Recombinant *in vitro* assembled hepatitis C virus core particles induce strong specific immunity enhanced by formulation with an oil-based adjuvant

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ABSTRACT

In the present work, immunogenicity of recombinant *in vitro* assembled hepatitis C virus core particles, HCcAg.120-VLPs, either alone or in combination with different adjuvants was evaluated in BALB/c mice. HCcAg.120-VLPs induced high titers of anti-HCcAg.120 antibodies and virus-specific cellular immune responses. Particularly, HCcAg.120-VLPs induced specific delayed type hypersensitivity, and generated a predominant T helper 1 cytokine profile in immunized mice. In addition, HCcAg.120-VLPs prime splenocytes proliferate *in vitro* against different HCcAg.120-specific peptides, depending on either the immunization route or the adjuvant used. Remarkably, immunization with HCcAg.120-VLPs/Montanide ISA888 formulation resulted in a significant control of vaccinia virus titer in mice after challenge with a recombinant vaccinia virus expressing HCV core protein, vvCore. Animals immunized with this formulation had a marked increase in the number of IFN- γ producing spleen cells, after stimulation with P815 cells infected with vvCore. These results suggest the use of recombinant HCV core particles as components of therapeutic or preventive vaccine candidates against HCV.

Key terms: challenge; HCV; immunity, adjuvant, capsid.

INTRODUCTION

Hepatitis C virus (HCV) is a leading cause of chronic liver disease, with over 170 million people infected worldwide. Complications from chronic hepatitis C infection include cirrhosis, hepatic decompensation, and hepatocellular carcinoma (Strader et al., 2004). To date, there is no effective vaccine against HCV infection and current therapies are not optimal (Pol and Bourliere, 2006). Thus, prophylactic and therapeutic approaches to prevent HCV-related liver disease are urgently needed.

HCV is a member of the genus Hepacivirus, belonging to the family Flaviviridae. The viral genome is a single positive-stranded RNA encoding for a large polyprotein (Choo et al., 1989). Among the viral structural proteins, the core protein (HCcAg) is considered the main protein component of nucleocapsids. Remarkably, HCV not only exists as enveloped virions but also as free, non-enveloped nucleocapsids in the sera of HCV infected patients (Ishida et al., 2001; Maillard et al., 2001).

The immunologic correlates of protective immunity, viral clearance and

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liver damage are not fully understood. The role of neutralizing antibodies in protective immunity against HCV infection is still uncertain (Zeisel et al., 2007, Dreux and Cosset, 2007). However, the envelope protein E2 is believed to contain important neutralizing epitopes (Youn et al., 2005). On the other hand, studies in humans and chimpanzees have indicated that failure to generate broad-specific cellular immune responses against HCV in the acute phase of infection is associated with chronicity (Timm and Roggendorf, 2007, Rushbrook et al., 2007). Indeed, multi-epitopic HCV-specific antiviral CD8+ and CD4+ T cell responses with a T helper 1 (Th1) profile of cytokine production has been reported in patients with self-limited infection (Lechner et al., 2000, Thimme et al., 2001, Gramenzi et al., 2005). Thus, the ability to mount an efficient cellular immune response has been proposed as an important mechanism involved in the control of HCV infection, whereas a defect of such responses leads to the chronic stage (Neumann-Haefelin et al., 2007). Therefore, a candidate HCV vaccine should mainly prime broad HCV-specific T helper (Th) and cytotoxic T lymphocytes (CTL) responses (Shiina and Rehmann, 2006).

One of the challenges facing the development of HCV vaccine is their extensive genetic variation (Moreau et al., 2008). HCcAg is among the most conserved proteins in HCV (Bukh et al., 1994) and is known to induce sensitization of both CD4+ and CD8+ lymphocytes (Lechner et al., 2000). Therefore, HCcAg could constitute an important vaccine component.

Virus-like particles (VLPs) are attractive as a recombinant protein vaccine because they have proved to be efficient in generating powerful immune responses (Lechmann et al., 2001, Storni et al., 2003). Previously, it has been reported that recombinant VLPs containing a C-terminal truncated HCcAg (HCcAg.120) induces a potent anti-HCcAg.120 immune response in mice (Álvarez-Obregon et al., 2001, Aguilar et al., 2003). These recombinant VLPs induce a Th1 biased cell response (Álvarez-Obregon et al., 2001) and resemble those observed for hepatitis B

virus core antigen, which induce strong mucosal immunogenicity due to their size, shape and stability, enabling antigen sampling and transport by M cells from nasopharyngeal epithelium to immune-inductive lymphoid tissues (Whinter and Innes, 1994, Aguilar et al., 2003). However, these VLPs contain bacterial nucleic acids (Lorenzo et al., 2001), which are known to be strong adjuvants that induce a Th1-biased cell response (Riedl et al., 2002). Recently, it has been shown that HCcAg.120 assembles *in vitro* into homogeneous VLPs (HCcAg.120-VLPs) in the absence of nucleic acids (Acosta-Rivero et al., 2005b). Interestingly, addition of nucleic acids to assembly reactions led to heterogenous VLPs formation (Acosta-Rivero et al., 2005).

In the present study, we reveal the icosahedral nature of HCcAg.120 *in vitro* assembled VLPs. Additionally, we characterize the immune response elicited in mice after administration of these HCcAg.120-VLPs, either alone or in combination with different adjuvants. Particularly, we have chosen Aluminum hydroxide and an oil-based adjuvant for favouring Th-2 or a balanced Th-1/Th-2 profile (Roohvand et al., 2007, Bungener et al., 2008), respectively, in order to comparatively study the ability for generating both humoral and cellular immune response against HCcAg.120-VLPs. Results presented here raise the possibility of taking advantage of particulate state and adjuvant formulation for increasing HCV core immunogenicity and avoiding or reversing any immunosuppressive effect potentially related to this antigen.

METHODS

Antigens and peptides

Endotoxin-free HCcAg.120, *in vitro* assembled into VLPs of approximately 30 nm, in the absence of nucleic acids, was produced for research use up to 90% of purity as previously described (Acosta-Rivero et al., 2005b). Particulate

recombinant small surface antigen of hepatitis B virus (HBsAg) was produced with more than 95% of purity (Aguilar et al., 2003) at the CIGB production facilities (CIGB, Cuba).

The following peptides (A1, A2 and A3), corresponding to HCcAg sequence from a Cuban isolate genotype 1b, were used for *in vitro* studies. Peptide A1, (aa 5-35) PKPQRKTKRNTNRRPQDVKFPGGGQIVGGVY; Peptide A2, (aa 51-70) KTSERSQPRGRRQPIKVR; Peptide A3, (aa 110-131) TDPRRRSRNLGKVIDTLTGFA. These peptides were produced by the Department of Peptide Synthesis (CIGB, Ciudad de La Habana, Cuba).

Human sera and viruses

Human sera have been previously employed (Álvarez-Lajonchere et al., 2002). Briefly, 129 patient's sera serologically positive to third-generation HCV enzyme immunoassays and positive for HCV RNA in serum by Reverse-Transcription Nested Polymerase Chain Reaction (RT-PCR) were used. In addition, 68 sera negative for HCV markers were used as negative control. All sera were negative for markers of hepatitis B virus (Tecnosuma International, Havana, Cuba).

The recombinant vaccinia virus vvCore (HCcAg-vaccinia), containing the gene-fragment coding for the first 176 aa of the HCcAg, has been previously described (Álvarez-Lajonchere et al., 2008).

Animals and immunization schedules

BALB/c (H-2d) female mice of 6 to 8 weeks old, 18-20 g of weight, were purchased from CENPALAB (Ciudad de la Habana, Cuba) and used for all *in vivo* evaluations. Maintenance and studies in mice were carried out according the Guiding Principles in the Care and Use of Laboratory Animals, endorsed by the American Physiological Society.

HCcAg.120-VLPs were used to induce specific immune responses in mice. Three groups of ten mice each were immunized intramuscularly (i.m.) as follow: Group 1 was i.m. immunized with phosphate-

buffered saline solution (PBS) (0.1 M NaCl, 2 mM KCl, 10 mM Na₂HPO₄, 1 mM KH₂PO₄ pH 7.4) (control group), Group 2 was i.m. immunized with 5 µg of HCcAg.120-VLPs in PBS, Group 3 was i.m. immunized with 50 µg of HCcAg.120-VLPs in PBS. All groups were boosted at 14 and 28 days after primary immunization. Delayed type hypersensitivity (DTH) response was assayed 21 days after the last immunization.

On the other hand, five groups of ten mice each were immunized either i.m. or intranasally (i.n.) as follow: Group 1 was i.m. immunized with 10 µg of HCcAg.120-VLPs in PBS, Group 2 was i.m. immunized with 10 µg of HCcAg.120-VLPs adsorbed (1:40, w/w) to Aluminum hydroxide (Alhydrogel®, Superfos Biosector, Kvistgard, Denmark), Group 3 was i.m. immunized with 10 µg of HCcAg.120-VLPs formulated (1:1, volume/volume) with Montanide ISA888 (SEPPIC, Paris, France), Group 4 was i.n. immunized with 10 µg of HCcAg.120-VLPs in PBS, Group 5 was i.m. immunized with Montanide ISA888 (control group). In addition, groups of ten mice each, were immunized i.m. as follow: Group 1 was immunized with 10 µg of HCcAg.120-VLPs in PBS, Group 2 was immunized with 10 µg of HCcAg.120-VLPs mixed with Montanide ISA888, Group 3 was immunized with Montanide ISA888 (control group). 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 were isolated on day 42.

Delayed Type Hypersensitivity (DTH) response

DTH response induced by HCcAg.120-VLPs was assayed by the footpad-swelling test 21 days after the third immunization, essentially as previously described (Acosta-Rivero et al., 2004). Briefly, ten mice were injected with 5 µg of HCcAg.120-VLPs into the right footpads. As a negative control, HBsAg was injected into the left footpads. The swelling response was measured using a calliper (Mitutoyo,

Kawasaki, Japan) 72 h after injection. The results are expressed as the mean difference between the measures in right and left footpads.

Enzyme-linked immunosorbent assay (ELISA)

The 96-well microtiter plates (Costar, Cambridge, USA) were coated with 100 μ L of HCcAg.120-VLPs (10 μ g/mL) 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 μ L of PBST containing 1 % skim milk at room temperature for 1 hour. Wells were washed three times with PBST and received 100 μ L of either mice or human sera at serial two-fold dilutions (1:50 up to 200,000) in PBST. The plates were incubated at 37°C for 1 hour. Subsequent steps were performed as previously described (Álvarez-Obregon et al., 2001). In the particular case of IgG1 or IgG2a subclass determination, two specific biotinylated monoclonal antibodies for IgG1 and IgG2a (Amersham, Little Chalfont, UK) were used at 1: 3,000 dilution. After one-hour incubation, a second conjugate of streptavidine and peroxidase (Amersham, UK) was used at 1:3,000 dilution in 100 μ L.

The antibody titers against HCcAg.120-VLPs are determined as the highest serum dilution giving twice the mean absorbance ($A_{492\text{nm}}$) value of negative controls (either control mice sera or sera from healthy individuals).

Lymphoproliferation assay

Lymphoproliferation assay was carried out as described previously (Álvarez-Obregon et al., 2001). Briefly, pools of spleen cells from five immunized mice per group were cultivated in triplicate by using 96-well round-bottom plates at 2×10^5 cells per well in 200 μ L of RPMI-1640 containing 10% heat-inactivated fetal calf serum (FCS), 2 mmol/liter L-glutamine, 50 units/mL penicillin, and 50 μ g/mL streptomycin. Cells were stimulated with 5 μ g/mL of

HCcAg.120-VLPs in triplicate. As negative controls, effector cells were stimulated with either recombinant HBsAg or medium alone. Concanavalin A (5 μ g/mL) was used as positive control. After stimulation for 5 days, [^3H]thymidine was added (1 μ Ci/well). Cells were incubated for an additional 10 hours and the [^3H]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 HBsAg. A SI ≥ 3 was considered as positive. Splenocytes from non-immunized mice were analyzed as controls, and they all showed a SI < 1.5 .

Analysis of Cytokine Production

Levels of interleukin 4 (IL-4) and interferon gamma (IFN- γ) in spleens cells from three immunized mice per group were determined with commercial kits from R&D Systems (Minneapolis, MN, U.S.A.), following the manufacturer's specification, as previously described (Dueñas-Carrera et al., 2002). The detection thresholds of IL-4 and IFN- γ were 7.8 and 9.4 pg/mL, respectively. Splenocytes were stimulated with 5 μ g/mL HCcAg.120-VLPs. Concanavalin A (5 μ g/mL) was used as mitogen for positive control of stimulation.

Assessment of antiviral immunity in vivo

To examine antiviral immunity, five immunized BALB/c mice per group were infected intraperitoneally (i.p) with 10^6 plaque-forming units (pfu) of either vvCore or WR strain of vaccinia virus one week after the last immunization. Five days later, ovaries were collected and the vaccinia titers were determined on BSC-40 cells as previously described (Álvarez-Lajonchere et al., 2006).

Ex vivo direct Enzyme-linked Immunospot (ELISPOT) assay for IFN- γ

ELISPOT assay for detecting IFN- γ secreting cells specific for HCV core

epitopes has been previously described (Dueñas-Carrera et al., 2002). Here, nitrocellulose-backed 96-well plates (MAHA S45; Millipore, Bedford, MA, USA) were coated with 100 μ L of 5 μ g/mL murine IFN- γ -specific mAb R46A2 (Pharmingen, San Diego, CA, USA) overnight at 4°C, washed three times with PBS and blocked using complete medium at 37°C for 1 h. Freshly isolated splenocytes from five immunized mice per group (2×10^5 to 10^6) were added to the wells in duplicates and stimulated with inactivated (25 μ g/ml of mitomycin C (Sigma) for 30 min at 37°C) 10^5 of P815 cells infected for 1 hour at 37°C with vvCore at a multiplicity of infection (m.o.i.) of 10. As negative control, inactivated (mitomycin C treated) 10^5 P815 cells infected with WR vaccinia virus were added, and as a positive control, Concanavalin A (Sigma-Aldrich) was added at a concentration of 5 μ g/mL. Cells were incubated for 20 h at 37°C in 5% CO₂. The plates were washed three times with PBS and five times with 0.05% Tween 20 in PBS (PBST), and then 0.5 μ g/mL of secondary biotin-conjugated antibody XMGI.2 (Pharmingen) was added and reacted at room temperature for 2 h. The wells were washed five times with 0.05% PBST and peroxidase-labelled streptavidine (Sigma) was added at a dilution of 1:1,000 for 1 h. The wells were washed again with 0.05% PBST and PBS and the spots were developed by adding 3,3'-diaminobenzidine (3,3',4,4'-tetraaminobiphenyl) tetrahydrochloride (Sigma) in 50 mmol/L Tris-HCl, pH 7.4, with 0.3% H₂O₂. After 15 min the wells were washed with tap water, dried and spot counted using a dissection microscope. The numbers of antigen-specific SFU per well were calculated by subtracting background values (stimulation with P815 cells infected with WR vaccinia virus).

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. To

compare differences between two groups the Mann-Whitney *U* test (two-tailed) was employed. $P < 0.05$ was considered significant.

RESULTS

Recognition of HCcAg.120-VLPs by sera from HCV-infected patients

The capacity of HCcAg.120-VLPs to react with anti-HCV antibodies present in serum from individuals infected with HCV was then analyzed (Figure 1). 119 out of 129 (92.2%) sera from HCV-infected patients showed specific reactivity against HCcAg.120-VLPs. On the other hand, 68 out of 68 (100%) sera from healthy individuals were not immunoreactive against HCcAg.120-VLPs.

DTH response

As shown in Figure 2, HCcAg.120-VLPs induced a DTH response specific for HCcAg.120. This DTH response was higher in mice immunized with 50 μ g of HCcAg.120-VLPs than in those immunized with 5 μ g of the recombinant protein. Antigen specificity of the swelling reaction was tested in PBS immune mice, where no swelling was induced after injection of HCcAg.120-VLPs.

Humoral immune response

To further evaluate the capacity of HCcAg.120-VLPs to induce a specific immune response in mice, HCcAg.120-VLPs either in PBS or in Alum or in Montanide ISA888 were injected into Balb/c mice. After the initial immunization and 2 boosts, the sera were analyzed for anti-HCcAg.120 antibodies. As shown in Figure 3A, mice immunized with HCcAg.120-VLPs formulated in Alum or in Montanide ISA888 but not in PBS, exhibited a strong HCcAg.120-specific serum IgG antibody response. No statistically significant differences were observed among groups immunized with HCcAg.120-VLPs, although HCcAg.120-specific serum IgG titers

Figure 1: Recognition of HCcAg.120-VLPs by anti-HCV human sera. Sera from 129 HCV RNA positive patients and 68 individuals negative to HCV markers were analyzed by ELISA. Results are expressed as Reciprocal antibody titer against HCcAg.120-VLPs, determined as the highest serum dilution giving twice the mean absorbance (A_{492nm}) value of negative controls (sera from healthy individuals).

Figure 2: Induction of DTH by injection of HCcAg.120-VLPs into the footpads of immunized mice. Mice were immunized with either 5 µg or 50 µg of HCcAg.120-VLPs. 21 days after the last immunization, 5 µg of HCcAg.120-VLPs were injected into the hind right footpads subcutaneously. As a negative control, HBsAg was injected into the left footpads. Error bars indicating the standard errors of the means are shown. Stat: One-way ANOVA with the Newman-Keuls post-test ($b, c > a^{***}$, $p < 0.001$; $c > b^*$, $p < 0.05$).

Figure 3: Induction of humoral immune responses by injection of HCcAg.120-VLPs into BALB/c mice. Antibody titers, detected in mice sera after immunization, of either total HCcAg.120-specific IgG (A) or HCcAg.120-specific IgG1 [b>a*, p<0.05] (B) or HCcAg.120-specific IgG2a [c>b*, p<0.05; c>a**, p<0.01] (C) are shown. 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. (HCcAg.120/PBS, +Alum, +MontISA888 correspond to groups of mice i.m. immunized with HCcAg.120-VLPs in PBS, Alum and Montanide ISA888, respectively); (+PBSin corresponds to group of mice i.n. immunized with HCcAg.120-VLPs in PBS).

obtained after administration of HCcAg.120-VLPs+Montanide ISA888 reached values above 1:80,000 (Fig 3A). Mice receiving only Montanide ISA888 (negative controls) showed no specific antibody response against HCcAg.120-VLPs (statistically significant differences with respect to the other groups, $p < 0.05$, Kruskal-Wallis non-parametric statistic test with Dunn's Multiple Comparison Test as post-test)

Specific antibodies induced by HCcAg.120-VLPs were also explored by the evaluation of the major IgG subclasses in sera for all the groups involved in the experiment. A statistically significant improvement in the HCcAg.120-specific serum IgG1 titers was observed in mice i.m. immunized with HCcAg.120-VLPs+Montanide ISA888 compared to mice i.n. immunized with HCcAg.120-VLPs/PBS (Fig 3B, $p < 0.05$, Kruskal-Wallis non-parametric statistic test with Dunn's Multiple Comparison Test as post-test). However, no significant differences were observed among i.m. immunized groups of mice. Interestingly, the HCcAg.120+Montanide ISA888 formulation induced the highest anti-HCcAg.120 IgG2a response (Fig 3C $p < 0.05$, Kruskal-Wallis non-parametric statistic test with Dunn's Multiple Comparison Test as post-test).

Proliferative responses of spleen cells

As shown in Figure 4A, HCcAg.120-VLPs immunization induced proliferative responses specific for HCcAg.120 in all immunized animals. However, no lymphoproliferative response was observed in the control group. Responses were independent of the type of adjuvant used or the inoculation route. In addition, when a control preparation of HBsAg was used to stimulate splenocytes *in vitro*, no proliferation was observed, indicating that the detected proliferative responses were specific for HCcAg.120-VLPs.

In addition, a set of non-overlapping synthetic peptides spanning aa 5-131 of HCcAg were tested for the ability to stimulate splenocytes *in vitro*. Interestingly, immunization of mice by i.n. route induced a lymphoproliferative response specific for peptide A2, whereas mice immunized with

HCcAg.120-VLPs/PBS by i.m. route did not show proliferative responses against any of the peptides evaluated (Fig 4A). On the other hand, mice immunized with antigen in Alum showed a similar proliferative response against peptide A2. It is interestingly to note that vaccination of mice with HCcAg.120-VLPs/Montanide ISA888 formulation induced a lymphoproliferative response against peptide A1 but not against peptides A2 and A3. No proliferative responses were observed against peptide A3, possibly because part of this peptide comprises a region (aa 120-131) that is absent in HCcAg.120.

In vitro levels of Interferon gamma and IL-4

On the other hand, splenocytes from BALB/c mice i.m. immunized with HCcAg.120-VLPs either in PBS or in Montanide ISA888 were harvested after the third immunization and stimulated with recombinant HCcAg.120-VLPs as described above. Analysis of IFN- γ levels in the culture supernatants revealed that HCcAg.120-VLPs induced IFN- γ secretion specific for HCcAg.120 in 2 of 3 mice (329 and 723 pg/mL) (Fig 4B). In addition, HCcAg.120-VLPs/Montanide ISA888 formulation induced high levels of IFN- γ in 2 of 3 mice (1,811 and 2,084 pg/mL). In control-immunized animals, we detected no production of IFN- γ . On the other hand, we were able to detect IL-4 (277 ± 31 pg/mL) only in the culture supernatants of splenocytes from mice inoculated with HCcAg.120-VLPs+Alum. When a control preparation of HBsAg was used to stimulate splenocytes *in vitro*, no cytokine production was observed (data not shown), indicating that responses were specific for HCcAg.120-VLPs.

Control of infection in a surrogate challenge model

To assess the biological relevance of the immunity induced by vaccination with HCcAg.120-VLPs formulations, we performed challenge experiments in which either a recombinant vaccinia virus

Figure 4: Induction of cellular immune responses by injection of HCcAg.120-VLPs into BALB/c mice. A) Proliferative responses of splenic lymphocytes from immunized mice against different antigens. A1, A2 and A3 are synthetic peptides comprising HCV core sequences corresponding to a HCV genotype 1b Cuban isolate. HCcAg.120 is a recombinant protein comprising the first 120 aa of HCV core antigen. HBsAg is a recombinant variant of HBV surface antigen. Spleens were pooled from five animals of each group. Data are shown as the mean Stimulation Index (SI). Error bars indicating the standard errors of the means are shown. SI ≥ 3 was considered positive. B) IFN- γ production by splenocytes from immunized mice after stimulation with HCcAg.120-VLPs. Cytokine levels were measured by ELISA in the supernatant of splenocytes activated with HCV structural proteins. Data represent the mean levels of IFN- γ $\pm$ SEM. ($P > 0.05$) no significant differences were found. Stat: Kruskal-Wallis non-parametric statistic test with Dunn's Multiple Comparison Test as post-test were used. HCcAg.120/PBS, +Alum, +MontISA888 correspond to groups of mice i.m. immunized with HCcAg.120-VLPs in PBS, Alum and Montanide ISA888, respectively; +PBSin corresponds to group of mice i.n. immunized with HCcAg.120-VLPs in PBS; MontISA888 corresponds to group of mice i.m. immunized with Montanide ISA888.

expressing HCcAg (vvCore) or WR vaccinia strain were inoculated into immunized BALB/c mice. As shown in Figure 5A, mice immunized with VLPs/Montanide ISA888 formulation showed a reduction of vvCore viral titers in their ovaries (pools of ovaries from each group). The reduction in viral titers between HCcAg.120/Montanide ISA888 and the other groups of mice was greater than $\log_{10}=3$ (Fig 5A). We also investigated the viral titer in pools of ovaries after challenge with WR vaccinia virus in animals immunized with HCcAg.120-VLPs either in PBS or in Montanide ISA888 compared with the corresponding negative controls. No reduction in titer of WR ($>1 \log_{10}$) was observed (Fig 5A).

Based on these results, viral titers in ovaries from each mouse immunized with either HCcAg.120/Montanide ISA888 or Montanide ISA888 as control were determined. Remarkably, the mean viral titer in mice immunized with HCcAg.120/Montanide ISA888 was significantly reduced as compared to that observed in control-immunized mice after vvCore challenge (Fig 5B). Furthermore, three of the five HCcAg.120/MontanideISA888 immunized mice had no detectable virus after challenge with vvCore. The other two animals in this group also had several logs of reduction in viral titers as compared with the control group (Fig 5B).

As a potential model for the induction of immunity against recombinant vaccinia virus infection, it is important to show that VLPs-mediated immunization also stimulates recognition of endogenously processed viral antigens. To that end, P815 target cells were infected with vvCore. P815 cells infected with WR vaccinia virus were used as control. By using this approach a significant increase in the vvCore-specific IFN- γ producing splenocytes was obtained in HCcAg.120/MontanideISA888 immunized mice compared with the control group (Fig 5B).

DISCUSSION

HCV core antigen seems to be a relevant target in the efforts for developing

therapeutic and preventive strategies against this pathogen. Previously, it has been reported that HCcAg.120 assembles *in vitro* into VLPs in the absence of nucleic acids (Acosta-Rivero et al., 2005b). In the present study, the presence of antibodies directed to HCcAg.120-VLPs was detected in a high percent of HCV infected patients.

Three intramuscular injections of HCcAg.120 (3, 6 or 12 μg) every 21 days to BALB/c and C57BL6 mice have previously induced specific immune response (Álvarez-Obregon et al., 2001). Thus, here we evaluated two different antigen amounts, 5 and 50 μg , and a reduced interval between doses. Particularly, we tested 50 μg in order to evaluate whether the use of this relatively high amount of protein antigen increases the immunogenicity or causes a non-responsive status in animals due to T cell exhaustion, as previously described for other antigens (Martinez-Donato et al., 2007). We choose a two-week interval between doses as has been previously employed successfully for other HCV core protein variants (Harase et al., 1995). In fact, HCcAg.120-VLPs induced a strong HCcAg.120-specific humoral immune response in BALB/c mice. HCV infected patients frequently make antibodies to HCcAg (Chiba et al., 1991). These antibodies may or may not have viral neutralizing activity, since high titer anti-HCcAg antibodies can coexist with viremia (Zhang et al., 1993). Indeed, HCV not only exists as enveloped virions but also as free, non-enveloped nucleocapsids in the sera of HCV infected patients (Ishida et al., 2001, Maillard et al., 2001). Lipo-viro-particles, comprising HCV core protein, HCV RNA and lipoproteins, purified from plasma of untreated chronically HCV-infected patients, bind and enter hepatocyte cell lines, suggesting the possibility that HCV nucleocapsids could contribute to HCV infectivity *in vivo* (André et al., 2002).

Induction of specific DTH response *in vivo* is mediated by antigen-specific CD4+ Th1 effectors (Jin et al., 2004). Possibly, on day 21 after the last immunization, HCcAg.120-specific CD4+ Th1 effectors were able to circulate through footpad tissue, as indicated by a strong DTH to

HCcAg.120-VLPs. Thus, data suggest that HCcAg.120-VLPs immunization in BALB/c mice leads to HCcAg.120 processing and presentation *in vivo* which induced an HCcAg.120-specific activated CD4⁺ Th1-cell response in mice.

Supporting the *in vivo* DTH results, HCcAg.120-VLPs induced proliferative

responses with a putative Th1 cytokine profile after *in vitro* stimulation (HCcAg.120-VLPs-dependent production of IFN- γ but not IL-4). Similarly, previous studies showed the induction of both proliferative and an IFN- γ producing Th1 response by HCcAg.120 immunization in BALB/c and C57 mice (Álvarez-Obregon et

Figure 5: Vaccinia virus titers in immunized BALB/c mice. Mice were immunized with different formulations containing HCcAg.120-VLPs or control preparations and then challenged with either vvCore or WR after the third immunization. Then mice (five per group) were sacrificed 5 days after challenge and vaccinia titers in ovaries were determined. (A) Data are shown for pools of ovaries from each group challenged with vvCore or WR and the vaccinia virus titers are indicated. (B) Data from individual mice immunized with HCcAg.120+MontISA888 or MontISA888 are shown. At left, individual vaccinia virus titer in ovaries from mice challenged with vvCore. Mean vvCore titer is also shown ($b > a^{**}$, $p < 0.01$). At right, *ex vivo* direct ELISPOT assay for determination of spleen cells secreting IFN- γ . Splenocytes were stimulated with P815 cells infected with vvCore. Data represent the mean values of spot forming units $\pm$ SEM from duplicate tests after subtracting the background value (stimulation with P815 cells infected with WR vaccinia virus) ($a > b^{*}$, $p < 0.05$). Stat: Two tailed Mann-Whitney *U* test was used.

al., 2001, Aguilar et al., 2003). However, as those VLPs contained bacterial nucleic acids (Lorenzo et al., 2001), which are potent inducers of Th1 response, the intrinsic quality of HCcAg.120-VLPs to induce this kind of immune response remained unanswered. Data shown here provide evidence about the ability of HCcAg.120-VLPs to generate strong cellular immune responses.

Analysis of proliferation and cytokine profiles of HCV-specific T cells showed that persons showing a strong HCV-specific T-cell proliferation with increased IFN- γ production are more likely to experience viral clearance (Rosen et al., 2002, Gramenzi et al., 2005). In addition, strong CD4 $^{+}$ T cell responses against HCcAg have been found to be associated with self limited infection (Botarelli et al., 1993, Missale et al., 1996). Besides, it has been shown that a strong Th1-like T cell response specific for HCcAg in HCV infected patients is related either with a benign course of the infection or with a successful immune responses against HCV (Missale et al., 1996, Lechner et al., 2000, Gramenzi et al., 2005). Therefore, our findings that HCcAg.120-VLPs can induce proliferative responses with production of IFN- γ , and they are also able to elicit a strong DTH response against HCcAg.120, are important features for a potentially effective HCV vaccine.

Adjuvants have been used with conventional vaccines to elicit an early, robust, and durable immune response, and they can modulate the immune response toward different T-helper (Th1 versus Th2) response (Cox and Coulter, 1997). Previously, HCV core protein formulated in Montanide720 (with a synergistic effect by inclusion of CpG) could induce balanced and strong Th1/Th2 responses (Roohvand et al., 2007). The higher induction of IgG2a antibodies and the tendency to obtain higher secretion of IFN- γ by cells from HCcAg.120-VLPs/Montanide ISA888 immunized mice suggest that this formulation induce a Th1-type response in BALB/c mice. In addition, HCcAg.120-VLPs/Montanide ISA888 immunization induced a

differential epitope proliferative response (positive to A1 but not to A2 and A3 peptides) regarding the other immunogens, suggesting that this formulation modulated the HCcAg.120-specific immune response in BALB/c mice. Peptides A1, A2 and A3 comprise HCV epitopes previously described in mice and humans (Harase et al., 1995, Kakimi et al., 1995, Jackson et al., 1997). Particularly, A1 and A2 comprise epitopes specific for mouse T CD4 $^{+}$ lymphocytes (Kakimi et al., 1995, Harase et al., 1995). Peptide A1 also encompasses an epitope specific for human B lymphocytes (Jackson et al., 1997). Differential immune recognition at the T cell level for a particular peptide after immunization by different routes, like the observed for peptide A2 (i.n. vs i.m.), could be related by the difference in cells responsible for antigen processing and presentation (Novak et al., 2004).

The protective efficacy of any experimental vaccine formulation can best be analysed in an *in vivo* bioassay model. We tested the ability to control infection after immunization with HCcAg.120-VLPs in a surrogate challenge model with recombinant HCcAg-vaccinia. By using this approach, it has recently been shown that immunization of BALB/c mice with HCcAg.120-VLPs, mixed with a DNA vaccine (containing the HCV structural region), induces a strong cellular immune response and specifically controlled viral infection after challenge with vvCore (Álvarez-Lajonchere et al., 2006). On the other hand, a previous study has reported that HCV-VLPs-induced immune responses protected mice from challenge with a recombinant HCV vaccinia expressing HCV structural proteins (Murata et al., 2003). Furthermore, adoptive transfer of splenocytes from immunized to naïve mice conferred protection against recombinant vaccinia challenge and the selective depletion of the CD4 $^{+}$ or CD8 $^{+}$ population abolished the protective immunity (Murata et al., 2003), suggesting the critical role of both CD4 $^{+}$ and CD8 $^{+}$ effector lymphocytes for this immunity. In this work, immunization with HCcAg.120-VLPs/Montanide ISA888 resulted in a $>3 \log_{10}$

reduction in vaccinia virus titer when challenged with vvCore, whereas no reduction was seen when challenged with WR vaccinia strain compared to control immunized mice. Data suggest that immunity against vvCore was mediated by the cellular immune response against HCcAg.

Interestingly, there was a marked increase in the number of IFN- γ producing spleen cells in mice immunized with HCcAg.120-VLPs/Montanide ISA888 compared to control immunized mice. The *ex vivo* direct ELISPOT assay using the P815 mastocytoma cell line infected with vvCore indicated that this formulation induces effector antigen-specific T cells (possible CD8+ cells), showing the ability to recognize naturally processed and presented target antigens on the surface of infected P815 cells. This is consistent with the induction of a Th1 response and protection from vaccinia virus infection in mice immunized with this formulation. This quality of effector T cells is important for peripheral viral infections, where protection requires activated T cells to extravasate into peripheral tissues (Kundig et al., 1996). It has been shown that HCV-specific activated effector T cells homing to the site of infection (liver) were required to provide a rapid antiviral and protective response (Shoukry et al., 2003). In addition, in acute HCV infection, an early and vigorous activated HCV-specific T-cell response was associated with viral clearance and recovery (Lechner et al., 2000, Grakoui et al., 2003). Although induction of strong anti-HCcAg.120 cellular immune responses may be important for protection in immunized mice, additional experiments are needed to precisely define the role of CD4+ and CD8+ responses in this observed protective immunity.

Recombinant proteins usually fail to induce significant immune responses unless given in strong adjuvants (Singh and Srivastava, 2003). Such adjuvants may enhance immune responses mainly via two mechanisms: prolonged antigen presentation due to a depot effect and activation of antigen presenting cells (APC), in particular dendritic cells

(Medzhitov and Janeway, 1997). On the other hand, VLPs can induce strong immune responses in the absence of adjuvants (Lechmann et al., 2001). Due to their particulate nature, they may efficiently reach the MHC class I pathway *in vivo* and induce CD8+ T cells or CTL responses (Moron et al., 2003). However, cellular immune responses induced by VLPs in the absence of certain adjuvants may fail to induce immunity against recombinant vaccinia challenge (Storni et al., 2002). In this regard, a preceding work provided evidences that for induction of protective T cell-mediated immunity (using a recombinant vaccinia virus model) antigen had to be presented for extended time periods by activated professional APC (Storni et al., 2003).

Montanide ISA888 formulations have been shown to induce strong and long term immunity (Bahnmann and Mesquita, 1987). Emulsions can have a depot effect and induce a slow release of the antigen as well as activate APC. Thus, this quality may provide HCcAg.120-VLPs/Montanide ISA888 formulation with the ability to induce immunity against vvCore challenge in BALB/c mice.

Data shown here provide evidence about the ability of HCcAg.120-VLPs to generate strong humoral and cellular immune responses and preferentially stimulate Th1-cell response. Additionally, HCcAg.120-VLPs may become very powerful immunogens if applied together with adjuvants that stimulate both innate and acquired immune system. Non-clinical stability and toxicology studies with these VLPs should render important information about their future as potential components of effective vaccine candidates against HCV. Particularly, the results related with the ability for eliciting cell mediated immune response are attractive for the evaluation of these VLPs in the rational design of therapeutic strategies against HCV. Different factors including optimal dose, immunization schedule and combination with antiviral therapies are expected to influence the future of this and other potential immunotherapeutic strategies and deserve detailed evaluation.

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