

Two Complex, Adenovirus-Based Vaccines That Together Induce Immune Responses to All Four Dengue Virus Serotypes[∇]

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Dengue virus infections can cause hemorrhagic fever, shock, encephalitis, and even death. Worldwide, approximately 2.5 billion people live in dengue-infested regions with about 100 million new cases each year, although many of these infections are believed to be silent. There are four antigenically distinct serotypes of dengue virus; thus, immunity from one serotype will not cross-protect from infection with the other three. The difficulties that hamper vaccine development include requirements of the natural conformation of the envelope glycoprotein to induce neutralizing immune responses and the necessity of presenting antigens of all four serotypes. Currently, the only way to meet these requirements is to use a mixture of four serotypes of live attenuated dengue viruses, but safety remains a major problem. In this study, we have developed the basis for a tetravalent dengue vaccine using a novel complex adenovirus platform that is capable of expressing multiple antigens de novo. This dengue vaccine is constructed as a pair of vectors that each expresses the premembrane and envelope genes of two different dengue virus serotypes. Upon vaccination, the vaccine expressed high levels of the dengue virus antigens in cells to mimic a natural infection and induced both humoral and cellular immune responses against multiple serotypes of dengue virus in an animal model. Further analyses show the humoral responses were indeed neutralizing against all four serotypes. Our studies demonstrate the concept of mimicking infections to induce immune responses by synthesizing dengue virus membrane antigens de novo and the feasibility of developing an effective tetravalent dengue vaccine by vector-mediated expression of glycoproteins of the four serotypes.

Dengue fever is a widespread mosquito-borne illness affecting an estimated 100 million people worldwide each year (9). While a majority of dengue virus infections are asymptomatic and go unnoticed, the disease can occasionally present with symptoms, such as severe headache, retro-orbital pain, or joint and muscle pain. The most severe cases can lead to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), manifesting as hemorrhage in multiple organs, including the central nervous system, as well as massive vascular damage, bleeding, and shock. Fatality rates for DHF and DSS can reach up to 15%, the majority of which are in the pediatric demographic (23, 24).

Dengue viruses are members of the flavivirus family of viruses. All four serotypes (dengue virus [DEN] serotype 1 [DEN1] to DEN4) are identical in gene structure, consisting of three structural proteins, capsid (C), premembrane (prM), and envelope (E), and seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (14). These genes are translated from the positive-stranded RNA genome as a single polyprotein that is cleaved into individual proteins by cellular and viral proteases. Despite the structural similarities, there is

variation in the genetic sequences of each serotype mainly in the membrane glycoproteins (prM and E) that results in antigenic distinction and lack of cross protection among the serotypes. Furthermore, subsequent infection with an alternate serotype may enhance disease severity, contributing to the onset of DHF or DSS. One possibility for this disease enhancement is that (nonneutralizing) antibodies may be aiding in viral entry into host cells, thereby enhancing the infection. Alternatively, broadly reactive cytotoxic T-lymphocyte (CTL) responses to DEN antigens, in the absence of neutralizing responses, may accelerate the destruction of host cells.

There is currently no licensed vaccine available for dengue virus infection. Because of the immune enhancement associated with subsequent DEN infections, a successful DEN vaccine must induce neutralizing responses against all four DEN serotypes simultaneously (tetravalent). The difficulties that hamper vaccine development include the preservation of the neutralizing epitopes of the native DEN glycoprotein antigens and the necessity to present antigens from all serotypes. If a dengue vaccine does not offer tetravalent protection, a vaccinated individual would be susceptible to development of DHF or DSS if infected by a serotype not targeted by the vaccine.

A great amount of effort has focused on traditional approaches to dengue vaccine development; however, these approaches have yet to generate a licensed tetravalent dengue vaccine. For example, live attenuated DEN vaccines have shown protective immunity in animal models (3) but have been

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unsuccessful in humans due to underattenuation or unbalanced immunity between serotypes (8, 17, 32). In addition, while several dengue virus chimera vaccines, including a yellow fever/dengue virus chimera, have produced protective immune responses in animals (4, 5, 11, 15), there are concerns regarding potential side effects from replication-competent hybrid viruses. However, a recent clinical trial demonstrated a monovalent yellow fever/dengue virus chimeric vaccine was well tolerated (10).

A number of studies have shown injection of DNA plasmids containing DEN sequences can be immunogenic and offer protection from DEN challenge in animal models (18, 19, 21, 27, 28). While this approach is simple, the potency of DNA vaccines continues to be a major limitation. Current efforts have focused on searching for effective adjuvants to enhance the immunogenicity of these vaccines.

Another encouraging platform includes the use of replication-defective recombinant adenovirus (Ad) vectors as vaccines. These vectors have the ability to express antigens at high levels in a wide variety of cell types, making them ideal for inducing potent immune responses. In addition, they have been studied as vaccine platforms for a wide variety of infectious agents, including dengue virus (16), tick-borne encephalitis virus (33), human immunodeficiency virus (2), Ebola virus (30, 31, 36), and Marburg virus (35, 37). However, the major limitation for the traditional Ad vector vaccine approaches is the amount of antigenic information that can be carried by a single vector. Specifically, in the Ad-based dengue vaccine, only a fraction of the E gene sequence of a single DEN serotype was expressed from the vaccine vector (16).

In the present report, we introduce a dengue vaccine candidate that is based on a pair of novel complex Ad vectors that can accommodate large antigenic sequences and multiple antigen inserts. To assure equal expression of antigens from all four serotypes, we engineered the prM and E antigens of the four serotypes of DEN into symmetrical ends of each vector's genome. This resulted in a pair of vectors expressing the prM and E glycoprotein complex of four DEN serotypes; if administered together, the vector pair would represent all four serotypes. Both prM and E were selected as antigens because several epitopes within the DEN E protein are known to play a significant role in the induction of both neutralizing antibodies (NAbs) and CTL responses (7, 22) and expression of the flavivirus prM protein is necessary for proper processing and expression of E (1, 20, 26).

In this study, our tetravalent vaccine was tested as single bivalent components. These vectors were able to express high levels of prM and E proteins *in vitro* and induced potent antibodies against all four DEN serotypes *in vivo*. Using an improved plaque reduction assay, we show that the antibody responses are indeed neutralizing and are against all four serotypes, presumably because the antigens are naturally expressed and associated with the membrane. Finally, the *de novo*-synthesized antigens induced broadly reactive cellular immune responses. These responses in combination with neutralizing immunity will be important in developing protective immunity against all four serotypes of DEN. In summary, these data demonstrate efficient induction of immune responses against multiple serotypes of dengue viruses by our cAdVax-based vaccines and support further investigation into combin-

ing the two components to form a tetravalent Ad-based dengue vaccine.

MATERIALS AND METHODS

Cell lines and viruses. HEK293 and Vero E6 cells were obtained from ATCC. Both cell lines were maintained in Dulbecco modified Eagle medium supplemented with 10% Cosmic calf serum (CCS) (HyClone) and 1× penicillin-streptomycin (P/S) (Cambrex). Mouse splenocytes were cultured in RPMI 1640 supplemented with 10% CCS, 10^{-5} M β -mercaptoethanol, 10 mM HEPES, 1× P/S, and 1× nonessential amino acids). Hybridoma cell lines 3H5-1, 5D4-11, and 1H10-6 were from ATCC. Hybridoma cell lines 13E7-9-10 and 2H2-9-15 were both obtained from the U.S. Army Medical Research Institute for Infectious Diseases. All hybridomas were maintained in HybriCare medium (ATCC) supplemented with 10% CCS and 1× P/S. Dengue viruses DEN1 (Hawaii), DEN2 (New Guinea C), DEN3 (H87), and DEN4 (814669) were kindly provided by Barry Falgout of the FDA Center for Biologics Evaluation and Research.

Construction of the cAdVaxD vaccines. Plasmids containing the prM-E gene sequences of all four dengue virus serotypes were kindly provided by the U.S. Naval Medical Research Center. The desired gene fragments (prM-E) were PCR amplified from each plasmid using primers specific for each serotype. The primers and their sequences were as follows: for DEN1 (GenBank accession numbers AF425619 and X76219), forward (5'-ATGGCTGTGACCATGCTCCTCATGC-3') and reverse (5'-TTACGCTGAACCATGACTCC-3'); for DEN2 (GenBank accession number AF038403), forward (5'-ATGGCGTTCCATTAACCACACG-3') and reverse (5'-CTAGCGCTGCACATAACTCC-3'); for DEN3 (GenBank accession number NC001475), forward (5'-ATGGCATCGCTGTCTCATGATG-3') and reverse (5'-CTAAGCTTGACCACGACCCC C-3'); and for DEN4 (GenBank accession number AF326573), forward (5'-ATGCTGAACATCCTGAACGGGAG-3') and reverse (5'-TTATGCTTGAAGTGTGAAGCCCAAGAAC-3'). Once the prM-E PCR fragments for each serotype were generated, they were each subcloned into a cAdVax arm. The prM-E sequences of DEN1 and DEN2 were inserted into the left and right arms of the cAdVaxD(1-2) vaccine construct, respectively. The prM-E sequences of DEN3 and DEN4 were inserted into the left and right arms of the cAdVaxD(3-4) vaccine construct, respectively. Open reading frames were sequenced to ensure that the cloned dengue virus sequences were correct. Once the cloning constructs were completed, the cAdVaxD vaccine vectors were constructed as described previously (29).

Immunoblot analyses. HEK293 cells were infected with cAdVaxD(1-2) or cAdVaxD(3-4) vectors at a multiplicity of infection (MOI) of 10 for 24 h. Cells were washed three times with phosphate-buffered saline (PBS) and lysates were prepared using MPERS buffer (Pierce) according to the manufacturer's instructions. Cell lysates (10 μ l) were separated on 4 to 12% NuPAGE gels (Invitrogen) and then transferred to polyvinylidene difluoride membranes. Membranes were then probed with anti-DEN monoclonal antibodies (MAbs). Antibodies used and specificities were as follows (from the U.S. Naval Medical Research Center): HMAF (hyperimmune mouse ascitic fluid, polyclonal), 2H3 (DEN2-E), 8C2 (DEN1-prM), 8B9 (DEN1-E), 8D21 (DEN3-E), and 1H10-6-7 (DEN4-E). Proteins reacting with the MAbs were visualized using WesternBreeze chemiluminescence Western blot immunodetection kit (Invitrogen).

Dengue indirect immunofluorescence assay. Vero cells were mock infected or infected with each cAdVaxD vector (MOI of 20) or each of the wild-type dengue viruses (MOI of 2). Two days postinfection, the cells were fixed with 4% formaldehyde, blocked, and permeabilized (PBS, 2.5% nonfat dry milk, 0.05% Tween 20, 0.5% Triton X-100), and probed with serotype-specific anti-DEN MAbs. MAbs specific for DEN1/DEN3 E (13E7-9-10), DEN2 E (3H5-1), DEN3 E (5D4-11), and DEN4 E (1H10-6) were prepared from undiluted supernatants of hybridoma cell cultures. The specificities of these MAbs are previously described (12, 13). After the cells were probed with the primary antibody, the cells were washed three times with PBS containing 10% CCS and probed with a phycoerythrin-conjugated anti-mouse immunoglobulin G secondary antibody (BD Pharmingen). After the cells were probed with the secondary antibody, the cells were washed again as described above and visualized with a fluorescence microscope (Olympus).

Immunization of mice with cAdVaxD vaccines and preparation of sera. CD-1 or C57BL/6 mice (Charles River Laboratory; Charleston, SC) were immunized intraperitoneally at weeks 0 and 8 with 10^8 PFU of cAdVaxD(1-2), cAdVaxD(3-4), S3G, or CHB3, prepared in 100 μ l PBS. S3G and CHB3, which are unrelated cAdVax-based severe acute respiratory syndrome and hepatitis B virus vaccines, respectively, served as negative controls. Due to a scheduling conflict, the cAdVaxD(1-2) and S3G groups were actually boosted 4 days prior to the

8-week collection point, whereas the cAdVaxD(3-4) and CHB3 groups of animals were boosted 2 days after the 8-week collection point.

After primary vaccination of CD-1 mice, blood was collected biweekly via retro-orbital extraction under light anesthesia (CO₂ inhalation). Serum from each blood sample was prepared as previously described (32). Small aliquots were supplemented with NaN₃ (0.05%) and stored at 4°C. All animals were maintained according to Institutional Animal Care and Use Committee standards and regulations.

Indirect enzyme-linked immunosorbent assay (ELISA). DEN2- or DEN4-infected Vero cell culture supernatants, coated directly onto the wells of 96-well flat-bottom plates (Nalge Nunc International), served as coating antigen. Each dengue virus serotype was propagated in Vero cells for approximately 7 to 10 days until a cytopathic effect was evident. The virus preparations were collected as cell culture supernatants and stored at -80°C. Titrations were performed using serotype-specific MAbs (3H5-1, DEN2; 1H10-6, DEN4) to optimize the amount of virus preparation used for coating the plates. These optimization procedures were performed for all virus preparations. The assays were then performed as previously described (37). Antibody titers for each mouse were determined by calculating the dilution of serum that corresponded to a signal of three times the background for that particular test.

DEN PRNT. Ten weeks after the booster immunizations (18 weeks after primary immunizations), the CD-1 mice described above were sacrificed, and blood was collected via cardiac puncture. Animal sera were then prepared as described above. To perform plaque reduction neutralization test (PRNT) analyses, 60 PFU of DEN1 (Hawaii), DEN2 (New Guinea C), DEN3 (H87), and DEN4 (814669) were each incubated with serial dilutions of heat-inactivated serum from vaccinated animals, followed by adsorption onto Vero cells in 12-well plates. After adsorption, the monolayers were covered with an agar/medium overlay (1% agar, 1× Eagle's minimum essential medium, 5% CCS, 100 mM L-glutamine, 1× nonessential amino acids, 50 mM sodium pyruvate, 1× P/S) and incubated for 7 days (DEN2, DEN3, and DEN4 infections) or 10 days (DEN1 infections) at 37°C and 5% CO₂. After infection, the agar overlays were removed and then fixed and blocked according to the protocol described above for indirect immunofluorescence. Fixed cells were then probed with MAb clone 2H2-9-10 that reacts with all four serotypes of DEN (12). This antibody was used undiluted as a hybridoma cell culture supernatant. After the cells were probed with the primary antibody, they were washed three times with PBS containing 0.05% Tween 20 and probed with an alkaline phosphatase-conjugated anti-mouse immunoglobulin G secondary antibody (Kirkegaard and Perry). The cells were washed three times more, and plaques were visualized by adding the 5-bromo-4-chloro-3-indolylphosphate (BCIP) or nitroblue tetrazolium substrate (Pierce). Viral plaques were counted, and the raw data were converted to percent neutralization of plaque formation.

Murine gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay. C57BL/6 mice were vaccinated with 10⁸ PFU of cAdVaxD(1-2), cAdVaxD(3-4), or CHB3 as described above. Four mice from each vaccine group were sacrificed 4 and 10 weeks after primary immunizations, and spleens were collected as previously described (36, 37). Splenocytes were isolated from each animal and diluted to a concentration of 4 × 10⁶ cells/ml in RP10C culture medium.

For the antigenic stimulant, a pool of consecutive peptides that spanned amino acids 1 to 110 of the DEN2 envelope protein was used (synthesized by Mimitopes [Victoria, Australia]). The peptide pool consisted of 20 total peptides, each a 15-mer with a 10-amino-acid overlap. Each peptide was individually dissolved in dimethyl sulfoxide at approximately 20 mg/ml. The peptide pool was then generated by combining all 20 overlapping peptides at a final concentration of 20 μg/ml in RP10C.

MultiScreen 96-well assay plates (Millipore) were coated with the rat anti-mouse IFN-γ capture antibody (BD Pharmingen) and incubated at 4°C overnight. The next day, the plates were washed three times with PBS and blocked with 200 μl/well of RP10C culture medium for 1 hour at room temperature. Next, the dengue virus peptide pool mixture was added to each well (50 μl per well), followed by an equal volume of splenocyte suspension (4 × 10⁶ cells/ml in RP10C). The plates were incubated for 18 h at 37°C, and ELISPOT assays were completed as previously described (36, 37).

RESULTS

Construction of two bivalent cAdVax expression vectors. All four serotypes of dengue virus are structurally identical (Fig. 1A). We engineered each cAdVax vector to express the prM and E genes from two different dengue virus serotypes: DEN1

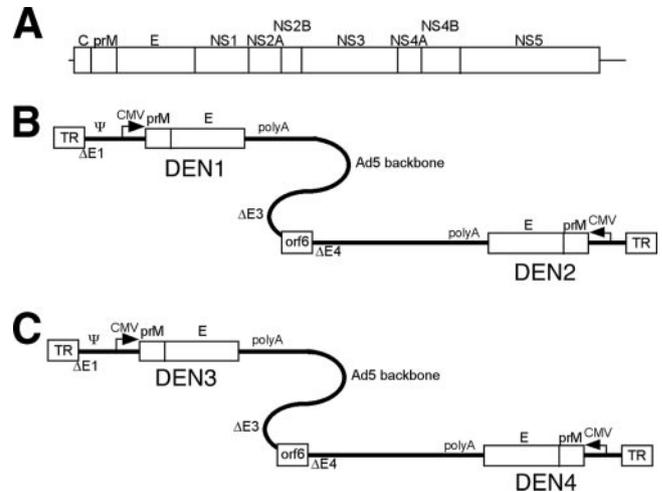


FIG. 1. Dengue virus and cAdVax vector genomes. (A) Schematic of the dengue virus positive-stranded RNA genome. (B) Schematic of the cAdVaxD(1-2) vaccine construct. cAdVaxD(1-2) contains DEN1 and DEN2 prM-E sequences in the left and right arms of the vector, respectively. (C) Schematic of the cAdVaxD(3-4) vaccine construct. cAdVaxD(3-4) contains DEN3 and DEN4 prM-E sequences in the left and right arms of the vector, respectively. TR, terminal repeat; CMV, cytomegalovirus.

and DEN2 or DEN3 and DEN4 (Fig. 1B and C). Each prM-E cassette was inserted into a left or right cAdVax arm: DEN1 and DEN2 cassettes were inserted into the left and right arms, respectively, of cAdVaxD(1-2) (Fig. 1B), and DEN3 and DEN4 cassettes were inserted into the left and right arms, respectively, of cAdVaxD(3-4) (Fig. 1C). Each bivalent vector was constructed, propagated, purified, and titrated by previously described methods (29, 36, 37).

cAdVax dengue vectors express high levels of dengue virus proteins in vitro. Following construction of the two bivalent vectors, we confirmed that each expressed the correct dengue virus antigens using Western blot analysis of HEK293 cell lysates that were infected with either cAdVaxD(1-2) or cAdVaxD(3-4). We found cAdVaxD(1-2) induced both DEN1 and DEN2 prM expression in vitro (Fig. 2A, lanes 3 and 2, respectively). The same series of Western blots also confirmed DEN1 and DEN2 E expression (Fig. 2A, lanes 4 and 1, respectively). We also detected both DEN3 and DEN4 E proteins in cAdVaxD(3-4)-infected cell lysates (Fig. 2B, lanes 1 and 3, respectively). Unfortunately, we were unable to detect prM protein expression for DEN3 or DEN4 serotypes due to a lack of serotype-specific antibodies for these proteins.

However, the expression of DEN3 and DEN4 E proteins may provide some indirect evidence that the prM proteins of both serotypes are also being expressed. Each prM-E cassette in our cAdVaxD vectors is designed to be expressed as a single polyprotein upon infection of target cells. Therefore, detection of DEN3 and DEN4 E (which is distal to prM) from cAdVaxD(3-4)-infected cell lysates indirectly indicates that the prM proteins from the same serotypes are also being expressed. Furthermore, both DEN3 and DEN4 E proteins appear at the same approximate molecular mass (50 to 60 kDa) as DEN1 and DEN2 E proteins do (Fig. 2A). Previous work with other related flaviviruses has shown that prM pro-

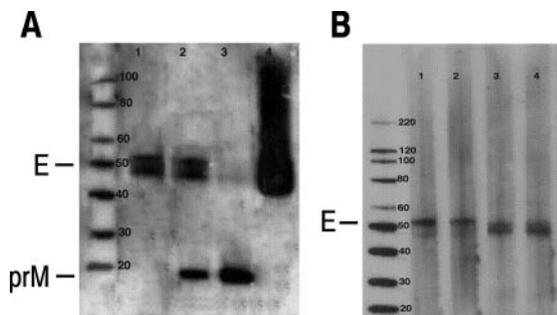


FIG. 2. cAdVaxD vectors express dengue virus proteins in infected cells *in vitro*. (A) Lysates of HEK293 cells infected with cAdVaxD(1-2) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western blotting was performed to analyze DEN1 and DEN2 prM-E expression. The results are a composite of multiple Western blots probed with serotype- and protein-specific antibodies (shown in parentheses): lane 1, DEN2 E (clone 2H3); lane 2, DEN2 E and DEN2 prM (DEN2 hyperimmune ascitic fluid); lane 3, DEN1 prM (clone 8C2); lane 4, DEN1 E (clone 8B9). The positions of molecular mass markers (in kilodaltons) are shown. (B) Lysates of HEK293 cells infected with cAdVaxD(3-4) were analyzed as described above for panel A for DEN3 and DEN4 prM-E expression. Results are a composite of multiple Western blots: lane 1, DEN3 E (clone 8D2-1); lane 3, DEN4 E (clone 1H10-6-7). Lanes 2 and 4 were probed with MAb clones 14A4-8 and 1A2-6, respectively. While these two clones are specific for dengue virus, the serotype specificity is undetermined.

tein expression is necessary for the proper processing and glycosylation of E (1, 20). Therefore, the appearance of DEN3 and DEN4 E proteins with a molecular mass similar to that of DEN1 and DEN2 E proteins is also indirect evidence that DEN3 prM and DEN4 prM are present in cAdVaxD(3-4)-infected cells.

To confirm our results from the Western blot analyses, we performed indirect immunofluorescence assays using serotype-specific monoclonal antibodies. As shown in Fig. 3A, the cAdVaxD(1-2) vector demonstrated positive staining by a DEN1/DEN3 E-specific MAb (13E7-9-10) and a DEN2 E-specific MAb (3H5-1). While the 13E7-9-10 MAb cannot distinguish between DEN1 E and DEN3 E, positive staining of the DEN1-infected control suggests that this MAb is able to demonstrate DEN1 E expression. In addition, lack of staining of the DEN2-infected lysates by 13E7-9-10 further confirms the MAb's specificity.

Similar to the results seen with cAdVaxD(1-2), cAdVaxD(3-4) also demonstrated positive expression of its respective gene inserts (Fig. 3B). The cAdVaxD(3-4)-infected cells were positively stained by a DEN3 E-specific MAb (5D4-11) and a DEN4 E-specific MAb (1H10-6). Specific binding by 5D4-11 and 1H10-6 to DEN3- and DEN4-infected cells confirmed the specificities of these MAbs. In addition, all negative-control mock-infected cells showed no detectable background nonspecific binding. Collectively, these data indicate that the E proteins from all four DEN serotypes are expressed from the cAdVaxD vectors, supporting the Western blots from Fig. 2. The lack of cross-reactivity among serotypes confirms the specific nature of the MAbs used in these experiments, consistent with their initial introduction and characterization (12, 13).

Vaccination of mice with the cAdVaxD vectors induces humoral immune responses. Mice were vaccinated as described in the legend to Fig. 4. Vaccination with S3G and CHB3

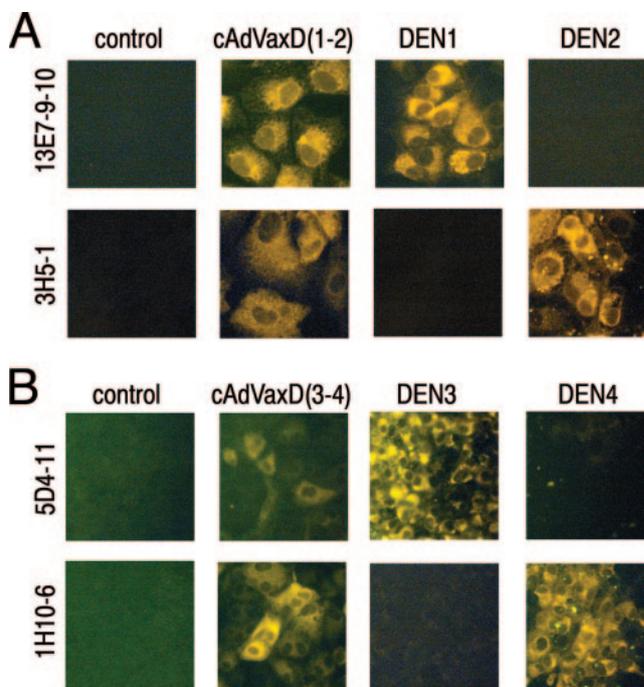


FIG. 3. Indirect immunofluorescent staining of DEN proteins expressed from cAdVax vectors. (A) Vero cells were mock infected (control) or infected with cAdVaxD(1-2) at a MOI of 20 or with wild-type DEN1 or DEN2 viruses at a MOI of 2 for 48 h. Following infection, the cells were fixed in formaldehyde and immunostained with serotype-specific MAb 13E7-9-10 (DEN1/DEN3 E) or 3H5-1 (DEN2 E). (B) Vero cells were mock infected (control) or infected with cAdVaxD(3-4) at a MOI of 20 or with wild-type DEN3 or DEN4 viruses at a MOI of 2 for 48 h. Cells were fixed and immunostained with serotype-specific MAb 5D4-11 (DEN3 E) or 1H10-6 (DEN4 E). Positive cells were detected using a phycoerythrin-conjugated secondary antibody and visualized with a fluorescence microscope.

(unrelated cAdVax-based severe acute respiratory syndrome and hepatitis B virus vaccines, respectively) served as negative controls. Sera from vaccinated mice were then collected and used to perform ELISAs for detection of dengue virus-reactive antibodies as described in Materials and Methods.

We found that animals vaccinated with cAdVaxD(1-2) produced high titers of DEN2-reactive antibodies, detectable as early as 4 weeks after primary immunization (Fig. 4A). These titers decreased slightly at 6 weeks and then showed a rebound effect at 8 weeks, which likely resulted from the booster immunization 4 days prior to this time point. Interestingly, we found almost identical results when measuring the DEN4-reactive antibody titers from the same mice (Fig. 4B). We considered the DEN4-reactive sera to be serotype cross-reactive, as DEN4 antigens are not expressed in the cAdVaxD(1-2) vaccine vector.

Animals vaccinated with cAdVaxD(3-4) produced high titers of DEN4-reactive antibodies (Fig. 4D). Evidence of the booster immunization is demonstrated by the increase in titers between weeks 8 and 10. As described above, we found that sera from these animals also reacted strongly with a dengue virus serotype not covered by this vaccine vector (i.e., DEN2 [Fig. 4C]). Sera from S3G or CHB3 control animals showed no reactivity against either dengue virus serotype.

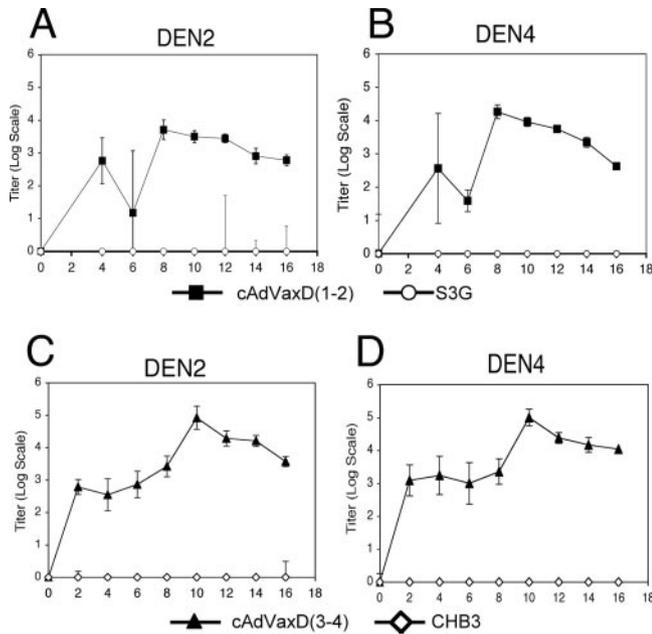


FIG. 4. Vaccination of mice with cAdVaxD vectors induces anti-DEN2 and anti-DEN4 antibody responses. CD-1 mice were vaccinated with 1×10^8 PFU of the indicated vaccines on weeks 0 and 8. After vaccination, serum samples were collected biweekly for antibody analyses using ELISA. The wells in 96-well plates were coated with culture supernatants from Vero cells infected with wild-type DEN2 (A and C) or DEN4 (B and D) and probed with serial dilutions of serum obtained from vaccinated animals at the indicated time points. Dilution curves were used to calculate antibody titers from individual animals. Each data point represents the mean \pm standard deviation (error bar) for at least five individual vaccinated mice. (A and B) Sera from mice vaccinated with cAdVaxD(1-2) or S3G were tested against DEN2 (A) or DEN4 (B) serotype. (C and D) Sera from mice vaccinated with cAdVaxD(3-4) or CHB3 were tested against DEN2 (C) or DEN4 (D) serotype.

These results indicate that the cAdVaxD vaccines induce the production of antibodies cross-reactive to dengue virus serotypes other than those contained within the respective vector. However, these antibodies are still specific for DEN, as the S3G and CHB3 control vaccines did not induce DEN-reactive antibody production. Additionally, these antibody responses were long lasting in the cAdVaxD-vaccinated mice, with anti-DEN antibody titers remaining elevated (average titer of 3.0 on a \log_{10} scale) for up to 32 weeks after the primary vaccination (data not shown).

Vaccination induced tetraivalent neutralizing antibodies. We next measured the neutralizing antibody responses from mice vaccinated as described above by using a plaque reduction neutralization test. We found the NAb responses showed a slightly more serotype-specific profile than the cross-reactive antibodies detected in the ELISAs described above. For example, the neutralizing activity against DEN1 and DEN2 serotypes was higher in sera from cAdVaxD(1-2)-vaccinated animals than in sera from cAdVaxD(3-4)-vaccinated animals (Fig. 5A and B). We found an inverse relationship when examining NAb responses against DEN3 and DEN4 serotypes. Here, the sera from animals vaccinated with cAdVaxD(3-4) were more effective at neutralizing DEN3 and DEN4 than sera

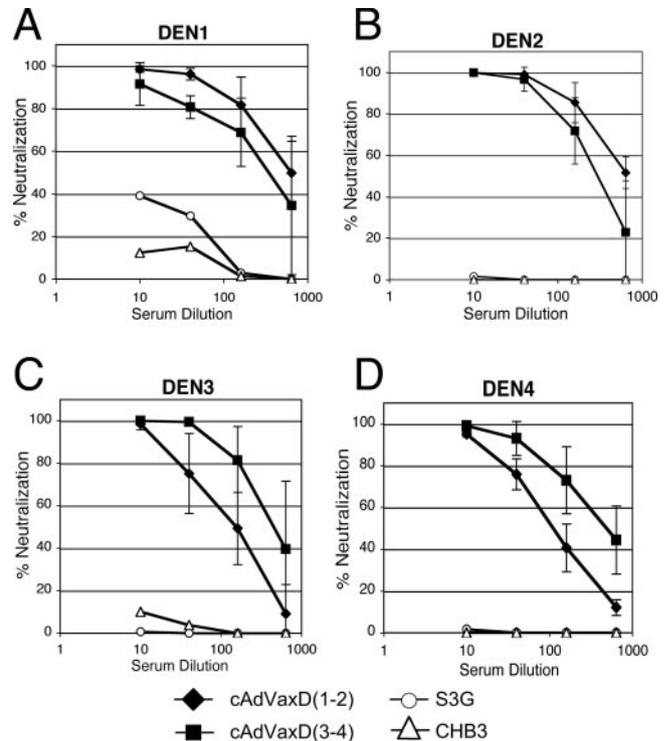


FIG. 5. Vaccination of mice with cAdVaxD vectors induces neutralizing antibody responses against all four DEN serotypes. Sera from mice vaccinated with cAdVaxD(1-2), cAdVaxD(3-4), S3G, or CHB3 were used to perform DEN PRNTs. Serial dilutions of sera that were collected 18 weeks after primary immunizations were incubated with each DEN serotype prior to infection of Vero cells. Seven days later, DEN plaques were visualized by immunostaining and then counted. Raw data were converted to percent inhibition of DEN plaque formation, and each data point represents the mean \pm standard deviation (error bar) for four individual animals. (A) DEN1 neutralizing activity; (B) DEN2 neutralizing activity; (C) DEN3 neutralizing activity; (D) DEN4 neutralizing activity.

from animals vaccinated with cAdVaxD(1-2) (Fig. 5C and D). These differences in serotype specificities were relatively small, as there was a high level of serotype cross-neutralization induced by both dengue vaccines. Both groups of cAdVaxD animal sera showed a much higher level of neutralizing antibody activity against all four DEN serotypes compared to control-vaccinated animal sera.

Serotype-independent cellular immune responses in mice vaccinated with cAdVaxD vectors. For detection of cellular immune responses induced by these vaccine candidates, C57BL/6 mice were each vaccinated (intraperitoneally) with 1×10^8 PFU of cAdVaxD(1-2), cAdVaxD(3-4), or CHB3 at weeks 0 and 8. Groups of mice were then sacrificed 4 or 10 weeks after the primary immunization to measure CTL activity.

Both groups of cAdVaxD-vaccinated mice had much higher CTL activity than the CHB3 control group did (Fig. 6). This was evident both 4 and 10 weeks after the primary immunization. Additionally, the CTL profiles of cAdVaxD(3-4)-vaccinated animals were similar to those of cAdVaxD(1-2)-vaccinated animals, even though the antigenic stimulant for the assay was derived from DEN2 E protein. This may indicate a

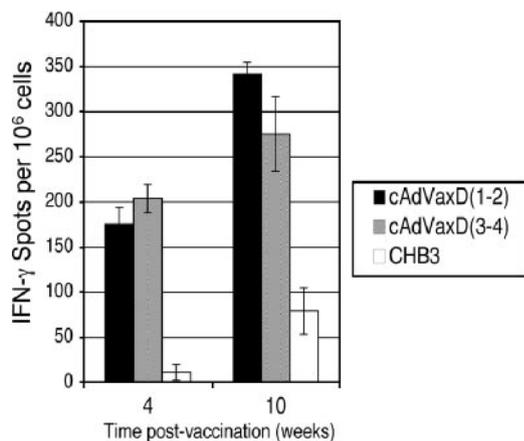


FIG. 6. Vaccination of mice with cAdVaxD vectors induces anti-dengue virus cellular immune responses. C57BL/6 mice were vaccinated with 1×10^8 PFU of cAdVaxD(1-2), cAdVaxD(3-4), or CHB3. Animals were sacrificed 4 and 10 weeks after primary immunizations, and isolated splenocytes from each animal were incubated with a pool of overlapping peptides derived from the DEN2 E protein (amino acids 1 to 110). After incubation, anti-mouse IFN- γ ELISPOT assays were performed to detect DEN-specific CTL activities. Results are presented as the number of spots formed per 10^6 cells, and each data point represents the mean plus standard deviation (error bar) for four individual animals.

serotype cross-reactive CTL response. An increase in CTL activities was detected in all three vaccinated groups from 4 to 10 weeks after the primary immunization. Whether this is due to the booster immunization (at week 8) or simply a time-dependent phenomenon is unknown. From these data, we can conclude that in addition to humoral immune responses, the cAdVaxD vaccines also induce cellular immune responses against dengue virus proteins in vaccinated mice and that this cellular response is potentially broadly reactive across serotypes.

DISCUSSION

One of the prerequisites for a tetravalent dengue vaccine is that it must induce direct neutralizing immune responses against all four serotypes; cross-reactive but nonneutralizing antibody responses do not prevent dengue virus infection and have even been implicated as a major causative factor in immune enhancement of the disease. This presents two major obstacles. First, the neutralizing target antigens (prM and E) must be in their natural conformations to present their receptor binding sites where the neutralizing epitopes reside. Protein antigens synthesized in nonmammalian cell culture are not fully glycosylated and are folded differently; even antigens produced in mammalian cells are denatured during the purification process. Therefore, these antigens often do not induce strong neutralizing responses. Second, it requires presenting antigens of all four serotype dengue viruses at similar ratios to prevent underrepresentation of any one of the four serotypes. This has been particularly difficult to achieve even with live attenuated virus vaccines because each virus is attenuated differently. Furthermore, each attenuated virus may behave differently in different people or populations. Overattenuation may cause a subtype to be underrepresented and cause im-

mune enhancement; underattenuation will cause infection in some individuals by the vaccine as seen in the recently halted phase III clinical trials (17).

In the current study, we present a novel dengue vaccine strategy that induces multivalent immune responses by de novo synthesis of antigens from all four serotypes of the virus. The present report is the first to describe a complex Ad vector system capable of expressing multiple dengue virus prM and E antigens from a single vector with the possibility of inducing tetravalent immune responses in animal models if used in combination. The cAdVax system is capable of accommodating a large amount of foreign antigen sequences and multiple antigen inserts, allowing it to induce the balanced expression of the neutralizing antigens of all four serotypes of the virus. To assure that the prM and E antigens are expressed at similarly high levels, we put the prM and E of each serotype into an identical immediate-early cytomegalovirus CMV/bovine growth hormone polyadenylation cassette and inserted each expression cassette into either end of the cAdVax vector. The potent cytomegalovirus promoter and /bovine growth hormone polyadenylation sequences provide high levels of antigen expression; the near symmetrical insertion of each cassette at both ends of the adenovirus genome provide each expression cassette with a similar flanking environment to assure similar levels of expression. Indeed, our results show similar levels of expression of the prM and E proteins and, more importantly, similar levels of neutralizing immune responses to the serotypes of the dengue virus in vaccinated animals.

Immune responses are induced when cells express the dengue virus antigens on their surfaces, mimicking dengue virus-infected cells to the immune system. In contrast to the protein subunit-based vaccines, the de novo-synthesized antigens are naturally formed, glycosylated (shown as diffuse bands on Western blots), and associated with cell membrane (as recognized by immunostaining). This provides persistent immune system stimulation and induces potent humoral and cellular responses. Furthermore, the antibodies produced are neutralizing against all four serotypes, presumably because the naturally formed membrane glycoproteins have the intact receptor binding sites that contain the neutralizing epitopes.

Because the antigens are synthesized from the dengue virus genes transferred into cells, the antigen synthesis may last for weeks and provide potent and continuous stimulation to the immune system and induce CTL responses as shown in ELISPOT assays. CTL responses are broadly reactive to all four serotypes and important in eliminating dengue virus infections with the presence of neutralizing immune responses. However, on the basis of epidemiology studies, the broadly reactive CTL response alone is not sufficient to prevent dengue virus infection by a different serotype but may contribute to enhancement of the diseases due to the massive number of host cells are infected by the virus in the absence of neutralizing antibodies. This further underlined the importance of inducing balanced neutralizing immune responses against all four serotypes of dengue virus.

A previous study described an Ad-based dengue vaccine vector that expressed only the ectodomain of the DEN2 E protein (16). That vector induced anti-DEN2 neutralizing antibody responses in mice; however, other serotypes were not tested. Comparisons between the experimental results from

the aforementioned study and those of this study are difficult due to differences in vector design, vaccination schedule, serum collection time points, and mouse strains.

A common criticism of Ad vector use in humans is the possibility of preexisting immunity to adenovirus, in particular adenovirus subtype 5. Adenovirus subtype 5 is the most common subtype used for vector-based gene therapy and vaccine studies. It is also one of the subtypes to which a vast majority of the world is already immune (25). The presence of circulating Ad neutralizing antibodies has the potential to limit an Ad-based vector vaccine's efficacy. However, a recent clinical trial found no correlation between preexisting Ad-neutralizing antibody titers and potency of an Ad-based influenza vaccine in humans (34). This is supported by data from the Merck/NIH Vaccine Research Center phase I/II human immunodeficiency virus vaccine trials; it was concluded that using a highly immunogenic antigen, in combination with a higher dose, is sufficient to overcome preexisting immunity (6). Furthermore, previous studies with our cAdVax-based vaccines indicated that subsequent booster vaccinations are not hindered by primary immunity at all. In fact, the booster vaccinations actually enhance immune responses against target antigens in animal models (35–37). This was also supported by the data presented here with our cAdVaxD vaccine vectors.

In summary, we have presented two cAdVax dengue vaccine vectors that can individually function as bivalent dengue virus vaccine candidates in mouse models of vaccination. Additionally, the data presented here indicate that these two vectors have the potential to create a tetravalent dengue virus vaccine candidate if administered in combination. As our studies progress, we will test these bivalent vectors in animal challenge models, both individually and as a two-component "tetravalent" mixture. Results from these studies will provide valuable insight into the potential for these vaccines not only to induce tetravalent anti-dengue virus immune responses but to actually protect from dengue virus infection in animal models.

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