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# MODIFICATION OF DENGUE VIRUS STRAINS BY PASSAGE IN PRIMARY DOG KIDNEY CELLS: PREPARATION OF CANDIDATE VACCINES AND IMMUNIZATION OF MONKEYS

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*Abstract.* Dengue (DENV) virus strains for each of the four DENV serotypes were modified by passage in primary dog kidney (PDK) cell cultures with final manufacture of vaccine lots in fetal rhesus monkey diploid cell cultures. "Strain sets" consisting of serially-passaged DENV were inoculated in rhesus monkeys along with unmodified parent viruses for each strain. Vaccine candidates were compared with unmodified parent viruses by measuring viremia and immune responses. All except one DENV-1 strain demonstrated reduced infection in monkeys after PDK cell passage. A DENV-3 strain lost all monkey infectivity after PDK cell passage. Twelve vaccine candidates were selected for Phase 1 human trials through this selection process.

## INTRODUCTION

Prior to the advent of cell culture technology for use in vaccine manufacture, early attempts to grow dengue viruses (DENV) for the specific purpose of vaccine preparation were of limited success. The first recorded attempt was by Simmons and others[1] who used DENV-infected mosquito suspensions that were inactivated with formaldehyde. Later, Hotta,[2] Sabin and Schlesinger,[3] and Wisseman and others[4] grew DENV in mouse brain for both live and inactivated vaccine preparations. The first recorded use of cell cultures for the intended purpose of preparing a DENV vaccine was by Eckels and others who isolated a small plaque, temperature-sensitive DENV-2 virus for use as a live-attenuated vaccine.[5] Halstead[6] and Halstead and Marchette[7] were the first to use primary dog kidney (PDK) cell cultures to passage DENV strains and to demonstrate that passaged virus was modified, measured by *in vitro* as well as animal markers. Studies by Bhamarapravati and others using PDK-modified DENV-2 and DENV-4 demonstrated that these viruses are attenuated for flavivirus-naive humans.[8,9] DENV-4 (strain 341750) passaged through PDK cell cultures for modification and terminally passaged in fetal rhesus monkey lung (FRhL) diploid cells for seed and vaccine lot production was tested in a small group of human volunteers and shown to be safe and immunogenic.[10]

Based on this initial success, a plan was devised to prepare candidate live-attenuated vaccines from selected DENV strains representing all four serotypes, and to follow a similar production and testing scheme used for DENV-4: at incremental PDK passages for each strain; prepare vaccine lots consistent with current Good Manufacturing Practices in FRhL cell cultures; test the production seed or vaccine in rhesus monkeys to measure infectivity and immunogenicity; and select candidate vaccines for human Phase 1 testing that demonstrated diminished infectivity in monkeys when compared with animals that received parental DENV.

Rhesus and other monkey species can be infected with all four DENV serotypes.[11–16] Infection can be monitored by measuring viremia and/or antibodies in peripheral blood. Disease symptoms are not readily apparent and febrile responses are variable and dependent on the virulence of the DENV strain used. Viremia and antibody responses can be useful when comparing modified DENV with unmodified "parental" strains. Decreased duration and lower levels of viremia when a DENV vaccine candidate is administered to monkeys are indicative of reduced viral replication that may be correlated with attenuation for humans.[17]

## MATERIALS AND METHODS

**Virus strains.** DENV were passaged in PDK cell cultures following isolation from patients with dengue illness. A typical passage consisted of inoculation of PDK cells that were grown to monolayers from frozen banks. Following inoculation, virus was incubated for seven days and supernatant culture fluids were harvested, clarified, and used for the next passage. Table 1 lists the strains that were adapted and passaged in PDK cells. After passage in PDK cells, virus strains were further adapted to FRhL cells for seed and vaccine production. This consisted of 3–4 additional passages for final vaccine lot preparation. Parental virus strains, also listed in Table 1, were derived from low cell culture passages in cells that were permissive for DENV replication. PDK cell passage of DENV-4 (341750) and DENV-2 (S16803) were performed at the University of Hawaii at Manoa.[7]

**Vaccine production.** DENV vaccines for all four serotypes were prepared in FRhL cell culture using a similar procedure. FRhL cells banked and pre-tested (see Table 2 for testing results) were removed from liquid nitrogen storage and plated in 150-$cm^2$ flasks in Eagle's minimum essential medium (EMEM) cell culture medium supplemented with non-essential amino acids, fetal bovine serum (FBS), and antibiotics. After the cells reached confluency, medium was removed and flasks were inoculated with DENV production seed diluted for a multiplicity of infection of 0.01; the inoculum was allowed to adsorb at 32°C for one hour. Following adsorption and feeding with fresh EMEM medium, flasks were returned to 32°C for four days. On day 4 post-inoculation, the medium from all flasks was discarded and cell monolayers were washed three times with 100 mL of Hanks' balanced salt solution. After washing, flasks were fed with EMEM medium containing 0.25% human serum albumin (HSA) replacing FBS. After an additional two days of incubation at 32°C, supernatant culture fluids were removed from all flasks and pooled. After sampling for safety tests, the remaining culture fluids were pooled and clarified by filtration through a 0.45-μm, non-protein–binding membrane filter.

TABLE 1
Dengue (DENV) virus strains used for development of live-attenuated vaccines*

| Serotype | Original isolate | Vaccine strain: passage from human isolate | PDK passages selected for vaccine preparation | FRhL passages for seed and vaccine preparation | Parental strain: passage from human isolate |
|---|---|---|---|---|---|
| DENV-1 (West Pac 74; 45AZ5) | Human isolate, Nauru, 1974 | 20 × FRhL (with plaque selection and mutagenization with 5-azacytidine); vaccine prepared at p-20 caused dengue fever in 2 volunteers | 10, 20, 27 | 1: master seed<br>2: production seed<br>3: vaccine lot | 9 × FRhL |
| DENV-2 (S16803) | Human isolate, Thailand, 1974 | 1 × mosquito; 4 × PGMK cells | 10, 20, 30, 40, 50 | 1: master seed<br>2: production seed<br>3: vaccine lot | 4 × PGMK; 2 × C6/36 |
| DENV-3 (CH53489) | Human isolate, Thailand, 1973 | 4 × PGMK cells; 5 × C6/36 cells | 10, 20, 30 | 1: master seed<br>2: production seed<br>3: vaccine lot | 4 × PGMK |
| DENV-4 (341750) | Human isolate, Columbia, 1982 | 1 × mosquito | 6, 10, 15, 20 | 1: pre-master seed (PDK-20 only)<br>2: master seed<br>3: production seed<br>4: vaccine lot | 1 × mosquito; 5 × PGMK; 4 × FRhL |

* PDK = primary dog kidney; FRhL = fetal rhesus monkey lung; PGMK = primary green monkey kidney.

The filtered fluids were pooled and mixed with an equal volume of stabilizer containing 15% lactose and 5% HSA. The bulk, stabilized fluids were stored at -70C until freeze-dried. For final fill, bulk, stabilized fluids were thawed rapidly at 35°C and aliquoted in 3-mL volumes in serum vials. Trays of vials were frozen to a temperature of -40°C for two hours in a freeze-dryer. After overnight drying, the temperature was raised slowly to 20°C and held at this temperature for two hours before backfilling with sterile, dry nitrogen and capping. Final, dried product was stored at -30°C.

**Vaccine testing.** All cell banks used for virus preparations as well as seed and vaccine lots were tested for the presence of contaminating agents. Virus harvest fluids were used to inoculate bacterial and mycoplasma media, a variety of cell cultures, as well as mice, guinea pigs, rabbits, and embryonated eggs. Reverse transcriptase sequences were probed in a hybridization assay. Tests performed on the FRhL cell banks, seeds, and vaccine lots are listed in Table 2.

**Rhesus monkey inoculation.** Adult male and female rhesus monkeys (*Macaca rhesus*) weighing 6–15 kg were immunized with DEN vaccine strain or parent viruses by subcutaneous inoculation of 0.5 mL in the upper arm. Blood for virus isolation and antibody tests was drawn from the femoral vein prior to inoculation and every day for 14 days following inoculation. Blood was also drawn at 30 and 60 days following immunization. Serum was separated and frozen at -80C.

TABLE 2
Pre-clinical testing of RFhL cell banks and DEN LAV seeds and vaccine lots*

| Test | FRhL cell banks | Master seed | Production seed | Vaccine (bulk) | Vaccine (final container) |
|---|---|---|---|---|---|
| Sterility | X | X | X | X | X |
| Mycoplasma | X | | X | X | |
| Reverse transcriptase | X | | X | | |
| Hemadsorption | X | | X | X | |
| Cell culture safety (4 cell lines) | X | | | X | |
| Embryonated egg safety | X | | | | |
| Animal safety: adult mice | X | | | X | |
| Animal safety: suckling mice | X | | | X | |
| Animal safety: guinea pigs | X | | | X | |
| Animal safety: rabbits | X | | | | |
| Tumorgenicity | X | NA | NA | NA | NA |
| Karyology | X | NA | NA | NA | NA |
| Monkey safety: neurovirulence | NA | | X (DEN-4) | | |
| Monkey infectivity/immunogenicity | NA | | X | | |
| Monkey efficacy | NA | | | | X (DEN-2, DEN-4) |
| Infectivity (plaque assay) | NA | X | X | X | X |
| General safety | NA | | | X | X |
| Residual moisture | NA | | | | X |
| Reconstituted pH | NA | | | | X |
| Reconstituted osmolality | NA | | | | X |
| Endotoxin | NA | | | | X |
| Identity (DEN) | NA | X | X | | X |

* FRhL = fetal rhesus monkey lung; DEN = dengue; LAV = live-attenuated vaccine; X = test performed; NA = not applicable.

Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, NRC Publication, 1996 edition. All procedures were reviewed and approved by the Institute's Animal Care and Use Committee, and performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

**Virus isolation by amplification in C6/36 cells.** Virus isolation by C6/36 cell culture amplification has been described.[18] Briefly, thawed sera diluted 1:3 in cell culture medium were used to inoculate 25-$cm^2$ flasks containing monolayers of C6/36 mosquito cells. Following adsorption of virus, flasks were maintained at 28°C in EMEM maintenance medium. After seven days, medium was changed and flasks incubated for an additional seven days. On day 14 post-inoculation, supernatant culture fluids were decanted and frozen at -80C after mixing with an equal volume of heat-inactivated FBS. Frozen specimens were later assayed for infectious virus by plaque assay.

**Plaque assays.** Infectious virus was titrated from amplified viremia isolates or directly from monkey sera by plaque assay in LLC-Mk2 cells.[18] Assays in C6/36 cells were performed as described by Putnak and others.[19]

**Neutralization tests.** DEN neutralizing antibodies were measured in monkey sera using a plaque reduction neutralization test similar to that described by Russell and others.[20] Parent viruses listed in Table 1 were used to measure the plaque reduction 50% endpoint ($PRNT_{50}$) in serum specimens. Assays were performed using LLC-$MK_2$ cell cultures.

## RESULTS

**DENV modification in PDK cells and vaccine lot production.** DENV strains selected for vaccine development had a variety of passage histories prior to PDK cell passage. In the case of DENV-4 341750, there was just one mosquito passage before inoculation of PDK cell culture, while DEN-1 (West Pac 74 strain) had a history of 20 FRhL cell passages prior to PDK cell passage (Table 1). With the exception of DENV-3, all strains adapted to grow in PDK cells after a small number of passages. For DENV-3, additional efforts were required to increase viral input in early passages to adapt this strain to PDK cells. As a general case, after adaptation to PDK cells, DENV titers were found to be $10^4$–$10^5$ plaque-forming units (PFU)/mL. Attempts to increase titers were not successful and alternative cell substrates were sought for vaccine production. DBS-FRhL-2 cells were selected for this purpose for several reasons: 1) DENV replicate to titers of $10^5$–$10^7$ PFU/mL, which allows manufacture of DENV vaccines in these cells; 2) the cells have been used for the preparation of several DENV vaccines that have been tested in Phase 1 clinical trials without adverse reactions that could be related to the vaccine cell substrate; 3) FRhL cells are normal, rhesus monkey lung diploid cells that have no tumorigenic potential and are free of reverse transcriptase activity and contaminating agents; 4) since the cells are "normal" diploid cells, there is no regulatory or other requirement to purify the vaccines; and 5) FRhL cell banks can be established at cell generations usable for vaccine manufacture starting with available, low passage cells.

Adaptation of PDK-passaged viruses to FRhL cells was uniformly successful for all strains of DEN virus and was not dependent on PDK cell passage. Viral titers from harvests of FRhL passages 1–4 ranged from $10^5$–$10^6$ PFU/mL. By the third to fourth FRhL cell passage, vaccine lots of all of the DEN strain set viruses were prepared and tested as listed in Table 2. Data are also provided in Table 2 for the FRhL cell bank testing, as well as the master and production seed testing. Results of these tests, required to ensure safety and absence of contamination, were negative, or fell within allowable specifications. For the DEN-4 341750 PDK-20 production seed, monkey neurovirulence tests were performed. Results of this study can be found in Hoke and others.[10] The DEN-4 production seed, as well as the DEN-4 parent virus that was used for comparison, were not neuropathogenic. Monkeys inoculated by the intrathalamic and intraspinal routes did not demonstrate central nervous system disease over the three-week observation period and tissue sections from inoculated organs exhibited a minimal number and severity of neurovirulence lesions.

**Rhesus monkeys inoculated with PDK cell-passaged DENV.** The infectivity of DENV passaged in PDK cells and designated as strain sets was compared with parental, unmodified viruses for each serotype. Table 3 lists the results of these studies in which the degree of infectivity for monkeys was measured by the number of days of viremia in sequentially drawn serum specimens over 14 days following inoculation. Parental virus inoculation of monkeys resulted in 6.8, 5, 3, and 4.7 mean days of viremia in groups of 3–4 monkeys inoculated with DENV-1, DENV-2, DENV-3, and DENV-4, respectively. For the DENV-2 parent, additional data has substantiated that infection with measurable viremia is very reproducible over time using similar monkeys and isolation techniques. Unfortunately, only partial data exists on viremia titers in monkey sera. Most of the data that exist come from experience with the DENV-2 parent virus where monkey viremic blood was titrated in mosquito cell culture. Peak viremia titers at 4–8 days post-inoculation resulted in titers reaching $10^5$ PFU/mL of serum.[18]

For each strain set, PDK passage resulted in modified DENV as shown by reduced capacity of the viruses to infect monkeys. For several of the strain sets we failed to detect viremia at the highest PDK cell passage. Inoculation of monkeys with DENV-1 at PDK cell passage 27 resulted in 0 days of viremia in four monkeys (0 isolations of a total of 56 daily specimens tested). A similar result was found for DENV-3 PDK-20 and PDK-30. Indeed, DENV-3 PDK-30 appeared to have lost infectivity for monkeys, i.e., there was neither viremia nor seroconversion in the monkeys inoculated with $10^6$ PFU of virus. The DENV-2 strain required the greatest number of PDK passages to attain modification of monkey infectivity. With this virus, at least 40 passages in PDK cell culture were required for reduced viremia. To contrast this experience, the DENV-4 strain 341750 only required six passages in PDK cells for a modified monkey infection. For another DENV-1 strain, 1009, even after 50 PDK passages there was no evidence of modified monkey infectivity when compared with parental virus (data not shown).

TABLE 3
DENV strain sets adapted to PDK cells and used for inoculation of rhesus monkeys*

| DENV strain | Viruses | Inoc: PFU/0.5 mL | Monkeys viremic/total (mean days of viremia) | Monkeys seroconverted/total (GMT $PRNT_{50}$ at 1–2 months post-inoculation) |
|---|---|---|---|---|
| DENV-1, 45AZ5 | PDK-0 (parent) | $3.3 \times 10^4$ | 4/4 (6.8) | 4/4 (760) |
| | PDK-10 (prod seed) | $7.0 \times 10^4$ | 4/4 (4.75) | 4/4 (1,030) |
| | PDK-20 (prod seed) | $1.7 \times 10^4$ | 4/4 (4.5) | 4/5 (640) |
| | PDK-27 (prod seed) | $1.8 \times 10^4$ | 0/4 (0) | 4/4 (50) |
| DENV-2, S16803 | PDK-0 (parent) | $5.0 \times 10^6$ | 4/4 (5) | 4/4 (600) |
| | PDK-10 (prod seed) | $3.8 \times 10^5$ | 4/4 (4.75) | 5/5 (570) |
| | PDK-20 (prod seed) | $2.2 \times 10^5$ | 4/4 (6.5) | 4/4 (920) |
| | PDK-30 (prod seed†) | $4.4 \times 10^5$ | 2/3 (3.3) | 4/4 (640) |
| | PDK-30 (prod seed†) | $2.1 \times 10^5$ | 3/3 (6.0) | 3/3 (640) |
| | PDK-40 (prod seed) | $1.0 \times 10^4$ | 2/4 (1) | 3/4 (90) |
| | PDK-50 (prod seed†) | $2.6 \times 10^6$ | 2/4 (1) | 4/4 (310) |
| | PDK-50 (prod seed†) | $5.9 \times 10^5$ | 3/4 (3.25) | 4/4 (280) |
| | PDK-50 (vaccine) | $1 \times 10^6$ | ND | 4/4 (270) |
| DENV-3, CH53489 | PDK-0 (parent) | $8.0 \times 10^3$ | 3/3 (3) | 3/3 (660) |
| | PDK-10 (prod seed) | $2.5 \times 10^6$‡ | 2/3 (1.3) | 3/3 (150) |
| | PDK-20 (prod seed) | $1.0 \times 10^6$‡ | 0/3 | 3/3 (130) |
| | PDK-30 (prod seed) | $9.3 \times 10^5$‡ | 0/3 | 0/3 (<10) |
| DENV-4, 341750 | PDK-0 (parent) | $1.0 \times 10^3$ | 3/3 (4.7) | 3/3 (420) |
| | PDK-6 (prod seed) | $1.7 \times 10^5$ | 1/4 (0.5) | 4/4 (250) |
| | PDK-10 (prod seed) | $2.9 \times 10^5$ | 1/4 (1.3) | 2/4 (90) |
| | PDK-15 (prod seed) | $5.5 \times 10^4$ | 1/4 (0.25) | 2/4 (40) |
| | PDK-20 (prod seed) | $5.5 \times 10^4$ | 1/4 (0.25) | 2/4 (70) |
| | PDK-20 (vaccine) | $1.2 \times 10^5$ | 1/3 (0.3) | 3/3 (50) |

* DENV = dengue virus; PDK = primary dog kidney; Inoc. = inoculum; PFU = plaque-forming units; GMT = geometric mean titer; $PRNT_{50}$ = 50% plaque reduction neutralization test; prod = production; ND = not determined.
† Two separate monkey experimental groups.
‡ Plaque assay performed in C6/36 cells.

## DISCUSSION

Early attempts to select DENV vaccine candidate viruses on the basis of laboratory markers thought to be associated with human attenuation failed. Selection of small plaque, temperature-sensitive variants from viruses grown in monkey cell cultures did not always predict attenuation for humans.[21,22] Stability of these selected populations was also not predictable.[23] A plaque-purified DENV-1 vaccine candidate, when administered to humans, rapidly changed phenotype with virus isolated from serum that had mixed plaque size phenotypes.[22] Selected viral populations based on plaque purification or other methods may set a genetic limitation for suitable, permissive cell receptors in subjects receiving this virus. To illustrate this, Halstead and Marchette[7] refer to the study by Eckels and others[23] using plaque-purified DENV-4 virus in a small number of individuals. Two were infected and experienced mild dengue fever while three others were not infected. Plaque-purified virus would decrease the chance for successful immunization, while heterogeneous viral populations that have not been purified would stand a better chance at infection and immunization of an individual. The same restriction may occur with infectious clone-generated virus that is derived from a consensus sequence.

Although laboratory markers of plaque size and temperature sensitivity have been used by others to monitor modification of DENV passaged through PDK cells, administration of the vaccine candidate to volunteers remains the final and sole test of viral attenuation. Monkey infectivity of PDK cell-passaged strains, as addressed in this report and others, appears to have some merit for predicting human reactogenicity and infectivity.[17] All of the DEN strains that exhibited monkey modification of infectivity were also, in varying degrees, modified and attenuated for humans. For all of the DEN strain sets that were PDK cell-adapted, there is a definite trend of increased modification and decreased infectivity with increased PDK cell passage.

Results of monkey testing also enabled the elimination of eight vaccine candidates from consideration for human testing: DENV-1 PDK-10, 20, 30, 40, 50; DENV-2 PDK-10, DENV-2 PDK-20; and DENV-3 PDK-30. The DENV-1 and DENV-2 candidates showed no evidence of modification of monkey infectivity compared with the DEN-2 parent virus, while the DEN-3 candidate lost all monkey infectivity.

Genetic sequences of the PDK cell-passaged DENV will eventually provide knowledge about mutations that are associated with PDK cell adaptation and human attenuation. In the near future, complete sequences will be known and correlation can be made with documented human clinical experience. These genome changes may be useful to design more immunogenic and less reactogenic vaccine candidate viruses through site-directed mutagenesis of infectious clones.

In conclusion, PDK cell passage appears to be an effective method to modify and attenuate DENV isolates. This is an unnatural host for DENV that probably places selection pressure on virus best suited for PDK cell replication, but not necessarily for replication in target cells in monkeys and humans.

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