Original Article

Evaluation of cellular responses for a chimeric HBsAg-HCV core DNA vaccine in BALB/c mice

Maryam Yazdanian, Arash Memarnejadian, Mehdi Mahdavi[1], Fatemeh Motevalli, Seyed Mehdi Sadat, Rouhollah Vahabpour, Hossein Khanahmad[2], Hoorieh Soleimanjahi[3], Agata Budkowska[4], Farzin Roohvand[1]

Departments of Hepatitis and AIDS, [1]Department of Virology, Pasteur Institute of Iran, Tehran, [2]BCG Research Center, Karaj Research and Production, Pasteur Institute of Iran, Karaj, [3]Department of Virology, Medical School, Tarbiat Moderes University (TMU), Tehran, Iran, [4]Department of Virology, Unit of Hepacivirus and Innate Immunity, Pasteur Institute, 25/28 Rue du Dr. Roux, Paris 75724, France

Abstract

Background: Fusion of Hepatitis B virus surface antigen (HBsAg) to a DNA construct might be considered as a strategy to enhance cellular and cytotoxic T-lymphocytes (CTL) responses of a Hepatitis C Virus core protein (HCVcp)-based DNA vaccine comparable to that of adjuvanted protein (subunit) immunization.

Materials and Methods: pCHCORE vector harboring coding sequence of HBsAg and HCVcp (amino acids 2-120) in tandem within the pCDNA3.1 backbone was constructed. The corresponding recombinant HCVcp was also expressed and purified in *Escherichia coli*. Mice were immunized either by adjuvanted HCVcp (pluronic acid + protein) or by pCHCORE vector primed/protein boosted immunization regimen. The cellular immune responses (proliferation, *In vivo* CTL assay and IFN-γ/IL-4 ELISpot) against a strong and dominant H2-d restricted, $CD8^+$-epitopic peptide (C39) (core 39-48; RRGPRLGVRA) of HCVcp were compared in immunized animals.

Result: Proper expression of the fused protein by pCHCORE in transiently transfected HEK 293T cells and in the expected size (around 50 kDa) was confirmed by western blotting. The immunization results indicated that the pCHCORE shifted the immune responses pathway to Th1 by enhancing the IFN-γ cytokine level much higher than protein immunization while the proliferative and CTL responses were comparable (or slightly in favor of DNA immunization).

Conclusion: Fusion of HBsAg to HCVcp in the context of a DNA vaccine modality could augment Th1-oriented cellular and CTL responses toward a protective epitope, comparable to that of HCVcp (subunit HCV vaccine) immunization.

Key Words: Cytotoxic T-lymphocyte response, Deoxyribonucleic acid vaccine, ELISpot, Hepatitis C virus, HCV core protein, HBsAg

Address for correspondence:
Dr. Farzin Roohvand, Department of Virology, Pasteur Institute of Iran, Pasteur Ave., Tehran 13164, Iran.
E-mail: rfarzin@pasteur.ac.ir
Received: 16.04.2013, **Accepted:** 28.08.2013

Access this article online

Quick Response Code:

Website: www.advbiores.net

DOI: 10.4103/2277-9175.148296

INTRODUCTION

Hepatitis C virus (HCV), one of the major causes of blood-borne acute and chronic hepatitis, holds a single-stranded positive-sense RNA genome that encodes at least 10 proteins, including three structural proteins (core, and envelope proteins E1 and E2), a small protein p7, and six nonstructural (NS) proteins (NS2,

Copyright: © 2015 Yazdanian. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

How to cite this article: Yazdanian M, Memarnejadian A, Mahdavi M, Motevalli F, Sadat SM, Vahabpour R, *et al*. Evaluation of cellular responses for a chimeric HBsAg-HCV core DNA vaccine in BALB/c mice. Adv Biomed Res 2015;4:13.

NS3, NS4A, NS4B, NS5A, and NS5B). An estimated 180 million people are infected with HCV around the world and annually 3-4 million individuals are newly infected. HCV causes a high rate of chronic infection, which can lead to liver cirrhosis and hepatocellular carcinoma. There is no effective vaccine against HCV infection and the optimal treatment, which is the combination of pegylated IFN-α with ribavirin eradicates the virus in less than 50% of the patients.[1,2] These facts imply the immediate need for development of an effective prophylactic and/or therapeutic vaccine against this infection. During prior years, great attempts and diverse strategies were employed to provide efficient vaccines against HCV infection by using different HCV antigens in various formulations and modalities such as recombinant protein (subunit), plasmid DNA, peptide, live vector-based and epitopic/polytopic vaccines.[2,3] Accumulated data from these previous studies suggested that cellular responses including HCV-specific cytotoxic T-lymphocytes (CTL) play a significant role in recovery of infected persons. In effect, CD8+ cell responses to viral epitopes accompanied with CD4+ helper activities and secretion of the Th1-type cytokines is shown to be efficient in HCV elimination, while the role of antibodies seems to be limited.[2] Accordingly, among different vaccine modalities, DNA vaccination against HCV was of interest because of possibility of inducing strong and specific cellular immune responses by DNA immunization.[3,4] Induction of weak immune responses by DNA immunization was, however, the major shortcoming of such vaccines, which has been conquered by several approaches[3] like coadministration of immunological adjuvant such as IL-23 and GM-CSF[5] or DNA and recombinant protein vaccine.[6] More recently, fusion of virus-like particle (VLP)-forming elements (like hepatitis B surface antigen; HBsAg), which encode viral structural proteins capable of assembling into particulate repeated arrays, are considered as a promising approach for the efficient delivery of antigenic targets.[7] The small hepatitis B surface antigen (HBsAg-S; comprising the 219 amino acid of the small envelope protein of hepatitis B virus), is highly immunogenic and capable of multimerization and self-assembly to form VLPs. The corresponding VLPs can act as a perfect adjuvant for antigens of other pathogens (in fused and chimeric forms) while also are capable of delivering them as immunogenic particles to antigen presenting cells (APCs) to elicit enhanced cellular and CTL responses.[8,9] In this context, fusion of HBsAg in various DNA vaccines was already employed to enhance cellular responses toward targeted antigens with different degrees of success.[7,10,11]

Among HCV proteins, core (HCVcp) is the most conserved protein among various HCV genotypes, which besides capsid protein, its nucleotide sequence might encode expression of other proteins (Core+1/ARFP) through ribosomal-frame shifts[12] and contains a number of T cell, and CTL determinants, while antibodies against it are also the first to be detected after onset of the infection.[6,13-16] These properties make HCVcp an attractive candidate for HCV vaccine formulations. However, it is shown that the full length core protein exerts immune-suppressive effects on antigen presenting (APCs) and T cells through its C-terminal region to reduce both innate and acquired immune responses.[2,17] In this context, truncated form of HCVcp representing the first 120 N-terminal residues (hydrophilic region), which contains both nuclear localization signals (NLS)[18] and conserved epitopes for cellular and CTL responses was considered for formulation of a number of HCV vaccine candidates.[2,19,20] Results of these previous studies, however, indicated that (as could be anticipated) HCVcp is a rather weak immunogen when used as a DNA vaccine (compared with adjuvanted subunit vaccines),[3,4,21,22] and therefore sounded for the need of improvements in the formulation of HCVcp-based DNA vaccine modalities.

In the present study, a DNA construct consisted of HBsAg fused to the HCVcp (amino acids 2-122) is considered for immunization of BALB/c mice in a DNA/protein prime/boost immunization regime and cellular immune responses toward a dominant H2-d restricted epitope within HCVcp were compared with that of protein immunization (adjuvantedHCVcp-based subunit vaccine) alone to evaluate the potential effects of HBsAg fusion in enhancement of immune responses of the DNA construct.

MATERIALS AND METHODS

Recombinant HCVcp protein and the H2-d restricted peptide

Expression of HCVcp (1-122) by pIVEX2.4a-core vector harboring the corresponding gene in BL21-AI strain of *Escherichia coli* under the control of an arabinose-inducible (araBAD) promoter[23,24] and detailed steps for protein induction by arabinose and purification through application of nitrilotriacetic acid (Ni-NTA) chromatography has been already described.[20,24] The protein concentration was determined by the BCA protein assay method (Pierce USA) and the endotoxin level, was measured by QCL-1000 Chromogenic Limulus amoebocyte lysate test (BioWhittaker). The strong and dominant H2-d restricted, $CD8^+$-epitopic peptide (C39) (core 39-48; RRGPRLGVRA) of HCVcp was synthesized with 95% purity (Biomatik Co., Canada) and used for all immune analyses throughout this study as described elsewhere.[17,20,25]

Construction of HCVcp-based DNA vaccine plasmid

The HCV core (amino acids 2-122) gene was amplified by polymerase chain reaction (PCR) from the same pIVEX2.4a-core plasmid,[23,24] which was also used for protein expression in *E. coli* in the present study (as noted before). The upstream primer contained a *BamH*I site (underlined) at its 5′ (5′TATGGATCCGGCACGATTCCCAAACC 3′) and the downstream primer harbored an *Eco*RV site (underlined) and two stop codons (bolded; TCA/TTA) successively at its 5′ site (5′AAGGATATCTCATTACTTACCCAAATTGC3′). Amplification was carried out using *Pfu* DNA polymerase (fermentas) and by the following protocol: Predenaturation at 94°C for 4 min, and 30 cycles of denaturation at 94°C, annealing at 55°C for 1 min and extension at 72°C for 1 min followed by 10 min at 72°C. The PCR-amplified fragment was treated with *BamH*I/*EcoR*V enzymes and the gel purified amplicon was inserted into the same restriction site of the pCHBS eukaryotic expression vector to produce chimeric pCHCORE vector harboring HBsAg and HCV core genes in tandem [Figure 1b]. Construction of pCHBS vector containing HBsAg coding sequence (ayw serotype; GenBank accession X02496, REGION: 157-837) in *Hind*III-*BamH*I sites within a pCDNA3.1 plasmid [Figure 1a] was previously described.[11] Confirmation of the pCHCORE vector was achieved by restriction analyses and sequencing reactions.

SDS–PAGE and western blotting analysis

Expression of the HBsAg-HCVcp fusion protein by pCHCORE was assayed in transiently transfected HEK 293T cells by western blotting analysis. In brief, cells were transfected with pCHCORE using lipofectamine (GIBCO-BRL, Scotland). Subsequently, transfected cells were resuspended in lysis buffer, containing 0.1 M Tris–Cl (pH 7.8) and 0.5% (V/V) Triton X-100. Cell lysate was used to detect HBsAg-core fusion protein using primary mouse anti-HBsAg polyclonal antibody followed by incubation with HRP-conjugated anti-mouse antibody (DAKO, Denmark). Accordingly, purified recombinant HCVcp was analyzed by SDS-PAGE and western-blot analysis as previously described.[20,22,23]

Immunization of mice

Six-to eight-week-old, BALB/c (H2d) female mice were purchased from Pasteur Institute of Iran (Karaj, Iran) and housed and handled according to international animal care ethics. The mice were divided into five groups containing eight mice each to receive different immunogens as summarized in Table 1. The mice were immunized either sub-cutaneously (s.c.) in the tail base with 50 μl of purified protein immunogen containing 5 μg of HCVcp formulated in pluronic acid (F127) as adjuvant according to the prior description[20] or intramuscularly (into the quadriceps muscles of leg) with 100 μg of the plasmid (pCHBS or pCHCORE) in the final volume of 50 μl (2 μg/ml). DNA vaccination was performed as heterologous prime/boost immunization using purified plasmid DNA prepared by EndoFree Plasmid Giga Kit (Qiagen, Australia). Control groups were injected by either phosphate buffered saline (PBS), F127 or pCHBS alone [Table 1].

Table 1: Groups of vaccinated mice, immunogen formulations and immunization regimens

Vaccinated groups	Vaccine formulation	Vaccination regimen*
1	Saline	Saline (0,10, 20)
2	F127 (8% W/V)	F127 (0,10, 20)
3	pCHBS	pCHBS (0, 21, 42)
4	HCVcp+F127	HCVcp+F127 (0, 10, 20)
5	pCHCORE	pCHCORE (0,21) HCVcp+F127 (42)

*Numbers in the parentheses indicate the days of animal immunization

Preparation of the splenocytes

Three weeks after the last immunization, spleens of the immunized mice were removed, smashed into a cell homogenizer, washed with saline supplemented with 2% fetal bovine serum (FBS) and splenocytes were isolated by removing the erythrocytes with 0.83% NH4Cl solution. Isolated cells were cultivated in complete RPMI-1640 medium, containing 10% FBS, 2 mM L-glutamine, 100 mg/ml streptomycin and 100 U/ml penicillin.

Antigen specific proliferation assay

Cultivated splenocytes were cultured in triplicate in a 96-well plate (1.5×10^5 cells/well) and stimulated with 2 μg/well of C39 peptide as recall antigen. Phytohemagglutinin-A (PHA) (5 μg/ml; Gibco) was used as a positive control and un-stimulated wells were used as negative controls. After 72 h of incubation at 37°C in 5% CO_2 humid incubator, the antigen specific proliferations were measured by 5-bromo-2 deoxyuridine (BrdU) incorporation into the stimulated splenocytes using a cell proliferation ELISA kit (Roche Diagnostic CmbH, Mannheim, Germany) following the manufacturer's recommendations. The stimulation index (SI) for cell activity was calculated using the following formula: SI = (OD stimulated − blank)/(OD nonstimulated − blank).

In vivo CTL assay

To prepare CTL targets, single cell suspensions of splenocytes from naive BALB/c mice were depleted of red cells and divided equally into two parts. The first suspension labeled with a high concentration (5 μM)

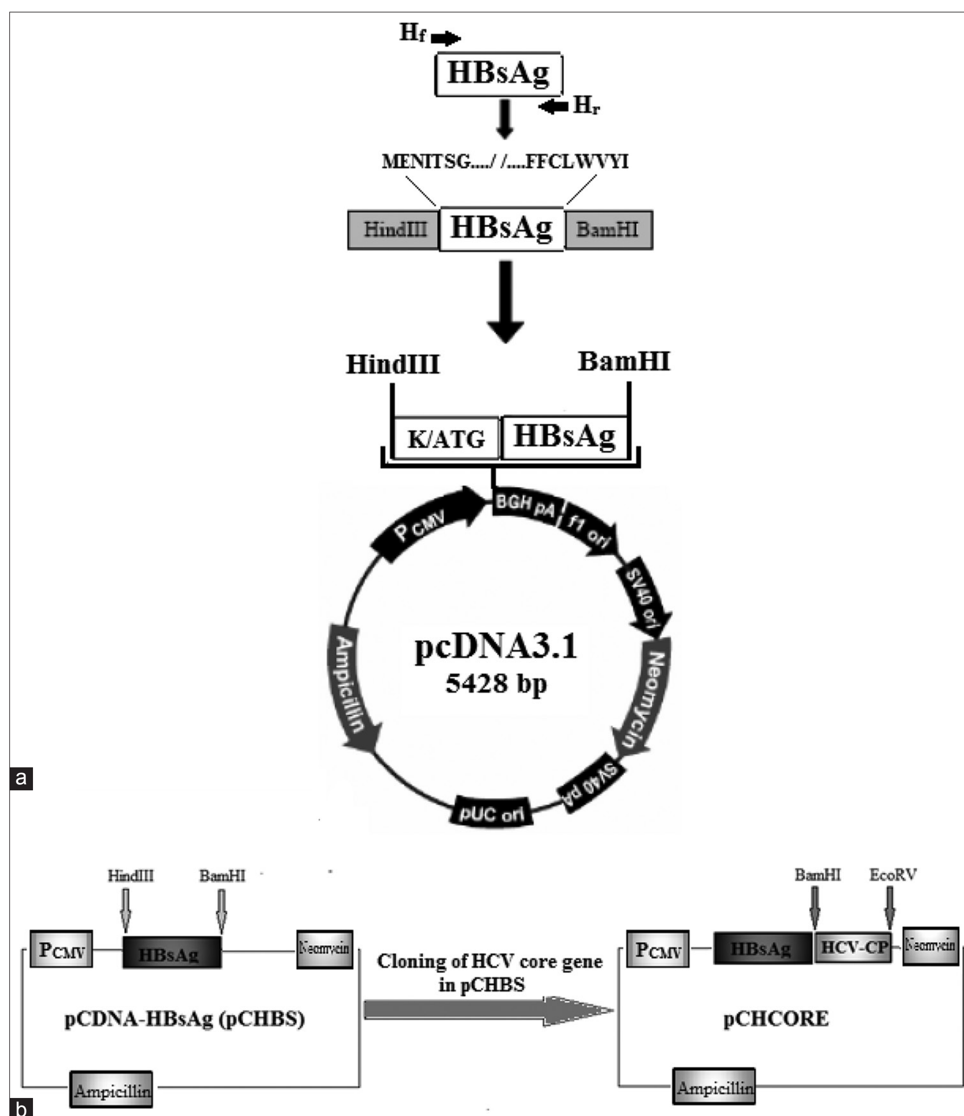

Figure 1: Schematic presentation of pCHBS and pCHCORE vector construction. (a) In a previous study[11] the HBsAg was PCR amplified and cloned into *Hind*III/*BamH*I restriction enzyme sites of pCDNA3.1 to produce pCHBS vector. K/ATG denotes Kozak/start codon. P_{CMV} and BGH pA show the CMV promoter and bovine growth hormone polyadenylation site, respectively. SV40 ori and SV40 pA denote to the same elements from SV40. (b) Schematic diagram for insertion of HCVcp (2-120) DNA sequence in pCHBS (HBsAg harboring pCDNA3.1 plasmid) to construct the pCHCORE vector

of Carboxy Fluorescein diacetate, Succinimidyl Ester (CFSE) (CFSE high population). The second suspension was labeled with a low concentration (0.5 µM) of CFSE (CFSE low population). The stimulating peptide was added to high CFSE tube to a final concentration of 10 µM (10 µg/ml) and incubated for 60-90 min at 37°C. Equal numbers of cells from each population were pooled and 100 µl of it containing 4-6 × 10^6 total cells were injected intravenously into each recipient mice. 20 h later spleens were harvested from the mice and the relative proportion of CFSE-high and CFSE-low cells was determined by flow cytometry using a PAS (ParTec) instrument and analyzed using FlowMax (Partec) software. Percent-specific lysis was calculated by [1−(r-unprimed/r-primed)] ×100 where r= %CFSE-low/%CFSE-high for each mouse.

ELISPOT assay for IFNγand IL-4 cytokines

The ELISPOT assay was used to determine IFNγ and IL-4 secreting cells among the mice spleen cells under the stimulation of peptide C39 using Mouse Elispot kits (Mabtech, Sweden) according to the manufacturer protocol. In brief, splenocytes (3 × 10^5 cells/well) were plated in triplicate onto either anti-IFNγ or anti IL-4 coated 96-well plates and stimulated for 48 h with epitopic peptide C39 (2 µg/well) in separate reactions. After washing steps, the secondary biotin-conjugated anti-IFN-γ/IL-4 detection antibody was added and incubated at room temperature for 1 h. The wells

were washed with PBS and substrate solution (BCIP/NBT) was added. After developing, the spots were counted using a dissection stereoscope (Nikon, Japan). The results were expressed as the numbers of spot-forming-cells (SFC) per 10^6 splenocytes. Cell mitogen PHA (phytohemaglotinin, Sigma chemicals) at a concentration of 2 μg/ml was used as positive control.

Statistical analysis

Student's t-test and one-way analysis of variance (ANOVA) analyses were employed to evaluate the differences of the change in the percent lysis, cell proliferation and the level of cytokine production among immunized groups. Values of $P < 0.05$ were considered significant.

RESULTS

Analysis of protein expression by recombinant constructs

Construction of pCHCORE vector by insertion of HCVcp (2-120) DNA sequence in pCHBS (HBsAg harboring pCDNA3.1 plasmid;[11] Figure 1a) is schematically presented in Figure 1b. Analysis by restriction enzymes followed by sequencing reactions approved the accuracy of the chimeric expression vector (results not shown). Analysis of the lysates of pCHCORE transfected HEK293T cells by western blotting indicated the *in vitro* expression of the HBsAg-HCVcp fusion protein with the expected size of around 50 kDa in the doublet shaped bands, which demonstrated the occurrence of both glycosysated and nonglycolsylated forms of the fused protein [Figure 2]. Accordingly and in agreement with our previous results,[20,23,24] coomassie blue-stained gel of SDS–PAGE and western blot analyses of arabinose-induced lysates of BL21-AI strain of *E. coli* cells harboring pIVEX2.4a-core vector, indicated a 21-kDa protein band corresponding to HCVcp (residues 2-122). SDS-PAGE and densitometry analysis of the Ni-NTA chromatography-based purified protein demonstrated over 85% purification and less than 25 endotoxin units per 50 μg of the protein as previously described[20] (results are not shown due to their presence in our previous publications, which are accordingly referred here).

Evaluation of cell proliferation for DNA and protein immunizations

To evaluate the lymphocyte proliferation response, splenocytes of immunized mice were stimulated with C39 peptide and incorporation of 5-bromo-2 deoxyuridine (BrdU) into the stimulated splenocytes was detected by ELISA as described in the method section. Results indicated that the lymphocytes obtained from the animals immunized with HCVcp and DNA vaccine proliferated significantly higher

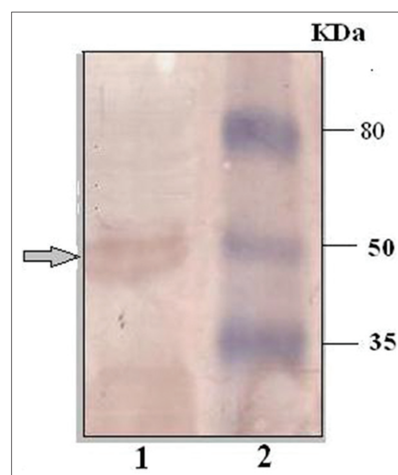

Figure 2: *In vitro* expression analysis of pCHCORE plasmid by western blotting. Assessment of the expression for HBsAg-HCVcp fusion protein in cell lysates of pCHCORE transfected HEK 293T cells was carried out by using anti-HBsAg polyclonal antibody. In lane 1 of the blotted membrane presentation of the doublet bands in expected sizes (≈50 kDa) (indicated arrows) are due to the presence of both glycosylated and nonglycosylated forms of HBsAg-HCVcp fusion protein. Lane 2: Protein MW marker

compared with control groups (saline, F127 and pCHBS) following *in vitro* stimulation with peptide C39 [Figure 3a]. Additionally, the proliferative response of animals that were immunized with pCHCORE had a significantly higher SI mean compared with those immunized with HCVcp + F127 ($P < 0.05$).

In vivo CTL assay

Results of CTL assay indicated a significant increase in specific lysis of C39 peptide-pulsed target cells by the splenocytes of pCHCORE and HCVcp-vaccinated groups compared with other control groups [Figure 3b]. Although The CTL activity against peptide C39 was highest in animals immunized with pCHCORE compared with HCV core protein but there was no significant difference between them [Figure 3b].

Ex vivo IFN-γ and IL-4 ELISpot assays

In accordance with the results of proliferation and CTL assays, data of *ex vivo* ELISpot assay also indicated a significant difference in the number of IFN-γ and IL-4-secreting cells in favor of pCHCORE and HCVcp immunized mice groups compared with the control groups [Figure 4]. Moreover, as shown in Figure 4, the group immunized with pCHCORE elicited significantly more spots of IFN-γ than HCVcp vaccinated groups. In contrast, the immunized group by HCVcp shows the IL-4 secreting responses significantly higher than the vaccinated group by pCHCORE. These results clearly indicated an elevated Th1-oriented response for our DNA vaccination strategy compared with that of protein immunization.

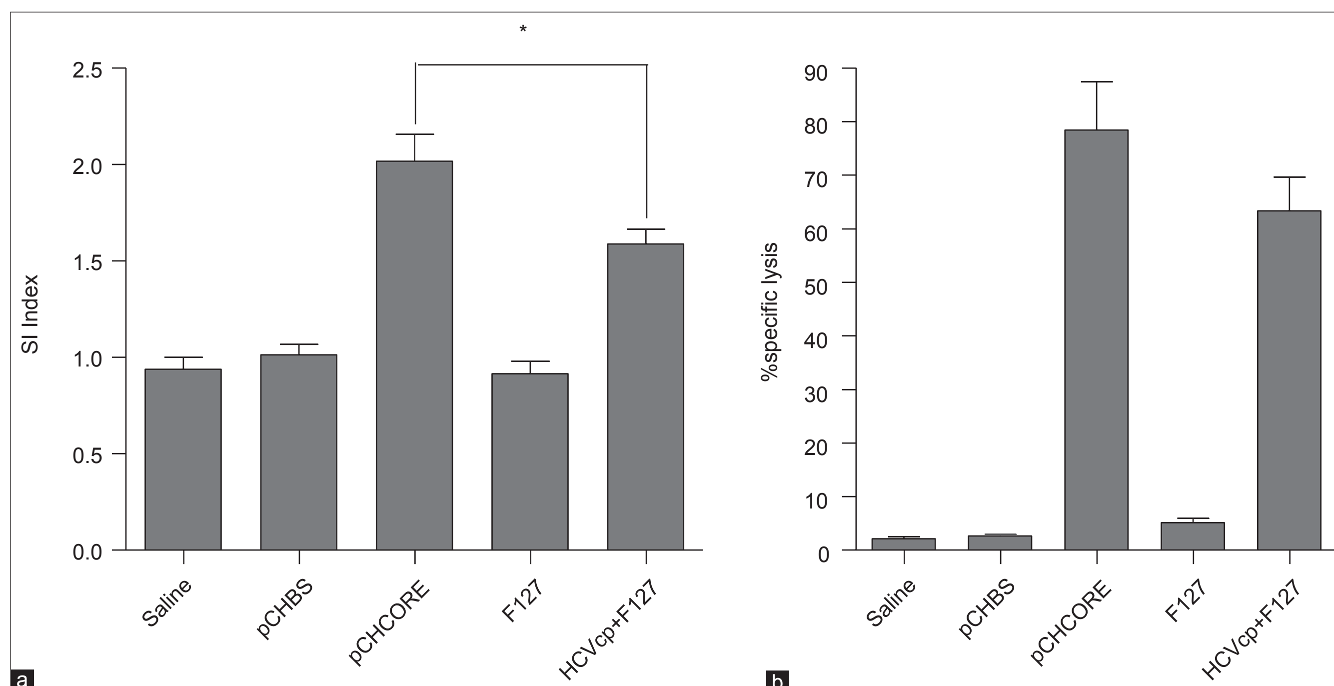

Figure 3: Lymphoproliferative and CTL responses to HCVcp and pCHCORE DNA vaccine immunizations. (a) Lymphocytes isolated from the spleens of vaccinated mice were cultured and pulsed with C39 peptide. The proliferative response was measured by cell proliferation ELISA, BrdU colorimetric kit (Roche Diagnostics, Germany) as per the manufacturer's protocol. The data represents mean SI of two determinations ± S.D. The figure is representative of two different experiments with similar observations (* indicates statistical significance, P value < 0.05). (b) *In vivo* CTL responses measurement of the relative proportion of CFSEhigh and CFSElow cells by flow cytometry via a FACS Calibur (BD Biosciences) indicated that Mice immunized with pCHCORE and HCVcp + F127 showed significantly higher specific lysis than control groups with no significant difference between both groups

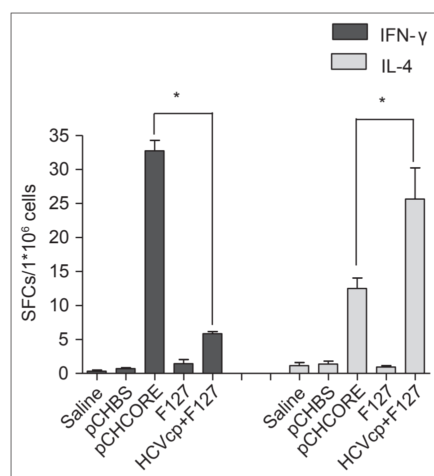

Figure 4: Enzyme-linked-immunospot assays. Mouse Elispot kit (Mabtech, Sweden) was used for detection of class I-binding C39 peptide specific IFN-γ and IL-4-releasing T cells in splenocytes of immunized mice. Results are shown as the numbers of spot-forming-cells per 10^6 splenocytes

DISCUSSION

Prior studies suggested that Th1 oriented cellular responses including HCV-specific CTL and induction of IFNγ secreting cells play a significant role in virus clearance and recovery of infected persons or vaccinated chimpanzees from HCV infection.[2] In this context, potential of DNA vaccination for induction of specific cellular immune responses made it attractive for development of HCV vaccines if the amount of the responses elicited by such vaccination strategy could be enhanced.[3,4] Accordingly, results of the preceding attempts to utilize HCVcp for immunization (which has been considered as a superior immunogenic antigen) indicated that HCVcp is a rather weak immunogen when used in the context of DNA vaccine.[3,4,21,22] To overcome this shortcoming, formulations based on a mixture of DNA vaccine harboring HCVcp together with recombinant HCV core particles[6] or coadministration of IL-23 and GM-CSF were considered to enhance the immune responses toward HCV-core DNA vaccination regimen.[5] We addressed this concern through the development of an HCVcp-based DNA vaccine harboring (N-terminally fused) HBsAg in a DNA-prime/protein-boosting immunization regimen and compared the cellular responses with that of HCVcp (subunit modality) immunization. To make our results comparable to that of the earlier studies, all the cellular responses throughout this study were analyzed for a conserved and dominant H2-d restricted, $CD8^+$-epitopic peptide (C39), which had been already employed for evaluation of immune responses toward a number of other HCVcp-based vaccines.[17,20,25] In fact, although a few prior studies reported the occurrence of cellular responses against C39 epitope following

immunizations by HCVcp-based DNA vaccines[17,25] but the strength of the immune responses (which were relatively weak) could not be weight against that of HCVcp (subunit) immunization in a single comparative study, the point that is addressed in the current investigation. The HCVcp subunit vaccine formulation utilized in the present study was based on the Pluronic F127 (F127) adjuvant, a nonionic copolymer surfactant and stabilizer that retains the native conformation of proteins at high concentration and was previously described for use with HCVcp immunization studies.[20] Our results indicated that both proliferation and CTL responses [Figure 3a and b] in pCHCORE-primed/HCVcp-boosted group are comparable to that of HCVcp immunized group and even slightly (but significantly) higher in case of proliferation responses [Figure 3a]. Accordingly, results of the cytokine h in our study revealed that the number of IFN-γ secreting cells in pCHCORE-primed/HCVcp-boosted group were significantly (and considerably) higher than HCVcp immunized group while the reverse results were observed for IL-4 secreting cells. Therefore, results of the present study clearly demonstrated strong and comparable (and even higher) Th1-oriented cellular responses for HCVcp immunization in the form of DNA rather than protein (subunit) vaccine modality. Although, earlier HCVcp immunization studies had also reported the Th1-oriented immune responses for DNA vaccine modalities but the immune responses were rather weak and not comparable to that of protein (subunit vaccine) immunization.[21,22] Therefore, the reason for these observations (strong Th1-oriented cellular responses for DNA immunization) in our study might be contributed to the fusion of HBsAg to HCVcp DNA sequence in the pCHCORE construct. This conclusion is in accordance with previous reports on the positive effect of HBsAg fusion for enhancement of immune responses to protective epitopes of HCV DNA vaccine constructs,[7,8,11] while in contrast with another report.[10] The enhanced cellular responses in case of pCHCORE immunization might be explained by secretion of HBsAg-HCVcp chimeric particles (VLPs), which allows their efficient uptake by APCs and presentation of the epitopes to MHC-I molecules as previously proposed.[7,8]

In summary, in the present study we clearly showed that fusion of HBsAg to HCVcp in the context of a DNA vaccine modality could augment Th1-oriented cellular and CTL responses toward a protective epitope, comparable to that of HCVcp (subunit HCV vaccine) immunization. Although in this study, HBsAg sequence was fused to the N-terminal of HCVcp, future investigations may address its localization at C-terminal or other regions of the protein to search for better potencies of augmented immune responses.

REFERENCES

1. Schaefer M, Capuron L, Friebe A, Diez-Quevedo C, Robaeys G, Neri S, *et al*. Hepatitis C infection, antiviral treatment and mental health: A European expert consensus statement. J Hepatol 2012;57:1379-90.
2. Roohvand F, Kossari N. Advances in hepatitis C virus vaccines, part one: Advances in basic knowledge for hepatitis C virus vaccine design. Expert Opin Ther Pat 2011;21:1-20.
3. Roohvand F, Kossari N. Advances in hepatitis C virus vaccines, part two: Advances in hepatitis C virus vaccine formulations and modalities. Expert Opin Ther Pat 2012;22:1-25.
4. Sällberg M, Frelin L, Weiland O. DNA vaccine therapy for chronic hepatitis C virus (HCV) infection: Immune control of a moving target. Expert Opin Biol Ther 2009;9:805-15.
5. Hartoonian C, Ebtekar M, Soleimanjahi H, Karami A, Mahdavi M, Rastgoo N, *et al*. Effect of immunological adjuvants: GM-CSF (granulocyte-monocyte colony stimulating factor) and IL-23 (interleukin-23) on immune responses generated against hepatitis C virus core DNA vaccine. Cytokine 2009;46:43-50.
6. Alvarez-Lajonchere L, González M, Alvarez-Obregón JC, Guerra I, Viña A, *et al*. Hepatitis C virus (HCV) core protein enhances the immunogenicity of a co-delivered DNA vaccine encoding HCV structural antigens in mice. Biotechnol Appl Biochem 2006;44:9-17.
7. Memarnejadian A, Roohvand F. Fusion of HBsAg and prime/boosting augment Th1 and CTL responses to HCV polytope DNA vaccine. Cell Immunol 2010;261:93-8.
8. Chen D, Edgtton K, Gould A, Guo H, Mather M, Haigh O, *et al*. HBsAg-vectored vaccines simultaneously deliver CTL responses to protective epitopes from multiple viral pathogens. Virology 2009;398:68-78.
9. Guilléna G, Aguilara JC, Dueñasa S, Hermidaa L, Guzmánb MG, Pentona E, *et al*. Virus-Like Particles as vaccine antigens and adjuvants: Application to chronic disease, cancer immunotherapy and infectious disease preventive strategies. Proc Vaccinol 2010;2:128-33.
10. Arashkia A, Roohvand F, Memarnejadian A, Aghasadeghi MR, Rafati S. Construction of HCV-polytope vaccine candidates harbouring immune-enhancer sequences and primary evaluation of their immunogenicity in BALB/c mice. Virus Genes 2010;40:44-52.
11. Memarnejadian A, Roohvand F, Arashkia A, Rafati S, Shokrgozar MA. Polytope DNA vaccine development against hepatitis C virus: A streamlined approach from in silico design to *in vitro* and primary *in vivo* analyses in BALB/c mice. Protein Pept Lett 2009;16:842-50.
12. Baghbani-arani F, Roohvand F, Aghasadeghi M, Eidi A, Amini S, Motevalli F, *et al*. Expression and characterization of *Escherichia coli* derived hepatitis C virus ARFP/F protein. Mol Biol 2012;46:226-35.
13. Tan SL, editor. Hepatitis C viruses: Genomes and molecular biology. Norfolk (UK): Horizon Bioscience; 2006.
14. McLauchlan J. Properties of the hepatitis C virus core protein: A structural protein that modulates cellular processes. J Viral Hepat 2000;7:2-14.
15. Schulzezur WJ, Schmitz H, Borowski E, Borowski P. The proteins of the Hepatitis C virus: Their features and interactions with intracellular protein phosphorylation. Arch Virol 2003;148:1247-67.
16. Christie JM, Chapel H, Chapman RW, Rosenberg WM. Immune selection and genetic sequence variation in core and envelope regions of hepatitis C virus. Hepatology 1999;30:1037-44.
17. Zhu W, Chang Y, Wu C, Han Q, Pei R, Lu M, *et al*. The wild-type hepatitis C virus core inhibits initiation of antigen-specific T-and B-cell immune responses in BALB/c mice. Clin Vaccine Immunol 2010;17:1139-47.
18. Cerutti A, Maillard P, Minisini R, Vidalain PO, Roohvand F, Pecheur EI, *et al*. Identification of a functional, CRM-1-dependent nuclear export signal in hepatitis C virus core protein. PloS One 2011;6:e25854.
19. Liao G, Wang Y, Chang J, Bian T, Tan W, Sun M, *et al*. Hepatitis B virus precore protein augments genetic immunizations of the truncated hepatitis C virus core in BALB/c mice. Hepatology 2008;47:25-34.
20. Roohvand F, Aghasadeghi MR, Sadat SM, Budkowska A, Khabiri AR. HCV core protein immunization with Montanide/CpG elicits strong Th1/Th2 and long-lived CTL responses. Biochem Biophys Res Commun 2007;354:641-9.

21. Dueñas-Carrera S, Alvarez-Lajonchere L, Alvarez-Obregón JC, Herrera A, Lorenzo LJ, Pichardo D, *et al*. A truncated variant of the hepatitis C virus core induces a slow but potent immune response in mice following DNA immunization. Vaccine 2000;19:992-7.
22. Major ME, Vitvitski L, Mink MA, Schleef M, Whalen RG, Trepo C, *et al*. DNA-based immunization with chimeric vectors for the induction of immune responses against the hepatitis C virus nucleocapsid. J Virol 1995;69:5798-805.
23. Roohvand F, Maillard P, Lavergne JP, Boulant S, Walic M, Andréo U, *et al*. Initiation of Hepatitis C Virus Infection requires the Dynamic Microtubule Network. J Biol Chem 2009;284:13778-91.
24. Maillard P, Lavergne JP, Sibe´ril S, Faure G, Roohvand F, Petres S, *et al*. Fc-γ receptor-like activity of hepatitis C virus core protein. J Biol Chem 2004;279:2430-7.
25. Hu GJ, Wang RY, Han DS, Alter HJ, Shih JW. Characterization of the humoral and cellular immune responses against hepatitis C virus core induced by DNA-based immunization. Vaccine 1999;17:3160-70.

Source of Support: This study received financial support from Pasteur Institute of Iran, **Conflict of Interest:** None declared.