

Proof of concept in non-human primates of the heterologous prime-boost strategy combining dengue-2 virus and recombinant proteins including domain III of the viral envelope protein

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ABSTRACT

Live attenuated viruses are the most advanced candidates against a dengue infection. They have been demonstrated to be immunogenic in preclinical and clinical studies. However, due to their replicative capacity, they require several doses to achieve the balanced immune response against the four serotypes. The use of a suitable combination using nonreplicative immunogens, without viral interference, can help to induce such a balanced response. This work dealt with the proof of concept of the heterologous prime-boost strategy combining in the same schedule in non-human primates of a live virus and the recombinant proteins containing the domain III of the viral envelope. These combinations may result in condensed immunization schedules for humans, thus reducing the number of doses with attenuated virus and the dose time spacing. In both studies, the humoral and cellular immune responses after the boost dose with each recombinant protein were evaluated. In the second study, additionally, the possibility of shortening the schedule was assessed, an advantage related with this prime-boost strategy. The boost effect was demonstrated by the neutralizing antibodies induced after recombinant protein immunizations. Additionally, it was confirmed that these neutralizing antibodies were long lasting, also the animals were able to mount a specific cellular immune response after the boost. This study won the Annual Award of the Academy of Sciences of Cuba in 2012.

Keywords: dengue, vaccine candidate, prime-boost, neutralizing antibodies, non-human primates

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RESUMEN

Prueba de concepto en primates no humanos de estrategia de inmunización contra dengue con virus del serotipo 2 y re-estimulación con proteínas recombinantes que portan el dominio III de la proteína de la envoltura viral. Los virus vivos atenuados son los candidatos vacunales más avanzados contra la infección por dengue. Sus propiedades inmunogénicas se han demostrado en estudios preclínicos y clínicos. Sin embargo, por su cualidad replicativa es necesario administrarlos en varias dosis para alcanzar el equilibrio entre las respuestas inmunológicas contra los cuatro serotipos del virus dengue. Las combinaciones adecuadas de inmunógenos no replicativos sin interferencia viral pudiera ayudar a alcanzar esta respuesta. En este trabajo se describe la prueba de concepto de la estrategia de inducción-potenciación de la respuesta inmune en un mismo esquema en primates no humanos tras la inmunización: primero con un virus vivo (dengue serotipo 2) y después con proteínas recombinantes que contenían el dominio III de la envoltura viral. Estas combinaciones pueden generar esquemas más cortos para la inmunización de seres humanos, con la reducción del número de dosis del virus atenuado y el espaciamiento entre ellas. En ambos estudios se evaluaron las respuestas inmune humoral y celular después de la dosis de refuerzo con cada proteína recombinante. En el segundo estudio se exploró, además, el acortamiento del esquema, una ventaja de este tipo de estrategias vacunales. El efecto potenciador se demostró mediante la inducción de anticuerpos neutralizantes después de la dosis de refuerzo con proteínas recombinantes. Adicionalmente se confirmó que estos anticuerpos neutralizantes tenían larga duración, y en los animales fueron hubo respuesta inmune celular específica después del refuerzo. Este estudio mereció el Premio Anual de la Academia de Ciencias de Cuba, en el año 2012.

Palabras clave: dengue, candidato vacunal, inducción-potenciación, anticuerpos neutralizantes, primates no humanos

Introduction

Dengue fever and dengue hemorrhagic fever are viral diseases transmitted to humans by the bite of infected mosquitoes belonging to the genus *Aedes*, subgenus *Stegomyia* [1]. There are four immunologically and antigenically distinct dengue virus (DENV) sero-

types, referred to as DENV-1 to 4. Dengue is endemic throughout large parts of the Americas and Asia, and is increasingly reported in Africa [2]. Currently, dengue disease is one of the most important arthropod borne diseases, with an incidence of 50-100 million

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infections and estimates of 500 000 cases in the severe forms of the disease [3].

Although urgently needed, a licensed vaccine for dengue is not yet available, and the priorities are vector control and eradication. Vaccine development strategies are focused on candidates able to protect against the four serotypes, to avoid the contribution of single serotype immune responses to the immunopathogenesis by secondary infections [4]. In this sense, live attenuated viruses are the most advanced vaccine candidates against the infection. Such vaccines have been immunogenic in human clinical trials mostly due to their replicative capacity [5-7]. However, and due to this same feature, reactogenicity in variable degrees has been reported in different studies [8]. In addition, two or three doses have been required to induce a balanced tetravalent immune response. To solve this problem, the administration of several spaced doses is required for current candidates based on this technology, including immunization programs that can take up to a year to be completed [9].

One of the attractive alternatives to solve the previous disadvantages is the use of a heterologous prime-boost strategy based on a combination of nonreplicative immunogens and a candidate attenuated virus in the same schedule. These combinations may result in condensed immunization schedules for humans, thus reducing the number of doses with attenuated virus and the time spacing. On the other hand, the use of a suitable combination using nonreplicative immunogens, without the viral interference phenomenon, can help to induce a balanced response against the four serotypes.

Results

This work is the proof of concept of the heterologous prime-boost strategy against DENV, aimed at combining in the same immunization schedule in non-human primates two types of candidates. We selected two formulations of recombinant proteins containing domain III of the envelope protein (E) from DENV-2 and a single dose of infective DENV-2, as a model of an attenuated viral strain. One of them included the PD5 recombinant protein (domain III, amino acids 286-426 of the E protein from DENV-2, fused to the C-terminus of the carrier protein P64k). As adjuvants were added the serogroup A capsular polysaccharide (CPS-A) from *Neisseria meningitidis* and aluminum hydroxide [10]. The second formulation is a fusion protein composed of domain III of the E protein and the capsid protein (DIIC) from DENV-2, as an

aggregate antigen by incubation with oligodeoxynucleotides [11]. In both studies in non-human primates was inoculated as prime the infective DENV-2, as a model of an attenuated viral candidate, and the animals were further immunized with one booster dose of the respective recombinant protein formulation. In the first study, using the PD5-CPS-A candidate, the schedule was 0 and 5 months for each immunization, as frequently used with attenuated vaccine candidates in humans. In contrast, in the second study, the time was condensed to only three months between the doses, applying as boost the DIIC protein in its aggregated form. A shorter immunization schedule is the significant advantage associated to this kind of strategy.

In both experiments, the humoral and cellular immune responses, induced after the infection with the DENV-2 and each recombinant protein formulation, were evaluated. The functionality of the antibodies of the different immunizations was evaluated by the *in vitro* plaque reduction neutralization test. An increase of antibody titers was evident after inoculation with preparations of recombinant proteins (Table).

As a result, in both studies were demonstrated the boost effects in terms of neutralizing antibodies induced firstly by the infection with the infective DENV-2 and after boost with each based protein formulations. It was also possible to confirm that these antibodies persisted at high levels for six months after the booster (Table).

Additionally, the ability of the immunized monkeys to develop a cell-mediated immune response after receiving each protein heterologous doses were also determined. The secretion of IFN- γ was measured in culture supernatants of PBMCs extracted from the blood of the immunized monkeys, upon its stimulation with protein antigens. Cytokine secretion was measured 6 months after each booster dose, either PD5-CPS-A or DIIC-C (Figure).

As shown in the Figure, upon stimulation with the viral antigen in both studies, high levels of IFN- γ were detected in the supernatants of cultured PBMCs from animals receiving booster (PD5-CPS-A or DIIC).

In summary, the results shown here demonstrated that heterologous prime-boost immunization supports the development of a safe immunization schedule, including only one dose of the infective virus and a recombinant subunit vaccine, mounting a long-lasting immune response against dengue virus. The model DENV-2 virus could be replaced latter by an attenuated vaccine candidate.

Table. Neutralizing antibody titers induced after vaccination of green monkeys against dengue, as measured by plaque reduction neutralization test*

Study	Immunogen		Days										
	Prime	Boost	0	30	60	90	120	150	180	210	240	270	330
1	DENV-2	PD5-CPS-A	< 10	35.8	93.7	nd	nd	70.1	789.9	493.2	447.3	442.2	349.9
2	DENV-2	DIII-C	< 10	33.6	109.4	160.9	879.4	880.7	852.7	584.9	nd	341.1	nd

* Results are given as geometric means of antibody titers and are representative of three independent experiments. The neutralizing antibody titer is the highest serum dilution that resulted in a 50 % reduction in the number of plaques produced by dengue virus serotype 2 (DENV-2). In both studies, the prime dose consisted of infection with DENV-2. In study 1, the boost (second dose) comprised the DENV-2 envelope protein PD5 fragment fused to the C-terminus of the carrier protein P64k and the capsular polysaccharide, both of *Neisseria meningitidis* (PD5-CPS-A). In study 2, the fusion protein composed of domain III of the E protein and the capsid protein DENV-2 (DIIC) was used as booster. nd: not determined. Prime immunizations were administered on day 0 in both groups. Bold numbers indicate the time of booster administrations.

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Relevance of the study

The heterologous prime-boost strategy assayed, combining attenuated candidates and based-protein formulations, could be advantageous replacing the attenuated virus vaccine dose by safer formulations. It also can reduce the immunization schedule.

Significantly, in this work was demonstrated for the first time the combination of one dose of the virus and subsequent administration of these recombinant proteins able to boost the humoral immune response in terms of the antiviral and neutralizing antibodies. The increase in antibody titers confirmed the proper folding of the domain III region in the context of the PD5 or DIIC proteins. This approach confirms that is feasible to obtain the domain III region as recombinant protein in *Escherichia coli*, with the proper formation of disulfide bonds.

Finally and according to the results, both recombinant protein formulations, PD5-CPS-A and the DIIC fusion protein, both formulated in alum as adjuvant, would be potential vaccine candidates to be tested in prime-boost schedules combined with infective DENV.

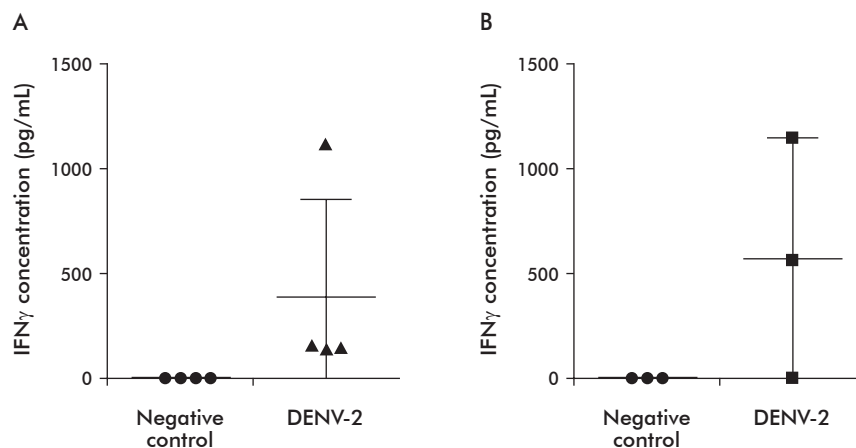


Figure. IFN- γ concentrations determined in culture supernatants of stimulated peripheral blood mononuclear cells (PBMCs). Culture supernatants from PBMCs of immunized animals were stimulated with the homologous virus and tested by ELISA. Data presented as means \pm standard deviations. A) PBMCs from animals primed with dengue virus serotype 2 (DENV-2) and boosted with PD5-CPS-A. B) PBMCs from animals primed with DENV-2 and boosted with DIIC. These experiments were done in triplicates, with the same results. Negative control: PBMCs from the same animals but stimulated with supernatants of uninfected cultures.