RESEARCH

Evaluation of different formulations of a dengue-2 chimeric protein and outer membrane vesicles from Neisseria meningitidis *in mice*

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ABSTRACT

New generation vaccines, particularly those based on recombinant proteins, are generally less reactogenic than traditional live attenuated vaccines. Nevertheless, in terms of immunogenicity, they require potent adjuvants to reach a proper immune response in the recipients. We had previously evaluated the potential capacity of PD5 protein (a vaccine candidate against dengue-2, composed by the P64k protein of *Neisseria meningitidis*, and the domain III of the dengue Envelope protein), as a vaccine candidate with Freund's adjuvant. In this work, we evaluated the adjuvant capacity of the outer membrane vesicles (OMV) from *N. meningitidis* on the immunogenicity of the PD5 protein. As a result, after three doses in mice, the groups immunized with three different formulations of OMV elicited high titers of antiviral and neutralizing antibodies against dengue-2 with predominant IgG1 levels. Additionally, in the protection study, the most statistical difference was obtained in one of the three groups immunized with OMV, specifically with one formulation which favors the possible association between the protein and vesicles.

Keywords: adjuvant, dengue virus, *Neisseria meningitidis*, outer membrane vesicles, recombinant protein

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RESUMEN

Evaluación en ratones de diferentes formulaciones de la proteína quimérica de dengue-2 y las vesículas de membrana externa de *Neisseria meningitidis*. La nueva generación de vacunas, específicamente las basadas en proteínas recombinantes, son menos reactogénicas que las vacunas vivas atenuadas tradicionales. Sin embargo, en términos de inmunogenicidad, estas requieren de potentes adyuvantes para lograr la respuesta inmune adecuada. Nosotros previamente hemos evaluado la capacidad potencial de la proteína PD5 (candidato vacunal contra el virus dengue-2, compuesto por la proteína P64k de *Neisseria meningitidis* y el dominio III de la proteína de la Envoltura de dengue), como un candidato de vacuna con adyuvante de Freund. En el presente trabajo nosotros evaluamos la capacidad adyuvante de las vesículas de membrana externa (VME) de *N. meningitidis* sobre la inmunogenicidad de la proteína PD5. Como resultado, después de tres dosis en ratones, los grupos inmunizados con tres formulaciones diferentes de VME generaron altos títulos de anticuerpos antivirales y neutralizantes contra dengue-2 con niveles predominantes de anticuerpos IgG1. Adicionalmente, en el estudio de protección, la mayor diferencia estadística fue obtenida en uno de los tres grupos inmunizados con VME, específicamente en el cual se favorece la posible asociación entre la proteína y las vesículas.

Palabras clave: adyuvante, virus dengue, *Neisseria meningitidis*, vesículas de membrana externa, proteína recombinante

*I*ntroduction

Dengue virus infections are a serious health problem; indeed, the cause of morbidity and mortality in the majority of tropical and subtropical regions of the world: mainly Southeast and South Asia, Central and South America, and the Caribbean [1]. An estimate of 100 million human infections and several hundred cases of dengue hemorrhagic fever/dengue shock syndrome are reported annually [2].

For the development of vaccine candidates against dengue (DEN) virus, the strategies based on recombinant subunit vaccines have been used by several groups [3-14]. Most of these strategies have focused on the Envelope (E) protein, which is the main target of neutralizing antibodies [15]. In this sense, different candidates have been evaluated in mice with successful results [3, 4, 7, 8, 12-14]. Most of them have used potent adjuvants to demonstrate the functionality of the protein; nevertheless these adjuvants can not be used in humans [7, 8, 12, 13].

Our group has previously reported the expression, characterization and immunological evaluation of the recombinant protein PD5, which contain the domain III of the E protein from DEN-2 fused to the protein carrier P64k [16]. This molecule completely protected in *Macaca fascicularis* monkeys against viral challenge when the Freund's adjuvant was employed in the formulation [10]. However, several authors [9, 5, 6, 11, 17] employing fragments of recombinant E protein did not protect monkeys when the aluminum hydroxide was used in the formulations. Nevertheless, we had evaluated the protein PD5 formulated in aluminum hydroxide combined with the outer membrane vesicles (OMV) from *Neisseria meningitidis* in *Chlorocebus aethiops sabaeus* monkey [18], but that for-

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mulation was not immunogenic in this species. Alternatively, in the present study we evaluated a different process of formulation to characterize the influence of OMV in the immunogenicity of recombinant protein PD5. These vesicles have been used in different heterologous antigen formulations with successful results [19-21]. On the other hand, as a vaccine against the homologous pathogen, its safety and immunogenicity in humans have been demonstrated in several clinical trials [22-25].

In the current study, the formulations of the protein PD5 combined with OMV were evaluated in mice. In all cases, as previously described for the OMV formulations, the components were adsorbed in aluminum hydroxide [22, 23]. The immunization schedule was performed; as well the humoral immune response and protection against the infective DEN-2 virus were determined.

Material and methods

Virus strains

An inactivated preparation from suckling mice brain infected with DEN-2 strain New Guinea C (highly passaged in mice) was used as antigen for antibody detection [26].

For animal immunization and virus challenge, a preparation of infective virus (10^5 PFU/mL) verified by plaque assay, was employed. It was obtained by homogenization in RPMI 1640 medium (Sigma Aldrich, Ayrshire KA, UK) of suckling mice brain infected with DEN-2 strain New Guinea C.

For the neutralization assay, cell-culture supernatant harvested from Vero cells infected with the DEN-2 strain SB8553 (kindly provided by Dr. M. J. Cardosa, University Sarawak, Malaysia) was used.

Recombinant protein and formulation process with the outer membrane vesicles

The design, cloning and expression of the recombinant protein were previously described [16]. Briefly, the fusion protein PD5 containing the domain III, coding for amino acid 286-426, of dengue E glycoprotein from strain Jamaica, fused to the carrier protein P64k. The purified protein was kindly provided by Dr. Carlos López from the Center for Genetic Engineering and Biotechnology (CIGB) [27]. Protein PD5, produced under GMP conditions and at high levels of purity, was employed for mice immunizations. The calculated percentage of purity was 97%.

Outer membrane vesicles were purified from the *N. meningitidis* serogroup B strain CU385 (B:4,7: P1.19,15; ST = 33) from the collection of the Finlay Institute, Havana, Cuba [23]. The OMV from *N. meningitidis* serogroup B were obtained by extraction with deoxycholate as previously described [28].

Three formulations were performed with the purified recombinant PD5 protein and OMV. Two different concentrations of the OMV were evaluated 0.5 and 1 mg/mL, these concentrations are corresponding to total proteins present in the vesicles. The formulations were identified as PD5-OMV(0.5) and PD5-OMV(1) respectively. The different concentrations of OMV were firstly mixed with 2.5 mg/mL of PD5 protein to favors the possible association between both antigens. The resultant formulations were subsequently adsorbed on aluminum hydroxide (alum) at a final concentration of 1.44 mg/mL. As the control formulation, the simple mixture of the three components PD5, OMV and alum was performed (PD5 + OMV), in similar conditions of concentration of the OMV and PD5 to the described above.

Enzyme linked immunosorbent assay

The anti-DEN-2 antibody levels in mice sera were determined by an amplified sandwich ELISA system. Briefly, 96 well polystyrene plates (Costar, USA) were coated with 100 µL/well of a mixture of human immunoglobulins (IgG) (5 µg/mL) highly reactive to DEN virus in coating buffer (0.16% Na_2CO_3, 0.29% $NaHCO_3$, pH 9.5). Plates were incubated 2 h at 37 °C and then, blocked with coating buffer containing 1% bovine serum albumin (BSA). After 1 h incubation at 37 °C, they were washed three times with PBS-T. An extract of suckling mouse brain infected with DEN-2 (100 µL/well) was used as antigen and incubated overnight at 4 °C. After three washes in PBS-T, 100 µL/well of sera from each group were tested by serial dilutions in PBS-T, starting at 1:1 000 and incubated 2 h at 37 °C. Plates were washed as aforementioned and 100 µL/well of 1:6 000 diluted anti-mouse IgG-HRP conjugate (Amersham-Pharmacia, UK), containing fetal calf serum, were added and the plates were incubated 1 h at 37 °C. After washing again with PBS-T, 100 µL/well of O-phenilendiamine 0.04% in substrate buffer (2% Na_2HPO_4, 1% citric acid, pH 5.0), were added. The plates were incubated 30 min at room temperature and the reaction was stopped with 50 µL/well of 12.5% H_2SO_4. Absorbance was read at 492 nm in a microplate reader (SensIdent Scan; Merck, Germany). An absorbance value of a two-fold increase compared to the mean of the control extract or the pre-immune serum plus two standard deviations was considered as positive result.

Besides, the anti-mouse IgG subclasses antibody in mice sera were determined by a similar ELISA system as mentioned above, but employing 1:5 000 diluted subclass anti-mouse IgG-HRP conjugate (Amersham-Pharmacia, UK).

Plaque reduction neutralization test

Neutralizing antibody titers were measured by plaque reduction neutralization test (PRNT) in BHK-21 cells as previously described [29]. The strain SB8553 was used in this test. The neutralizing antibody titer was identified as the highest serum dilution that reduced the number of virus plaques in the test by 50% or more. The monoclonal antibody 4G2 was used as positive control [30].

Mice immunization

Groups of 20 female 5-6 week-old Balb/c mice (CENPALAB, Cuba) were injected by the intraperitoneal (i.p.) route on days 0, 15, and 30 with the different formulations containing 15 µg of the PD5 purified protein and OMV with alum as adjuvant. Similarly, negative control mice received 15 µg of OMV at a final concentration of aluminum hydroxide of 1.44 mg/mL. As positive control, one dose of 10^5 PFU/mL of infective DEN-2 virus (strain New Guinea C), obtained from infected suckling mouse brain, was inoculated by the same route. Ten mice

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from each group were bled two weeks after the last dose; and sera were collected for further immunological analysis. The maintenance and care of the experimental animals used in this research complied with the Cuban Institute of Health guidelines for the humane care and use of laboratory animals.

Animal protection study

One month after the last dose, ten remaining animals (non-bled) were injected intracranially (i.c.) with 20 μL of a suspension of DEN-2 (strain New Guinea C) virus-infected suckling mouse brain containing 100 median lethal doses (LD_{50}). Mice were observed daily for 22 days. Morbidity and mortality were recorded.

Statistical analysis

Direct or transformed (Log10) data that passed the normality test (D'Agostino & Pearson omnibus normality test) and showed variance homogeneity (Bartlett´s test) were analyzed by ANOVA parametric tests. Data that not fulfill normality and /or variance homogeneity test, even after transformations were analyzed by nonparametric test. The analysis of data from ELISA was assessed using a Newman-Keuls multiple comparison test. The analysis of data from PRNT was assessed using a Kruskal-Wallis non-parametric test with Dunn's multiple comparison test. Data from protection assay were analyzed by the log-rank test. In all cases, the GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com was employed.

Results

Antiviral antibody response after immunization

Three formulations, containing the recombinant protein PD5 and OMV, were inoculated in mice to evaluate the humoral immune response induced (as stated in Materials and methods section). Two addi-tional groups were immunized with PD5-alum and OMV-alum, respectively. After the third dose, ten mice per group were bled to obtain the sera. To determine the presence of antiviral antibodies, each serum was evaluated by a capture ELISA system. In all groups, high levels of anti-DEN-2 antibodies were detected, with statistically significant differences compared to the negative control group ($p < 0.001$). Additionally, all the formulations containing the PD5 protein elicited levels of antibodies similar to those induced in the control group immunized with DEN virus (Figure 1).

Antiviral IgG subclasses were also determined by the same capture ELISA system using pools of sera from each group. As shown in figure 2, in groups immunized with the protein PD5, the predominant pattern was IgG1 subtype. However, in the groups immunized with OMV as adjuvant, the ratio IgG1:IgG2a was lower than that observed in the PD5-alum group. In contrast, the pool sera from mice inoculated with the infective virus showed high levels of IgG2a compared to those of IgG1, reflected as the ratio IgG1:IgG2a < 1 (Figure 2).

Neutralizing antibodies after immunization

The functionality of the antibodies elicited by the different formulations was measured by PRNT. Figure 3

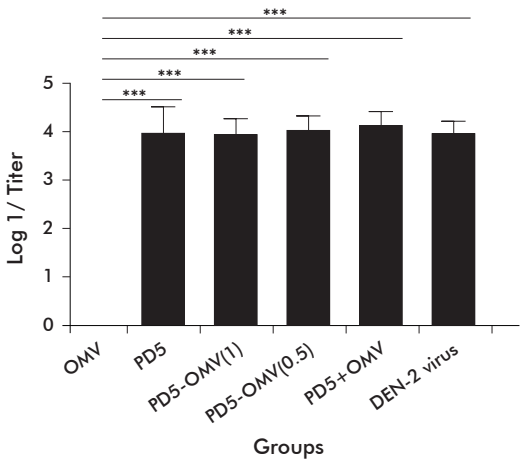

Figure 1. Antibody IgG response against DEN-2 virus after mice immunization with the different formulations. The results are given as logarithm of the antibody titers. The significant degree is referred as ***$p < 0.001$. The analysis of data from ELISA was assessed using a Newman-Keuls multiple comparison test ($n = 10$ per group). Data represent the mean ± standard deviation, ($n = 10$ per group).

shows the neutralizing antibody response in each group. In contrast to antiviral antibody response, in the groups inoculated with formulations of OMV, independently of the formulation process, high levels of neutralizing antibodies were elicited with statistically significant differences with respect to the negative control group ($p < 0.01$) (Figure 3). In case of the viral group, a low response of neutralizing antibodies was detected.

Protection study

One month after the last dose, the 10 remaining mice from each group were inoculated by the i.c. route with a live neuroadapted DEN-2 virus. Figure 4 shows the analysis of morbidity and mortality data following challenge and for 22 subsequent days. Animals immunized with the DEN-2 virus achieved a 100% protection, however during the observation period they showed symptoms of illness (Figure 4A and 4B).

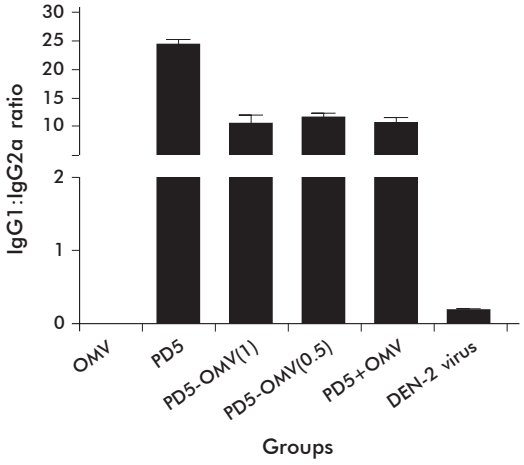

Figure 2. IgG1: IgG2a antibody ratios. The antiviral antibody subclasses were measured by an ELISA system using pools of sera from each group ($n = 10$ per group).

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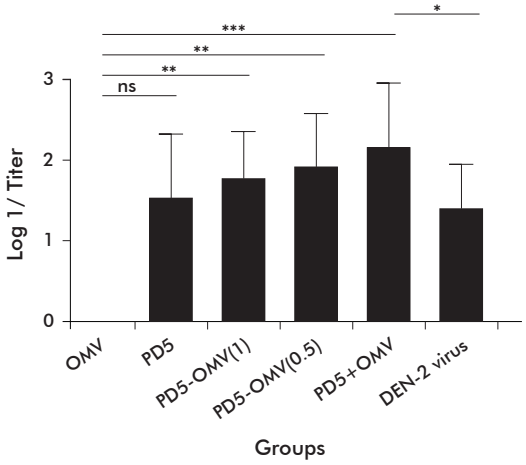

Figure 3. Neutralizing antibody response in mice after three doses measured by PRNT. The results are given as the logarithm of the antibody titers (n = 10 per group). The analysis of data was assessed using a Kruskal-Wallis non-parametric test with Dunn's multiple comparison test. The significant degree is referred as *p < 0.05; **p < 0.01; ***p < 0.001 and ns means non statistical significant differences. Data represent the mean ± standard deviation, (n = 10 per group).

Unlike to the results of the humoral immune response, only mice immunized with formulations PD5-OMV(1) and PD5-OMV(0.5) showed statistically significant differences with respect to the negative control group in terms of percentage of survival, ($p < 0.01$). The group immunized with the simple mixture of the formulation components (PD5 + OMV) did not show statistically significant difference compared to the negative control group (Figure 4B). At the end of the observation period, 40% and 20% of animals immunized with PD5-OMV(1) and PD5-OMV(0.5) survived, respectively. The group that received protein PD5 alone had 20% of survival, however did not show significant statistical differences with the OMV group (Figure 4B).

*D*iscussion

In a previous work, we had evaluated a PD5-OMV formulation absorbed in aluminum hydroxide in monkeys. As a result, the PD5-OMV formulation was not able to induce antiviral and neutralizing antibodies in this model. Besides, it did not protect i*n vivo* protection assay of viral challenge [18]. For this reason, in the present work we evaluated other formulation processes to enhance the immunogenic capacity of PD5 when it is combined with OMV.

The OMV from *N. meningitidis* have been widely employed [22-25] and their adjuvant capacity has been demonstrated [19-21] .

One of the proposed mechanisms through which OMV may exert this adjuvant effect is by acting as ligands for several receptors that stimulate the innate immunity such as the Toll-like receptors [31]. In fact, different groups have reported vaccine formulations combining the OMV and heterologous antigens from malaria [19], *Haemophilus influenzae* type b [32], and pneumococcus [33], with successful results.

The current work aimed at evaluating the influence of OMV on the immunogenicity of the recombinant protein PD5. In terms of antiviral antibodies no differences were observed among the groups immunized with recombinant protein. On the other hand, the functional antibody response was higher in the groups immunized with formulations containing the protein PD5 and OMV. These evidences suggest the proper antigenic presentation of the protein PD5 in the context of OMV independently of the formulation process.

In the case of the viral control group, the neutralizing antibody titers were low. However, similarly low antibody titers have been reported by other authors for the same serotype [34], and for DEN-1 [7] and DEN-4 [14], after immunization with one viral infective dose. A possible explanation could be related with the short-lived viremia produced in mice after virus immunization by the intraperitoneal route [35], and consequently, with the induction of a limited immune

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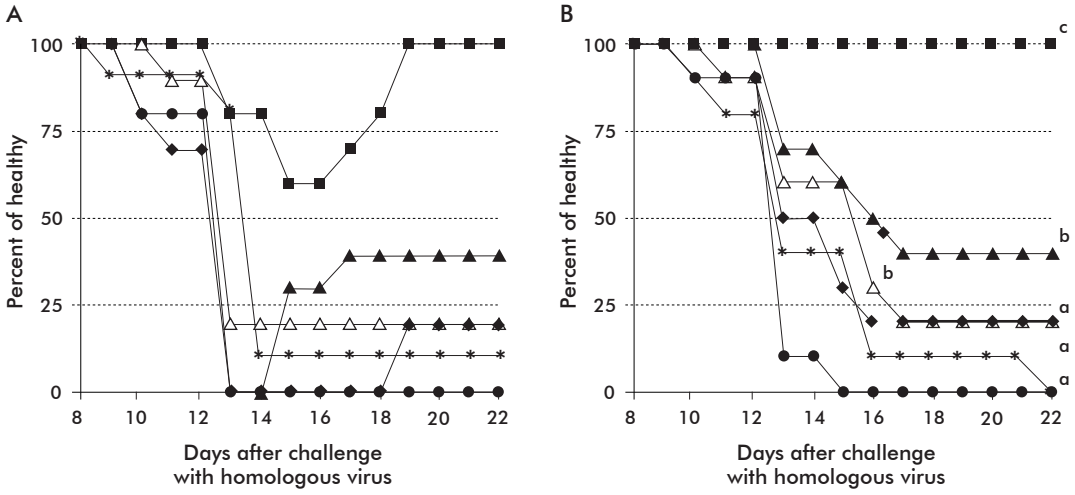

Figure 4. Protection study of mice immunized with PD5 formulations. A month after the last immunization, animals were challenged intracerebrally with 100 LD50 of lethal DEN-2 virus. Data from challenge assay were analyzed by the log-rank test. Different letters mean statistically significant differences compared with OMV group (a, ns; b, $p < 0.01$; c, $p < 0.001$) (n = 10 per group). (A) Percentage of healthy mice post-challenge. (B) Percentage of survival at each day post-challenge. (●) OMS, (◆) PD5, (▲) PD5-OMV(1), (△)PD5-OMV(0.5), (∗) PD5+OMV; (■) DEN-2 virus.

response. However, this response was sufficient to induce total protection after homologous viral challenge.

Moreover, it has also been described that OMV as adjuvant modulates the immune response induced by different antigens measured by the ratio IgG1:IgG2a [36]. Consistently, in the current work, the ratio IgG1:IgG2a of formulations of the protein PD5 and OMV were lower than that determined in the PD5-alum group, though the typical Th2 pattern was kept. This behavior could be explained by the soluble nature of the protein, mainly inductor of humoral immune response since the domain III is the principal target of neutralizing antibodies [15]. Additionally, in all the formulations, the alum was the base adjuvant being a potent Th2 inducer.

The protection study against the homologous infective virus was the other parameter measured. Despite the difference of this animal model with respect to the human disease, significant differences in the percentages of survival compared to the negative control group were attained as other indicator of a functional immune response. In the challenge assay, only the groups immunized with the formulations based on the interaction between the PD5 protein and the OMV demonstrated statistical differences with respect to the negative control group ($p < 0.01$). On the contrary, when both components were simply mixed together with alum, the induced immune response was unable to protect the animals despite the highest neutralization titers detected in this group (Figure 4B).

Various candidates containing the domain III of the E protein have been evaluated by different groups and their protective capacity has been assessed in the encephalitis mouse model [3, 4, 7, 8, 13, 14]. However, the best results in percentage of survival have been obtained when potent adjuvants, not suitable for human use, have been employed [7, 8, 13]. On the other hand, a tandem domain III candidate against the four dengue serotypes was developed by Chen and coworkers, although in this study, the protective capacity of the humoral response was only elicited in mice; and excluded the cell-mediated mechanisms [12]. Only Simmons and coworkers achieved an 80% survival rate against viral challenge with formulations in alum, but they used a very small number of animals per group in their study [4]. Of the candidates mentioned above, only two have been assayed in monkeys using alum, and did not protect against viral challenge [10, 17].

In general, the percentages of survival obtained in this study could have been affected by the high severity of the challenge assay, manifested by the fact that, in spite of the 100% survival rate obtained in the virus-immunized group, several mice in this group became ill during the observation period. On the other hand, considering the similarity in the humoral immune response induced by the formulations containing PD5, we can suggest that a possible association between OMV and PD5 may have favored another mechanism of the immune system that was responsible for the protection obtained. Several groups have reported the lack of correlation between neutralizing antibodies and protection [5-8, 37-39]. In this sense, cell-mediated immune mechanisms could have played a role in protecting against the virus, in fact, some modulation in the IgG subclass was detected.

The lack of correlation between neutralizing antibodies and protection was clearly evident in the virus-immunized group. Accordingly, in this case, the cellular immune response was reflected in the ratio of IgG1:IgG2a, indicating a potent protector mechanism in this animal model. In general, the percentages of survival obtained in the study were lower than those previously reported by our group [8].

Taking into account, the evidences of the present study support the OMV as one of the possible adjuvant formulation for PD5. Further studies in monkeys would be needed to confirm the capacity of PD5-OMV formulation to provide complete protection of monkeys against viral challenge.

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