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REPORT

Nucleocapsid-like particles, an alternative and safe vaccine strategy against Dengue virus based on the induction of cell-mediated alone adaptive immune responses

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ABSTRACT

The development of an effective and safe vaccine against the Dengue virus has been limited by the absence of a definite correlate of protection, together with the possibility of amplification of the viral infection by the neutralizing antibody response of low affinity and concentration. However, the cellular immune response offers possibilities to develop protection safely, and may be able to prevent the development of the severe form of the disease. The present work focused on the capsid protein, which is the main target of the $CD4^+$ T cell response, and against which the $CD8^+$ T cell response is generated during the natural infection in the absence of its recognition by antibodies in the native virus. The capsid protein of serotype 2 was obtained recombinant and it was observed that it formed particles similar to nucleocapsids (PSN-2) when incubated with oligodeoxynucleotides (ODN). These PSN-2 induced a functional and protective cellular immune response in BALB mice. Subsequently, proteins were obtained for the remaining serotypes and their corresponding PSN. The tetravalent formulation of the PSN was evaluated immunologically in BALB mice and monkeys. It was possible to induce an immune response mediated by IFN-gamma-secreting cells after viral stimulus *in vitro*, in addition to controlling the viral load after the challenge.

Keywords: Dengue virus, capsid proteins, cell-mediated immune response, vaccines

RESUMEN

Las Partículas Semejantes a Nucleocápsidas: una estrategia vacunal alternativa y segura contra los virus del dengue, basada solo en la generación de respuesta inmune celular. El desarrollo de una vacuna eficaz y segura contra el virus Dengue se ha visto limitado por la ausencia de un correlato de protección definido, junto a la posibilidad de amplificación de la infección viral por la respuesta de anticuerpos neutralizantes de baja afinidad y concentración. Sin embargo, la respuesta inmune celular ofrece posibilidades de desarrollar protección de forma segura, y pudiera ser capaz de evitar el desarrollo de la forma severa de la enfermedad. El presente trabajo se focalizó en la proteína de cápsida, que es el principal blanco de la respuesta de células T $CD4^+$, y contra la cual se genera respuesta de células T $CD8^+$ durante la infección natural en ausencia de reconocimiento por los anticuerpos en el virus nativo. Se obtuvo por vía recombinante la proteína de la cápsida del serotipo 2 y se observó que la misma formó partículas semejantes a nucleocápsidas (PSN-2) al ser incubada con oligodesoxinucleotidos (ODN). Estas PSN-2 indujeron una respuesta inmune celular funcional y protectora en ratones BALB/c, en ausencia de respuesta inmune humoral. Posteriormente, se obtuvieron las proteínas para los restantes serotipos y sus correspondientes PSN. La formulación tetravalente de las PSN se evaluó inmunológicamente en ratones BALB/c y en monos, y se logró inducir una respuesta inmune mediada por células secretoras de IFN gamma ante el estímulo viral *in vitro*, además de controlar la carga viral tras el reto. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba para el año 2018.

Palabras clave: Diversidad genética, marcadores moleculares, *Nicotiana*, polimorfismo, tabaco

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Introduction

Dengue fever is a disease caused by a viral complex of the four serotypes of Dengue virus (DV), which is a *Flavivirus* of the *Flaviviridae* family [1]. Infection by any of the four serotypes can lead to dengue fever (DF), or cause a more severe form of the disease known as dengue hemorrhagic fever (DHF) that is sometimes accompanied by dengue shock syndrome (DSS). Several studies propose that severe manifestations during a secondary infection are triggered mainly by a phenomenon named as Antibody-Dependent Amplification (ADA) [2, 3]. This consists on the exacerbation of the antibody response against the virus due to a pre-existing cross-reactivity among viral serotypes of antibodies generated during the primary infection. It leads to an increase in viral load, because virus-antibodies complexes become concentrated on the surface of the target cells, facilitating their infection.

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Studies in mice published in recent years also point to the protective role of cell-mediated immunity against DV [4-6]. Since DV is a non-cytopathic virus that stimulates the expression of major histocompatibility class I (MHC) molecules in the cells it infects, the cellular immune response must be an important mediator of the reaction of the adaptive immune system against this pathogen. Recent findings points toward the possible safe generation of this type of response without immunopathogenic effects during a secondary infection in both mice and humans [7, 8]. Hence, in this setting, having a vaccine candidate able to induce a protective cellular response against the four viral serotypes independent of the antibody response could be advantageous over other vaccine candidates based on the generation of antiviral antibodies. It could also be devoid of the sensitization seen in vaccinated individuals with tetravalent formulations based on antibody responses with short-term or not equivalent immune responses.

Among the possible antigens with the potential to induce only a cellular immune response, is the DV capsid protein (C) which does not have exposed regions on the surface of the mature virion [9, 10]. This also explains why the antibodies generated against this protein never mediate the ADA phenomenon. Taking into account this background, the Dengue Vaccine project run in collaboration between the Center for Genetic Engineering and Biotechnology, CIGB, and the Institute of Tropical Medicine "Pedro Kouri", developed a genetic construct expressing the recombinant C protein of VD2. It was shown that this protein form particles similar to viral nucleocapsids (NLPs-2) when incubated with oligodeoxynucleotides (ODNs). Therefore, this work was aimed to evaluate the immunogenicity and protective capacity of NLPs-2 in mice, as a proof of concept, and then to evaluate the immunogenicity and protective capacity these NLPs for the other three DV serotypes in mice and monkeys.

Main results

In this work it was demonstrated that NLPs-2 were able to induced IFN-γ-secreting $CD4^+$ and $CD8^+$ cell mediated immune responses, which significantly contributed to control the viral load after challenge.

Our strategy took advantage of the native function of the nucleocapsid protein of protecting the viral RNA molecule. Based on this, the capsid protein was incubated with 45-bp-long ODNs at a 1:3 protein-ODN molecular ratio according to the procedure described by Wengler *et al*. [11]. Then, the NLPs-2 were obtained, which were further characterized by electron microscopy with reverse staining ([12]). The NLPs-2 obtained were spherical in shape with an average diameter of 27.6 ± 3.2 nm, and up to 192.5 ± 22.2 particles were visualized per field.

Then, the capacity of the particles to mount a functional cellular immune against DV was evaluated by immunizing Balb/C mice with NLPs-2 (10 μg of protein, administering three doses at one-week interval between doses). A placebo formulation was included containing just a similar amount of ODNs as that forming the particles. All the immunizations were adjuvanted in alum. A group of animals was immunized with a single dose of DV2 as positive control of the experiment.

Antibodies reactive to the recombinant protein or DV2 were detected by ELISA 30 days after the third dose. Animals immunized with NLPs-2 generated anti-capsid antibody titers statistically significant and higher than those detected in the negative control group. Noteworthy, these antibodies did not recognize the virus, nor neutralized the viral infection in vitro (data not shown). Immunization with the NLPs-2 formulation induced spleen cells capable of secreting high levels of IFN-γ (1578.8 ± 180.5 pg) after in vitro stimulation, 30 days after the last dose [12]. Notably, the NLPs-2 generated immune responses at the same level of that attained by the DV (1950.1 ± 317.8 pg/mL) ($p > 0.05$).

The depletion of $CD4^+$ and $CD8^+$ cell populations very significantly reduced IFN-γ secretion ($p \pm 0.001$), as compared to the levels detected in culture supernatants of total splenocytes [12]. The same behavior was also seen in animals immunized with DV2. After 30 days from the last dose, all the animals were challenges by intracranial route with 50 LD_{50} of a neuro-adapted DV2 viral strain. At the end of the experiment, all the animals developing an immune response against DV2 survived, while 90 % of the animals from the negative control group developed encephalitis symptoms and died. At the same time, 80 % of the animals previously immunized with the NLPs-2 formulation were able to control viral encephalitis and survived (Figure 2B). It was also observed that inoculation prior to viral challenge with an MAb depleting $CD4^+$ cell populations led to the appearance of encephalitis symptoms and the death of 90 % of the animals, while a similar Mab eliminating the $CD8^+$ cell populations decreased survival in about 40 % survival as compared to survival rates seen in animals not treated with the MAbs [12]. Overall, these results indicated that the CD8+ protective response was somewhat dependent on the $CD4^+$ response, at least in the mice model.

Subsequently, we proposed to follow the same strategy by generating NLPs for the remaining DV three serotypes 1, 3 and 4, and to evaluate their immunogenicity and protective capacity in mice and non-human primates. For this, *Escherichia col*i BL21 (DE3) cells were transformed with the respective expression plasmids pACC1, 3 and 4, which were previously obtained at the Dengue Virus lab at the CIGB. These constructs carried the genes coding for the capsids of the respective DV serotypes. Consequently, recombinant proteins expression was induced when cultures were at the exponential growth phase. Gene expression under the control the *pT7lac* promoter was induced by adding IPTG to the medium. A protein band of an approximate 30-kDa molecular weight was detected by 15 % SDS-PAGE for the three DV serotype constructs, accounting for 15-18 % of total bacterial proteins. The bands were immunodetected in a Western Blot assay by using the MAb 8H8 antibody which recognizes a conserved epitope on the four DV serotype variants (data not shown). Then, the proteins were purified in a single step of ion exchange chromatography as previously described for the C-2 protein [13]. Proteins were obtained 91.25 ± 1.5 % pure, with a yield of 89.75 ± 3.9 %. Afterwards, the proteins of the four DV serotypes were incubated with the immunostimulatory 39M ODN

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(5'-GGGG**GACGATC**GTCGGGGGAT**CG**ACTCT**CG**AG**CG**TTCTC-3'), previously designed and of proven immunoenhancing activity due to immunstimulatory CpG motifs (bold and underlined nucleotides in the ODN sequence) [14]. Similarly to what was observed for the DV2 capsid protein, the other capsids effectively formed NLPs.

At this point, a tetravalent formulation named TetraNLP was prepared by mixing at equal amounts the NLPs of the four serotypes, and it was further evaluated for immunogenicity in Balb/C mice. As negative control, one group received a placebo preparation containing just the same amount of the 39M ODN administered in the tetravalent formulation. All formulations were adjuvanted in alum. As positive controls, four groups were immunized with each viral serotype. The schedule included four immunizations on days 0, 7, 21 and 51.

TetraNLP formulation induced high titers of anti-recombinant antibodies against the four capsid proteins and those antibodies neither recognized the DV, nor neutralized the viral infection in vitro (data not shown). Then, the cellular immune responses generated by TetraNLP were measured 30 days after the last dose, and the frequency of IFN-γ-secreting cells was determined by ELISpot assay. It was shown that all the animals immunized with the TetraNLP formulation mounted a positive response, which was statistically similar to that observed in the animals immunized with the recombinant viruses ([15]).

Once the induction of a cell-mediated immune response specific to the recombinant DVs was demonstrated in animals immunized with TetraNLP, its protective capacity was studied in the viral encephalitis model in mice. Animals immunized with this formulation had a significantly lower viral load than animals immunized with the placebo formulation ($p < 0.05$). No infectious viral particles were detected in animals receiving the viral preparations (DVs 1-4)[15].

Since the ability of the TetraNLP formulation to induce a protective cellular immune response was proven in mice, we set out to evaluate the immunogenicity of this formulation in non-human primates. Two groups of African green monkeys received two preparations of this formulation, which contained 5 μg of each capsid protein in the form of NLPs obtained after incubation with 2.5 μg or 25 μg of the 39M ODN, respectively. That is, one group received 20 μg of TetraNLP formulated with 10 μg of the 39M ODN, while the other received 20 μg of TetraNLP formulated with 100 μg of the 39M ODN. These two groups would allow us to study the possible immunostimulatory effect of ODN 39M in monkeys immunized with TetraNLP. As control, a group of animals was immunized with a placebo formulation containing 100 μg of the 39M ODN. All the formulations were adjuvanted in alum and the animals received three doses on days 0, 60 and 120, respectively. The humoral and cellular immune responses were evaluated 30 days after the last dose.

In agreement with the results observed in mice, the sera of the monkeys immunized with TetraNLP recognized the four DV capsid proteins, and as expected, those antibodies neither recognized the recombinant DVs, nor neutralized the infection in vitro for any of the viral serotypes (data not shown). Nevertheless, when the cellular immune response was evaluated by measuring the levels of IFN-γ in the culture supernatant of PBMC of the animals stimulated in vitro with each DV, the secretion of the antiviral cytokine was shown dependent on the amount of ODN 39M administered in the form of NLP [15]. Animals of the negative control group did not show a specific immune response to any protein, despite the high dose of 39M ODN administered.

Considering the results of the cellular immune response generated in non-human primates following the administration of TetraNLP formulations containing either 100 μg of the 39M ODN, we decided to evaluate the protective capacity of this preparation. For this, immunized monkeys were challenged 15 days after evaluating the cellular immune response (45 days after the last dose) with 10^2 plaque-forming units (p.f.u.) of DV3 serotype, strain Nicaragua. This was the strain against which the lowest IFN-γ-secreting response was obtained. It was observed that all the animals in the negative control group (placebo) developed viremia, which lasted for 4.5 days at a maximum viral load of $10^{3.3}$ p.f.u./mL. On the contrary, transient viremia was detected in animals immunized with TetraNLP, which lasted for 2.7 days, with a maximum load of $10^{2.5}$ p.f.u./mL. A significant reduction in viral load was detected in animals receiving TetraNLP by comparing viremia with that of animals in the placebo group, specifically on days five, six, seven and eight after challenge ($p = 0.011$, $p = 0.04$, $p = 0.037$ and $p = 0.037$, respectively) [15]. This indicated that the immune responses induced by the TetraNLP formulations containing the NLPs of the capsids of the four DV serotypes and the 39M ODN were able to mount effective immune responses, which were able to control viral infection after challenge in the monkey model.

Scientific relevance and social and economic impact

Dengue and severe dengue become increasingly important as health problems, with around 350 million cases of Dengue, of which 96 million develop some degree of severity. In Cuba, several well-defined epidemics have occurred, the most significant ones on 1981 and 1997 [16]. Despite the numerous efforts that are being made to eliminate the transmitting vector, the availability of an effective vaccine would provide a solution of high social and economic impact. Recently, the Dengvaxia® vaccine (Sanofi Pasteur, France) was tested in children against the four DV serotypes, but unfortunately, it was banned from being administered to children younger than nine years-old due to safety and protective efficacy issues. This poses a threat of infection for this highly susceptible age group devoid of vaccination.

Consistent with the efforts to circumvent these limitations, our results demonstrate the protective capacity in mice and monkeys of the TetraNLP formulation as alternative vaccine candidate against DV, which does not induces detrimental antiviral antibody responses. Moreover, this last aspect provides this candidate unique advantages over the current vaccine candidates under development. In fact, and as detected so far, the absence of such ADA effects dependent

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on antibodies increases the chances of TetraNLP to provide an effective immune response to fight DV infection without antibody-mediated side effects. Moreover, TetraNLP induced a functional cellular immune response, both in mice and monkeys, which significantly decreased viral load after challenge and it is not expected to sensitize against viral infection. These two key safety elements of this candidate makes possible to advance into the more advanced clinical trials, faster than for candidates based on the induction of neutralizing antibodies, at least attending to the abovementioned concerns. Another aspect to consider is that NLPs do not generate sterilizing immunity; but, noteworthy, its ability to reduce viral load could be relevant in two important clinical scenarios: to change a severe dengue case into a classic dengue fever, or also a classic dengue into a sub-clinical viral infection. Having a vaccine candidate with these potential vaccination outcomes is very significant, considering the global expansion of the disease during the last years and the costs involved in vector control as the ultimate way to control dengue epidemics.

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Conflicts of interest statement

The authors declare that there are no conflicts of interest.