**ABSTRACT**

Two synthetic peptides comprising aa regions in the NS4 protein (aa 1689-1735) and the hypervariable region I (HVR I, aa 384-414) in the HCV E2 protein were conjugated to the P64k protein, a previously demonstrated carrier protein. These peptides were also conjugated to the Co.120 protein, a truncated HCV core variant, to evaluate for the first time its ability as a carrier for B cell epitopes. Five micrograms of free peptides or conjugates, without an adjuvant, were administered subcutaneously to mice to evaluate the immune response of anti-HCV peptides. After four doses at weeks 0, 3, 6, and 10, only the animals vaccinated with the conjugates had a positive antibody response against HCV peptides. Mice immunized with the conjugated P64k elicited the strongest antibody response against both NS4 and HVR I peptides (p<0.01). Particularly, the mean antibody titers against the HVR 1 peptide reached 1:39,000 in mice immunized with the conjugated P64k. Unfortunately, anti-HVR I antibodies elicited by both, Co.120 and P64k conjugates only recognized the homologous HVR I sequence. Our results indicate that conjugation to carrier proteins could be a feasible strategy to induce a strong antibody response against the HVR I that is potentially able to neutralize the homologous isolate of HCV.

Keywords: HCV, HVR I, antibody response, carrier protein, P64k

**RESUMEN**

La inmunización con péptidos sintéticos de HCV conjugados a la proteína P64k generó una fuerte respuesta de anticuerpos en ratones. En el presente trabajo, dos péptidos sintéticos que abarcan secuencias de aa contenidas en la proteína NS4 (aa 1689-1735) y la región hipervariable I (HVR I, aa 384-414) comprendida en la proteína E2 del VHC fueron conjugados a la proteína P64k, una proteína previamente descrita como transportadora. Estos péptidos también se conjugaron a la proteína Co.120, una variante truncada de la cápsida del VHC, para evaluar por primera vez su capacidad como portadora de epitopos de células B. Se administraron 5 µg de conjugado o péptidos individuales, sin adyuvante, por vía subcutánea a ratones con el objetivo de evaluar la respuesta inmune contra ambos péptidos. Después de 4 dosis en las semanas 0, 3, 6 y 10, solo los animales vacunados con los conjugados evidenciaron una respuesta positiva de anticuerpos contra los péptidos del VHC. Los ratones inmunizados con los conjugados que contenían P64k, indujeron la respuesta de anticuerpos más fuerte frente a ambos péptidos (p<0.01). Particularmente, el título promedio de los anticuerpos contra la HVR I alcanzó el valor de 1:39,000 en los ratones inmunizados con el conjugado que contenía P64k. Lamentablemente, los anticuerpos anti-HRV I generados por los conjugados de Co.120 y P64k solo reconocieron la HVR I homóloga. Nuestros resultados indican que la conjugación a proteínas transportadoras pudiera ser una alternativa viable para inducir una fuerte respuesta de anticuerpos contra la HVR I, potencialmente capaz de neutralizar el aislamiento homólogo del VHC.

**Palabras Claves:** HCV, HVR I, respuesta de anticuerpos, proteína transportadora, P64k

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**Introduction**

The Hepatitis C virus (HCV) is the main causative agent of parenterally transmitted non-A non-B hepatitis [1]. The HCV genome is a positive-stranded RNA, encoding a single polyprotein precursor of approximately 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 [2].

Given the global impact on public health of hepatitis C, the development of an effective preventive or therapeutic vaccine is a priority. However, obstacles such as viral heterogeneity, antigenic masking, and the absence of reliable animal models make this task difficult [3]. The pathogenesis of chronic hepatitis induced by the HCV as well as the immune mechanisms responsible for the elimination of this infection are still not fully understood. The course of HCV infection depends on the extent to which both specific and non-specific immune responses are induced. Induction of high-titer, long-lasting, and cross-reactive anti-envelope antibodies (Abs) and a vigorous multispecific cellular immune response, including both helper and cytotoxic T lymphocytes (CTL), might be necessary for an effective anti-HCV vaccine [4]. Particularly, the induction of an early and strong antibody (Ab) response against the hypervariable region I (HVR I) has been related to self-resolution of HCV infection [5]. This is a highly heterogeneous region under selective pressure that contains a neutralizing epitope [6]. Different approaches have either failed to induce significant Ab responses against this region or this response has been limited to the homologous sequence [7, 8].

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**Corresponding author**
The present study evaluates the immunogenicity of HVR I (aa 384-414) and NS4 (aa 1689-1735) synthetic peptides alone or conjugated to carrier proteins, after immunization in mice without an adjuvant. Furthermore, the use of the HCV core as a carrier protein for HCV peptides was investigated for the first time. We also used P64k, a Neisseria meningitidis protein [9], as a carrier for HCV peptides.

Materials and Methods

Co.120 protein: Recombinant Co.120 is an Escherichia coli-derived protein containing the first 120 amino acids (aa) of the HCV viral polyprotein. It was purified by a combination of washed pellet procedures and gel filtration chromatography as previously described [10].

P64k: Recombinant P64k was obtained as described previously [11] and was kindly donated by Dr. Ricardo Silva from the Vaccine Department, Center for Genetic Engineering and Biotechnology.

Synthetic peptides

The following synthetic peptides, covering the HVR I of different isolates, were used here (Table 1):

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Genotype</th>
<th>Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 a</td>
<td>THYTVGSAAGYTVYGFVS LLAAGKGNV</td>
<td>1a</td>
<td>HCV-1</td>
</tr>
<tr>
<td>1 b</td>
<td>THYTVGQAATTMNRLVS MFASGPSQKI</td>
<td>1b</td>
<td>HCV- BK</td>
</tr>
<tr>
<td>2 a</td>
<td>THYTVGGSTAHNRTLIG MFSQGARQKI</td>
<td>2a</td>
<td>HC-J6</td>
</tr>
<tr>
<td>2 b</td>
<td>TSYGGQEGATMYVAGAF LFTGGKAGNL</td>
<td>2b</td>
<td>HC-J8</td>
</tr>
<tr>
<td>SS</td>
<td>TTTTVGGQASHQVHSLTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>TGYTGYGTAAARGYVSQF TGFLTSGPSQKQGL</td>
<td>1b</td>
<td>Cuban</td>
</tr>
</tbody>
</table>

All these peptides comprised aa 384-410 in the viral polyprotein, except the one corresponding to the Cuban isolate (R) that covered aa 384-414. One peptide (SS) was designed to comprise consensus HVR-1 aa sequence from different genotypes, according to the frequency of appearance of the amino acids in each position. Specifically the R peptide was used to conjugate experiments.

The other peptide that was conjugated to Co.120 and P64k peptides comprised the sequence in the NS4 protein (aa 1689-1735):

SGRPVIPDREVLYQEFDEMEECASHLPYIE QGMQLAEQFQKALGL. This peptide was kindly donated by Dr. Larramendi from the Diagnostic Division, CIGB.

HVR I peptides were purchased by the Department of Peptide Synthesis (CIGB, Havana, Cuba). They were synthesized according to the Solid-Phase method [12] on 4-methylbenzhydrylamine (MBHA) resin (1 mmol/g, Fluka, Switzerland), using the tert-butyloxycarbonyl/Benzyl strategy. Peptide-resin was cleaved with fluoride hydrogen (HF) using the "Low-High" procedure in the presence of the appropriate scavengers and washed three times with ether [13]. Peptides were extracted with 30% acetic acid. The peptides were purified on reverse phase high performance liquid chromatography (HPLC) (Vydac C18, 10x250 mm) and solubilized in water at a concentration of 1 mg/mL.

Preparation of the protein-peptide conjugates

Conjugates were obtained as previously described [14]. Briefly, 5 mg of Co.120 or P64k were solubilized in 1 mL of 0.2 M K$_2$HPO$_4$ pH 8.0. Solid succinic anhydride (1 mg) was added and the solution was stirred until all anhydride was dissolved. The pH was kept at 8.5-8.6 using 3.0 M NaOH. The mixture was dialyzed against water at pH 4-5. Then 7 mg of 1-ethyl-3-(dimethylamino)propyl) carbodiimide (EDAC) was added to the activated protein and stirred for 10 minutes at 4°C. Five mg of the peptide were solubilized in PBS at 5 mg/mL. The succinyl-protein and peptide solutions were mixed for 3 h at room temperature with gentle stirring.

The conjugates were purified by gel filtration through Sephadex CL-4B (Pharmacia, Sweden) equilibrated in PBS. The conjugate concentration was determined using the Bradford method [15].

Human sera

Human sera obtained from blood donors and chronic patients were previously screened for the presence of anti-HCV Abs by UMEELISA HCV from the Centro de Inmunooensayo (Havana, Cuba). The anti-HCV positive sera were also confirmed by Ortho HCV 2.0 ELISA (Ortho Diagnostic Systems, Raritan, NJ). Negative human sera for anti-HCV AbS were employed as controls. Additionally, all the human sera employed in this work had been previously tested by ELISA for the presence of anti-P64k Abs with negative results.

Immunoblotting assay

For immunoblotting, the samples were resolved by SDS-PAGE and the proteins were electrophoretically transferred to a nitrocellulose membrane (HYBOND C, Amersham, UK). The binding of the IgG was detected as previously described [16]. Briefly, the membrane was blocked for 1 h at room temperature with the phosphate-buffered saline solution (PBS) (0.1 M NaCl, 2 mM KCl, 10 mM Na$_2$HPO$_4$, 1 mM KH$_2$PO$_4$, pH 7.4) containing 5% skimmed milk and then incubated with the MAb SS-HepC.1 [17] directed against the residues 5-35 of the HCV core protein, for 1 h at 37°C. After washing, the membrane was made to react with a 1/5000 dilution of horseradish peroxidase-conjugated anti-mouse IgG (Amersham, UK) for 1 h at 37°C. Immuneactivity was detected by using 200 mg/mL 9-amino-4-ethyl carbazole (Sigma, St. Louis, USA) and 0.1% H$_2$O$_2$ in 50 mM NaAc, pH 5.3.

Immunization schedule

BALB/c female mice 6 to 8 weeks old (18-20 g of weight) were purchased from CENPALAB (Havana, Cuba). The housing, maintenance, and care of the animals were in compliance with all relevant guidelines and requirements. Mice were subcutaneously injected at 0, 3, 6 and 10 weeks with 100 µL of the immunogen, containing 5 µg of conjugates or free peptides. Blood samples were collected from the retro-orbital sinus of different isolates, were used here (Table 1):

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HCV peptides conjugated to P64k elicited strong antibody response

Enzyme-linked immunosorbent assay (ELISA)
To detect murine Abs, 96-well microtiter plates (Costar, Cambridge, MA, USA) were coated with 100 µL (1µg/mL) of NS4 or HVR I peptides diluted in coating buffer (50 mM carbonate buffer, pH 9.6), and incubated overnight at 4°C. The wells were washed three times with 0.05% Tween 20 in PBS (PBST) and blocked with 200 µL of PBST containing 1% skimmed milk (Oxoid, Basingstoke, Hampshire, England) for 1 h at room temperature. After three washings with PBST, 100 µL of serial two-fold dilutions of individual mouse sera in PBST were added and incubated at 37°C for 1 h. The plates were washed three times with PBST, and 100 µL of horseradish peroxidase-conjugate goat anti-mouse IgG (Amersham, Little Chalfont, Bucks, UK) diluted 1:3000 was added at 37°C for 1 h. Positive reactions were visualized with o-phenylenediamine (Sigma, St Louis, USA) in 0.1 M citric acid, 0.2 M NaH₂PO₄ pH 5.0 and 0.015% H₂O₂ as the substrate; the reaction was stopped with 50 µL of 2.5 M H₂SO₄. Optical density (OD) at 492 nm was measured in a plate reader (SenslIndScan, Merck, Germany). To evaluate the detection of peptides and conjugates by human sera, a similar ELISA was carried out using an anti-human IgG conjugate (Sigma, St Louis, USA), diluted 1:30000.

Statistical Methods
A Student t test was used for group comparison. Significant differences were considered for p values under 0.05.

Results
Analysis of carrier-peptide conjugates
Synthetic peptides comprising aa sequences of the HVR I and NS4 (aa 384-414 and 1689-1735 in HCV polyprotein, respectively) were chemically conjugated to P64k and Co.120 carrier proteins, to produce the conjugates: P64k-R, P64k-NS4, Co.120-R and Co.120-NS4. The detection of synthetic peptides, either free or conjugated to the P64k protein, by human sera was studied through ELISA. Forty-three human sera from HCV infected patients were studied. The R peptide was similarly detected either alone or conjugated to P64k (41 vs. 44%). However, P64k-NS4 was detected by 15 human sera more than the free peptide (44 vs. 79%). The percent of coincidence, for sera detecting both, the free and conjugated peptide, was 79 for the free R peptide and its P64k conjugate and 55 for the free NS4 and that conjugated to P64k (Table 2).

On the other hand, the Co.120-R and Co.120-NS4 conjugates were analyzed by SDS-PAGE and Western blot (Figure 1). Several bands above the Co.120 protein size were specifically detected by the mAb SS HepC.1 in the conjugate samples but not in the control protein.

Conjugates induced anti-peptide antibody response in mice
The immunogenicity of NS4 and R peptides, free or conjugated, was evaluated in mice. Both, anti-NS4 and anti-R Ab responses were tested 2, 5, 8, and 12 weeks after the first immunization. In the group immunized with P64k-NS4 five weeks after the primary immunization just one mouse seroconverted against NS4, but three weeks later all mice showed anti-NS4 Ab response. Mice immunized with the NS4 free peptide did not show any response during the study. The titers raised by each immunogen are presented in Figure 2A. The Ab response elicited by P64k-NS4 against the NS4 peptide was significantly greater than the free NS4 (p<0.01).

Five weeks after the primary immunization 71% of the mice immunized with P64k-R showed a specific anti-R Ab response. Three weeks later, this group showed 100% seroconversion. The mice immunized with P64k-R showed 100% seroconversion. The mice immunized with P64k-R showed 100% seroconversion. The mice immunized with P64k-R showed 100% seroconversion.

Table 1. Detection of free HCV peptides or those conjugated to the P64k protein by anti-HCV positive human sera

<table>
<thead>
<tr>
<th></th>
<th>P64k-NS4</th>
<th>NS4</th>
<th>P64k-R</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of positive sera</td>
<td>34</td>
<td>19</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td>% of detection</td>
<td>79</td>
<td>44</td>
<td>44</td>
<td>41</td>
</tr>
<tr>
<td>% of coincidence</td>
<td>55</td>
<td>55</td>
<td>79</td>
<td>79</td>
</tr>
</tbody>
</table>

Reactivity of 43 human sera positive to HCV, diluted 1:10, against free peptides or those conjugated to P64k was evaluated by ELISA. The cut-off value to consider a positive reaction was established as twice the mean OD 492nm of the negative control human sera.

protein at 0, 2, 5, 8, and 12 weeks after the primary immunization. The mice were euthanized after the final blood samples were taken.

...'
immunized with the peptide alone did not show a detectable Ab response.

Twelve weeks after primary immunization, the anti-R Ab response elicited by P64k-R was significantly greater than that induced by the free R (p<0.001). In fact, P64k-R induced a strong Ab response (mean Ab titer of 1:39000), Figure 2B. However, the anti-HVR I Ab response elicited by the conjugated P64k-R was isolate restricted (Fig. 3 A). The detection of a panel of carriers for human immunization due to the reactivity of the pre-immune serum.

Discussion

Some of the classical carrier proteins such as BSA have a mammalian origin, thus affecting their immunogenicity in animals [18]. This motivates the search for new carrier molecules, as well as the need for different carriers for human immunization due to the immune suppression induced by the carrier protein [19]. More than just carrier proteins, some synthetic molecules such as the immunostimulating complex (ISCOMS), multiple antigen peptides (MAPs) and constructs with fatty acids [20], have been developed with this aim.

In this study, we include a Neisseria meningitidis protein expressed in E. coli, which has been used before as a carrier, and is suitable for weak immunogens [9]. Here, we found neither an anti-NS4 nor an anti-HVR I Ab response after immunization with the free peptides without the adjuvant. In previous experiments, the NS4 peptide formulated in aluminum hydroxide induced an Ab response after three doses in mice [9], indicating that this region might contain a CD4+ T cell epitope.

Immunization with the P64k-based conjugates strongly increased the anti-HCV Ab response compared to free peptides. Remarkably, the P64k-R elicited a potent Ab response against the HCV HVR I. Previously, a strong anti-HVR I Ab response has been induced by immunization with synthetic peptides formulated in complete and incomplete Freund adjuvant [27]. In a previous study, Goto et al. demonstrated that immunization without the adjuvant with an HVR I peptide conjugated to KLH also elicited a positive Ab response, although with lower Ab titers than the values observed here, in animals vaccinated with the P64k-R conjugate [28]. Interestingly, the Ab response induced by Goto et al. was enough to protect against the challenge with the HCV homologous isolate [28]. Abs targeted at the HVR-I have been shown to inhibit viral attachment to susceptible cells, suggesting that these Abs might neutralize HCV-infectivity.
fection [8]. Moreover, an epitope mapping of Abs detected in the acute, self-limiting and chronic HCV infection has indicated that an early appearance of Abs targeted at the N-terminus of HVR I is associated with the acute, self-limiting infections of HCV [5]. Nevertheless, Abs specific to the HVR I are frequently virus isolate-restricted [29]. Unfortunately, the sera induced by the P64k-R conjugate only detected the peptide based on the Cuban viral isolate sequence. Abs targeted at the HVR I, reactive with variants from isolates corresponding to different genotypes, have been generated after immunization with DNA constructs expressing HCV E2 but infrequently with recombinant proteins [6, 30]. Additionally, aspects such as the form of immunization and the animal model used, have been said to influence the specificity of the Abs against the HVR I [31].

In the mid 80s, recombinant hepatitis B virus cores (HBc) gave way to icosahedral virus-like particles (VLPs) as a basic class of non-infectious carriers of foreign immunological epitopes. The recombinant HBc particles were used to display immunodominant epitopes of the hepatitis B, C, and E viruses, as well as some bacterial and protozoan protein epitopes. The practical applicability of the HBc particles as carriers is due to their ability to express themselves at high levels and to correct self-assembly in heterologous expression systems [21].

Similarly, the HCV core variant Co.120, used here as a carrier, self-aggregates to form virus-like particles [22]. This core variant is highly immunogenic in different animal models [10, 23]. The core antigen is one of the most conserved HCV proteins among the various genotypes, and several B and T cell determinants within this protein have been characterized [24, 25]. Moreover, the association of T-helper (Th) cell response to the HCV core protein with a benign clinical course has also been suggested [26]. Additionally, in the sequence of this protein Th epitopes for mice and humans [32] are reported. These characteristics make this protein interesting as a carrier for HCV B cell epitopes. In the experimental conditions evaluated here, we demonstrated for the first time that lower amounts of HCV peptides conjugated to Co.120 elicited a slightly increased Ab response compared to free peptides. Perhaps, immunization with equal amounts of the conjugated peptide compared to the free peptide might evidence a greater enhancing effect. The analysis of other aspects such as the conjugation method, or the protein-peptide ratio also deserves further research. Unfortunately, immunization with Co.120-R also failed to induce Abs detecting the HVR I from heterologous isolates.

Here, in spite of the isolate-specificity of Abs, the strong humoral response induced against the HVR I by immunization with the P64K-R conjugate is truly encouraging and might be potentially protective against the homologous HCV isolate. Strategies targeted to induce strong and cross-reactive humoral responses against the HVR I could be effective, in the near future, to protect against HCV infection.

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