Original Article

Design and expression of fusion protein consists of HBsAg and Polyepitope of HCV as an HCV potential vaccine

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Abstract

Background: Hepatitis C virus (HCV) infection is a serious public health threat worldwide. Cellular immune responses, especially cytotoxic T-lymphocytes (CTLs), play a critical role in immune response toward the HCV clearance. Since polytope vaccines have the ability to stimulate the cellular immunity, a recombinant fusion protein was developed in this study.

Materials and Methods: The designed fusion protein is composed of hepatitis B surface antigen (HBsAg), as an immunocarrier, fused to an HCV polytope sequence. The polytope containing five immunogenic epitopes of HCV was designed to induce specific CTL responses. The construct was cloned into the pET-28a, and its expression was investigated in BL21 (DE3), BL21 pLysS, BL21 pLysE, and BL21 AI *Escherichia coli* strains using 12% gel sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Finally, the identity of expressed fusion protein was confirmed by Western blotting using anti-His monoclonal antibody and affinity chromatography was applied to purify the expressed protein.

Results: The accuracy of the construct was confirmed by restriction map analysis and sequencing. The transformation of the construct into the BL21 (DE3), pLysS, and pLysE *E. coli* strains did not lead to any expression. The fusion protein was found to be toxic for *E. coli* DE3. By applying two steps inhibition, the fusion protein was successfully expressed in BL21 (AI) *E. coli* strain.

Conclusion: The HBsAg-polytope fusion protein expressed in this study can be further evaluated for its immunogenicity in animal models.

Key Words: Expression, hepatitis C virus, polytope vaccine

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INTRODUCTION

Around 170–200 million people are chronically infected by hepatitis C virus (HCV) but there is not any approved vaccine for HCV infection.[1] There are a number of problems such as high-sequence divergence, cytotoxic T-lymphocyte (CTL) escape

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genotypes, and emergence of quasispecies in the course of infection for developing an effective HCV vaccine.[2] The HCV genome, 9600 base pair in length, contains 5' UTR, one open-reading-frame encoding a polyprotein precursor (3000 amino acids) and 3' UTR, respectively. The polyprotein cleaved into three structural proteins (E1, E2, and Core) and seven nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B, and p7). Among HCV proteins, Core and NS3 contain protective epitopes for eliciting cellular responses while envelope proteins, E1and E2, are important in generating neutralizing antibodies.[3] Many studies demonstrated both therapy-linked, and spontaneous HCV clearance are associated with strong, multi-specific and sustained T cell responses against HCV antigens. In contrast, individuals with chronic infection demonstrate relatively weak and narrowly focused $CD8^+$ T cell responses.[4] Classical vaccines used total antigen to induce T-helper 1 (Th1) response and CTLs provocation. Although they have been achieved success in some pathogens, observable competence has not been shown yet in chronic viral infections like HCV. Insufficient number of protective CTL epitopes in intact antigen and existence of repressive epitopes which interfere with protecting epitopes in whole antigen are indicated as the most reasons of inefficacy. Polyepitope recombinant vaccines as a suitable strategy can overcome this obstacle.[5-7] The peptide and epitopic vaccines are utilized as attractive tools to enhance mainly T-cell immunity in prophylactic and therapeutic approaches against cancer disorders and viral infections.[8] There are some advantages for multi-epitope vaccines including (a) the possibility of co-delivery of multiple critical epitopes from various antigens, (b) the engineering of the target immunogen based on accessible *in silico* algorithms,[9] and (c) the possibility of exploiting isolated subdominant epitopes instead of dominant ones in the context of polyepitope.[10,11] This application might be especially important for immunotherapy of chronic infections, like HCV, with exhausted $CD8^+$ T-cell response toward dominant epitopes.[12] In previous studies, we evaluated two novel polyepitope DNA-based HCV vaccines that showed their eligibility by *in vitro* and primary *in vivo* analysis. First, a polyepitope encoding four immunodominant $CD8^+$ CTL epitopes derived from both structural (E2, core), and NS antigens (NS3) and the latter encoded one murine and two potentially subdominant human restricted epitopes derived from structural antigens (core, E1, and E2) in various combinations with three different immune stimulatory sequences including hepatitis B surface antigen (HBsAg) gene.[9,10] The aim of this study was cloning and expression of a novel recombinant fusion protein as an HCV polyepitope vaccine. The designed fusion protein which contains HBsAg as an immunocarrier and five immunogenic epitopes of HCV can be evaluated for *in vivo* induction of specific CTL responses. This structure has an extra core (39-48aa) epitope in comparison with previous structures.

MATERIALS AND METHODS

Design and cloning of hepatitis B surface antigen-polyepitope construct

Five different immunogenic epitopes of HCV proteins ($Core_{132–142}$, $E2_{405–414}$, $E2_{614–622}$, $NS3_{1406–1415}$ and $Core_{39–48}$) were selected by different online web servers "RankPep", "BIMAS" and "SYFPEITHI" and fused to the HBsAg sequence (GenBank Accession No.: X02496) The sequence composed of HBsAg and five epitopes was synthesized by Biomatik Inc. (http://www.biomatik.com) and the construct was received in pUC57 vector.[7,9,10] The HBsAg-polyepitope fragment, 860 bp in length, was amplified using 5′-TTACCATGGGAGACAT CACATCAGGATTCCTAGGACC-3′ and 5′-ATTCTCGAGGCCCCGCACGCCCAGCCG-3' as forward and reverse primers, respectively. Pfu DNA polymerase (Fermentas, Lithuania) was used and thermal cycling program was as follow: 94°C/4 min, for 30 cycles 94°C/30 s, 56°C/30 s and 72°C/2 min and then 15 min at 72°C for final extension. The amplified fragment was analyzed on 1% agarose gel electrophoresis and then purified by gel extraction kit (Fermentas, Lithuania). The PCR product and pET-28a expression vector were digested by *Nco*I and *Xho*I restriction enzymes. Finally, PCR product was cloned into pET-28a expression vector then transformed to TOP10F' *Escherichia coli* strain. The fidelity of the construct was confirmed by restriction map analysis and sequencing.

Expression and identification of hepatitis B surface antigen-polyepitope fusion protein

The construct was transformed in two *E. coli* BL21 strain cells (DE3 and AI, Invitrogen, USA) for protein expression. Different conditions were investigated (induction time, temperature and inducer concentration). The expression of pET28a-HBsAg-polyepitope (p28 hp) was induced by addition of 0.2% w/v arabinose at mid-log phase. The expression was carried out at 37°C for 3 h in 5 ml Luria Broth containing 30 μg/ml kanamycin with vigorous shaking (200 rpm). The harvested cells were centrifuged at 4000 g for 20 min/4°C and lysed in 5x sample buffer (2.5 ml Tris-HCl 1 M, 4 ml Glycerol 89%, 2 ml sodium dodecyl sulfate [SDS] 10%, 0.5 ml betamercapto-ethanol, 6 mg bromo-phenol blue adjusted to pH 6.8). The extracted

samples were analyzed in 12% gel poly-acrylamide gel electrophoresis (PAGE) (Bio-Rad, USA) by coomassie brilliant blue staining. For western blot analysis, samples were transferred to nitrocellulose membrane (Sigma-Aldrich, Germany) by a wet blotting system (Bio-Rad, USA). Immunoblotting was performed using anti-His mouse monoclonal antibody followed by HRP-conjugated anti-mouse antibody as secondary antibody.

Plasmid stability test

The plasmid stability test was used to identify the toxicity of fusion protein. A single colony, resulted from BL21 (DE3) transformation by p28 hp, was cultured in 2 ml LB medium with vigorous shaking (200 rpm) at 37°C for an overnight. The 5 ml of prewarmed media (LB) was inoculated by 100 µl of the overnight cultures and incubated at 37°C with vigorous shaking (200 rpm) until an OD_{600} of 0.5 was reached. The 10^5 dilution of culture was prepared and 100 µl was added to four plates that differ in the LB agar additives [plate-1; LB agar medium, plate-2; LB agar medium and Kanamycin (30 µg/ml), plates-3; LB agar medium and isopropyl-beta-D-thiogalactopyranoside (IPTG) (1 mM), plates-4; LB agar medium, IPTG (1 mM), and Kanamycin (30 µg/ml)]. The plates were incubated at 37°C for an overnight and colony forming unit was determined for each plate.

Purification of expressed hepatitis B surface antigen-polyepitope fusion protein

Protein purification was performed under denaturing conditions at 4°C. 50 ml of culture was centrifuged at 4000 g for 20 min. The pellet was resuspended in 7 ml of lysis buffer (20 mM TrisHCl, 100 mM NaCl, 8 M urea adjusted to pH 8.0), sonicated and clarified by centrifugation. The clarified supernatant was applied to Ni-NTA affinity chromatography (Qiagen, USA) which was equilibrated as manually described. The column was washed twice with washing buffers (20 mM TrisHCl, 100 mM NaCl, 8 M urea adjusted to pH 7 and 6.3). The bound protein was eluted from the resin with a pH gradient of elution buffers (20 mM TrisHCl, 100 mM NaCl, 8 M urea adjusted to pH 5.9 and 4.5). The collected fractions were analyzed on 12% gel SDS-PAGE by coomassie brilliant blue staining.

RESULTS

Design and cloning of hepatitis B surface antigen-polyepitope construct

The HBsAg-polyepitope fragment was designed as previously described [Table 1][7,9,10] and successfully amplified by PCR using designed primers [Figure 1a] and ligated into the pET-28a expression vector. Finally, the accuracy of construct was confirmed by restriction map analysis by *Pvu*II, *Hinc*II, *Bgl*II, and *EcoR*V restriction enzymes as well as sequencing [Figure 1b].

Table 1: The selected epitopes and their MHC dependency

Epitope name	Sequence	Protein	Amino acid position	MHC dependency
C132	DLMGYIPLVGA	Core	132-142	A2.1/H-2^d
E614	RLWHYPCTI	E2	614-622	A2.1
N1406	KLSGLGLNAV	NS3	1406-1415	A2.1
E405	SGPSQKIQLV	E2	405-414	H-2^d
C39	RRGPRLGVRA	Core	39-48	H-2^d

MHC: Major histocompatibility complex

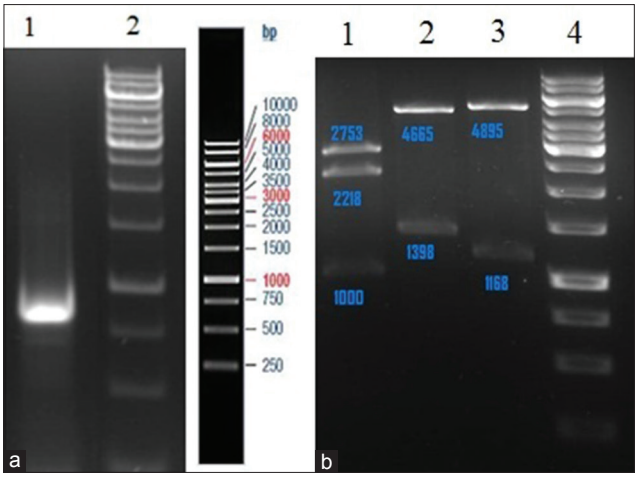

Figure 1: (a) Gel electrophoresis of PCR product on 1% agarose gel. (Lane 1: PCR product [860 bp], Lane 2; 1 kb DNA ladder [Fermentas, Lithuania]) and (b) restriction map analysis of p28 hp construct. (Lane 1: Digestion with *Pvu*II restriction enzyme, Lane 2: Digestion with *Hinc*II restriction enzyme and Lane 3: Digestion with *Bgl*II and *EcoR*V restriction enzymes and Lane 4: 1 kb DNA ladder [Fermentas, Lithuania])

Expression and purification of hepatitis B surface antigen-polyepitope fusion protein

The p28 hp was transformed into BL21 (AI) *E. coli* strain and induced by 0.2% w/v arabinose. The expression was only observed at 37°C. The best expression level was obtained 3 h after induction [Figure 2a; Lane 5]. As expected, a 34 kDa band was observed on 12% gel SDS-PAGE by coomassie brilliant blue staining [Figure 2a; Lanes 4–7]. The protein was successfully purified by Ni-NTA affinity chromatography [Figure 2b; Lane 7].

The observed band was identified on crude lysis by anti-His mouse monoclonal antibody [Figure 3].

Plasmid stability test

Since 4 h after induction, the OD_{600} decreased, plasmid stability test was performed to identify the toxicity of the fusion protein. The 10^5 dilution added to four plates differing in the LB agar additives. The colony number for each plate was determined as listed in Table 2.

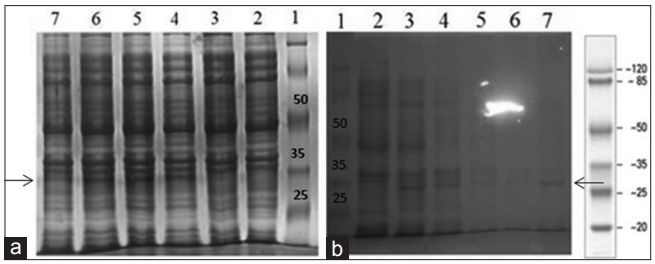

Figure 2: (a) Expression levels at different times were investigated: A 34 kDa band was observed on 12% gel sodium dodecyl sulfate-polyacrylamide gel electrophoresis 2, 3, 4, and 5 h after induction (Lane 1: Pierce Prestained protein marker [Cat. No. 26612, Thermo Fisher Scientific, USA], Lane 2: Un-induced sample, Lane 3: 1 h after induction, Lane 4: 2 h after induction, Lane 5: 3 h after induction, Lane 6: 4 h after induction and Lane 7: 5 h after induction) and (b) purification of recombinant protein. (Lane 1: Pierce Prestained protein marker [Cat. No. 26612, Thermo Fisher Scientific, USA], Lane 2: Un-induced sample, Lane 3: Crude lysis, Lane 4: Superflow, Lane 5: Wash buffer-1, Lane 6: Wash buffer-2 and Lane 7: Elution buffer)

Table 2: The colony count based on plasmid stability test

Culture medium	Number of colonies (CFU)
LB agar medium	64
LB agar medium + kanamycin (30 μg/ml)	43
LB agar medium + IPTG (1 mM)	180
LB agar medium + kanamycin (30 μg/ml) + IPTG (1 mM)	130

CFU: Colony-forming unit, LB: Luria broth, IPTG: Isopropyl-beta-D-thiogalactopyranoside

DISCUSSION

To produce an efficient anti-HCV vaccine, most efforts are focused on provoking cellular immunity. Virus-specific T cell response plays an important role in the resistance or clearance of HCV infection.[13,14] Some studies have been reported more difficulty with HCV-specific T $CD8^+$ cells induction than the HCV-specific $CD4^+$ cells by peptide vaccination.[15] Different strategies are investigated such as DNA vaccines, recombinant protein vaccines, and polyepitope vaccines. DNA vaccine development suffers from (a) delivery complication and potential integration of DNA into host genome leading to mutagenesis, (b) induction of autoimmune responses, and (c) induction of immunologic tolerance.[16,17] Polyepitope recombinant vaccines are a new generation of vaccines which selected conserved sequences in several epitopes and arranged their nucleotide sequences in a simple structure that can provoke contemporaneously immune responses against several important epitopes. Polyepitope-based vaccines are well-tolerated like recombinant protein vaccines and can induce HCV-specific T-cell immunity through the direct presentation of vaccine peptide to the T-cell receptor by HLA molecules. Therefore, the use of selected epitopes allows the development of vaccines that are well-defined chemical entities, and safer as well as easier to develop as pharmaceutical products.[18-21]

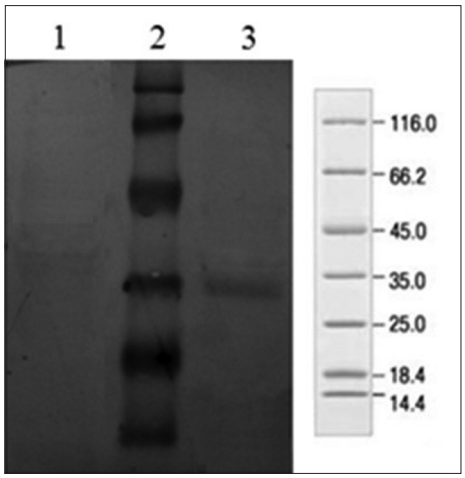

Figure 3: Western blot analysis of expressed fusion protein using anti-His mouse monoclonal antibody. A band of about 34 kDa was observed [Lane 1: Prestained protein marker (Cat. No. SM1811, Thermoscientific, USA), Lane 2: Un-induced sample, Lane 3: Crude lysis]

Envelope protein 2 (E2) has displayed the binding of HCV particles to the host cells via receptor binding as a major target of the immune response in HCV-infected patients.[22,23] On the other hand, trial vaccines included the two HCV envelope glycoproteins have until now limited success in the chimpanzee, the only animal model for HCV infection.[24] Extra to E2 protein, more conserved core protein have logically used to expand the immune response of the vaccine for including of the specific CTL epitopes.[25-27] NS3 protein as a targeted antigen in therapy-linked or spontaneously resolved HCV patients has recently considered and thus, as a potential target in vaccine development studies has been shown.[28,29] Polyepitope vaccine studies were recently described for HCV by application of epitopes derived from both structural and NS proteins. Therefore, three HCV proteins, E2, Core, and NS3 were selected for epitope prediction.

The epitopes have been selected by three online servers based on sequence similarity to a set of peptides known to bind to a given major histocompatibility complex (MHC) molecule, published motifs and takes into consideration the amino acids in the anchor and auxiliary anchor positions as well as other frequent amino acids, and a predicted half-time of dissociation to HLA class I molecules. The E614 and NS1406 epitopes, as HLA-A2 epitopes, were selected from E2 and NS3 antigens to locate beside C135 epitope which cross-reacted with HLA-A2 (a human HLA-epitopes) and H-2d (a BALB/c mice HLA-epitopes). Moreover, the two H-2d-restricted epitopes (E405 and C39) were further used to enhance CTL responses in BALB/c mice. These selected epitopes have been already addressed by several independent studies to be part of the dominant CTL epitopes in the course of HCV infection or vaccination.[7,9,10] All immune-dominant

sequences had following features: (a) selected epitopes were CTL epitopes as the immune-dominant epitopes implicated in the HCV-specific immune response induction, (b) partial sequence conservation in either 1a or 1b HCV strains, which are most prevalent and therapy-resistant genotypes (c) there are not any spacer between five epitopes to prevent from composing additional epitopes. In order to increase the exposure of polyepitope as viral-like particles (VLP), it was fused to HBsAg as a fusion protein. The reports have been shown that the use of VLP particles as an antigen carrier increase immune responses by different mechanisms such as better epitope presentation to antigen-presenting cells and stabilizing antigenic sequence.[7,9,30] The HBsAg is currently applied as the only licensed HBV vaccine because of the presence of multiple T-helper epitopes. Although the external hydrophilic loop of HBsAg is considered as a preferred site for insertion of foreign antigens, antibody rather than T cell responses has been obtained against epitopes.[3,31,32] Therefore, polyepitope sequence designed to bind at the N-terminal of HBsAg.

It must be mentioned that the polyepitope construct was first cloned into the pQE-60 plasmid to express the fusion protein in M15 *E. coli* strain. Unfortunately, no detectable protein band was found in this system (data not shown). Alternatively, a pET expression system was selected for further expression study. Therefore, the polyepitope construct was subcloned into the pET-28a vector and expression was induced in the BL21 (DE3) *E. coli* strain by IPTG. It was identified that the OD_{600} of host cells decreased after 4 h of IPTG addition, and no band was found on SDS-PAGE analysis. Therefore, it was assumed the recombinant protein may be toxic for BL21 (DE3) *E. coli* strain. The plasmid stability test was used to analyze the toxicity of HBsAg-polyepitope fusion protein. As interpretation, the colony existence in the no additive LB agar plate shows all viable cells. The colony existence in LB agar plate containing only antibiotic shows cells carry the plasmid. The colony existence in the LB agar plate containing only IPTG shows cells that have lost the plasmid or mutations in the plasmid and finally the colony existence in LB agar plate containing both antibiotic and IPTG shows only mutants have the plasmid but have lost the ability of the target gene expression. As listed in Table 1, the colonies excessively grow either in the IPTG-LB agar plate or antibiotic-IPTG-LB agar plate. If the desired protein is not toxic, the number of colonies on IPTG-containing plates and plates containing both antibiotic and IPTG must be <2% and 0.01% of the colonies either on the no additive plates or only antibiotic LB plates, respectively.[32,33] In the case of a toxic protein, the fraction of cells that have lost recombinant plasmid will be illustrated by increased colonies on the IPTG-LB agar plate and a decrease on the antibiotic plate. Therefore, the designed fusion protein has demonstrated to be a toxic protein. The recombinant proteins expressed in *E. coli* can interfere normal function of the cells and hence may be lethal but the toxicity degree varies depends on the protein.[33-35] Although, the lysogen BL21 (DE3) contains a single copy gene for T7 RNA polymerase under the inducible *lac*UV5 promoter, but reported that it can be produced even in the absence of inducer (IPTG). Therefore, plasmids that contain sufficiently toxic proteins may be unstable or accumulate mutations and cannot be express desired proteins. It is reported that many of the regulated promoters such as *lac* promoters are not so strong and show low expression level before the addition of inducer which leads to plasmid instability.[36-38] Different approaches are reported for toxic protein expression in *E. coli* systems including; (a) applying a lower copy number plasmid such as pET expression system (b) addition of1% glucose represses induction of the *lac* promoter by lactose, which is present in the most rich media (c) usage of compatible hosts such as BL21 (DE3) pLysS or pLysE *E. coli* strain and (d) employing a more tightly regulated promoter like *araBAD* promoter.[39] As discussed, if recombinant plasmid genes are toxic for the host, it is better to use a low copy number plasmid because plasmid loss can increase in high copy number plasmids. Therefore, the first used expression system, pQE-60 as a high copy number plasmid, is not suitable for HBsAg-polyepitope expression as we observed. With these qualities, the p28 hp was transformed into BL21 (DE3) pLysS *E. coli* strain and the expression was induced by 1 mM IPTG. In addition, basal expression levels were repressed by adding 1% glucose to the growth medium. It is reported that glucose lowers cyclic-AMP levels in the cell which lead to a decrease in transcriptional activation.[33-35,40] Regrettably, it was still not observed a band for fusion protein (data not shown). Although the T7 lysozyme expression in BL21 (DE3) pLysS strain was demonstrated to obviously reduce basal expression of target protein by binding toT7 polymerase present before induction, it was also known to reduce expression level after induction and in some cases, lead to noticeably lower yield because it keeps even on to hinder T7 RNA polymerase after induction.[41-43] A hopeful strain was developed which contains the chromosomal copy of the T7 RNA polymerase is under the control of the pBAD promoter. Expression systems in which the sequence for T7 RNA polymerase has been controlled by the pBAD promoter under the arabinose operon have relatively low basal expression and can make them useful for maintaining and expressing toxic genes. Therefore, the BL21-AI *E. coli* strain was

utilized as host for expression. Similarly, 1% glucose was added as a repressor for basal expression but the expression was induced by 0.2% w/v arabinose. The SDS-PAGE analysis was successfully showed a band around 34 kDa on 12% gel. In comparison with BL21 (DE3) pLysS, BL21-AI has a 4-fold lower basal level but a similar expression level. It is also reported that using of an expression system under the control of the T7 promoter, BL21-AI *E. coli* strain, should be the first choice to investigate if BL21 (DE3) pLysS and pLysE *E. coli* strains fail to maintain the target gene.[33,44-46] Finally, the expressed protein was identified by Western blot analysis using anti-His mouse monoclonal antibody. Since a His-tag was added to C-terminal of the construct, the Ni^{+}-affinity chromatography was used for purification as popularly for identifying of the *E. coli* expressed recombinant proteins. In conclusion, we designed and expressed a novel fusion protein as an HCV polyepitope vaccine which contains several MHC class I dependent epitopes and HBsAg as an immunopotentiator. The purified HBsAg-polyepitope fusion protein can be more evaluated to evoke Th-1 and CTLs responses *in vivo*.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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