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Preparation of hepatitis C virus structural and non-structural protein fragments and studies of their immunogenicity

Marija Mihailova [a,b,*], Melanie Fiedler [a], Mechthild Boos [a], Ivars Petrovskis [b], Irina Sominskaya [b], Michael Roggendorf [a], Sergei Viazov [a], Paul Pumpens [b]

[a] *Institute of Virology, Essen University Hospital, Hufelandstr. 55, 45122 Essen, Germany*
[b] *Biomedical Research and Study Center, University of Latvia, Ratsupites str. 1, 1067 Riga, Latvia*

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Abstract

Plasmids pQE-60 and pQE-30 containing 6× His-tag sequence were used for expression of fragments of HCV structural and non-structural proteins in *Escherichia coli* (*E. coli*). The following fragments were used: core (1–98 aa), NS3 (202–482 aa), and tetramer of hypervariable region 1 (HVR1) of E2 protein. The constructed plasmids directed high levels of expression of HCV proteins in *E. coli* JM109. After purification by the metal-affinity chromatography on nickel–nitrilotriacetic acid (Ni–NTA) agarose, the His-tagged HCV proteins were used for immunization of BALB/c mice. All three proteins were able to induce high levels of specific antibodies and, in the case of the NS3 and HVR1 tetramer, also to mount vigorous cell-proliferating responses. High immunogenicity of the tested fragments of HCV proteins shows them as good candidates for inclusion into the future HCV vaccine preparations.

Keywords: Hepatitis C virus; Recombinant protein purification; Immunogenicity

Hepatitis C virus (HCV)[1] is one of the main etiological agents of chronic liver disease, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [1]. It is assumed that about 170 million persons are chronically infected with HCV and 3–4 million persons are newly infected each year worldwide [http://www.who.int/media-centre/factsheets/fs164/en/index.html]. HCV contains a single-stranded positive-sense RNA genome of approximately 9600 nucleotides. It encodes a polypeptide precursor consisting of about 3010 amino acid (aa) residues, which is cleaved by host and viral proteases to produce structural (core, E1 and E2) and non-structural proteins (NS2, NS3, NS4A/B and NS5A/B) [2]. HCV is characterized by substantial genome heterogeneity, and at least six major genotypes have been defined on the basis of nucleotide and amino acid sequences of conserved and non-conserved genome regions [3]. Presently, combined therapy with pegylated interferon alpha (IFN-α) and ribavirin remains the only available option for treatment of patients with chronic hepatitis C. Unfortunately, many patients, especially those infected with HCV type 1 strains, often demonstrate no sustained virological and biochemical responses to the therapy [4]. Thus, there is an urgent need for development not only of new effective therapies, but also of prophylactic and therapeutic vaccines to control the HCV infection. To achieve this goal, better knowledge of the correlates of immunity to HCV and of the features of the immune response to different viral proteins is needed.

In the current study, we expressed and purified fragments of HCV structural (core and hypervariable region 1 (HVR1), 27 amino acid-long N terminal segment of E2 glycoprotein) and non-structural (NS3) proteins, and determined their immunogenicity in BALB/c mice. The humoral

* Corresponding author. Fax: +371 7442407.
E-mail address: mary@biomed.lu.lv (M. Mihailova).

[1] *Abbreviations used:* HCV, hepatitis C virus; HVR1, hypervariable region 1; Ni–NTA, nickel–nitrilotriacetic acid; FCS, fetal calf serum.

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response, in terms of kinetics, specificity, intensity, and the IgG subclass distribution of anti-HCV antibody production, was determined. The cellular immune reactivity was assessed by estimating the specific lymphoproliferative response. The choice of the proteins was made based on their assessment as attractive candidated for inclusion into HCV vaccines.

Materials and methods

Generation of recombinant HCV 6× His-tagged proteins

For cloning of different HCV protein fragments plasmids pQE-60 (Qiagen) and pQE-30 (Qiagen) containing the T5 promoter and 6 × His-tag coding sequence at 3′ or 5′ to the cloning region, respectively, were used. Fragments of core 1–98 aa, NS3 202–482 aa and HVR1 were chosen for construction of His-tagged HCV proteins. N-terminal part of HCV core (1–98 aa) was chosen because this fragment contains almost all known B and T helper epitopes [http://hcv.lanl.gov/content/immuno/immuno-main.html]. NS3 202–482 aa fragment contains several T helper and CTL epitopes some of which are also immunodominant and recognized in a great majority of HCV patients [http://hcv.lanl.gov/content/immuno/immuno-main.html]. The HCV core fragment nt 1–294 (1–98 aa) was obtained by polymerase chain reaction (PCR) with primers containing terminal *Nco*I and *Bgl*II restriction sites using cDNA of HCV AD78 isolate genotype 1b (GenBank Accession No. AJ132996) as a template, and ligated into pQE-60 plasmid restricted with the same endonucleases. The resulting construct was named pQE/core98.

The HVR1 region is highly variable both between HCV types and subtypes. In our study, we used four different HVR1 sequences to obtain a HVR1 tetrameric molecule that would be able to induce broadly cross-reactive immunity. The HCV HVR1 tetramer molecule was created by ligation of four synthetic oligonucleotides corresponding to two HVR1 "mimotopes" R9 and G31 [5,6], and two HVR1 sequences from field isolates of HCV genotype 1b [Khudyakov, unpublished data], respectively:

R9: QTTVVGGSQSHTVRGLTSLFSPGASQN;
G31: THTVGGSVARQVHSLTGLFSPGPQQK;
YK-5829: TTTVSGGHASQITRGVTSFFSPGSAQK;
YK-5807:VTYTTGGSQARHTQGVASFFTPGPAQK.

The sequences of HVR1 were codon-optimized for expression in *E. coli* using *E. coli* codon frequency usage table [http://psyche.uthct.edu/shaun/SBlack/codonuse.html]. Glycine spacers were introduced between each two peptides with the formation of the following tetrameric structure: R9–G–G–G31–G–G–YK-5829–G–G–YK-5807. The DNA encoding this tetrameric molecule was cloned into the vector pCR2.1 (Invitrogen) and the resulting plasmid was then used for PCR amplification of the HVR1 tetramer region with primers containing terminal *Bam*HI and *Hin*dIII restriction sites, and cloned into pQE-30 plasmid restricted with *Bam*HI/*Hin*dIII endonucleases. The resulting construct was named pQE/HVR1tetramer.

The fragment corresponding to the HCV NS3 nt 604–1446 (202–482 aa) was obtained by PCR introducing *Nco*I and *Bgl*II restriction sites, using plasmid pFK-I_{389}/NS3-3′/ET (HCV Con1 isolate, subtype1b) [7] as a template, and cloned into pQE-60 plasmid. The resulting construct was named pQE/NS3/202-482.

Recombinant clones were selected on ampicillin plates and subjected to direct colony polymerase chain reaction screening, using insert-specific primers to identify recombinants harbouring cloned fragments of HCV. The inserts were further verified by restriction analysis of plasmid DNAs.

Expression and purification of HCV 6× His-tagged proteins

Protein expression was carried out in the *Escherichia coli* (*E.coli*) strain JM109. Bacterial cells were grown at 37 °C in 2× YT medium (16 g/l bacto-peptone (Difco), 10 g/l yeast extract (Difco), and 5 g/l NaCl), supplemented with 100 μg/ml ampicillin, to an OD_{540} of 0.5, and protein expression was induced with 1 mM IPTG. Induction was continued for 4 h at 30 °C.

Purification of the proteins was performed using metal-affinity chromatography on nickel–nitrilotriacetic acid (Ni–NTA) agarose. Bacteria from the induced culture were centrifuged in a Sorvall GS3 rotor at 6000 rpm for 30 min at 4 °C. Cells were resuspended in lysis buffer (6 M GuHCl, 10 mM Tris–HCl, and 100 mM NaH_2PO_4, pH 8.0) at 5 ml per gram wet weight and stirred for 1 h at room temperature. Lysate was centrifuged at 10,000 rpm for 30 min at room temperature to pellet the cellular debris. 50% Ni–NTA resin (Qiagen) was added at 2 ml per every 4 ml of cleared lysate and mixed gently avoiding foaming by shaking on a rotary shaker for 4 h at room temperature. After incubation the suspension was packed into a polypropylene column (5 ml capacity, Qiagen). After collecting the flow through, the column was washed extensively with washing buffer (8 M urea, 10 mM Tris–HCl, and 100 mM NaH_2PO_4, pH 6.3). Proteins were eluted with elution buffer 1 (8 M urea, 10 mM Tris–HCl, and 100 mM NaH_2PO_4, pH 5.9) and elution buffer 2 (8 M urea, 10 mM Tris–HCl, and 100 mM NaH_2PO_4, pH 4.5) subsequently for determination of optimal elution buffer. All of the fractions were analysed by SDS–PAGE. Aliquots containing GuHCl (cleared lysate, flow through, and washing fractions) were treated by trichloroacetic acid precipitation prior to protein electrophoresis. It was determined that the optimal buffer for elution of the core98/6× His and NS3/202-482/6× His proteins was buffer 1 with pH 5.9, and for 6× His/HVR1tetramer protein-buffer 2 (pH 4.5). The homogeneous fractions containing the investigated protein were pooled together and subjected to gradual dialysis against PBS. Final protein probes were aliquoted and stored at −20 °C.