. Eleven of 12 pups nursed by the vaccinated mother (PRNT titer of 1:160) survived viral infection, whereas none of 12 pups, receiving 50,000 PFU virus, from the control mother survived. Limited DEN-2 virus replication occurred in the survived pups since DEN-2

ELISA antibody (1:100) was detected in the serum of all survived pups at 25 days postchallenge.

Discussion

The use of plasmid vectors to express prM/M and E proteins that are able to stimulate a protective immune response is a novel and promising approach for the development of flavivirus vaccines (Chang et al., 2001). It is also an important step toward the development of a stably transformed cell line that constitutively produces and secretes noninfectious subviral particles as the potential source of a biosynthetic subunit vaccine and serodiagnostic antigen. Vaccination with the recombinant plasmid results in endogenous expression of prM and E proteins and stimulates anti-prM and E antibody responses in the host. We have designed a recombinant eukaryotic expression plasmid that contains optimized transcriptional and translational elements that enhanced the expression and secretion of JE and WN virus prM/M and E proteins into the culture medium of transiently transformed cells (Chang et al., 2000; Davis et al., 2001). Initially, the same strategy was applied to construct a recombinant DEN-2 plasmid, pCBD2-14-6, consisting of the authentic DEN-2 prM and E gene regions. Antigenic mapping of DEN-2 proteins expressed by the transformed COS-1 cells indicated that the prM and E proteins had a similar fluorescence intensity and MAb reactivity compared to virus-infected cells (Table 1). However, the staining pattern observed with plasmid-transformed cells was a globular pattern of intensely stained, large inclusion bodies as compared to the virus-infected cells, which showed a more evenly dispersed or diffused staining pattern.

Surprisingly, these pCBD2-14-6-transformed COS-1 cells failed to secrete a DEN-2 antigen detectable by Ag-capture ELISA, and plasmid vaccination failed to stimulate anti-DEN-2 virus Nt antibody in immunized mice (Table 3). Studies with recombinant vaccinia viruses expressing C-terminally truncated E proteins suggested that the first 392 amino acids of the DEN-4 virus E sequence are critical in maintaining proper antigenic structure of the E protein and that the C-terminal 20% of DEN-4 E contains a membrane retention signal (Men et al. 1991). A similar membrane retention signal may also present in the C-terminal region of DEN-2 E and could have caused an intracellular accumulation of viral protein and a resultant pattern of globular fluorescent staining in the pCBD2-14-6-transformed, acetone-fixed cells. This IFA staining pattern was not observed in the JE or WN recombinant plasmid-transformed cells (Chang et al., 2000; Davis et al., 2001). We, therefore, constructed two additional plasmids, pCB9D2-1J-4-3 and pCB8D2-2J-2-9-1, for which 10 or 20% of the C-terminal DEN-2 E was replaced with the corresponding region of JE virus E protein. The pCB9D2-1J-4-3 plasmid secreted very low levels of prM and E proteins, detectable

only by Western blot in transiently transformed COS-1 cells; only pCB8D2-2J-2-9-1-transformed cells secreted DEN-2 virus prM/M and E proteins that could be detected both by Ag-ELISA and Western blot (Fig. 1 and Table 2).

Particle assembly and secretion is influenced by prM and E interaction. Studies on TBE virus have indicated that interactions between prM and the ectodomain of E are involved in prM-mediated intracellular transport of prM-E heterodimers. Interactions between prM and both a predicted stem region (H2) and a first transmembrane region (TM1) of E are factors stabilizing the prM-E interaction, presumably leading to particle formation (Allison et al., 1999). Replacement of the C-terminal 20% of DEN-2 E with the corresponding JE E sequence to construct pCB8D2-2J-2-9-1 resulted in DEN-2 prM and chimeric E secretion, most likely in a particulate form [based on the immunogenic quality of this antigen (Table 3)]. The major portion of the prM and E proteins expressed by pCBD2-14-6- and pCB9D2-4-3-transformed COS-1 cells remained membrane-bound (Table 2 and Fig. 1). This result substantiated the observation that a membrane retention sequence, similar to an unidentified sequence in DEN-4 virus E, is also present in the C-terminal stem region of the DEN-2 E protein. It also provided evidence that H2 and TM1 of JE E could interact with DEN-2 prM, perhaps stabilizing the prM-E interaction leading to prM/M and E protein secretion. The prM processing resulted in M protein-containing virion occurs at the latest stage during flavivirus maturation.

The prM protein is essential for maintaining proper conformation and secretion of the E protein during prM-E maturation (Aberle et al., 1999; Allison et al., 1995). For Murray Valley encephalitis virus, the ectodomain of E protein interacting with prM has been estimated to be between amino acids 200 and 327 (Guirakhoo et al., 1992). Recombinant vaccinia viruses expressing C-terminally truncated E proteins suggest that the first 392 amino acids of the DEN-4 virus E sequence is required for proper antigenic structure (Men et al., 1991). We believe that proper prM and E interactions as well as the structural integrity of E are maintained in the protein expressed by all three DEN-2 constructs. Replacement of the C-terminal 20% of E in pCB8D2-2J-2-9-1, conserving 397 amino acids of authentic DEN-2 E, was expected to have a minimum effect on the antigenic structure of E and to preserve prM-E interactions. Interactions of the prM and stem-anchor region of E are not virus-species specific. Replacing the stem-anchor region of DEN-2 E with the corresponding JE sequence had no effect on MAb reactivity (Table 1), indicating that this region is antigenically inert, but is required for prM/M and E secretion, and possibly assembly of prM/M-E containing EPs.

The plasmid construct responsible for secreting EPs of TBE virus prM and E protein was superior to constructs producing primarily intracellular or soluble forms of E protein in terms of extent and functionality of the antibody response (Aberle et al., 1999). Here we demonstrated that the ability of DEN-2 plasmid DNA to stimulate Nt antibody

was correlated with the secretion of prM/M and E (Fig. 1, Tables 2 and 3). The morphology and physical character of secreted prM and E were not investigated in this study. It is likely that prM/M and E secreted by the pCB8D2-2J-2-9-1 construct forms EPs, which represent a potent immunogen with multiple, protective antigens presented on the particle surface.

Previous attempts to develop a DEN-2 virus DNA vaccine has resulted in various degrees of success (Kochel et al., 1997; Konishi et al., 2000a; Simmons et al., 2001). Vector components in those studies are similar to the present study, in which RNA transcripts of the DEN insert are regulated by the cytomegalovirus early gene promoter and bovine growth hormone poly(A) termination signal. However, the Nt-inducing efficiency of DNA vaccine in mice is significantly higher in our study than previous reports. Of the prM and E gene regions reported in the previous publications, one construct is similar to our 100% DEN-2 plasmid (Konishi et al., 2000a), and the other contains only 91% of the E gene (Kochel et al., 1997). Kozak's consensus sequence used in our construct could enhance the translation efficiency of the DEN-2 insert (Chang et al., 2000). The transmembrane signal peptide sequence (derived from JE virus) used in our construct has improved cleavage probability and could influence the proper translocation as well as processing of prM/M and E proteins. Signal peptide differences in various constructs may account for the difference in protein translocation, cleavage site presentation, and correct topology of prM and E proteins (Chang et al., 2001). However, it is highly probable that the higher Nt-inducing efficiency of the 80% DEN-2 E construct in mice in the present study is due to the synergistic effects of transcriptional and translational elements that increase both the secretability and the particle formation of DEN-2 prM and E proteins. Coimmunization of the immunostimulatory CpG motif-containing pUC19 plasmid, including plasmid-expressing murine GM-CSF in the vaccine regimen, or replacing the C-terminal 43 amino acids of E with lysosome-associated membrane retention sequence, improved the antibody response to the DEN-2 vaccine (Porter et al., 1998; Raviprakash et al., 2001).

Age-dependent resistance to viral infection in outbred mice has been observed in flaviviruses, including four serotypes of DEN, YF, St. Louis encephalitis, and JE viruses. The development of a consistent challenge protocol to address protective efficacy is essential for future multivalent DNA vaccine trials in mice. Previously, we observed that maternal antibodies could provide passive protection in neonatal mice from JE virus induced encephalitis by ip challenge (Chang et al., 2000). The protective efficacy in the baby correlated with the mother's serum Nt antibody titer. A similar strategy of immunizing female mice with the 80% DEN-2 E-20% JE E construct indicated that, indeed, maternal antibody capable of Nt DEN-2 virus could also provide passive protection in neonatal mice from DEN-2 virus ip challenge. Thus, enhancement of the vaccine potential of

Table 4

Oligonucleotides used to construct DEN-2 virus prM-E expression plasmids, and the junction region of chimeric DEN-2 and JE E indicated

100% DEN-2-prM-E: D2KasI-438 ^a CD2NotI-2402		5' TGTGCA <u>GGCGGCTT</u> CCATTTAACCAACGTAACG 5' TCGA <u>GGCGGCGCTT</u> CAACTAATTAGGCCTGCACCATGACTC	
90% DEN-2-E & 10% JE-E: T7 CD2BstXI-2244 JE-2281 CSP6		5' CTTATCGAAATTAATACGACTCACTATAGG 5' ATAGATTGCT <u>CCAAACACTTGGTGG</u> 5' ACTCCATAGGAAAAGCCGTTCAACC 5' GCGAGCTCTAGCATTTAGGTGACACTATAG	
90–10 Junction:	Leu His Gln Val Phe Gly Gly Ala Phe Arg Thr CTC CAC CAA GTG TTT GGT GGT GCC TTC AGA ACA	DEN-2← →JE	
80% DEN-2-E & 20% JE-E: T7 CD2BsmBI-2097 JEBsmBI-2175 CSP6		5' CTTATCGAAATTAATACGACTCACTATAGG 5' GAATT <u>CGTCTC</u> ACTTCCTTTCTTAAACCAAGTTGAGCTTC 5' GGAATT <u>CGTCTC</u> CGGAAGCACGCTGGGCAAGG 5' GCGAGCTCTAGCATTTAGGTGACACTATAG 3'	
80–20 Junction:	Asn Trp Lys Lys Gly Ser Thr Leu Gly Lys Ala AAC TGG TTT AAG AAA GGA AGC ACG CTG GCC GCC	DEN-2← →JE	

^a Restriction enzyme sites encoded in oligonucleotides were highlighted by bold, italics, and underlined functions.

flavivirus DNA can be achieved by manipulation of an expression plasmid for enhancing transcription and translation, promoting correct polyprotein processing and assembly, and targeting prM/M and E proteins for secretion.

We have constructed recombinant plasmids which secrete prM and E proteins for DEN-1 and DEN-3, similar to the 80% DEN-2 E construct in this study (G. J. Chang, unpublished results). We have also established DEN-1, -2, and -3 plasmid-transformed cell lines that constitutively express and secrete prM/M and E proteins for these three viruses. Objectives of an ongoing study are to obtain both DEN-4 and YF plasmid constructs and to establish DEN-4 and YF prM/M and E antigen-producing cell lines. Using candidate DNA vaccines for the four DEN serotypes, YF, JE, and WN viruses, a future study will address the vaccine potential of the formulated combination vaccine cocktails, based on the disease prevalence in different geographic regions. Noninfectious prM/M and E antigen-producing cell lines can provide biosynthetic subunit vaccines as well as standardized, noninfectious serodiagnostic antigens.

Materials and methods

Cell culture and virus strain

COS-1 cells (ATCC, Manassas, VA; 1650-CRL) were grown at 37°C with 5% CO₂ in Dulbecco's modified Eagle's minimal essential medium (DMEM, GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone Laboratories, Inc., Logan, UT), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 20 ml/l 7.5% NaHCO₃, 100 U/ml penicillin, and 100 µg/ml streptomycin. Vero and C6/36 cells were grown

under the same conditions used for COS-1 cells. DEN-2 virus strain-16681 was used for cDNA cloning, IgG ELISA, and the PRNT. Virus was propagated in C6/36 cell culture. Virus used for immunological or biochemical studies was purified by precipitation with 7% polyethylene glycol (PEG-8000; Fisher Scientific, Fair Lawn, NJ), followed by ultracentrifugation on 30% glycerol–45% potassium tartrate gradients (Obijeski et al., 1976).

Mouse brain adapted DEN-2 New Guinea C strain virus was used in the passive challenge experiment. This strain was propagated in suckling mouse by intracranial injection, and a 10% suspension of homogenized mouse brain was used for the challenge study.

Plasmid construction

Genomic RNA was extracted from 150 µl C6/36 cell-culture medium infected with DEN-2 (16681) using the QIAamp Viral RNA Kit (Qiagen, Santa Clarita, CA). Extracted RNA, resuspended in 80 µl DEPC (Sigma, St. Louis, MO), was used as a template in a reverse transcriptase-PCR (RT-PCR) for the amplification of DEN-2 virus prM/M and E genes. Primer sequences (Table 4) were designed based on the published sequence (Kinney et al., 1997). Restriction enzyme site *KasI* was incorporated at the 5'-terminus of the cDNA amplicon. An in-frame termination codon, followed by a *NotI* restriction site, was introduced at the 3'-terminus of the cDNA amplicon. The DEN-2 virus cDNA amplicon was digested with *KasI* and *NotI* enzymes and inserted into the *KasI* and *NotI* sites of a pCBJESS vector to form the 100% DEN-2 E plasmid, pCBD2-14-6. The pCBJESS vector was derived from the pCBamp plasmid which contained the cytomegalovirus

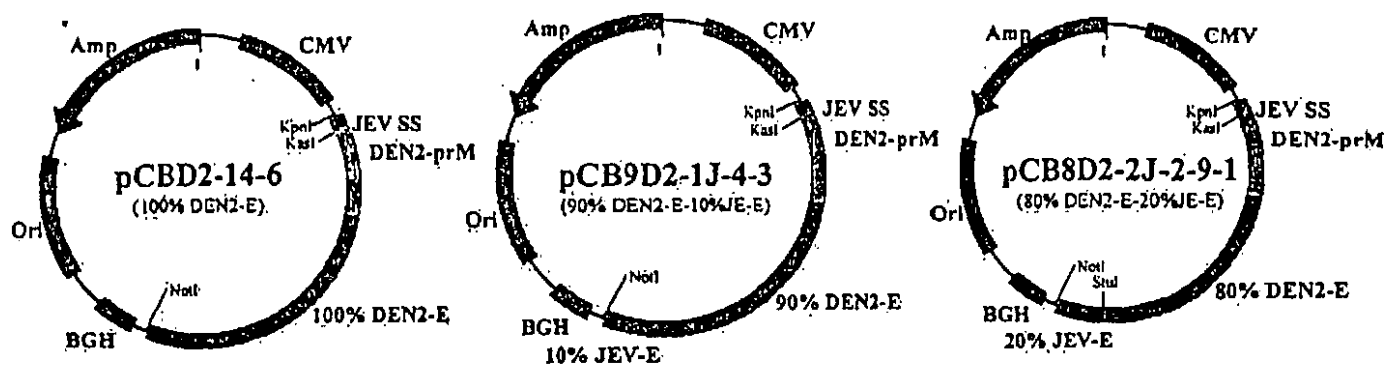


Fig. 2. Schematic representations of plasmid vectors pCBD2-14-6 (100% DEN2-E), pCB9D2-1J-4-3 (90% DEN2-E-10% JE-E), and pCB8D2-2J-2-9-1 (80% DEN2-E-20% JE-E). These plasmids include the human cytomegalovirus (CMV) early gene promoter, JE virus signal sequence, DEN-2 virus prM and E gene region (amino-terminal 100, 90, or 80%), JE virus E gene region (carboxyl-terminal 10 or 20%), and bovine growth hormone poly(A) signal (BGH).

early gene promoter, translational control element, and engineered JE signal sequence element (Davis et al., 2001).

To construct the 90 and 80% DEN-2 E plasmids, the 100% DEN-2 plasmid, pCBD2-14-6, and the JE plasmid, pCBE1-14, were used as the PCR templates to amplify DEN-2 and JE DNA sequence, respectively (Chang et al., 2000). Primer sets to obtain the DEN-2 and JE gene fragments are listed in Table 4. T7 and SP6 priming sites are found in the pCBamp plasmid, derived from original pCDNA-3 plasmid (Invitrogen, Carlsbad, CA). PCR-amplified DNA fragments for the 90% DEN-2-10% JE E gene were digested with *Bst*XI restriction endonuclease, ligated with T4 DNA ligase, digested with *Ksa*I and *Not*I enzymes, and inserted into the *Ksa*I and *Not*I sites of the pCBE1-14 vector to obtain the plasmid pCB9D2-1J-4-3. PCR-amplified DNA fragments for the 80% DEN-2-20% JE E gene were digested with *Bsm*BI, ligated with T4 DNA ligase, digested with *Ksa*I and *Not*I enzymes, and inserted into the *Ksa*I and *Not*I sites of the pCBE1-14 vector to obtain the plasmid pCB8D2-2J-2-9-1. Schematic representations of the three plasmid constructs are shown in Fig. 2. The 90% DEN-2-10% JE E and the 80% DEN-2-20% JE E junction regions are shown in Table 4.

Automated DNA sequencing was performed on an ABI Prism 377 Sequencer (Applied Biosystems/Perkin-Elmer, Foster City, CA) according to the manufacturer's recommended procedures. The recombinant plasmids that had correct prM and E sequences were identified.

Transient expression of DEN-2 recombinant antigen in COS-1 cells by electroporation

COS-1 cells were electroporated separately with each DEN-2 plasmid or green fluorescent protein expression plasmid control (pEGFP, Clontech, San Francisco, CA) using the protocol described previously (Chang et al., 2000). Electroporated cells were seeded onto 75-cm² culture flasks and incubated at 37°C with 5% CO₂. Six hours following electroporation, the growth medium was replaced with a maintenance medium containing 2% fetal bovine serum. Tissue-culture medium and cells were harvested

separately 48 h following electroporation for antigen characterization.

Epitope mapping using DEN-2 E-specific monoclonal antibodies

Forty-eight hours following electroporation, adherent cells were trypsinized, resuspended in phosphate-buffered saline solution (PBS) containing 5% goat serum, spotted on a 12-well spot-slide, and air dried. Cells adhered to the spot-slide were fixed with acetone for 10 min at -20°C and allowed to air dry. E-protein-specific monoclonal antibodies were used to detect protein expression by the indirect IFA, as described previously (Chang et al., 2000).

Characterization of the recombinant DEN-2 virus antigen

Tissue-culture medium was harvested 48 h following electroporation. Antigen-capture (Ag-capture) ELISA was used to detect secreted DEN-2 virus antigen in the culture medium of transiently transformed COS-1 cells. MAb 4G2 and horseradish peroxidase conjugated MAb 6B6C-1 were used to capture the DEN virus antigens and detect captured antigen, respectively (Chang et al., 2000; Hunt et al., 2001).

Recombinant antigen in the medium was concentrated by precipitation with 10% PEG-8000. The precipitant was resuspended in TNE buffer (50 mM Tris, 100 mM NaCl, 10 mM EDTA, pH 7.5) to 1/100th of the original volume, clarified by centrifugation, and stored at 4°C. Recombinant antigen concentrated by PEG precipitation and resuspended in TNE buffer was extracted with 4% ethanol to remove residual PEG (Hunt et al., 2001). Ethanol-extracted antigens, hydrophobic membrane proteins from the transformed cells, and gradient-purified DEN-2 virions were analyzed under non-denaturing conditions on a NuPAGE, 4-12% Bis-Tris gradient gel in an Excel Plus Electrophoresis Apparatus (Invitrogen Corp.), followed by electroblotting onto nitrocellulose membranes using a Excel Plus Blot Unit (Invitrogen Corp.). DEN-2 virus-specific protein was detected by Western blot using DEN-2 virus-specific MAbs 1A6A-8 (E-specific) and 1A2A-1 (capsid-specific), as well as rabbit antiserum-specific for DEN-2 prM and mouse

serum-specific for a peptide composed of amino acids 1–34 of the DEN-2 M protein, and normal mouse ascitic fluid as a negative control (Murray et al., 1993; Roehrig et al., 1998).

Forty-eight hours following electroporation, transformed cells for each plasmid were trypsinized and aliquots containing 5×10^6 cells were resuspended in PBS. These cell samples were processed for membrane protein extraction using the Mem-PER Mammalian Membrane Protein Extraction Reagent Kit (Pierce) following the manufacturer's protocol. Both hydrophobic and hydrophilic proteins were isolated. This procedure was developed for enrichment of integral membrane protein found in the hydrophobic phase. Both hydrophobic and hydrophilic fractions were analyzed by Ag-capture ELISA for DEN-2 recombinant antigen; the hydrophobic fraction was also examined by Western blot as described above.

Mouse vaccination and challenge

Groups of 10 three-week-old female ICR outbred mice were used in the study. Mice were injected im with pCBD2-14-6, pCB9D2-1J-4-3, pCB8D2-2J-2-9-1, or pEGFP on weeks 0 and 3 with a dose of 100 μ g in a volume of 100 μ l per mouse. The plasmid DNA was purified from XL-1 blue cells with EndoFree Plasmid Giga Kits (Qiagen) and resuspended in PBS, pH 7.5, at a concentration of 1.0 μ g/ μ l. Mice that received 100 μ g pEGFP were used as plasmid-vaccinated controls. Mice were bled every 3 weeks following injection, and the DEN-2 virus-specific antibody response was evaluated by indirect ELISA and PRNT.

For evaluation of passive protection by maternal antibody, pups were obtained from mating of nonimmunized males with immunized females 9 weeks following a single vaccination of pCB8D2-2J-2-9-1 (100 μ g/100 μ l in PBS) at 3 weeks of age. Pups from unvaccinated females were used as the challenge control. Pups were challenged, ip, at 0 to 2 days after birth with 50,000 PFU/50 μ l of NGC virus and observed daily for 3 weeks. Postchallenge serum was collected from mice that survived challenge and was screened by the IgG-ELISA at 1:100 and 1:400.

Serological tests

Pre- and postvaccination serum specimens were tested for antibody binding ability to purified DEN-2 virus by ELISA, for Nt antibody by PRNT, and for antibodies that recognize purified DEN-2 virus proteins by Western blotting. PRNT was performed with Vero cells, as previously described (Chang et al., 2000), using DEN-2 (16681) and JE (Nakayama) viruses. Endpoints were determined at a 90% plaque-reduction level.

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25

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USDA ISSUES LICENSE FOR WEST NILE VIRUS DNA VACCINE FOR HORSES

WASHINGTON, July, 18, 2005—The U.S. Department of Agriculture today announced that it has issued a license to Fort Dodge Laboratories, Inc. of Fort Dodge, Iowa, a division of Wyeth, for a vaccine to protect horses from viremia caused by the West Nile virus (WNV).

The horse vaccine, which was developed through a collaboration of the Centers for Disease Control and Fort Dodge, is the first fully licensed WNV DNA vaccine for animals in the United States. The vaccine contains genes for two WNV proteins, and therefore, does not contain any whole WNV, live or killed. Once the horse's cells begin making proteins from the virus, they trigger a protective immune response.

In granting full licensure, USDA's Center for Veterinary Biologics determined that the vaccine's safety and efficacy have been satisfactorily demonstrated. Studies looking at how long the vaccine maintains its immunity are ongoing. However, the label contains a caution that vaccinated horses may not be eligible for export, as current commercially used tests may not be able to differentiate between this vaccine, conventional vaccines and horses that have been exposed to the actual virus.

WNV, a mosquito-borne disease, was first detected in the United States in 1999. The virus, which can cause encephalitis or inflammation of the brain in animals and, in some cases, humans, has been found in Africa, western Asia, the Middle East and the Mediterranean region of Europe. It has spread to all 48 of the Continental United States.

WNV infection in horses may include both central nervous system and peripheral nervous system signs. From the first introduction of the virus in 1999 through December 2004, there have been 22,602 reported equine cases. Approximately one-third of horses that become ill with WNV die or must be euthanized.

This technology represents a new generation of vaccines. Traditional vaccine development involves either passing a disease-producing virus through a different species or cell type until it no longer causes disease but does create immunity, or by killing the virus in such a manner that allows it to produce immunity but no disease in the recipient. DNA vaccines, by contrast, use specific fragments of a pathogen's unique genetic material to stimulate a targeted immune response from the host.

Once licensed by USDA, a product of this nature can be marketed to the public. For information on the vaccine, contact Ft. Dodge Laboratories, Inc., at 1-800-685-5656.

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Noninfectious Recombinant Antigen for Detection of St. Louis Encephalitis Virus-Specific Antibodies in Serum by Enzyme-Linked Immunosorbent Assay

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Proper surveillance of virus activity and a timely response to viral outbreaks depend upon the rapid diagnosis of viral infections. The immunoglobulin M (IgM) antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) is a fast, sensitive test routinely used for the diagnosis of the medically important West Nile and St. Louis encephalitis flaviviruses. However, the suckling mouse brain-derived (SMB) antigen used in this assay is tedious to prepare and has a risk of exposing personnel to live virus and hazardous chemicals. We report the development of a St. Louis encephalitis virus (SLEV) noninfectious recombinant antigen that is a safe and easily produced alternative antigen for use in diagnostic assays. The expression plasmid pCB8SJ2, containing the premembrane and envelope structural protein-encoding regions of SLEV, was constructed to express secreted extracellular virus-like particles (VLPs) from CHO cells. Blind-coded human serum panels were assembled from patients having recent SLEV, West Nile virus (WNV), Powassan virus, or La Crosse encephalitis virus infections to assess the sensitivity and specificity of assays with SLEV VLP or SMB antigen. MAC-ELISAs with either antigen had comparable sensitivity for the detection of IgM antibodies against SLEV. Importantly, when these two antigens were tested against a human serum panel from patients having recent WNV or Powassan virus infections, the SLEV VLPs were less likely than SMB antigen to detect flavivirus cross-reactive IgM antibodies. An optimized IgG antibody capture ELISA (GAC-ELISA) with both WNV and SLEV VLPs was developed to circumvent the frequently observed higher background in the antigen-capture IgG-ELISA (ACG-ELISA). For the detection of IgG antibodies against WNV, the GAC-ELISA resulted in a statistically significant higher performance accuracy ($P = 0.003$) than the ACG-ELISA when the WNV VLP antigen was used in both assays. However, no statistical difference was observed in the assay performance of the GAC-ELISA with SLEV VLP or the ACG-ELISA with SLEV SMB antigen.

St. Louis encephalitis virus (SLEV), a member of the genus *Flavivirus* in the family *Flaviviridae*, was the leading cause of epidemic flaviviral encephalitis in the United States prior to the introduction of West Nile virus (WNV) in late 1999. This mosquito-borne arbovirus, transmitted primarily by *Culex* species, is distributed throughout the lower 48 states, with periodic epidemics occurring mostly in the Midwest and Southeast. Between 1964 and 2002, over 4,500 human cases of SLEV infection were reported, with a national average of 118 reported cases per year (G. L. Campbell, Centers for Disease Control and Prevention, personal communication). The last major outbreak, from 1974 to 1977, involved 35 states and over 2,500 reported cases (16, 17). More recent outbreaks occurred in Florida in 1990 (226 reported human cases) (2, 9), Louisiana in 1998 and 2001 (18 and 71 cases, respectively) (3, 4), and Texas in 2002 (19 cases; G. L. Campbell, Centers for Disease Control and Prevention, personal communication). Most SLEV infections are not clinically apparent or only present as a nonspecific flu-like illness; therefore, the vast majority remain undiagnosed. Infection may progress to severe disease,

such as meningoencephalitis with a case fatality rate of 5 to 20% (24). The elderly are especially at risk for progression to severe disease and death.

Intensive surveillance of flaviviral outbreaks provides valuable information applicable to the control and prevention of virus activity. A standardized IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) protocol was previously developed for the rapid screening of human serum samples for various arboviral infections (15). This assay serves as a valuable tool for the presumptive diagnosis of acute flaviviral infections and facilitates the processing of numerous serum samples. However, the use of arbovirus antigens prepared from virus-infected suckling mouse brains in this diagnostic assay may expose the personnel to infectious agents and hazardous chemicals during antigen production. In addition, the procedure for this antigen preparation is time consuming, costly and tedious. During natural flavivirus infection, empty virus-like particles consisting of viral premembrane, membrane (M), and envelope (E) structural proteins are produced in addition to infectious virus (21). These noninfectious virus-like particles (VLPs) do not contain nucleocapsid but are structurally and physiochemically similar to infectious virion particles. We have developed an expression system to produce secreted extracellular VLPs for several flaviviruses in mammalian cell culture. African green monkey kidney (COS-1) cells trans-

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TABLE 1. Oligonucleotides used for cloning of 100% and 80% SLEV premembrane-E protein-encoding cDNA into expression plasmids

Construct and primers	Oligonucleotide sequence ^a
pCDSLE4MU4 SLE-AfeI-463	5' AAAAGAAAA <u>ACCGCT</u> TTGCAGTTATCAAC CTATCAGGGGAA
cSLE-NotI-2447	5' AAAAGAAAA <u>ACCGCCGC</u> TCAGGCTTGCA CGCTGGTTGCCA
pCB8SJ2 T7	5' CTTATCGAAATTAATACGACTCACTA TAGG
cSLE-BsmBI-2150	5' AATGCT <u>CGTCTC</u> GGCTTCCTCTTTGTGCCA GTGGTAGTTA
JE-BsmBI-2175	5' GGAATT <u>CGTCTC</u> GGAAGCACGCTGGGC AAGG
cSP6	5' GCGAGCTCTAGCATTTAGGTGACACT ATAG

^a Restriction endonuclease sites are indicated by bold, italicized, and underlined font.

formed with expression plasmids containing the premembrane and E structural proteins of either Japanese encephalitis virus (JEV) or WNV secreted VLPs into tissue culture fluid at high antigen capture (AC) enzyme-linked immunosorbent assay (ELISA) titers (6, 8, 12). Replacement of the C terminus of dengue virus type 2 E protein (amino acids E-397 to E-495) with the JEV homologous region was necessary, however, for high-level secretion of dengue virus type 2 VLPs (7). This region is believed to contain a putative membrane retention signal located between amino acids E-397 and E-436 that prevents efficient budding of VLPs from the cytoplasmic membrane.

Noninfectious recombinant antigen prepared by polyethylene glycol precipitation of VLPs secreted into tissue culture fluid by a stably transformed COS-1 cell line was shown to be a safe and sensitive alternative to virus-infected suckling mouse brain-derived (SMB) antigens for diagnosis of JEV and WNV infections (8, 12). The WNV VLP antigen was shown to have sensitivity comparable to that of WNV SMB antigen for detecting IgM and IgG antibodies against WNV (8). In 2001, public health service laboratories in the United States replaced the traditional WNV SMB antigen with WNV VLPs for use in diagnostic assays (8, 19).

Prior to the introduction of WNV in 1999, SLEV was the only mosquito-borne flavivirus resulting in significant human morbidity and mortality in the United States. Both WNV and SLEV are members of the JEV serocomplex. The recent expansion of WNV across the United States and the subsequent cocirculation with SLEV in some regions has complicated serodiagnosis due to the presence of flavivirus group-reactive antibodies elicited by either viral infection. However, antibodies produced during SLEV infection were generally found to be more cross-reactive than those produced during WNV infection (14). Simultaneous testing of these human serum samples for WNV and SLEV antibodies, thus, is necessary in order to identify the virus responsible for the most recent infection.

We present here a procedure for the production and purification of SLEV VLPs in tissue culture. A SLEV premembrane and E protein expression plasmid, pCB8SJ2, was constructed for transient expression of viral structural proteins in Chinese hamster ovary (CHO) cells, and the secreted VLPs were partially purified and concentrated by ultracentrifugation.

Four human serum panels were assembled to compare the sensitivity and specificity of SLEV VLPs and SMB antigen in detecting IgM and IgG antibody. These serum panels consisted of sera from patients infected with two members of the JEV serocomplex, SLEV and WNV, as well as two North American arboviruses, Powassan virus (a member of the tick-borne flaviviruses), and La Crosse virus, a member of the family *Bunyaviridae*. Additionally, an optimized IgG antibody capture (GAC)-ELISA for both SLEV and WNV was developed in order to circumvent frequently observed higher background absorbance against normal CHO cell antigen in the antigen capture IgG ELISA (ACG-ELISA).

MATERIALS AND METHODS

Cell culture and virus strain. CHO-K1 cells (CCL-61; American Type Culture Collection, Manassas, Va.) were grown in Dulbecco's modified Eagle's medium/F12 (Gibco Laboratories, Grand Island, N.Y.). Human embryonic kidney (293) cells (ATCC CRL-1573), African green monkey kidney (COS-1) cells (ATCC CRL-1650), and C6/36 mosquito cells were grown in Dulbecco's modified Eagle's medium (Gibco Laboratories). All media were supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah), 1 mM sodium pyruvate, 0.1 mM minimal essential medium nonessential amino acids, penicillin (100 U/ml), and 100 µg of streptomycin per ml, and all cells were grown at 37°C with 5% CO₂. SLEV (strain MSI-7) propagated in C6/36 cell culture was used for RNA extraction, reverse transcription-PCR (RT-PCR), cDNA cloning, and protein analysis. For use in the Western blot, virus was purified by precipitation with 7% polyethylene glycol 8000 (Fisher Scientific, Fair Lawn, N.J.), followed by ultracentrifugation on 30% glycerol-45% potassium tartrate gradients (18).

Construction of expression plasmids. The pCDSLE4MU4 plasmid was constructed by inserting the SLEV premembrane and E genes into pCDNA-3 (Invitrogen Corp., Carlsbad, Calif.) as previously described for other flaviviruses (5, 6, 8, 12). Briefly, the QIAamp Viral RNA kit (Qiagen Inc., Valencia, Calif.) was used according to the manufacturer's recommended procedures to extract genomic RNA from 140 µl of C6/36 cell culture supernatant containing SLEV, MSI-7 strain. Extracted RNA was used as the template for production of cDNA containing the SLEV premembrane and E protein-encoding sequences with the Qiagen OneStep RT-PCR kit (Qiagen Inc.).

Primer sequences containing the appropriate restriction enzyme sites for cloning (Table 1) were designed based on the published sequence (23). The cDNA was digested with AfeI and NotI and then ligated into AfeI- and NotI-digested pCDNA-3. This SLEV premembrane-E plasmid and the pCDJE2-7 (6) plasmid were used as the PCR templates to amplify SLEV and JEV cDNA sequences, respectively, with the appropriate primer sets listed in Table 1. The PCR products for the SLEV premembrane and 80% E and 20% JEV E protein-encoding sequences were digested with BsmBI and ligated. This ligation product was then PCR amplified with primers T7 and cSP6. This amplicon was digested with KpnI and NotI and then inserted into the KpnI and NotI sites of the pCBamp plasmid (6) to create pCB8SJ2 (Fig. 1).

Automated DNA sequencing was performed on a CEQ 8000 genetic analysis system (Beckman Coulter, Fullerton, Calif.) according to the manufacturer's recommended procedures. The recombinant plasmids that had the correct sequence were identified.

Electroporation of tissue culture cells with plasmid DNA. For transformation, tissue culture cells were grown to 90 to 100% confluence in 150-cm² culture flasks, trypsinized, and resuspended in ice-cold phosphate-buffered saline (PBS) to a final density of 1.5×10^7 cells/ml. For each reaction, 0.5 ml of this cell suspension was electroporated with 30 µg of plasmid DNA in a 0.4-cm electrode gap cuvette with a Bio-Rad Gene Pulser II (Bio-Rad Laboratories, Hercules, Calif.) set at 250 V and 975 µF. Two separate electroporation reactions were combined and seeded onto a single 150-cm² culture flask containing 40 ml of growth medium. Tissue culture medium was harvested 48 h following electroporation, clarified by centrifugation at 10,000 rpm for 30 min at 4°C in a Sorval F-16/250 rotor, and stored at 4°C for further analysis. The AC-ELISA was performed with flavivirus E-specific, group-reactive monoclonal antibodies 4G2 (11) and 6B6C-1 conjugated to horseradish peroxidase (20) (Jackson Immunochemical Laboratories, Inc., West Grove, Pa.) to capture and detect secreted VLPs, respectively, as previously described (6, 12).

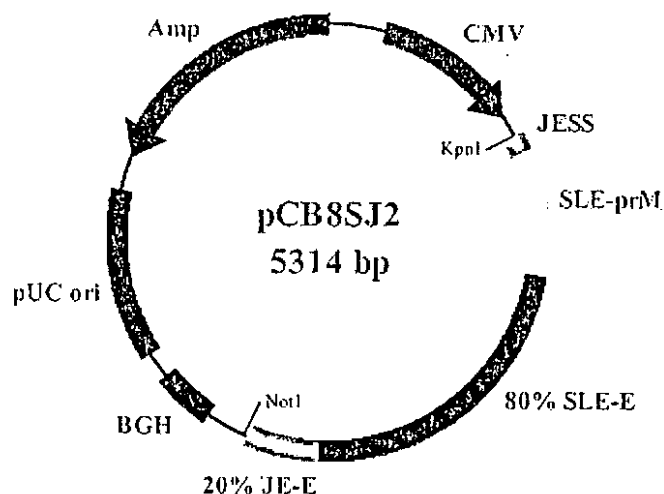


FIG. 1. pCB8SJ2 plasmid map for expressing SLEV structural proteins. This plasmid contains the human cytomegalovirus (CMV) early gene promoter, Japanese encephalitis virus (JEV) signal sequence (JESS), SLEV premembrane (prM) and envelope (E, N-terminal 80%) gene regions, and JEV E (C-terminal 20%), and bovine growth hormone poly(A) signal (BGH) for expression of premembrane and recombinant E proteins in tissue culture cells.

Characterization of SLEV extracellular VLPs. The SLEV VLPs were concentrated and partially purified from clarified tissue culture medium by ultracentrifugation at 19,000 rpm for 8 to 16 h in a Beckman Coulter type 19 rotor at 4°C. The pellet was resuspended in TN buffer (50 mM Tris, 100 mM NaCl, pH 7.5) to 1/50th the original volume, aliquoted into 1-ml samples, and stored at -70°C. For use in assays, aliquots were thawed once and stored at 4°C for up to 1 week, then discarded. For comparison of noninfectious recombinant antigen purification and concentration protocols, SLEV VLPs were also precipitated from clarified tissue culture medium with 10% polyethylene glycol 8000 for 4 to 12 h at 4°C (12). The precipitant was collected by centrifugation at 10,000 rpm for 30 min at 4°C in a Sorval F-16/250 rotor, and the pellet was resuspended as above. SLEV VLPs and gradient-purified virions were analyzed under nondenaturing conditions on a NuPAGE 4 to 12% Bis-Tris gradient gel in an XCell SureLock Mini-Cell electrophoresis apparatus (Invitrogen Corp.), followed by electroblotting onto nitrocellulose membranes with an XCell II Blot module (Invitrogen Corp.). SLEV proteins were detected by Western blot with mouse hyperimmune ascitic fluid (HIAF) against SLEV (MSI-7) and flavivirus E-protein cross-reactive monoclonal antibody 4G2. Total protein was detected with the Owl Pro-Blue kit (Owl Separation Systems, Portsmouth, N.H.) as per the manufacturer's recommended protocol.

Human serum. Serum specimens were obtained from the Diagnostic and Reference Laboratory, Arbovirus Diseases Branch, Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention. Panels were assembled by selecting serum specimens collected from 1999 to 2003 having neutralizing antibody titers to WNV ($n = 18$), SLEV ($n = 12$), Powassan virus ($n = 5$), or La Crosse virus ($n = 5$), as determined by the gold standard 90% plaque-reduction neutralization test. The flavivirus responsible for the most recent infection was defined as that virus having a neutralizing antibody titer at least fourfold greater than any other virus tested. Positive-to-negative absorbance ratios of these specimens for MAC- or ACG-ELISA with SLEV SMB, WNV SMB, or WNV VLPs were determined by Arbovirus Diseases Branch's diagnostic laboratory and are listed in Table 2.

ELISA protocols. SLEV VLPs and normal CHO cell culture antigen were prepared as described above. Lyophilized preparations of WNV VLP antigen and normal COS-1 cell culture antigen, prepared as previously described (8), were resuspended in 0.5 ml of distilled water. Antigens were independently titrated against a positive control serum sample with a twofold dilution series and standardized by selecting a dilution that yielded an absorbance of 0.8 to 1.2 at 450 nm (A_{450}).

For detecting the presence of virus-specific IgM in serum panels with the VLPs, the MAC-ELISA was performed as described previously (15) with some modifications. Briefly, the inner 60 wells of Immulon II HB flat-bottomed 96-well plates (Dynatech Industries, Inc., Chantilly, Va.) were coated overnight at 4°C in a humidified chamber with 75 μ l of goat anti-human IgM (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) diluted 1:2,000 in coating buffer (0.015 M

sodium carbonate, 0.035 M sodium bicarbonate, pH 9.6). Wells were blocked with 300 μ l of StartingBlock (PBS) blocking buffer (Pierce, Rockford, Ill.) according to the manufacturer's recommended procedure. Dried plates were stored with desiccant at 4°C until use. In our hands, the use of plates that had been coated with capture antibody, blocked, and then dried as per the manufacturer's recommended procedure had similar activity in detecting antiviral IgM and IgG antibodies after storage at 4°C for up to 2 weeks.

Patient sera and positive and negative antibody controls were diluted appropriately in wash buffer (PBS with 0.5% Tween 20), added to wells (50 μ l/well), and incubated at 37°C for 1 h in a humidified chamber. Test and negative human control sera were diluted 1:400 as recommended (13). Positive control sera for SLEV and WNV were diluted 1:3,000 and 1:200, respectively. Positive and negative control antigens were tested with each patient serum sample in triplicate by diluting appropriately in wash buffer and adding 50 μ l to appropriate wells for incubation at 4°C overnight in a humidified chamber. Horseradish peroxidase-conjugated 6B6C-1 was diluted 1:6,000 in PBS containing 0.5% Tween 20 and 5% nonfat dry milk, and 50 μ l was added to each well and incubated at 37°C for 1 h in a humidified chamber. Bound conjugate was detected by adding 75 μ l of 3,3',5,5'-tetramethylbenzidine (TMB; Neogen Corp., Lexington, Ky.) substrate and incubating at room temperature for 10 min. The substrate reaction was stopped with 50 μ l of 1 N H_2SO_4 and reactions were measured at an A_{450} with an EL 312e Bio-Kinetics microplate reader (Bio-Tek Instruments, Inc., Winooski, Vt.).

For detection of virus-specific IgG in serum samples with VLPs, the GAC-ELISA was performed essentially as described above for the modified MAC-ELISA with the following exception: plates were initially coated with goat anti-human IgG (Kirkegaard & Perry Laboratories), diluted 1:2,000, to capture IgG antibodies from patient serum.

Test validation and calculation of positive/negative absorbance ratio values. Test validation and positive-to-negative ratio values were determined according to the procedure of Martin et al. (14). Briefly, an internal positive and negative serum control were included in each 96-well plate for test validation. For a testing plate to be considered valid, the average A_{450} for the positive serum control reacted with positive viral antigen must be at least two times greater than the average A_{450} for the same positive serum control reacted with the negative tissue culture fluid antigen. Each patient serum sample was validated in the same manner. This verifies that significant A_{450} values against viral antigen are not due to nonspecific binding of serum antibodies to tissue culture fluid components.

Positive values for each specimen were determined as the average A_{450} for the patient serum sample reacted with positive viral antigen. Negative values were determined for individual 96-well plates as the average A_{450} for the normal human serum control reacted with the positive viral antigen. A specimen was classified as a validated-positive sample if it had a positive/negative ratio of ≥ 3.0 .

Statistical analysis. The receiver operating characteristic (ROC) curve, a plot of sensitivity versus specificity, was applied to determine the discriminatory accuracy of tests in question with MedCalc statistical package (Berkley, Calif.) (22). The accuracy is measured by the area under the ROC curve (AUC). A parametric method with a maximum likelihood estimator was used to calculate the AUC and 95% confidence interval (26). The AUC measures the discriminatory power, that is, the ability of a test to correctly classify those with or without disease. An AUC approaching 1 indicates that the test is highly sensitive as well as highly specific; an AUC approaching 0.5 indicates that the test is neither sensitive nor specific. We used the following criteria to define the disease state and the assigned numerical value was applied to transform the positive/negative ratio value for ROC analysis: 0 = positive/negative absorbance ratio of < 2 (definitive negative), 1 = positive/negative ratio of ≥ 2 and < 3 (presumptive positive), 2 = positive/negative ratio of ≥ 3 and < 4 (positive), and 3 = positive/negative ratio of ≥ 4 (definitive positive). The comparative ROC was used to calculate the significance level and determine an assay's performance.

Calculation of positive and negative prediction value. A positive/negative ratio of < 3 or ≥ 3 for a given specimen was classified as assay negative or positive, respectively. A 2 by 2 table was prepared which categorized four quadrants as true positive, true negative, false negative, and false positive. The positive prediction value is the percentage of true positives/(true positives + false positives), and the negative prediction value is the percentage of true negatives/(false negatives + true negatives).

RESULTS

Transient expression, concentration, and characterization of SLEV VLPs. Tissue culture cells transiently transformed by

TABLE 2. ELISA detection of IgM and IgG antibodies in human serum samples with various diagnostic antigens

Panel	Specimen		PRNT ₉₀ ^a		Positive/negative ratio ^b								
	No.	Day post onset	SLEV	WNV	IgM				IgG				
					SLEV		WNV		SLEV		WNV		
					VLP	SMB ^c	VLP	SMB ^c	VLP	SMB ^d	VLP	SMB ^d	VLP ^d
SLEV	1	8	1,280	160	8.27	2.64	10.80	3.08	9.82	4.69	23.10	7.89	
	2	0	320	10	7.33	9.03	2.41	2.23	1.83	2.58	1.17	2.42	
	3	0	1,280	20	8.42	8.28	4.29	4.61	1.68	3.23	1.49	4.07	
	4	5	2,560	40	10.70	7.82	3.50	2.46	2.67	3.45	1.66	2.70	
	5	0	160	10	9.96	8.97	2.92	2.25	2.59	2.74	1.75	3.28	
	6	6	10,240	80	10.60	9.11	2.89	2.67	5.30	2.49	3.37	3.61	
	7	18	160	40	6.40	10.50	6.68	8.60	3.93	19.30	5.20	14.20	
	8	4	640	20	12.40	9.82	14.90	7.54	2.29	4.56	2.14	6.72	
	9	0	80	<10	8.11	8.47	2.79	2.31	1.45	3.53	1.13	2.16	
	10	0	1,280	20	9.81	8.68	11.10	7.00	5.06	5.35	2.67	3.63	
	11	0	320	20	8.56	9.08	8.27	5.32	2.04	5.85	1.90	5.61	
	12	21	1,280	10	11.80	8.71	43.70	8.71	2.08	3.94	2.96	5.13	
No. positive					12	11	8	7	4	9	3	9	
WNV	1	10	80	320	2.73	2.00	8.38		13.60	6.80	21.00		3.90
	2	0	40	160	3.37	2.80	7.88		9.65	5.70	17.50		2.30
	3	12	40	160	1.70	2.00	18.66		11.20	4.70	19.90		2.80
	4	6		1,280	1.28	2.10	9.27		1.66	2.40	7.16		2.70
	5	0	40	1,280	2.62	3.40	34.70		1.84	2.90	14.30		3.00
	6		10	320	1.29	2.50	8.61		5.77	3.60	13.60		3.60
	7		10	640	1.98	2.70	18.60		4.09	4.70	9.60		3.00
	8	14	40	640	2.02	2.70	19.50		1.67	3.10	5.32		3.70
	9	24	320	1,280	4.36	9.80	33.00		13.00	5.00	23.60		5.70
	10	9	40	5,120	3.45	4.30	33.80		2.66	3.00	11.60		4.80
	11	3	80	1,280	3.34	3.60	23.90		2.95	7.44	18.20		7.76
	12		10	1,280	2.66	4.20	33.40		2.36	7.05	14.90		5.27
	13	0	40	2,560	2.12	2.70	26.30		2.64	2.40	18.40		4.80
	14	4	20	640	2.16	4.10	37.10		2.20	2.60	12.50		4.44
	15	9	80	320	3.69	3.40	8.13		11.30	4.10	21.30		2.60
	16	16	40	1,280	2.14	5.00	28.80		3.01	3.00	15.30		5.10
	17		160	2,560	1.48	2.30	5.76		12.80	8.40	20.30		6.20
	18	4	20	640	1.49	2.70	16.50		1.71	5.57	6.66		5.87
No. positive					5	8	18		9	14	18		14
Powassan virus	1	19	20		1.81	2.00	1.25	0.92	1.11	2.70	1.32	1.70	
	2	15	<10	<10	2.41	5.00	1.83	3.10	1.37	4.30	1.62	3.00	
	3				1.12	0.60	1.05	0.51	0.90	*	0.84	1.50	
	4				1.62	3.10	1.90	2.90	1.09	2.80	1.05	1.90	
	5	5			1.23	1.10	1.13	0.77	1.90	*	2.17	*	
No. positive					0	2	0	1	0	1	0	1	
La Crosse Virus	1	30			1.03	0.88	0.97	0.47	1.14	0.89	1.35	0.90	
	2	34			1.06	0.86	1.02	0.94	1.06	1.50	1.06	1.40	
	3	24			1.12	1.00	2.29	9.60	0.97	1.20	0.96	1.10	
	4	62			0.99	0.88	1.05	0.52	0.89	0.82	0.92	0.90	
	5	10			1.08	1.10	1.03	1.30	1.15	0.92	1.05	1.00	
No positive					0	0	0	1	0	0	0	0	

^a PRNT₉₀, plaque reduction neutralization test; titers represent 90% plaque reduction endpoints reported by the reference laboratory.
^b Values represent ratios calculated by the method indicated in Materials and Methods. Unless specified, the IgM or IgG capture ELISA protocol was used. Positive ratios of ≥3 are shown in bold.
^c Ratios reported by the reference laboratory.
^d Ratios reported by the reference laboratory. The antigen capture IgG-ELISA (ACG-ELISA) protocol was used. *, specimens failed the test validation due to high background absorbance against the normal cell antigen.

electroporation with plasmid DNA, pCDSLE4MU4, encoding the full-length SLEV premembrane and E proteins expressed these proteins intracellularly, as determined by the indirect immunofluorescence antibody assay (data not shown), but did not secrete VLPs into the tissue culture fluid efficiently. The AC-ELISA titers for SLEV antigen in tissue culture fluid were less than 1:4 at 48 h after plasmid transformation for multiple

attempts (data not shown). However, transient expression with pCB8SJ2 (Fig. 1), containing a replacement of the C-terminal 20% of the SLEV E protein with that of JEV, resulted in the dramatically enhanced secretion of SLEV VLPs. Three types of tissue culture cells were compared for optimal secretion when transformed with pCB8SJ2. CHO cells were found to be the optimal cell line for this purpose. VLP secretion measured

by the AC-ELISA was higher in CHO cells (1:32) than COS-1 cells (1:4). Plasmid-transformed CHO and 293 cells secreted VLPs to similar titers; however, CHO cells attached to tissue culture flasks more tightly than 293 cells, a characteristic that is preferable for process and production purposes. All further production of SLEV VLPs was therefore conducted with CHO cells.

A comparison of two noninfectious recombinant antigen purification protocols was made to determine if a simpler ultracentrifugation procedure could be used to prepare VLP antigen. Equal volumes of transiently transformed CHO cell tissue culture fluid were prepared by either precipitation with 10% polyethylene glycol or ultracentrifugation. Both concentration methods yielded similar titers of VLPs. However, the SLEV antigens concentrated by ultracentrifugation were cleaner than by polyethylene glycol precipitation. In a total protein staining gel, a continuous smear of protein was observed for samples prepared by polyethylene glycol precipitation, making the identification of discrete individual bands difficult, whereas discrete protein bands could be easily discerned from samples prepared by ultracentrifugation (data not shown). Therefore, the ultracentrifugation was used to prepare the SLEV VLP antigen.

The purity and protein identity of the resuspended preparation were analyzed by a total protein stain (Fig. 2A) and a Western blot with SLEV HIAF and the flavivirus group-reactive, E-specific monoclonal antibody 4G2. SLEV HIAF detected protein bands corresponding to the size for SLEV pre-membrane and E proteins (~20 and 55 kDa, respectively) for both purified SLEV (MSI-7) and SLEV VLPs (Fig. 2B, lanes 1 and 2, respectively). Monoclonal antibody 4G2 detected the E protein as an appropriately sized band in the SLEV VLP sample (Fig. 2B, lane 4). Nonspecific binding was not detected with either antibody preparation when reacted with CHO negative antigen prepared identically to VLPs (Fig. 2B, lanes 3 and 5).

Comparison of ELISA formats for detection of IgG in WNV diagnosis. Several attempts to utilize ACG-ELISA with SLEV and WNV VLPs in this study resulted in a high background absorbance against normal CHO cell antigens (13). A panel of 18 serum samples (Table 2), collected between 2002 and 2003 from WNV-infected patients, was used to develop the GAC-ELISA for detection of WNV IgG antibody (Table 2). For this panel, 14 of 18 samples were previously determined to be positive (positive/negative ratio values of ≥ 3.0 ; range, 3.0 to 7.76; average, 4.80) by the ACG-ELISA. Testing this panel by GAC-ELISA resulted in 18 positive samples (positive/negative ratio values of ≥ 3.0 ; range, 5.32 to 23.6; average, 15.1).

The ROC analysis was applied to compare the performance accuracy of two protocols. The WNV panel was designated the disease panel and the remaining three serum panels, SLEV, Powassan virus, and La Crosse virus, were combined as the nondisease panel (Table 3). The AUCs of 0.95 and 0.70 for GAC- and ACG-ELISA, respectively, indicated that the GAC-ELISA protocol had higher sensitivity and specificity than the ACG-ELISA (Table 3 and Fig. 3). The test performance between the two protocols was statistically significant at the level of 0.003. The GAC-ELISA had a higher positive prediction value than the ACG-ELISA (86 versus 64%, respectively) and,

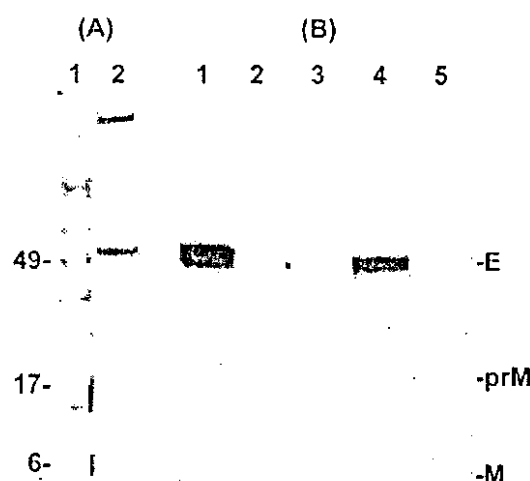


FIG. 2. Detection of SLEV structural proteins in virions and extra-cellular virus-like particles (VLPs). (A) Total protein stain of SLEV VLPs partially purified and concentrated by ultracentrifugation (lane 2). Lane 1 contains SeeBlue Plus2 prestained protein standards with molecular masses indicated to the left (in kilodaltons). (B) Reactivity of purified SLEV virion protein (lane 1), pelleted SLEV VLPs (lanes 2 and 4), and pelleted normal CHO tissue culture fluid (lanes 3 and 5) with mouse hyperimmune ascitic fluid against SLEV strain MSI-7 (lanes 1-3) or flavivirus group-reactive E-protein specific monoclonal antibody 4G2 (lane 4 and 5). Bands corresponding to envelope (E), pre-membrane (prM), and cleaved membrane (M) proteins are indicated to the right.

additionally, the GAC-ELISA had a negative prediction value of 100%, compared to 78% with ACG-ELISA.

Detection of SLEV viral antibody by ELISA. A panel of 12 SLEV-infected serum samples, confirmed by the plaque reduction neutralization test, was used in this study (Table 2). The IgM antibody was tested by the MAC-ELISA with SMB antigen or VLPs. Use of SMB antigens in the MAC-ELISA detected 11 of 12 positive samples (positive/negative ratio values of ≥ 3.0 ; range, 7.82 to 10.5; average, 8.95). Replacing SMB antigens with SLEV VLPs in the MAC-ELISA detected 12 of 12 positive samples (positive/negative ratio values of ≥ 3.0 ; range, 6.40 to 12.4; average, 9.36). ROC analysis with various combined panels as the nondisease panel indicated that there was no statistically significant difference in the performance of MAC-ELISA with either VLPs or SMB antigen, with the single exception of SLEV against the combined WNV and Powassan virus panel (Table 4, $P = 0.05$). Compared to SMB antigen, this analysis also indicated that SLEV VLP had a higher positive predictive value and negative predictive value (Table 4).

TABLE 3. ROC curve determination of sensitivity and specificity of WNV IgG detection by antigen-capture IgG or IgG antibody-capture ELISA

ELISA	AUC (95% CI) [P]	Predictive value (%)	
		Positive	Negative
ACG	0.70 (0.53-0.84) [0.003]	64	78
GAC	0.95 (0.83-0.99)	86	100

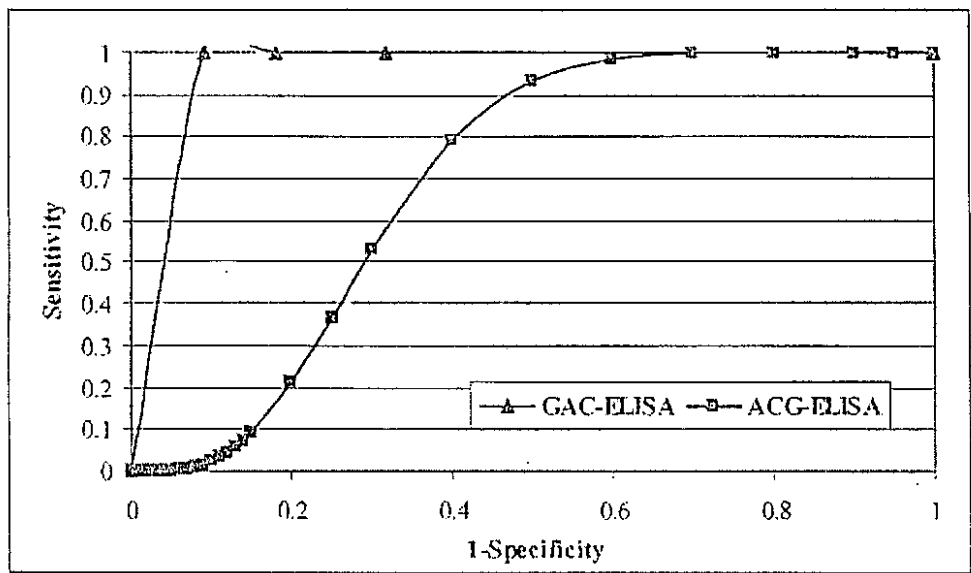


FIG. 3. Comparative ROC curves corresponding to the GAC- and ACG-ELISA results with WNV VLPs as the antigen, as shown in Table 3.

When the same panel was tested for the presence of IgG antibody by ACG-ELISA, use of SLEV SMB antigen detected nine positive specimens (75%, Table 2). However, the higher absorbance against normal CHO cell antigens prohibited the use of VLPS with the ACG-ELISA protocol. Thus, the GAC-ELISA protocol developed and verified with the WNV panel was applied to test IgG antibodies in the SLEV panel. This protocol greatly reduced the background absorbance against normal cell antigen but also reduced the number of IgG-positive specimens to 33% (4 out of 12; Table 2). However, ROC analysis with various combined panels as the nondisease panel indicated that there was no statistically significant differences between the performance of GAC- and ACG-ELISA (Table 5).

Detection of cross-reactive antibodies. As shown in Table 2, use of SLEV SMB antigen in either MAC- or ACG-ELISA for WNV-infected patients detected cross-reactive IgM and IgG antibodies in 8 (44%) and 14 (78%), respectively, of the 18

WNV-positive serum samples. However, the SLEV VLPs detected cross-reactive antibody in only 5 (28%) and 9 (50%) of the same 18 samples by MAC- and GAC-ELISA, respectively. Cross-reactive IgM antibodies in 8 of 12 (67%) SLEV-infected serum samples were detected by WNV VLPs, compared to 7 of 12 (58%) detected by WNV SMB antigen. The WNV VLPs detected cross-reactive IgG antibody in 3 of 12 (25%) SLEV-infected samples by GAC-ELISA and WNV SMB antigen detected 9 of 12 (75%) of these samples by ACG-ELISA.

SLEV and WNV VLPs were used to test against WNV, SLEV, and Powassan virus panels to determine the extent of IgM and IgG cross-reactivity against flaviviruses commonly associated with human disease in North America in MAC- and GAC-ELISAs, respectively. The comparative ROC analysis (Table 6) indicated that SLEV VLPs performed significantly better than WNV VLPs in MAC-ELISA (AUCs of 0.97 and 0.79, respectively), and WNV VLPs performed significantly better than SLEV VLPs in GAC-ELISA (AUCs of 0.94 and

TABLE 4. IgM antibody detection by the IgM antibody-capture ELISA with either SLEV VLP or suckling mouse brain-derived antigens

Sera ^a		Antigen	AUC (95% CI) [P]	Predictive value (%)	
Disease panel	Nondisease panel			Positive	Negative
SLEV	WNV + POWV + LACV	SMB	0.86 (0.71–0.95) [0.08]	52	95
		VLP	0.98 (0.88–0.99)	71	100
SLEV	WNV + POWV	SMB	0.82 (0.66–0.93) [0.05]	52	93
		VLP	0.98 (0.86–0.99)	71	100
SLEV	POWV + LACV	SMB	0.93 (0.74–0.99) [0.23]	85	89
		VLP	1.00 (0.84–1.00)	100	100
SLEV	WNV	SMB	0.81 (0.63–0.93) [0.06]	58	91
		VLP	0.97 (0.84–0.99)	71	100
SLEV	POWV	SMB	0.87 (0.62–0.98) [0.14]	85	75
		VLP	1.00 (0.80–1.00)	100	100
SLEV + WNV	POWV + LACV	SMB	0.87 (0.72–0.95) [0.81]	90	42
		VLP	0.88 (0.74–0.96)	100	43
SLEV + WNV + POWV	LACV	SMB	0.97 (0.86–1.00) [0.09]	100	26
		VLP	0.86 (0.71–0.95)	100	22

^a POWV, Powassan virus; LACV, LaCrosse virus.

30

TABLE 5. Comparison of IgG antibody detection with either SLEV VLP or suckling mouse brain-derived antigens

Sera ^a		ELISA	Antigen	AUC (95% CI) [P]	Predictive value (%)	
Disease panel	Nondisease panel				Positive	Negative
SLEV	WNV + POWV + LACV	ACG	SMB	0.59 (0.43–0.75) [0.89]	38	81
		GAC	VLP	0.58 (0.41–0.73)	31	70
SLEV	WNV + POWV	ACG	SMB	0.51 (0.33–0.68) [0.92]	38	73
		GAC	VLP	0.52 (0.34–0.69)	31	64
SLEV	POWV + LACV	ACG	SMB	0.87 (0.66–0.97) [0.97]	90	75
		GAC	VLP	0.88 (0.67–0.97)	100	56
SLEV	WNV	ACG	SMB	0.56 (0.37–0.74) [0.84]	39	57
		GAC	VLP	0.58 (0.39–0.76)	31	53
SLEV	POWV	ACG	SMB	0.74 (0.48–0.92) [0.34]	90	57
		GAC	VLP	0.88 (0.63–0.98)	100	38
SLEV	LACV	ACG	SMB	1.00 (0.80–1.00) [0.15]	100	63
		GAC	VLP	0.88 (0.63–0.98)	100	38
SLEV + WNV + POWV	LACV	ACG	SMB	1.00 (0.91–1.00) [0.03]	100	31
		GAC	VLP	0.83 (0.68–0.93)	100	18
SLEV + WNV	LACV	ACG	SMB	1.00 (0.90–1.00) [0.07]	100	42
		GAC	VLP	0.88 (0.73–0.97)	100	22
SLEV + WNV	POWV	ACG	SMB	0.76 (0.58–0.89) [0.24]	96	36
		GAC	VLP	0.88 (0.73–0.97)	100	23

^a See Table 4, footnote a.

0.58, respectively) in detecting flavivirus cross-reactive antibodies.

DISCUSSION

An expression plasmid was constructed for the extracellular secretion of SLEV VLPs by CHO cells. As reported previously in our dengue virus type 2 study (7), use of the native viral full-length premembrane and E protein-encoding sequence resulted in accumulation of these proteins intracellularly, but VLPs were not efficiently secreted. Replacement of the C-terminal 20% of the SLEV E protein (amino acids E-405 to E-501) with the corresponding JEV E region increased secretion of VLPs by at least eightfold. This result substantiates the previous observation and suggests the presence of a putative membrane retention signal in the C-terminal region of the E protein. This region consists of two α-helical segments (H1 and H2) and two transmembrane α-helices (T1 and T2) involved in the proper E-E and premembrane-E hetero- or homodimer interactions necessary for secretion. The finding that the dengue virus type 2 90%-JEV 10% E protein construct did not secrete VLPs implicates H1 and/or H2 in forming the proper interactions required for efficient VLP formation and budding (7). Identification of the specific amino acid(s) involved in these interactions is currently being investigated for SLEV and all four dengue virus serotypes.

Three tissue culture cell lines were compared for their ability to secrete SLEV VLPs when transiently transformed with pCB8SJ2 plasmid DNA. COS-1 cells, previously shown to efficiently secrete JEV, WNV, and dengue virus type 2 VLPs (7, 8, 12), did not secrete SLEV VLPs efficiently in this study. Both CHO and 293 cells secreted SLEV VLPs at equally high titers, but 293 cells were found to be impractical for production and preparation due to their weak adherence to tissue culture flasks. CHO cells had additional advantages over COS-1 cells for their use in production of flaviviral VLPs, including their shorter generation time and higher cell density per 150-cm² culture flask. These factors decrease both the time and amount of medium and materials required to produce the large quantities of antigen necessary for supporting nationwide surveillance.

Secretion of VLPs into tissue culture fluid and ultracentrifugation as the only purification and concentration procedure additionally reduced the time and effort required for noninfectious recombinant antigen production compared to previously described protocols with polyethylene glycol precipitation. This simple pelleting procedure provided a product of higher purity which could be used as a diagnostic antigen as well as a protein preparation readily examined by immunochemical techniques without the prerequisite ethanol extraction necessary for removal of residual polyethylene glycol (12). The presence of polyethylene glycol in a sample results in

TABLE 6. Flavivirus cross-reactive IgM and IgG antibody detection by IgM and IgG antibody-capture ELISAs with SLEV and WNV VLP antigens

ELISA	Sera ^a		Antigen	AUC (95% interval)	Predictive value (%)	
	Panel 1	Panel 2			Positive	Negative
MAC	SLEV	WNV + POWV	SLEV	0.97 (0.84–0.99)	70.6	100
	WNV	SLEV + POWV	WNV	0.79 (0.62–0.91)	69.2	100
GAC	SLEV	WNV + POWV	SLEV	0.58 (0.39–0.76)	30.7	63.6
	WNV	SLEV + POWV	WNV	0.94 (0.81–0.99)	85.7	100

^a See Table 4, footnote a.

aberrant protein band migration in gel electrophoresis. Additionally, polyethylene glycol precipitates much of the serum protein present in tissue culture fluid and results in a continuous smear in a total protein stain, making identification of discrete bands difficult.

Electron microscopy, density gradient analysis, and hemagglutination assays indicated that JEV VLPs secreted from a stably transformed COS-1 cell line mimicked the virion structure of JEV (12). Epitope mapping of the E proteins of JEV and dengue virus type 2 VLPs with panels of E-specific monoclonal antibodies, including the conformation-dependent antibodies 4G2 and 6B6C-1, suggested that the E protein present in these particles was properly folded and similar to the E protein of virion particles (6, 7). In the current study, Western blot examination of pelleted tissue culture fluid preparations from pCB8SJ2-transformed CHO cells with SLEV HIAF revealed protein bands corresponding in size to native virus pre-membrane and E proteins, and the E-specific monoclonal antibody 4G2 detected a single E protein band. These results supported the expectation that SLEV pre-membrane and E proteins were expressed and secreted by CHO cells and that these proteins presumably self-assembled into particle form.

Several flaviviruses, including JEV, SLEV, and dengue virus type 2, contain N-linked glycosylation sites in the E proteins. SLEV MSI-7 has two potential glycosylation sites in the E protein, and the degree of glycosylation in the proteins may affect their migration in acrylamide gels. The Western blot analysis of SLEV MSI-7 virions with HIAF detected a doublet (Fig. 2B, lane 1) at ≈ 50 kDa. These bands were previously investigated and found to correspond to slow-migrating glycosylated and fast-migrating nonglycosylated forms of the E protein, designated E and E₀, respectively (25). Heterogeneous glycosylation of SLE virions has been demonstrated, and glycosylation does not appear to be essential for epitope conformation and recognition by monoclonal antibodies (25). Comparison of SLEV VLPs with purified virus in a Western blot with either HIAF or monoclonal antibody 4G2 (Fig. 2) revealed that the fast-migrating, potentially nonglycosylated E₀ form is apparently the dominant form present in SLEV VLPs. This presumptive nonglycosylated E₀ was readily detected by SLEV HIAF, monoclonal antibody 4G2, or virus-specific IgM and IgG antibodies present in human serum.

The IgG-ELISA developed previously by Johnson et al. (13) has been employed successfully to detect anti-flaviviral IgG antibody in patient specimens (8, 13). In our experiments, however, use of this antigen capture format with both WNV and SLEV VLPs resulted in a high background absorbance against normal CHO cell antigens, precluding the screening of serum panels at the recommended serum dilution of 1:400. Additionally, as noted in Table 2, ACG-ELISA results were not interpretable for two Powassan virus-infected serum samples tested on SMB antigens. Johnson et al. noted that this background absorbance of antibody-positive serum reacted on normal mouse brain antigen became a significant problem when specimens were tested at a dilution of less than 1:400.

To circumvent this problem, we employed the GAC-ELISA, a protocol similar in format to MAC-ELISA (15). This IgG antibody capture ELISA format, described by Erdman et al. (10), uses plate-bound goat anti-human IgG to capture serum IgG antibody to reduce nonspecific background absorbance.

One potential disadvantage of the GAC-ELISA is the capture of IgG antibodies of unknown specificity as well as virus-specific antibodies. High concentrations of IgG antibodies of unknown specificity may compete with and inhibit the capture of the relatively small amounts of virus-specific IgG antibodies, thereby reducing the sensitivity of the assay. Although there was no statistically significant difference between the performance of either test, concerns about the lower number of positively detected by the GAC-ELISA with SLEV VLPs compared to the ACG-ELISA with SLEV SMB antigen (Tables 2 and 5) were raised. However, the GAC-ELISA had higher sensitivity and specificity than the ACG-ELISA when WNV VLPs were used to detect antiviral IgG antibody in WNV-infected patient serum samples (Tables 2 and 3).

These apparently contradictory results might indicate that the detection of a greater number of IgG-positive serum samples in the ACG-ELISA contributed to the presence of cross-reactive nonstructural proteins in the SMB preparation. Testing of the Powassan virus and La Crosse virus serum panels with the SLEV and WNV VLPs in both MAC- and GAC-ELISAs resulted in specificities of 100%, whereas the specificities of the corresponding ELISA format with the SMB antigens with these panels were lower. Both IgM and IgG serum antibodies contribute to the neutralization of virus in the plaque reduction neutralization test; therefore, for samples which are found to have a recent viral infection by plaque reduction neutralization test but which do not have detectable titers of specific IgG antibody by ELISA, neutralization must be due to IgM antibodies. For the two SLEV-infected samples (2 and 5 in Table 2) that tested negative for SLEV-specific IgG antibody by either GAC-ELISA or ACG-ELISA, SLEV-specific IgM antibody was detected with either the SLEV SMB or VLP antigen.

MAC-ELISAs with either VLP or SMB antigen as the serodiagnostic antigen had comparable sensitivity and specificity (Table 4) for the detection of serum IgM antibody produced in response to SLEV infection. The positive predictive value as well as the negative predictive value for a given test are important determinants when the assay is implemented as the tool for disease surveillance. The positive and negative predictive values determine the true positive and negative disease states, respectively, for a given specimen under the specified assay condition. In general, VLPs had a higher positive predictive value and negative predictive value than SMB antigens, and therefore selecting VLPs as the antigen of choice should enhance the performance of disease surveillance to determine the disease prevalence.

The ease of production, safety, adequate sensitivity for detection of true positives, and low cross-reactivity with anti-WNV IgM antibody make the use of SLEV VLPs in MAC-ELISA screening of patient serum samples preferable to that of antigens derived from suckling mouse brain preparations. The use of a stably transformed cell clone to constitutively secrete VLPs would also allow standardization and large-scale production of this flaviviral noninfectious recombinant antigen. A cell line stably transformed with the SLEV pre-membrane-E (80% SLEV-20% JE) protein-coding region is currently being developed in our laboratory.

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

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

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

WN fever is a mosquito-borne flavivirus infection that is transmitted to vertebrates primarily by various species of *Culex* mosquitoes. Like other members of the Japanese encephalitis (JE) antigenic complex of viruses, including JE, St. Louis encephalitis (SLE), and Murray Valley encephalitis viruses, WN virus is maintained in a natural cycle between arthropod vectors and birds. The virus was first isolated from a febrile human in the West Nile district of Uganda in 1937 (38). It was soon recognized as one of the most widely distributed flaviviruses, with its geographic range including Africa, the Middle East,

western Asia, Europe, and Australia (18). Clinically, WN fever in humans is a self-limited acute febrile illness accompanied by headache, myalgia, polyarthropathy, rash, and lymphadenopathy (28). Rarely, though, acute hepatitis or pancreatitis has been reported, and cases in elderly patients are sometimes complicated by encephalitis or meningitis (7).

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

MATERIALS AND METHODS

Cell culture and virus strain. COS-1 cells (American Type Culture Collection, Manassas, Va.; 1650-CRL) were grown at 37°C with 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Inc.,

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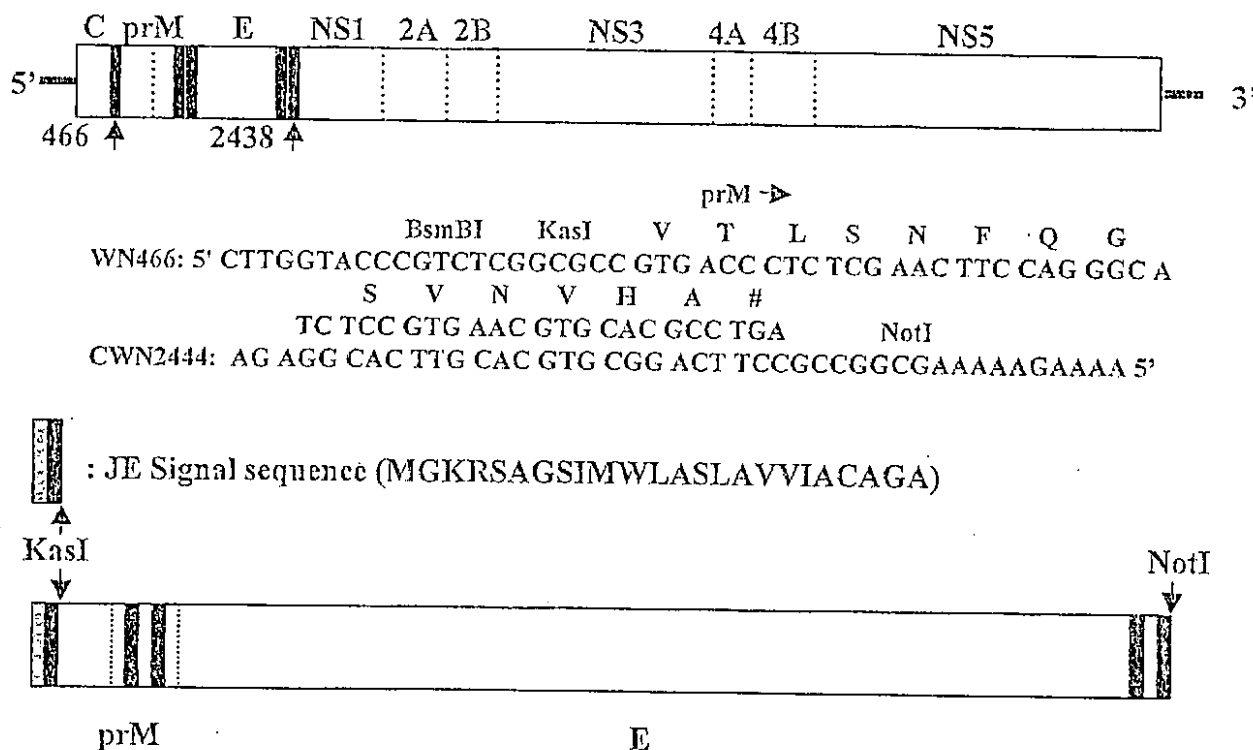


FIG. 1. Map of the WN virus genomic region (top) and oligonucleotides used in RT-PCR to construct the transcription unit for the expression of WN virus prM and E coding regions (bottom). Potential transmembrane helices of viral proteins are indicated by black boxes.

Logan, Utah), 1 mM sodium pyruvate, 1 mM nonessential amino acids, 17 ml per liter of 7.5% NaHCO₃, 5 ml per liter of 1 M HEPES (Bio Whittaker, Walkersville, Md.), 100 U of penicillin per ml, and 100 µg of streptomycin per ml. Vero cells were grown under the same conditions used for COS-1 cells. Two WN virus strains (NY99-6480, a mosquito isolate, and BC787, a bird isolate), isolated from the outbreak in New York in 1999, were used for challenge experiment or mosquito inoculation. The NY99-6480 strain was propagated in the Vero cell culture. The BC787 strain was passed once in suckling-mouse brain. The NY99-6480 strain used for immunological or biochemical studies was gradient purified by precipitating in 7% polyethylene glycol 8000 (PEG-8000; Fisher Scientific, Fair Lawn, N.J.) followed by ultracentrifugation on 30% glycerol–45% potassium tartrate gradients (30).

Construction of plasmid expressing WN virus prM and E proteins. Genomic RNA was extracted from 150 µl of Vero cell culture medium infected with strain NY99-6480 using a QIAamp viral RNA kit (Qiagen, Santa Clarita, Calif.). Extracted RNA, resuspended in 80 µl of diethyl pyrocarbonate-treated water (Sigma, St. Louis, Mo.), was used as a template in a reverse transcriptase-PCR (RT-PCR) for the amplification of WN virus prM and E genes. Primer sequences (Fig. 1) were designed based on the published sequence (22). Restriction enzyme sites for *BsmBI* and *KasI* were incorporated at the 5' terminus of the cDNA amplicon. An in-frame termination codon followed by a *NotI* restriction site was introduced at the 3' terminus of the cDNA amplicon. The RT-PCR and molecular cloning protocols used are essentially identical to those reported previously (8).

The WN virus cDNA amplicon was digested with *KasI* and *NotI* enzymes, and the resulting 998-bp (nucleotides –1470 to 2468) fragment of the cDNA was inserted into the *KasI* and *NotI* sites of a pCBJESS vector to form an intermediate plasmid, pCBINT. pCBJESS was derived from the pCBamp plasmid, which contained the cytomegalovirus early gene promoter and translational control element and engineered JE virus signal sequence element (8) (Fig. 1). The cDNA amplicon was subsequently digested with *BsmBI* and *KasI* enzymes, and the remaining 1,003-bp fragment (nt –466 to 1469) was inserted into the *KasI* site of pCBINT vector to form pCBWN (Fig. 2). Automated DNA sequencing was performed on an ABI Prism 377 sequencer (Applied Biosystems/Perkin Elmer, Foster City, Calif.) according to the manufacturer's recommended procedures. The recombinant plasmid which had a correct prM and E sequence was identified (22).

Immunochemical characterization of the recombinant WN virus antigen. COS-1 cells were electroporated with plasmid pCBWN using the protocol de-

scribed in a previous publication (8). Electroporated cells were seeded onto 75-cm² culture flasks or in a 12-well tissue culture dish containing one sterile coverslip per well. All flasks and 12-well plates were kept at 37°C in a 5% CO₂ incubator. Forty hours following electroporation, coverslips containing adherent cells were removed from the wells, washed briefly with phosphate-buffered saline (PBS), fixed with acetone for 2 min at room temperature, and allowed to air dry. The flavivirus E protein-specific MAb 4G2 (17), WN virus mouse hyperimmune ascitic fluid (HIAF), and normal mouse serum (NMS) at a 1:200 dilution in PBS were used as the primary antibodies to detect protein expression by an indirect immunofluorescence antibody assay (IFA), as described previously (8).

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

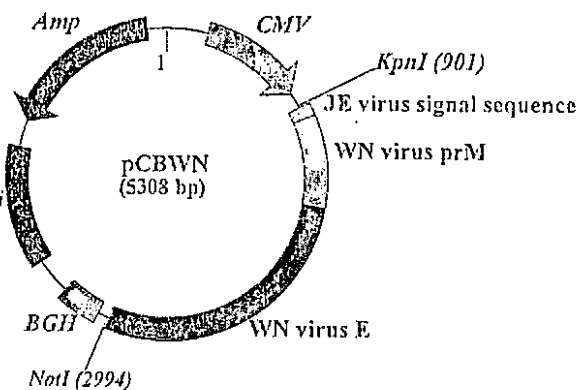


FIG. 2. Map of the recombinant WN virus plasmid pCBWN. The transcription unit contains the human cytomegalovirus early gene promoter (CMV), JE virus signal sequence, WN virus prM and E gene region, and bovine growth hormone poly(A) signal (BGH).

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

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

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

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

Mice were bled every 3 weeks following injection, and the WN virus-specific antibody response was evaluated by Ag-capture ELISA and PRNT. Challenge experiments were performed by two methods. Half of the mouse groups were challenged intraperitoneally (i.p.) at 6 weeks postvaccination with 1,000 50% lethal doses (LD₅₀) (1,025 PFU/100 μ l) of NY99-6480 virus. The LD₅₀ was determined previously by i.p. inoculation of 10-week-old adult ICR mice (data not shown). The remaining mice were each exposed to the bites of three *Culex tritaeniorhynchus* mosquitoes that had been infected with NY99-6480 virus 7 days prior to the challenge experiment. Mosquitoes were allowed to feed on mice until they were fully engorged. Mice were observed twice daily for 3 weeks after challenge.

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

Two days prior to virus challenge, 12 horses (4 vaccinated and 8 control) were relocated into a biosafety level 3 containment building at Colorado State University. The eight unvaccinated control horses were the subset of a study that was designed to investigate WN virus-induced pathogenesis in horses and the potential of horses to serve as amplifying hosts (M. L. Bunning, R. A. Bowen, B. Davis,

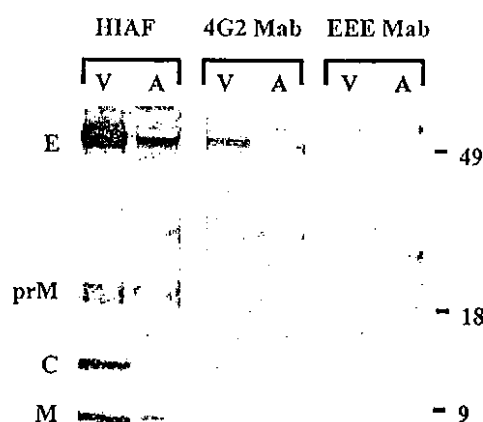


FIG. 3. Comparison of Western blot reactivity between NRA produced by pCBWN-transformed COS-1 cells (A) and gradient-purified WN virion proteins (V). WN virus-specific mouse HIAF, flavivirus E-specific cross-reactive MAb 4G2, and eastern equine encephalitis virus monoclonal antibody (EEE MAb) were used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

N. Kumar, M. Godsey, D. Baker, D. Hettler, and C. J. Mitchell, submitted for publication). Horses were each challenged by the bite of 14 or 15 *Aedes albopictus* mosquitoes that had been infected by NY99-6425 or BC787 virus 12 days prior to horse challenge. Mosquitoes were allowed to feed on horses for 10 min. Horses were examined for signs of disease twice daily. Body temperature was recorded, and serum specimens were collected twice daily from days 0 (day of infection) to 10 and then once daily through day 14. Pulse and respiration were recorded daily after challenge. The collected serum samples were tested by plaque titration for detection of viremia and by MAC- or IgG ELISA and PRNT for antibody response. Vaccinated horses were euthanized by pentobarbital overdose at 14 days after virus challenge and necropsied for gross pathological and histopathological examination, and their carcasses were incinerated within the containment facility.

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

RESULTS

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

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

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

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

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

and 4. NRA was concentrated by PEG precipitation, resuspended in lyophilization buffer, and lyophilized for preservation. For diagnostic test development, one vial of lyophilized NRA was reconstituted with 1.0 ml of distilled water and titrated in the MAC- or indirect IgG ELISA using WN virus-positive and -negative reference human sera (18, 24). Dilutions to 1:320 and 1:160 of the NRA were found to be the optimal concentrations for use in MAC- and IgG ELISA, respectively. These dilutions resulted in a P/N OD₄₅₀ ratio of 4.19 and 4.54 for MAC and IgG tests, respectively (Table 1). The WN virus SMB antigens produced by the NY99-6480 and Eg101 strains were used at a 1:320 and 1:640 dilution for MAC-ELISA and a 1:120 and 1:320 dilution for IgG ELISA. The negative control antigens, PEG precipitates of the culture medium of normal COS-1 cells and normal SMB antigen, were used at the same dilutions as the respective NRA and SMB antigens. Human serum specimens, diluted to 1:400, were tested concurrently in triplicate with virus-specific and negative control antigens. For the positive test result to be valid, the OD₄₅₀ for the test serum reacted with viral antigen (P) had to be at least twofold greater than the corresponding OD value of the same serum reacted with negative control antigen (N).

The reactivities of NRA and NY99-0648, Eg101, and SLE virus SMBs were compared by MAC- and IgG ELISAs using 21 coded human serum specimens (Table 1). Of the 21 specimens, 19 had similar results on all three antigens (8 negative and 11 suspect or positive). Eighteen specimens were also tested separately using SLE virus SMB antigen. Only 3 of 13 Eg-101 SMB-positive specimens were suspect or positive in the SLE virus MAC-ELISA (Table 1). None of the WN antigen-

negative specimens was positive by SLE virus MAC-ELISA. This result provided further evidence that anti-WN virus IgM did not cross-react significantly with other flavivirus antigens (39) and was specific to diagnose acute WN virus infection regardless of the antigen (NRA or SMB) used in the test. All of the specimens were also tested concurrently by indirect IgG ELISA, and 10 of 21 specimens were positive with all three antigens.

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

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

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

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

^a Titers were calculated using an absolute P/N ratio cutoff of 3.0.

^b Random sample 9 in Table 1.

^c Random sample 7 in Table 1.

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

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

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

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

^a Groups of 10 ICR mice were immunized via a single i.m. injection or i.m. followed by electrotransfer (E).

^b Serum neutralization titers of postvaccination (p.v.) pooled sera were determined as described in Materials and Methods. Week 9 sera were collected from the surviving mice 3 weeks after virus challenge.

^c Mice were challenged by i.p. injection of 1,000 LD₅₀ of virus or by exposure to the bite of three virus-infected mosquitoes (Mosq.).

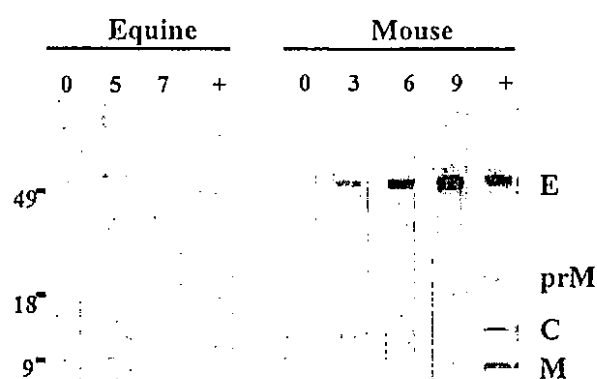


FIG. 4. WN virus-specific reactivity of pre- and postchallenge serum specimens obtained from mice and horses immunized with WN virus DNA vaccine. Pooled serum specimens from the mice and horses used in the experiments were tested at a 1:25 dilution by Western blot analysis using purified WN virion as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

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

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

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

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

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

^a Viremia titer was expressed as log₁₀ Vero cell PFU/ml of serum.

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

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

DISCUSSION

We previously designed a recombinant eukaryotic expression plasmid that contained an optimal genetic constellation to enhance the expression and secretion of JE virus prM and E

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

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

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

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

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

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

As a safe alternative to the traditional SMB antigen preparation and to streamline production efforts, we have cloned

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

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

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ADDENDUM IN PROOF

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

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