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# Heterologous prime-boost strategy in non-human primates combining the infective dengue virus and a recombinant protein in a formulation suitable for human use

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ABSTRACT

*Objective:* The aim of the present work was to test the concept of the heterologous prime-boost strategy combining an infective dengue virus with a recombinant chimeric protein carrying domain III of the envelope protein.
*Methods:* Two studies in monkeys, combining recombinant protein PD5 (domain III of the envelope protein from dengue-2 virus, fused to the protein carrier P64k) and the infective dengue virus in the same immunization schedules were carried out. Humoral and cell-mediated immunity were evaluated.
*Results:* In the first study, monkeys received four doses of the protein PD5 and were subsequently infected with one dose of dengue virus. Antibody response measured after virus inoculation was significantly higher compared to that in non-primed monkeys and comparable to that elicited after two doses of infective virus. In a second study, monkeys were infected with one dose of the virus and subsequently boosted with one dose of the recombinant protein, reaching high levels of neutralizing antibodies, which were still detectable 14 months after the last immunization. In addition, the cellular immune response was also recalled.
*Conclusions:* The results obtained in the present work support the approach of heterologous prime-boosting, in either order prime or boost, combining the chimeric protein PD5 (formulated in alum-CPS-A) and an infective dengue virus. The latter could potentially be replaced by an attenuated vaccine candidate.


## 1. Introduction

Dengue epidemics caused by the four dengue virus serotypes DEN-1 to DEN-4 are a major problem in most tropical and subtropical regions. According to estimates, as many as 100 million dengue infections occur every year worldwide.[1,2] The incidence of dengue fever and dengue hemorrhagic fever is rising and there is currently no vaccine available to prevent the disease.

Live attenuated viruses are the most advanced vaccine candidates against the infection.[3–5] Such vaccines have been immunogenic in human clinical trials mostly due to their replicative capacity. However, and due to this same feature, reactogenicity in variable degrees has been reported in different studies.[5–7] In addition, two or three doses have been required to induce a balanced tetravalent immune response.[4,5,7]

As an alternative, to avoid the aforementioned disadvantages, the subunit vaccine strategy has been one of the goals to which the efforts of different groups have been directed.[8–18] Specifically, our group has developed different constructs based on the protein P64k of *Neisseria meningitidis* and domain III of the envelope (E) protein from dengue virus. The safety and the carrier capacity of the protein P64k have previously been proved in humans with successful results.[19,20] The recombinant fusion molecules of serotypes 1–4 expressed in *Escherichia coli* have been purified[21] and successfully evaluated in mice.[17,22,23] In addition, DEN-1 and DEN-2 constructs, adjuvanted in Freund's adjuvant, were found to be immunogenic and conferred protection in monkeys against challenge with the homologous infective virus.[14,16] Moreover, when formulations suitable for human use were evaluated, only the combination with the serogroup A capsular polysaccharide (CPS-A) from *N. meningitidis* adsorbed in aluminum hydroxide, exhibited partial protection in monkeys.[18]

While safety is one of the crucial attributes of vaccine candidates based on recombinant proteins, the induction of a

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proper functional immune response with formulations suitable for human use has yet to be proved.

Based on the advantages and disadvantages of the subunit and the attenuated virus candidates, the present work had testing of the concept of the prime-boost strategy, combining the two types of candidates, as its main goal. We selected a formulation containing the recombinant chimeric protein PD5 (domain III, amino acids 286–426 of the E protein from DEN-2 virus, fused to the C-terminus of the carrier protein P64k), suitable for human use, which was able to partially protect monkeys against viral challenge.[18] In turn, a single dose of infective DEN-2 virus was considered as a model of an attenuated viral strain. Some of the results of this work are an extension of a previous study on the immunological evaluation of two formulations of PD5 in monkeys.[18] The kinetics of antibody response in vaccinated monkeys after challenge was measured and compared with that elicited in monkeys receiving one or two doses of the infective virus. Furthermore, a second study was conducted in monkeys to determine the boosting capacity of one formulation of PD5 after one dose of infective virus.

## 2. Materials and methods

### 2.1. Animals

Healthy adult green monkeys (*Chlorocebus* (formerly *Cercopithecus*) *aethiops sabaeus*) were obtained from CENPALAB (Havana, Cuba). All animals were screened for previous exposure to dengue virus by ELISA and plaque reduction neutralization test (PRNT), and to P64k protein by ELISA. Animals were considered naive with respect to both antigens when antigen-specific antibodies were undetectable by ELISA (titer <1:100) and PRNT (titer <1:10). Monkeys were maintained in accordance with the Cuban guidelines for the care and use of laboratory animals.

### 2.2. Viruses

Preparation from suckling mouse brain infected with dengue-2 virus (strain New Guinea C) was used as sucrose–acetone antigen[24] for immunoassay tests. A similar preparation obtained from brain of non-inoculated mice was used as negative control. For the PRNT, cell culture supernatant harvested from Vero cells infected with DEN-2 virus, strain SB8553 (kindly provided by Dr M.J. Cardosa, University Sarawak, Malaysia) was used. A viral stock for the challenge study was prepared with the DEN-2 strain SB8553 in Vero cells using fresh supplemented RPMI medium (5% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 100 U of penicillin–streptomycin). The supernatant was harvested 144 h later, then aliquoted, stored at −70 °C and titrated by plaque formation on BHK-21 cells.

### 2.3. Analysis of the antibody response

The anti-DEN IgG antibodies stimulated by immunization were monitored by an amplified sandwich ELISA system as previously described.[18] Absorbance was read at 492 nm in a microplate reader (SensIdent Scan; Merck, Germany). Titers were defined as the dilution of serum giving twice the absorbance value of the negative control serum.

The functionality of the antibodies was measured by neutralization of DEN-2 infectivity by a PRNT on BHK-21 cell culture as previously described.[25]

### 2.4. Virus detection by ELISA

Flat-bottomed 96-well plates (Costar, USA) were coated with anti-DEN human IgG (5 μg/ml) for 2 h at 37 °C. Plates were then blocked with 2% bovine serum albumin (BSA) and incubated for 1 h at 37 °C. After two washes with phosphate-buffered saline/Tween-20 (PBS-T), culture supernatant was added in triplicate to each well and plates were incubated for 2 h at 37 °C. Three additional washes were performed and then polyclonal hyperimmune mouse ascitic fluid, diluted 1:2000 in PBS-T with 1% normal mouse brain antigen was added to the plates. After 1 h of incubation at 37 °C, plates were washed again three times and then anti-mouse IgG–peroxidase conjugate (Sigma, USA) was added. Plates were incubated for 1 h at 37 °C, washed three times, and 0.04% substrate solution (*O*-phenylenediamine in buffer 2% $Na_2HPO_4$, 1% citric acid, and 30% $H_2O_2$, pH 5.0) was added. The reaction was stopped 30 min later by the addition of 12.5% $H_2SO_4$ and the absorbance was read at 492 nm in a microplate reader (SensIdent Scan). A value of absorbance of two-fold the absorbance of the supernatant from an uninfected culture was considered as positive.

### 2.5. Virus detection by flow cytometry

Vero cells were removed from the flask using a cell scraper and distributed into triplicate tubes at $10^6$ cells per tube. The cells were fixed using 3% formaldehyde in phosphate-buffered saline (PBS) for 15 min, and 0.1% Triton X-100 was employed to permeabilize fixed cells. After washing with PBS, the cells were incubated with the monoclonal antibody 4G2 for 30 min at 4 °C. Cells were then washed and incubated with fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG for 30 min at 4 °C. After incubation cells were washed again, suspended in PBS and then analyzed using a FACScan flow cytometer PASIII (Partec GmbH, Münster, Germany). Non-infected cells were used as negative control and cells infected with DEN-2 virus (strain SB8553) at 0.001 multiplicity of infection were used as positive control. A value of three-fold the value of non-infected cells was considered as positive.

### 2.6. Measurement of cell-mediated immunity

Blood was obtained by venepuncture at days 0 and 60 after the dose with infective virus DEN-2 and at days 0 and 30 after the dose with PD5–CPS-A formulation. The peripheral blood mononuclear cells (PBMC) were isolated over Ficoll-Paque™ Plus (Amersham Biosciences AB, Uppsala, Sweden) density gradient centrifugation. Cells were washed twice with PBS–2% FBS (PAA Laboratories, Canada) and resuspended at $2 \times 10^6$ cells/ml in RPMI-1640 medium (Sigma Aldrich, Ayrshire, UK) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco), 2 mM glutamine (Glutamax, Gibco), $5 \times 10^{-5}$ M 2-mercaptoethanol (Sigma St. Louis, MO) and 5% FBS. Finally $2 \times 10^5$ cells/well were cultured in 96-well round bottom plates with the antigens (3 $\log_{10}$ plaque-forming units (PFU) of DEN-2 antigen or mock preparation). Concanavalin A (ConA; Sigma St. Louis, MO) was used as a positive control. In all the experiments three wells were plated for each antigen. After four days of culture, culture supernatants were collected and stored a −20 °C.

The culture supernatants previously stimulated with each antigen were analyzed in duplicate for interferon-γ (INF-γ) concentration by ELISA using monoclonal antibody pairs (Mabtech INF-γ; Mabtech, Sweden). The ELISA protocol recommended by the manufacturer was used with slight modifications. The lower limit of detection of cytokine in this assay was 4 pg/ml.

### 2.7. Study design 1

The recombinant protein preparation, animal immunization and viral challenge have been described in detail elsewhere.[18]