INVESTIGACIÓN

Immunization with HCV synthetic peptides conjugated to the P64k protein elicited strong antibody response in mice

✉ Liz Alvarez-Lajonchere,[1] Gillian Martínez,[1] Juan Morales,[1]
Julio C Aguilar,[2] Santiago Dueñas-Carrera[1]

[1]HCV Department, [2]Clinical Studies Department. Vaccine Division,
Centro de Ingeniería Genética y Biotecnología. P.O. Box 6162,
Havana City, Cuba. Fax: (53-7) 214764; E-mail: liz.lajonchere@cigb.edu.cu

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 I 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

Biotecnología Aplicada 2003;20:209-213

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 (RHV 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 RHV I alcanzó el valor de 1:39000 en los ratones inmunizados con el conjugado que contenía P64k. Lamentablemente los anticuerpos anti-RHV I generados por los conjugados de Co.120 y P64k solo reconocieron la RHV 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 RHV I, potencialmente capaz de neutralizar el aislamiento homólogo del VHC.

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

*I*ntroduction

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].

1. Kiyosawa K, Tanaka E, Sodeyama T. Hepatitis C virus and hepatocellular carcinoma. In: Hepatitis C virus and hepatocellular carcinoma. Curr Stud Hematol Blood Transf. Basel: Karger 1998;62:161-80.

2. Grakoui A, Wychowski C, Lin C, Feinstone SM, Rice CM. Expression and identification of hepatitis C virus polyprotein cleavage products. J Virol 1993;67:1385-95.

3. Lechmann M,Liang TJ. Vaccine develpment for hepatitis C. Semin Liver Dis 2000;20:211-26.

4. Lechner F, Wong DK, Dunbar PR, Chapman R, Chung RT, Dohrenwend P, et al. Analysis of successful immune responses in persons infected with hepatitis C virus. J Exp Med 2000;191:1499-512.

✉ 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 1

Peptide	Sequence	Genotype	Isolate
1 a	THVTGGSAGHTVSGFVS LLAPGAKQNV	1a	HCV-1
1 b	THVTGGAQAKTTNRLVS MFASGPSQKI	1b	HCV-BK
2 a	THTVGGSTAHNARTLTG MFSLGARQKI	2a	HC-J6
2b	TYSSGQEAGRTVAGFAG LFTTGAKQNL	2b	HC-J8
SS	TTTVGGQASHQVHSLTG LFSPGAKQNV		
R	TGTYVTGGTAARGVSQF TGLFTSGPSQKIQL	1b	Cuban

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 proteins comprised the sequence in the NS4 protein (aa 1689-1735):

SGRPAVIPDREVLYQEFDEMEECASHLPYIE QGMQLAEQFKQKALGL. 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, Zwitzerland), 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_2HPO_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 8-8.5 using 3.0 M NaOH. The mixture was dialyzed against water at pH 4-5. Then 7 mg of 1-ethyl-3-(dimethylaminopropyl) 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 succinic-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 UMELISA HCV from the Centro de Inmunoensayo (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_2HPO_4, 1 mM KH_2PO_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. Immunoreactivity was detected by using 200 mg/mL 9-amino-4-ethyl carbazole (Sigma, St. Louis, USA) and 0.1% H_2O_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

5. Zibert A, Kraas W, Meisel H, Jung G, Roggendorf M. Epitope mapping of antibodies directed against hypervariable region 1 in acute self-limiting and chronic infections due to hepatitis C virus. J Virol 1997;71:4123-7.

6. Zibert A, Schreier E, Roggendorf M. Antibodies in human sera specific to hypervariable region 1 of hepatitis C virus can block viral attachment. Virology 1995; 208:653-61.

7. Lee SW, Cho JH, Lee KJ, Sung YC. Hepatitis C virus envelope DNA-based immunization elicits humoral and cellular immune responses. Mol Cells 1998;8:444-51.

8. Choo QL, Kuo G, Ralston R, Weiner A, Chien D, Van Nest G, et al. Vaccination of chimpanzees against infection by the hepatitis C virus. Proc Natl Acad Sci USA 1994;91:1294-8.

9. Gonzalez S, Alvarez A, Caballero E, Vina L, Guillen G, Silva R. P64k meningococcal protein as immunological carrier for weak immunogens. Scand J Immunol 2000; 52:113-116.

10. Dueñas-Carrera S, Morales J, Acosta-Rivero N, Lorenzo LJ, García C, Ramos T, et al. Variable level expression of hepatitis C virus core protein in a prokaryotic system. Analysis of the humoral response in rabbit. Biotecnologia Aplicada 1999;16: 226-31.

11. Guillén G, Alvarez A, Silva R, Morera V, Gonzales S, Musacchio A, et al. Expression in Escherichia coli of the ludA gene: Protein sequence análisis and immunological characterization of the P64k protein from Neisseria meningitidis. Biotechnol. Appl Biochem 1998;27:189-96.

12. Houghten RA, DeGraw ST, Bray MK, Hoffmann SR, Frizzell ND. Simultaneous multiple peptide synthesis: the rapid preparation of large numbers of discrete peptides for biological, immunological, and methodological studies. BioTechniques 1986;4:522-6.

13. Tam JP, Heath WF, Merrifield RB. S_N2 deprotection of synthetic peptides with a low concentration of HF in dimethyl sulfide: evidence and application in peptide synthesis. J Am Chem Soc 1983;105: 6442-55.

14. Deen C, Claassen E, Gerritse K, Zegers ND, Boersma WJA. A novel carbodiimide coupling method for synthetic peptides: Enhanced antipeptide antibody responses. J Immunol Methods 1990;129:119-125.

15. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-254.

16. Lorenzo LJ, García O, Acosta-Rivero N, Dueñas-Carrera S, Martínez G, Alvarez-Obregón JC, et al. Expresión and immunological evaluation of the Escherichia Coli-derived hepatitis C virus enverlope E1 protein. Biotechnol Appl Biochem 2000; 32:137-143.

17. Falcon V, Garcia C, de la Rosa MC, Menendez I, Seoane J, Grillo JM.. Ultrastructural and immunocytochemical evidences of core-particle formation in the methylotrophic Pichia pastoris yeast when expressing HCV structural proteins (core-E1). Tissue Cell 1999;31:117-125.

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

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 4ºC. 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 37ºC 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 37ºC for 1 h. Positive reactions were visualized with o-phenylenediamine (Sigma, St Louis, USA) in 0.1 M citric acid, 0.2 M NaH $_2PO_4$ pH 5.0 and 0.015% H as the substrate; the reaction was stopped with 50 µL of 2.5 M H_2SO_4. Optical density (OD) at 492 nm was measured in a plate reader (SensIdent Scan, 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.

The cut-off value to consider a positive Ab response was established as twice the mean OD_{492nm} of the negative control sera (sera from mice before immunization or from humans negative for anti-HCV Abs). Seroconversion was considered for animals showing a positive Ab response in sera diluted 1:50.

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.

Table 1. Detection of free HCV peptides or those conjugated to the P64K protein by anti-HCV positive human sera

	P64k-NS4	NS4	P64k-R	R
Number of positive sera	34	19	19	18
% of detection	79	44	44	41
% of coincidence	55	55	79	79

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.

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 im-

18. Geerligs HJ, Weijer WJ, Welling GW, Weling WS. The influence of different adjuvants on the immune response to a synthetic peptide comprising amino acid residues 9-21 of herpes simplex virus type 1 glycoprotein D. J Immunol Meth 1989; 124:95-102.

19. Fattom a, Cho YH, Chu C, Fuller S, Fries L, Naso R. Epitopic overload at the site of injection may result in suppression of the immune response to combined capsular polysaccharide conjugate vaccine. Vaccine 1999;17:126-133.

20. Beekman NJ, Schaaper WM, Turkstra JA, Meloen RH. Highly immunogenic and fully synthetic peptide-carrier constructs targetting GnRH. Vaccine 1999;17:2043-2050.

21. Pumpens P, Grens E. HBV core particles as a carrier for B cell/T cell epitopes Intervirology 2001;44:98-114.

22. Lorenzo LJ, Dueñas-Carrera S, Falcón V, Acosta-Rivero N, González E, de la Rosa MC, et al. Assembly of truncated HCV core antigen into virus-like particles in E. coli. Biochem Bioph Res Commun 2001; 281:962-965.

23. Alvarez-Obregon JC, Dueñas-Carrera S, Valenzuela C, Grillo JM. A truncated HCV core protein elicits a potent immune response with a strong participation of cellular immunity components in mice. Vaccine 2001;19:3940-3946.

24. Jackson P, Petrik J, Alexander GJM, Pearson G, Allain J-P. Reactivity of synthetic peptides representing selected regions of hepatitis C virus core and envelope proteins with a panel of hepatitis C virus-seropositive human plasma. J Med Virol 1997;51:67-79.

25. Wang Y-F, Brotman B, Andrus L, Prince AM. Immune response to epitopes of hepatitis C virus (HCV) structural proteins in HCV-infected humans and chimpanzees. J Infect Dis 1996;173:808-821.

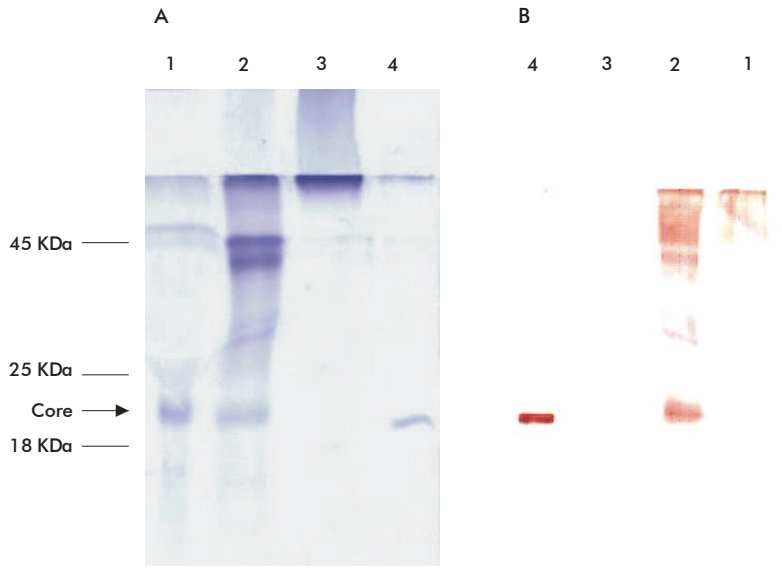

Figure 1. Analysis of Co.120-based conjugates by SDS-PAGE (A) and Western blot (B). Lane 1: Co.120-R, Lane 2: Co.120-NS4, Lane 3: P64k-NS4, Lane 4: Co.120. The MAb SS-HepC.1 was used for specific detection in Western blot.

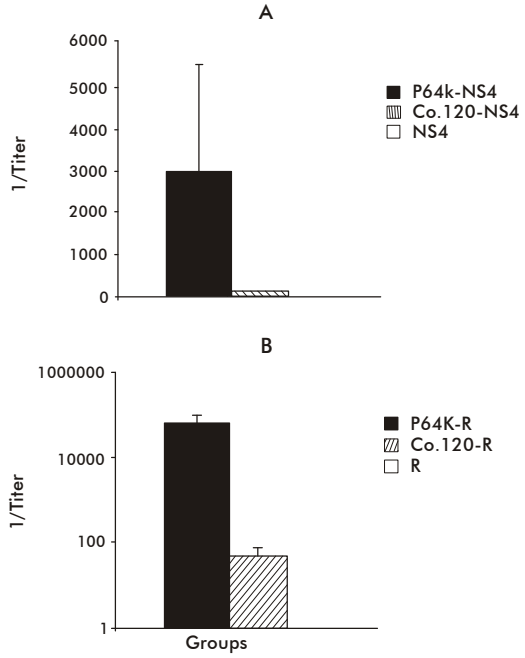

Figure 2: Anti-HCV peptide response in mice. The results are shown as the reciprocal mean antibody titer induced against NS4 (A) and R (B) peptides, twelve weeks after primary immunization. The titer was determined by ELISA as the greatest dilution where the serum reactivity was at least twice the reactivity of the pre-immune serum.

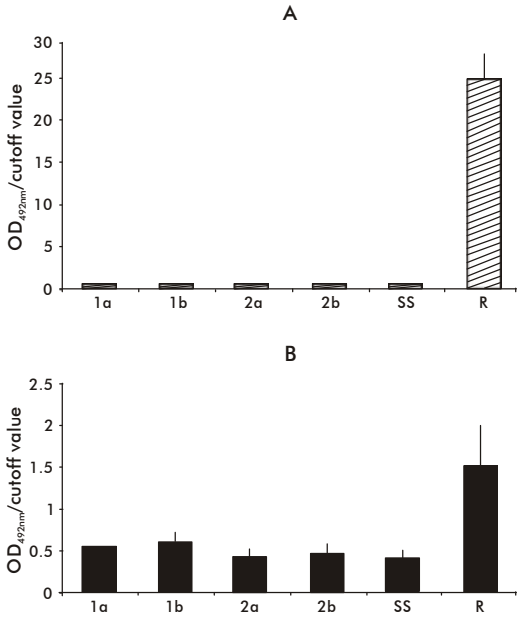

Figure 3. Anti-HVR I response in P64k-R (A) and Co.120-R (B) immunized mice. ELISA plates were coated with HVR-I peptides from different genotypes: 1a, 1b, 2a, 2b, one consensus sequence (SS) and the peptide present in the immunogen (R). The results are shown as $OD_{492\ nm}$/cut-off value. The error bars indicate the standard deviation of the mean.

munized 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 HVR I corresponding to different virus genotypes by the P64k-R anti-sera was studied by ELISA. The panel of peptides was designed to have isolates represented from genotypes 1a, 1b, 2a and 2b. We also evaluated a peptide comprising a HVR I consensus sequence. The sera from mice immunized with P64k-R only detected peptide R used for immunization (Fig.3 A).

On the other hand, the Ab response elicited by the Co.120 conjugates was evaluated as well. Mice immunized with Co.120-NS4 showed 57% seroconversion by week 12. In regard to anti-R Ab response, at week 12, only 42% of mice immunized with Co.120-R were positive. The titers raised by each immunogen are presented in Figure 2A and 2B. Moreover, immunization with Co.120-R, as previously observed after inoculation with P64k-R, elicited an isolate restricted Ab response (Figure 3B).

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-1 have been shown to inhibit viral attachment to susceptible cells, suggesting that these Abs might neutralize HCV in-

26. Botarelli P, Brunetto MR, Minutello MA, Calvo P, Unutmaz D, Weiner AJ, et al. T-lymphocyte response to hepatitis C virus in different clinical courses of infection. Gastroenterology 1993;104:580-587.

27. Puntoriero G, Meola A, Lahm A, Zucchelli S, Ercole BB, Tafi R, et al. Towards a solution for hepatitis C virus hypervariability: mimotopes of the hypervariable region 1 can induce antibodies cross-reacting with a large number of viral variants. EMBO J 1998;17:3521-3533.

28. Goto J, Nishimura S, Esumi M, Makizumi K, Rikihisa T, Nishihara T, et al. Prevention of hepatitis C virus infection in a chimpanzee by vaccination and epitope mapping of antiserum directed against hypervariable region 1. Hepatol Res 2001;19(3):270-83.

29. Kato N, Nakazawa T, Ootsuyama Y, Sugiyama K, Ohkoshi S, Shimotohno K. Virus isolate-specific antibodies against hypervariable region 1 of the hepatitis C virus second envelope protein, gp70. Jpn J Cancer Res 1994;85:987-991.

30. Dueñas-Carrera S, Alvarez-Lajonchere L, Cesar Alvarez-Obregon J, Perez A, Acosta-Rivero N, Vazquez DM, et al. Enhancement of the immune response generated against hepatitis C virus envelope proteins after DNA vaccination with polyprotein-encoding plasmids. Biotechnol Appl Biochem 2002;35:205-212.

31. Fournillier A, Depla E, Karayiannis P, Vidalin O, Maertens G, Trepo C, et al. Expression of noncovalent hepatitis C virus envelope E1-E2 complexes is not required for the induction of antibodies with neutralizing properties following DNA immunization. J Virol 1999;73:7497-7504.

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.

Acknowledgments

We would like to thanks Dr. Ricardo Silva for the donation of the P64k protein and Dr. Sonia Gonzalez for the constructive reading of the manuscript.

Received in January, 2003. Accepted for publication in October, 2003.

32. Lechmann M, Ihlenfeldt HG, Braunschweiger I, Giers G, Jung G, Matz B, et al. T- and B-cell responses to different hepatitis C virus antigens in patients with chronic hepatitis C infection and in healthy anti-hepatitis C virus—positive blood donors without viremia. Hepatology 1996;24(4):790-5.