REPORT

New alternatives for the development of vaccine preparations: contributions to the knowledge on the interaction of recombinant protein viral antigens with nucleic acids

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ABSTRACT

Currently, there are no effective tools to fight multiple pathogens due to the insufficient knowledge on their components and the limited arsenal of vaccine alternatives. In the present paper, combinations of recombinant viral antigens and nucleic acids are studied using electron microscopy, electrophoresis, nuclease digestion and sucrose or cesium chloride gradients. We describe new data on morphology, size, and the increased stability of protein particles composed of viral antigens in close association with nucleic acids. We demonstrate, for the first time, that particles of recombinant HCV viral core proteins are able to interact with plasmid molecules for DNA immunization, forming complexes of particles with increased density and size. Additionally, the increased immunogenicity of these recombinant viral protein-plasmid DNA mixtures for DNA immunization was evidenced when administered in animal models, compared to the individual components. In addition, we discuss the possible mechanisms behind the enhancement of the immune response, based on the experimental evidence of the interaction between mixture components and their properties. The use of recombinant viral proteins interacting with nucleic acids as the active principle, and simultaneously as an adjuvant or molecular vehicle for DNA vaccines, has important implications for the development of new effective strategies to prevent and treat diseases caused by different pathogens.

Keywords: vaccine, adjuvant, viral capsid, particles, DNA immunization

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RESUMEN

Actualmente no se cuenta con herramientas efectivas para combatir múltiples patógenos debido en gran medida al insuficiente conocimiento de sus componentes y al limitado arsenal de alternativas vacunales. En el presente trabajo, se estudian mezclas de antígenos proteicos virales recombinantes y ácidos nucléicos mediante microscopía electrónica, migración electroforética, digestión con nucleasas y gradientes de sacarosa ó cloruro de Cesio. Se aportan elementos novedosos acerca de la morfología, tamaño, y el incremento de la estabilidad de las partículas proteicas formadas por el antígeno viral en asociación con ácidos nucléicos. Se demuestra por primera vez que partículas proteicas recombinantes de una cápsida viral son capaces de interactuar con un plasmidio para la inmunización con ADN, formándose complejos con densidad incrementada y mayor tamaño de las partículas. Adicionalmente, se estudia la inmunogenicidad de estas mezclas de antígenos proteicos virales recombinantes y plasmidios para la inmunización con ADN en modelos animales, demostrándose por primera vez la potenciación de la respuesta inmune inducida por la administración de estas mezclas, con relación a la generada por los componentes individuales. Se plantean los posibles mecanismos de potenciación de la respuesta inmune, basados en las evidencias experimentales de interacción entre los componentes de las mezclas y sus propiedades. El empleo de antígenos proteicos virales recombinantes, capaces de interactuar con ácidos nucléicos, como principio activo y simultáneamente como adyuvante o vehículo molecular para vacunas de ADN, tiene implicaciones importantes para el desarrollo de nuevas estrategias efectivas para la prevención y el tratamiento de enfermedades ocasionadas por diferentes patógenos.

Palabras clave: vacuna, adyuvante, cápsida viral, partículas, inmunización con ADN

*I*ntroduction

The available arsenal of vaccine preparations to fight the multiple pathogens affecting man and relevant animal species is still limited. In most cases, the knowledge on characteristics and components of pathogens is also scarce, reducing the possibilities of developing effective alternatives to prevent and treat diseases. Particularly, highly variable viruses (like the hepatitis C virus; HCV) represent important challenges to the

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host, and frequently to the immune response associated to the protection or clarification of the resulting infection involving both humoral and cellular immunity. In this regard, DNA immunization arises as a promising approach to generate effective preventive or therapeutic interventions [1]. However, DNA vaccines have frequently failed to elicit strong immunity in humans against a variety of pathogens [2], despite the positive results obtained after DNA vaccination in animal models. The efficient DNA uptake by the immunologically relevant cell populations is probably the key event to induce strong immune response by DNA vaccination. Many alternative approaches to avoid DNA degradation by interstitial nucleases and to facilitate cellular uptake have been evaluated (reviewed by Donnelly e*t al*. [3]).

Viral core proteins are able to interact with nucleic acids and naturally protect viral genetic material from degradation [4, 5]. In addition, protein particles are generally better immunogens than smaller molecules [6]. Thus, the use of recombinant viral core proteins to encapsulate DNA vaccines is an interesting approach, both to protect the plasmid DNA molecule (delivery vehicle) and as an immunogen, also coordinated if the viral protein and the antigens expressed by the DNA construct are derived from the same pathogen.

We studied recombinant HCV core protein-plasmid DNA mixtures for DNA immunization mixtures using electron microscopy, electrophoretic mobility assays, nuclease digestion and sucrose or cesium chloride gradients. Also, the immunogenicity of recombinant HCV or the hepatitis B virus (HBV) core protein, mixed with a plasmid for DNA immunization, was evaluated in animal models.

Materials and methods

Plasmids

The pIDKE2 [7] is a pAEC-K6-based plasmid DNA immunization construct expressing the first 650 aa of the HCV polyprotein from a genotype 1b-Cuban isolate. The parental pAEC-K6 vector [8] was used as a mock for immunization experiments.

Antibodies

The mouse monoclonal antibody mAb SS-HepC.1, specific for the HCV core (HCcAg) [9], was used to detect it. Sera containing anti-HCcAg.120 IgG antibodies obtained in mice immunized with HCV virus-like particles containing HCcAg.120 were used in immunoelectron (IEM) and immunofluorescence microscopy analysis.

Recombinant proteins

Recombinant HCcAg Co.120 [10], E1.3 40 [11] and E2C [12] were obtained from recombinant *Escherichia coli* cells with over 85% purity by a combination of washed pellet procedures and gel filtration chromatography. Co.120 comprises the first 120 aa of the HCV core protein, whereas E1.340 and E2C encompass aa 192-340 and 384-605 in the viral polyprotein, respectively. The recombinant HCcAg Co.173, comprising the first 173 aa of the HCV core protein, was obtained from *Picchia pastoris* cells, as previously described [13]. The hepatitis B core antigen (HBcAg) was kindly donated by Dr. Alexis Musacchio.

Peptides

Synthetic peptides, previously described [14], covering the hypervariable region I (HVR-1) (aa 384-410 in the viral polyprotein) of different viral isolates, were used: THTVGGSTAHNARTLTGMFSLGARQKI (genotype 2a, isolate HC-J6), TYSSGQEAGRTVAGFAGLFTTGAKQNL (genotype 2b, isolate HC-J8), THVTGGSAGHTVSGFVSLLAPGAKQNV (genotype 1a, isolate HCV-1), THVTGGAQAKTTNRLVSMFASGPSQKI (genotype 1b, isolate HCV-BK). Peptide TGTYVTGGTAARGVSQFTGLFTSGPSQKIQL (Cuban isolate) comprises HVR-1 (aa 384-414) from a genotype 1b-Cuban isolate. Additionally, two other peptides comprising HVR-1 consensus sequences were used: consensus-1, TTTVGGQASHQVHSLTGLFSPGAKQNV; consensus-2, THVTGGSAARTTSGLTSLFSPGASQNL.

Yeast and bacterial strains and growth conditions

The *P. pastoris* strain MP-36/C-E1.339 expresses the entire HCcAg and the first 148 aa of the HCV E1 protein as previously described [15]. The MP-36 strain was used as a negative control. MP-36/C-E1.339 and MP-36 strains were grown using already established conditions [16]. The BL21(DE3)pLysS strain was used to express the HCcAg.120 as previously reported [17].

Vaccinia viruses and cell lines

The recombinant vvCore is a vaccinia virus derived from the Western reserve (WR) strain [18], expressing aa 1-176 of the HCV polyprotein. African green monkey kidney cells BSC-40 [19] were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and used to determine vaccinia virus titers in mice ovaries.

Purification of the HCcAg.120

The HCcAg.120 was purified as previously described [20, 21].

HCcAg.120-pIDKE2 plasmid interaction studies

In vitro protein-nucleic acid assembly assays were carried out following previously described procedures [21, 22]. Ten micromolar purified HCcAg.120 in refolding buffer (100 mM NaCl, 1% Glycerol, 20 mM Tris, pH 7.0) was mixed with an equal volume of the pIDKE2 plasmid at a 100:1 protein-nucleic acid molar ratio. Denatured HCcAg.120 was resuspended in refolding buffer containing tRNA (Sigma, St Louis, USA) at 100:1 protein-nucleic acid molar ratio under RNAse-free conditions, and used as the control. The reactions were incubated at 30 ºC for 10 min followed by 15 min on ice and centrifuged at 16,000 g for 15 min at 4 ºC.

RNAse and DNAse digestion

Sucrose density gradient fractions containing nucleocapsid-like particles (NLPs) that banded at 1.27 g/mL

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were dialyzed against TEN buffer (1 mM EDTA, 150 mM NaCl, 50 mM Tris HCl, pH 8.0). Co-purified but unprotected nucleic acids were digested at 37 ºC for 30 min. with RNAse and/or DNAse I (both from bovine pancreas, 10 µg/mL each; Boehringer-Mannheim). Similar conditions were used to completely digest free DNA and RNA purified from *P. pastoris* cells. After the RNAse/DNAse I digestion, the mixture was briefly treated with diethyl-pyrocarbonate to avoid cleavage after the sample-loading step. RNA and DNA content were measured by UV spectroscopy at 260 and 280 nm, respectively, and by gel electrophoresis. The 1 kb Plus Ladder (Gibco-BRL) was used as the molecular weight marker.

Equilibrium density gradient centrifugation

Five hundred microliters of either HCcAg.120-pIDKE2 plasmid mixtures or refolded HCcAg.120 samples were applied to cesium chloride (CsCl) or sucrose density gradients and assayed by Dot blot as previously reported [23].

Transmission electron microscopy

Refolded HCcAg.120, pIDKE2 plasmid, and HCc Ag.120-pIDKE2 plasmid and HCcAg.120-tRNA mixtures were fixed in glutaraldehyde, negatively stained with uranyl acetate and further analyzed by transmission electron microscopy as previously described [23].

Detection of HCV core protein in hepatocytes

For the IEM analysis, samples of liver tissue from either HCV-infected patients or healthy individuals were fixed with 4% (v/v) paraformaldehyde containing 0.2% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) at 4 ºC for 3 h and washed with 0.1 M phosphate buffer. Further processing and detection were carried out as previously described [23].

Animals and immunization schedule

Pathogen-free, 6 to 8-weeks-old BALB/c female mice (18-20 g of weight) were purchased from CENPALAB (Centro Nacional para la Producción de Animales de Laboratorio, Havana City, Cuba) and used for all *in vivo* studies. Groups of ten animals each were immunized with different combinations of plasmid pIDKE2 and either Co.120, Co.173, or HBcAg recombinant proteins, or the pAEC-K6 mock plasmid DNA in control groups. Mice were injected in the quadriceps muscle with 50 µg of plasmid DNA and 5 µg of protein, according to the immunogen studied, in a 100 µL final volume of 0.9% NaCl solution and following different immunization schedules (on weeks 0-3, 0-3-7 or 0-3-7-12-16, respectively). The plasmid-protein mixtures were prepared just before inoculation. Mice were bled at different time points after the primary immunization. Immunization schedules were carried out in duplicate.

Enzyme-linked immunosorbent assay (ELISA)

To detect murine antibodies against HCV structural proteins, an ELISA was carried out as previously described [7]. The cut-off value to consider a positive antibody (Ab) response was established as twice the mean absorbance at 492 nm of the negative control sera (sera from pAEC-K6-immunized mice).

Lymphoproliferation assay

Spleen cells were obtained from mice of both immunized and control groups, 4 or 14 weeks after primary immunization. T cell proliferation assays were made with individual spleens or pools of spleens from three animals per group, as previously described [7]. Data was expressed as the Stimulation Index (SI), defined as the ratio between the mean c.p.m. obtained with stimulation and the background of RPMI 1640 medium value. Lymphoproliferative responses were considered positive for SI values above 3.

Titration of vaccinia viruses in ovaries of challenged mice

Seven and twenty-one days after the final DNA vaccination, according the immunization schedule, 5 mice per group were intraperitoneally challenged with 10^6 plate forming units of a vaccinia virus expressing HCV core protein (vvCore), as previously described [18].

Statistical methods

The normal distribution of the data was analyzed by using a Kolmogorov-Smirnov test. Data were analyzed using an ANOVA test with Newman-Keuls' test as a post-test for parametric analyses, or a Kruskal-Wallis' test with Dunn's Multiple Comparison as the post-test for non-parametric analyses. Significant differences were considered for $p < 0.05$.

Results and discussion

Protein particle and nucleic acid interactions and characterization

Here we demonstrate that protein particles, similar to the HCV nucleocapsid, obtained from a recombinant *P. pastoris* strain do interact with nucleic acids. Digestion with both DNAses and RNAses modified the behavior of the particles in sucrose gradients, although no changes in size and spherical morphology were observed. DNA and RNA molecules of different sizes were observed interacting with the protein particles. Particularly, RNA species of approximately 100 bp long were resistant to the treatment with nucleases, suggesting that they were encapsulated, and thus, non accessible. These encapsulated RNA molecules stabilize the already existing particles. Nucleic acid molecules that are un-encapsulated but associated to the particles contribute to particle stability. It is noteworthy, that the interaction between monomers of the HCV core protein and nucleic acids, even with those species that are no longer encapsulated, direct the accurate assembly of multimers [24]. Data from this and previous studies using the *P. pastoris* expression system [9, 25], provided evidence that may help to elucidate some aspects of the HCV nucleocapsid assembly mechanism. Various intermediate products of the HCcAg multimerization process may exist *in vivo*. The mature HCcAg predominantly interacts with cellular membranes where capsid assembly takes place. To a certain extent, the immature HCcAg may contribute to the HCc Ag multimerization intermediate products but does not support the correct assembly of nucleocapsids.

On the other hand, we demonstrated that a truncated variant of the HCV core, obtained from a recombinant

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E. coli strain, interacts with a plasmid for DNA immunization encoding for the HCV structural antigens. Interestingly, the buoyant density of the protein particles increased after adding the plasmid DNA. Moreover, delayed electrophoretic mobility of the protein-DNA samples were observed in agarose gels, indicating a direct interaction between the particles and the nucleic acids. The mixture of protein with plasmid DNA, or tRNA, induced the formation of larger-size and more heterogeneous particles than those observed in the absence of nucleic acids by electron microscopy [21].

Immunogenicity studies

The administration of mixtures composed of DNA immunization plasmids coding for HCV structural antigens and the HCV core protein in mice induced increased immune responses, compared to those triggered by the individual or alternate administration of their components. In fact, the administration of the protein-plasmid mixtures induced antibodies against the HCV core, E1 and E2 antigens, including antibodies against the HVR-I of different HCV isolates. Additionally, the antibodies elicited by these preparations recognized viral antigens isolated from the liver of HCV-infected patients. Moreover, strong lymphoproliferative responses specific against the HCV structural antigens were elicited. This evidence, together with the demonstration of controlled viremia in a surrogate challenge model with recombinant vaccinia viruses, shows the ability of these preparations to induce strong cellular immune responses [18].

Additionally, we demonstrated the enhancement of the antibody response (Figure 1A) against the HBcAg antigen after the administration, in mice, of mixtures of HBcAg particles and DNA immunization plasmids coding for HCV antigens [26]. Moreover, we demonstrated that preparations based on the mixture of the HBV core protein with a plasmid expressing the HCV structural antigens also showed enhanced immune antibody response (Figure 1B) against HCV antigens [26]. This result shows that the enhancement of the immune response can occur even if the recombinant viral protein is not encoded by the plasmid for DNA immunization, mixed with the protein. This result opens an important perspective for the future development of combined vaccines.

The putative mechanism for this enhanced immune response against the un-encoded recombinant protein antigen is based on evidence of the interaction between the components of the protein-DNA mixtures and their properties [1, 18]. DNA protection, facilitation of DNA uptake by antigen presenting cells, and contribution to immunogenicity due to the nature and size of the particle, are the probable features involved in the adjuvant action shown by HCV core particles. Additionally, CpG motifs in plasmid DNA may contribute to the adjuvant effect of the plasmid [1].

Conclusions

Here, we demonstrated for the first time that recombinant particles based on a viral core protein are able to interact with a plasmid for DNA immunization, forming molecular complexes devoid of antibodies or other molecules in their preparation. Additionally, new data on the morphology, size and enhanced stability of the protein particles in close association with nucleic acids were given. Finally, we also demonstrated the synergic enhancement of the immune response elicited by administering a mixture of recombinant protein particles based on a viral core protein and a plasmid for DNA immunization in mice.

The generation of new alternatives for the development of vaccine preparations is a priority in facing currently relevant health problems. This study is included in this global effort, opening the possibility of using recombinant viral capsids as the active principle and simultaneously as vehicles and/or adjuvants for DNA immunization plasmids. The results of this study may significantly contribute to the rational design of new vaccine preparations against several pathogens.

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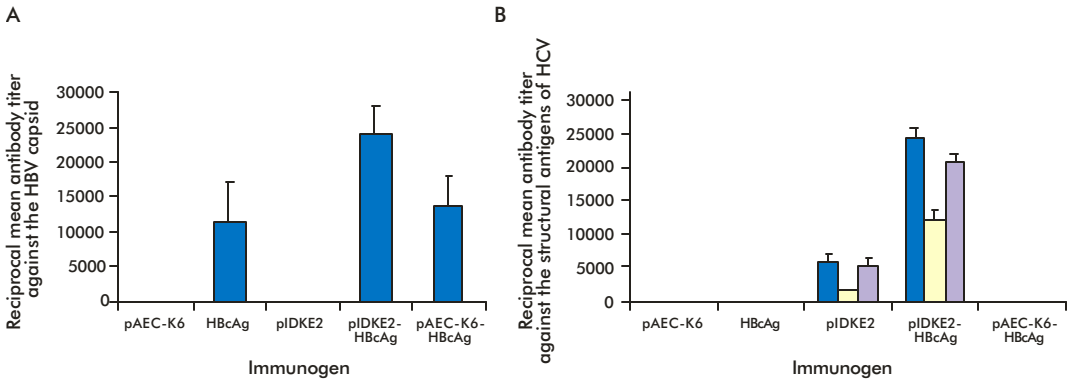

Figure 1. Immunization with plasmids for DNA immunization and the HBcAg protein, administered individually or mixed. Animals were immunized with 50 μg of DNA and 5 μg of protein by the intramuscular route. A. Antibody response against the HBV core. B. Antibody response against the HCV structural antigens. Results are shown as reciprocal mean antibody titers against the core (Blue bars), E1 (Yellow bars) and against E2 (Violet bars). HBcAg-HBV core antigen. pAEC-K6- plasmid for DNA immunization used as the negative control (mock). pIDKE2- plasmid for DNA immunization expressing HCV structural antigens.