REPORT

Development of a technological framework for using virus-like particles as adjuvants in prophylactic and therapeutic vaccines: demonstration of effect in animal models and humans

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ABSTRACT

Developing an effective vaccine entails: 1) proper antigenic presentation to the immune system, inducing a response of adequate intensity 2) a long duration for said response 3) the ability to steer this response towards the immune system pattern most suited for the elimination of the pathogen. Among the current crop of adjuvants, only aluminum-based gels and some oil emulsions are compliant with established regulatory and safety requirements and, not surprisingly, have received extensive use. The present work describes the development of a technological framework for the inclusion of virus-like particles (VLP) as adjuvants in vaccine preparations, allowing the induction of a functional immune response in humans that achieves seroconversion and protective antibody levels without the need for additional adjuvants. This technological framework comprises both the production and formulation of antigens as virus-like particles and the use of these VLP as adjuvants for soluble antigens, obtaining high levels of induction of the Th1 cell response which, in combination with the humoral response, have been shown to confer a fully functional immunoprotective status in animal models. Some of the novel aspects of the present work are the development of a common methodology for the obtention and formulation of different VLP; the demonstration that VLP-containing formulations are able to bias response towards a Th1 pattern; the finding that VLP can be used as adjuvants stimulating a fully functional response in humans to soluble antigens; the safety of VLP-adjuvanted formulations and, lastly, the induction of a functional, protective response in animal models and humans.

Keywords: adjuvant, preventive vaccine, therapeutic vaccine, vírus-like particles, animals, humans, vaccine formulation

*I*ntroduction

Vaccine development, even with *a priori* knowledge of the antigens required to induce a protective or therapeutic response, is limited by the availability of formulations providing: 1) proper antigenic presentation to the immune system, inducing a response of adequate intensity, 2) a long-lasting response and 3) the ability to steer the immune system towards the Th pattern most suited for the elimination of the pathogen. In addition, in the case of therapeutic vaccines for chronic diseases, the formulation must be able to break down immunological tolerance. Despite the large number of adjuvants and antigen presentation systems described in the literature, only aluminum-based gels and some oil emulsions have complied with all regulatory parameters and safet y requirements and have therefore been extensively used.

Developing a therapeutic vaccine against chronic hepatitis B (HBV) infections constitutes an attractive strategy. Such a product would fill the niche opened by the 400 million chronic HBV carriers that currently exist throughout the world, for whom the available treatments show little or no efficacy together with undesirable side effects further limiting their use [1-3].

A prophylactic and/or therapeutic vaccine against type 1 Human Immunodeficiency Virus HIV-1), in addition, would have a massive impact. Worldwide estimates for the number of HIV-infected persons hover above 33 million, with an average of 2.7 million new infections taking place per year and over 2 million yearly deaths. According to UNAIDS, antiretroviral therapy reached a coverage of 42% by the end of 2008. This achievement, however, is marred by the growing dissemination of isolates resistant to first-line antiretroviral drugs which, together with the current world economic crisis, threaten to minimize these successes and revert this situation [4]. An anti-HIV vaccine, therefore, still represents the best alternative in the long term, according to the experts.

Hepatitis C (HCV) infections continue to represent a significant public health problem; approximately 3% of the world population is infected with this virus, which often results in the appearance of cirrhosis and liver cancer. No vaccine is available and current therapies, in addition to being costly and producing a number of adverse events, have a success rate lower than 50% [5]. It then follows that the obtention of a therapeutic vaccine against this pathogen would be significant for Cuba from both economic and social standpoints.

Dengue Fever and Dengue Hemorrhagic Fever are increasingly recognized as an emergent health problem. From 50 to 100 million cases of Dengue Fever and 250 000 to 500 000 cases of the hemorrhagic form of the disease are reported each year in the world

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[6]. Four well-defined epidemics have taken place in Cuba, of which the most important are those of 1981 and 1997. In spite of the large effort undertaken to curb transmission by controlling the mosquito vector, an effective vaccine would constitute a total solution with a large social and economic impact. No vaccines are commercially available, and the most advanced vaccine candidates along the development pipeline in institutions around the world are based on the use of attenuated viral strains. Such an approach, however, presents a number of risks, the main of which is the possibility of a reversion; in addition to the high reactogenicity of these candidates according to actual data already obtained in clinical trials. Hence, a Dengue vaccine formulation based on recombinant proteins constitutes an attractive alternative.

In order to contribute to the development of new vaccine candidates against diseases which represent very complex targets due to the type of immune response that has to be generated, we have developed a methodology for the obtention and formulation of different virus-like particles. It addition, the capacity of formulations containing these VLP to steer the immune response towards the most adequate immune pattern for a specific pathogen has been examined.

Results and discussion

First, we demonstrated the adjuvant effect of VLP on the immune response to co-administered soluble antigens, allowing the induction of a functional response in humans. Then, the safety profile of formulations adjuvanted with VLP was examined, followed by the study of the induction of protective and functional responses in animal models and humans.

Anti-HBsAg seroconversion to protective antibody levels with a formulation containing HBsAg and HBcAg virus-like particles without additional adjuvants

The nasal immunization route represents an ideal choice for the induction of mucosal immune responses, which represent the best alternative to protect against sexually transmitted or infectious respiratory diseases. The use of this route, however, has been hampered by the lack of adequate adjuvants. Most attempts have employed the B subunit of cholera toxin or *Escherichia coli* enterotoxin which, while being potent mucosal adjuvants, also display marked neurotoxicities when administered by this route. Our group has demonstrated that VLP formed solely by HBV surface and core antigens (HBsAg and HBcAg, respectively) can induce a seroprotective response without additional adjuvants (Table 1) [7]. As shown in figure 1, most of the immunized volunteers generated protective antibody levels (anti-HBsAg titers larger than 10 mIU/mL).

Development of the production and formulation of antigens as virus-like particles. Their use as adjuvants for soluble antigens.

A truncated variant of the core protein of HCV was produced in a recombinant *E. coli* strain and self-assembled *in vitro* into spherical VLP. The process took place in the absence of nucleic acids, according to chromatographic and electron microscopy studies. The particles, with a mean diameter of 30 nm, had a homogeneous size distribution and morphology, similar to that of particles detected in serum and hepatocytes of HCV-infected patients [8]. The particles were included into a formulation together with a DNA immunization plasmid expressing the structural proteins of HCV to increase the immunogenicity of the latter. This formulation, codenamed CIGB-230, has displayed very low reactogenicity during pre-clinical toxicological testing [9].

Table 1. Antibody response as percentage seroconversion rates against HBsAg – HBcAg in healthy volunteers immunized intranasally with 50 μg of HBcAg and 50 μg of HBsAg

Group/time	Vaccine candidate			Placebo		
	Day 0	Day 30	Day 90	Day 0	Day 30	Day 90
N	9	8	8	10	9	9
Anti HBsAg	-	8 (100%)	8 (100%)	-	-	-
Anti HBcAg	-	2 (25%)	6(75%)	-	-	-

An outside group has evaluated in clinical trials a V3 loop-based multi-antigen peptide (MAP) as a prophylactic vaccine candidate against HIV-1. This candidate was well tolerated, and although most of the volunteers immunized with the 500 μg dose elicited neutralizing antibodies against the homologous strain, they failed to neutralize heterologous isolates. The solution the authors proposed to this problem, the use of MAP cocktails, is impractical, as the total peptide mass required to elicit neutralizing antibodies to a reasonable number of strains would be too large for inoculation into humans (once again illustrating the obstacle to vaccine development posed by the variability of HIV-1). In the context of this situation, we developed a method for conjugating MAP (also using V3 loop-based MAPs for the proof-of-concept) to HBsAg VLP carriers, thus managing to significantly increase the cross-reactivity of the obtained sera above that of their unconjugated counterparts [4, 10, 11].

Virus-like particles based on the capsid protein of Dengue-2 Virus (PSN2) were mixed with a soluble fusion antigen formed by the P64k protein from *Neisseria meningitidis* and domain III of the envelope protein from Dengue-4 Virus (PD24). BALB/c mice were then immunized with the PD24-PSN2 mixture, using animals immunized solely with either PSN2 or PD24 as controls, together with a placebo group receiving P64k and a positive control group inoculated with infectious

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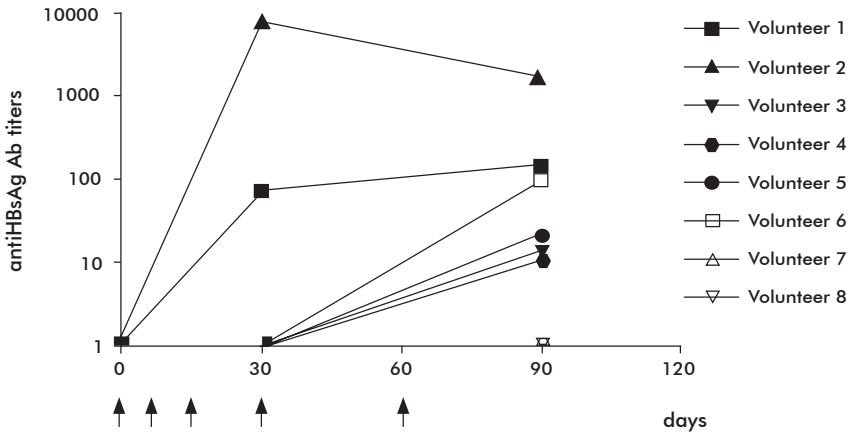

Figure 1. Anti-HBsAg antibody response kinetics in healthy adults immunized intranasally with a mixture of HBsAg and HBcAg at 0, 7, 15, 30 and 60 days. Blood samples were drawn in days 30, 60 and 90. Vertical arrows indicate the intranasal inoculations.

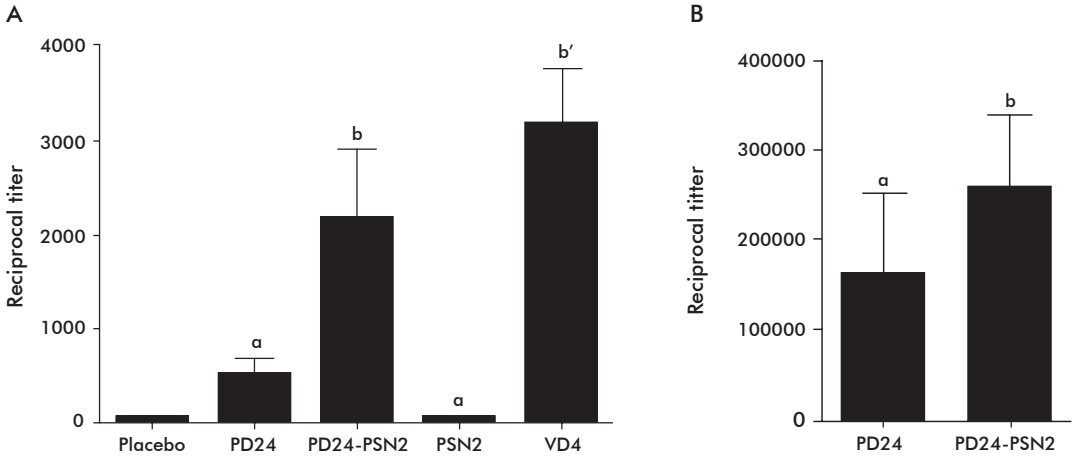

Figure 2. Charts for the humoral immune response determined by ELISA, 15 days after the last dose. A) Anti-VD4 antibody response. The presence of statistically significant differences was assessed with a single-way ANOVA, using the Newman-Keuls post-hoc test for multiple comparisons between group means ($a \neq b$: $p < 0.01$; $a \neq b'$: $p < 0.001$). B), Anti-PD24 antibody response. The presence of statistically significant differences was assessed with a non-parametric Mann-Whitney U test ($a \neq b$: $p < 0.05$). The figures represent the mean plus the standard deviation ($n = 10$ per group). The data are representative for two independent experiments

VD4. The appearance of VD4-reactive antibodies was evaluated by ELISA (Figure 2). There were no detectable antiviral titers in mice immunized with P64k alone or PSN2. However, the antiviral titers in animals receiving the PD24-PSN2 mixture were higher than in those immunized only with PD24 ($p<0.01$, one-way ANOVA and Newman-Keuls multiple comparisons test), and similar to those of the positive control group (Figure 2).

Strong induction of a Th1 cellular response on top of the humoral response

The preparation based on HCV capsid particles with the DNA immunization plasmid expressing structural antigens from HCV was shown to induce both humoral and cell-based specific immune responses, which were particularly strong against the capsid antigen. This evidences how the particulate nature of the antigen represents a major determinant for its immunogenicity [9].

One month after the last dose of the immunization scheme described above for Dengue Virus antigens, splenocytes from 6 mice per group were stimulated *in vitro* with infective VD4. Culture supernatants were then analyzed by ELISA to determine the concentration of IFN-γ. The levels of secretion of this cytokine in the splenocytes from the mouse group immunized with the PD24-PSN2 preparation (10165.17 ± 2250.68 pg/mL) was similar to that of splenocytes from the positive control group (12525.15 ± 4465.56 pg/mL) and higher than in the group immunized with PD24 alone (6084.71 ± 1365.37 pg/mL) [6].

Demonstration in animal models and humans of the induction of a functional and protective response

The administration of a preparation based on particles of the HCV capsid protein and the DNA immunization plasmid expressing structural antigens from HCV has been shown to protect mice in a model of challenge with a surrogate virus expressing HCV antigens [12].

In the case of Dengue antigens, after the immunization with the vaccine candidates described above, the mice were challenged with a preparation of infectious Dengue 4 virus (DV4). All animals immunized with the PD24-PSN2 vaccine preparation survived, whereas only 50% of mice survived in the group immunized with PD24. This evidences the capacity of PSN2 to enhance and potentiate the protective capacity of PD24 (Figure 3).

The data lead us to conclude that it is possible to take advantage of the attractive characteristics of VLP as immunogens and immunomodulators to develop a technological framework consisting on antigens with adjuvant properties that can be further used in the development of attractive vaccine candidates for pathogens whose transmission is difficult to prevent, such as HIV, HCV or Dengue, or causing diseases with a difficult therapy, such as HBV, HCV and HIV.

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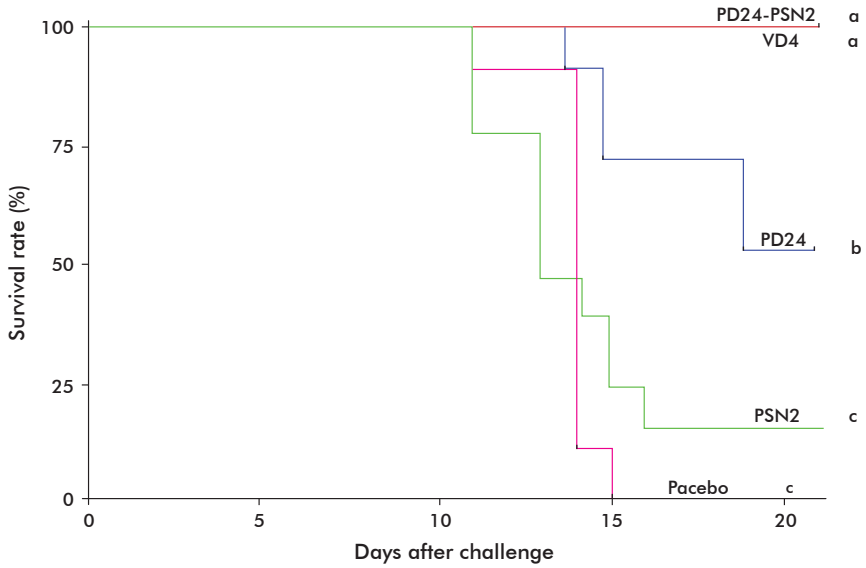

Figure 3. Chart representing survival rates after a lethal intracranial inoculation with VD4. Groups sharing the same letter have statistically indistinguishable levels of protection. The data are representative for two independent experiments.