

Generation and characterization of recombinant vaccinia viruses expressing a hepatitis C virus Core protein, genotype 1b, individually or as a polyprotein

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ABSTRACT

Hepatitis C virus Core is an intriguing protein with important roles in life cycle of this pathogen. In the present work, recombinant vaccinia viruses expressing a genotype 1b HCV Core protein, individually (vvCore) or as a polyprotein Core-E1-E2 (vvRE), were generated and characterized. In general, viral titer of recombinant vaccinia viruses expressing the Core protein in BSC40 infected cells was similar to the one detected in BSC40 cells infected with the Western Reserve vaccinia virus control. Additionally, intraperitoneal inoculation of BALB/c mice with 10^6 plaque forming units (pfu) of vvCore or vvRE effectively infected animals. Viral load detected in ovaries of infected mice was similar for recombinant viruses or vaccinia virus control. Moreover, up to 10^8 pfu of recombinant vaccinia viruses were inoculated to BALB/c mice without lethal consequences. Remarkably, after single inoculation of recombinant vaccinia viruses, specific antibodies or IFN-gamma secreting cells against the HCV Core antigen were not detectable. The generated recombinant vaccinia viruses, expressing the HCV Core, are valuable instruments for the development of surrogate challenge models and for the future investigation of *in vitro* and *in vivo* aspects related to this viral antigen.

Keywords: HCV, cellular immunity, IFN gamma, vaccinia, replication

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RESUMEN

Generación y caracterización de virus vaccinias recombinantes que expresan una proteína de la cápsida del virus de la hepatitis C de genotipo 1b, individualmente o como poliproteína. La proteína de la cápsida del virus de la hepatitis C es un antígeno interesante con importantes funciones en el ciclo de vida de este patógeno. En el presente trabajo se generaron y caracterizaron virus vaccinias recombinantes que expresan la proteína de la cápsida del VHC de genotipo 1b, individualmente (vvCore) o como poliproteína Cápsida-E1-E2 (vvRE). En general, el título viral de los virus vaccinias recombinantes, que expresan la proteína de la cápsida en células BSC40 infectadas, fue similar al detectado en las células BSC40 infectadas con el virus vaccinia control Western Reserve. Adicionalmente, la inoculación intraperitoneal de ratones BALB/c con 10^6 unidades formadoras de placas (ufp) de vvCore o vvRE infectó de forma efectiva los animales. La carga viral detectada en los ovarios de los ratones infectados fue similar para los virus recombinantes y el virus vaccinia control. Además, hasta 10^8 ufp de virus vaccinias recombinantes fueron inoculadas a ratones BALB/c sin consecuencias letales. Resultó interesante que después de una sola inoculación de virus vaccinias recombinantes no fue posible detectar una respuesta específica de anticuerpos o de células secretoras de IFN-gamma contra el antígeno de la cápsida del VHC. Los virus vaccinias recombinantes generados, que expresan la proteína de la cápsida del VHC, son instrumentos valiosos para el desarrollo de modelos sustitutos de reto y para futuros estudios *in vitro* e *in vivo* de diferentes aspectos relacionados con este antígeno viral.

Palabras claves: VHC, inmunidad celular, IFN ganma, vaccinia, replicación

Introduction

Hepatitis C virus (HCV) is the major causative agent of parenteral non-A, non-B hepatitis [1]. More than 85% of infected patients develop chronic hepatitis with the risk of progression to cirrhosis and hepatocellular carcinoma [2]. Current treatments for chronic HCV infection are generally effective in less than 50% of cases [3, 4]. Therefore, the development of a vaccine against HCV infection is highly desirable. However, the absence of a feasible animal model and a consistent cell culture system to investigate and propagate HCV isolates, from different genotypes, have been important obstacles to progress in prophylactic and therapeutic strategies.

Some surrogate models have been developed to study the HCV life cycle and the immune response eli-

cited against this pathogen [5, 6]. Recombinant viral vectors have been frequently employed as tools for these purposes. Particularly, vaccinia viruses, recombinant for HCV proteins, have been widely evaluated for *in vitro* and *in vivo* study of cellular immunity against HCV antigens [7, 8]. In addition, vaccinia viruses have also been used to study the effect of HCV proteins expression in the host [9, 10].

HCV contains a positive-stranded genome encoding a single polyprotein precursor of about 3000 amino acids (aa) that is cleaved by both host and viral proteases to generate three putative structural proteins (Core, E1 and E2) and at least six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B) [11]. HCV Core protein is one of the most conserved and immu-

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nogenic proteins of this virus [11]. This antigen is likely to be the main component of the HCV capsid. Additionally, HCV Core bears three nuclear localization signals and is able to bind nucleic acids [12]. These features support demonstrated or theoretical roles of this protein in HCV life cycle [13]. Particularly, some studies suggest that HCV Core protein has oncogenic properties that affect normal cell functions as proliferation and death [14, 15]. In addition, HCV Core protein seems to interact with gC1qR and inhibits T lymphocyte proliferation [16].

In this work, two recombinant vaccinia viruses expressing a genotype 1b HCV Core, individually or as a polyprotein Core-E1-E2, were generated. *In vitro* and *in vivo* replication of these recombinant vaccinia viruses was characterized and compared with the parenteral vaccinia virus, non-expressing the HCV antigens. Moreover, immune response against HCV Core was studied in mice inoculated with recombinant vaccinia viruses. Novel elements about the expression of HCV Core protein in mammalian cells infected with recombinant vaccinia viruses, as well as tolerability of high viral load inoculation in mice, are shown.

Materials and methods

Recombinant proteins and peptides

Co.120 is a protein containing the first 120 amino acids (aa) of HCV polyprotein, obtained from recombinant *Escherichia coli*. It was purified by a combination of washed pellet procedures and gel filtration chromatography, as previously described [17]. E2.680 is a protein comprising aa 384 to 680 of HCV polyprotein (corresponding to HCV E2 antigen), obtained from a recombinant yeast [18].

P132-142 is a synthetic peptide comprising aa 132-142 (DLMGYIPLVGA) of HCV polyprotein from a genotype 1b Cuban isolate. This peptide was purchased from the Department of Peptide Synthesis at the Center for Genetic Engineering and Biotechnology, Havana, Cuba (CIGB) and used to investigate anti-HCV IFN- γ secretory immune response. It was synthesized according to the solid-phase method [19] on 4-methylbenzhydrylamine resin (1 mmol/g; Fluka, Buchs, Switzerland).

Plasmids

pHLZ [20] is a shuttle plasmid vector for vaccinia virus, gently donated by Dr. Carlos Duarte (AIDS Department, CIGB). This plasmid comprises a *LacZ* gene under the control of 7.5 vaccinia promoter, a multicloning site for insertion of heterologous genes under the control of a synthetic early/late promoter. This unit is flanked by two regions homologous to vaccinia hemagglutinin locus.

pIDKCo [21] and pIDKE2 [22] are plasmids for DNA immunization expressing the first 176 and 650 aa, respectively, of HCV polyprotein from a genotype 1b Cuban isolate [23]. These plasmids were used as source of sequence coding for HCV antigens to be inserted in pHLZ.

Synthetic DNA linker

The synthetic DNA fragment used as adapter for cloning the region encoding for the HCV structural

region in the plasmid pHLZ was obtained from the Synthesis Department (CIGB). Briefly, two primers (5'GATCTCAGCCACTAGCGAAT 3' and 5'CTAGATTTCGCTAGTGGCTGA3') were synthesized and hybridized, generating a linker with ends corresponding to *Xba* I and *Bgl* II restriction endonucleases sites.

Viruses and cell lines

The Western Reserve (WR) vaccinia virus strain [24] was used to generate recombinant vaccinia viruses for the HCV Core protein. WR was also used as a negative control in HCV antigen expression studies and immune response evaluation of recombinant vaccinia viruses. In addition, WR was used as a positive control in all the *in vitro* and *in vivo* replication studies.

FPCoE1 is a recombinant fowlpox virus, derived from FP9 strain [25], expressing a protein variant encompassing aa 79-338 (Core and E1) of the HCV polyprotein [26]. FPCoE1 and FP9 viruses were used as positive and negative control immunogens for ELISPOT assays, respectively, as previously described [26, 27].

African green monkey kidney cells BSC40 [28] were grown in monolayers and used for *in vitro* studies and *ex vivo* determination of vaccinia virus titer in mouse ovaries. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) (v/v), 100 μ g/mL streptomycin and 100 U/mL penicillin. During vaccinia virus infections, FBS was used at 2% (v/v). Cells were maintained at 37°C with 5% CO₂.

P815 cells, a BALB/c mice mastocytome cell line (ECACC/ACC1) was used as presenting cells in ELISPOT assay.

Generation of recombinant vaccinia viruses and *in vitro* expression studies

For generation of recombinant vaccinia viruses, BSC40 cells were infected for 2 h at a multiplicity of infection (MOI) of 1 with WR. Then, the cells were transfected with 10 μ g of plasmid DNA. Transfections were carried out using Lipofectamine (Invitrogen, New Zealand) as liposomal transfection reagent, according to the manufacturer's instructions.

Viruses were harvested 48 h post-infection by scraping BSC40 infected/transfected cells into 2% DMEM. These suspensions were frozen/thawed three times to release the viruses from the cells. These extracts were stored at -80°C and used for further purification of recombinant viruses. Briefly, six wells dishes of BSC40 cell monolayers were infected with virus extracts dilutions (10⁻¹, 10⁻², and 10⁻³). Then, the cells were overlaid with 3 mL of 2% DMEM, containing 1.0% low melting point agarose. After 48 h, a second overlaid containing 0.3 mg/mL of 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal) (Dalton, Toronto Canada) was added. Twenty four h later, recombinant blue plaques were visualized, picked up, and placed into 0.5 mL of 2% DMEM. A minimum of six plaques were picked up for further plaque purification. All recombinant viruses were plaque purified five times as described above. Virus plaques from the last round were amplified in 25 cm² culture flasks, seeded with BSC40 cells. The viral titer

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was determined on BSC40 cells infected with 1 mL of 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} and 10^{-9} dilutions of the virus in duplicates. After 48 h, the cells were stained with 1% crystal violet in methanol and viral plaques were counted. The viral titer was expressed as plaques forming units (pfu)/mL.

To determine the presence of HCV proteins in the infected cells with the possible recombinant viruses, BSC40 cells seeded in 60 mm plates were infected with the parental virus WR or the possible recombinant viruses at MOI=5. Culture samples were taken at different time points to perform sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot assays for confirming the presence of the corresponding HCV proteins, indicating that double crossover events have occurred.

Sample processing for SDS-PAGE studies

BSC40 cells infected with vaccinia virus were scraped and resuspended in lysis buffer (20 mM Tris-HCl pH 8.0, 0.5% triton X-100, 100 mM NaCl, 0.2 mM Phenylmethyl-sulfonyl fluoride), incubated 10 min on ice, and then centrifuged at $1000 \times g$ for 10 min. The pellet and supernatant were both used for electrophoresis in denaturing buffer (10 mM Tris-HCl, 1 mM EDTA, 1% SDS, 5% β -mercaptoethanol, 20% glycerol, pH 8.0). In other cases, the infected cells were directly resuspended in denaturing buffer.

SDS-PAGE and Western blot

Samples were separated in 15.0% sodium dodecyl sulphate-polyacrylamide gel electrophoresis [29] and stained with 0.1% Coomassie brilliant blue R250 (CBB, Sigma, St Louis, USA) in 30% methanol, 10% acetic acid in H₂O or electro transferred [30] to a nitrocellulose membrane (Hybond-C, Amersham, UK). The transferred sheet was blocked for 1 h at room temperature with phosphate-buffered saline (PBS: 0.1 M NaCl, 2 mM KCl, 10 mM Na₂HPO₄, 1 mM KH₂PO₄, pH 7.2) containing 5% skimmed milk, then incubated for 1 h at 37 °C with 1 μ g/mL of monoclonal antibody (Mab) SS-HepC.1 [31], directed against the residues 5-35 of the HCV Core protein, or an anti-E2 polyclonal serum [32], diluted 1:1000. After washing with PBS, the membrane was reacted with 1:1000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Amersham, UK) for 1 h, at 37 °C. After washing, immunoreactivity was detected using the ECL[®] system (Amersham, UK). The SDS-PAGE Molecular Weight Standards Broad Range from Bio-Rad (California, USA) and the protein Co.120 [17], with an electrophoretic pattern corresponding to 21 kDa, were used as controls.

Cumulative growth of recombinant viruses

For analysis of virus replication, BSC40 cells were infected with 0.01 MOI of recombinant vaccinia viruses or the parental virus WR. Samples of cells and supernatants were taken and analyzed separately, at different time points. Cell samples were frozen-thawed three times. All samples were sonicated and assayed in duplicates on BSC40 monolayers.

Virus inoculations in mice

BALB/c (H-2d) female mice 6 to 8 weeks old (18-20 g of weight) were purchased from the National Centre

for Laboratory Animal Production, Havana, Cuba, and used for all *in vivo* studies. The housing, maintenance, and care of the animals were in compliance with all relevant guidelines and requirements.

Infectivity of vaccinia viruses *in vivo* was evaluated by single intraperitoneal injection of 10^6 pfu (200 μ L) of the virus in study, in PBS. Groups of 8 mice were inoculated with vvCore, vvRE or WR. Five days after virus inoculation, 5 animals per group were euthanized for determination of viral load in ovaries. Blood extraction and ELISPOT assay were performed at week 5 in the remaining 3 animals. Two other groups, 3 animals each, were immunized twice at weeks 0 and 3 with 2.5×10^7 pfu of FPCoE1 or FP9, as additional control immunogens for ELISPOT assay.

The effect of high viral load inoculation to mice was evaluated by intraperitoneal injection of 5×10^7 , 8×10^7 or 1×10^8 pfu (200 μ L) of the vaccinia virus in study, in PBS. Groups of 12 mice were employed. Animals were followed-up during 15 days after virus inoculation, and then euthanized.

Determination of vaccinia virus titer in the ovaries of inoculated mice

Five days after intraperitoneal injection of 10^6 pfu of the vaccinia virus in study, mice were sacrificed, and the ovaries were removed, homogenized, sonicated, and assayed for viral titer determination by serial 10-fold dilutions on BSC40 indicator cell plates. After a 2-days culture, the medium was removed, the BSC40 cells monolayer was stained with 1% crystal violet (Merck, Darmstadt, Germany) in methanol for 10 min, and the number of plaques per well was counted.

Enzyme-linked immunosorbent assay

To detect murine antibodies to HCV Core protein, 96-well microtiter plates (Costar, Cambridge, MA, USA) were coated with 100 μ L per well of Co.120 (5 μ g/mL) diluted in coating buffer (50 mM carbonate buffer, pH 9.6) during 14 h at 4°C. The wells were washed three times with 0.05% Tween 20 in phosphate buffered saline pH 7.5 (PBST) and blocked with 200 μ L of PBST containing 1% skimmed milk (Oxoid Ltd, England) for 1 h at 25 °C. After three washes with PBST, mouse sera at double-serial dilutions in PBST, from 1:50 to 1:200, were added (100 μ L/well), and the plates were incubated at 37 °C for 1 h. The plates were washed three times with PBST, and then 100 μ L of the horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma, St Louis, USA), diluted 1: 20 000, were added per well and the plates were incubated for 1 h at 37 °C. Positive reactions were visualized with 0.05% o-phenylenediamine (Sigma, St Louis, USA) in 0.1 M citric acid, 0.2 M NaH₂PO₄, pH 5.0 and 0.015% H₂O₂ as substrate. The reaction was stopped with 50 μ L of 2.5 M H₂SO₄. Measurement of Absorbance (A_{492nm}) was made in a SensIdent Scan plate reader (Merck, Darmstadt, Germany).

The cut-off value to consider a positive antibody response was established as twice the mean A_{492nm} of the negative control sera (sera from WR inoculated mice). As positive control, a pool of sera from animals immunized with Co.120 and high Ab titer against this antigen [31] was used, 1:5000 diluted in PBST.

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Enzyme linked immunospot assay (ELISPOT)

At week 5 of the immunization schedule, spleens from 3 animals per group were removed, pooled and homogenized. The splenocytes were treated once with 0.83% NH₄Cl for erythrocytes lysis. Then, splenocytes were washed three times and suspended with RPMI complete medium (RPMI 1640 supplemented with 10% of FBS, 2 mM glutamine, 100 µg/mL streptomycin, 100 U/mL penicillin). Splenocytes were incubated at 2x10⁶ cells/mL for 7 days with the peptide P132-142 (5 mg/mL) at 37 °C and 5% CO₂. On day 4, 10 U/mL of human IL-2 was added (CIGB, Cuba).

MultiScreen-HA 96-well plates (Millipore, Molsheim, France) were coated overnight at 4 °C with 100 µL/well (5 µg/mL) of anti-mouse interferon-gamma (IFN-γ) R4 (Pharmingen, San Diego, CA, USA) in 50 mM carbonate/bicarbonate buffer, pH 10.6. The plates were blocked for 2 h at 37 °C with RPMI 1640 containing 10% FBS, and washed three times with PBS. Later on, splenocytes (4x10⁴) were incubated with 10⁵ P815 cells at 37 °C for 18 h, in a 200 µL final volume of complete RPMI 1640 medium per well. Previously, P815 antigen presenting cells had been pulsed with the peptide P132-142 (1 µM), or incubated only with medium without peptide, for 1 h, then treated with 30 µg/mL mitomycin C (Sigma, St. Louis, USA) and washed with RPMI 1640 medium. Plates were washed with PBST and incubated with 100 µL (0.5 µg/mL) of biotinylated anti-mouse IFN-γ (XMGI.2; Pharmingen). The plates were washed six times with PBST and development was performed using HRP-streptavidin complex (Amersham, Little Chalfont, Bucks, UK) at 1:800 dilution in PBST for 1 h at 28 °C. After a final wash with PBS, IFN-γ spot-forming cells were detected by the addition of 0.33 mg/mL Amino ethyl carbazole (Sigma, St Louis, USA) in sodium acetate 0.1 M pH 5.2, 0.05% of hydrogen peroxide solution. Spots were counted with a stereomicroscope (Leica Microscopy Systems, Heerbrugg, Switzerland). Each condition was studied in duplicates (2 wells). The results were expressed as the numbers of spot-forming cells (SFC)/10⁶ splenocytes. Positive results were considered for values above 250 SFC/10⁶ splenocytes, exceeding twice the number of SFC/10⁶ splenocytes detected in the negative control (P815, not charged with peptide P132-142, in the presence of medium) for each group and in the external negative control groups (mice immunized with WR or FP9).

Statistical methods

Normal distribution was analyzed by using the Kolmogorov-Smirnov' test. To compare differences among groups, a One-way ANOVA with the Newman-Keuls' Multiple comparison post test was used for parametric analysis. For non-parametric analysis, a Kruskal-Wallis' test with Dunn's Multiple comparison test was used. *p* values <0.05 were considered significant.

Results

Generation of recombinant vaccinia viruses

With the aim to generate vaccinia viruses expressing the HCV Core protein individually, or as a polyprotein

Core-E1-E2, two plasmids for recombination with the vaccinia virus genome were generated.

The plasmid pDKCo was digested with *Xba*I and *Eco*RV endonucleases, and the fragment of 544 bp, comprising the HCV core sequence, was inserted into the pHLZ plasmid, previously digested with restriction enzymes *Bgl*II and *Sma*I, using a DNA linker with *Xba*I/*Bgl*II endings. The resultant plasmid, pVACore, contained the DNA sequence coding for aa 1-176 of the HCV Core protein under the control of a vaccinia virus early/late promoter (Figure 1A).

The second plasmid was obtained following the same strategy, using pDKCE2 instead of pDKCo as a source of the encoding sequence. In the resultant plasmid, pVACRE, the inserted fragment of 1971 bp, obtained from the digestion of pDKCE2, comprised the coding sequence for aa 1-650 of the HCV polyprotein (Figure 1B). The identity of both plasmids, pVACore and pVACRE, was confirmed by restriction endonuclease analysis (Figures 1A and 1B, respectively) and DNA sequencing.

For generation of recombinant vaccinia viruses, BSC40 cells were infected with WR vaccinia strain and then, the cells were transfected with either pVACore or pVACRE. After 48 h, the cells were harvested and five selection rounds were performed in solid media

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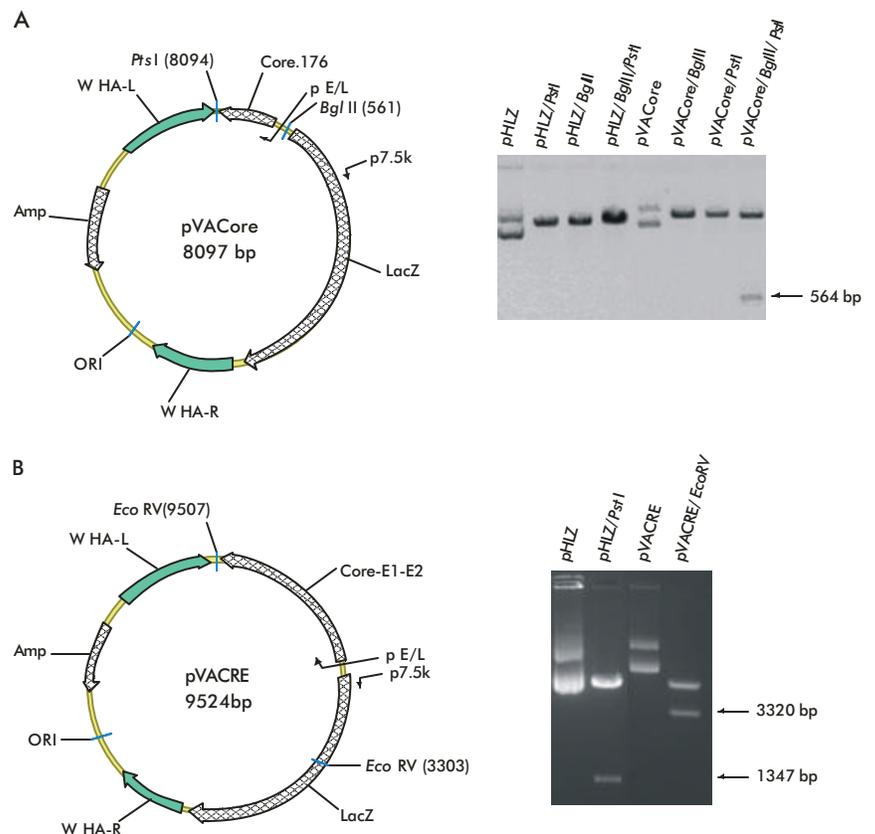


Figure 1. A schematic representation of the plasmids (A) pVACore and (B) pVACRE, LacZ: LacZ gene, HA-vv (R) and HA-vv (L): homologous sequence with regions in the vaccinia hemagglutinin locus, ORI: replication origin for bacteria, P E/L: synthetic early/late promoter for vaccinia, P7.5: promoter 7.5 of vaccinia, Co. 176: HCV sequence corresponding to aa 1-176 of the Core protein. The restriction analyses for both plasmids are shown in the figure. The arrows indicate differential fragments between the parental and the recombinant plasmids.

containing X-gal. After the fourth round, all plaques were blue, indicating the presence of 100% recombinant vaccinia viruses. Virus plaques from the fifth round were amplified in culture flasks seeded with BSC40 cells. Viral products from this last step were named vvCore, for BSC40 cells transfected with pVACore, or vvRE for BSC40 cells transfected with pVACRE.

To determine the presence of HCV proteins in the cells infected with the possible recombinant viruses vvCore and vvRE, BSC40 cells were infected with them or with the parental virus WR as negative control. Culture samples were taken at different time points after infection and analyzed by SDS-PAGE and Western Blot using a MAb specific for the N-terminus of the HCV Core antigen or an anti-E2 polyclonal serum. HCV Core was detected 24 and 48 h post-infection with vvCore or vvRE in BSC40 cells (Figures 2 and 3, respectively). In addition, expression of HCV E2 was detected in cells infected with vvRE (Figure 3C). No signals for HCV Core or E2 were detected in BSC40 cells infected with negative control virus WR.

In vitro and *in vivo* replication

Recombinant vvCore and vvRE viruses were compared with WR concerning *in vitro* replication to evaluate potential effects related to HCV sequences. BSC40

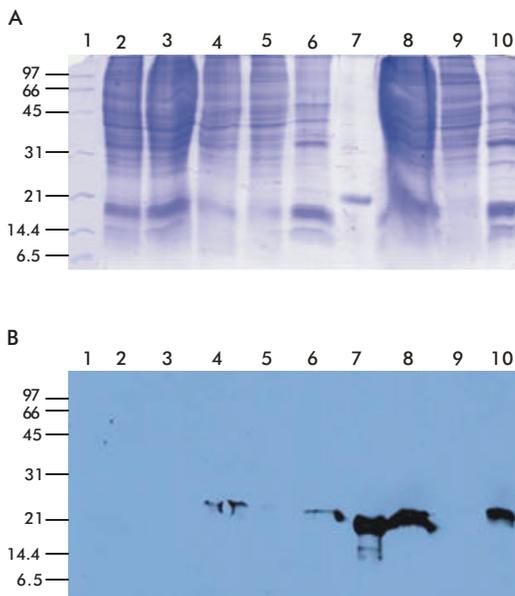


Figure 2. Expression of the HCV Core protein in cells infected with vaccinia virus vvCore. BSC40 cells were infected with recombinant or parental virus at a multiplicity of infection of 5. A. At 24 and 48 h following infection, protein extracts were prepared and proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lane 1, Molecular Weight Marker (Bio-Rad, California, USA), Lanes 2-3, lysis supernatant and lysis pellet from WR infected cells after 48 h, respectively; Lanes 4-6, whole cells, lysis supernatant and lysis pellet from vvCore infected cells after 24 h, respectively; Lane 7, Co.120 (positive control); Lanes 8-10, whole cells, lysis supernatant and lysis pellet from vvCore infected cells after 48 h, respectively. B. After electrophoretic transfer to membrane, the HCV Core protein was detected with the Mab SS HepC.1. Numbers on the left of the panel indicate molecular weights (in kDa) according to the Molecular Weight Marker (Bio-Rad, California, USA).

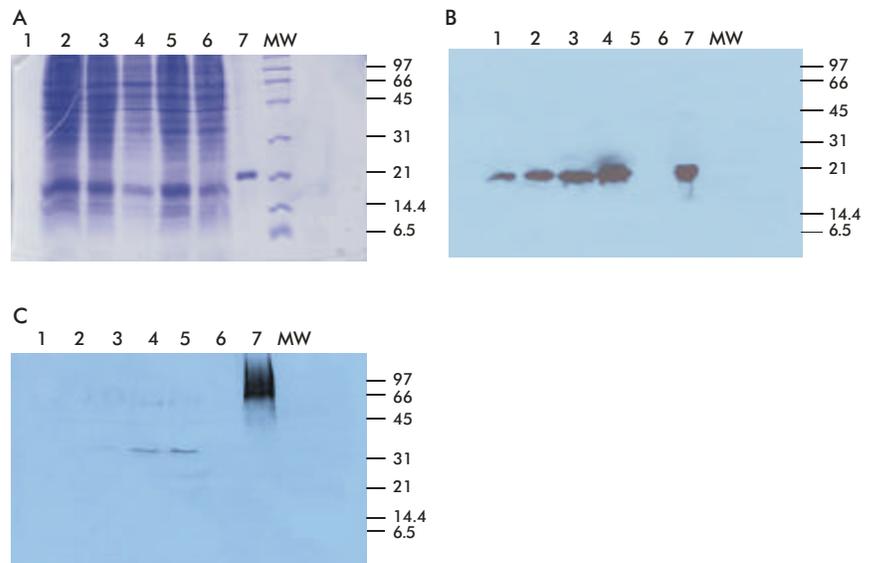


Figure 3. Expression of HCV Core and E2 proteins in cells infected with vaccinia virus vvRE. Proteins in whole cell extracts of BSC40 infected with vvRE after 21 h (lane 1), 24 h (lane 2), 45 h (lane 3), 48 h (lane 4), or infected with WR after 48 h (lane 5), were resolved by SDS-PAGE (A) and analyzed by Western blot using the Mab SS HepC.1 (B) or an anti-E2 polyclonal serum (C). Recombinant Co.120 (A and B) or E2.680 (C) proteins were used as positive control in lane 6. MW (Molecular Weight Marker, Bio-Rad, California, USA) was run in lane 7. Numbers on the right of the panel indicate molecular weights (in kDa) according to MW.

cells were infected with vaccinia viruses and culture samples were taken at 18, 24, 42 and 48 h to determine viral titer. Only at 18 and 24 h, vvRE titer values in the intracellular fraction were statistically different (Kruskal Wallis and Dunn's Multiple Comparison test) to those observed for WR (Figure 4A). At 42 and 48 h there were no statistical differences among the vaccinia viruses in study with respect to viral titer at the intracellular fraction. Viral titer at the extracellular fraction was similar among the different vaccinia viruses at all time points in study (Figure 4B).

On the other hand, BALB/c mice were inoculated with vaccinia viruses to comparatively evaluate the vvCore and vvRE *in vivo* replication with respect to WR control. Animals were inoculated with 10^6 pfu of viruses under investigation. Figure 5 shows that, 5 days after viral inoculation, all viruses were detected at similar levels in the ovaries of inoculated mice ($p > 0.05$, one way ANOVA and Newman-Keuls Multiple comparison test).

To evaluate whether the administration of higher viral doses of a recombinant vaccinia virus expressing the HCV Core could be lethal for BALB/c mice, animals were inoculated with 5×10^7 , 8×10^7 or 1×10^8 pfu of vvCore. One group of mice was inoculated with 1×10^8 pfu of WR control. Animals were observed during 15 days post-infection. No animal died during this time (data not shown).

Immune response against HCV Core antigen

Additionally, we evaluated whether single inoculation (10^6 pfu) of vaccinia viruses was able to elicit anti-HCV Core immune response or not. Antibodies against HCV Core were neither detected in sera from mice inoculated with control nor recombinant vaccinia

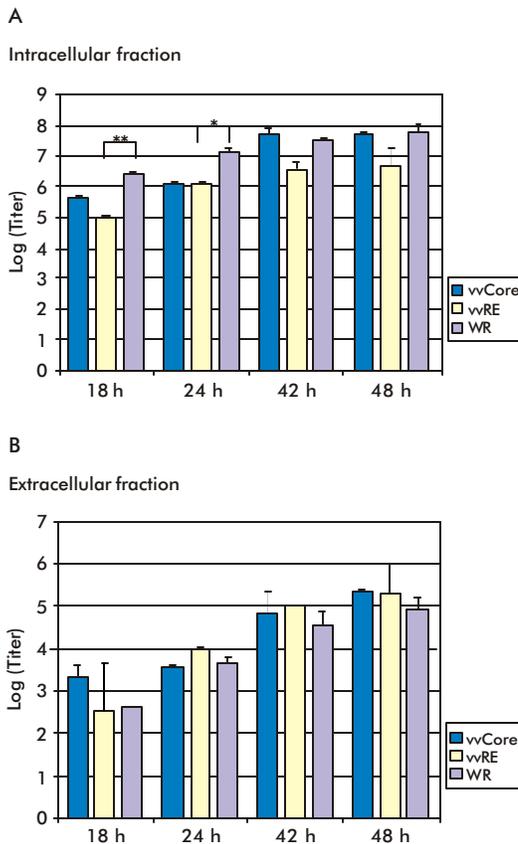


Figure 4. Cumulative growth of vvRE, vvCore and WR at intracellular (A) and extracellular (B) fractions. BSC40 cells were infected with vaccinia virus at a multiplicity of infection of 0.01. At the indicated times, cell and culture supernatants were collected. The cells were harvested and disrupted by freezing-thawing. Viral titres at cell and culture supernatant were determined by viral plaque assay on a monolayer of BSC40 cells. Data represent the mean from two independent sets of experiments. Error bars indicate the standard deviation of the mean. * denote $p < 0.05$ and ** denote $p < 0.01$ according to Kruskal-Wallis test with Dunn's Multiple Comparison test.

viruses despite a positive control serum confirmed by ELISA was working properly (data not shown). Moreover, vaccinia viruses also failed to elicit a detectable IFN-gamma secretion response, determined by ELISPOT against a conserved and immunodominant epitope in HCV Core protein, five weeks after viral inoculation (Figure 6). However, animals immunized with a HCV recombinant fowlpox virus, FPCoE1, showed a positive response in this assay.

Discussion

Several cell culture systems [5] and mouse xenograft models with HCV-infected human liver fragment [33] have been reported to support HCV replication. However, their reliability and simplicity as models of HCV replication are still in question. Recently, Lindenbach *et al.*, describe a full-length genotype 2a HCV genome that replicates and produces virus particles that are infectious in cell culture [34]. Nevertheless, this work was restricted to a particular isolate of genotype 2a. Unfortunately, despite progress in HCV research, the knowledge about the expression

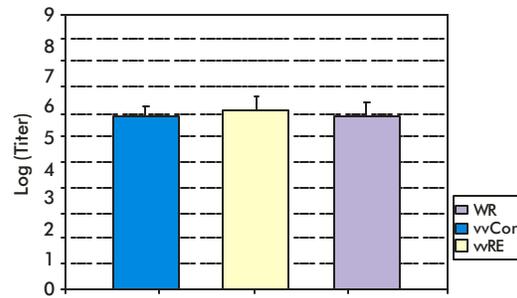


Figure 5. Vaccinia virus titres in ovaries of inoculated mice. Five days after inoculation of 10^6 pfu of recombinants and parental virus the mice ovaries were extracted, disrupted and assayed for viral titer determination in BSC40 cells. Bars indicate the mean titres from 5 individual mice per group. Error bars represent standard deviation of the mean. No statistically significant differences were found among groups (One-way ANOVA with the Newman-Keuls Multiple comparison post test, $p > 0.05$).

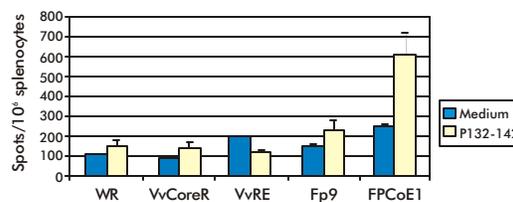


Figure 6. IFN- γ secretion response determined by Enzyme linked immunospot assay 5 weeks after inoculation. The experiment was performed with splenocytes pools from 3 animals per group. Splenocytes were incubated for 7 days with the peptide P132-142 ($5 \mu\text{g}/\text{mL}$). Later on, 4×10^4 splenocytes were incubated with 1×10^5 P815 cells charged or not with P132-142 peptide at 37°C for 18 h, in a $200 \mu\text{L}$ final volume of complete RPMI 1640 medium per well. The bars represent the spots/ 10^6 splenocytes. Positive results were considered for values above $250 \text{ SFC}/10^6$ splenocytes, exceeding twice the number of $\text{SFC}/10^6$ splenocytes detected in the negative control for each group (P815, not charged with peptide P132-142, in the presence of medium) and in the external negative control group (WR or Fp9).

and characteristics of HCV proteins, as well as their effect on host cells is still limited.

In this work, novel recombinant vaccinia viruses expressing HCV Core protein, individually or as a Core-E1-E2 polyprotein, from a HCV genotype 1b Cuban isolate, were generated. The recombinant viruses were obtained by using a plasmid vector allowing the homologous recombination with the non essential hemagglutinin vaccinia gene, which results in inactivation of this gene. This strategy has been previously used to obtain recombinant vaccinia viruses [35]. In addition, the expression cassette employed could be also flanked by segments of the vaccinia thymidine kinase (TK) gene instead of hemagglutinin gene, although the cell line used should be TK- [36].

HCV Core protein variants of similar sizes were detected in cells infected with vvCore or vvRE, indicating that the HCV Core antigen is processed from the Core-E1-E2 polyprotein. Moreover, we found similar behavior with respect to replication in vaccinia viruses expressing HCV Core individually or as a Core-E1-E2 polyprotein. Previous reports have described changes at the replication levels for some recombinant vaccinia viruses, including differential plaque size and morphology [37]. In the present work, differences

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in viral titer between vvRE and WR were observed *in vitro* at early stages of replication but disappeared at 48 h of cell culture. This phenomenon might be cell-specific and has no probable biological significance since no difference in viral replication was observed *in vivo* among the different viruses, five days post-infection. The vaccinia virus dose chosen for mice inoculation has been previously used in other studies with recombinant vaccinia virus [38, 39].

Previously, HCV core recombinant vaccinia viruses have shown an increased virulence in mice [40]. Particularly, an increased viral titer was detected in mice liver by Large *et al.* [40], compared to parental or other HCV recombinant viruses. Here, we determined the viral titer in ovaries, the preferential organs for vaccinia virus replication in mice, as demonstrated by Binder and Küding [41]. We found similar viral titres in ovaries from mice inoculated with control or recombinant vaccinia viruses.

On the other hand, Large *et al.*, have also found an increased mortality in mice after inoculation of a HCV core recombinant vaccinia virus, in comparison with the parental vaccinia virus and a vaccinia virus recombinant for HCV nonstructural proteins [40]. However, we found no lethal effect after single inoculation with high doses of vvCore. A possible explanation for these conflicting results could be related to differences in the HCV core sequence used to generate the recombinant vaccinia viruses, since we employed a HCV genotype 1b isolate sequence and Large *et al.* used the sequence of a HCV genotype 1a isolate [40]. The identity of the HCV core sequence present in the vaccinia virus described by Large, *et al.* and the viruses obtained in the present work is 92.6% at the nucleotide level and a 98.2% at aa level. Different works have previously shown that point mutations in conserved regions of other HCV antigens like NS5A and E2, as well as the presence of certain motifs in isolates from some genotypes, influence in differential viral behavior in aspects like interaction with the co-receptor CD81, or resistance to IFN- α [42, 43]. Thus, critical genotype or isolate specific sequence motif in the HCV core might be important for viral replication in this system.

Data previously obtained by Zhang suggest that host immune response does not contribute to HCV-Core induced mortality [44]. Interestingly, we were not able to detect any humoral or cellular immune responses against Core in mice inoculated with the HCV recombinant vaccinia viruses. Particularly, the synthetic peptide P132-142, used in this work to investigate the anti-Core IFN- γ secreting response, comprises a highly conserved and immunodominant

epitope of HCV Core, recognized by both murine and human cytotoxic T lymphocytes (CTL) [45]. The murine epitope is restricted to the H-2d haplotype and has been mapped to the decapeptide LMGYIPLVGA [45]. Induction of a specific CTL response against this epitope has been described in BALB/c H-2d mice, after the administration of a peptide vaccine candidate [46]. Moreover, the reliability of the assay was confirmed by the positive response against P132-142 in control FPCoE1-immunized mice.

Several reports have shown positive humoral and cellular immune responses against not HCV antigens in mice inoculated with recombinant vaccinia viruses [47, 48]. Remarkably, Harrington *et al.* detected immune response against the heterologous antigen from 7 to 60 days after inoculation of 2×10^6 pfu of the recombinant vaccinia virus [48]. However, Large *et al.* have already found that vaccinia viruses, recombinant for HCV Core protein, failed to elicit cellular immune response in mice, 5 days after single viral inoculation with 5×10^7 pfu [40]. Large *et al.* suggested that the HCV Core protein was the responsible for the failure in eliciting immune response by suppressing the host response [40]. This fact is in agreement with negative effects associated with the HCV Core protein by affecting many cellular functions [49] related to immune systems, including, cell proliferation, signal transduction systems, and metabolism of lipids [50]. Nevertheless, Liu *et al.* found no modulatory effects of the HCV Core protein of genotype 1b on induction of virus-specific immune responses [51]. It would be interesting to uncover the molecular means by which the recombinant HCV Core protein can escape immune recognition under certain conditions. In our case, we can not rule out that immune response is being generated against other relevant epitopes in HCV Core [52] not evaluated in this work.

The vaccinia viruses generated in the present work are now available as analytical tools for producing antigen presenting cells to be used in the *in vitro* evaluation of cellular immunity against HCV Core. In addition, these viruses can be used in surrogate challenge models against HCV, particularly for genotype 1b isolates. In fact, one of these viruses has been recently used for the evaluation of a DNA vaccine candidate [53]. Finally, recombinant vaccinia viruses expressing the whole or truncated variants of HCV core might be very useful to elucidate the mechanisms behind some intriguing effects of this antigen during viral infection.

Acknowledgements

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