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Expression and immunoreactivity of HCV/HBV epitopes

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Abstract

AIM: To develop the epitope-based vaccines to prevent Hepatitis C virus (HCV)/Hepatitis B virus (HBV) infections.

METHODS: The HCV core epitopes C1 STNPKPQRKTKRNTNRRPQD (residuals aa2-21) and C2 VKFPGGGQIVGGVYLLPRR (residuals aa22-40), envelope epitope E GHRMAWDMMMNWSP (residuals aa315-328) and HBsAg epitope S CTTPAQGNSMFPSCCCTKPTDGNC (residuals aa124-147) were displayed in five different sites of the flock house virus capsid protein as a vector, and expressed in *E. coli* cells (pET-3 system). Immunoreactivity of the epitopes with anti-HCV and anti-HBV antibodies in the serum from hepatitis C and hepatitis B patients were determined.

RESULTS: The expressed chimeric protein carrying the HCV epitopes C1, C2, E (two times), L3C1-I2E-L1C2-L2E could react with anti-HCV antibodies. The expressed chimeric protein carrying the HBV epitopes S, I3S could react with anti-HBs antibodies. The expressed chimeric proteins carrying the HCV epitopes C1, C2, E plus HBV epitope S, L3C1-I2E-L1C2-L2E-I3S could react with anti-HCV and anti-HBs antibodies.

CONCLUSION: These epitopes have highly specific and sensitive immunoreaction and are useful in the development of epitope-based vaccines.

Key words: HCV; HBV; Epitope-based vaccine; Recombinant; Immunoreactivity

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INTRODUCTION

hepatitis C virus (HCV) and Hepatitis B virus (HBV) are the causative agents of hepatitis C and B. Exposure to HCV or HBV causes acute hepatitis, leading to chronic hepatitis, liver cirrhosis, hepatocellular carcinoma and even death. The worldwide prevalence is estimated to be around 170 million individuals (3%) infected with HCV and 350 million individuals (7%) infected with HBV[1,2]. HCV and HBV infections are social and economic issues.

HCV is a member of the Flaviviridae family, possessing a linear single-stranded RNA genome of 9.4 kb[3]. The HCV genome contains a single open reading frame (ORF) encoding a polyprotein that is cleaved into the mature viral core, envelope and non-structural proteins[4]. The core protein is the most conserved and contains highly conserved epitopes[5]. The envelope protein E1 is the most variant region and contains the major neutralizing epitopes[6-11]. HBV is a double-stranded circular DNA virus[12]. The surface antigen of HBV (HBsAg), the major antigen protein, consists of large, middle and small proteins encoded by ORF S and preS, and have been successfully used as a hepatitis B vaccine. Since HCV and HBV infections share a similar route, i.e., mainly infected individuals through serum or seral products, it is very important to develop HCV/HBV covalent vaccines to simultaneously protect individuals from HCV and HBV infections.

It has been previously demonstrated that the epitope-presenting system based on the flock house virus (FHV) capsid protein is useful in displaying foreign epitopes[13]. In the present study, epitopes derived from the HCV core, envelope protein and HBsAg were displayed in this system, immunoreactivities were determined, and the possibility to develop epitope-based vaccines was discussed.

MATERIALS AND METHODS

Epitopes and epitope-presenting system

The epitopes studied in this study were derived from the HCV core region residues aa2-21C1 STNPKPQRKTKRNTNRRPQD (C1) and aa22-40, VKFPGGGQIVGGVYLLPRR (C2), the envelope region residues aa315-328, GHRMAWDMMMNWSP (E), and the HBsAg residues aa124-147 CTTPAQGNSMFPSCCCTKPTDGNC (S).

The epitope-presenting system was developed using the

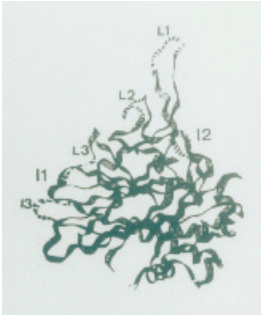

Figure 1 Three-dimensional structure of FHV capsid protein.

FHV capsid protein as a vector (FHV-RNA2 system)[13]. The FHV capsid protein expressed in the recombinant system could self-assemble into virus-like particles (VLPs). Six sites on the vector protein outer surface could be chosen for insertion of foreign epitopes which have little influence on the protein structure (Figure 1). The epitopes were inserted into L3 (C1), I2 (E), L1 (C2), L2 (E) and I3 (S), by means of genetic recombination engineering on plasmid pET-3. Four recombinant plasmids were constructed, pET-Wt carrying the bare vector protein gene, pET-I3S carrying the vector gene and the epitope S, pET-L3C1-I2E-L1C2-L2E carrying the vector gene and the epitopes C1, C2, E, and pET-L3C1-I2E-L1C2-L2E-I3S carrying the vector gene and the epitopes C1, C2, E and S.

Expression of chimeric proteins carrying epitopes

The recombinant plasmids were transformed into *E. coli* BL21(DE3) cells. The chimeric proteins carrying the epitopes were expressed under the control of T7 promotor[14] in TB/ampicillin media at 37 ℃. Cells were harvested by centrifugation at 3 000 r/min for 10 min at 4 ℃ (Beckman rotor type J-20). Cell lysis was accomplished by ultrasonication and centrifuged at 10 000*g* for 30 min. The chimeric proteins in the deposit were dissolved in 8 mol/L urea and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). To purify the expressed chimeric proteins, the band containing the chimeric proteins was cut-off from the gel and the proteins were recovered by further electrophoresis procedures. The purified chimeric proteins were stored at -20 ℃ and used as an antigen in ELISA and Western blot tests.

Serum samples

Serum samples used in this study were collected from patients with hepatitis C (anti-HCV positive or HCV-RNA positive) or hepatitis B (anti-HBs positive), from the Kunming Infectious Disease Hospital, Kunming, China.

ELISA

Recombinant chimeric proteins carrying HCV/HBV epitopes purified by PAGE were diluted to 5 mg/L in carbonate/bicarbonate buffer (pH 9.6) and used as coating antigens in ELISA test. One hundred microliters of the protein solution was added to each well of 96-well microtiter plates. The plates were incubated overnight at 4 ℃ and then blocked with 2.5 g/L bovine serum albumin in 10 mmol/L of phosphate-buffered saline containing 0.01% Tween 20 (PBS-T), at 37 ℃ for 1 h and washed five times with (PBS-T). Sera from hepatitis C or B patients were diluted to the ratio 1:10 and 100 μL was added to each well of the blocked plates. The mixture was incubated at 37 ℃ for 1 h. After washing, 100 μL of horseradish peroxidase-conjugated rabbit anti-human immunoglobulin G (Sigma BioSciences, St. Louis, MO, USA) was added to each well, and the plates were incubated for 1 h at 37 ℃. After incubation and washing, ortho-phenylenediamine dihydrochloride (OPD, Sigma BioSciences, St. Louis, MO, USA) was added, and the color was measured at 455 nm with a Titertek plate reader.

When the cut-off value was (SS-NC)/NC ≧ 2, it was defined as positive. In the formula, SS is the OD value of the serum sample, NC is the value of the negative control.

Western blot

In the Western blot test, the expressed chimeric proteins were separated in 10% SDS-PAGE and transferred onto the nitrocellulose membrane. After incubation with the patient's serum (in 1∶400 dilution) and horseradish peroxidase-conjugated rabbit anti-human immunoglobulin G, the immobilized antigens (epitopes) were detected with 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma BioSciences, St. Louis, MO, USA).

RESULTS

Expression and purification of chimeric proteins

The chimeric proteins Wt, I3S, L3C1-I2E-L1C2-L2E and L3C1-I2E-L1C2-L2E-I3S were expressed in inclusion body form in transformed cells with the corresponding recombinant plasmids pET-Wt, pET-I3S, pET-L3C1-I2E-L1C2-L2E-I3S or pET-L3C1-I2E-L1C2-L2E-I3S, and analyzed by 10% SDS-PAGE (Figure 2). The results showed that the chimeric proteins were highly expressed.

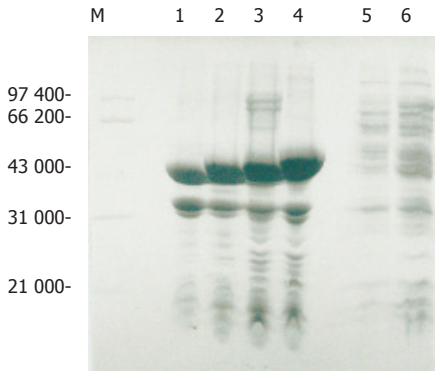

Figure 2 Recombinant proteins expressed in *E. coli* cells. M: protein molecular mass standard; lane 1: recombinant vector protein Wt; lane 2: chimeric protein I3S; lane 3: L3C1-I2E-L1C2-L2E; lane 4: L3C1-I2E-L1C2-L2E-I3S; lane 5: supernatant of BL21 cells transformed by pET-L3C1-I2E-L1C2-L2E; lane 6: non-transformed BL21 cells.

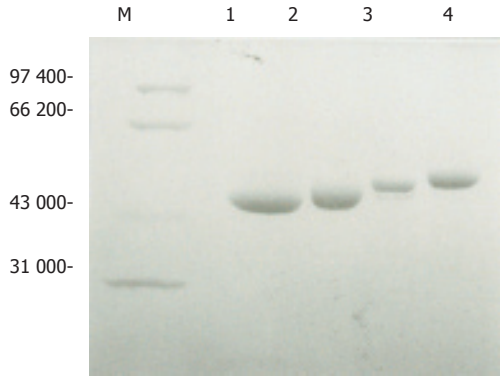

Figure 3 Expression of purified expressed proteins. M: protein molecular mass standard; lane 1: recombinant vector protein Wt; lane 2: chimeric protein I3S; lane 3: L3C1-I2E-L1C2-L2E; lane 4: L3C1-I2E-L1C2-L2E-I3S.

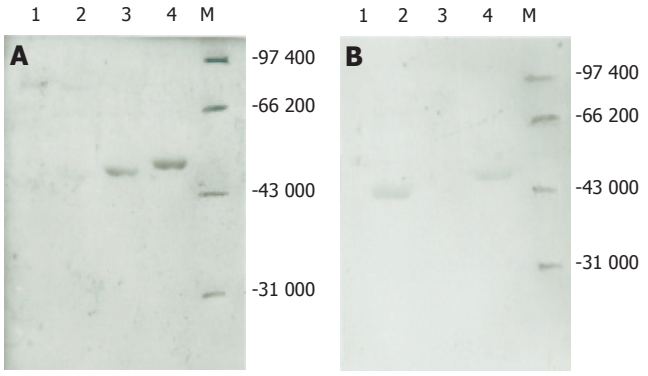

Figure 4 Western blot of expressed proteins using anti-HCV+ **(A)** and anti-HBsAg+ **(B)** sera as detecting antibodies. M: protein molecular mass standard; lane 1: recombinant vector protein W; lane 2: chimeric protein I3S, lane 3: L3C1-I2E-L1C2-L2E; lane 4: L3C1-I2E-L1C2-L2E-I3S.

Table 1 Detection of immunoreaction of expressed proteins as antigen with anti-HCV antibodies in serum from hepatitis C patients by ELISA

Protein	Patients	
	Anti-HCV$^+$/HCV-RNA$^+$ (%)	Anti-HCV$^-$/HCV-RNA$^+$ (%)
Wt	0	0
13S	2/66 (3.1)	1/24 (4.1)
L3C1-12E-L1C2-L2E	63/66 (95.5)	7/24 (29.2)
L3C1-12E-L1C2-L2E-I3S	63/66 (95.5)	9/24 (37.5)

The contents of chimeric proteins were estimated possessing about 35.1% (Wt), 35.5% (I3S), 34.2% (L3C1-I2E-L1C2-L2E), and 39.7% (L3C1-I2E-L1C2-L2E-I3S) of the full cell proteins. After purification, the chimeric proteins possessed about 43.8 kD (Wt), 46.5 kD (I3S), 49.7 kD (L3C1-I2E-L1C2-L2E) and 52.5 kD (L3C1-I2E-L1C2-L2E-I3S) of molecular weight, respectively as expected (Figure 3).

ELISA test

Using purified chimeric proteins as a coating antigen, 66 anti-HCV$^+$/HCV-RNA$^+$ and 24 anti-HCV$^+$/HCV-RNA$^-$ serum samples from hepatitis C patients were determined (Table 1). The results showed that the chimeric proteins carrying HCV epitopes, L3C1-I2E-L1C2-L2E and L3C1-I2E-L1C2-L2E-I3S could react with ant-HCV antibodies with a high specificity and susceptibility. The reactivity rates of chimeric proteins L3C1-I2E-L1C2-L2E and L3C1-I2E-L1C2-L2E-I3S were 95.5% and 95.5% with anti-HCV$^+$/HCV-RNA$^+$ sera, 29.2% and 37.5% with anti-HCV$^+$/HCV-RNA$^-$ sera, respectively. No serum samples reacted with the expressed vector protein Wt. Few serum samples reacted with chimeric protein I3S, implying that the patients were co-infected with HBV

Western blot test

Using sera from patients with hepatitis C (anti-HCV positive by ELISA kit) or B (anti-HBs positive by ELISA kit) as detecting antibody, the chimeric proteins carrying HCV epitopes L3C1-I2E-L1C2-L2E and L3C1-I2E-L1C2-L2E-I3S could be recognized by anti-HCV antibody (Figure 4A), and the chimeric protein carrying HBV epitopes S, I3S and L3C1-I2E-L1C2-L2E-I3S could be recognized by anti-HBV antibody on Western blot (Figure 4B). Ten serum samples from hepatitis C or B patients were detected respectively and the results in each group were similar.

DISCUSSION

Due to the host defense mechanism and virus genome RNA instability, HCV seems to escape immune pressure by mutation and results in high genetic heterogeneity[15,16]. The humoral immune response to neutralizing antibodies appears to be restricted and isolate-specific. HCV isolates obtained can be classified into at least six major clades (clades 1 to 6) and more than 70 subtypes[3]. Development of HCV vaccines is largely hampered for these characteristics of the virus.

Recent studies indicate that when a virus epitope is present in an appropriate vector system, the epitope can be displayed on the exposed surface of the vector protein with high immunogenicity[17]. Sequencing and immunological analysis showed that the residues aa1-40 in HCV core region are the most conserved and the residues aa315-328 in E1 region are highly conserved too[18]. HBsAg is the major antigenic protein of HBV. Mature HBsAg can self-assemble into VLPs and induce effective immune response. The residues aa120-160 of the large protein S exposed on the outer surface of VLPs are defined as a determinant. This determinant induces cross neutralizing antibodies which protect infections against different HBV subtypes[19-21]. Further studies indicate that the "a" determinant is mainly located within a double-looped structure formed by disulfide bridges between cysteines at 124, 137 and at 139-147[22,23]. The epitope S (aa124-147) studied in this report contains the key residues of the "a"

determinant. The epitopes displayed in the FHV-RNA2 system in this study had a high reactivity to specific anti-HCV or anti-HBV antibodies, indicating the importance of these HCV and HBV epitopes.

HCV/HBV epitopes, multiple-presented in a fusion form, cross react with HCV and HBV antibodies[24]. DNA immunization with fusion genes encoding different regions of the HCV E2 fused to the HBsAg gene elicits immune responses to both HCV and HBV. The antibody responses induced by the same epitopes are also demonstrated[25]. These results suggest that these epitopes contribute to the development of epitope-based vaccines.

Since HCV and HBV have a similar infection route with a high co-infection rate, development of HCV/HBV covalent vaccines is of great importance. Previous studies on HIV-1[13], HCV[26] and rotavirus[27-29] single epitopes and the present study demonstrated that the FHV-RNA2 system can be used to study the foreign epitope characteristics and to develop epitope-based vaccines. Since no sustainable cell culture system can be used, whether the antibodies elicited by these epitopes neutralize HCV and HBV infectivity or protect individuals against HCV and HBV infection remains to be studied.

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