REPORT

A new tetravalent Dengue vaccine formulation based on four chimeric envelope domain III-capsid proteins induces a functional immune response in mice and non-human primates

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ABSTRACT

It is crucial in dengue a tetravalent vaccine candidate effective against the four dengue virus (DENV) serotypes, considering the lack of long lasting cross-protective immunity among infections with either serotype, and the fact that secondary heterotypic infections can led to severe forms of the disease. In this work, it was described for the first time the generation and characterization of the protein chimeric variants domain III-capsid (DIIIC) of DENV serotypes 1, 3 and 4, based on previous evidences of the ability of DIIIC-2 protein aggregated with oligodeoxynucleotide (ODN) 39M to induce a protective immunity in mice and monkeys. The recombinant proteins DIIIC-1, 3 and 4 aggregated with the ODN 39M, independently or combined with DIIIC-2 in a tetravalent formulation were evaluated in mice, demonstrating the induction of a humoral and cellular immune response able to protect against the four viral serotypes. The tetravalent DIIIC formulation was further evaluated in monkeys administered through different routes, demonstrating the induction of functional humoral and cell immune responses, results that opens the doors to clinical trials to this new tetravalent formulation against dengue. This research granted the 2015 Award of the Cuban National Academy of Sciences.

Keywords: dengue virus, tetravalent vaccine, domain III, capsid, DIIIC, protective response

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RESUMEN

Nueva formulación vacunal tetravalente contra el dengue basada en la combinación de cuatro proteínas quiméricas dominio III de la envoltura-cápsida que induce una respuesta inmune funcional en ratones y primates no humanos. La ausencia de inmunidad cruzada protectora de larga duración entre los serotipos del virus dengue, unido al hecho de que una inmunidad heterotípica predispone a las formas más severas de la enfermedad, hace necesario el desarrollo de un candidato vacunal tetravalente como única alternativa posible en el desarrollo de una vacuna efectiva contra el virus dengue (DENV). En este trabajo se describió, por primera vez, la obtención de las variantes quiméricas Dominio III-cápsida (DIIIC) de los serotipos DENV 1, 3 y 4 según resultados previos que muestran que la proteína recombinante DIIIC del serotipo 2, agregada con el oligonucleótido (ODN) 39M es capaz de inducir una respuesta inmune protectora en ratones y monos. Las proteínas DIIIC-1, 3 y 4, agregadas con el ODN 39M y adyuvadas en alúmina de manera independiente o combinadas con DIIIC-2 en una formulación tetravalente, se evaluaron en ratones BALB/c e indujeron respuestas inmune humoral y celular protectoras contra los cuatro serotipo virales. Adicionalmente, se demostró la capacidad de la formulación tetravalente DIIIC para inducir respuestas inmune humoral y celular funcionales en primates no humanos, al ser administrada por diferentes vías, lo que abre las puertas a estudios clínicos de esta nueva formulación como candidato vacunal contra el dengue. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba para el año 2015.

Palabras clave: dengue, vacuna tetravalente, dominio III, cápsida, DIIIC, respuesta protectora

*I*ntroduction

Currently, there is no effective vaccine available against Dengue virus (DENV) infection and it is associated mosquito-borne dengue disease which affects about 390 million people every year [1]. Particularly, some Dengue epidemics have occurred in Cuba since 1977, with the most severe in 1981 and 1997. But Dengue disease remains as a serious health problem in spite of the numerous and sustained efforts to eradicate both, the vector and the disease.

An effective vaccine against Dengue implies the generation of immunity against the four DENV serotypes. This is necessary due to the lack of long-lasting and cross-protective immune response among serotypes. Besides, the heterotypic

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immunity makes individuals susceptible to a most severe dengue subsequent infection by a heterologous serotype. In fact, the most advanced candidate is composed of chimeric attenuated strains showing preliminary efficacy results lower than 60 % [2-4].

Therefore, in this work, a tetravalent vaccine candidate was generated for the first time based on the fusion of the DENV envelope domain III (DIII) and capsid protein (DIIIC) of all the four DENV serotypes, formulated as an aggregate with oligodeoxynucleotides (ODN). DIII of the viral envelope protein mediates the virus attachment to its cellular receptor and bears neutralizing epitopes. On the other hand, the capsid protein was able to induce cell-mediated protective immunity in mice and monkeys [5, 6] and it was demonstrated that in the presence of ODNs it forms particulate aggregates with immunogenic potential. Previous evidences on the induction of a functional and protective immune response in mice and monkeys by administering the DIIIC protein from DENV2 formulated as an aggregate [7], supported the extension of this DIIIC design to the rest of DENV serotypes.

DIIIC chimeric variants for DENV serotypes 1, 3 and 4 were obtained by recombinant procedures, purified and subjected to antigenic characterization. Furthermore, their immunogenicity and protective efficacy was studied in mice, once formulated together with the previous DIIIC-2 chimeric protein and aggregated with ODN 39M, as a tetravalent vaccine candidate (Tetra-DIIIC). The immunogenicity of the tetra-DIIIC vaccine candidate formulation was evaluated in non-human primates using different administration routes. This research granted the 2015 Award of the Cuban National Academy of Sciences.

Main results

Recombinant chimeric molecules comprising DIIIC proteins against DENV serotypes 1, 3 and 4 were generated by cloning their respective fusion genes into the pET28a plasmid expression vector. These genetic constructions called ACDC-1, 3, 4 plasmids contain a 6-His-tag at the N-terminal sites of the fusion proteins. The recombinant proteins were expressed under the control of the T7 promoter in the BL21(DE3) strain of *Escherichia coli* using IPTG as inductor. SDS-PAGE revealed the presence of a band of approximately 28 kDa that was also immunoindentified with anti-DENV hyperimmune murine ascitic fluids (HMAF) [8].

The three chimeric proteins were purified and antigenically characterized by measuring their reactivity against an anti-dengue murine and human panel of sera. As shown in figure 1, DIIIC-1 and DIIIC-3 were highly recognized by both panel of sera assayed, indicating a proper folding of domain III region in the context of the homologous capsid protein. In the specific case of DIIIC-4, the reactivity was lower than that measured for the rest of the proteins. Similar results have been obtained with several tetravalent recombinant candidates against DENV where the serotype 4 was less immunogenic [9-11].

Subsequently, the characterization of DIIIC proteins by transmission electron microscopy revealed

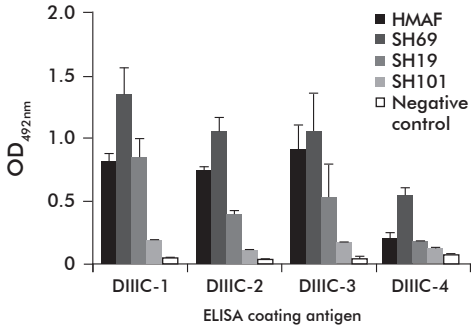

Figure 1. Reactivity of DIIIC recombinant proteins against anti-dengue virus (DENV) mouse and human sera, measured in ELISA. Mouse sera: polyclonal hyperimmune ascitic fluid (HMAF) produced against the homologous DENV serotype. Human sera: SH69, SH 19, SH 101 (sera collected in the Havana 2000 DENV epidemic). Negative control: negative purification. Results are the means and standard error of the mean of three independent experiments.

the presence of aggregates once they were incubated with ODN39M, an oligonucleotide of proven immunostimulatory capacity [7]. The microscopy showed particle of 50-55 nm depending of ODN addition. The particle nature of these aggregates could favor their internalization by dendritic cells and their transport to lymph nodes where the induction of an effective immune response takes place.

Subsequently, the chimeric DIIIC proteins were evaluated, either separate or all combined with DIIIC2 in a tetravalent formulation, in BALB/c mice. For this purpose, the proteins were incubated with ODN 39M at the protein: ODN ratio promoting the 50 % of protein precipitation and the presence of soluble and insoluble species of protein-ODN. Afterward the DIIIC preparation was adjuvanted with alum and inoculated in mice by the intraperitoneal route on days 0, 15 and 45. Fifteen days after the third dose, the humoral immune response against each recombinant protein was determined. All animals seroconverted and the administration of the tetravalent formulation generated high anti-DIIIC IgG titers against the four recombinant proteins. The results shows a lack of antigenic competition in the tetravalent formulation with similar titers of humoral responses against each chimeric protein to that obtained with the respective monovalent formulations (Figure 2).

Furthermore, the functionality of the humoral immune response was determined by means of its neutralizing activity (Table 1), showing a similar response related to the percentage of responders for the tetravalent and monovalent formulations against DENV serotypes 1, 2 and 3. No neutralizing antibodies were detected against the DENV4 protein for any of the formulations assayed.

One month after the administration of the last dose, the cell-mediated immune response was determined by measuring the mice splenocytes secretion of interferon gamma (IFNγ) in response to the stimulation with the recombinant DIIIC proteins [12]. When DIIIC-1, 2 and 3 proteins were used for stimulation,

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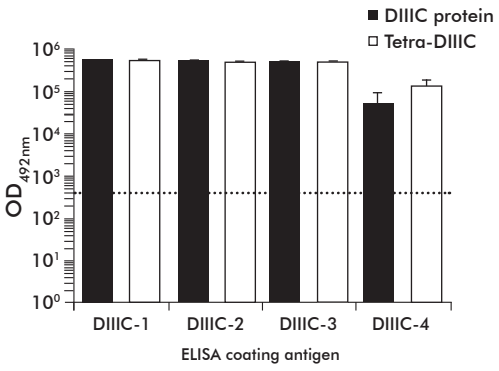

Figure 2. Antibody response to recombinant dengue virus proteins. Mice were immunized with the monovalent and tetravalent formulation of DIIIC proteins. Fifteen days after the third dose, animals were bled and the anti-DIIIC IgG antibody response was measured by ELISA. Data represent the geometric mean with 95 % confidence interval (n = 7). The dotted line indicates the cutoff value determined as two times the average values of the placebo animals. No statistical difference were detected between monovalent and tetravalent immunization groups using Kruskal-Wallis and Dunn's multiple comparison tests. The results are representative of two independent experiments.

Table 1. Neutralizing antibodies responses generated in mice after immunization with DIIIC monovalent and tetravalent formulations*

Groups	Neutralizing antibodies titers GMT			
	DENV1	DENV2	DENV3	DENV4
DIIIC monovalent	1238a	4200a	2924a	6b
Tetra DIIIC	399a	2009a	3114a	6b
VD	298a	1737a	648b	550a

* Neutralizing antibody titers are the highest serum dilution that resulted in a 50% reduction in the number of plaques produced by the viruses. The titers were detected 30 days after the last immunization using the cell line LLCMK2 and the dengue virus (DENV) strains (DENV1: West Pacific 74; DENV2: S16803; DENV3: CH53489; DENV4: TVP 360). Responders were considered when titers ≥1:10. The statistical analyses were performed inside each serotype, where different letters represent statistical differences; using the Tukey multiple comparison for serotypes 1 and 3 and Dunn multiple comparison for serotypes 2 and 4. GMT: geometric mean titer. All the aniamls were responders, except for 10 % and 25 % of the animals immunized with DIIIC monovalent or Tetra-DIIIC, respectively, against DENV4.

all the animals immunized with the monovalent and tetravalent formulations showed a positive response. In the case of serotype 4 the secretion of IFNγ was lower, with only 5 out of 7 and 6 out of 7 animals showing a positive response in the monovalent and tetravalent groups, respectively. The low immunogenicity of DENV-4 has been extensively described in mouse experiments with other dengue vaccine candidates such as the recombinant DENV-4 DIII fused to maltose binding protein [8], and a vaccine candidate based on dengue E protein, expressed in *Drosophila* S2 cells [13]. Taken together, we postulate that DIII of DENV-4 is not an immunodominant region in BALB/c mice.

The tetravalent formulation was able to induce a protective response against the four DENV serotypes in the DENV murine encephalitis model, in spite of the lower response detected against DENV4. Animals receiving the tetravalent formulation reduced the viral loads in the brain, with statistical differences with respect to the placebo group ($P < 0.05$), and statistically similar to the positive control groups ($P > 0.05$), indicating a solid protection [12].

Subsequently, the tetravalent DIIIC formulation adjuvanted in alum was evaluated in non-human primates, using different immunization routes (subcutaneous, intradermal or intramuscular route). The schedule comprised three immunizations at two-month intervals, in groups of three animals. After immunization, the animals developed antiviral antibodies with neutralizing activity against the four DENV serotypes (Figure 3 and Table 2). The formulation administered by the intradermal route trended to generate the lowest antibody response in terms of percentage of responders, maybe due to the 10-fold lower vaccine dose used by this route in comparison with the other immunizations.

Additionally, the frequency of IFNγ-secreting cells and the cytotoxic capacity of peripheral blood mononuclear cells (PBMCs) were measured by ELISPOT and LDH release in immunized monkeys, following the *in vitro* stimulation with each recombinant chimeric protein [12].

Results showed that the immunization with Tetra -DIIIC induces a memory immune response of

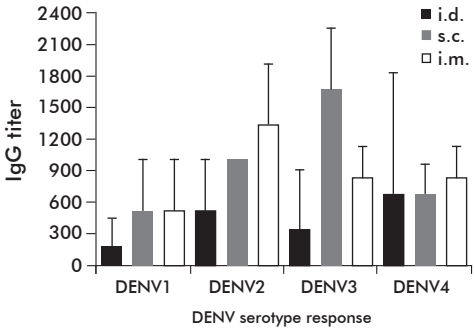

Figure 3. Anti-viral antibody response induced by the tetravalent formulation of DIIIC proteins in monkeys. Animals were immunized with the tetravalent formulation by the intradermal route (i.d.), subcutaneous route (s.c.) or intramuscular route (i.m.) on days 0, 60 and 120 (n = 3). The IgG anti-DENV response was determined by ELISA, 1 month after the third dose. Data represent mean ± standard error of the mean.

Table 2. Neutralizing humoral response induced by the administration of Tetra-DIIIC formulation in non-human primates using different administration routes, 30 days after the last immunization*

Immunization route	DENV serotype and IgG response							
	DENV1	R(%)	DENV2	R(%)	DENV3	R(%)	DENV4	R(%)
s.c.	43.2	66.7	290.6	100	198.0	100	55.8	100
i.d.	23.4	33.3	175.1	100	83.2	33.3	389.0	66.7
i.m.	29.1	100	392.0	100	58.4	100	83.2	100

* Titers were detected by plaque reduction neutralization test (PRNT) in Vero cells with dengue virus (DENV) strains (DENV1: Jamaica; DENV2: SB8553; DENV3: Nicaragua; DENV4: Dominica). The end-point neutralization titer was calculated as the highest serum dilution tested that reduced the number of plaques by at least 50 %, and responders were considered when titers ≥1:10. Data represents geometric mean titer (GMT) and the percentage of responder animals. R(%): percentage of responder animals.

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cytotoxic IFNγ-secreting cells, with higher responses attained in animals immunized intramuscularly after PBMCs stimulation in vitro with DIIICs. This result confirms the ability of Tetra-DIIIC to generate a functional cellular immune response by vaccination in non-human primates.

Relevance of the study

A new tetravalent vaccine candidate is proposed against dengue, with promising immunogenicity and protection results in animal models. The DIIIC vaccine formulation is able to activate a functional immune response, involving both the humoral and the cellular arms of immunity, in mice and non-human primates against the four DENV serotypes. Moreover, the recombinant nature of the antigens makes its design safer than attenuated viral strain vaccines to be administered in children younger than 1 year old. The results obtained pave the way towards a future clinical testing of the Tetra-DIIIC vaccine.