

## Ultrastructural and Immunological Characterization of Hepatitis C Core Protein-DNA Plasmid Complexes

<sup>1</sup>Nelson Acosta-Rivero, <sup>1</sup>Yaraima Aguilera, <sup>1</sup>Viviana Falcon, <sup>1</sup>Joanna Poutou, <sup>1</sup>Alexis Musacchio  
<sup>1</sup>Liz Alvarez-Lajonchere, <sup>1</sup>Ivis Guerra, <sup>1</sup>Julio C. Alvarez-Obregón, <sup>1</sup>Yalena Amador-Cañizares  
<sup>1</sup>Gillian Martinez-Donato, <sup>1</sup>Jeny Marante, <sup>1</sup>Julio C. Aguilar, <sup>2</sup>Yordanka Soria, <sup>1</sup>Felix Alvarez  
<sup>1</sup>Angel Perez, <sup>1</sup>Maria C. de la Rosa, <sup>1</sup>Juan Morales, <sup>3</sup>Juan B. Kouri and <sup>1</sup>Santiago Dueñas-Carrera  
<sup>1</sup>Biomedicine Division, <sup>2</sup>Bioterio Department, Center for Genetic Engineering and Biotechnology  
P.O. Box 6162, C.P. 10600, C. Habana, Cuba  
<sup>3</sup>Centro de Investigación y de Estudios Avanzados (CINVESTAV-IPN), México City, México

**Abstract:** Recently, it has been shown that a truncated HCV core (HCcAg) variant, covering the first 120 aa (HCcAg.120), interacts with plasmid DNA vaccine (pIDKE2), encoding the HCV structural proteins (HCcAg, E1 and E2). In the present work, HCcAg.120-pIDKE2 complexes, forming heterogeneous packaged structures, were visualized using a negative stain/rotary shadow technique. Interestingly, 72 hours after intramuscular injection of HCcAg.120-pIDKE2 complexes in Balb/c mice, E2 protein was immunolabeled in muscle cells. In fact, HCcAg.120-pIDKE2 complexes induced anti-HCV humoral and cellular immune responses in mice when inoculated by both, parenteral or mucosal routes, although intranasal administration generally rendered weaker results. On the other hand, data demonstrated that Alum enhanced the HCV-specific IgG antibody production. However, the analysis of the HCV-specific cellular immune response showed that HCcAg.120-pIDKE2 delivered in PBS by the intramuscular route induced the strongest HCV-specific lymphoproliferative response, especially against E1 and induced viremia control in a vaccinia virus surrogate challenge model. These results support the use of HCcAg.120-pIDKE2 complexes in the rational design of therapeutic or preventive vaccine strategies against HCV infection.

**Key words:** Hepatitis C virus, Core antigen, DNA vaccine, immune response

### INTRODUCTION

Hepatitis C virus (HCV) infection is considered a major health problem affecting an estimated 170 million people worldwide<sup>[1]</sup>. Approximately 50 to 80% of HCV patients develop chronic hepatitis, which may be complicated by cirrhosis and/or hepatocellular carcinoma<sup>[1]</sup>. At present, there is no vaccine available to prevent HCV infection and current therapies are not optimal<sup>[2]</sup>. Thus, prophylactic and therapeutic approaches to prevent liver damage caused by HCV infection are urgently needed.

HCV is a member of the Flaviviridae family with a positive strand RNA of 9.6 kb<sup>[3]</sup>. The viral genome is translated into a single polyprotein of 3,000 amino acids in host cells. A combination of host and viral proteases are involved in polyprotein processing to give at least nine different proteins<sup>[4,5]</sup>. The structural proteins of HCV are believed to comprise the core protein (HCcAg) and two envelope glycoproteins: E1 and E2<sup>[5]</sup>.

Because HCV is an enveloped virus, neutralizing determinants likely reside on the surface of the

envelope. The envelope protein E2 of HCV contains highly variable sequences within the N-terminal region (HVR1), which are thought to contain neutralizing B-cell epitopes<sup>[6,7]</sup>. However, the role of neutralizing Abs in protective immunity against HCV infection is still uncertain<sup>[6]</sup>.

Studies in humans and chimpanzees have indicated that failure to generate multispecific cellular immune responses against HCV in the acute phase of infection is associated with chronicity<sup>[8,9]</sup>. Conversely, patients with strong HCV-specific cellular immune responses have lower levels of viremia and these responses correlate with recovery<sup>[10-12]</sup>. Of the cellular immune responses, the induction of Th1 type response, which has been linked to viral clearance in HCV, is probably important for a HCV vaccine<sup>[13]</sup>. Therefore, an ideal HCV vaccine may need to induce strong humoral responses against the envelope proteins and to prime broad HCV-specific T helper (Th) and cytotoxic T lymphocytes (CTL) responses<sup>[14]</sup>.

Previously, it has been reported that a recombinant C-terminal truncated HCcAg (HCcAg.120) interacts *in vitro* with a plasmid DNA vaccine (pIDKE2) leading to

**Corresponding Author:** Santiago Dueñas-Carrera, Biomedicine Division, Center for Genetic Engineering and Biotechnology, Ave. 31 e/158 y 190, P.O. Box 6162, C.P. 10 600, La Habana, Cuba, Tel: (53 7) 271 4764, Fax: (53 7) 271 4764

heterogenous virus-like particles (VLPs) formation<sup>[15]</sup>. In addition, HCcAg.120 has recently been shown to enhance the immune response elicited by pIDKE2 plasmid co-delivered in Balb/c mice<sup>[16]</sup>. In the present study, HCcAg.120-pIDKE2 complexes were characterized at the ultrastructural level. Besides, novel elements about the immunogenicity of these protein-DNA complexes, including the influence of adjuvant and administration route are described.

## MATERIALS AND METHODS

**Antigens and antibody:** Endotoxin-free HCcAg.120 was produced for research use only up to 90% of purity as previously described<sup>[17]</sup>. Recombinant truncated E1 (E1.339) and E2 (E2.680) proteins have been previously described<sup>[18,19]</sup>. Particulate recombinant small surface antigen of hepatitis B virus (HBsAg) was produced with more than 95% of purity at the CIGB production facilities (CIGB, Cuba)<sup>[20]</sup>. A mouse monoclonal antibody against the residues 580-600 of E2 (mAb SS-HepC.3) was used to detect E2.680 in immunoelectron microscopy experiments<sup>[21]</sup>.

**Plasmids:** pIDKE2<sup>[22]</sup> is a plasmid for DNA immunization containing the gene-fragment coding for the first 650 aa of the HCV polyprotein (including HCcAg, E1 and E2). pAEC-K6 plasmid<sup>[22]</sup> was used as negative control.

**Vaccinia viruses and cell lines:** The recombinant vaccinia virus vvRE, that expresses aa 1-650 of HCV polyprotein, is a vaccinia virus derived from the Western Reserve (WR) strain of vaccinia virus<sup>[23]</sup>. African green monkey kidney cells BSC-40<sup>[24]</sup> were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FBS and were used for determination of vaccinia virus titer in mice ovaries.

**HCcAg.120-pIDKE2 plasmid interaction:** *In vitro* protein-nucleic acid assembly reactions were carried out using conditions previously described<sup>[15]</sup>. Briefly, 5 µg of purified HCcAg.120 was mixed with an equal volume of pIDKE2 plasmid (50 µg). The reactions were incubated at 30 °C for 10 min followed by 15 min on ice.

**Transmission electron microscopy (TEM):** Either pIDKE2 plasmid or HCcAg.120-pIDKE2 complexes were processed for TEM using a negative stain/rotary shadow technique. Fifteen microliter drops of freshly prepared samples were placed on glow-discharged collodion/carbon-coated 400-mesh copper grids for 3 min. The solution was wicked off with filter paper and grids were rinsed in double-distilled H<sub>2</sub>O and allowed to dry. Rotary shadowing was performed using 1 in. of 0.008-inch Pt/Pd 80/20 wire at 12° angle. Grids were

imaged in a Jeol JEM 2000EX electron microscope operated at 80 kV.

**Immunoelectron microscopy (IEM):** The muscle tissue samples were fixed with 4% (v/v) paraformaldehyde containing 0.2% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) at 4 °C for 3 h and washed with 0.1 M phosphate buffer. Fixed cells were dehydrated as previously described<sup>[21]</sup>, embedded in Lowicryl and polymerized by exposure to ultraviolet light at room temperature for 72 h. Ultrathin sections of liver biopsies were incubated with anti-E2 mAb in phosphate buffer, for 45 min at RT. The sections were rinsed three times for 30 min at RT with 0.1% BSA in PBS (BSA-PBS) and incubated for 1 h at RT with gold-labeled anti-mouse IgG (Amersham, England) diluted 1:100 in BSA-PBS. As control, the primary antibody was substituted by normal mouse serum. All sections were stained with saturated uranyl acetate and lead citrate and examined with a JEOL/JEM 2000 EX transmission electron microscope (JEOL, Japan).

**Immunization schedule:** HCcAg.120-pIDKE2 complexes were used to induce specific immune responses in mice. On one hand, four groups of ten female Balb/c mice (6 to 8 weeks old) each, were immunized either intramuscularly (i.m.) or intranasal (i.n.) as follow: Group 1 was i.m. immunized with HCcAg.120-pIDKE2 (5 µg of HCcAg.120/50 µg of pIDKE2) in phosphate-buffered saline (PBS) solution (0.1 M NaCl, 2 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM KH<sub>2</sub>PO<sub>4</sub> pH 7.4), Group 2 was i.m. immunized with HCcAg.120-pIDKE2 adsorbed to Aluminum Hydroxide (Alum), Group 3 was i.n. immunized with HCcAg.120-pIDKE2 in PBS, Group 4 was i.m. immunized with 50 µg of pAEC-K6 adsorbed in Aluminum Hydroxide. All groups were boosted at 14 and 28 days after primary immunization. Serum samples were taken before immunization and on day 42 by retro-orbital puncture. Splenocytes from 5 mice per group were isolated on day 42. Five animals per group received two additional doses of the respective immunogen at weeks 6 and 12 after primary immunization. These mice were challenged with a recombinant vaccinia virus expressing HCV Core, E1 and E2 proteins, 2 weeks after the last immunization.

**Enzyme-linked immunosorbent assay (ELISA):** The 96-well microtiter plates (Costar, Cambridge, MA) were coated with 100 µL of either E1.339 (10 µg mL<sup>-1</sup>) or E2.680 (10 µg mL<sup>-1</sup>) diluted in coating buffer (50 mM carbonate buffer, pH 9.6) at 4°C overnight. After three washes with PBS containing 0.05 % Tween 20 (PBST), the wells were blocked with 100 µl of PBST containing 1 % skim milk at room temperature for 1 hour. Each well received 100 µl of the mice sera in PBST at serial two-fold dilutions (starting from 1:50) and the plates were incubated at 37°C for 1 hour.

Subsequent steps were performed as previously described<sup>[22]</sup>. The cut off value to consider a sample as positive was established as twice the mean absorbance (O.D) value of control mice sera (animals inoculated with pAEC-K6 adsorbed in Alum) from three determinations. Antibody titer was considered as the maximal serum sample dilution rendering a positive reaction in ELISA.

**Lymphoproliferation assay:** This assay has been described previously<sup>[25]</sup>. Briefly, pools of spleen cells from five immunized mice per group were cultivated in triplicate by using 96-well round-bottom plates at  $2 \times 10^5$  cells per well in 200  $\mu$ l of RPMI-1640 containing 10% heat-inactivated fetal calf serum (FCS), 2 mmol L<sup>-1</sup> L-glutamine, 50 units mL<sup>-1</sup> penicillin and 50  $\mu$ g mL<sup>-1</sup> streptomycin. Cells were stimulated with 5  $\mu$ g mL<sup>-1</sup> of either E1.339 or E2.680 in triplicate. As negative controls, effector cells were stimulated with either recombinant HBsAg or medium alone. Concanavalin A (5  $\mu$ g mL<sup>-1</sup>) was used as positive control. After stimulation for 5 days, [<sup>3</sup>H]thymidine was added (1  $\mu$ Ci/well). Cells were incubated for an additional 6 hours and the [<sup>3</sup>H]thymidine incorporation into DNA was measured after harvesting the plates. Lymphocyte stimulation index (SI) was calculated as the mean counts per minute (cpm) measured after antigen stimulation divided by the mean cpm of cells incubated with medium alone. A SI of >3 was considered as positive. Splenocytes from unimmunized mice were analyzed as controls and they all showed a SI of <1.5.

**Vaccinia virus titer in the ovaries of challenged mice:** At 15 days after the final protein-DNA complexes administration, mice (5 per group) were challenged intraperitoneally with  $10^6$  plate forming units (pfu) of vaccinia virus expressing HCV structural proteins (vvRE). Five days after the challenge, mice were sacrificed and the ovaries were removed, homogenized, sonicated and assayed for viral titer by serial 10-fold dilutions on a plate of BSC-40 indicator cells. After 2 days of culture, the medium was removed, the BSC-40 cell monolayer was stained with 1 % crystal violet (Merck, Darmstadt, Germany) for 10 min and the number of plaques per well was counted.

**Statistical procedures:** To compare differences among groups, a One-way ANOVA with the Newman-Keuls post-test or Kruskal-Wallis non-parametric statistic test with Dunn's Multiple Comparison Test as post-test were used.  $P < 0.05$  was considered significant.

## RESULTS

**TEM characterization of HCcAg.120-pIDKE2 complexes:** Either pIDKE2 plasmid (arrowheads) or HCcAg.120-pIDKE2 complexes (arrows) were visualized in TEM using the negative stain/rotary

shadow technique (Fig. 1). HCcAg.120-pIDKE2 complexes forming heterogeneous packaged structures were observed (Fig. 1, arrow). The size ranged between 80 nm and 300 nm.

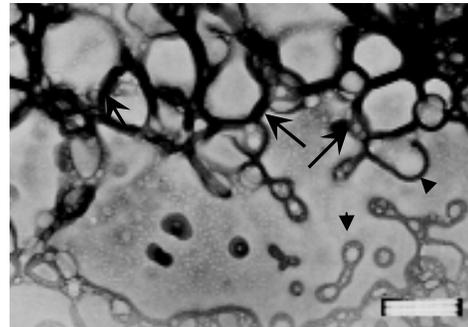


Fig. 1: Transmission electron microscopy image of HCcAg.120-pIDKE2 complexes using rotary shadowing technique. Arrows: HCcAg.120-pIDKE2 complexes, arrowhead: pIDKE2 plasmid. (Bar=200 nm)

**TEM characterization of HCV expression:** HCV expressing cells are shown in Fig. 2. Seventy-two hours after inoculation of HCcAg.120-pIDKE2, E2 protein was immunolabeled in muscle cells. In addition, VLPs were rarely observed. These particles were scarcely detected and immunolabeled with anti-E2 antibodies (Fig. 2B, arrows). No immunolabeling was observed in muscle cells from non-immunized mice (Fig. 2A).

**Effect of the administration route and adjuvant on the immunogenicity of HCcAg.120-pIDKE2 complexes:** To evaluate the effect of the immunization route and adjuvant on the immunogenicity of HCcAg.120-pIDKE2 in mice, HCcAg.120-pIDKE2 either in PBS or in Alum were i.m. or i.n. injected into Balb/c mice. Particularly, we focused on the immune response elicited against envelope antigens, since it is only generated after protein production occurs *in vivo* in cells incorporating functional pIDKE2 molecules.

**Total serum envelope-specific IgG response:** Presence of anti-envelope antibodies (against E1 and E2) in immunized mice was detected by an anti-envelope ELISA. The HCcAg.120-pIDKE2/Alum formulation induced the highest anti-E1 and anti-E2 IgG responses (Fig. 3). These responses showed significant differences compared to that detected in mice immunized with HCcAg.120-pIDKE2/PBS by the i.n. route. However, no significant differences were observed with respect to the group immunized with HCcAg.120-pIDKE2/PBS by the i.m. route (Fig. 3).

**Envelope-specific lymphoproliferative responses:** Strong anti-envelope proliferative responses were detected in splenocytes from all mice immunized with HCcAg.120-pIDKE2 (Fig. 4).

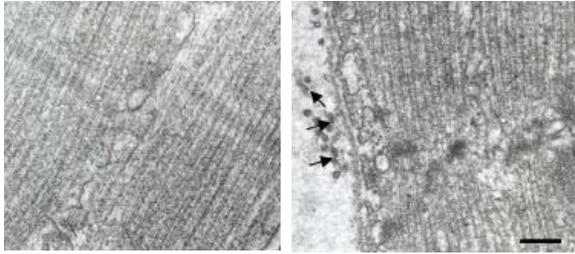


Fig. 2: Immunolabeling of E2 by IEM. A) Muscle cells from non-immunized mice, no immunostaining was observed using anti-E2 mAbs. B) Immunostaining with anti-E2 mAbs and gold-labeled anti-mouse IgG, detected E2 in muscle cells and VLPs. (Bar=200 nm)

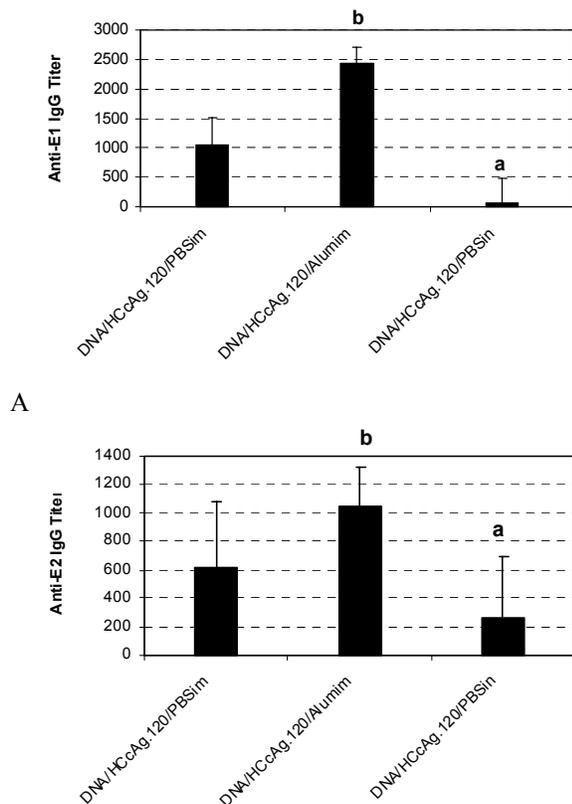


Fig. 3: Induction of humoral immune response by injection of HCCAg.120-pIDKE2 into Balb/c mice. Titters of either total E1-specific IgG (A) [b>a\*\*, (\*\*p<0.01)] or total E2-specific IgG (B) [b>a\*\*, (\*\*p<0.01)] response in sera of mice after immunization are shown. DNA: pIDKE2 plasmid; im: intramuscularly; in: intranasally. Results are shown as the reciprocal of the serum dilution equivalent to twice the mean optical density (O.D.492 nm) of negative control. Errors bars indicating the standard errors of the means are shown. Stat: \*\* Kruskal-Wallis non-parametric statistic test with Dunn's Multiple Comparison Test as post-test were used. P<0.05 was considered significant

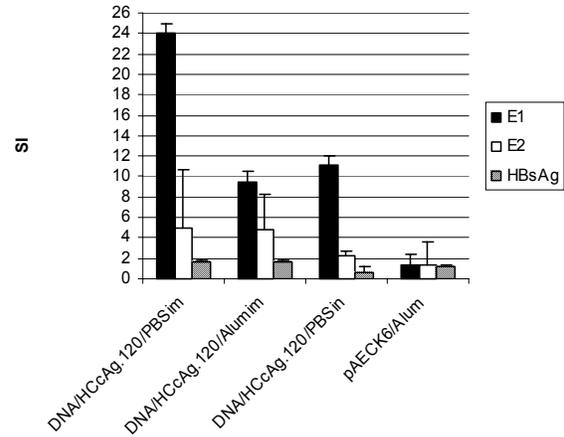


Fig. 4: Proliferative responses of splenic lymphocytes from immunized mice. DNA: pIDKE2 plasmid; im: intramuscularly; in: intranasally. Data are shown as the mean SI. Errors bars indicating the standard errors of the means are shown. SI of  $\geq 3$  is considered positive. Groups are indicated on the x-axis

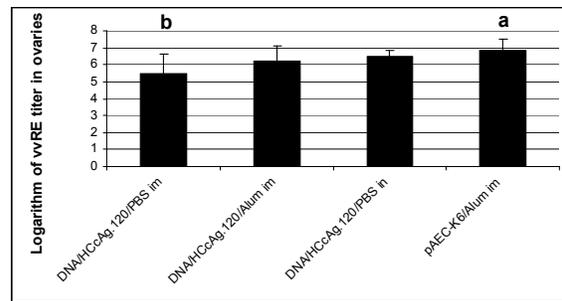


Fig. 5: Viremia control in a surrogate vaccinia virus challenge model. Results are shown as the mean logarithm of vvRE, a vaccinia virus expressing HCV structural antigens, in ovaries of immunized mice. Error bars indicate the standard deviation of the mean from 5 individual mice. Stat: \* One-way ANOVA with the Newman-Keuls post-test were used. P<0.05 was considered significant. b<a\*\*, (\*\*p<0.01)

Note that there was a trend for the anti-E1 proliferative response to be higher than the anti-E2 proliferative response in all groups of immunized mice. In mice immunized with HCCAg.120-pIDKE2/PBS by the i.n. route, anti-E1 but not anti-E2 proliferative responses were detected. Remarkably, the anti-E1 proliferative response obtained in mice i.m. immunized with HCCAg.120-pIDKE2/PBS was at least two fold higher than that observed in the other groups of mice (Fig. 4).

**Viremia control in a vaccinia virus surrogate challenge model:** Two weeks after the final immunization, five animals per group were challenged with a recombinant vaccinia virus expressing the HCV structural proteins. Only the administration of

HCcAg.120-pIDKE2/PBS by i.m. route could significantly contribute to viremia control in challenged mice (Fig. 5). Viral titer in ovaries of these mice was 1.5 log lower ( $p < 0.01$ ) than the value detected in negative control animals (pAEC-K6/Alum group), although no statistical differences were observed with respect to groups immunized with HCcAg.120-pIDKE2 in Alum or administered in PBS by i.n. route.

## DISCUSSION

Recently, it has been shown that a truncated HCcAg variant, comprising the first 120 aa (HCcAg.120) of this antigen, with a 32 aa N-terminal fusion peptide (6xHistag-XpressTMepitope), interacts with nucleic acids<sup>[15]</sup>. In fact, *in vitro* interaction between HCcAg.120 and pIDKE2 plasmid has been previously demonstrated by delayed electrophoretic mobility. Formation of large and heterogenous VLPs, characterized by a buoyant density of 1.30-1.34 g mL<sup>-1</sup>, has been described<sup>[15]</sup>.

In the present study, HCcAg.120-pIDKE2 complexes were visualized by TEM, showing the rounded shape of these complexes. In addition, HCV expression *in vivo* was monitored by TEM. Immunolabeling of E2 indicates early expression of HCV proteins in muscle cells, after protein-DNA complexes inoculation in mice. Besides, VLPs with an average size of 40 nm were immunolabeled with anti-E2 antibodies indicating the presence of E2 on the surface of such structures. However, it was very difficult to detect these particles, suggesting that the assembly process might be inefficient.

The HCV envelope proteins are believed to contain important epitopes<sup>[7]</sup>. Therefore, many studies have focused on the induction of anti-envelope humoral and cellular immune responses in mice and monkeys<sup>[19,22,26,27]</sup>. Our data show that HCcAg.120-pIDKE2 complexes were immunogenic *in vivo*, inducing strong anti-HCV humoral and T cell proliferative immune responses, directed against envelope antigens. This is in agreement with a previous work, showing that mixtures of HCcAg and pIDKE2 elicited stronger and more diverse humoral and cellular immunity than the immunization with the individual components in Balb/c mice<sup>[16]</sup>. This kind of mixture also controlled the infection after the challenge with a recombinant vaccinia virus expressing the HCV core<sup>[16]</sup>. In the present work, i.m. injection of HCcAg.120-pIDKE2 complexes induced viremia control after challenge with a recombinant vaccinia virus expressing HCV Core, E1 and E2.

It should be noted that HCcAg.120-pIDKE2 administration by the i.n. route showed a trend to be less immunogenic than inoculation of HCcAg.120-pIDKE2 by the i.m. route. Probably, immune response against antigens encoded by pIDKE2 is not correctly activated in mucosa since a previous study has

demonstrated that individual administration of HCcAg.120 protein by the i.n. route induces strong immune responses in Balb/c mice<sup>[20]</sup>.

On the other hand, results shown here demonstrated that Alum enhanced the HCV-specific IgG antibody production after HCcAg.120-pIDKE2 administration in mice. However, the analysis of the HCV-specific cellular immune response showed that HCcAg.120-pIDKE2 complexes delivered in PBS by the i.m. route induced the strongest HCV-specific lymphoproliferative response, especially against E1. This result might be suggesting that formulation of protein-DNA complexes in conjunction with Alum can not only enhance the specific anti-HCV antibody response, but also influence the bias of the immune response to a Th2 pattern. Previously, Kwissa *et al.* showed that a CTL response was not elicited when AIPO4 was used in formulations containing protein and a DNA vaccine<sup>[28]</sup>. This is consistent with the observed failure in controlling vaccinia virus infection, in the surrogate challenge model, after administration of HCcAg.120-pIDKE2 formulated in Alum, in contrast to inoculation in PBS. Indeed, several evidences indicate that in the vaccinia virus challenge model, protective response is mainly mediated by the cellular immune response induced against the antigen shared by the virus and the immunogen used<sup>[29-31]</sup>.

Several human studies have presented evidence that a strong HCV-specific CD4+ T cell proliferative response is associated with either viral clearance in acute hepatitis C<sup>[32-34]</sup> or in chronic HCV infection successfully treated with IFN<sup>[35]</sup> or with a benign carrier state<sup>[36,37]</sup>. The importance of HCV specific CD4+ T cell proliferative response in viral control has also been demonstrated in patients with HCV recurrence after loss of virus-specific CD4+ T cell response<sup>[33]</sup>. In general, persons able to mount a strong cellular immune response are more likely to experience viral clearance<sup>[13,37,38]</sup>. Therefore, our findings that HCcAg.120-pIDKE2 complexes can induce not only anti-HCV envelope antibodies, but strong cellular immunity, suggest their potential use as components of HCV vaccine candidates. Moreover, the ability of HCV Core protein for binding nucleic acids and forming protein-DNA complexes might be particularly useful for the development of DNA vaccine formulations against different pathogens.

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