

INDUCTION OF BIVALENT IMMUNE RESPONSES BY EXPRESSION OF DENGUE VIRUS TYPE 1 AND TYPE 2 ANTIGENS FROM A SINGLE COMPLEX ADENOVIRAL VECTOR

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Abstract. There are approximately 100 million new cases of dengue (DEN) virus infection each year. Infection can result in illness ranging from a mild fever to hemorrhaging, shock, or even death. There are four serotypes of dengue virus (DEN1–4), and immunity to one serotype does not cross protect from infection with other serotypes. Currently there are no approved vaccines for dengue fever. In this report, we describe the construction of a bivalent dengue virus vaccine using a complex recombinant adenovirus approach to express multiple genes of DEN1 and DEN2 serotypes. In vaccinated mice, this vector induced humoral immune responses against all four dengue serotypes as measured by enzyme-linked immunosorbent assay. However, the neutralizing antibody responses were specific for DEN1 and DEN2 serotypes. Expansion of this vaccine development platform towards the DEN3 and DEN4 serotypes can lead towards the development of an adenovirus-based tetravalent dengue vaccine.

INTRODUCTION

Dengue (DEN) virus infection can range in clinical manifestation from a mild febrile illness known as dengue fever (DF) to more severe disease such as dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS).¹ It is estimated that each year there are approximately 100 million cases of DF worldwide, with 500,000 cases of DHF and 25,000 deaths attributed to dengue virus infection.² Dengue viruses are mosquito-borne pathogens transmitted primarily by *Aedes aegypti* and *Ae. albopictus* species. These particular species are found worldwide in tropical and subtropical regions, as well as in the Gulf Coast Region of the United States. Because of the widespread distribution of these mosquito vectors, more than 2.5 billion people now live in areas where dengue virus is considered endemic.³

There are four characterized, antigenically distinct serotypes of dengue virus (DEN1–4), all of which belong to the family Flaviviridae. The viral genome consists of a positive single-stranded RNA nearly 11 kb in length, which is translated into a single polyprotein that is cleaved by both host cell and viral proteases to produce 10 different viral proteins. There are three structural proteins: capsid (C), premembrane (prM), and envelope (E); and seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5.⁴

Despite having been identified more than 60 years ago, there is still no specific antiviral drug or approved vaccine for DF. Additionally, although immunity from one dengue serotype is long-lived, it does not protect against infection by other serotypes, and may in fact enhance the severity of a secondary infection with a different serotype through a phenomenon known as antibody-dependent enhancement of infection.¹ Therefore, the need for a tetravalent dengue vaccine that protects against all four serotypes is of utmost importance to such complications. Live attenuated dengue viruses have shown inconsistent results in animal models and human trials over the years as either monovalent or tetravalent vac-

cine formulations.^{5–8} Most of these studies showed a great deal of variation between immune responses to each serotype when human volunteers were vaccinated with tetravalent formulations. Additionally, this approach carries with it considerable safety issues such as under-attenuation or reversion to pathogenic state. Other dengue vaccine approaches have used separately produced and purified viral subunits.^{9–12} This approach usually involves production of the recombinant proteins in non-mammalian cell cultures, and can result in proteins that are either not fully glycosylated or are folded differently than the viral proteins expressed during infection. As a result, these synthetic proteins used in the vaccine may not induce a completely neutralizing antibody response to the heavily glycosylated viral proteins.

The use of live, chimeric dengue viruses as a potential vaccine was first demonstrated in the early 1990s.¹³ This approach is based on creation of a chimeric (intertypic) dengue virus that generally involves the use of a common serotype backbone that expresses structural genes of a different serotype. Animal studies have reported induction of tetravalent anti-dengue neutralizing antibody responses in mice as well as protection from viral challenge in non-human primates using various chimeric dengue vaccine platforms.^{14,15} Other researchers have investigated creating a flavivirus chimeric vaccine composed of a yellow fever virus backbone and dengue virus structural proteins.^{16,17} This platform also offered protection from dengue virus challenge in both rodent and non-human primate studies. Although offering promising data, a chimeric dengue virus is still a live attenuated virus carrying the same safety issues mentioned above for live attenuated virus vaccines. Furthermore, the mixture of multiple live-attenuated chimeric dengue viruses may enhance the potential for recombination among the replication-competent hybrids.

Naked DNA vaccines have also shown the ability to elicit neutralizing antibody responses in mice and offer protection from dengue virus challenge in monkeys using monovalent constructs.^{18–20} Other DNA vaccine studies have investigated tetravalent formulations.^{21,22} The development of DNA vaccines for dengue virus is an encouraging platform, although the potency of immune induction may limit the ef-

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ficacy of DNA vaccines. For example, several studies show vaccination of non-human primates with dengue DNA vaccine constructs offered only partial protection from dengue infection after virus challenge.^{20,23,24}

Replication-defective recombinant adenovirus (rAd) vectors have been shown to drive high transgene expression levels and to infect a wide variety of dividing and non-dividing target cells. Recombinant Ad-based vaccines have been studied in regard to several members of the flavivirus family, including hepatitis C virus,²⁵ tick-borne encephalitis virus,²⁶ and DEN-2 virus.²⁷ First-generation rAd vectors are often limited by the genome space available for exogenous DNA insertion. For example, the rAd-based dengue vaccine mentioned above expressed only the ectodomain of the E protein of a single serotype.²⁷ Because much of the world's population has been subjected to natural wild-type adenovirus infection, there is a possibility that circulating Ad-neutralizing antibodies may limit a rAd-based vaccine vector's efficacy. Furthermore, these Ad-neutralizing antibodies would have the potential to lower the level of dengue neutralizing antibodies induced by the vaccine, putting the vaccinated individual at risk for immune enhancement of infection. However, results from two independent clinical trials have suggested that pre-existing adenovirus immunity in the human volunteers had no bearing on the efficacy of the respective vaccine vectors under study.^{28,29} Whether the immune enhancement phenomenon will influence an Ad-based dengue vaccine is unknown.

We previously described the development of a novel complex rAd-based vaccine platform (cAdVax) used to construct monovalent or trivalent Marburg virus vaccines^{30,31} and a bivalent Ebola virus vaccine.³² We applied the cAdVax platform to develop a bivalent dengue virus vaccine expressing the prM, E, and NS1 genes of DEN1 and DEN2 (cAdVax-DEN1/DEN2). This vector simultaneously expresses antigens from DEN1 and DEN2 serotypes in infected target cells *in vitro*. We found that vaccinating mice with the cAdVax-DEN1/DEN2 vaccine induced antibody responses reactive to all four dengue serotypes as measured by enzyme-linked immunosorbent assay (ELISA). However, the neutralizing antibody responses induced by this vaccine were DEN1 specific and DEN2 specific. The results from these studies indicate that 1) it is possible to express multiple antigens from two different dengue serotypes in a single Ad vector, 2) doing so can induce a bivalent neutralizing antibody immune responses in mice, 3) this approach can be used to develop a second bivalent cAdVax vector expressing antigens from the remaining two dengue serotypes, and 4) a combination of two bivalent cAdVax dengue vectors could serve as a candidate tetravalent dengue vaccine.

MATERIALS AND METHODS

Cell lines. HEK293 (human embryonic kidney) and Vero E6 (African green monkey kidney) cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). Both cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS; HyClone; Logan, UT). Hybridoma cell lines 15F3-1, 3H5-1, 5D4-11, and 1H10-6 were obtained from ATCC and maintained in HybriCare medium (ATCC) supplemented with 10% FBS. Hybridoma cell lines 13E7-9-10 and 2H2-9-15

were obtained from the United States Army Medical Research Institute for Infectious Diseases (Fort Detrick, MD). These two cell lines were also maintained in HybriCare medium supplemented with 10% FBS.

Construction of the cAdVax-DEN1/DEN2 vaccine. For construction of cAdVax-DEN1/DEN2, wild-type dengue viruses DEN1 (Hawaii; accession nos. AF425619 and X76219) and DEN2 (New Guinea C; accession no. AF038403) were obtained from Dr. Barry Falgout (U.S. Food and Drug Administration Center for Biologics Evaluation and Research, Rockville, MD). Wild-type viruses DEN3 (H87) and DEN4 (814669) were also obtained from Dr. Falgout. The viruses were propagated in Vero cells and cDNAs for DEN1 and DEN2 were generated by reverse transcription-polymerase chain reaction (RT-PCR). The PCR was then used again to generate the prM-E-NS1 cassette for both dengue serotypes with desired restriction sites for the subsequent cloning steps. The PCR fragments generated were subcloned into the cAdVax shuttle vectors 2pL-Ad and 2pR-Ad. The DEN1 prM-E-NS1 cassette was inserted into the multiple cloning site *Bam* HI/*Eco* RV of 2pL-Ad, and same cassette for DEN2 was inserted into the multiple cloning site *Xho* I/*Eco* RV of 2pR-Ad. Both transgene cassettes were identically flanked by the cytomegalovirus (CMV)*ie* promoter and bovine growth hormone (BGH) polyA at the 5' and 3' ends, respectively. Open reading frames (ORFs) were sequenced to ensure that no mutations occurred during the PCR and subcloning processes. Once each cloning construct was verified, the cAdVax-based dengue vaccine vector was constructed as described previously.³³ This vector was based on a modified Ad5sub360, which contains deletions in E1, E3, and almost all E4 ORFs with the exception of ORF6.

cAdVax vector propagation, confirmation by sequencing analysis, and titration. cAdVax vectors were propagated in HEK293 cells, using previously described procedures.³³ Briefly, HEK293 cells, which provide Ad5 E1a and E1b functions *in trans*, were transfected with the recombinant cAdVax-DEN1/DEN2 vector genomic DNA using LipofectamineTM Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Transfected cells were maintained until Ad-related cytopathic effects were observed (typically 7–14 days post-transfection), at which point the vector-containing cells were harvested, as previously described.^{33,34} After several rounds of single-plaque selection, the DNAs of candidate vaccine vector clones were analyzed by restriction endonuclease digestion and sequencing analysis. This confirmed that the vaccine preparations contained the correct dengue gene sequences.

The correct cAdVax vector clones were re-amplified in HEK293 cells and purified by ultra-centrifugation in cesium chloride gradients as previously described.³³ All vectors were titrated on HEK293 cells in 12-well plates by infecting triplicate wells with serial dilutions of the vectors. The resulting titers were scored as plaque-forming units (pfu)/mL.

Dengue indirect immunofluorescence assay. To visualize protein expression from the cAdVax-DEN1/DEN2 vector, Vero cells were infected with cAdVax-DEN1/DEN2 at a multiplicity of infection (MOI) of 20, or with wild-type dengue viruses at an MOI of 2. Three days post-infection, the cells were fixed with 4% formaldehyde, permeabilized with detergent (phosphate-buffered saline, 2.5% non-fat dry milk, 0.05% Tween-20, 0.5% Triton-X 100), and probed with sero-

type-specific monoclonal antibodies (MAbs) against dengue virus. The MAbs specific for DEN1 NS1 (clone 15F3-1), DEN1/DEN3 E (clone 13E7-9-10), or DEN2 E (clone 3H5-1) were prepared from supernatants of the hybridoma cell cultures. The hybridoma cell lines were maintained in culture media until they reached a cell density of approximately 1.5×10^6 cells/mL. The supernatants were then cleared by centrifugation at $600 \times g$ for 5 minutes. Cleared supernatants were then aliquotted and stored at -20°C . The MAb preparations were used undiluted for these assays. The specificities of these antibodies have been previously described.^{35,36} To visualize protein expression, either fluorescein isothiocyanate-conjugated anti-mouse IgG (Sigma, St. Louis, MO) or phycoerythrin-conjugated anti-mouse IgG (BD Pharmingen, San Diego, CA) were used as secondary antibodies. Cells were observed under a ultraviolet fluorescent microscope (IX70; Olympus, Tokyo, Japan).

Immunoblot analysis. HEK293 cells were infected with cAdVax-DEN1/DEN2, DEN1, or DEN2 for 24 hours. Cells were washed three times with PBS and lysates were prepared using MPERS buffer (Pierce, Rockford, IL) according to the manufacturer's instructions. Cell lysates (10 μL) were separated on 4–12% NuPAGE gels (Invitrogen) and then transferred to polyvinylidene fluoride membranes. Membranes were then probed with MAbs against dengue virus. Antibodies used and specificities were as follows (from U.S. Naval Medical Research Center, Bethesda, MD): 8B9 (DEN1-E), 3H1 (DEN2-E), 8C2 (DEN1-prM), and 7E11 (DEN NS1). Proteins reacting with MAbs were visualized using the WesternBreeze Chemiluminescent Western blot immunodetection kit (Invitrogen).

Immunization of mice with cAdVax-DEN1/DEN2 vaccines. Outbred six-week old CD-1 mice (Charles River Laboratory; Charleston, SC) were immunized intraperitoneally at weeks 0 and 4 with 1×10^8 pfu of cAdVax-DEN1/DEN2 or control vaccine prepared in 100 μL of PBS/10% glycerol. An adenovirus-based hepatitis C virus vaccine (HC3) was used as a negative control. Blood was collected at two-week intervals from the mice by retro-orbital extraction under light anesthesia (CO_2 inhalation). Sera were prepared from each animal to determine the titer of antibodies to dengue virus. Each animal was analyzed independently. Vaccinated mice were visually monitored for any adverse effects resulting from immunization. Particular attention was paid to food and water intake, coat texture (ruffled coats are often a sign of illness), and excessive weight loss or gain. All animals were maintained and treated under the standards and regulations of the Institutional Animal Care and Use Committee.

Preparation of animal sera. Sera were prepared from each blood sample by incubating the blood at room temperature for approximately four hours to allow for clotting and an overnight incubation at 4°C . The next day, clots were removed and blood was centrifuged at $2,000 \times g$ for 10 minutes. Supernatants were transferred to sterile tubes and the serum was stored at -80°C . Small aliquots were stored short-term at 4°C . Sodium azide was added as a preservative to these samples to a final concentration of 0.05%.

Indirect ELISA. Titers of antibodies against dengue virus in mouse sera were determined by an indirect ELISA. The antigen consisted of wild-type DEN1, DEN2, DEN3, or DEN4 viruses collected from infected Vero cell culture supernatants. Viruses were propagated in Vero cells until ap-

proximately 50% of cells showed the cytopathic effects of dengue virus replication (7–10 days). Each virus preparation was subsequently harvested as cell culture supernatant and stored at -80°C . Virus preparations were coated directly onto 96-well flat-bottom plates (Nunc-Immuno Plate MaxiSorp Surface; Nalge Nunc International, Rochester, NY). Optimum dilutions of virus-containing supernatants used for coating the plates were determined by titration of each serotype with positive control MAbs 15F3-1 (DEN1), 3H5-1 (DEN2), 5D4-11 (DEN3), or 1H10-6 (DEN4). The MAbs were collected from hybridoma cell culture supernatants as stated above. Because of variations in viral stock titers, the optimization procedures were performed for all virus preparations. Assays were performed as previously described.³¹ Antibody titers were determined by calculating the dilution of serum that corresponded to a signal of three times the background for that particular test.

Dengue plaque reduction neutralization assay. The same mice used in the ELISA experiments described above were killed nine weeks after the primary immunization and animal sera were collected by cardiac puncture after killing. Serial dilutions of heat-inactivated sera from vaccinated or control animals were pre-incubated for 30 minutes at 37°C with wild-type dengue virus (60 pfu per dengue virus serotype per serum dilution) before infection of Vero cell monolayers in six-well plates. After infection, the monolayers were covered with an agar/medium overlay and incubated for seven days at 37°C . The agar overlays were removed and the cells were fixed in 4% formaldehyde, followed by a blocking and permeabilization step in a milk/detergent buffer (PBS, 2.5% non-fat dry milk, 0.05% Tween-20, 0.5% Triton-X 100). After blocking and permeabilization, the plates were probed with the primary antibody 2H2-9-15. This MAb, which reacts with all four serotypes of dengue virus, was prepared as a hybridoma supernatant and used undiluted as a primary antibody. After primary antibody, the plates were washed three times with PBS, 0.05% Tween-20 and probed with the secondary antibody, an alkaline-phosphatase conjugated anti-mouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The plates were then washed three times and dengue plaques were visualized by adding the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium (Pierce). For the neutralization studies, non-immune animals were used as negative controls. Raw data (number of plaques per well) were converted to percent inhibition of dengue plaque formation for each dilution of serum used.

Statistical analysis. An unpaired Student's *t*-test was applied using Microsoft (Redmond, WA) Excel[®] software.

RESULTS

Construction of an adenovirus expression vector that contains dengue genes. Our novel cAdVax technology allows for insertion of multiple transgene cassettes into a recombinant adenovirus backbone.^{31–33} This replication-defective Ad platform differs from earlier Ad-based vectors in that it contains, in addition to deletions in the adenoviral E1, E3, and E4 (except ORF6) genes, multiple insertion sites throughout the adenovirus backbone. These modifications enable the vector to accommodate relatively large amounts of exogenous DNA

(up to 7 kb) and render the vector deficient for replication (Figure 1).

We constructed a bivalent cAdVax-based dengue vaccine candidate that expresses the prM, E, and NS1 proteins from the DEN1 and DEN2 serotypes (Figure 1B). The dengue E glycoprotein is considered a crucial antigen for inducing protective immunity,^{21,37} and the dengue non-structural proteins, including NS1, are associated with induction of cellular immune responses.³⁸ Therefore, including both proteins in the vaccine vector increases the likelihood for induction of an immune response that would be both humoral and cellular in nature. We included the prM protein is to increase the proper expression, processing, and cellular localization of the E glycoprotein.^{39,40} For cAdVax-DEN1/DEN2 construction, the prM-E-NS1 DNA sequences were obtained from wild-type DEN1 (Hawaii strain) and DEN2 (NGC) viruses using RT-PCR. The prM-E-NS1 sequence of DEN1 was incorporated into the left end of the Ad vector genome, and the same sequences of DEN2 were inserted on the right end (Figure 1B). The vector was then propagated, purified, and titrated as described in the Materials and Methods.

Bivalent dengue protein expression from a single adenovirus vector. After completing construction of the vaccine vector, we confirmed expression of dengue virus proteins upon infection of target cells *in vitro*. Vero cells were mock infected (control), infected with wild type DEN1 or DEN2 at an MOI of 2, or infected with cAdVax-DEN1/DEN2 at an MOI of 20. Three days after infection, cells were fixed and probed with MAbs specific for DEN1 NS1 (Figure 2A), DEN1/DEN3 E (Figure 2B), or DEN2 E (Figure 2C). Dengue virus proteins were then visualized under a fluorescent microscope using fluorescein isothiocyanate (Figure 2A)– or phycoerythrin (Figure 2B and C)–conjugated secondary antibodies.

When we used the antibody to DEN1 NS1 protein, Vero cells infected with wild type DEN1 or cAdVax-DEN1/DEN2 (Figure 2A, panels 2 and 4) stained positive. Control and DEN2-infected cells showed no DEN1 NS1 expression, as expected (Figure 2A, panels 1 and 3). Fluorescent staining with the DEN1/DEN3 E protein MAb showed similar results with DEN1-infected cells and cAdVax-DEN1/DEN2-infected cells staining positive (Figure 2B, panels 2 and 4) and no

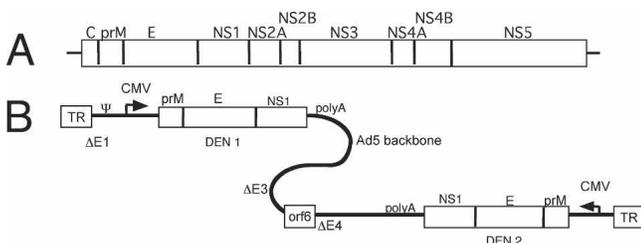


FIGURE 1. Dengue (DEN) virus and cAdVax vector genomes. **A**, Schematic showing the mature viral proteins within the polyprotein open reading frame encoded by the dengue virus positive-stranded RNA genome. The four serotypes of dengue virus are identical in gene structure, but vary in sequence. C = capsid, prM = pre-membrane, E = envelope; NS non-structural protein. **B**, Schematic of cAdVax-DEN1/DEN2 vaccine construct. Each dengue prM-E-NS1 transgene sequence was inserted into identical cytomegalovirus (CMV)/ie/bovine growth hormone (BGH) polyA cassettes, which were then used to construct the cAdVax vector. TR = terminal repeat; Ad5 = adenovirus subtype 5; orf6 = adenovirus E4 gene open reading frame 6.

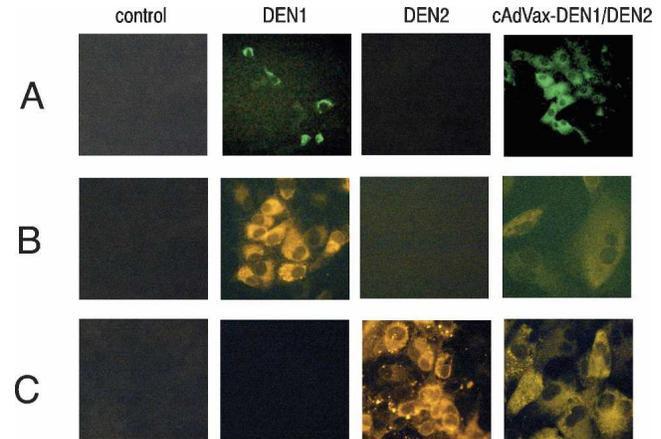


FIGURE 2. Expression of dengue (DEN)1 and DEN2 antigens from a single cAdVax vector. Vero cells were mock infected or infected with cAdVax-DEN1/DEN2 or wild-type dengue viruses of each serotype at a multiplicity of infection of 20 or 2, respectively. At three days post-infection, the cells were fixed in 4% formaldehyde and probed with monoclonal antibodies (MAbs) specific for dengue virus proteins of each serotype: **A**, DEN1 NS1 (Mab clone 15F3-1); **B**, DEN1/DEN3 E (Mab clone 13E7-9-10); **C**, DEN2 E (Mab clone 3H5-1). Proteins were visualized with a fluorescein isothiocyanate (**A**)– or phycoerythrin (**B** and **C**)–conjugated secondary antibody under an ultraviolet fluorescent microscope. Magnification: row A \times 300; row B \times 400; row C \times 400.

fluorescence in the control or DEN2-infected cells (Figure 2B, panels 1 and 3). This particular antibody clone is specific only for DEN1 and DEN3 E proteins,³⁵ and because our cAdVax-DEN1/DEN2 vector contains no DEN3 genes, it was suitable for detection of DEN1 E in this assay. The DEN1 specificity is supported in that this antibody did not react with DEN2-infected cells. Finally, when we used antibody to DEN2 E protein, we found a similar staining pattern in DEN2-infected cells and cAdVax-DEN1/DEN2-infected cells (Figure 2C, panels 3 and 4). Control and DEN1-infected cells showed no DEN2 E protein fluorescent staining (Figure 2C, panels 1 and 2). Collectively, these data show that the cAdVax-DEN1/DEN2 vector co-expresses dengue virus proteins from two different serotypes *in vitro*.

To support the results from the immunofluorescence assays above, we performed Western blot analyses of cAdVax-DEN1/DEN2-infected cells (Figure 3). HEK 293 cells were uninfected (lane 5) or infected with cAdVax-DEN1/DEN2 (lane 2), DEN1 (lane 3), or DEN2 (lane 4) viruses. After infection, cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blots were performed to detect the dengue virus proteins. Interestingly, the DEN1 E protein-specific antibody was unable to detect any reactive proteins in cAdVax-DEN1/DEN2-infected cell lysates or in control lysates. However, the E protein was readily detectable in DEN1-infected cell lysates (Figure 3A). This was in contrast to the immunofluorescence data, where the DEN1 E protein was detectable in cAdVax-DEN1/DEN2 infected cells (Figure 2). The DEN2 E protein-specific antibody (3H1) detected protein bands in cAdVax-DEN1/DEN2-infected cells and DEN2-infected cells only, as expected (Figure 3B). Although DEN1 E protein was not detectable in these Western blots, we were able to detect DEN1 prM using Mab 8C2. This antibody detected proteins in cAdVax-DEN1/DEN2-infected cells and DEN1-infected

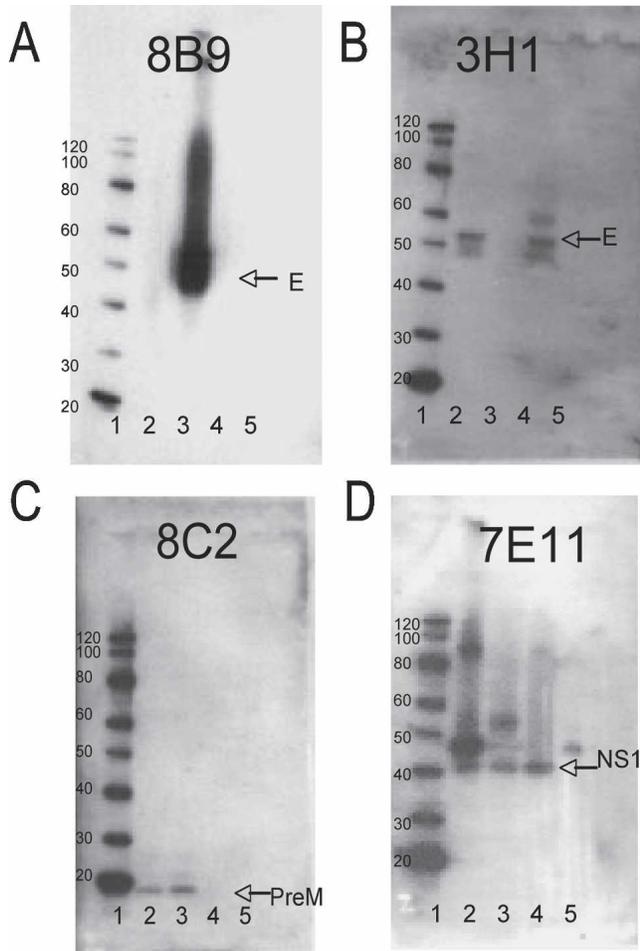


FIGURE 3. Detection of dengue (DEN) virus proteins in vector-transduced cell lysates. HEK 293 cells were either uninfected (lane 5) or infected with cAdVax-DEN1/DEN2 (lane 2), DEN1 (lane 3), or DEN2 (lane 4) for 24 hours. Cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and dengue virus proteins were detected by Western blot using monoclonal antibodies specific for DEN1 envelope (E) protein (A), DEN2 E protein (B), DEN1 pre-membrane (PreM) (C), or non-structural protein 1 (NS1) (D). The NS1-specific antibody reacts with all four serotypes of dengue virus NS1. Lane 1, Magic Mark (Invitrogen, Carlsbad, CA) molecular mass standard.

cells only. Finally, we detected the dengue virus NS1 protein in all three infected cell lysates (Figure 3D). The antibody used for this blot (7E11) is not serotype specific, and therefore detected both DEN1 and DEN2 NS1 proteins. Interestingly, the NS1-specific antibody also appeared to detect a high molecular weight protein of approximately 90 kD in the cAdVax-DEN1/DEN2-infected cell lysate.

Induction of humoral immune responses cross-reactive with all four dengue serotypes by vaccination of mice with cAdVax-DEN1/DEN2. The cAdVax-mediated cellular expression of dengue antigens should mimic the expression that would occur during a natural dengue infection. We hypothesize that *in vivo*, this presentation of dengue antigens to the immune system will induce an immune response to dengue viruses, all without the clinical manifestations of an actual dengue infection.

To test the immunogenicity of our dengue virus vaccine vector, we vaccinated CD-1 mice IP with 1×10^8 pfu of

cAdVax-DEN1/DEN2 at weeks 0 and 4. Sera from all animals were collected biweekly for antibody titer analyses. For a negative control, sera from animals vaccinated with HC3 were used. These animals were vaccinated with the same dose, route, and schedule as those receiving the cAdVax-DEN1/DEN2 vaccine. ELISAs were performed using each of the four dengue virus serotypes as immune targets. We found, in addition to reactive antisera for DEN1 (Figure 4A) and DEN2 (Figure 4B), that our bivalent vaccine induced cross-reactive antibodies with DEN3 (Figure 4C) and DEN4 (Figure 4D). The antibody titers to dengue virus against all four serotypes were detectable as early as two weeks after immunization, and continued to increase over time (Figure 4). Control sera showed some cross-reactivity with each dengue virus serotype as well, although considerably lower than the test group. This low level of reactivity appeared to be non-specific and may be caused by serum components within the dengue virus culture supernatants that were used as immune targets. The differences in DEN3 reactivity between cAdVax-DEN1/DEN2-vaccinated and HC3-vaccinated animal sera were relatively small in earlier collection points (Figure 4C). However, by eight weeks post-primary immunization, the cAdVax-DEN1/DEN2-vaccinated animal sera showed a DEN3-reactive profile well above the control animal sera, similar to that of the other three serotypes.

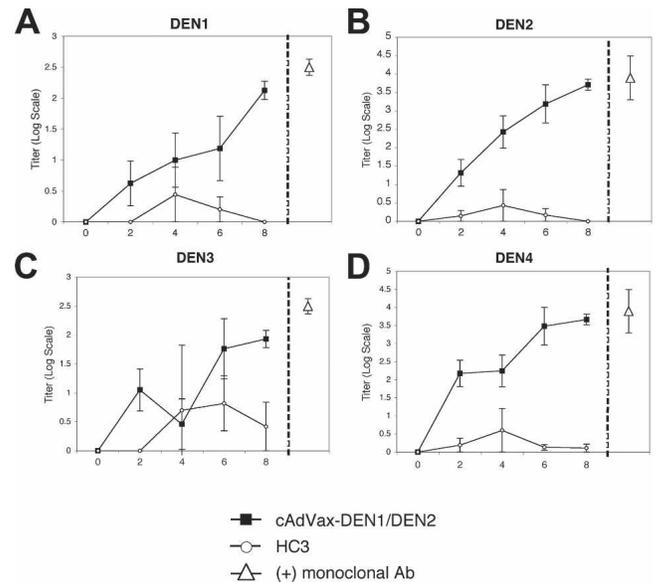


FIGURE 4. Induction of cross-reactive antibodies to dengue (DEN) virus in mice vaccinated with cAdVax-DEN1/DEN2. CD-1 mice were vaccinated with 1×10^8 plaque-forming units of cAdVax-DEN1/DEN2 (solid squares) at weeks 0 and 4. Control mice (open circles) received the same dose and schedule of the adenovirus-based hepatitis C virus (HC3) control vaccine. Blood samples were collected at two-week intervals after the primary immunization. Titers of antibody (Ab) to dengue virus were determined by enzyme-linked immunosorbent assay using 96-well plates coated with culture supernatants of Vero cells infected with wild-type DEN1 (A), DEN2 (B), DEN3 (C), or DEN4 (D) viruses as described in the Materials and Methods. Monoclonal antibodies specific for each dengue serotype were used as a positive control (open triangles). The monoclonal antibodies were 15F3-1 (A), 3H5-1 (B), 5D4-11 (C), and 1H10-6 (D). Titers were defined as the dilution of serum that produced a positive signal three times that of the background. Each point represents the mean titer of six animals. Error bars show the standard deviation.

Interestingly, the end point (eight weeks) antibody titers in cAdVax-DEN1/DEN2-vaccinated animals were lower against DEN1 than against DEN2 (Figure 4A and B). This may represent an imbalanced expression between the DEN1 and DEN2 transgene cassettes from the cAdVax vector. However, the same trend was also seen when comparing the DEN3-reactive and DEN4-reactive antibody titers from the same animals, with antibody titers against DEN3 lower than the titers against DEN4 (Figure 4C and D). Regardless, the antibody responses against all four serotypes were well above those of control-vaccinated mice. Therefore, we can conclude that vaccinating mice with cAdVax-DEN1/DEN2 induces production of antibodies to dengue virus that cross-react with all four dengue serotypes.

Induction of serotype-specific neutralizing antibody responses by vaccination of mice with cAdVax-DEN1/DEN2.

The sera from cAdVax-DEN1/DEN2 vaccinated animals above reacted with all four dengue virus serotypes in the ELISA experiments. However, ELISA-reactive antibodies do not directly correlate with neutralizing antibodies. In fact, it is the cross-reactive, non-neutralizing antibodies that are thought to contribute to antibody dependent enhancement of infection.¹ Therefore, to better assess the efficacy of our bivalent vaccine vector, we measured the dengue-specific neutralizing activity of the humoral immune response. The mice from the experiment described above were killed at nine weeks post-primary immunization and sera were collected to perform neutralization assays against wild-type DEN1, DEN2, or DEN3 viruses. Equal volumes of serum from five animals were pooled and used to perform the experiments. Thus, it should be noted that neutralizing responses of individual animals could not be determined. For controls, sera from non-immune animals were used. The three dengue virus serotypes were each incubated with serial dilutions of animal sera, followed by a plaque reduction neutralization test. Sera from animals vaccinated with cAdVax-DEN1/DEN2 had significantly higher neutralizing activity against DEN1 (Figure 5A)

and DEN2 (Figure 5B) compared with the control sera (considered non-specific neutralizing activity). Interestingly, the DEN2 neutralizing activity appeared to be higher than the neutralizing activity against DEN1. Although background activity in the control sera appeared relatively high, it remained statistically significantly lower than the cAdVax-DEN1/DEN2-vaccinated animals. Sera from cAdVax-DEN1/DEN2-vaccinated animals had no specific neutralizing activity against a dengue virus serotype (DEN3) not carried in the vaccine vector (Figure 5C). These results are consistent with other reports on the serotype specificity of dengue virus-neutralizing antibodies.⁴¹ Therefore, we can deduce that although cAdVax-DEN1/DEN2 vaccination of mice induces DEN3-reactive antibodies as measured in ELISAs (Figure 4C), these same antisera do not possess neutralizing activity against this serotype of dengue virus (Figure 5C).

DISCUSSION

In this study, we constructed and investigated a bivalent dengue vaccine candidate based on the same cAdVax technology used to construct previously described Marburg virus³¹ and Ebola virus³² vaccines. This approach for vaccine development entails expression of dengue viral antigens *de novo* in host cells. This should provide the immune system with a replica of the viral antigen(s) that would be encountered during a natural infection, without the detrimental effects of an actual dengue virus infection. In this manner, naturally occurring epitopes such as viral receptor binding sites would be presented to the immune system. This is potentially much more advantageous than using recombinant proteins or subunit vaccines, in which epitopes presented to the immune system are not necessarily the same as those carried by the wild-type virus because of improper glycosylation or folding.

Previous investigators show using an Ad vector expressing only the ectodomain of the DEN2 E antigen could be a potential vaccine tool.²⁷ In that study, Jaiswal and others found that their monovalent DEN2 vaccine vector was able to generate neutralizing antibody titers in mice that could neutralize 50% of DEN2 infectivity (PRNT₅₀) as high as PRNT₅₀ = 73 after three injections. The titers were calculated as the inverse of the serum dilution able to neutralize 50% of DEN2 infectivity. Our bivalent cAdVax-DEN1/DEN2 vaccine induced DEN2-specific neutralizing antibodies in mice as well. Although we did not calculate PRNT₅₀ antibody titers in this report, results in Figure 5B show that a serum dilution factor of 640 is still able to neutralize approximately 80% of DEN2 infectivity. This potent neutralizing antibody response, coupled with the DEN1-neutralizing capabilities shown in Figure 5A, make cAdVaxDEN1/DEN2 an attractive vaccine candidate. Whether the higher levels of DEN2 neutralizing antibody responses in our studies compared with those of the previous report were caused by the presence of DEN2 prM and NS1 genes or the prM, E, and NS1 genes of DEN1 remains to be seen. Direct comparison of the two studies is difficult because of differences in vector design, transgenes, vaccination schedules, doses, and mouse strains used.

The non-neutralizing, cross-reactive antibodies induced by the cAdVax-DEN1/DEN2 vector could be a cause for concern (Figure 4). Our bivalent vector induced antibodies in mice that reacted with all four dengue serotypes in the

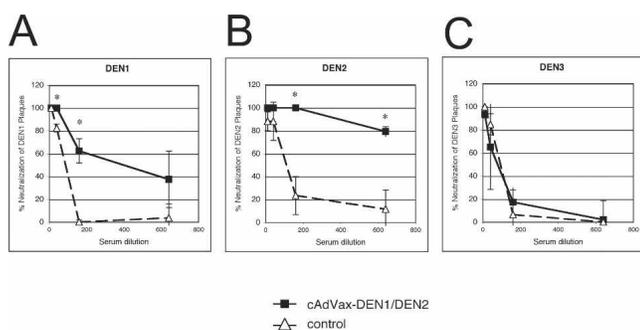


FIGURE 5. Induction of dengue (DEN)1- and DEN2-specific neutralizing antibodies in mice vaccinated with cAdVax-DEN1/DEN2. Pooled sera from mice vaccinated with cAdVax-DEN1/DEN2 (five weeks post-booster immunization) or unvaccinated controls were used to perform neutralization assays against DEN1 (A), DEN2 (B), or DEN3 (C) wild-type viruses. Serial dilutions of sera were incubated with 60 plaque-forming units of each virus serotype, followed by infection of Vero cell monolayers under an agar/medium overlay. After seven days of infection, dengue viral plaques were visualized by immunostaining and counted. Results are presented as percent inhibition of dengue plaque formation and are representative of three independent analyses of the pooled mouse sera. (Statistical analysis was performed using an unpaired Student's *t*-test. **P* < 0.001). Error bars show the standard deviation.

ELISA. The DEN3-reactive antisera shown in Figure 4C can be considered non-neutralizing because sera from the same mice did not neutralize DEN3 infectivity compared with control sera (Figure 5C). We hypothesize the same trend to exist for DEN4, although these experiments were not conducted. The presence of non-neutralizing, cross-reactive antibodies induced by our vaccine vector could cause problems if vaccinated individuals were subsequently infected with an alternate serotype (DEN3 or DEN4), which could potentially result in antibody-induced enhanced infection. This possibility re-emphasizes the requirement for a tetravalent dengue vaccine to avoid DHF or DSS risks in vaccinated persons. Because the previous Ad-based dengue vaccine study only tested the DEN2 serotype,²⁷ no conclusions for that vaccine vector can be made in this regard.

Results from the ELISA experiments showed that antibody titers to DEN1 virus were slightly lower than the antibody titers to DEN2 virus in cAdVax-DEN1/DEN2-vaccinated mice. A similar trend was noticed when testing for neutralizing antibodies, where there appeared to be an overall higher level of neutralizing antibody activity against DEN2 when compared with that of DEN1. These trends may be the result of the vector expressing each serotype's antigens at different levels. The NS1 Western blot results in Figure 3D showed a high molecular weight band in the cAdVax-DEN1/DEN2-infected cell lysates. A possible explanation for this could be improper processing of the DEN1 prM-E-NS1 cassette. These viral proteins are translated as a polyprotein and then cleaved by viral and cellular proteases into the individual components. The prM protein of this cassette appears to be cleaved off and expressed normally, as shown in Figure 3C. However, the E protein of the DEN1 cassette was only weakly detectable in immunofluorescence staining (Figure 2) and was undetectable in Western blots (Figure 3). The NS1 protein of this cassette was detectable in immunofluorescence and showed the high molecular weight protein in Western blot, which is consistent with the size of an E-NS1 polyprotein. An uncleaved DEN1 E-NS1 polyprotein could be the reason that neutralizing antibody titers to DEN1 in vaccinated mice were lower than those for DEN2. Further investigation into these possibilities is necessary for confirmation. Alternatively, there may be differences in the immunogenicity of DEN1 and DEN2 antigens in CD-1 mice. Differences in flavivirus immune responses have been documented in inbred versus outbred strains of mice.⁴² Nonetheless, there were still effective levels of DEN1-specific neutralizing antibodies in vaccinated mice. To conclusively confirm these antibody titers are indeed sufficient, for immunity, animal challenge experiments must be done in future studies.

We included the dengue virus NS1 antigen to stimulate a strong anti-dengue virus cellular immune response. However, because the scope of this study focused on humoral immune responses, experiments to detect anti-dengue virus cytotoxic T lymphocyte (CTL) activities were not performed. Because our research into cAdVax-based dengue vaccines continues, we will investigate the effects of these vaccines on CTL activity. Previous reports show that expression of the DEN2 E protein from an Ad vector is sufficient to induce anti-dengue virus CTL activity in mice.²⁷ Other investigators reported isolation of dengue E protein-specific CTL clones from human donors, indicating that the E protein itself contains CTL epitopes.⁴³ Therefore, we hypothesize our cAdVax vectors

also induce cellular immune responses, but further investigation is required. Whether expression of NS1 protein increases the cellular responses compared with expression of only E protein is currently being studied.

We previously reported that our cAdVax-based Marburg and Ebola virus vaccines were safe in mouse models of vaccination.^{31,32} These vaccine candidates showed no adverse effects in livers or spleens of vaccinated animals when tissue sections were analyzed by staining with hematoxylin and eosin. Although safety studies were not carried out for our cAdVax-based dengue vaccine candidate described in this report, we predict a similar safety profile for several reasons. For example, it is well established that the glycoproteins of the filovirus family (Marburg and Ebola viruses) have considerable toxicity. The extensive vascular damage seen in a filovirus infection can be attributed to glycoprotein GP.⁴⁴ Dengue virus glycoproteins do not have the same level of toxicity as those of the filoviruses. Additionally, during the animal experiments in this study, no signs of distress were noted in cAdVax-vaccinated animals in regard to eating, posture, or social interaction. The dosage of vaccine administered to the mice in this study (two injections of 1×10^8 pfu) could be considered high when proportioned for use in humans. Requirement of such a dose of vaccine could potentially cause logistical problems in terms of mass quantities necessary for human use and cost of production for such quantities. However, improvements in bioreactor design make large-scale good manufacturing practice production of rAd vectors at high titers easily achievable. Furthermore, the dose of vaccine given to mice in this study may not be necessary to generate high antibody titers to dengue viruses. Dose-response studies will provide data on the possibility of lowering the dose of vaccine while maintaining high antibody titers.

The cAdVax system is an effective vaccine platform because of vector genome packaging size, high-level and long-lasting expression of transgenes, infection of multiple cell types (including antigen-presenting dendritic cells), overall safety of replication-deficient Ad vectors, and ease of production of large quantities of Ad vector vaccines. The bivalent cAdVax-DEN1/DEN2 vaccine vector paves the way towards construction of a second bivalent dengue vaccine carrying genes for the DEN3 and DEN4 serotypes. The combination of these two vaccine candidates would create a potential tetravalent dengue vaccine able to induce effective immune responses across all four serotypes of dengue virus. A major advantage to such an approach is that at most this vaccine candidate would consist of two-bivalent components (DEN1/DEN2 and DEN3/DEN4), whereas other tetravalent vaccine platforms require up to four separate components. This raises questions in regard to the balance of immune responses to each serotype after vaccination. For example, several clinical trials using live attenuated dengue virus in a tetravalent formulation have shown imbalanced immune responses to the individual serotypes.^{5,8,45} Other tetravalent dengue vaccine approaches that involve the pooling of multiple components, such as recombinant protein subunit vaccines, have also produced imbalanced serotypic immune responses in test animals.⁴¹ Our proposed two-component tetravalent dengue vaccine would still be a multiple component vaccine, and therefore the balance of immune responses to each serotype would have to be carefully monitored, especially in light of the potential immunity imbalance demonstrated by our biva-

lent vector results shown in Figure 5. Additionally, pre-existing immunity to adenovirus may affect the overall efficacy of an Ad-based dengue vaccine. For example, if circulating Ad-neutralizing antibodies were to lower the immune response induced by an Ad-based dengue vaccine, the vaccinated person may be put at risk for immune enhancement of subsequent dengue virus infection. These possibilities must be considered for advancement of such a vaccine into human clinical trials. However, the recent clinical trial data mentioned earlier seem to indicate that pre-existing Ad immunity does not affect the efficacy of other Ad-based vaccines.^{28,29}

In conclusion, we have described a novel, bivalent dengue vaccine candidate that expresses DEN1 and DEN2 proteins simultaneously. Despite the possibility of expressing an improperly processed DEN1 polypeptide, this vector induced bivalent neutralizing antibody responses in vaccinated mice. Clearly, the balance of dengue virus protein expression from this vector must be leveled before its incorporation into a two-component tetravalent dengue virus vaccine. Nevertheless, the data in this report indicate using a rAd-based vaccine platform for development of a multivalent dengue virus vaccine is quite possible.

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