

Evaluation of a Native Preparation of HCV Core Protein (2-122) for Potential Applications in Immunization, Diagnosis and mAb Production

MR Aghasadeghi¹, SM Sadat¹, A Budkowska², AR Khabiri³, S Amini¹, G Bahramali¹, SR Naddaf³
*F Roohvand¹

¹Dept. of Hepatitis and AIDS, Pasteur Institute of Iran, Tehran, Iran

²Unite Hepacivirus, Institut Pasteur Paris, France

³Dept. of Parasitology, Pasteur Institute of Iran. Tehran, Iran

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Abstract

Infection with hepatitis C virus (HCV) is a worldwide problem. Among HCV proteins, core antigen (Ag), besides its importance for diagnostic application is a prime candidate for component of a vaccine. Herein, we report results of studies on production of the hydrophilic domain of core Ag (2-122) in native conformation by an arabinose induction system in *E.coli* and the primary characterization of this recombinant protein for applications in diagnosis, immunization and mAb production. Recombinant core (r-Core) was able to detect anti-core antibodies in HCV positive serum samples in a dilution rate of 1/3200. It was also capable to elicit a potent anti-HCV humoral immune response in BALB/c mice. Finally, we established two stable clones of hybridoma which shown to produce specific and sensitive mAbs against the core protein. HCV core was able to elicit a broad range of antibody specificities depending on the immunogen conformation. Therefore, it may be possible to get new mAbs with higher affinities towards native conformation of core Ag.

Keywords: Hepatitis C, HCV core protein, Monoclonal antibody, Total core Ag, Iran

Introduction

Hepatitis C virus (HCV) identified in 1989 as the causative agent of non-A Non-B hepatitis, remains a major public health problem in Iran and in the world (1-3). More than 170 million people worldwide are chronically infected with HCV, which is responsible for development of cirrhosis and more than 100 000 cases of liver cancer per year. There is no protective vaccine against hepatitis C and the most potent available anti-viral treatment (IFN- α) can eradicate the virus only in about 45% of the patients (4). HCV has a single-stranded positive-sense RNA genome of approximately 9500 nucleotides that encodes a polypeptide, which is cleaved by host and viral proteases to produce structural (Core, E1, E2) and non-structural proteins with various enzymatic activities (5). HCV has a high

mutation rate and based on the heterogeneity of the genome, six major genotypes had been defined by 1993 (6). Since then, 11 genotypes and more than 70 subtypes of HCV have been described (7). Among HCV proteins, the core antigen (Ag) is a strongly basic and evolutionary most conserved protein among various genotypes that elicits a rapid antibody (Ab) response after the onset of the disease (5, 6, 8, 9). Thus, the measurement of HCV core Ab titer in serum is a general assay to screen for HCV infection (10, 11).

During viral morphogenesis, HCV core protein (HCVcp) is cleaved by cellular signal peptidase into truncated core proteins of 191 and 173 with molecular weights of 21 and 19 kDa, respectively (9,12). Three main domains can be identified in the 21 kDa core structure (13, 14).

The hydrophilic domain one consists of the first 121 amino acids, contains immunodominant conformational and sequential epitopes, and is a promising candidate for induction of antiviral immune responses (13, 15). HCVcp is a multi-functional protein and besides its structural function in formation of HCV nucleocapsid (16), its role has been implicated in the regulation of cell signalling, lipid metabolism, apoptosis and induction of cell transformation (17-21). HCVcp may also play a role in the suppression of the T cell immune responses during HCV infection via its interaction with dendritic cells (22), lymphotoxin β receptor (23) and C1q receptor (24, 25). In transgenic mouse model HCVcp leads to immune suppression, liver steatosis and hepatocarcinoma (26, 27). Thus, the core Ag would be potentially an important target for the induction of immune responses against HCV for vaccine formulations (28).

Current diagnosis of HCV infection is based on detection of antibodies against HCV proteins using recombinant and/or synthetic viral antigens (10, 11). These tests, however, are unable to identify subjects at the early stage of infection (window period of 2-12 months) (29). Recently, a test for quantitative determination of HCV-core antigen, based on production of high-affinity monoclonal antibodies directed against the HCVcp has been proved a precise and specific indirect marker of HCV replication (30, 31). Thus, availability of such mAbs is important to design an ELISA for core Ag screening in blood donors and for monitoring of the viral load during anti-viral therapy (32).

In this study, we report developing of an inexpensive and simple source for purified HCVcp hydrophilic-domain one (amino acids 2-122) in native conformation and providing data on encouraging primary results on potential application of this protein for induction of humeral response, diagnostic assays and mAb production.

Materials and Methods

Cloning, expression and purification of recombinant core protein The hydrophilic sec-

tion of HCV-core protein, amino acids 2-122 was produced in *E. coli* BL21-AI recombinant expression system (Invitrogen, USA). To this end, the corresponding DNA fragment after PCR amplification (by the following primers: Forward: 5'gtgcaccATGggcaccgattcccaaact 3' and Reverse:5' gggtatcgatattccttacccaaattgcega 3') was inserted into *Nco I-Sma I* sites of the pIVEX 2.3 vector, a T7 promoter based plasmid (Roche) and the resulting chimeric construct after sequencing confirmation was transformed into *E. coli* (Fig. 1) (33). The original source of the core gene was already described (34). Protein expression was induced by addition of 2% arabinose (35) to mid-log phase culture of bacteria at 37 °C and after 3 h incubation, the cell pellet was saved at -20 °C for purification steps. HCVcp was purified in native condition on a nickel-nitrilotriacetic acid (NNTA)-agarose column according to the manufacturer protocol (Qiagen, Germany) with some modification. Briefly, the cell pellet was solubilized by lysis buffer containing 5 mM imidazole and after 30 minute incubation on ice and sonication, the lysate was applied to the NNTA column (Qiagen, Germany) and following three times washing steps, purified protein was eluted by 250 mM imidazole elution buffer. High concentration of imidazole was subsequently exchanged to phosphate buffer by overnight dialysis at 4 °C. Purified r-Core protein was analyzed by 12% SDS-PAGE and western blotting according to standard protocols (36) described later in the following procedures.

Evaluation of diagnostic value of r-HCV core protein

To evaluate the potential application of the produced r-HCV core Ag in the measurement of core Ab titer in the blood serum of HCV positive humans, we performed ELISA on 11 positive blood sera obtained from Tehran blood transfusion centre (all proved positive by Anti-HCV kit, Asia-lion Biotech. Co). To this end, ELISA plates (Flat bottom 96-well polyvinyl chloride plate-Nunc, Denmark) were coated with r-Core antigen (3 μ g/ml) in 50mM carbonate buffer (pH; 9.5) at 4 °C overnight. After

three times washing steps with PBS and blocking with Skim milk (2% w/v), diluted human sera (1/ 3200) was added (in duplicate) to each well and incubated for 2 h at room temperature. After several washing steps with PBS, a horse-radish peroxidase (HRP)-labeled anti-human IgG (Sigma, USA) was added with the 1: 5000 dilutions and incubated for one h at rt. After several washing steps, the TMB, a ready to use substrate (Sigma, USA) was added and by development of the color, the reaction was stopped by addition of 50 µl of 2.5 M H₂SO₄. Finally, absorbance was read at 450 nm. Normal human serum was used as negative control. The positive cut-off value was taken as twice the mean values of the negative control sera.

Mice immunization against HCV core protein

Ten female BALB/c mice aged 6-8 weeks were given 5 µg of purified HCV antigen in complete freund's adjuvant intraperitoneally (i.p). One month later, the same dose of antigen in incomplete freund's adjuvant was injected i.p. as a booster. Blood samples were obtained from the retroorbital sinus of mice at weeks 0, 3, 6 and stored at -20 °C prior to analysis.

Evaluation of Anti-Core humoral response in mice by ELISA

HCV core Abs in mice sera was measured by an indirect ELISA. Briefly, ELISA plates were coated with r-Core Ag (3 µg/ml) in 50mM carbonate buffer (pH; 9.5) at 4° C overnight. After three times washing steps with PBS and blocking with Skim milk (2% w/v), diluted mice sera (10⁻³) was added in duplicate to each well and incubated for 2 h at room temperature. After several washing steps with PBS, a horse radish peroxidase (HRP)-labeled anti-mouse IgG (Sigma, USA) was added as 1: 5000 dilutions and incubated for one h at room temperature. After several washing steps, the TMB, ready to use substrate (Sigma, USA) was added and by development of the color the reaction was stopped by addition of 50 µl of 2.5 M H₂SO₄. Finally, Absorbance was read at 450 nm. Serum of a normal mouse (not injected by r-Core) was used as negative control. The posi-

tive cut-off value was taken as twice the mean values of the negative control sera.

Preparation of murine monoclonal antibodies

After six weeks, the best Ab responder mouse (mouse number two in Fig. 4a) was injected intravenously into the base of tail with the same dose of antigen in phosphate buffer saline (PBS) and was sacrificed three days later. Spleen cells of immunized mice were harvested and fused to X63 myeloma cell lines in the presence of poly ethylene glycol (PEG) 1500 as fusing agent according to the method of Goding (37). After 12-14 days, the culture supernatants of all clones were screened by ELISA in which the solid phase was coated with the antigen used for immunization and those producing specific antibodies were sub-cloned at least twice by limiting dilution.

ELISA tests for mAb producing clones

This test was the same as ELISA for mice serum mentioned above, except for the mice sera, which were replaced by hybridoma supernatants (1: 10 dilution). For sub-class determination of mAbs, hybridoma supernatants (1:10 dilution) were utilized in an ELISA kit (Sigma, USA) with the same procedure.

SDS-PAGE and Western blotting

Purified core antigen and lysate of bacteria expressing r-Core protein were analyzed by SDS-PAGE according to the method of Laemmli (36) in discontinuous buffer system in slab gel using 12% separating gel. The antigens were transferred from the gels to nitrocellulose membranes by means of a Fast Transfer Apparatus (Pharmacia LKB) following standardized protocols. Efficiency of transfer was verified by membrane staining with a ponceau S red solution (0.3% w/v trichloroacetic acid). Nitrocellulose membranes containing antigens were blocked and strips incubated at room temperature overnight with 0.2 dilutions of supernatant culture of mAb producing hybridoma clones and washed three times. The strips were future incubated with proxidase conjugated goat anti-mouse IgG (Sigma, USA) and developed with diaminoben-

zamidine tetra hydrochloride (DAB) (0.06% w/v) and H₂O₂ (0.1% v/v).

Statistical analysis All data of the experiments were recorded as computerized database and a descriptive statistical analysis was performed by the SPSS package (SPSS, ver 8.0.1, 1998).

Results

Characterization of recombinant C-Terminally His-tagged core protein (r-Core) Expression of heterogeneous gene in pIVEX2.3 system results to a C-terminally His-tagged protein (Fig. 1). This property facilitates purification of the completely expressed protein, i.e. excluding the un-terminated ribosomal released ones. Analysis of the core protein expressed in this system in a 12% SDS-PAGE indicated that a 17 kDa protein corresponding to HCV core domain one (residues 1-122), after arabinose induction was produced. Purification of the protein in completely native condition, i.e. without application of urea, resulted to a homogenous 17 kDa protein band which was in accordance to the calculated theoretical value (Lab. Works Software, UK) determined for the HCVcp amino acid 2-122 in the present expression system (Fig. 2. a). Western blot analysis of the core protein with a standard mAb (Alexis biochemicals, USA) resulted to a very specific interaction even with crude lysate, indicating the immunogenic properties of the naturally purified r-HCV protein expressed in pIVEX2. 3 system (Fig. 2. b). By application of the present system, we were able to achieve yields around 3.5 mg/l of the purified core protein, measured by both optical densities at 280nm (33) and BCA protein measurement kit (Pierce, USA).

Detection of anti-core in human sera by r-HCV core The results of ELISA indicated that all 11 HCV positive human sera used in this study could be screened by our r-HCVcp. According to Fig.3, all 11 sera diluted to 1/3200 presented values above cut-off value. This primary result implies to the diagnostic potential of this r-cp for screening of HCV positive blood samples. More studies that are detailed are re-

quired to measure the sensitivity and specificity to address the real diagnostic value of this protein and its potential for inclusion in a cocktail of HCV proteins in Ab detection assays.

Evaluation of specific antibody response to r-Core protein in mice Sera of the mice immunized with r-Core and control animals were obtained as described above. As it can be seen in Fig. 4a, Sera of the negative control groups did not show any specific reactivity to the r-Core Ag, while almost all the immunized mice with r-Core demonstrated IgG antibody response 3 wk after the first injection. However, titers of Ab rose rapidly after the second injection in all immunized mice. The time course of the anti-core antibody response in the immunized mice is shown in Fig. 4 a. Moreover, the same ELISA test for serial serum dilutions of the mouse number 2 (the high responder mouse) indicated that dilution rates around 1/8000 were still detectable (above cut-off value) by 3 µg/ml of r-Core Ag (Fig. 4b).

Characterization of mAb producing hybridomas Results of the fusion of spleen cells from the BALB/c mice immunized with HCV core antigen with X-63 cells is summarized in Table 1. As it is shown, this fusion finally, yield 19 hybridomas secreting antibodies specific for r-Core protein. All these Ab producing clones were sub-classed (Table 1). However, only three clones, HC1-B2, HC2-C4 and HC3-D1 were finally stable after limiting dilutions steps (Table 2). Results of the characterization of these 3 hybridomas are summarized in Table 2.

Immunoblotting To further confirm the sensitivity of produced mAb clones to recognize the core antigen, lysate of bacteria expressing r-Core protein and purified core antigen was fractionated by SDS-PAGE and blotted on to the nitrocellulose filters as described in the method section (Fig. 5). As shown in Fig. 5 only the mAb producing clones HC1-B2, HC2-C4 revealed a band of 17 kDa as a result of interaction with core antigen. These mAbs also recognized the core antigen in ELISA test (Table 1).

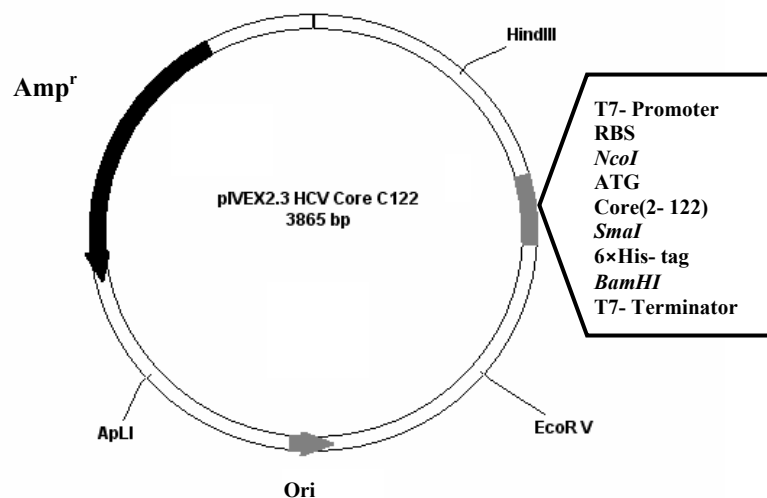


Fig.1: Expression vector map (pIVEX2.3 HCV Core C122) RBS: Ribosomal Binding Site, ATG: translation initiation codon, Core 2-122: Sequence of gene corresponding to amino acid 2-122 of HCV core protein

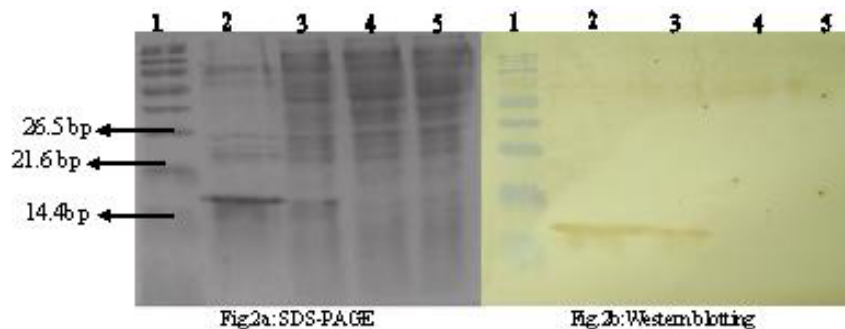


Fig. 2: Electrophoresis and Western blotting of bacterial samples, before and after induction. Lane 1: Protein ladder marker (Pre-Stained), Lane 2: r-Core protein purified from bacterial lysate, Lane 3, 4 and 5: belong to bacterial lysate after and before arabinose induction, respectively.

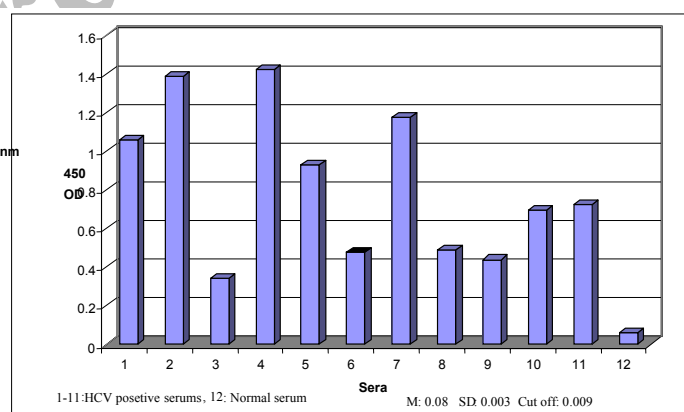


Fig. 3: Result of ELISA assay for detection of anti-core in human sera by r-HCV Core. Samples 1-11 belong to HCV positive human sera. Sample 12 belong to HCV negative normal human serum.

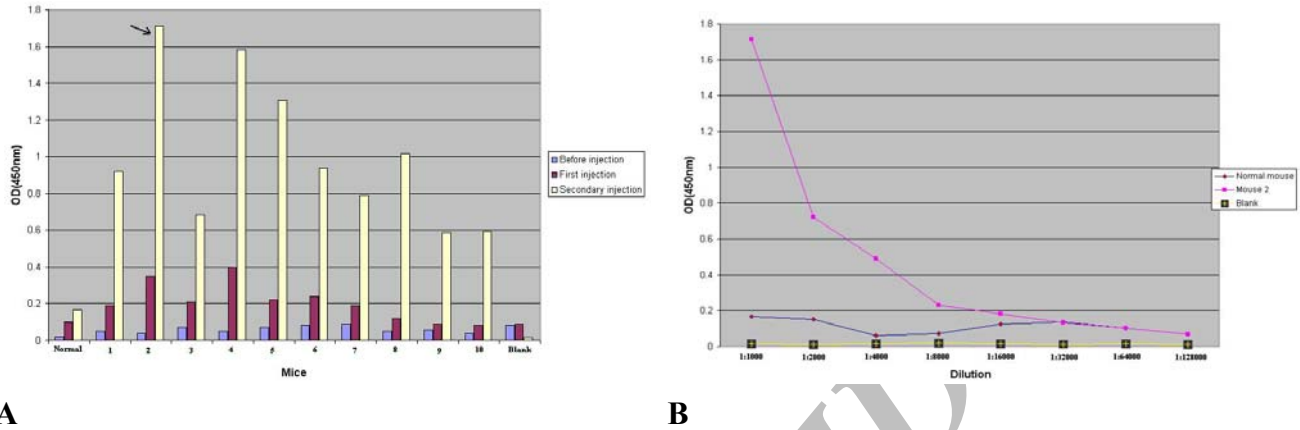


Fig. 4: Anti-HCV Core humoral immune response induced after immunization with r-Core in Balb/c mice.

A: Time course of anti- core IgG response. Control sample belong to normal mouse, i.e.: Un injected mouse). Samples 1-10 belong to 10 different mice immunized with r-Core Ag. Blank sample was simply PBS.

B: Evaluation of antibody detection in mouse number 2 for different serum dilution.

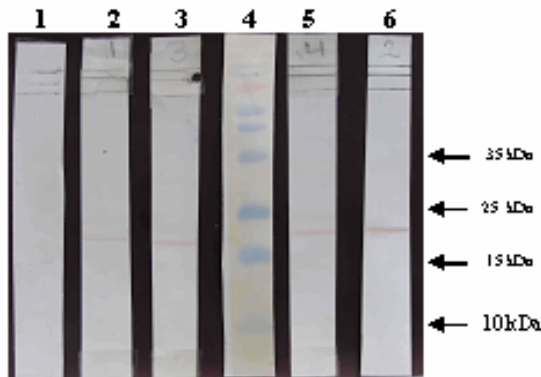


Fig. 5: Confirmation of mAb producing clones by Western blotting:

Lane 1: bacterial lysate (before induction), lane 2 and 5: bacterial lysate (after induction), lane 3 and 6: Ni-NTA purified and concentrated r-Core. Lane 4: Protein ladder marker (Pre-Stained).

Lane 1-3: belongs to western blotting of HC1-B2 and lane 5 and 6 to HC2-C4 mAb producing clones.

Table 1: Summary of the primary selection steps for mAb producing hybridomas

Plate No.	*No. of hybrid	**Specificity to clone	Class of Ab.
HC1	47	2	γ_1 , 2 α , 2b
HC2	46	6	2 α , γ_1
HC3	32	8	2 α , 2b
HC4	43	3	2 α , M

*: Number of hybride clones primary determined by microscopic observation.

**.: Number of clones producing specific antibody toward r-HCV Core.

Table 2: Characterization of mAb producing hybridomas:

Hybrid	Class of Ab.	Subclass	Specificity to core-Ag in ELISA	Sensitivity to core-Ag in Western blot
HC1-B2	IgG	2 α	+	+
HC2-C4	IgG	γ	+	+
HC3- D1	IgG	2b	+	-

Discussion

In the present study, we reported efficiency of a simple and high yield T7 promoter based plasmid for production and one-step purification of HCVcp by arabinose induction. Arabinose induction system has been used for expression of a few other proteins like p⁵³ (38). HCVcp has already been expressed in insect (39) and animal cells (40) with low yields which could not provide enough protein for different applications. Expression of HCV core Ag in *E. coli* by IPTG Induction/expression systems has also been reported by several groups (41, 42), but to our knowledge we report the first successful attempt in expression of hydrophilic domain of Core Ag in an arabinose induction system (35) in *E. coli*. Moreover, in most of the previous studies recombinant protein has been purified in denatured form and by application of high concentration of urea. In addition, previous studies on Immunogenicity (13,15) or mAb production (43-46) for Core protein have been based on either full length Core (residues 1-173 and 1-191) which may aggregate and hide some epitopes or on denatured protein preparations which may lose the native conformation.

However, our purification method permitted us to save the soluble fraction of the HCVcp expressed in *E. coli* in native conformation. Availability of HCVcp in its native conformation may provide additional epitopes, which might increase the potential application of this protein for diagnosis, vaccination or mAb production.

One of the main components of routine kits for Current diagnosis of HCV, is Core protein (10, 11). Our primary results on detection of Anti-core antibodies by this new r-Core protein indicates that it may have great potential of application in such diagnosis assays which may be determined by more detailed studies.

It is generally considered that HCV proteins may be poor immunogens. However, we found that our r-Core provoked a strong and specific immune response in mice. These results are in accordance with a recently published paper that

exploited r-Core (amino acids: 1-120) protein in a completely different expression and purification system (15).

HCVcp produced in mammalian cells is mainly found in the cytoplasm in association with membranes, but it can be also found in cell nucleus. These two forms of HCVcp are recognized by different monoclonal antibodies (47-49). Thus, natural HCV nucleocapsids (16) and nucleocapsid-like particles (50) might express conformational epitopes different from continuous, linear epitopes present on synthetic core peptides of limited length and denatured recombinant core proteins produced so far. Thus, production of new mAbs with better diagnostic value might be possible by exploiting r-Core protein in native conformation.

In the present study, we have established and partially characterized two relatively stable clones of mAbs to the native HCV core protein.

We got promising results by ELISA and western blot regarding the specificity and sensitivity of these clones toward HCVcp (Fig. 5). Although production of mAbs to recombinant HCVcp have already been reported (43-46), however, to our knowledge, generation and characterization of mouse monoclonal antibodies raised against native recombinant HCVcp have not been reported, yet. In addition, mAbs obtained by Ferns et al (44) were all reported as conformational since the Lno-LIA HCV III Assay, based on core peptide detection, failed to recognize any of them. Among the three monoclonal antibodies obtained by Moradpour et al. (46), one was directed against a strain-specific conformational epitope located within the first 82 amino acids. It is supposed that the immunodominant domain of HCV core is able to elicit a broad range of antibody specificities depending on the immunogen conformation (45). Therefore, it may be possible to get new mAbs with higher affinities towards Core Ag in native conformation. Availability of such mAbs will be critical for improvement of ELISA used for total core Ag screening in blood donors and

monitoring of the viral load during anti-viral therapy (32).

All together, the present study was undertaken to provide an efficient and simple system for expression and purification of hydrophilic immunodominant region of HCVcp in native conformation and to investigate its potential application for diagnosis, mAb preparation and immunogenicity. Our data provided, promising results regarding the first steps of above-mentioned applications and further experiments for evaluation of CTL responses in mice and detailed characterization of mAbs are currently undertaken.

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