REPORT

New evidence on the virus capsid as a vaccine candidate against the Dengue 2 virus without the induction of neutralizing antibodies

Carlos López[1], ✉ Laura Lazo[1], Lázaro Gil[1], Lisset Hermida[1], Gerardo Guillén[1], Guadalupe Guzmán[2], Susana Vázquez[2], Lídice Bernardo[2], Aída Zulueta[1], Jorge Martín[1], Iris Valdés[1], Jorge Sánchez[1], Aracelys Blanco[1], Yaremis Romero[1], Ivón Menéndez[1], María C de la Rosa[1], Gabriel Márquez[1], Ricardo Silva[1], Viviana Falcón[1], Manuel Selman-Housein[1]

[1]Centro de Ingeniería Genética y Biotecnología, CIGB
Ave. 31 e/ 190 y 158, AP 6162, CP 10 600, Playa, Ciudad de La Habana, Cuba
[2]Instituto de Medicina Tropical "Pedro Kourí"
E-mail: laura.lazo@cigb.edu.cu

*I*ntroduction

Dengue is the most widely extended human viral disease transmitted by arthropods. The agent producing this disease is the dengue virus (DV) belonging to the *Flavivirus* genus of the *Flaviviridae* family, which is transmitted by the bite of the *Aedes aegypti* mosquito [1].

A large number of strategies are now under study for the production of an effective and safe vaccine against dengue, although up to now no single vaccine has proven to be completely effective. The most advanced vaccine candidates in the world are formed by attenuated strains of the virus, which have the drawback that there is a possibility of reversion to virulence as well as its reactogenicity that has been detected in human beings [2]. Taking into account these disadvantages, the variant of a formulation based on recombinant proteins is an attractive strategy for the development of a vaccine candidate. The envelop protein is the main target of the host immune response, which can induce high titers of neutralizing antibodies. However, the vaccine candidates based on this protein have the potential risk of inducing antibody dependent enhancement (ADE) if an effective neutralizing response against the four serotypes is not obtained [3]. Recent studies indicate a correlation between the generation of a cytotoxic cellular response and the protection to viral challenge in different animal models [4]. Hence, we proposed the development of the capsid protein as a vaccine candidate against this virus, since it is incapable of inducing anti-viral antibodies, thereby discarding the risk of inducing ADA.

*R*esults

The present paper is the first study on cloning and expressing the recombinant Dengue 2 virus capsid protein in *Escherichia coli*. The protein was obtained with a molecular weight of 15 kDa and accounted for the 15% of the total bacterial proteins; it was semi-purified up to 60% purity by ion exchange chromatography, and characterized through gel filtration chromatography detecting high molecular weight aggregates.

An immunization schedule in Balb/c mice using the semi-purified and aggregated preparation was designed to evaluate the immunogenic and protective capacity of the recombinant protein. This first schedule produced 44% protection in mice after the intracranial inoculation with VD2, while no neutralizing antibodies were detected [5]. Similar protection percentages in mice have been correlated with a complete protective response in the monkey model.

After this result was achieved, a purification procedure was developed with which we were able to obtain the protein with more than 90% purity. Because of the dimeric nature of the pure protein, an *in vitro* process for obtaining particles was standardized. Through electron microscopy it was shown that obtaining the particles from the protein depended on the neutralization of the positive charges of the dimers, and virus-like particles of 25 nm in diameter were obtained (Figure 1). The diameter of the particles produced *in vitro* agrees with the diameter of the native viral nucleocapsid

A new immunologic assessment was carried out in mice with the new pure preparation and the particles. It was shown that the purification procedure and the process used to obtain the particles did not affect the immunogenicity of the recombinant protein. Furthermore, there was an increase in protection with the particulated antigen as compared to the dimeric protein.

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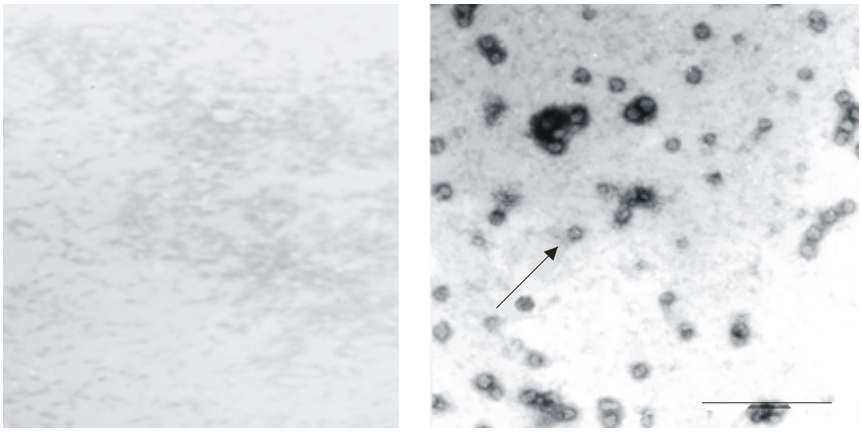

Figure 1. Analysis by electron microscopy of negative staining. The rod is equivalent to 200 nm.
A. Desalted dimeric capsid in assembly buffer.
B. Desalted dimeric capsid in assembly buffer after incubation with oligonucleotides (the arrow points to a typical particle).

✉ Corresponding author

In order to characterize the immune response induced in mice, a T $CD8^+$ cell selective elimination study was carried out in immunized mice before inoculating with the virus. This experiment made it possible to define that the protection conferred by this protein is not mediated by $CD8^+$ T cells, since there was no statistically significant difference between the percentage of mice protected within the group where this cell population had been eliminated and that in which it was not.

Finally we assessed the immuno-potentiating capacity of the capsid protein on other antigens. For this, we carried out immunization schedules in which the chimeric protein PD-24 was co-immunized with the Dengue 2 virus capsid protein obtained by recombinant procedures, pure and particulated *in vitro*. The PD-24 protein is a fusion between the P64K protein of *Neisseria meningitidis* and the domain III of the envelop protein of the Dengue 4 virus. It was observed that the percentages of protection (Figure 2), as well as the levels of anti-viral antibodies induced by the chimeric protein were higher when the mice were immunized with preparations containing the capsid.

This research showed for the first time that a protection against the DV is possible without the induction of antiviral antibodies using the capsid protein as a vaccine candidate. The importance of the cell response in the protection against the virus was therefore clearly shown. At the same time, this was the first report of having obtained particles of the capsid protein of the DV *in vitro* and it is shown that its assembly depends on the neutralization of the positive charges of the dimers.

Furthermore, we standardized a process to obtain particles *in vitro* with nucleic acids of "non-defined" sequences, which made it possible to obtain virus-like particles with a diameter that is similar to that reported for the native capsid. We demonstrated that it is possible to obtain virus-like particles with the use of bio-degradable polymers that neutr alize the positive charges of the capsid; this is of utmost importance in the development of vaccine formulations since it eliminates the risks of using nucleic acids. These experiments also show that there must be regulation mechanisms in viral morphogenesis that would allow the binding of the capsid to the viral RNA, which have not yet been described.

The results demonstrating the protection induced by the capsid protein were published [5] and are protected by the application of an international patent that also protects the use of the capsid as an immunopotentiator in vaccine formulations [6].

Relevance of the study

Dengue fever and dengue hemorrhagic fever are of increasing importance as health problems. Between 50 and 100 million cases of the former and 250 000 to 500 000 cases of the hemorrhagic form are reported in the world each year. There have been 4 well defined epidemics in Cuba, of which those of 1981 and 1997 are noteworthy. Considering that there is no cross-protection between the 4 viral serotypes, our population is prone to be affected by this disease. In spite of the large amount of effort focused on eliminating the transmitting agent, the possibility of having an effective vaccine would be the complete solution, of great social and economic impact. At present there is no vaccine available on the market, and the more advanced candidates worldwide, are formed by attenuated strains of the virus. Taking into account its possible reversion, as well as the reactogenicity that has been shown in human beings, the variant of a formulation based on recombinant proteins is an interesting alternative. Within this variant, the possibility of having a vaccine candidate that can avoid the risk of antibody dependent immuno-amplification is of utmost importance, since this event has the highest influence on hemorrhagic fever of the DV.

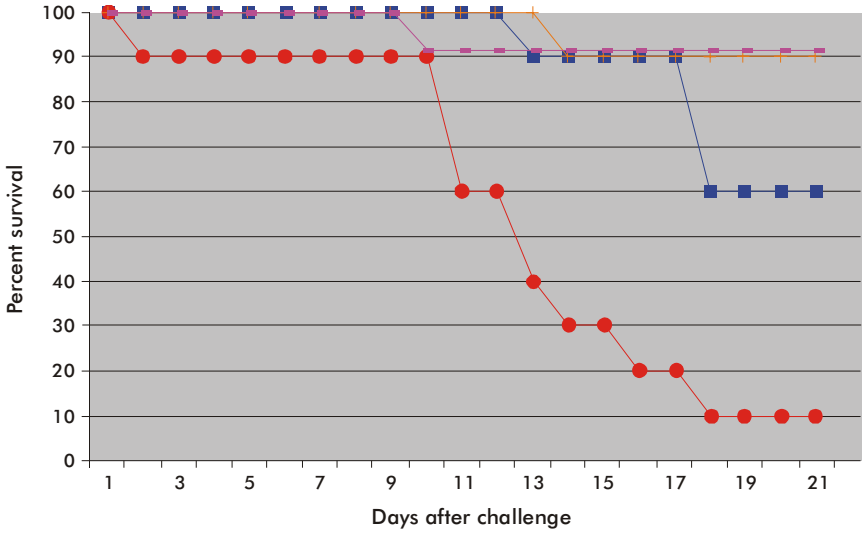

Figure 2. Protection after the inoculation with 100 medium lethal dose (100 DL50) of VD4 after immunizing with: PD-24 adjuvanted in alum (■); PD-24 and the capsid of DV2 in alum (■); negative control (●); Dengue Virus 4 (+).

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