Development of a Purified, Inactivated, Dengue-2 Virus Vaccine Prototype in Vero Cells: Immunogenicity and Protection in Mice and Rhesus Monkeys

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The feasibility of a purified, inactivated dengue (DEN) vaccine made in Vero cells was explored. A DEN-2 virus candidate was chosen for production of a monotypic, purified, inactivated vaccine (PIV). Virus was harvested from roller bottle culture supernatants, concentrated, and purified on sucrose gradients. The purified virus was inactivated with 0.05% formalin at 22°C. After inactivation, the virus retained its antigenicity and was immunogenic in mice and rhesus monkeys, in which it elicited high titers of DEN-2 virus–neutralizing antibody. Mice were completely protected against challenge with live, virulent virus after receiving two 0.15-μg doses of PIV. Monkeys vaccinated with three doses ranging as low as 0.25 μg demonstrated complete absence or a significant reduction in the number of days of viremia after challenge with homologous virus. These results warrant further testing and development of PIVs for other DEN virus serotypes.

Dengue (DEN), which is transmitted by mosquitoes, is an acute viral disease of humans. It is endemic in the tropics and subtropics, worldwide, where an estimated 100 million cases occur annually (reviewed in [1]). DEN is characterized clinically by biphasic fever, rash, and hematopoietic depression. Patients commonly exhibit malaise, arthralgia, myalgia, and headache (reviewed in [2]). Infrequently, more severe disease, manifest by hemorrhage, may progress to lethal shock [3–5]. Although relatively rare, DEN hemorrhagic fever and DEN shock syndrome may cause death in children. There is no vaccine to protect against DEN, and attempts to prevent disease by vector control have proven largely ineffective.

DEN viruses are members of the family Flaviviridae [6], which includes >60 members, many of which are important human pathogens. Within the DEN group, there are 4 serotypes, DEN-1–4. Although these viruses share genetic and antigenic features, infection with one serotype does not afford long-term protection against all serotypes [7]. Like the family prototype, yellow fever virus, DEN viruses are enveloped, single-stranded RNA viruses ~50 nm in size [1]. Their 10.5-kb genome encodes 10 proteins: 3 virion structural proteins at the 5′ end of the RNA, beginning with capsid, matrix (M) and its precursor prematrix (prM), and envelope (Env), and followed by 7 nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (reviewed in [8]). Virus-neutralizing antibody (VNA), which is thought to play a primary role in immunity and protection, is directed against the Env protein [9–10].

Currently, only two flavivirus vaccines have been licensed by the US Food and Drug Administration for human use: live, attenuated yellow fever virus (strain 17D; developed by Theiler et al. [11] in the 1930s) and purified, formalin-inactivated Japanese encephalitis virus (developed in the 1960s in Japan [12]). Both vaccines are safe and effective, eliciting high titers of VNA and conferring solid protection [13] (reviewed in [14]). Also of note is a purified, formalin-inactivated vaccine developed in the 1970s in Austria. The vaccine is used successfully against tickborne encephalitis virus in many European countries [15].

Much work has been done using recombinant cDNA technology to develop second-generation flavivirus vaccines (reviewed in [16, 17]). Full-length infectious cDNA clones, which are now available for yellow fever [18], Japanese encephalitis [19], and DEN-4 [20] viruses, offer the exciting possibility of making genetically engineered, attenuated vaccines, while in vitro expression systems allow production of recombinant subunit antigens. Although promising, these technologies have yet to yield practical alternatives to existing flavivirus vaccines.

The early history of DEN vaccines dates back nearly 70 years to mostly unsuccessful attempts to prevent virus transmission with crude vaccines prepared by treating infectious human plasma with ox bile or formalin (reviewed in [21]). In the 1940s, candidate attenuated DEN vaccines, which were partially efficacious, were developed by serial passage of wild viruses in mouse brain [22]. More recently, several attenuated DEN vaccine candidates have been made by passage of wild viruses in primary and diploid cell cultures [14, 23–30]. Some of these have shown promise, although it has been difficult to find candidates that are both suitably attenuated and immuno-

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genic. Other concerns are that these viruses may undergo reversion to virulence and be transmitted by the vector. Because most DEN virus isolates replicate poorly in cell culture, inactivated vaccines have generally been thought to be impracticable. Nevertheless, the possibility of using viruses adapted to continuous cell lines that can be grown in large-scale cultures has prompted reexploration of the inactivated vaccine approach for DEN.

In the present study, a process was developed for preparing a purified, inactivated vaccine (PIV) prototype for DEN-2 virus. DEN-2, strain S16803, was adapted to Vero cells, a pathogen-free, continuous cell line used for production of licensed vaccines. The virus was propagated in roller bottle cultures, purified, and inactivated. The PIV was used to immunize mice and rhesus monkeys. We investigated whether this approach might form the basis for producing a vaccine against all 4 serotypes of DEN virus.

Materials and Methods

Cells. African green monkey kidney cells (Vero p-129 B; gift from Pasteur-Mérieux Serums et Vaccins, Marcy l'Etoile, France) were used for virus production. A frozen, master cell bank was prepared at the Salk Institute (Swiftwater, PA) and tested for adventitious agents, tumorigenicity, reverse transcriptase, and identity. LLC-MK_2 cells were used for plaque-titration of viruses (see below). *Aedes albopictus* cells (clone C6/36) were used for virus amplification (see below).

Viruses. DEN-2 virus (strain S16803; from N. Marchette, University of Hawaii at Manoa, Honolulu), which had been isolated from a DEN fever patient by passage in C6/36 cells, was passaged three times in Vero cells. A master seed was made at passage two and a production seed at passage three. Vaccine lots were prepared at Vero cell passage level four.

Antibodies. DEN-2 New Guinea C strain hyperimmune mouse ascitic fluid (HMAF) reactive against all structural and nonstructural antigens of DEN-2 virus was prepared as previously described [31]. Mouse ascitic fluids containing monoclonal antibodies (MAbs) specific for Env antigen (2H3, 3H5, and 4E5), prM/M antigen (14E9), and NS1 antigen (7E11) have been described [10, 32, 33].

Virus production in Vero cell cultures. Vero cells (passage 139) stored in liquid N_2 were thawed rapidly at 35°C. One milliliter of cell suspension containing $\sim 10^7$ cells was used for each 150-cm^2 flask (T150; Corning, Corning, NY). Cells were fed with 50 mL of growth medium (Eagle MEM [EMEM], 10% heat-inactivated [56°C, 30 min] fetal bovine serum [FBS], 2 m*M* L-glutamine [all from BioWhittaker, Walkersville, MD], and 100 U of streptomycin and neomycin) and incubated at 35°C. Upon confluency, cells were trypsinized, and one flask was subcultured into each 490-cm^2 roller bottle (Corning). Roller bottle cultures were fed with 100 mL of growth medium and incubated at 35°C on a roller apparatus set to rotate at 0.5 rpm. Under these conditions, Vero cells formed monolayers, which were 90%–100% confluent within 5 days. Monolayers were infected at an MOI of 0.01 plaque-forming units (pfu)/per cell. Virus was allowed to adsorb for 1 h at 35°C, and then the monolayers were fed with 100 mL of maintenance medium (the same as growth medium, except containing 0.25% human serum albumin in place of FBS) and incubated at 35°C. Two days after inoculation, cultures were washed three times with serum-free EMEM and refed with serum-free EMEM or EMEM containing 0.25% human serum albumin. Culture supernatants were pooled for virus harvest 5 days after inoculation, when cytopathology was ~75%.

Virus concentration by tangential flow ultrafiltration. Supernatants from infected cell cultures were clarified by centrifugation at 1500 rpm for 10 min in a Sorvall (Norwalk, CT) RT 6000 B centrifuge using a H1000 rotor and then filtered through a 0.45-μm filter (CN type, Nalgene; Nalge, Rochester, NY). The filtered supernatant was concentrated by tangential flow ultrafiltration using a low protein–binding, 100-kDa cutoff membrane (Omega 100K screen channel; Filtron, Northborough, MA). Concentration was carried out at 4°C using a flow rate of 400 mL/min, a filtration rate of ~100 mL/min, and a pressure of 20–30 psi. The processing time for a 15- to 20-fold reduction in volume was ~10–15 min. Following concentration, samples were held briefly at 4°C prior to sucrose gradient ultracentrifugation.

Sucrose gradient ultracentrifugation. Virus was purified on sucrose gradients as described previously [34] with minor modifications. Sucrose gradients (15 mL) were made in 25.4 × 88.9 mm (40 mL) ultracentrifuge tubes (Ultra-clear; Beckman, Fullerton, CA) by stepwise addition of the following wt/wt sucrose solutions in PBS without Ca and Mg (pH 7.4; BioWhittaker): 2 mL of 60%, 2 mL of 55%, 2 mL of 50%, 2 mL of 45%, 2 mL of 40%, 2 mL of 35%, 2 mL of 30%, and 1 mL of 15%. A smooth gradient was formed by allowing the tubes to stand for 2–4 h at room temperature. Up to 25 mL of concentrated virus was added to each tube and centrifuged in a SW 28 rotor (Beckman) at 17,000 rpm for 18 h at 4°C. Following centrifugation, 1-mL fractions were collected from the bottom of the tubes and assayed for total protein and for virus-induced hemagglutination (HA) of goose red blood cells. Gradient fractions positive by the HA test (see below) were pooled and diluted to ≤10% sucrose with medium (M199; GIBCO BRL, Grand Island, NY) or PBS. Prior to formalin inactivation, virus pools were filtered through a 0.22-μm low protein–binding filter (GV type; Millipore, Bedford, NY).

Virus inactivation with formalin. Formalin (37% formaldehyde, ACS grade; Fisher Scientific, Pittsburgh) was diluted 1:40 in PBS, the pH was adjusted to 7.4 with 1 *N* NaOH, and the solution was sterilized by passage through a 0.22-μm CN filter (Nalgene). One volume of this solution was added to 50 vol of purified virus for a final formalin concentration of 0.05%. Inactivation was carried out in a sterile 50-mL polypropylene tube (Corning) at 22°C in a constant-temperature water bath. After 48 h, virus was filtered through a 0.22-μm GV type filter and transferred to a fresh container. Aliquots were taken at regular intervals to measure infectious virus and virus antigen. Free formaldehyde in the samples was neutralized by the addition of 1 vol of a 1:8 dilution of a 35% (wt/vol) aqueous solution of sodium bisulfite to 100 vol of sample. For plaque assay, one-half of each sample was transferred to a tube containing an equal volume of heat-inactivated FBS and frozen at −80°C. At the completion of inactivation, free formaldehyde in the bulk culture was neutralized by addition of an equimolar amount of sterile 10% (wt/vol) sodium bisulfite.

C6/36 cell virus amplification assay. C6/36 cells at passage level 15–30 were grown at 28°C as monolayers in 25-cm^2 flasks

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(T25) with EMEM, nonessential amino acids (BioWhittaker), 10% heat-inactivated FBS, streptomycin, and neomycin. Upon confluency, the growth medium was removed, and duplicate flasks were inoculated with samples from each formalin inactivation time point. In addition, a minimum of 10 flasks was inoculated with each final vaccine lot (representing at least 4% of the total lot volume). After adsorption for 1 h at 35°C, cells were refed and incubated at 28°C. Cells were refed after 7 days, and at 14 days, the culture medium was harvested and plaqued to detect infectious virus (see below).

Virus plaque titration assay. Virus infectivity titrations were done by plaquing on LLC-MK_2 cell monolayers [35].

Virus plaque reduction neutralization (PRNT) assay. Anti–DEN-2 VNA was measured in sera from immunized mice and monkeys by use of an assay [36] modified by inclusion of a heat-labile factor from normal (not DEN immune) human plasma, which enhances antibody-dependant neutralization [37]. The reciprocal of the serum dilution that resulted in a 50% reduction in the number of virus plaques ($PRNT_{50}$) was calculated as the end point. The DEN-2 virus used in the assay was strain S16803 adjusted to a dose of 75–100 pfu.

Virus HA and HA-inhibition (HAI) assays. Virus HA and HAI assays were done as previously described [38]. Antigen for the HAI assay was prepared from the mouse-adapted DEN-2, New Guinea C strain.

Antigen spot-blot assay. To detect and quantitate antigen in inactivated virus preparations, an antigen spot-blot assay was done. Samples were diluted serially, 2-fold, and spotted onto nitrocellulose papers. The papers were air-dried, blocked with 5% casein in PBS, and incubated with anti–DEN-2 HMAF followed by an enzyme-linked secondary antibody as previously described [39].

SDS-PAGE and Western blotting. Proteins were solubilized at 22°C for 10 min in SDS-PAGE sample buffer containing 1% SDS, 66 m*M* TRIS-HCl, pH 6.8, 1% glycerol, and 0.7% bromphenol blue and then electrophoresed on 12.5% polyacrylamide gels [40]. Resolved proteins were transferred electrophoretically to Immobilon-P membrane sheets (Millipore, Bedford, MA). Proteins were detected by staining with colloidal gold (BioRad, Hercules, CA), and viral antigens were identified immunologically using a nonisotopic modification [39] of the Western blot [41].

Protein assay. Total protein was determined using a commercially available kit (BioRad) essentially as described by Bradford [42]; bovine serum albumin was used as a standard.

Mouse immunization and protection assays. Female, 5- to 6-week-old ICR mice (Jackson Laboratory, Bar Harbor, ME) were immunized with PIV adjuvanted with 0.1% aluminum hydroxide (alum) (Alhydrogel; Superfos Biosector, Vedbak, Denmark) in PBS. Inoculations (0.1 mL) were given subcutaneously with a 22-gauge needle in the region of the hind quadriceps. Two inoculations were given 1 month apart. Blood for serology was obtained 1 month after the first and 2 weeks after the second inoculation by retroorbital puncture using sterile, glass capillary pipettes.

For protection assays, 3-week-old, female BALB/c mice were inoculated subcutaneously in the hindquarters with PIV in a 0.1% suspension of alum in PBS (0.1 mL). Mice were boosted with an equivalent dose 2 weeks later. At 6 weeks of age, immunized mice were inoculated intracranially using a 26-gauge needle with $\sim 10^5$ pfu of live, mouse-adapted DEN-2 virus (New Guinea C strain) in 30–50 μL of Hanks' balanced salt solution. Challenged mice were monitored daily for morbidity and mortality for up to 21 days and scored according to morbidity status (MS) as follows: 0 = healthy; 1 = ruffled coat, lethargic; 2 = partial hind-limb paralysis; 3 = complete hind-limb paralysis, wasting. For comparison among vaccine and control groups, a mean MS was calculated as follows: [sum of all MSs × (no. of mice scored on a given day/total no. of surviving mice on that day)]/total no. of days of illness.

Table 1. Master and production seeds for DEN purified, inactivated vaccines prepared in Vero cells.

Virus (strain)*	Master seed (pfu/mL)†	Production seed (pfu/mL)‡
DEN-1 (West Pac 74)	2.5×10^6	2.1×10^7
DEN-2 (S16803)	2.9×10^6	3.0×10^6
DEN-3 (CH53489)	1.1×10^6	3.1×10^6
DEN-4 (TVP-360)	1.3×10^7	3.0×10^7

* DEN virus isolates that replicated to highest titers on Vero cells were selected for making master and production seeds.
† Prepared at Vero cell passage 2; titered on LLC-MK_2 cell monolayers.
‡ Prepared at Vero cell passage 3; titered on LLC-MK_2 cell monolayers.

Immunization and challenge of rhesus monkeys. Adult male and female rhesus monkeys (6–15 kg) were immunized with PIV adjuvanted with 0.1% alum in PBS. Control monkeys received 0.1% alum in PBS. Subcutaneous inoculations (0.5 mL) were given in the upper arm with a 22-gauge needle. Inoculations were given at 0, 1, and 6 months. Blood for serology was obtained from the femoral vein before each PIV inoculation, on the day of challenge, and at intervals following each of these inoculations. Eight weeks after the third PIV inoculation, all monkeys, including controls, were challenged with 10^4 pfu of DEN-2 virus strain S16803 (production seed). Blood was drawn on 14 consecutive days following challenge, and serum was separated and frozen at −80°C for virus isolation and assay.

Statistics. Where appropriate, data were analyzed for statistical significance by Fisher's exact test.

Results

DEN virus growth and purification. Several DEN virus isolates representing all 4 serotypes were screened for their ability to replicate to high titers in Vero cell cultures. Those viruses with the highest infectivity titers, as measured by plaque titration of the culture supernatants, were selected for preparation of master (second passage) and production (third passage) seeds (table 1).

Following the scheme outlined in figure 1, a DEN-2 virus candidate was chosen for the preparation of several pilot lots of monotypic PIV in roller bottles. Production of virus in the presence and absence of serum supplements was compared. Table 2 lists the results of virus production in cells maintained in the presence of human serum albumin (lot WR-3) and virus production in cells maintained in serum-free medium (lot WR-4). Initial yields and final recoveries of virus, based on pfu and HA unit determinations, were not significantly different, but the

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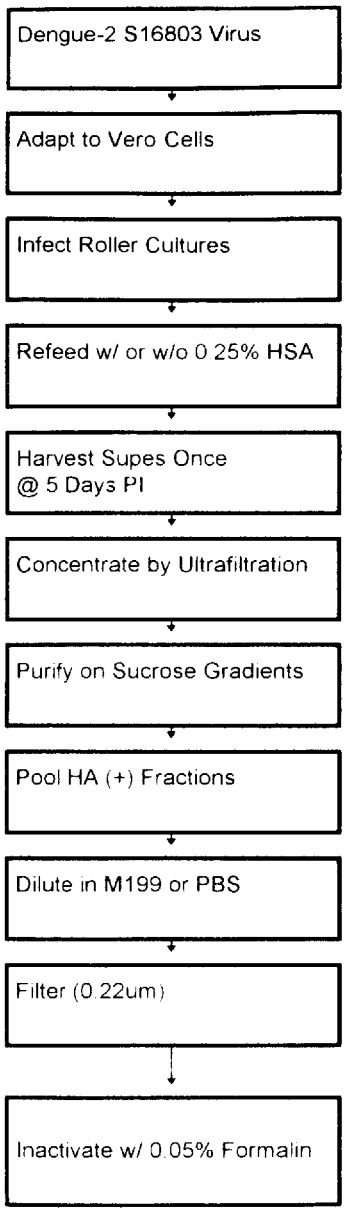

Figure 1. Production scheme for DEN-2 purified, inactivated vaccine (PIV). w/, with; w/o, without; HSA, human serum albumin; Supes, supernatants; PI, postinoculation; HA, hemagglutination

purity of lot WR-4, based on a specific activity determination (i.e., pfu per milligram of protein), was ~5-fold greater. Figure 2 shows the results of the sucrose gradient purification used to prepare virus for lot WR-4. The gradient had one peak of HA activity with a trailing shoulder; about 95% of contaminating protein was removed from the antigen-containing peak. Analysis of lot WR-4 by SDS-PAGE demonstrated a high degree of purification with only two major proteins detected by gold staining (figure 3A). These two proteins comigrated with virion Env and prM/M antigens, which were detected by Western blotting with anti–DEN-2 HMAF (figure 3B), MAbs reactive with Env antigen (figure 3C), and MAbs reactive with prM/M antigen (figure 3D). In addition, lot WR-4 contained a trace amount of the dimer form of NS1, which reacted with anti-NS1 MAb (see figure 3D) but was not seen on the gold-stained gel.

DEN virus inactivation. Purified DEN-2 virus was inactivated with 0.05% formalin at 22°C. These conditions were established from previous work using unpurified DEN virus (Dubois DR, unpublished results). The results of inactivation of a typical PIV lot (WR-2) are listed in table 3. Residual virus was quantitated by direct plaque assay after neutralization of formalin. In addition, samples were subjected to passage on C6/36 cell monolayers in order to amplify low levels of virus. No infectious virus was recoverable after 84 h, and inactivation was continued for at least 208 h for a suitable margin of safety. There were no losses in antigenicity during this period (measured in a spot-blot assay using anti–DEN-2 HMAF; data not shown). An aliquot (4%) of each PIV was tested by C6/36 amplification assay and found to be negative for live virus. From these results, we conclude that the inactivation conditions used were suitable for inactivating purified DEN virus while preserving its antigenicity.

Immunogenicity and protective efficacy of purified, inactivated DEN-2 virus in mice. Immunogenicity of the PIVs was tested in adult mice (table 4). Mice developed significant titers of DEN-2 VNA after one inoculation with PIV WR-3 or WR-4. Titers increased ~4- to 10-fold after a second dose. Two inoculations were required for development of HAI antibody.

The protective efficacy of the PIVs was then tested in juvenile mice. All vaccinated mice remained healthy after virus challenge (mean MS of 0). In comparison, control mice became sick (mean MS of 1.70 and 1.25 in two experiments) beginning at 6–7 days after challenge. The mortality rates among the controls were 47% (8/15, $P < .02$) and 80% (2/10, $P < .001$) in the two experiments. These experiments demonstrate that mice could be solidly protected against disease with two doses of PIV WR-4, each containing 0.15 μg of protein.

Immunogenicity of purified, inactivated DEN-2 virus in rhesus monkeys. Immunogenicity of PIV WR-4 was determined in groups of rhesus monkeys given doses ranging from 0.25 to 75 μg; the results are shown in table 5. All monkeys except DA86 were nonimmune when given the first inoculation. DA86, who had a low level of DEN-2 VNAs, will be discussed separately. Of the 17 other monkeys, 10 responded to the first dose of PIV with $PRNT_{50}$ titers of 10–160, with 6 of 7 nonresponders in the low-dose range (0.25–2.5 μg). Following the second PIV dose, the seroconversion rate increased to 100%. Four weeks after the second dose, the geometric mean $PRNT_{50}$ titer (GMT) for the whole group of monkeys was 370. In the higher-dose range (7.5–75 μg), the GMT was 560; in the lower-dose range, it was 220. Prior to the third dose at 182 days, VNA had decayed to undetectable levels in 5 of 17 monkeys. Four weeks after the third dose of PIV, all monkeys regained measurable VNAs: GMT = 400 for all monkeys, 930 for those given doses of 7.5–75 μg, and 150 for those given

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Table 2. Purification of DEN-2 strain S16803 grown in Vero cells in the presence of 0.25% human serum albumin (lot WR-3) or no serum protein supplement (WR-4).

Virus-infected cell supernatant	Total volume (mL)	Total pfu* (pfu/cell)	% yield (pfu)	Total HAU†	% yield (HAU)	Total protein (mg)	% yield (protein)	Specific activity (pfu/mg protein)
From lot WR-3								
Day 5 supernatant	950	6.7×10^9 (11.2)	100	60,800	100	1710	100	3.9×10^6
Filtron concentrate	55	3.5×10^9	52	88,000	145	1364	80	2.6×10^6
Sucrose gradient pool	50	4.6×10^9	70	40,000	65	70	4	6.7×10^7
After filtration	50	2.4×10^9	36	15,000	25	23.5	1.4	1.0×10^8
From lot WR-4								
Day 5 supernatant	960	1.7×10^{10} (28.3)	100	61,440	100	139	100	1.2×10^8
Filtron concentrate	75	1.2×10^{10}	71	120,000	195	62	45	1.9×10^8
Sucrose gradient pool	50	1.1×10^{10}	65	40,000	65	9.7	7	1.1×10^9
After filtration	50	4.0×10^9	24	20,000	32	7.5	5.4	5.3×10^8

NOTE. HAU, hemagglutinin units.
* Plaque-forming units (pfu)/cell calculated based on 6×10^7 Vero cells/490-cm^2 roller bottle.

0.25–2.5 μg. At the time of challenge (8 weeks after dose three), the GMT was 130 for all monkeys, 190 for those given doses of 7.5–75 μg, and 80 for those given 0.25–2.5 μg. Challenge consisted of inoculation of all monkeys, including a group of control animals that had received three doses of PBS-alum over the same time course, with 10^4 pfu of DEN-2 virus strain S16803. Serum was obtained daily for a total of 14 days following challenge and, for each monkey, it was tested for virus by amplification in mosquito cells. Sera that were found to be positive in this assay were titrated by direct plaque assay to determine the levels of circulating virus. Table 5 lists the results on 10 days after challenge; the 4 days for which data are not shown were days on which no virus was recoverable. All monkeys in the PBS–alum control group became infected for a mean of 7 days, with virus titers reaching 5.0 $\log_{10}$ pfu. In the PIV-vaccinated groups, no animal had >3 days of viremia, however, 11 of 17 monkeys had at least 1 day of viremia; 4 of the 9 given 7.5–75 μg of PIV were viremic

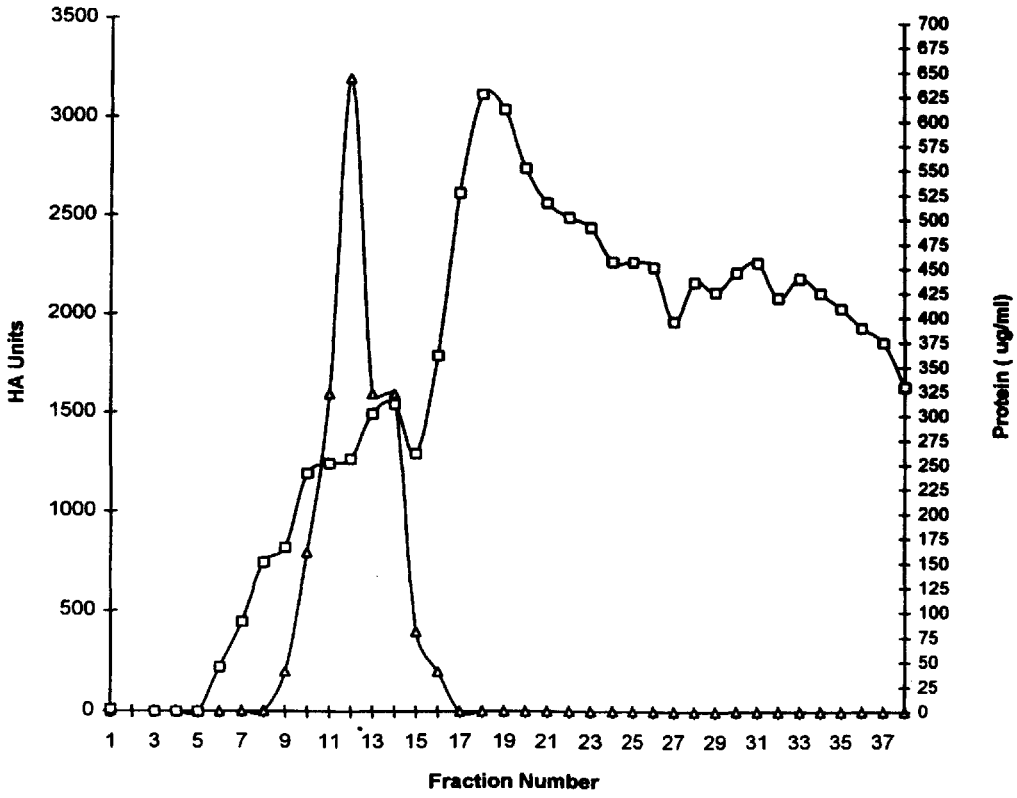

Figure 2. Purification of DEN-2 virus by sucrose gradient ultracentrifugation. 23 mL of concentrated virus culture fluids was applied to 15-mL, 15%–60% sucrose gradient and centrifuged at 17,000 rpm for 18 h at 4°C. 1-mL samples were collected from bottom of tube and assayed for virus hemagglutinin (△) and total protein (□). Data shown are for purified, inactivated vaccine (PIV) lot WR-4 (see table 2). HA, hemagglutination.

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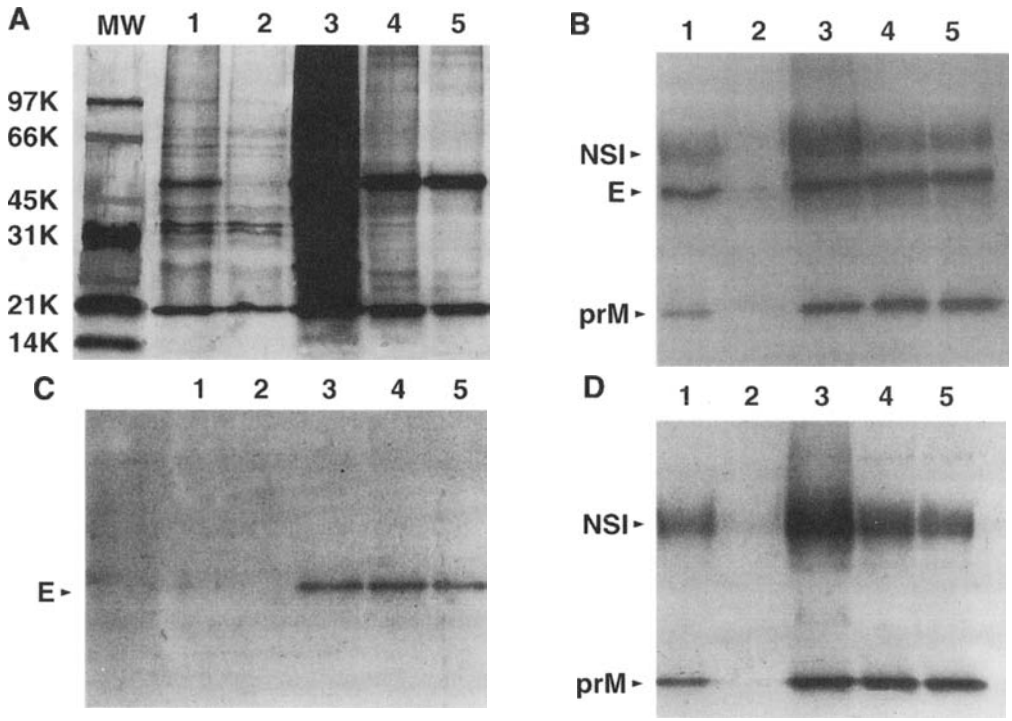

Figure 3. Analysis of DEN-2 virus by SDS-PAGE and Western blotting. Samples obtained during virus purification were subjected to SDS-PAGE, and resolved proteins were transferred to membranes. Proteins were visualized by staining with colloidal gold (**A**), and antigens were visualized by reaction with DEN-2 polyclonal antiserum (**B**), monoclonal antibodies 2H3, 3H5, and 4E9 reactive against envelope (E) antigen (**C**), and MAbs 14E9 and 7E11 reactive against precursor prematrix (prM)/matrix and nonstructural (NS)1 antigens (**D**). Lanes: 1, virus supernatants harvested from Vero cells; 2, filtrate (flow through) after ultrafiltration of virus fluids; 3, virus concentrate (retentate) after ultrafiltration; 4, sucrose gradient–purified virus pool; 5, filtered sucrose gradient–purified virus pool; MW, molecular weight. Protein MW standards are at left: 97 kDa = rabbit muscle phosphorylase B; 66 kDa = bovine serum albumin; 45 kDa = hen egg white ovalbumin; 31 kDa = bovine carbonic anhydrase B; 21 kDa = soybean trypsin inhibitor; 14 kDa = hen egg white lysozyme. Data are for purified, inactivated vaccine lot WR-4 (see table 2).

for a mean of <1 day; 7 of 8 given 0.25–2.5 μg were infectious, with a mean of 2 days of viremia.

Another observation that is probably dose-related is that, in general, viremic monkeys that had been given the lower doses had higher serum titers of virus when quantitated by plaque assay (table 5). Higher titers of circulating virus after challenge may also account for the higher postchallenge VNA levels in animals that were originally vaccinated with the lower doses of PIV. Sera obtained 10 weeks after challenge from the low-dose group had the highest levels of VNAs (GMT = 6000); the groups given higher doses of PIV had a GMT of 2500, and all groups combined had a GMT of 3900.

Monkey DA86 had preexisting DEN-2 VNAs when he was inoculated with 0.25 μg of WR-4 PIV. Of note, this monkey mounted the highest VNA response after each of the three PIV inoculations. No detectable virus was found following challenge, and serum VNA levels were not significantly different before and after challenge.

Discussion

Despite the existence of safe and effective inactivated virus vaccines, it frequently has been contended that such an approach is neither feasible nor practical for DEN because of the relatively poor growth of these viruses in culture and their questionable antigenic stability after purification and formalin fixation. To reevaluate this and to determine an effective dose in animals, a DEN-2 virus candidate was chosen for the production of several pilot lots of PIV. A continuous cell line (Vero) demonstrated to be pathogen-free and nontumorigenic was chosen as a potentially suitable production substrate [43]. After virus adaptation, roller bottle cultures were used for large-scale virus propagation, with titers reaching 10^7 pfu/mL culture fluid. These titers were maintained without serum supplements, a feature that is highly desirable from a vaccine production standpoint. The ability to maintain virus yields in the absence of serum supplements should allow candidate DEN PIVs to more easily meet accepted purity standards for inactivated vaccines made in continuous cell lines [44].

The initial step in downstream processing was to concentrate the culture supernatants by ultrafiltration. This method was fast and efficient and resulted in high virus recoveries, as determined by pfu and HA assays. Virus purification was done by ultracentrifugation of concentrated virus fluids on sucrose gradients, a method that can be scaled-up for the processing

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Table 3. Formalin inactivation of DEN-2 virus for purified, inactivated vaccine lot WR-2.

Time (h)	Inactivated DEN-2		Control DEN-2, direct assay (pfu/mL)
	Direct assay (pfu/mL)	Amplified assay	
0	6.8×10^7	+	5.0×10^7
6	4.7×10^6	+	ND
12	9.2×10^5	+	4.2×10^7
18	3.5×10^5	+	ND
24	8.0×10^4	+	4.1×10^7
36	1.2×10^4	+	ND
48	2.7×10^3	+	ND
60	7.7×10^2	+	ND
72	1.0×10^2	+	1.9×10^7
84	7.0×10^1	+	ND
96	<50	−	1.8×10^7
108	<50	−	ND
156	<50	−	ND
208	<50	−	ND

NOTE. DEN-2 purified virus was or was not (control) inactivated by addition of 0.05% formalin and held at 22°C. Samples were taken at intervals indicated and assayed for infectious virus by direct plaque assay or by amplification in C6/36 cells (see Materials and Methods). ND, not done.

of large sample volumes by batch or continuous flow. After centrifugation, a single peak, often with a trailing shoulder, was detected by HA assay. This peak, which coincided with a visible band in the gradient, most likely consisted of whole virions and coreless particles. The degree of purification achieved by this method was 25-fold for virus grown in cells maintained with human serum albumin and 4-fold for virus grown in serum-free medium. However, the final specific activity of virus grown without serum supplements was higher. The effectiveness of purification was demonstrated by gel electrophoresis. DEN virus Env and prM/M were the major proteins detected in PIV lots by Western blotting and gold staining. NS1 dimer [45], which appears to partially copurify with virions, was seen on Western blots but not on gold-stained gels, which suggests that it was present only in trace amounts.

The inactivation kinetics of DEN virus were typical of those seen with other viruses inactivated with formalin; 22°C was chosen over higher temperatures on the basis of results of previous studies indicating the lower temperature of inactivation was optimal for retention of immunogenicity. This was confirmed in the present study by evaluation of antigen before and after formalin fixation. There was no loss of antigenicity as determined by spot-blot assay using polyclonal antisera. These data suggest that formalin is a suitable inactivating agent for purified DEN viruses. The possible loss of key neutralizing or protective epitopes in the formalin-fixed DEN virion needs to be studied to limit the possibilities of inducing an aberrant or incomplete immune response in recipients of a DEN PIV. We plan to map the Env protein using MAbs so that data on epitope modification can be generated. Unfortunately, there is currently no animal model in which to study DEN immunopathogenesis.

Mice are routinely used for initial assessment of DEN vaccine candidates. These animals can be immunized and protected with small amounts of immunogen. In a previous study with recombinant DEN-4 Env protein, submicrogram doses of this immunogen were sufficient to protect mice against lethal challenge [46]. Similarly, the DEN-2 PIV (WR-4) was completely protective after two 0.15-μg doses.

Monkeys have also been used to evaluate DEN vaccine candidates, both live and subunit immunogens. In the study referenced above, DEN-4 Env protein that was highly efficacious in mice did not protect monkeys from challenge and was poorly immunogenic even after immunization with three doses exceeding 100 μg/dose. In the current study, DEN-2 PIV given as a single dose at a concentration as low as 7.5 μg was nearly 90% immunogenic in rhesus monkeys. PIV given at concentrations as low as 0.25 μg, although not immunostimulatory after one dose, appeared to prime monkeys when they were given a second dose at the same concentration 4 weeks later. After three doses, all monkeys in the dose range tested had evidence of at least partial protection from infection with 10^4 pfu of unmodified DEN-2 virus. Although there was not a clear protective dose effect after three doses, the animals that were given the higher doses (7.5–75 μg) had fewer days of viremia, and their virus titers did not exceed 1.2 $\log_{10}$. The VNA titer at the time of challenge was also higher (GMT = 190) in this group of animals than in the group given 0.25–2.5 μg (GMT = 80). All monkeys except CH137 and DA86 mounted substantial VNA responses after challenge, which may indicate that chal-

Table 4. Immune response of mice vaccinated with DEN-2 purified, inactivated vaccines (PIVs).

PIV doses × μg/dose*	Neutralization ($PRNT_{50}$)	Hemagglutination inhibition titer
WR-3		
1 × 47	160	<10
1 × 15.5	340	<10
2 × 47	800	40
2 × 15.5	1400	160
2 × 4.7	350	80
WR-4		
1 × 15	280	<10
1 × 5	230	<10
2 × 15	1200	160
2 × 5	2500	320
2 × 1.5	900	160

NOTE. Mice were inoculated with 1 or 2 doses of indicated PIV with aluminum hydroxide. Assays were done on pooled sera from 5 mice/group. See table 2 for details on vaccine lots WR-3 and WR-4. $PRNT_{50}$, plaque reduction neutralization 50% end point.

* Total protein administered subcutaneously in 0.1 mL of vaccine.

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Table 5. Neutralizing antibody responses and protection from viremia in rhesus monkeys following vaccination with DEN-2 purified, inactivated vaccine lot WR-4 and challenge with DEN-2 virus strain S16803.

Vaccine dose (ug)	Monkey	Inoc 1, day 0	Inoc 2, day 28	Day 56	Inoc 3, day 182	Day 210	Challenge, day 238	Day 308	Viremia on days 1–10 after challenge									
									1	2	3	4	5	6	7	8	9	10
75	4B6	<10	20	610	70	720	120	ND	–	ND	ND	ND	ND	ND	ND	ND	ND	ND
	CH137	<10	160	1040	140	900	210	150	–	–	–	–	–	–	–	–	–	–
	882C	<10	10	800	80	1000	150	4800	–	–	–	–	+/<1.2	–	–	–	–	–
25	033A	<10	80	1100	110	1000	170	7200	–	–	+/<1.2	–	–	–	–	–	–	–
	891558	<10	80	1170	210	680	150	3600	–	–	–	+/<1.2	+/<1.2	–	–	–	–	–
	68A	<10	<10	100	20	850	100	2300	–	–	–	–	–	–	–	–	–	–
7.5	3MI	<10	10	320	40	900	150	5300	–	–	–	–	–	–	–	–	–	–
	547A	<10	20	980	250	950	330	3900	–	–	–	–	+/1.2	–	–	–	–	–
	6PR	<10	160	320	50	1680	800	1860	–	–	–	–	–	–	–	–	–	–
2.5	84456	<20	30	1220	<10	1200	120	6400	–	–	–	+/4.1	–	–	–	–	–	–
	931C	<20	20	960	70	140	90	10,600	–	–	–	–	–	–	–	–	–	–
	B652	<20	<20	1230	<10	200	90	7600	–	–	–	+/4.1	+/4.1	–	–	–	–	–
0.75	3JT	<20	<20	330	<10	80	40	6700	–	–	–	+/4.4	+/5.5	+/5.2	–	–	–	–
	P991	<20	<20	170	20	150	70	5200	–	–	–	–	+/4.0	+/4.7	–	–	–	–
	907T	<20	<20	60	10	100	120	4400	–	–	–	–	+/<1.2	–	–	–	–	–
0.25	DA86	20	700	1950	300	8800	1320	1350	–	–	–	–	–	–	–	–	–	–
	159D	<20	<20	30	<10	20	60	4800	–	–	–	+/3.4	+/4.7	+/4.8	–	–	–	–
	N839	<20	<20	40	<10	400	60	4600	–	–	–	+/1/2	+/2.7	–	–	–	–	–
Untreated	CH139	<10	ND	ND	ND	ND	<10	2300	–	–	+/1.5	+/3.5	+/4.8	+/5.0	+/4.2	–	–	+/<1.2
	E42	<10	ND	ND	ND	ND	<10	520	+/3.9	+/<1.2	+/1.2	+/1.3	+/3.5	+/4.3	+/3.4	+/2.0	–	–
	E363	<10	ND	ND	ND	ND	<10	410	–	+/1.5	+/<1.2	–	–	+/2.3	+/<1.2	+/3.5	+/2.4	–

NOTE. Data are plaque reduction neutralization 50% end points or isolation of virus by amplification in mosquito cells (+ or −)/direct plaque assay of virus from serum (log_{10} pfu/mL). Samples not found viremic by amplification in mosquito cells (−) were not titrated by direct plaque assay. No virus was recoverable on days 11–14 after challenge. Monkey 4B6 died 2 days after challenge. Untreated (control) monkeys were given PBS–aluminum hydroxide on inoculation (Inoc) days. ND, not done.

lenge virus replicated. However, there is no correlation with titers after challenge and viremia that might explain this phenomenon. Monkey DA86 had serologic evidence of DEN infection before PIV inoculation and did not have an increase in serum VNA before or after challenge. Since prior DEN exposure is a real-life vaccination scenario, follow-up studies are indicated in monkeys to expand further on the observation in monkey DA86.

The present study demonstrates that DEN-2 virus, which is grown, purified, inactivated, and adjuvanted using conventional methodologies, results in a highly immunogenic product. Although all DEN viruses may not be as immunogenic, a PIV made using a strain of DEN-1 showed similar immunogenicity in monkeys after two doses. DEN-2 PIV WR-4 also appears comparable in purity, immunogenicity, and protective efficacy to other PIVs prepared for tickborne encephalitis and Japanese encephalitis flaviviruses using similar methodologies [47–49]. Future work will be devoted to producing and testing monovalent PIVs for all 4 DEN virus serotypes using the strains that have been identified. Eventual combination into a tetravalent product is the ultimate goal for producing a vaccine that will confer complete protection against DEN.

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