

Testing of Novel Dengue Virus 2 Vaccines in African Green Monkeys: Safety, Immunogenicity, and Efficacy

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Abstract. The immunogenicity and safety of three novel host-range vaccines containing deletions in the transmembrane domain of dengue virus serotype 2 (DV2) E glycoprotein were evaluated in African green monkeys. The shorter transmembrane domains are capable of functionally spanning an insect but not a mammalian cell membrane, resulting in production of viral mutants that have reduced infectivity in mammalian hosts but efficient growth in insect cells. Groups of four monkeys received one dose each of test vaccine candidate with no booster immunization. After immunization, levels of viremia produced by each vaccine were determined by infectious center assay. Vaccine recipient immune response to wild-type DV2 challenge was measured on Day 57 by enzyme-linked immunosorbent assay and plaque reduction neutralization test. Two vaccines, DV2ΔGVII and DV2G460P, generated neutralizing antibody in the range of 700–900 50% plaque reduction neutralization test units. All three vaccine strains decreased the length of viremia by at least two days. No safety concerns were identified.

INTRODUCTION

Dengue virus (DV) is a member of the flavivirus family and is transmitted by mosquitoes most commonly found in tropical and sub-tropical environments. Dengue virus exists in four serotypes, DV 1–4, all of which are genetically distinct. Infection with any of the DV1–4 serotypes is sufficient to cause dengue fever. Dengue fever is characterized by headache, fever, and rash. The fever associated with dengue is classically biphasic in which the fever returns for an additional time after its initial resolution.^{1,2} Although high fevers are associated with dengue fever, the illness is typically resolved in 10–14 days with few lasting effects. However, more severe forms of dengue disease, dengue hemorrhagic fever and dengue shock syndrome, are of greater concern. These two forms are usually caused by a secondary heterotypic infection with a different strain of the four closely related but antigenically distinct serotypes.^{3–6} Protection against homotypic reinfection is complete and probably lifelong.^{5,4,7–9} Cross-protection between serotypes is limited, and heterotypic infection is typically associated with higher risk of dengue hemorrhagic fever or dengue shock syndrome.^{10,11} Consequently, there remains a critical need to develop a tetravalent vaccine to confer a balanced and long lasting protection against all four dengue serotypes.^{12,13}

Arbovax Inc. (Raleigh, NC), in collaboration with North Carolina State University, is developing a novel strategy to produce a DV tetravalent vaccine. This vaccine technology is based on studies in Sindbis virus (SV).^{14,15} In SV, it was shown that large truncations of the envelope 2 transmembrane domain (TMD) are tolerated in insect but not mammalian cells. Because insect cells have less cholesterol than the mammalian cells, their transmembrane domains are thinner in cross section; viruses with shortened TMDs can span an insect cell membrane but not the mammalian cell membrane, resulting in a preferential growth in insect cells.¹⁵ This host-derived difference in response to shortened TMD was used to develop a method for production of viral mutants with truncated TMD

that are capable of efficient growth in invertebrate cells but attenuated for productive replication in vertebrate cells.^{15,16}

This difference is considered beneficial for several reasons. First, these host-range (HR) mutant viruses can easily be grown in laboratory conditions in insect cells. This ease of growth does not put additional selective pressure on the virus, thus minimizing the chances of a reversion to the wild-type (WT) phenotype. Second, the deletions are large (4–5 amino acids) and severely limit the ability of these mutants to revert. Third, limiting replication of the virus in mammalian cells enables vaccination with a live virus without producing disease. DV2 HR deletion mutants were found to be stable *in vitro* for four sequential passages in host cell lines,¹⁷ and reverse transcription–polymerase chain reaction (RT-PCR) analysis of virus amplified from African green monkey (*Chlorocebus aethiops*) serum 3–5 days post-vaccination showed no changes in the sequence at the mutation site. There is also little to no risk of the virus becoming mosquito borne through human interactions because levels of viremia present in the serum should be insufficient for contraction by the mosquito.^{18,19}

The hypothesis that vaccination with these HR mutants would provide efficient immunogens resulting in high levels of specific neutralizing antibody, similar to what is seen after infection with WT DV, was tested in this study. A targeted and rational method of deleting amino acids in the TMD of the envelope glycoprotein was used to create DV serotype 2 mutants (Patent no. 6,589,533) (Table 1).^{17,20} As shown in the SV model, deleting amino acids in the TMD of the E protein of DV2 would make these domains shorter and therefore capable of spanning an insect cell membrane but not a mammalian cell membrane. Deletions of this type resulted in the production of mutant viruses that demonstrate reduced infectivity in mammalian hosts but retain efficient growth in insect hosts, producing an HR phenotype, consistent with our results in SV. Only mutants displaying a ≥ 100 -fold differential in titer compared with production of virus from insect cells were considered to be HR mutants (Table 1).

In mice, two deletion mutants, DV2ΔLIG and DV2ΔGVII, with preferential growth in insect cells (Table 1) initiated a good neutralizing antibody response compared with the WT

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TABLE 1
Virus titers and sequences for WT DV2, host-range mutant viruses and DV2 LAV

Virus	Strain	E-T1 sequence	Titer (ffu/mL)	
			Vero	C6/36
WT DV2	16681	₄₅₂ SWTMKILIGVIITWIG ₄₆₇	1×10^6	1×10^7
DV2ΔLIG	16681	SWTMKI-----VIITWIG	1×10^1	3×10^4
DV2ΔGVII	16681	SWTMKILI-----TWIG	1×10^1	4×10^3
DV2G460P	16681	SWTMKILIPVIITWIG	2.5×10^1	5×10^2
DV2 LAV	S16803	SWTMKILIGVIITWIG	$1 \times 10^{4-5}$	ND
WT DV2	S16803	SWTMKILIGVIITWIG	$1 \times 10^{4-5}$	ND
WT DV2	NGC*	SWTMKILIGVIITWIG	5.5×10^5	2×10^5

*NGC = New Guinea C.

control and were considered to be good candidates to be tested in non-human primates (NHP).²⁰ A third HR mutant, DV2G460P, was created by using an alternative strategy of functionally shortening the TMD of the E protein at specific sites using a proline substitution method to kink the helix (Patent no. 13/173,895).^{17,20} The three HR mutants were confirmed by phenotype and sequence analysis. These three HR vaccine strains exhibit higher virus production in the insect host compared with the mammalian host (Table 1) and are produced *in vitro* in insect cells. After inoculation into the mammalian host, it was expected that these vaccine candidates would produce low amounts of viremia but still generate strong immune responses, as shown for other strains of live-attenuated DV.^{21,22} Initial studies were performed to evaluate immunogenicity, safety, and protection after challenge of these three DV2-specific vaccine strains in an NHP model.²⁰

African green monkeys were chosen as the NHP model for this study. Shortage of rhesus and cynomolgus macaques have led to the use of new primate species such as owl monkeys and African green monkeys.²³ Recent studies have demonstrated that African green monkeys provide a potential model for preclinical assessment of novel candidates for dengue vaccines.^{24,25} In addition, the mammalian cell line used to propagate DV in culture, Vero, is derived from African green monkeys and has been extensively used in research and vaccine development and production.^{26,27} Previous studies demonstrated that when infected with DV2, African green monkeys showed viremia in the range of 1–3 days and neutralizing antibody response in the range of 100–500 50% plaque reduction neutralization test (PRNT₅₀) titers, which is similar to those observed in related experiments in rhesus and cynomolgus monkeys.²⁴ The African green monkey has been among the most important NHP models for biomedical research. The need for use of the African green monkey model stems from the recognition that this system is an alternative to the rhesus macaque and has some important advantages. At present, rhesus monkeys are in short supplies and require greater financial resources to conduct a thorough study. In contrast, African green monkeys, which are abundant in the Caribbean region, are disease free, smaller, and easier to handle. African green monkeys have been shown to be an under-used NHP model for dengue.^{24,25,27}

MATERIALS AND METHODS

Cell culture and virus mutagenesis. C6/36 (*Aedes albopictus*, no. CRL-1660; American Type Culture Collection, Manassas, VA) and C7-10 cells (provided by Victor Stollar) were maintained in minimal essential medium (MEM) containing Earl's

salts supplemented with 10% fetal bovine serum, 5% tryptose phosphate broth, and 2 mM L-glutamine (C6/36 medium).²⁸ Vero cells (African Green monkey kidney, no. CRL-1660; American Type Culture Collection) were maintained in 1× MEM supplemented with 10% fetal bovine serum, 5% tryptose phosphate broth, 2 mM L-glutamine, 10 mM HEPES, pH 7.4, and 1× MEM nonessential amino acids (1:100 dilution of nonessential amino acids, no. 11140; Gibco, Carlsbad, CA) (Vero medium). A full-length cDNA clone of DV serotype 2 (DV2; Thai strain 16681, GenBank no. U87411) in pGEM3z+ was obtained from the Walter Reed Army Institute of Research (WRAIR) (Silver Spring, MD) for these studies.²⁹ Details of construction methods of mutant have been reported.²⁰

Primate model and study design. African green monkeys provide an excellent potential model for preclinical assessment of novel candidates for dengue vaccines.^{24,25} In the present study, 20 African green monkeys were used. All animals were prescreened for IgM or IgG against dengue 1–4 by ELISA and for neutralizing antibody (NAb) against all serotypes of DV by using a PRNT. Monkeys that were positive by either or both ELISA or PRNT were excluded from the study. Four monkeys each were assigned to one of five treatment groups to evaluate viremia and antibody responses and to test vaccine protection after a subsequent challenge with live DV2 strain S16803 WT virus (DV2 WT). The treatment groups were vaccine 1 (DV2ΔGVII), vaccine 2 (DV2G460P), vaccine 3 (DV2ΔLIG), negative control (cell filtrate with diluent), and positive control (DV2 strain S16803 live-attenuated virus [LAV], primary dog kidney 50 passage).

Blood samples were collected and clinical observations were made at baseline and at 1, 2, 3, 5, 7, 14, 30, and 57 days after vaccine administration. After blood collection on day 57, animals received live DV2 challenge virus (strain S16803 DV2 WT) before continued blood collection and clinical observations at 58–64, 71, and 142 days post-vaccine administration. A total of 45 mL of blood was obtained from each monkey by the end of study at day 142. Serum was collected from blood samples, which were used to measure viremia and NAb response. Vaccine viremia was measured on days 1, 2, 3, 5, and 7 post-vaccination, and on days 58–64 post-challenge. The NAb response was measured on days 0, 5, 7, 14, 30, 57, 71, and 142. Total IgG and IgM levels were also measured for both pre-challenge and post-challenge samples.

Clinical observation involved measuring packed cell volume/hematocrit, leukocyte count, and platelet count. Animals were also observed for any changes in their body weight, body temperature, respiration rate, and heart rate. Animals were observed in their home cages twice a day for the duration of

the study. More frequent monitoring took place if adverse effects were noted. Clinical examinations took place daily for the first week of the study, bi-weekly through day 30, and every month after that up to day 74.

All animal work was performed by RxGen (Hamden, CT) and adhered to guidelines stipulated by the St. Kitts Department of Agriculture and the U.S. Department of Health and Human Services. RxGen is the organizational institution responsible for the oversight of the study and conducts its primate research through a contractual relationship with the St. Kitts Biomedical Research Foundation (St. Kitts, West Indies) and the Axion Research Foundation (Hamden, CT). RxGen and the St. Kitts Biomedical Research Foundation meet all applicable standards for veterinary care, operation, and review as contained in the National Institutes of Health Guide for the Care and Use of Animals. The St. Kitts facility maintains an animal research committee that reviews the protocols and inspects the facilities as required by the Guide. The Foundation has an approved assurance filed with the Office of Laboratory Animal Welfare, as required by the Guide, no. A4384-01. All studies were conducted in accordance with these requirements and all applicable codes of practice for the care and housing of laboratory animals. Approval of the study protocol by the institutional Animal Care and Use Committee was obtained before initiation of the study.

Vaccines and challenge virus. The DV2 mutants were grown in the *Ae. albopictus* mosquito-derived C6/36 cell line. Cells were divided at a ratio of 1:3 one day before infection. Subconfluent monolayers of C6/36 cells were infected at a multiplicity of infection of approximately 0.03 focus-forming units (ffu)/cell or mock infected. Virus was diluted in 1× MEM medium and each 75-cm³ flask was infected with 1.0 mL of diluted virus or medium for 1.5 hour at room temperature with slow rocking. Fifty T75 cm³ flasks of C6/36 cells were infected with each of the vaccine candidates to ensure that each monkey was injected with at least 10³–10⁴ total ffu of virus/animal. After the initial infection, 4.0 mL of C6/36 media was added to each flask. Flasks were incubated for 7 days at 28°C. Virus was harvested by centrifugation of the supernatant at 4,000 rpm for 10 minutes in a table top centrifuge. Infected and uninfected mosquito cell supernatants were concentrated by tangential flow filtration by using a 1-MDa cut-off membrane as per manufacturer's recommendations (Pall Corporation, Port Washington, NY) and purified twice on 12% and 35% step iodixanol gradients to remove serum albumin and further concentrate the virus. Protein purity was evaluated by using 4–12% bis-Tris gradient gels (Invitrogen, Carlsbad, CA) and stained with SYPRO Ruby protein gel stain (Invitrogen). Purified virus concentrations were determined by using an EZ-Q protein determination kit (Invitrogen).

Vaccine strains and negative controls were administered subcutaneously in a total volume of 0.5 mL iodixanol solution (33% in phosphate-buffered saline diluent). Doses injected for each HR mutant vaccine strain were as follows: DV2ΔLIG (2.5 × 10³ ffu/monkey), DV2ΔGVII (7.5 × 10³ ffu/monkey), and DV2G460P (7.5 × 10⁴ ffu/monkey). Fluorescent focus assays were performed because HR mutants do not form visible plaques on Vero cells. Dosages were determined by estimating protein mass per milliliter of the vaccine candidates at the time of injection and later calculating titers by back titration of the samples. This method was adopted to

preserve the infectivity of the virus strains because they were not stabilized with any additives. The positive control (DV2 LAV) was administered in doses of 5 × 10⁴ plaque-forming units (pfu)/monkey. Sequences of mutagenized viruses and control TMDs are shown in (Table 1). All monkeys were challenged with DV2 WT at a dose of 1 × 10⁵ pfu/monkey (Table 1) on day 57.³⁰

Although the parental strain of HR DV2 mutants was 16681, the attenuated DV2 LAV was passaged through primary dog kidney (LAV50) and DV2 WT challenge virus, both S16803 strain, were used as controls because they were available as vaccine ready lots with known and confirmed titers from WRAIR. These viruses have also been well characterized, extensively studied for primate responses, and have been found to be efficacious in generating antibodies.^{31–35} The response of monkey hosts to these DV2 LAV and DV2 WT S16803 dosages is also well documented.^{36–38} No virus booster immunizations were used in the because on the basis of mouse data, it was expected that the vaccine strains would generate sufficient immune response in one dose.¹⁷ Cell supernatant from uninfected mosquito C6/36 cells was used as the negative control inoculum to monitor for any effects of mosquito antigens in the animals. The protocol designed for use in this study concentrated on gathering data that could assess immunogenicity, protection from challenge, and safety in African green monkeys to support further preclinical validation of these novel candidate dengue vaccines administered in a single dose.

Infectious center assay. This is the first study in African Green monkeys in which viremia has been measured by using an infectious center assay (ICA)³⁹ and mosquito C7-10 cells as indicator cells. The DV HR vaccine candidates do not form plaques on Vero cells and produce smaller plaques in general. Therefore, an assay of infectious centers (IC) on mosquito cells was chosen instead of a traditional mammalian cell plaque assay because this assay is more sensitive and facilitates direct counting of plaque areas produced by DV2 mutants that are not detected by a typical plaque assay.^{40–42} It has been established using DV2 that 1 pfu correlated with approximately 5 ICs, which is within the same standard error of the plaque assay.⁴² The ICA was also chosen to facilitate isolation of individual ICs to determine the sequence of the virus after replication in the primate host. This method of virus isolation made it possible to determine if reversion of the mutants occurred during the viremic period in the host animal.

For the ICA, monkey serum samples from blood collected 0–7 days post-vaccination were serially diluted 1:10 in completed 1× MEM. Two milliliters of log phase C7-10 cells suspended in 1× MEM complete medium (5 × 10⁶ cells/mL) were inoculated with 200 μL of diluted serum and rocked gently at room temperature (25°C) for 1.5 hours. Two milliliters of warm 1× MEM containing 1% low-melting temperature agarose (SeaPlaque Agarose; FMC BioProducts, Rockland, ME) was then added and mixed with each infection, and infections were gently poured onto a layer of cooled 1% agarose in 1× MEM to create a soft agar suspension. After 3–5 days, ICAs were stained with 1× MEM complete medium containing 1% agarose and 0.06% neutral red stain. Control virus of known concentration was titered in the same assay to confirm the detection level of each assay. Infectious centers were then counted to determine titer, picked, and

amplified to confirm the presence of mutant virus in post-vaccination monkey serum.

Plaque reduction neutralization test. Before the PRNT, monkey serum samples were heat inactivated for 30 minutes at 56°C and then diluted serially 1:2 in completed 1× MEM to form dilutions ranging from 1:10 to 1:5,120. The PRNTs were conducted as described with some modifications.⁴³ For this assay, DV2 strain New Guinea C (NGC; provided by Vance Vorndam, Dengue Branch, Centers for Disease Control and Prevention, Atlanta, GA) was used instead of the parental strain of the HR mutants (16681). NGC produces larger plaques that are therefore easier to quantify, which improves the accuracy and clarity of the assay. Fifty plaque-forming units of WT DV2 NGC was then added and mixed into each dilution and incubated at room temperature (25°C) for 15 minutes. After this incubation, sub-confluent Vero plates were inoculated with each dilution of serum/virus mixture and incubated at 37°C for 1.5 hours. Inoculum was then removed from the plates and 1× MEM containing 1% agarose was added to cover each well. After incubating for 7 days at 37°C, plates were stained for 4 hours at 37°C with phosphate-buffered saline diluent containing 1% agarose and 0.06% neutral red stain. After staining, plaques were counted and serum dilutions in which 50% of WT added was neutralized were determined by PRNT₅₀.

Analysis of viruses from ICAs by RT-PCR. To confirm that the desired deletions remained intact in the monkey serum samples after virus replication *in vivo*, RNA was extracted from each IC, amplified in C7-10 cells, reverse transcribed, and amplified by PCR. The RNA was extracted from the supernatant of cultured cells by pelleting the virus at 50,000 rpm in a SW55Ti rotor (Beckman Coulter, Brea, CA) for one hour. The pelleted virus was extracted as described.¹⁴ The RNA pellet was resuspended in 10 µL of diethylpyrocarbonate-treated water and analyzed by electrophoresis on a 1% agarose gel. The extracted RNA was reverse transcribed and amplified by PCR using the One-Step RT-PCR Kit (QIAGEN, Valencia, CA). Primers were designed for use in the RT-PCR by analyzing the folded DV2 RNA structures to optimize RNA binding accessibility.⁴⁴ The products generated in the RT-PCR (approximately 640 basepairs) were extracted with phenol/chloroform, precipitated, and sequenced to confirm the identity and presence of deletions. Some of the RT-PCR products were of insufficient quantity and had to be amplified by nested PCR. These were either sequenced directly or sub-cloned into the pDrive cloning vector and transformed in QIAGEN EZ Competent cells by using the QIAGEN PCR Cloning Plus Kit (QIAGEN). Sample DNAs were then amplified, extracted, and sequenced for verification (Eurofins MWG Operon, Huntsville, AL).

Dengue IgG and IgM ELISA. A dengue ELISA was performed in addition to PRNT to determine any differences in the amounts of specific neutralizing antibody titers present compared with the total amount of Ig produced. Total IgG and IgM measurements were made by using Dengue IgG and IgM ELISA Kits (IBL-America, Minneapolis, MN). In brief, serum samples were diluted 1:21 by adding 10 µL of the sample to 200 µL of sample diluents. One hundred microliters of diluted samples and kit controls were dispensed in appropriate wells of ELISA plates coated with purified DV antigen and incubated at room temperature for 20 minutes. Liquid was removed from the wells and the wells were

washed three times with 350 µL of the 1× wash buffer, followed by addition of 100 µL of the enzyme conjugate to each well. After incubation of 20 minutes at room temperature, the wells were washed three times with the wash buffer and incubated with 100 µL of 3, 3', 5, 5' tetramethyl benzidine substrate for 10 minutes at room temperature. The reaction in the plate was stopped by the addition of 100 µL of stop solution (1N HCl). Optical density was read at 450 nm by using an ELISA reader (GENios; Tecan, Männedorf, Switzerland) within 15 minutes and a reference filter at 630 nm.

RESULTS

Pre-challenge viremia. Animals were screened for DV-specific antibodies before use. Selected animals were injected with 10e3 to 10⁴ ffu/mL of DV2ΔGVII, ΔLIG, G460P, LAV, or vehicle alone (mock). Serum samples from days 1, 2, 3, 5, 7, and 14 post-inoculation were assayed for viremia in the monkey hosts by using an ICA. Viremia was found to peak on days 2–3 post-infection for all vaccine viruses. Average viremia for four monkeys per group is shown in Figure 1. Although each monkey responded differently to the inoculation, each group followed a notable bimodal trend. The amount of viremia detected in the test animals is similar to that with test DV2 vaccines reported in the literature and to the control vaccine DV2 LAV provided by Dr. Robert Putnak (5.0 × 10⁴ pfu/mL, WRAIR).^{36,45,46} Viremia was seen in two distinct peaks, a primary peak at days 2–3 and a second peak between days 5 and 7. Because of the bimodal nature of the viremia and the lack of data for days 8–13, duration of viremia could not be determined. Although the maximum titer of the two peaks of each of the viruses differs in quantity and day of onset, the amount of virus for all vaccine strains on days 1–14 was in the range of approximately 4 × 10⁴ ICU/mL/monkey (approximately 8 × 10³ pfu/mL) (Figure 1).

The total amount of viremia could not be determined because blood was not collected during days 7–14 and viremia

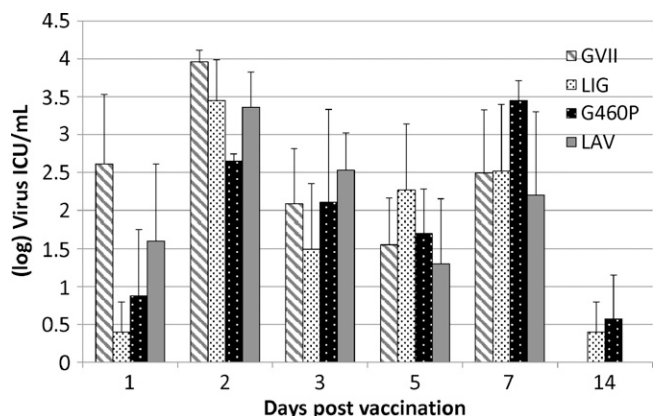


FIGURE 1. Post-vaccination viremia analysis of serum samples by infectious center assay from study day 1 through 14. Virus titer is shown as log infectious units (ICU)/mL. Error bars indicate SEM. No significant differences were seen between any of the viruses through the period measured. The mock injected animals had no detectable viremia. Viremia was still detectable at day 7 post-vaccination. No viremia as detected by day 14. For dengue virus serotype 2 and its mutants, 1 plaque-forming unit is < 10 ICUs. LAV = live attenuated virus.

was still seen on day 7. Previous studies in African green monkeys did not find or predict that DV2 viremia would be longer than 7–8 days. For this reason, viremia was not sampled past day 7 again until day 14.^{24,25} By day 14, the vaccine virus had been completely cleared for each vaccine strain. Using the Student's *t*-test (Welch corrected), we found that there were no significant differences seen between the viremia levels among the different vaccine groups on any of the days tested. These data demonstrate that the test vaccine strains produced equivalent amounts of viremia for the days tested as that produced by the well-established vaccine model DV2 LAV control and are attenuated for replication in the animal host as proposed.^{21,30}

Evaluation of ICs by sequence analysis has confirmed that DV2ΔGVII virus from all four monkeys at day 2 (peak titer) had no reversions, which demonstrated the genetic stability in the animal host of the largest deletion. The DV2G460P mutant produced a delayed viremia (day 3 versus day 2; Figure 1). No ICs were seen in the assay of serum from the mock-infected animals.

Pre-challenge NAb titers. Detection of neutralizing IgM and IgG titers post-vaccination began with the day 0 post-infection samples and included days 5, 7, 14, 30, and 57 (Table 2). The PRNT₅₀ was used to test for NAb production and was performed on each individual sample in each group on the days reported. Samples with an antibody titer < 10, which was the limit of the detection of the assay, were assigned an arbitrary value of 5, and the calculated geometric mean titer (GMT) from each group of animals is shown in (Table 2). The PRNT₅₀ represents the inverse of the serum dilution in which 50% of the control DV2 virus was inhibited.⁴⁷ As with the viremia data, individual monkey NAb titers demonstrated that each monkey responded differently to the inoculation, although each group followed a notable trend. The NAb production (pre-challenge) appears

to peak on day 14 for the DV2 LAV, DV2ΔLIG, and DV2G460P test vaccine strains, which is the day when IgG was first detected (Figure 2). Mutant DV2ΔGVII NAb peaked on day 7. All strains showed NAb titers on day 30 with the peak trailing to PRNT₅₀ of 10 at day 57 (LAV). The control strain DV2 LAV produced peak NAb titers equivalent to those seen previously, approximately 300 PRNT₅₀ on day 14.³⁸ As shown in (Table 2), levels of NAb are highest for all vaccine strains at day 14, which coincides with the end of the viremia (Figure 1). It is difficult to determine if IgM and IgG are contributing to the NAb response because no statistical differences in IgM levels were determined to occur for the days that IgM was measured (Figures 2 and 3). However, IgM levels were measured before detection of IgG, on day 14, and are short-lived. This finding might be contributing to the level of detectable NAb because some neutralization was seen before day 14. By day 57 post-vaccination, no levels of either NAb were detectable for any group with the exception of LAV (PRNT₅₀ = 10).

Post-challenge viremia. Post-challenge viremia was determined for 7 consecutive days (days 57–64) after challenge of NHPs by IC assay (Figure 4). Data showing viremia after challenge were plotted as the averages of the viremias assayed in all four monkeys of each of the vaccination groups. Statistical analysis (Student's *t*-test, Welch corrected) of the data showed that for each day post-challenge, there was no viremia difference within each group until day 6, when significant differences were seen. On day 6, differences in DV2ΔGVII, G460P, and LAV were significantly different from mock ($P < 0.001$). Mutant DV2ΔLIG was considered significantly different ($P < 0.03$). On day 7, all vaccination groups were significantly different from mock ($P < 0.02$). As was seen with the post-vaccination viremias, infection of the mock-vaccinated group displayed a bimodal peak of viremia,

TABLE 2
Pre-challenge neutralizing antibody (Nab) titers for all monkeys as determined by plaque reduction neutralization assay (PRNT₅₀)

Treatment	Animal ID	PRNT ₅₀ on days post vaccination					
		0	5 (IgM)	7 (IgM)	14 (IgM,IgG)	30 (IgG)	57
DV2ΔGVII	R044	< 10	< 10	< 10	< 10	< 10	< 10
	X927	< 10	640	40	320	640	< 10
	Y537	< 10	< 10	2560	640	< 10	< 10
	Y596	< 10	160	2560	640	320	< 10
	GMT	< 10	40	190.3	160	47.6	< 10
DV2ΔLIG	R238	< 10	40	< 10	640	< 10	< 10
	U781	< 10	640	640	640	40	< 10
	R277	< 10	< 10	< 10	40	160	< 10
	X912	320	< 10	< 10	160	640	< 10
	GMT	14.1	28.3	16.8	226.3	67.3	< 10
DV2G460P	R230	< 10	< 10	< 10	640	< 10	< 10
	X144	20	< 10	< 10	640	< 10	< 10
	X395	< 10	2560	< 10	< 10	< 10	< 10
	X898	40	< 10	< 10	320	640	< 10
	GMT	11.9	23.8	< 10	107	< 10	< 10
DV2 LAV	X403	< 10	< 10	< 10	40	< 10	< 10
	X667	< 10	< 10	< 10	640	320	80
	Y340	< 10	640	160	640	640	< 10
	Y557	< 10	40	20	640	80	< 10
	GMT	< 10	28.3	16.8	320	95.1	10.0
Mock	R349	< 10	< 10	< 10	20	40	< 10
	X073	< 10	40	< 10	160	< 10	< 10
	X161	< 10	80	< 10	80	< 10	< 10
	X608	< 10	< 10	< 10	10	< 10	< 10
	GMT	< 10	16.8	< 10	33.6	< 10	< 10

NAb titers are expressed as the geometric mean (GMT) of antibody titers for all the four monkeys in a group. Both IgM and IgG were detected on day 14. The limit of detection of this assay was < 10. NAb titers for DV2ΔGVII peaked on day 7, DV2G460P on days 5 and 14 and DV2ΔLIG and LAV on day 14. DV2ΔGVII and G460P produced higher levels of NAb than DV2 LAV.

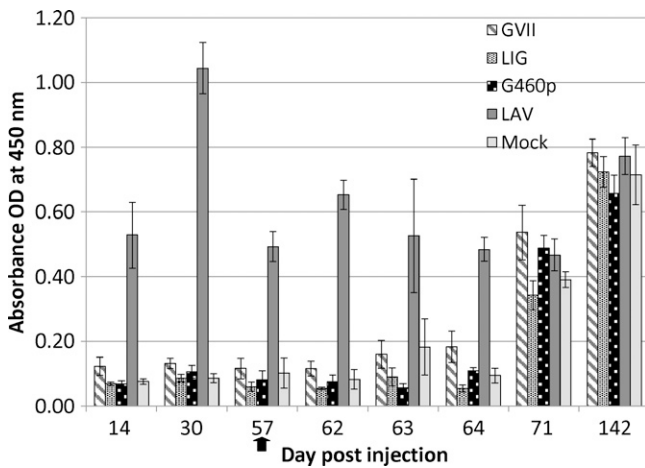


FIGURE 2. Total IgG levels pre-challenge and post-challenge determined by enzyme-linked immunosorbent assay. **Arrow** indicates the day of viral challenge (day 57). Animal serum was tested for IgG against dengue virus pre- (days 14, 30, and 57) and post- (days 62–142) virus challenge. The mean for four animals per group is shown. Error bars indicate SEM. Animals vaccinated with live attenuated virus (LAV) were found to have significantly higher titers of IgG at day 14 ($P < 0.03$), day 30 ($P < 0.002$), day 57 ($P < 0.002$), day 62 ($P < 0.0002$), and day 64 ($P < 0.0002$) compared with mock and other vaccination animal groups.

which might have extended past day 7 post-challenge. No samples were obtained until day 14 post-challenge because the extended viremia was unexpected. It is expected that the mock-vaccinated animals would respond to the challenge with WT levels of viremia (Figure 4).⁴⁶ Without samples from days 7 through 14, it was not possible to determine the length of viremia. However, statistical analysis using a Student's *t*-test indicated that vaccinated animals cleared the challenge virus infection at a significantly earlier time point compared with mock-vaccinated animals (average = 3.5 days for vaccinated groups versus 7 days for mock-vaccinated groups; $P < 0.02$).

Post-challenge NAb titers. To determine the amount of neutralizing antibody present after challenge, assays of DV2

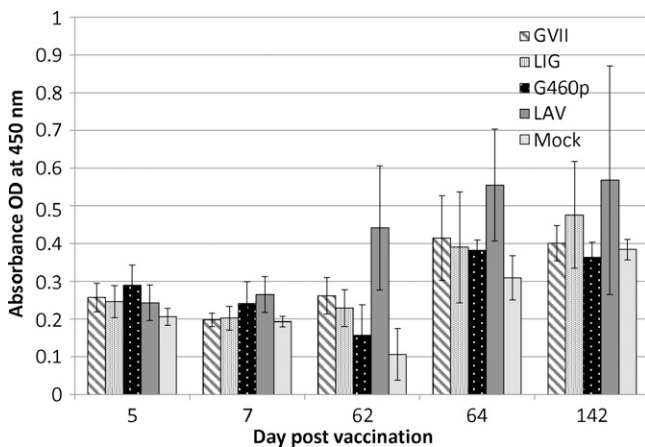


FIGURE 3. Total IgM levels pre-challenge and post-challenge determined by enzyme-linked immunosorbent assay. Each bar indicates the average IgM optical density (OD) measured at 450 nm for four monkeys in each group, and error bars indicate SEM ($n = 4$). No statistical difference was seen between the amounts of IgM measured over the trial period of 142 days. LAV = live attenuated virus.

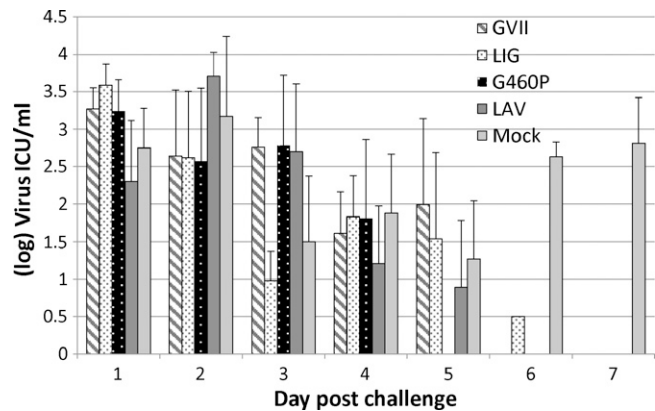


FIGURE 4. Post-challenge viremia. Dengue virus serotype 2 viral infectious centers isolated from green monkey serum from day 1 through day 7 post-challenge were evaluated. Results are shown as log infectious units/mL. Error bars indicate SEM. On day 6 post-challenge, differences in GVII, G460P, and live attenuated virus (LAV) were all extremely significantly different from mock ($P < 0.001$). LIG was considered significantly different ($P < 0.03$). On day 7, all vaccination groups were significantly different from mock ($P < 0.02$). Vaccination results in a reduced length of viremia compared with mock-vaccinated animals ($P < 0.02$).

NAb titers began at day 57 post-inoculation and continued for days 59, 61–64, and 71. A terminal blood collection was performed at day 142. Results from the PRNTs are shown in (Table 3). The NAb begins to appear 4 days post-challenge. With the exception of DV2ΔLIG (with NAb levels that peaked at day 14 post-challenge), all virus strains show a peak of NAb on days 4 or 5, which decreased through day 6 and began to increase on day 14 post-challenge. Maximal values of NAb titers, including those seen for DV2ΔLIG, were seen at day 14 post-challenge. All vaccine strains elicited a NAb titer higher than that for the mock group (approximately 380 PRNT₅₀) by day 14 post-challenge. At day 7 post-challenge (day 64 post-vaccination), mock-infected monkeys had low levels of IgG (Figure 2), although the viremia was high ($> 10^3$ ICU/mL). This finding is consistent with the observations that viremia precedes antibody production during dengue.⁷ The bimodal peaks of neutralization produced after vaccinations were reproducible in the mock group after challenge. All NAb titers returned to higher levels at day 14 post-challenge and were found to be associated with an increasing IgG response but the IgM measured remained more constant (Figures 2 and 3). The PRNT data for the post-challenge virus samples demonstrated that levels of NAb detected for DV2ΔGVII, on average, exceeded antibody titers produced by the control virus DV2 LAV. Taken together, these observations suggest that the mutant DV2ΔGVII qualitatively protected better than the other HR candidates from virus challenge.

Total antibodies pre-challenge and post-challenge. Both IgG and IgM were detected pre-challenge and post-challenge by ELISA. This finding suggests that IgG and IgM may contribute to neutralizing titers. Figure 2 (IgG) and Figure 3 (IgM) illustrate the total optical density values measured at 450 nm for IgG and IgM for each of the virus groups pre-challenge and post-challenge. Figure 2 shows that IgG was not present in large quantities in any of the monkeys vaccinated with the HR mutants before challenge on day 57 compared with the group vaccinated with the DV2 LAV control, which showed

TABLE 3
Post challenge neutralizing antibody (NAb) titers for all monkeys as determined by PRNT₅₀

Treatment	Animal ID	PRNT ₅₀ on days post challenge							
		0	2	4	5	6	7	14 (IgG)	85
DV2ΔGVII	R044	< 10	< 10	< 10	< 10	< 10	40	320	320
	X927	< 10	< 10	< 10	< 10	< 10	< 10	5120	640
	Y537	< 10	< 10	2560	5120	< 10	< 10	640	160
	Y596	< 10	< 10	1280	80	< 10	< 10	640	40
	GMT	< 10	< 10	95.1	56.6	< 10	< 10	905.1	190.3
DV2ΔLIG	R238	< 10	< 10	40	< 10	80	20	320	1280
	U781	< 10	< 10	< 10	< 10	< 10	320	160	160
	R277	< 10	< 10	< 10	< 10	< 10	< 10	1280	320
	X912	< 10	< 10	< 10	< 10	< 10	< 10	640	640
	GMT	< 10	< 10	< 10	< 10	10	20	452.5	452.5
DV2G460P	R230	< 10	< 10	< 10	< 10	< 10	< 10	160	320
	X144	< 10	< 10	< 10	40	< 10	20	640	1280
	X395	< 10	< 10	< 10	2560	< 10	10	1280	160
	X898	< 10	< 10	< 10	< 10	< 10	N/A	2560	640
	GMT	< 10	< 10	< 10	40	< 10	10	761.1	452.5
DV2 LAV	X403	< 10	< 10	< 10	80	320	1280	1280	320
	X667	80	< 10	< 10	40	< 10	80	160	80
	Y340	< 10	< 10	80	640	< 10	80	160	40
	Y557	< 10	< 10	2560	10	80	20	5120	160
	GMT	10	< 10	47.6	56.6	28.3	113.1	640	113.1
(Mock) Challenge	R349	< 10	< 10	< 10	< 10	< 10	40	320	40
	X073	< 10	< 10	160	< 10	< 10	< 10	640	640
	X161	< 10	< 10	< 10	640	80	< 10	160	160
	X608	< 10	< 10	< 10	40	< 10	< 10	640	1280
	GMT	< 10	< 10	11.9	28.3	10	< 10	380.5	269.1

NAb titers are expressed as geometric mean (GMT) of antibody titers for all the four monkeys in a group. The limit of detection of this assay was 10. The day 7 post challenge sample for monkey X898 was not received, and therefore could not be tested in this assay.

significantly higher IgG levels. After day 57 (challenge), levels of IgG did not increase significantly in the test HR vaccine monkey groups until days 71–142. However, total IgG against all mutant viruses tested peaked at day 71 and remained stable to the end of the study at day 142. Animals vaccinated with LAV were found to have significantly higher titers of IgG at day 14 ($P < 0.03$), day 30 ($P < 0.002$), day 57 ($P < 0.002$), day 62 ($P < 0.0002$), and day 64 ($P < 0.0002$) compared with mock and other vaccination animal groups. After virus challenge, there appeared to be no significant difference in IgG titers between the LAV and mutant virus groups. IgM showed no significant difference for any of the vaccination groups on the days tested.

Clinical results. Throughout the study, no major clinical concerns related to experimental vaccines were identified as part of performed assessments. Descriptive clinical observations, including evaluation of the injection site for erythema, animal weight, rectal temperature, heart rate, and respiratory rate, were recorded for each animal during the study. In addition, hematocrits, leukocyte counts, and platelet counts were determined for each collection of blood (Supplemental Tables 1–3). No erythema was observed at injection sites, and no fever was observed after experimental vaccine administration. Clinical observations made after viral challenge at day 57 highlighted modest but significant differences between treatment groups. Minimal body temperature increases were observed in the initial four days after viral challenge across all treatment groups. On day 62 (5 days post-viral challenge), a spike in temperatures was observed for animals that had received DV2ΔGVII and G460P vaccines. Body temperatures steadily decreased toward baseline levels over the next 2–3 days, and no other clinical abnormalities were observed during this time. This spike in temperature was preceded by viremia at day 58, followed by a spike in NAb on days 61 and 62. Analysis of the data suggests that these small spikes are not significantly

different from that seen in the DV2 LAV control at day 7. Minor changes in specific complete blood count measures were noted, but these changes, which included reduced platelet counts, were not consistent with a vaccine-specific safety concern because similar findings were observed in control groups. The slightly reduced platelet counts were likely a result of repeated sedation and blood collection as part of the study protocol, rather than a specific effect of test vaccine delivery because decreases in platelet counts were also observed in negative control treated animals. No pyrexia or other clinical signs were observed after administration of the experimental vaccines and positive (DV2 LAV) or negative controls.^{24,25} Similarly, no major changes in heart rate or respiratory rate were observed as a result of experimental vaccine administration compared with control groups (Supplemental Figure 1).

DISCUSSION

To study the ability of the DV2 HR mutants to effectively reduce the amount and length of viremia induced by virus challenge, it was necessary to deviate from the current rhesus macaque model and develop new assays for accurate determination of dengue viremia. The African green monkey model is not well established for the study of dengue but can become an asset to the field of arbovirology and the development of vaccines. As such, it was imperative to determine with as much accuracy as possible 1) the viremic periods pre-challenge and post-challenge, 2) NAb titers pre-challenge and post-challenge, 3) total antibody levels and IgG and IgM levels, and 4) the ability to protect against virus challenge without a booster immunization.

There was a noted inability of the HR mutants to plaque on Vero cells, and a fluorescent assay to detect viremia was

cumbersome and had a high margin of error. For these reasons, viremia was measured by ICA using C7-10 mosquito cells,^{48,49} which provided reliable and reproducible titers. The NAb titers were determined by standard PRNT on Vero cells with DV2 NGC⁴⁶ as the test strain because the DV2 cDNA clone strain 16681 also does not plaque well on Vero cells. Total IgG and IgM levels were determined by using commercially available ELISAs.

This study provides evidence that this novel method of creating HR mutants with preferential growth in insect cells for the production of DV2 live attenuated vaccines is notable compared with other methods of virus attenuation for several reasons. First, targeted molecular manipulation and selection are sufficient to produce a virus that has a limited host range and maintains the integrity of the virus structure, enabling production of a robust immune response. Second, all vaccine candidates initiated limited replication and produced a viremia comparable to that of the known attenuated DV2 LAV strain. Third, all DV2 HR mutants tested significantly shortened the length of challenge viremia, and the mutant DV2ΔGVII had higher levels of neutralizing antibody after challenge compared with those of the LAV control, which showed two possible markers of attenuation.⁵⁰⁻⁵² In 1988, Innis and others postulated that decreased virulence in monkeys was indicative of attenuation in humans.⁵⁰ Decreased virulence was measured by reduction in the duration and magnitude of viremia. However, at the time no assays were sensitive enough to determine titers of viremia in the monkey serum. This parameter was instead estimated by presence of NAb detected, which showed the (past) presence of virus.

One particularly interesting observation was the bimodal aspect of the vaccine viremia. The initial peak viremia at days 2-3 was expected. However, a second peak of infection was seen during days 5-7 post-vaccinations (Figure 1). A significant amount of viremia was still detected on day 7, suggesting that viremia was longer than 7 days, but shorter than 14 days, which was the next day analyzed. The first peak might represent replication of the virus at the site of injection, perhaps in dendritic cells, which then migrate to the lymph nodes, causing the second burst of replication.¹³ This bimodal trend was also observed after administration of pathogenic WT challenge virus (Figure 4).¹³ A bimodal fever is a diagnostic feature of dengue fever that has been observed since the disease was described.^{2,53} This secondary peak in fever associated with dengue fever could be associated with a secondary burst of viral replication, although more study in humans is needed to sufficiently address this hypothesis. There was no indication of a bimodal distribution of viremia in the rhesus monkey model of DV infection. Combined with the pre-challenge and post-challenge viremia data, this observation gives credence to the methods used to evaluate dengue infection in the African green monkey.

Another critical observation was that all three test vaccine strains elicited much less DV2 specific IgG than the LAV control. When total IgG was compared with levels of NAb, virus neutralization post-vaccination appears to be controlled first by IgM until the appearance of IgG on day 14. Examination of the IgG ELISA data shows that much larger quantities of IgG were elicited by the control LAV than the test vaccines. Because the low level of DV2-specific IgG induced by the test vaccines was neutralizing to a similar extent as the LAV on days 14 and 30 (Table 2), it can be concluded that

the mutant viruses are engaging the immune system in some manner distinguishable from that of the LAV. This is a significant finding because it is believed that high levels of non-neutralizing or sub-neutralizing antibodies contribute to antibody-dependent enhancement, leading to abnormal immune response and resulting in the more severe dengue hemorrhagic disease.⁵³⁻⁵⁵

The observation that HR mutants of DV2 can be protective without induction of high levels of IgG is significant because this finding is not typical. From this finding, it can be postulated that the immune response elicited by HR epitopes produced a much more specific and targeted immune response, possibly caused by some aspect of antigen presentation. DV2ΔGVII and ΔLIG differ by one amino acid in the E TMD and G460P kinks the TMD, which might affect the geometry of the ectodomain with respect to the E transmembrane domain.¹⁷ Recent studies indicate that virus conformation, and specifically use of live virus, is necessary to produce a protective immune response for DV in humans.⁵⁶ Taken together, these data strongly suggest that virus conformation and the use of a whole, live virus vaccine may play a critical role in the establishment of an effective neutralizing response to DV.

In the present study, virus neutralization was assessed by evaluating NAb and total DV2-specific IgG and IgM. Comparison of total IgG and IgM levels post-challenge showed that IgM was elicited before IgG, as has been reported.⁵⁷⁻⁵⁹ IgM levels post-vaccination were not found to be significantly different for the days tested (Figure 2). Although total IgG levels generated by the mutants appeared low, excellent neutralization of the challenge virus was seen for DV2ΔGVII and good neutralization was seen for G460P. Of the three HR vaccine strains tested, post-challenge virus NAb levels elicited were shown to be DV2ΔGVII > G460P > ΔLIG. All vaccine candidates induced NAb levels > 190 PRNT₅₀ values by the end of the study. A minimal seroconversion PRNT₅₀ value ≥ 10 is generally accepted as a positive response, which puts these values into the high-responding NAb category.^{21,30} This response was accomplished without eliciting high levels of IgG, which is paradoxical. However, there is evidence that a large number of antibodies are not required for virus neutralization.⁶⁰⁻⁶³ This phenomenon has been shown to be the case for one E1-neutralizing monoclonal antibody for Sindbis virus.^{60,61} There may be differences in antibody affinity or avidity of NAb generated to the conformations exposed by the mutant viruses.

A critical aspect of an effective and safe dengue vaccine is the ability to initiate a strong balanced neutralizing antibody response in the absence of significant amounts of non-neutralizing or sub-neutralizing antibodies. Our results indicate the potential for this method of generating live, HR mutants of DV to produce a successful vaccine. Antibody titers at maintenance levels were not determined because no additional blood collections were performed past 142 days.

All HR vaccine candidates were highly effective in neutralizing the challenge virus viremia compared with the control DV2 LAV. High titers of NAb were observed without use of a booster immunization, which is unconventional. Although viremia of the WT challenge virus in the mock-vaccinated animals showed no significant difference from the vaccine groups until day 6 when levels of viremia in the mock-infected animals were still significantly higher than those of the vaccine groups.

To reiterate a critical point, all test vaccines were able to protect African green monkeys from viremia to the same extent as the LAV vaccine control, with much less measurable IgG in the serum. This enhanced protection may stem from two sources. First, the mutations constructed may confer slight differences in immunogenicity than that expressed by the parental DV2 16681 strain. It was proposed that this property of the mutants might be the result of conformational differences transduced to the E ectodomain from the mutated TMD α -helix such that the epitopes of the mutants are presented to the host immune system in a more efficient manner or that new epitopes are exposed. The particular effects of the conformation of the virus structure on the immune response of the monkeys is difficult to predict without further experimentation. However, this should not impede development of additional HR mutants for vaccines. Second, it was demonstrated that a large amount of DV2 HR mutant virus particles are non-infectious, and these particles may contribute to immunogenicity of the vaccines.¹⁷ Although it is well established that killed DV will elicit an immune response, the effects of adding non-infectious virus to infectious virus in a vaccine are not known. One simple explanation could be that the additional antigen may serve as an adjuvant.

Overall, with the endpoints assessed in the present studies provided by a detailed analysis of the complete blood count data, no significant safety concerns adversely impact preclinical development of these vaccines. In conjunction with viremia and antibody response results, these data have provided great insights into the safety and protection of these test viruses. This study has provided important data, which strongly support continued preclinical development of these mutants as vaccine candidates. These data substantiate development of similar vaccine strains for DV1, DV3, and DV4, which are currently being developed for testing singly and in a tetravalent combination. Virus production of the vaccine strains will be moved from C6/36 cell line to Sf9 (*Spodoptera frugiperda*), which is now being approved for vaccine production.⁶⁴

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