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Highly efficient production of a dengue pseudoinfectious virus

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ABSTRACT

Dengue is a major infectious disease that affects people living in tropical and subtropical regions around the world. The causative agents are dengue virus serotype 1, 2, 3, and 4 (DENV1, 2, 3, and 4). Developing a vaccine for dengue is a high priority for public health, but traditional methods have faced numerous obstacles due to the unique immunopathogenesis of dengue virus infection. Here, we report a novel dengue vaccine candidate based on dengue pseudoinfectious virus (PIV) produced by the incorporation of a dengue subgenomic replicon into viral particles in highly efficient packaging cells. The subgenomic replicon was constructed by deleting the capsid protein (C) gene from the dengue viral genome and optimizing the signal peptide sequence of pre-membrane protein (prM) to facilitate the formation of viral particles. Packaging cells were developed for inducible expression of a bi-protein Cpr, where the protein pr is the "pr" segment of viral protein prM that holds the protein C on the endoplasmic reticulum (ER). When the replicon was introduced into the packaging cells, protein C was released from the bi-protein Cpr by a replicon-encoded viral protease. Coordinate expression of viral structural proteins by the replicon and packaging cells led to the incorporation of the replicon into viral particle to produce PIVs. Animal tests showed that the dengue PIV vaccine was highly immunogenic and the immune response protected mice challenged with a hundred-fold LD_{50} inoculation of dengue virus. The method described here has the potential to be applied to vaccine development for other flaviviruses.

1. Introduction

Dengue virus (DENV) infection is the most widespread vector-borne human viral infection in the world, with an estimated incidence of up to 390 million cases annually [1]. The predominant disease caused by DENV is dengue fever, a self-limited febrile illness often accompanied by minor hemorrhagic manifestations [2]. Every year approximately 250,000–500,000 cases of DENV infection develop into a more serious form called dengue hemorrhagic fever/shock syndrome (DHF/DSS), which has fatality rates as high as 20% [3]. The four dengue serotypes (DENV1, 2, 3, and 4) co-circulate in affected areas and any one is able to cause the full spectrum of dengue disease, from mild fever to DHF/DSS. While DENV infection appears to provide life-long immunity against re-infection by the same serotype, it only induces limited cross-serotype protective immunity, and people exposed to secondary DENV infections often have residual cross-reactive antibodies and T-cells from the initial infection that can actually exacerbate the disease [4,5]. A high frequency of severe dengue disease has also been observed following primary infections of infants born to dengue-immune mothers when the infant's maternal antibodies are reduced to sub-neutralizing levels [6]. Due to antibody-dependent enhancement (ADE) of dengue infections, an effective dengue vaccine must induce and maintain high levels of virus-neutralizing antibodies to ensure long-term protection against all four serotypes. Since the 1920s, tremendous effort has been spent on DENV vaccine development using a diverse array of potential immunogens, including inactive (killed) virus, attenuated live virus, chimeric virus, recombinant poxvirus, recombinant subunits, and the DNA sequence encoding a putative antigen. However, these approaches have faced challenges in producing long-term protective immunity against all four DENV serotypes with acceptable safety, stability, and universality [7–9]. Therefore, there are numerous efforts underway to develop and test new candidate vaccines [10,11].

The use of pseudoinfectious viruses (PIV) is a new direction for the development of a dengue vaccine. PIV has been demonstrated to induce high immunogenicity in a safe manner [12]. Furthermore,

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the unique characteristics of PIV single-round infections minimize the effect of viral growth interference among serotypes, since a dominant serotype in a tetravalent dengue live viral vaccine is associated with its higher replication potential and predominance of viremia [13,14]. As a result, dengue PIV vaccines can potentially induce a balanced immune response to all four DENV serotypes. The major challenge for the application of PIV in dengue vaccine development is the production of high titers of dengue PIV by stable cell lines.

The goal of the present study was to create a highly efficient DENV PIV production system based on stably transfected cells. High efficiency of PIV formation was achieved by coordinated expression of viral structural proteins by DENV replicon and packaging cells to incorporate the replicon into viral particles to form PIVs. Animal test results show that DENV PIV vaccine is highly immunogenic and protects mice challenged with a hundred-fold LD50 inoculation of dengue virus. The implication of these findings for dengue and other flavivirus vaccines is discussed.

2. Materials and methods

2.1. Creation of DENV2 replicons

DENV2 replicon cDNA plasmids were derived from full-length cDNA clone plasmids of DENV2 NGC [15]. The construct is based on plasmid pRS424, which is a yeast-bacterium shuttle vector used for the assembly and mutagenesis of full-length infectious DENV cDNAs in yeast cells [16,17]. The "infectious" cDNA copy of DENV2 in the construct was used as a basis for creating the replicon in this study. The primers used to create a PCR fragment containing a deletion of the viral capsid protein (C) coding sequence with or without an optimized signal peptide of membrane precursor protein (prM) are shown in Fig. 1. The PCR products generated with primer pairs D2/dc(5′u) and D2/dc(3′A), D2/dc(5′u) and D2/dc(3′B), or D2/dc(5′u) and D2/dc(3′C) contained corresponding deletions as shown in Fig. 1. For construction of a replicon with an optimized signal peptide sequence for protein prM, the first PCR product was generated with primers D2/dc(5′u) and L4(3′) with D2/ΔC(A) replicon cDNA as a template. The second PCR product was generated with primers PQAQA and D2/1431R with DENV2 cDNA plasmid as the template. A fused PCR product was created with primers D2/dc(5′u) and D2/1431R and the first and second PCR products as templates. Mutations (Fig. 1) were introduced into the pRS424/D2 by homologous recombination between a PCR fragment bearing the mutations and a linearized plasmid DNA in yeast cells as described previously [18]. Positive clones were confirmed by DNA sequencing. The replicon RNAs were produced by *in vitro* transcription, and the replicon RNA was introduced into BHK21 cells or BHK21-based packaging cells by electroporation [15]. To verify the replication of the DENV replicon in normal cells, re-suspended cells were seeded onto 3 chamber slides (BD Falcon, Bedford, MA) and DENV antigen-positive cells were identified by immunofluorescence analysis [15] at 48 h, 96 h or 1 week post transfection. For the packaging cells, one tenth of the replicon-transfected cells were seeded onto chamber slides and rest were plated onto one well of a six-well plate. The cells in the six-well plate were examined daily for cytopathic effect (CPE) and the supernatant was collected for testing PIV production.

2.2. Development of packaging cell lines

The packaging cells were created to provide viral protein Cpr for replicon encapsidation. The plasmid pCpr was developed based on the commercial pLVX-Tight-Puro vector (Clontech) using standard methods. Briefly, the DENV2 Cpr cDNA was synthesized with codons optimized for high expression in mammalian cells. An internal ribosome entry site (IRES) DNA fragment and an enhanced green fluorescent protein (EGFP) DNA fragment were created by PCR using pLVX-tet-off advanced vector plasmid DNA and pEGFP/C1 as templates, respectively. The three DNA fragments (C-pr, IRES, and EGFP) were fused together by PCR to form the expression cassette Cpr-IRES-EGFP. The cassette was clone into a pLVX-Tight-Puro vector to yield plasmid pCpr as shown in Fig. 2. The plasmid pCpr clones were further confirmed by DNA sequencing.

Lentiviral particles containing pLVX-tet-off advanced vectors, pLVX-Tight-Puro-Luc Control Vectors or pCpr vectors were produced by using the Lentiphos HT Packaging System (Clontech) following the manufacturer's instructions. To create inducible expression cells, 5×10^5 BHK21 cells were seeded into six-well plates in fresh growth medium and incubated overnight at 37 °C in a humidified incubator under 5% CO_2. The following day the cells were transduced with pLVX-tet-off lentiviral particles. Two days following transduction, the antibiotic Geneticin (G418) (Sigma) was added at a concentration of 400 μg/ml for selection of G418-resistant cell clones. These clones were then analyzed for inducibility of expression by transduction with pLVX-Tight-Puro-Luc lentiviral vectors, and 24 h following transduction cells were grown in medium in the presence (1 μg/ml) or absence of doxycycline. Luciferase activity was measured after 48 h of induction using bioluminescence imaging with a Xenogren IVIS instrument (Caliper Life Sciences, Hopkinton, MA) based on the manufacturer's protocol. The BHK-Tet-Off cell clone displaying the highest fold of induction was used to establish a stable BHK cell line expressing the DENV2 structural gene cassette Cpr by transduction with pCpr lentiviral particles as described above. At 48 h following the second transduction, cells were subjected to limited dilution into two 96-well plates in fresh growth medium containing 400 μg/ml of G4185, 1 μg/ml of doxycycline, and 5 μg/ml of puromycin. When cell clones in the 96-well plate were visible, cell culture medium was replaced with doxycycline-free medium with 10% tetracyline-free FBS. After 48 h of induction, several high-expression clones were selected based on levels of green florescence under a microscope. To select the most efficient packaging cell line, the high-expression cell clones were electroporated with DENV2 replicon RNA and cultured without doxycycline to determine whether they were able to produce PIV. The titers of PIV (in infectious units [IU] per milliliter) present in harvested culture fluids (CFs) were determined by flow cytometry [19]. The expression of Cpr mRNA in the packaging cells was confirmed by sequencing after reverse transcription-PCR amplification of total RNA isolated from the cells.

2.3. DENV PIVs growth curve

The PIVs growth curve in packaging cell line BHK/Cpr was analyzed in doxycycline-free growth medium supplemented with 10% tetracycline-free FBS. Packaging cells were seeded at a concentration of 1×10^6 per 100-mm dish. After a 24 h incubation at 37 °C, cells were infected with DENV PIV at an MOI of 0.01 IU/cell, and then incubated in doxycycline-free growth medium. For comparison, BHK-21 cells were infected by wild-type parent DENV2 under the same conditions. Aliquots of the culture fluid were removed daily and stored at −80 °C. The titer of DENV PIVs in each sample was determined as described above.

2.4. Animal tests

For animal studies, DENV PIVs were purified and concentrated by ultracentrifugation as described previously [20]. DENV PIV safety as a vaccine candidate was investigated by intracranial (1.c.) inoculation of virus or PIV into 3- to 4-day-old AG129 mice (parent mice were kindly provided by Herbert W. Virgin IV, Washington University School of Medicine). Groups of ten 2- to 3-day-old

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