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Identification of mutations in a candidate dengue 4 vaccine strain 341750 PDK20 and construction of a full-length cDNA clone of the PDK20 vaccine candidate☆

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ABSTRACT

Dengue 4 virus strain 341750 serially passaged 20 times in primary dog kidney (PDK) cells was shown to have reduced infectivity for rhesus monkeys but was immunogenic and protected the monkeys from challenge with low passage parent dengue 4 virus. The dengue 4 PDK20 virus was also shown to be attenuated for human volunteers. We compared the genomic nucleotide sequences of low passage parent and PDK20 attenuated vaccine strains and identified 11 nucleotide (nt) substitutions in the PDK20 genome. Five mutations caused amino acid changes in viral proteins E (N366N/S), NS1 (E146Q), NS4B (S/L112L and A240V), and NS5 (F/L790L). Silent mutations occurred in genes encoding NS1 (nt 2609), NS3 (nt 6113, 6230 and 6239) and NS5 (nt 8081 and 8588). A full-length cDNA clone of the dengue 4 strain 341750 PDK20 was constructed and RNA transcripts of the clone were infectious in monkey kidney (LLC-MK_2) and *Aedes albopictus* (C6/36) cells. The sequence analysis and availability of an infectious clone provide molecular tools to investigate the basis for the attenuation of dengue 4 virus.

1. Introduction

Dengue viruses belong to the *flavivirus* genus in the *Flaviviridae* family, and are endemic to tropical and subtropical areas of the world that are inhabited by the *Aedes* mosquito vector placing an estimated 2.5 billion people at risk [1]. The four distinct but closely related virus serotypes (dengue 1, -2, -3 and -4) cause a spectrum of human illness ranging from a mild flu-like syndrome with rash called dengue fever to severe and sometimes fatal dengue hemorrhagic fever/dengue shock syndrome, which is more common among infants and young children [2]. A primary infection with one serotype appears to confer life-long immunity against re-infection with the same serotype, but does not produce long-lasting cross-protective immunity against other serotypes. Dengue hemorrhagic fever occurs most frequently in patients experiencing a secondary dengue infection, and one mechanism proposed to explain this phenomenon is antibody dependent enhancement of infection, in which antibodies developed against the first infecting serotype virus enhance a later infection with a different serotype [3]. Other factors proposed to contribute to the disease process include infecting strain, virus burden and cross-reactive T lymphocytes [4–6]. Because of the association of dengue hemorrhagic fever with secondary dengue infections, safe and effective vaccines that provide simultaneous protection against all four dengue virus serotypes are sought. A greater understanding of the molecular genetics of dengue virulence is also a high priority.

The *flavivirus* genome consists of single-stranded positive sense RNA approximately 11 kb long that is capped at the 5′ end and lacks a 3′ polyadenylated tail. The genome encodes a single long open reading frame flanked by 5′ and 3′ non-coding regions of variable size depending on the flavivirus. Three structural proteins, capsid (C), premembrane (prM), and envelope (E), and seven nonstructural (NS) proteins are co- and post-translationally cleaved from the polyprotein precursor by viral and cellular proteases, and occur in the polyprotein in the order C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 [7,8].

Live-attenuated viruses are widely used for the prevention of human viral diseases. Attenuation of virulent wild viruses has gen-

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erally been accomplished by serial passage of virus in cells from non-natural hosts. To develop a live-attenuated vaccine, low passage dengue 4 virus strain 341750 was serially passaged in primary dog kidney (PDK) cells and tested in monkeys [9]. By the 15th PDK passage, the virus was less infectious for monkeys compared to the low passage parent virus but remained immunogenic. The 30th PDK passage virus was non-infectious for monkeys. A vaccine lot was prepared from the 20th PDK passage by amplification in primary fetal rhesus lung cells to increase the titer, and was shown to protect vaccinated rhesus monkeys from challenge with the homologous parent dengue 4 strain and a heterologous dengue 4 strain H241 [9]. The dengue 4 PDK20 vaccine was shown to produce mild symptoms in human volunteers, and five of eight (63%) volunteers developed anti-dengue neutralizing antibody responses [10]. The PDK20 candidate was therefore selected for further clinical trials [11]. The aim of the present study was to identify the nucleotide and amino acid changes that occurred in the dengue 4 virus during progressive attenuation and also to construct a full-length infectious cDNA clone of dengue 4 vaccine strain PDK20.

2. Materials and methods

2.1. Viruses

Dengue 4 virus strain 341750 was isolated in 1982 from a patient in Colombia, South America by one passage in mosquitoes [12]. Virus was passaged 5 times in primary green monkey cells and 4 times in fetal rhesus lung (FRhL) cells to become the low passage parent strain and was passaged 6 and 20 times in PDK cells to become PDK6 and PDK20 attenuated vaccine strains. Each was then amplified three (PDK6) or four (PDK20) times in FRhL cells to make vaccine [12].

2.2. Cells

Vero and LLC-MK_2 cells were grown at 37 °C in a humidified incubator under 5% CO_2 in Eagle's minimal essential medium supplemented with 10% fetal bovine serum and 50 μg of gentamicin sulphate per ml of culture medium. *Aedes albopictus* C6/36 mosquito cells were grown at 28 °C in the same medium supplemented with 1× nonessential amino acids, 0.1 mM sodium pyruvate and 25 mM HEPES, pH 7.5.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Dengue 4 strain 341750 low passage parent, PDK6 and PDK20 virus strains were provided in lyophilized form by Ken Eckels, Walter Reed Army Institute of Research. Virus was suspended in 1.0 ml sterile water, RNA was prepared using a QiaAmp viral RNA kit (Qiagen) and an aliquot of RNA was combined with 50 pmol 3′ end primer (Table 1) and denatured for 3 min at 72 °C and chilled on ice. For reverse transcription, the RNA–primer mixture was added to a 100 μl reaction mixture that contained 1× RT buffer, 0.5 mM each deoxynucleotide triphosphate (dNTP), 10 mM DTT and 40 U RNasin RNase inhibitor (Promega). This mixture was divided into halves; to one 50 U Superscript II reverse transcriptase was added and the other served as a negative control. Both reactions were incubated for 1–2 h at 40 °C and terminated after incubation for 5 min at 95 °C. To amplify DNA by the polymerase chain reaction (PCR), 1 μl cDNA was added to a 49 μl reaction mixture that contained 1× PCR buffer, 0.2 mM each dNTP, 25–50 pmol each PCR primer, and either 2.5 U of high-fidelity *Pfu* DNA polymerase (Stratagene) to produce three overlapping DNA fragments for assembling the infectious clone, or 2.5 U Expand DNA polymerase (Boehringer Mannheim) to produce six overlapping DNA fragments for sequence analysis. Primers are given in Table 1. The PCR mixture was subjected to denaturation at 94 °C for 5 min followed by 35 temperature cycles each being 94 °C for 10 s, 45 °C for 30 s and either 68 °C (Expand) or 72 °C (*Pfu*) for 6 min. Products were visualized by 0.8% agarose gel electrophoresis.

Table 1
Forward (F) and reverse (R) primers used to amplify the dengue 4 genome.

Primer	Gene	Sequence
1F[a]	5′NCR	5′-AGAACCTGTTGGATCAACAACACCAAT-3′
2235F[b]	E	5′-GGAAAGGCTGTGCACCAGGTTTTTGGA-3′
3040F	NS1	5′-AAAACCAGACCTGGCAGATAGAGAAAG-3′
5057F	NS3	5′-GGATGAGGACATTTTTCGAAAGAAAAG-3′
7331F	NS4B	5′-CATGCTACTAGTCTTGTGTGCTGGACA-3′
8568F	NS5	5′-CCAATGGTGACTCAGTTAGCCATGACA-3′
2357R	E	5′-TGAAGTGTTTGAGTTCGTGCCAATCCA-3′
3484R	NS2A	5′-TGTCCGGCCGTCACCTGTGATTTGACC-3′
3853R	NS2A	5′-CAGTGATATTCCATCAATGAGTTCCAT-3′
5339R	NS3	5′-TGATGACAAAAGTCTTGTTGTGAAGGGT-3′
7523R	NS4B	5′-CAGTCCAGCTCCCGCCAAGTAACTTCC-3′
8841R	NS5	5′-TCACAGTTCACTGGCTGATGTCCATCC-3′
9771R	NS5	5′-CTATCAGTTCATCCTGGTTTCTACATGG-3′
10649R	3′NCR	5′-AGTTGTTAGTCTGTGTGGACCGACAAG-3′
97R[c]	5′NCR	5′-CCAGAGATCTGCTCTCTATTCAAACAAAC-3′
10590F	3′NCR	5′-ACATCAATCCAGGCACAGAGCGCCGCA-3′

[a] Primer pairs 1F-3484R, 3040F-8841R, 7331F-10649R were used to amplify three overlapping fragments to construct the dengue 4 infectious clone. Primer 10649R was used to initiate reverse transcription of purified genomic RNA.
[b] Primers pairs 1F-2357R, 2235F-3853R, 3040F-5339R, 5057F-7523R, 7331F-9771R and 8568F-10649R were used to amplify overlapping DNA fragments to derive the genome sequence.
[c] Primer 97R was used to initiate reverse transcription of the RNA genome after its circularization and primer pair 10590F-97R amplified the fragment encoding the 5′–3′ junction.

2.4. DNA sequencing

Oligonucleotide primers were designed from a published sequence of dengue 4 strain 814669 (GenBank accession number M14931). To obtain 5′ and 3′ termini DNA for sequence analysis, the genome was circularized to form a junction which served as a template for RT-PCR [13]. First, the 5′ cap structure was removed by treating genomic RNA with 5 U tobacco acid pyrophosphatase in 1× buffer (Epicenter), 40 U RNAsin and 0.2 mM ATP in a 20 μl reaction volume for 1 h at 37 °C. Decapped RNA was extracted once with Aqua-Phenol (Ambion) and once with chloroform, after which RNA was precipitated with 0.3 M sodium acetate/ethanol. The RNA was resuspended in water and ligated by incubation at room temperature for 3 h in a 20 μl reaction mixture containing 5 U T4 RNA ligase and 1× ligase buffer (New England Biolabs), 10% DMSO, 1 mM ATP and 40 U RNAsin. Ligated RNA templates were reverse transcribed to cDNA using a 5′-end reverse primer and amplified by PCR using 3′-end forward and 5′-end reverse primers (Table 1).

Prior to starting all sequence reactions, unincorporated PCR primers were removed by incubating the PCR product with 2 μl Exozapit reagent (Boehringer Mannheim) for 15 min at 37 °C followed by heat-inactivation of the reaction mixture for 15 min at 80 °C. Sequence analyses of PCR products were performed on an ABI PRISM 3100 Genetic Analyzer using the ABI version 3.1 sequencing kit (Applied Biosystems). Each 20 μl sequence reaction contained 20 ng PCR product or 200 ng plasmid, 5–10 pmol primer and 7 μl sequence kit reagent. Sequence analysis was performed using Sequencher software (Gene Codes Corp).

2.5. Plaque assay

Virus titers were determined by quantitative plaque assay of virus on LLC-MK_2 cells in six-well plates. Serial dilutions of virus were adsorbed to cell monolayers for 1.5 h at room temperature after which the cells were overlaid with 3 ml of melted 1.0% SeaPlaque agarose (FMC Bioproducts) in Earle's balanced salt solution without phenol red and with 0.357% sodium bicarbonate, 10.0%