Original Article

Different protein expression systems can influence the direction of the immune responses against HCV core protein in animal model

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ABSTRACT

Introduction: *Hepatitis C virus* (HCV) infection is a major public health problem which influences about 170 million people worldwide. Different types of vaccines have been designed using HCV structural proteins to control the viral infection. The core nucleocapsid protein is one of the most conserved proteins and a desirable target for HCV vaccines, especially the protein-based vaccines. In current study, we generated the core protein recombinantly in prokaryotic (*Escherichia coli*) and eukaryotic (*Leishmania* tarentolae) expression systems and compared the humoral immune responses stimulated by each protein in a BALB/c mice model. **Methods:** The expression of HCV core protein was performed using the prokaryotic pET-28a/ BL21 expression system and also the eukaryotic LEXSY expression system. The recombinant core proteins expressed in *E. coli* and *Leishmania* were purified using reverse staining method and affinity chromatography under native conditions, respectively. The purified core proteins were detected by SDS-PAGE and Western blotting using anti-His antibody and were assessed by NanoDrop spectrophotometer. Finally, the abilities of both recombinant core proteins to induce the effective humoral immune responses were evaluated using indirect ELISA. **Results:** Our data indicated a clear band of ~ 21 kDa for the purified HCV core proteins in both expression systems, confirmed by SDS-PAGE and Western blotting. The mice immunization with both recombinant core proteins was able to produce high levels of antibody isotypes (IgG1 and IgG2a) in comparison with the controls ($p < 0.05$). In addition, the level of IgG2a response was significantly higher in the group immunized with the core protein purified from *leishmania* compared to the protein generated from E. coli ($p < 0.05$). **Conclusion:** The recombinant core protein generated by the *leishmania* expression system could induce a Th1-biased immune response with respect to the increase of IgG2a to IgG1 ratio.

KEYWORDS: *Hepatitis C virus*, LEXSY expression system, *E. coli* expression system, Core, Humoral immune responses.

INTRODUCTION

The development of a safe and effective *Hepatitis C virus* (HCV) vaccine has attracted a special interest for prevention of other viral infections [1-6]. HCV belongs to the *Flaviviridae* family and has a positive single strand RNA in its genome [7, 8]. The HCV polyprotein is post-translationally divided into three structural proteins including the core nucleocapsid protein and the two envelope glycoproteins (E1 and E2) and seven nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B and P7) [7, 8]. Among different HCV proteins, the core protein is a suitable target for development of HCV vaccines. It is highly conserved among different HCV genotypes and is also a strong immunogen. Previous studies have indicated that HCV core protein generated in prokaryotic expression systems can be used to improve the screening assays [7]. Many studies have produced HCV core protein in different expression systems such as bacteria [9, 10], insects [8, 11, 12], and yeast [13, 14]. In recent years, the protozoan *Leishmania* tarentolae has been known as a novel eukaryotic expression system for generation of various recombinant proteins with special structures and functions [15]. In this expression system, a group of inducible or constitutive vectors have been designed to target the proteins of interest to intracellular compartments or for secretion. Furthermore, the strains expressing the recombinant proteins are shown to be stable, easy to culture and suitable for production in large scales [16]. In the current study, we

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generated HCV core protein in two expression systems of *Escherichia coli* and *L. tarentolae*. We subsequently evaluated and compared the resulted recombinant proteins for their ability to induce robust humoral immune responses in a BALB/c model. Our goal was to determine the effects of the above-mentioned expression systems on the protein structure and folding associated with shifting of the humoral immune responses.

MATERIALS and METHODS

Expression and purification of the HCV core protein in *E. coli* by reverse staining method

The BL21 (DE3) bacterial strain harboring pET-core (Accession number: AB047640, [17]) was inoculated in 100 ml TY2X medium and grown to an optical density of 0.7-0.8 at 600 nm. The expression of core protein was induced by adding 1 mM IPTG and the pellet was harvested at 3 h post-induction. The bacterial pellets was dissolved in lysis buffer and placed on ice for 30 min. The lysates were sonicated for 20 min and the samples were dissolved in sample buffer and boiled for 5 min. The core proteins were analyzed by SDS-PAGE. An imidazole-SDS-Zn reverse staining method was used for the purification of the core protein as previously described [18]. The purified protein was concentrated and dialyzed against PBS (dialysis membrane, MWCO: 3.500 kDa). The protein concentration was assessed using NanoDrop spectrophotometer at 280 nm. The recombinant protein was kept at -20°C until use.

HCV core protein expression and purification in *Leishmania* by affinity chromatography using Ni-NTA column

The linearized pLEXSY-I-core plasmid (~ 10 µg, previously provided to Hepatitis and AIDS Department, Pasteur Institute of Iran) was electroporated into 4 × 107 log phase parasites (T7-TR LEXSY host) in 2 mm cuvettes at 450 V and 500 µF using Bio-Rad Gene PulserEcell. After two pulses, the recombinant stable transfectants were selected on M199-agar containing 50 µg/ml of bleomycin (Jena Bioscience, Germany). For genomic analysis, the recombinant clones were cultured in M199 media containing 50 µg/ml of bleomycin at 26° C. The genomic DNAs were prepared by the DNeasy® Blood and Tissue Kit (GF-1). The integration of pLEXSY-I-core into the genome was performed by diagnostic PCR using the primer pairs (core forward/ core reverse) and also 5'odc forward/ utr1 (aprt) reverse primers according to Jena Bioscience manual. The recombinant T7-TR LEXSY host harboring the pLEXSY-I-core was cultured in M199 media containing 50 µg/ml of bleomycin at 26°C. The induction of the protein expression was performed by 10 µg/ml tetracycline in final concentration. For obtaining an optimal expression, different times of the cell harvest were checked (i.e., 24, 48 and 72 h). The best time for the cell harvest was found to be 48 h after the induction. The expression of the core protein in *Leishmania* was confirmed by SDS-PAGE of the cell extracts. Finally, the recombinant core protein was purified by affinity chromatography on Ni-NTA resin column using 6xHis-tag, according to the manufacturer's instructions (Qiagen). The purification of the recombinant protein was done under native conditions and assessed using NanoDrop spectrophotometer at 280 nm. The recombinant protein was kept at -20°C until use.

Western blot analysis

The purified core proteins were analyzed using SDS-PAGE followed by staining with Coomassie brilliant blue as well as Western blotting. For the Western blot analysis, the core protein resolved on the gel was transferred onto protran nitrocellulose transfer membrane (Schleicher and Schuell Bioscience, Germany). The membrane was pre-equilibrated with TBST solution (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20) containing 2.5% bovine serum albumin (BSA) overnight and then was incubated with anti-His antibody (1:10000, Qiagen) for 2 h at room temperature. After 3 washes with TBST, the membrane was incubated with anti-mouse IgG-HRP (1: 10000, Sigma) for 1.5 h at room temperature. The immunoreactive protein bands were visualized using peroxidase substrate 3, 3'- Diaminobenzidine (DAB, Sigma).

Mice immunization

The immunization experiments were performed on female 6-8-week-old BALB/c mice (Pasteur Institute of Iran, Tehran, Iran). The mice were maintained under specific pathogen-free conditions and were used under the guidelines of the Pasteur Institute Animal Care and Use Committee. Four groups of 5 mice were selected and immunized subcutaneously at the footpad. The groups 1 and 2 (G1 and G2) were injected 3 times with 10 µg of the recombinant core protein purified from *E. coli* (B-core) plus Freund adjuvant (50: 50 v/v), the recombinant core protein purified from *Leishmania* (L-core) plus Freund adjuvant (50: 50 v/v), respectively. The control groups were injected with Freund adjuvant (G3) and PBS (G4). Table 1 shows the immunization regimens of the 4 groups.

Antibody responses

The mice groups were bled from retro-orbital at 3 and 6 weeks after the last immunization. The sera were then pooled for each group and stored at -20 °C until use. The levels of IgG1 and IgG2a antibodies were measured by indirect ELISA. Briefly, 96-well flat-bottom ELISA plate (Greiner) were coated overnight at 4° C with the recombinant HCV core proteins (namely, the B-core and L-core; 10 µg/ ml) as an antigen diluted in PBS (pH 7.2). The plates were then rinsed with washing buffer (0.5% (v/v) Tween-20 in PBS) and were incubated with blocking buffer (1% BSA in PBS) for 2 h at 37 °C. The diluted pooed sera (1:50 in dilution buffer 0.5% (v/v) Tween-20 in blocking buffer), were added to the plates which were incubated for 2 h at 37 °C. After rinsing with washing buffer, the plates were incubated with biotin-conjugated goat anti-mouse IgG1 or IgG2a (diluted 1:1000 in 1% BSA/PBS-Tween, Southern biotechnology Association Inc, USA) for 2 h at 37°C. The plates were washed and incubated with streptavidin-horseradish peroxidase diluted in PBS (1:10000; Sigma) at 37°C for 1 h. The detection was performed with 100 µl of O-Phenylenediamine (OPD, Sigma) as the substrate in citrate phosphate buffer (pH 4.5), followed by incubation for 30 min at 37°C. The enzyme reaction was stopped by 1 M H2SO4 and the absorbance was measured at 492 nm.

Statistical analysis

The differences in the levels of antibody responses between the control and the test groups were determined by one-way ANOVA (GraphPad Prism version 5.0 software). Such differences were considered statistically significant when the p-value was < 0.05.

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Table 1. Mice immunization using the recombinant core proteins generated in *E.coli* (B-core) and *Leishmania* (L-core)

Groups	Regimen	Day 0	Day 14	Day 28
G1	Protein + adjuvant	B-core protein + complete Freund	B-core protein + incomplete Freund	B-core protein + incomplete Freund
G2	Protein + adjuvant	L-core protein + complete Freund	L-core protein + incomplete Freund	L-core protein + incomplete Freund
G3	Control	complete Freund adjuvant	incomplete Freund adjuvant	incomplete Freund adjuvant
G4	Control	PBS	PBS	PBS

RESULTS

Confirmation of B-core and L-core in SDS-PAGE and Western blotting

Our data showed a dominant band of ~ 21 kDa for the recombinant core proteins expressed in both *E. coli* and *Leishmania* expression systems. The integration of the core gene in the genomic DNA of the recombinant *Leishmania* promastigotes was confirmed by PCR analysis. The expected ~ 1100 bp band was only observed from the transformed cells indicating the correct integration of the expression constructs into the odc locus. The expected PCR product of the core was appeared as a 573 bp fragment for the core positive clones. The best time for the expression of the core was determined at 48 h after the induction by tetracycline. Immobilized metal-affinity chromatography (IMAC) was applied to purify the recombinant core protein in LEXSY expression system. This method was based on the interaction between a transition metal ion (e.g., Ni 2+) immobilized on a matrix and specific amino acid side chains (e.g., Histidine). Indeed, the core protein containing polyhistidine sequences (His-tag) was significantly purified by the affinity chromatography under native conditions (i.e., 300 mM Imidazole). However, the reverse staining method based on Zn-Imidazole-SDS was more efficient than affinity chromatography for purification of the full length core protein in *E. coli* expression system. Both purified core proteins migrated as a ~ 21 kDa protein in SDS-PAGE. Western blot analysis was performed using anti-His antibody to ensure the proper expression of the recombinant core protein. Specific bands with the expected size (~ 21 kDa) were detected in the blots of the purified proteins from both systems (Fig. 1).

Stimulation of serum antibody response by protein-based immunizations through subcutaneous administration

We evaluated the potency of the two purified core proteins (B-core and L-core) for induction of humoral immune responses in a mice model. As shown in Fig. 2, all the mice receiving B-core and L-core developed significantly core-specific IgG1 and IgG2a immune responses as compared to the control groups (G3 and G4, $p < 0.05$). The ratio of IgG2a:IgG1 is a useful indication to compare the relative Th-bias between the groups. The humoral immune responses in the group which was immunized with L-core (G2) were directed toward IgG2a induction in comparison with the control groups, similar to the group immunized with B-core (G1). In contrast, the IgG1/IgG2a ratio was significantly increased in the group immunized with B-core (G1) as compared to the other groups ($p < 0.05$). In mice, the presence of IgG2a antibodies is an indicative of a Th1-biased response since the Th1 cytokines are necessary for this isotype shifting in the B cells. As described in Fig. 2, the group immunized with L-core elicited a Th1-biased immune response with respect to the increase of IgG2a/IgG1 ratio in contrast to the group immunized with B-core which induced a Th2-biased immune response. All the members of the immunized groups did not show any sign of suffering from toxicity and remained healthy. In addition, the humoral immune responses were stable at the sixth week after the last immunization.

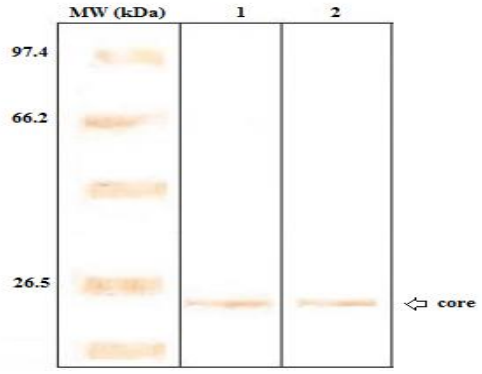

Fig. 1. Identification of core proteins by Western blotting using anti-His antibody: Lane 1: B-core, Lane 2: L-core. The weight of the molecular mass markers (Premixed protein molecular weight marker: 14.4-97.4 kDa) is indicated as MW.

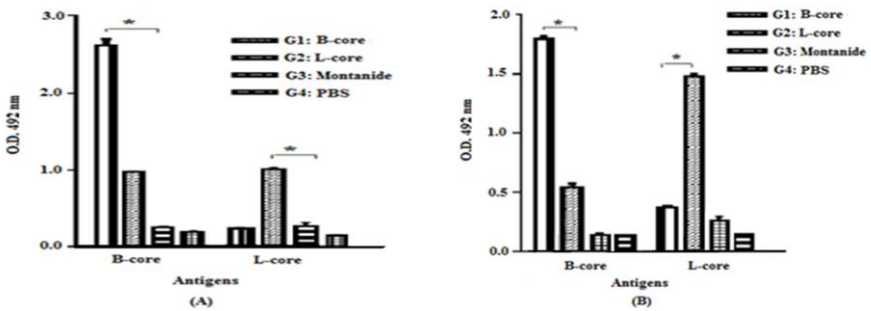

Fig. 2. Analysis of IgG1 (A) and IgG2a (B) antibody levels with respect to the coated antigens (B-core and L-core) using ELISA at 3 weeks after the third immunization. Data shows antibody responses of the pooled mice sera with independent experiments. The error bars indicate mean ± SD (* $p < 0.05$).

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DISCUSSION

HCV core antigen is a highly conserved protein and a major target to develope HCV preventive vaccines [19]. The generation of the core protein has been attempted in various expression systems [9, 10, 12-14]. In this regard, the bacterial expression systems possess several advantages including less time of production, high-level yields and cost-effectiveness as compared to other eukaryotic systems that use insect cells or yeasts [7]. For example, the expression of HCV core in *E. coli* BL21 using pGEX4T-2 expression vector has been performed using IPTG inducer as a recombinant fusion protein (e.g., GST-core). In these studies, HCV core has been purified through affinity chromatography [7]. This method required further isolation and purification processes. In the current study, pET-28a/BL21 bacterial system was used for the core protein expression. The core protein migrated as a ~ 21 kDa protein during the electrophoresis. In addition, the purification of the recombinant core protein was performed using reverse staining method.

On the other hand, *Leishmania* (LEXSY) expression system has been used as an alternative to other eukaryotic systems because of its simple genetic manipulation, suitable protein folding and low-cost of production [20]. For example, Laminin (LM)-332, a large heterotrimeric glycoprotein, produced in L. tarentolae has shown similar cell adhesion activity to LM-332 which was purified from mammalian cells, indicating its proper folding and assembly [21]. Moreover, this system is suitable to express recombinant proteins with a tag, allowing easy purification and detection of the recombinant protein [22]. Herein, LEXSY system was used to express the recombinant HCV core protein with a 6X-His-tag. Our results indicated the great benefit of the established L. tarentolae for production of the recombinant core protein. A ~ 21 kDa protein band was detected by SDS-PAGE and Western blotting, similar to that in the bacterial system. We then evaluated the humoral immune responses against the purified core proteins in both systems. The results showed that the adjuvanted core proteins generated in both systems induced a statistically significant increase of serum anti-core IgG2a and IgG1 isotypes as compared to the control groups. Based on our results, the core protein produced in *Leishmania* system could increase the shifting of the humoral immune responses toward IgG2a. In contrast, the core protein generated in the bacterial system could enhance the shifting of the humoral immune responses toward IgG1. Indeed, HCV core protein generated in the *Leishmania* expression system indicated a Th1-type response with respect to high IgG2a/IgG1 ratio as compared to HCV core protein generated in the bacterial system. Meanwhile, there was no severe reaction at the injection sites after the core protein-based immunizations. Briefly, these data indicated that the alteration of the structure and folding are likely effective parameters in switching of the antibody responses.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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