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REPORT

Heterologous prime-boost strategy based on the combination of a tetravalent vaccine of recombinant proteins and live-attenuated virus as a promising vaccine strategy against dengue virus

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ABSTRACT

Dengue, is one of the most important emerging disease around the world. Currently, only one vaccine has been approved and licensed against DENV, Dengvaxia®; however, its use is limited due to evidences of the risk of cause severe dengue under particular circumstances. For that, the development of new vaccine and/or immunization strategies continue be a priority to the scientific community. Vaccine candidates based on recombinant proteins are considered alternative approaches to solve the main disadvantages of attenuated vaccines, but these usually requiring adjuvants by their lower immunogenicity. This work describes the results in prime-boost schedules combining two different vaccine candidates: recombinant proteins and live attenuated virus. Firstly, we evaluated the capacity of Tetra DIIIC vaccine candidate to boost the memory immune response previously generated in DENV-immune monkeys. As results, the administration of Tetra DIIIC eight months later of the infection recalls the DENV specific memory B- and T-cell response. A second study, we tested in monkeys the combination of Tetra DIIIC with the LATV (TV005). This study demonstrates that animals Tetra DIIIC primed and later boosted with TV005 develop neutralizing antibodies against the four DENV serotypes, and the immune response induced reduces significantly LATV viremia. All these results highlight the possibility to combine the Tetra DIIIC vaccine candidate with LATV in a prime-boost strategy, and support these strategies as alternative approaches solving the troubles associated with each individual antigen. This work received the Annual Award of the Cuban Academy of Sciences for the year 2019.

Keywords: Dengue virus, vaccine, heterologous prime-boost, recombinant protein, monkeys, live-attenuated virus

RESUMEN

Esquemas de inmunización complementaria basados en la combinación de una formulación tetravalente de proteínas recombinantes y virus vivos atenuados como estrategia vacunal prometedora contra el virus dengue. El dengue es una de las enfermedades emergentes más importantes, con solo una vacuna aprobada y licenciada para su control (Dengvaxia®), su uso limitado por el riesgo de generar dengue severo. Por tales razones, el desarrollo de nuevas vacunas y las estrategias de inmunización es prioritario. Los candidatos vacunales de proteínas recombinantes aportan enfoques alternativos que podrían resolver las desventajas asociadas a las vacunas atenuadas, las que requieren de adyuvantes por su baja inmunogenicidad. En este trabajo se presentan los resultados de los esquemas de sensibilización-refuerzo que combinan dos tipos de candidatos: las proteínas recombinantes y los virus vivos atenuados tetravalentes (LATV). Primeramente, se evaluó la capacidad de la vacuna Tetra DIIIC para reforzar la respuesta inmune de memoria previamente generada contra a virus dengue (VDEN) en monos inmunizados. Su administración ocho meses tras la infección reactivó la respuesta de células B y T de memoria específicas contra VDEN. En un segundo estudio se evaluó la combinación Tetra DIIIC-candidato vacunal LATV TV005 en monos. Se demostró que los animales sensibilizados con el Tetra DIIIC y luego reactivados con el TV005 desarrollan anticuerpos neutralizantes contra los cuatro serotipos del VDEN, y la respuesta inmune inducida reduce significativamente la viremia de los inmunizados con LATV. Estos resultados resaltan la posibilidad de unir a ambos tipos de candidatos vacunales en una estrategia de sensibilización-refuerzo, como alternativa para resolver los problemas asociados a cada antígeno individual. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba para el año 2019.

Palabras clave: Virus dengue, vaccine, heterologous prime-boost, recombinant protein, monkeys, live-attenuated virus

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*I*ntroduction

Dengue is among the most significant human diseases due to its morbidity and mortality. It is estimated that over 2 billion people live in risk areas and every year there should be around 390 million dengue infections, 96 of them showing some severe disease symptom [1].

The etiological agent is a viral compound of four different viral serotypes of Dengue virus (DENV-1 to 4). It is transmitted to humans by the bite of mosquitoes from the *Stegomya* genera [2]. The infection by any of the four viral serotypes could be asymptomatic, develop dengue fever or cause the most severe stage of the disease known as severe Dengue [3].

Currently, only one live-vaccine has been licensed, Dengvaxia®, produced by Sanofi Pasteur, and registered in 20 countries [4,5]. The vaccine was attenuated by substituting the genes coding for viral proteins prM and E of 17D Yellow Fever virus by the equivalent DENV proteins. The resulting CYD chimeric viruses have demonstrated to be immunogenic in humans, further inducing a neutralizing antibody response against DENV-1 to 4 [6]. However, the cellular immune response detected was weak due to the lack of the viral capsid proteins and non-structural (NS) proteins 3 and 5. These proteins have been identified as the main targets of the cellular immune response against DENV [7].

Recent efficacy studies of Dengvaxia® demonstrated higher hospitalization rates at endemic areas among vaccinated people than for the unvaccinated [8]. Hence, the World Health Organization (WHO) has recommended the use of the vaccine only in people older than nine and in areas with over 80% of disease prevalence [9]. Therefore, the development of an effective vaccine or immunization strategies remain as a goal to achieve.

Just two other vaccine candidates against DENV are in Phase II clinical trials, both live-attenuated viruses [10, 11]. They have proven to be safe and immunogenic in humans but they are reactogenic, latently capable to revert to virulent versions of the virus and generally require two or three shots in a-year-long schedules. Besides, their protective efficacy still remains to be proven. Other vaccine candidates are based on recombinant proteins, which do not show the disadvantages of live-attenuated vaccines, but are less immunogenic and require the addition of adjuvants, or their combination with other candidates in complementary vaccine schedules for an efficacious immune response.

In this work, we present the results of the DENV vaccine approach using complementary immunization schedules which combine a formulation of a tetravalent vaccine formulation with live-attenuated viruses as promising vaccine strategy against Dengue. This work received the Annual Award of the Cuban Academy of Sciences for the year 2019.

*M*ain results

Two non-human primates' studies were done to demonstrate that it is possible to combine complementary vaccine schedules of two different types of vaccine candidates [12, 13]. They were the Tetra DIIIC candidate based on recombinant proteins [14] and the TV005 live-attenuated candidate [15].

In the first study, three groups of non-human primates were immunized with an infective strains either DENV-1 to 4 strains (10^3 or 10^4 p.f.u.), respectively. Virus replication was previously verified through viremia detection by viral isolation in Vero cells. Hence, viremia was detected in all the inoculated animals with mean peak duration of 4.7 days, from three to four days, respectively. Eight months after infection, the animals received a complementary immunization with the Cuban tetravalent vaccine candidate Tetra DIIIC (the four recombinant domain III-capsid proteins (DIIIC-1–DIIIC-4) are produced in *Escherichia coli*). Animals were bled before and after the immunization to evaluate the humoral and cellular immune response induced. The antibody response was determined by an indirect ELISA against the four viral serotypes. Antiviral antibodies were detectable from day 0 (Tetra DIIIC administration day) in all the animals against the four viral serotypes, regardless the inoculated serotype. More importantly, 30 days after the complementary Tetra DIIIC dose the antiviral antibody response was shown significantly increased (Figure 1) [12].

Additionally, the functionality of the antibody response was evaluated in an *in vitro* (PRNT) neutralization assay in Vero cells specific against each viral serotype. Similarly to antibodies, there was a rise in the neutralizing antibody response following the administration of the Tetra DIIIC formulation, regardless the viral serotype used for infection (Figure 2) [12]. These results suggested that the DIII region included in the DIIIC recombinant proteins is capable of calling the B cell memory response cells specific against that protein region generated during the viral infection. Therefore, this supports the potential use

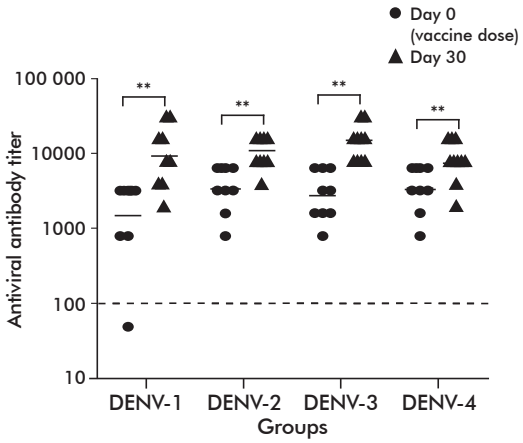

Figure 1. Antiviral antibody response against tetravalent formulation of DIIIC proteins of Dengue virus (DENV). Nine rhesus monkeys were inoculated with DENV-1, DENV-3 or DENV-4 and eight months later they were immunized with a tetravalent formulation of DIIIC proteins (Tetra DIIIC). Anti-DENV IgG antibodies were measured by ELISA the day of vaccine dose (day 0) and 30 days after the dose. Antiviral antibody titers, including all DENV-immune monkeys (n = 9). In all cases, the data represent the geometric mean titers of two independent experiments. The statistical analysis was performed using the Wilcoxon signed rank test (**: $p < 0.01$, two-side p-value). The dashed line indicates the cutoff value (two times the lowest dilution of sera). Reproduced from: Gil *et al*. Clin Transl Immunol. 2017;6:e148.

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of this viral fragment in vaccine candidates based on recombinant proteins. Moreover, they suggested that the Tetra DIIIC formulation could be administered in DENV-positive individuals, to increase the maturation and affinity of the humoral immune response. This could also help on avoiding the typical possible secondary or tertiary complications seen during the infection with a heterologous serotype.

The immunization capacity of the Tetra DIIIC formulation to recall the cellular response previously generated by the viral infection was evaluated by measuring the IFNγ levels in culture supernatants of animals' peripheral blood mononuclear cells (PBMCs). It was done following their stimulation *in vitro* with viral antigens, in samples taken on the administration day and 30 days after. Similarly to the antibody response, the cellular immune response increased significantly after the administration of the Tetra DIIIC formulation (Figure 3) [12]. This is remarkable since the role played by IFNγ against DENV has been widely demonstrated and its secretion correlates with protection and the subclinical stage of the disease. Therefore, it was shown that the Tetra DIIIC formulation is able to reinforce the cell-mediated immunity generated by the previous viral infection, further supporting the use of the capsid protein. This protein has been identified as the main target for the cytotoxic T CD4+ response, higher producers of IFNγ, induced during the natural infection. In fact, the capsid protein is part of the DIIIC recombinant proteins. Therefore, these cells can be efficiently recruited and expanded after the administration of the vaccine formulation.

In a second study in non-human primates [13], a combination was evaluated containing the Tetra DIIIC candidate and the tetravalent TV005 formulation of live-attenuated viruses by molecular procedures. This last was jointly developed by the National Institutes of Health (NIH) in US [15], and then licensed to the Vietnamese biotech company VABIOTECH.

Rhesus macaques were divided in four groups, two of them administered with the combination of one or two doses of the Tetra DIIIC vaccine candidate, respectively, both groups further receiving a dose of the live-attenuated virus TV005 formulation. A third group was immunized solely with the TV005 formulation and a fourth with a Placebo formulation (which contains all components of Tetra DIIIC minus the recombinant proteins). Then, animals were evaluated for the humoral and neutralizing antibody immune responses induced by each schedule, by procedures as described above. The PRNT neutralizing antibody levels detected against each viral serotype are shown in Figure 2 in reference [13].

On day 90 (Figure 2 in reference [13]), a month after the last immunization with the Tetra DIIIC formulation, only animals administered with two doses of that candidate showed neutralizing antibodies *in vitro* against the four viral serotypes. Otherwise, following the administration of the TV005 formulation, the neutralizing antibody response was boosted in all groups immunized with a single TetraDIIIC dose, except for the one receiving DENV-3. Animals immunized with two doses of Tetra DIIIC showed increased neutralizing antibody titers only for serotype 4. No quantitative differences were found in the levels of neutralizing

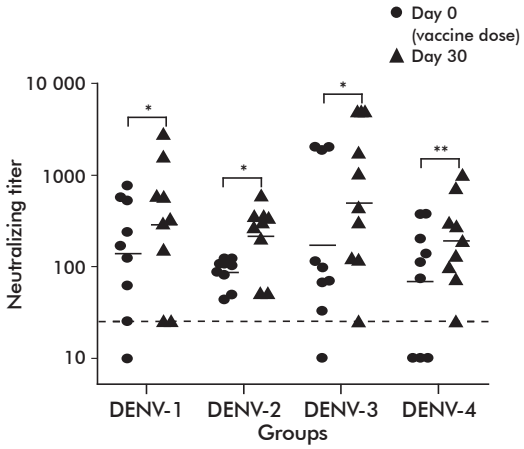

Figure 2. Neutralizing antibody response against Dengue virus (DENV) by tetravalent formulation of DIIIC proteins. Nine rhesus monkeys were inoculated with DENV-1, DENV-3 or DENV-4, and eight months later they were immunized with a tetravalent formulation of DIIIC proteins (Tetra DIIIC). Neutralizing antibodies were measured by a plaque-reduction neutralization test using Vero cells and the viral strains: DENV-1 Jamaica, DENV-2 SB8553, DENV-3 Nicaragua and DENV-4 Dominica, the day of vaccine dose (day 0) and 30 days after the dose. Neutralizing antibody response, including all DENV-immune monkeys (n = 9). In all cases, the data represent the geometric mean titers of two independent experiments. The statistical analysis was performed using the Wilcoxon signed rank test (* $p < 0.05$; ** $p < 0.01$, two-side p-value). The dashed line indicates the cutoff value (two times the lowest dilution of sera). Reproduced from: Gil *et al*. Clin Transl Immunol. 2017;6:e148.

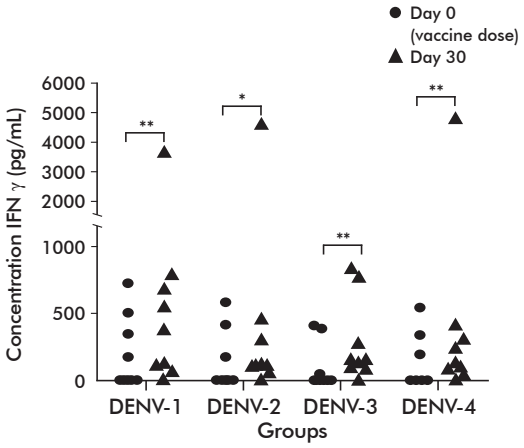

Figure 3. Cellular immune response against Dengue virus (DENV) by tetravalent formulation of DIIIC proteins. Nine rhesus monkeys were inoculated with DENV-1, DENV-3 or DENV-4 and eight months later they were immunized with a tetravalent formulation of DIIIC proteins (Tetra DIIIC). The day of vaccine dose (Day 0) and 30 days after the dose, PBMCs from the animals were stimulated *in vitro* with each DENV and IFNγ concentrations in culture supernatants were determined by ELISA. Cytokine concentration data in all DENV-immune monkeys represent the median of two independent experiments (n = 9). The statistical analysis was performed using the Wilcoxon signed rank test (*:$p < 0.05$; ** $p < 0.01$, two-side p-value). Reproduced from: Gil *et al*. Clin Transl Immunol. 2017;6:e148.

antibodies between the groups receiving either Tetra DIIIC/TV005 or TV005. This was probably caused by the disparate nature of both immunogens, which could differentiate the antibody response against each one, due to the differential exposure of different sets of epitopes and the distinctive affinity maturation of these antibodies.

Moreover, the replication capacity of the live-attenuated viral strains was evaluated after the administration of the TV005 vaccine candidate, ten days after that inoculation and by viral titration in Vero cells. Hence, animals receiving just the dose of TV005 candidate showed a viremia of short duration (1-2 days) for DENV-1, 2 and 4 serotypes, with titers in the range $10^{1.6}$ -$10^{2.1}$ p.f.u./mL. Viremia levels were similar to those reported in immunized humans for this vaccine candidate [15]. The absence of viremia for DENV-3 was probably not detected for DENV-3 due to the prevalence of immunity in animals immunized against this serotype. Animals vaccinated with Tetra DIIIC, either one or two doses, interestingly showed a significant reduction in the replication levels of viral strains included in the TV005 formulation (Table 2 in reference [13]).

Lastly, the protective capacity of the Tetra DIIIC/TV005 combination was assessed, in a complementary immunization schedule 77 days after the last immunization. For this experiment, both the group receiving 2×DIIIC+TV005 and Placebo were split into four individual subgroups, each challenged with a different viral serotype (3 or 4 animals each subgroup). In case of groups DIIIC+TV005 and TV005 (n = 4 each) exclusively with DENV-2 were challenged in order to test the protective capacity of TV005 against this serotype. In all cases, serum samples were collected daily during a 10 day post-challenge period, quantifying viremia on Vero cells. As shown in figure 5, all the animals in the placebo group showed detectable viremia following the viral challenge, with a mean duration of 4.5 for DENV-1, 3.75 for DENV-2, 2.75 for DENV-3 and 3.67 days for DENV-4, respectively (Figure 3 in reference [13]).

All the animals administered with Tetra DIIIC, either one or two doses, and then with TV005 were protected against the infective viral challenge (Figure 3 in reference [13]), despite the undetectable vaccine viremia (Table 2 in reference [13]). These results suggest that there is a low replication rate of the TV005 vaccine candidate, in spite of the Tetra DIIIC immunization reduces the replication capacity of the virus strains contained on it. That seems to be enough to reinforce the previous vaccine-induced immunity of the Tetra DIIIC candidate, further protecting against the viral challenge.

Hence, the reduced replication of the viral live-attenuated TV005 vaccine candidate caused by Tetra DIIIC has important clinical implications. This recombinant formulation could provide a potential solution to reactogenicity problems of vaccine candidates based on live-attenuated viruses, as TV005. Moreover, the Tetra DIIIC formulation reduces the replication capacity of the live-attenuated virus candidate without interfering with the immunogenicity of this last as shown here.

Therefore, the Tetra DIIIC/TV005 combination is an attractive alternative for the development and the clinical evaluation of the vaccine candidates complementary administered. This combination could improve the safety profile of the TV005 vaccine and to boost the immune response with no severe adverse events as those reported for DENV infections.

Relevance of the study

These results are the proof of concept of the complementary combination of recombinant protein vaccine candidates, as Tetra DIIIC, with live-attenuated viral vaccine candidates, as TV005, or even live DENV virus strains.

The main relevance resides on the first demonstration ever that the tetravalent DIIIC candidate is able to enhance the humoral and cellular immune responses generated by infective viruses in non-human primates. Besides, the Tetra DIIIC formulation is also able to induce a functional humoral immune response that reduces the viremia caused by a live-attenuated vaccine candidate virus, without affecting the protective capacity of the combination of both types of candidates against the viral challenge.

Therefore, this supports the use of the tetravalent DIIIC candidate in complementary immunization schedules, to boost the immunity after a natural viral infection in humans or to limit the reactogenicity of live-attenuated viruses. This is quite relevant in the current vaccine scenario to face the global expansion of this pathogen and its vector with no effective antiviral strategy available yet.

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