

Immunoreactivity of HCV/HBV epitopes displayed in an epitope-presenting system

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Abstract

It has been demonstrated that the immunodominant region of the HCV core protein and the hepatitis B surface antigen (HBsAg) have high degree of reactivity. In order to construct a chimeric protein that carries HCV and HBV epitopes and possesses immunogenicity to both HCV and HBV, four epitopes derived from residues aa2–21 (epitope C1), aa22–40 (epitope C2) of the core protein, residues aa315–328 (epitope E) of E1 protein of HCV, and residues aa124–147 (epitope S) of HBsAg were chosen to be displayed in a conformation-specific manner on the outer surface of the Flock House virus capsid protein and expressed in *E. coli* cells. The reactivity of these epitopes with antisera from hepatitis C and hepatitis B patients and induction of immune response in guinea pigs were determined. The results showed that when displayed in this system, the chimeric protein carrying only epitope S could react with anti-HBsAg positive human sera, elicit an anti-HBsAg response in guinea pigs. The chimeric protein carrying epitopes C1, C2 and E could react with antibodies to different HCV genotypes, elicit an anti-HCV response in guinea pigs. The chimeric protein carrying epitopes C1, C2, E, and S could react with antibodies against HCV and HBV, elicit anti-HCV and anti-HBsAg responses in guinea pigs. The results suggested that these epitopes displayed in this form could be considered for development of epitope-based vaccines against HCV/HBV infections.

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1. Introduction

Hepatitis C virus (HCV) and hepatitis B virus (HBV) are the major causes of chronic liver diseases worldwide, leading to cirrhosis and hepatocellular cancer. It was estimated that about 170 million persons are infected with HCV (Koff, 2003), 350 million persons infected with HBV (Kao and Chen, 2002). Since HCV and HBV share similar transmission routes, coinfections are very common (Chen et al., 2003; Liaw, 1995; Crespo et al., 1994; Rodriguez et al., 1992). Simultaneous prevention of hepatitis C and B has great importance.

The linear, single-stranded positive-sense HCV RNA genome of 9.4 kb contains a single open reading frame encoding a polypeptide which is cleaved into the individual mature viral proteins by host and virus-specific proteinases. Three structural proteins have been identified, the core (nucleocapsid) protein (aa1–191), envelope proteins, E1 (aa192–383) and E2 (aa384–809). Previous studies revealed that the HCV core protein is the most conserved, containing several epitopes mainly concentrated in N-terminus. The glycoproteins E1 and E2 are the most variable (Buratti et al., 1997). The HBV small envelope protein (HBsAg-S, 226aa) has the capacity to self-assemble with host-derived lipids into empty envelope particles without the participation of nucleocapsids (Ganem, 1996). Residues 120–160 aa of the HBsAg-S is an important cross neutralizing epitope and could induce cross neutraliz-

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ing antibody, protecting adults from infections by different HBV subtypes (Gerin et al., 1983; Moynihan et al., 2000; Stirk et al., 1992). The “a” determinant is located within a double-looped structure with the antigenically important residues between aa 122 and 150 of HBsAg-S and consists of a limited number of epitopes (Stirk et al., 1992; Gavilanes et al., 1982; Howard et al., 1988). The subviral HBsAg particles have been used successfully worldwide for hepatitis B vaccination.

In the previous study a foreign epitope-presenting system using the Flock House virus (FHV) capsid protein as a vector was constructed (Tisminetzky et al., 1994), and its usefulness in development of epitope-based vaccines was demonstrated. In order to evaluate possibility to develop of a combined vaccine that has immunogenicity against both HCV and HBV, in this study four highly conserved epitopes derived from the HCV core protein, E1 protein and the HBsAg within the “a” determinant were chosen to be inserted in five different outer loops on the surface of the FHV capsid protein, and expressed in *E. coli* cells. The reactivity of these epitopes with antisera from hepatitis C and/or B patients and induction of immune response in guinea pigs were determined.

2. Materials and methods

2.1. Epitopes, epitope-presenting system and expression

Four epitopes were studied in this paper. Three epitopes were derived from the HCV strain HCVJ (genotype 1b) (Kato et al., 1990), epitope C1, corresponding to the residues aa2–21 (STNPKPQRKTKRNTNRRPQD), epitope C2, corresponding to the residues aa22–40 (VKF-PGGGQIVGGVYLLPRR), of the core protein, and epitope E, corresponding to the residues aa315–328 (GHRMAWDM-MMNWSP) of the E1 protein. The other epitope, designated epitope S, was derived from the HBV clone pFDW294 (subtype adw) (Estacio et al., 1988), corresponding to the residues aa124–147 (CTTPAQGNSMFPSCCCTKPTDGNC) of the HBsAg-S protein. The specific oligonucleotides representing the corresponding epitopes were synthesized and inserted into the Bsu36 I cloning site at the position of L3, L1, L2, I2, and I3 sites on the vector protein by means of genetic engineering (Peng et al., 2005).

The epitope-presenting system was developed from the FHV capsid protein as a vector (Tisminetzky et al., 1994). The FHV capsid protein has six sites on the outer surface that can be used for insertion of foreign epitopes. The FHV capsid protein-coding gene was put into the plasmid pET-3, producing the expression plasmid pET-FHV-RNA2. The HCV and HBV epitopes were displayed in different sites of the vector protein, and four recombinant plasmids that direct the expression of the HCV and/or HBV epitopes were constructed by genetic recombination techniques as described previously (Tisminetzky et al., 1994), pET-FHV-Wt, pET-FHV-I3S, pET-FHV-L3C1-I2E-L1C2-L2E and

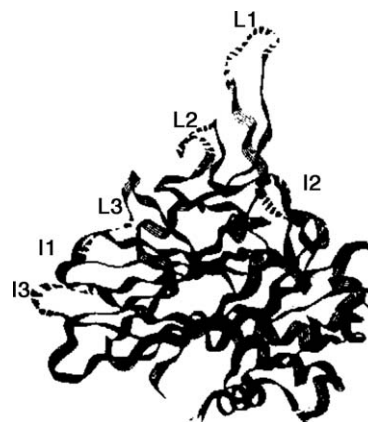


Fig. 1. Three-dimensional structure of FHV capsid protein showing six sites on the outer surface that can be chosen for insertion of foreign epitopes. The epitope C2, E, C1, E, and S are inserted in sites L1, L2, L3, I2 and I3, respectively.

pET-FHV-L3C1-I2E-L1C2-L2E-I3S. Plasmid pET-FHV-Wt carried the wild type FHV capsid gene, pET-FHV-I3S carried the FHV capsid gene with the HBV epitope S inserted in I3 site, pET-FHV-L3C1-I2E-L1C2-L2E carried the FHV capsid gene with the HCV epitope C1 inserted in L3 site, epitope E in I2 site and L2 site, and epitope C2 in L1 site, and pET-FHV-L3C1-I2E-L1C2-L2E-I3S carried the FHV capsid gene with the HCV epitope C1 inserted in L3 site, E in I2 and L2 sites, C2 in L1 site, and HBV epitope S in I3 site (see Fig. 1).

The above recombinant plasmids were transformed into *E. coli* BL21(DE3) cells and expressed in Terrific broth (TB) medium supplemented with 200 µg/ml ampicillin, at 37 °C for 12 h, without any chemical induction. Cells were harvested by centrifugation at 4 °C, 3000 rpm for 10 min (Beckman rotor type J-20). Harvested cells were lysed by ultrasonication. The sonicated solution was then centrifuged at 10 000 rpm for 30 min (Beckman rotor type J-20), and the deposit containing chimeric protein was dissolved in 8 M urea. The expressed proteins were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (12% SDS-PAGE).

2.2. Protein purification and concentration determination

The expressed proteins were purified by SDS-PAGE. Briefly, the proteins were separated on 10% SDS-PAGE and dyed by 150 mM KCl. According to the molecular weight of the protein, the band of gel containing only the interesting chimeric protein was cut off and put into a column filled with polyacrylamide gel (15%) on its bottom. Electrophoresis was performed at 20 mA, 4 °C for 12 h, and the chimeric protein was enriched in the vial that was linked to the column bottom. Sodium dodecyl sulphate (SDS) binding to the chimeric protein was removed by 20 mM KCl sedimentation (Suzuki and

Terada, 1988). The protein was further dialyzed in 10 mM phosphate-buffered saline (PBS) (pH 7.4) for desalination and renaturation.

The concentrations of the expressed proteins were determined by Lowry's method (Lowry et al., 1951).

2.3. Human serum

Human sera used in this study were collected from Chinese patients hospitalized with liver diseases. The HCV-RNA positive, anti-HCV positive but HCV-RNA negative sera, and HBsAg positive, anti-HBs positive sera were used to detect immunoreactivity of expressed chimeric proteins and antisera collected from the inoculated guinea pigs. The 5'UTR of HCV RNA was detected by nest RT-PCR and the HCV genotypes were defined by 5'UTR sequence divergence analysis as previously reported (Chen et al., 2002).

2.4. Animals and immunization

Guinea pigs were used in this study were purchased from China Medical Primates Center, Kunming, China. Sixteen guinea pigs at about 3 weeks of age were seldom divided into four groups and injected subcutaneously at the leg with 100 μ g in 100 μ l solution (15 mM Tris, 150 mM NaCl, pH 7.0) each of the chimeric proteins in the absence of adjuvant. Each animal took three times of injection at 2-week and then 1-week interval. Guinea pigs were bled from the heart at the 10th day post the last injection and antibody levels were measured by ELISA with the same batch of human antiserum above described.

3. ELISA

To test immunoreactivity of the epitopes with anti-HCV and anti-HBV antibodies, an enzyme-linked immunosorbent assay (ELISA) was performed using the purified proteins as coating antigens. Briefly, 100 μ l of the purified chimeric proteins (1 μ g/well) in sodium bicarbonate buffer (pH 9.4) was added in each well of the microtiter plate (Flow Laboratories Inc., McLean, VA, USA), and the plate was incubated at 4 °C overnight. After washing, the plate was blocked with 100 μ l each well of bovine serum albumin (BSA, 0.5%) in phosphate-buffered saline (PBS, pH 7.5) and Tween20 (0.01%), for 1 h at room temperature. The plate was then washed and each well was added with 100 μ l of the serum sample collected from the patient with hepatitis C or hepatitis B, at an appropriate dilution in PBS with BSA (0.05%) and Tween20 (0.05%). The plate was then incubated for 1 h at 37 °C. After washing steps, bound antibody was detected with a rabbit anti-human immunoglobulin (IgA, IgG and IgM) antibodies conjugated to horseradish peroxidase and OPD (*o*-phenylene diamine dihydrochloride) (Sigma BioSciences, St. Louis, MO, USA) in a citrate phosphate buffer. Results were read as positive

when the optical density at 455 nm of the serum value was 1.5-fold that of the negative control plus 1 standard deviation.

The commercial diagnostic ELISA kits (Sino-American Biotechnology Co., Henan, China) were used to detect anti-HCV antibodies in hepatitis C patients, HBsAg and anti-HBs antibodies in hepatitis B patients, and antibodies against HCV and HBsAg in guinea pigs inoculated with the expressed chimeric proteins. ELISA was performed following the affixed instructions with the kits. The procedures were the same to the above described for detection of the expressed proteins.

3.1. Western blot

Western blot was performed as previously described (Kato et al., 1990). Briefly, chimeric proteins were separated on 12% SDS-PAGE, transferred onto nitrocellular membrane and detected with anti-HCV or anti-HBsAg antibody in hepatitis C or hepatitis B patient serum (at 1:400 dilution). Bound antibody was detected with rabbit anti-human immunoglobulin (IgA, IgG and IgM) antibody conjugated to horseradish peroxidase and DAB (3,3'-diaminobenzidine tetrahydrochloride, Sigma).

4. Results

4.1. Expression of chimeric proteins

The recombinant plasmids pET-FHV-Wt, pET-FHV-I3S, pET-FHV-L3C1-I2E-L1C2-L2E and pET-FHV-L3C1-I2E-L1C2-L2E-I3S were transformed into competent *BL21(DE3)* cells and expressed. The recombinant proteins were expressed in inclusion body form in the transformed cells. The expressed proteins, recombinant vector protein Wt, chimeric proteins I3S that carries the epitope S, L3C1-I2E-L1C2-L2E that carries the epitopes C1, C2, and E, and L3C1-I2E-L1C2-L2E-I3S that carries the epitopes C1, C2, E and S, had molecular weight of 43.8, 46.5, 49.7, 52.5 kDa, and possessed 35.1, 35.5, 34.2, 39.7% of the total proteins of the transformed cells, respectively, as expected (see Figs. 2 and 3).

4.2. Reactivity of the chimeric proteins with human serum

The immunological reactivity of the chimeric proteins was detected by ELISA and Western blot. Using the chimeric proteins as coating antigen, 152 sera collected from hepatitis C patients (120 anti-HCV antibody positive and 32 HCV-RNA positive but anti-HCV negative) and 84 sera collected from hepatitis B patients (anti-HBs positive) were examined by ELISA. The results showed that the chimeric proteins carrying the HCV epitopes could specifically reacted with the anti-HCV antibodies (Table 1), the chimeric proteins carrying HBV epitope S could specifically reacted with anti-

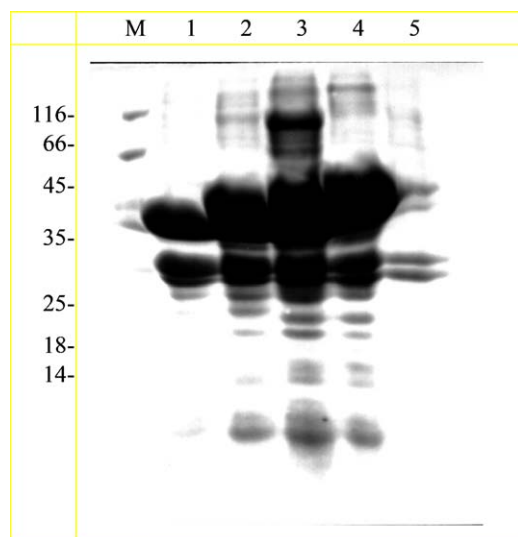


Fig. 2. The chimeric proteins expressed in *E. coli* BL21(DE3) cells, prior to purification. Protein molecular weight standard (M), recombinant vector (control) protein Wt (1), chimeric proteins carrying the HCV and HBV epitopes, I3S (2), L3C1-I2E-L1C2-L2E (3), L3C1-I2E-L1C2-L2E-I3S (4), and supernatant of lysate of BL21(DE3) cells transformed with recombinant plasmid pET-FHV-Wt (5).

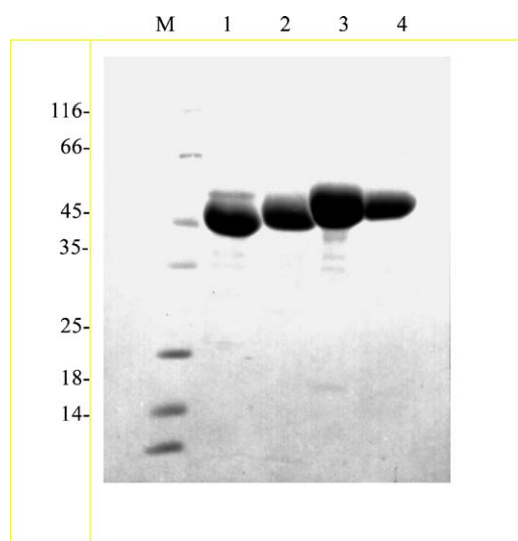


Fig. 3. SDS-PAGE (10%) of purified chimeric proteins. Protein molecular weight standard (M), recombinant vector (control) protein Wt (1), chimeric proteins carrying the HCV and HBV epitopes, I3S (2), L3C1-I2E-L1C2-L2E (3), L3C1-I2E-L1C2-L2E-I3S (4).

Table 1
Reactions of chimeric proteins as coating antigen with anti-HCV antibodies in sera from hepatitis C patients by ELISA

Chimeric protein	serum	
	Anti-HCV ⁺ (120)	HCV-RNA ⁺ / anti-HCV ⁻ (32)
Wt	1 (0.83%)	0
I3S	3 (2.5%)	0
L3C1-I2E-L1C2-L2E	116 (96.67%)	2 (6.25%)
L3C1-I2E-L1C2-L2E-I3S	117 (97.5%)	3 (9.76%)

Table 2

Reactions of chimeric proteins as coating antigen with anti-HBV antibodies in sera from hepatitis B patients by ELISA

Chimeric protein	Anti-HBs ⁺ serum (84)
Wt	0
I3S	78 (92.86%)
L3C1-I2E-L1C2-L2E	3 (3.57%)
L3C1-I2E-L1C2-L2E-I3S	80 (95.24%)

HBs antibodies (see Table 2). Few serum samples derived from the anti-HCV⁺ patients reacted with the chimeric protein I3S, and derived from the anti-HBs⁺ patients reacted with the chimeric protein L3C1-I2E-L1C2-L2E, implying the patients from whom the sera were collected were coinfecting with HCV and HBV. No anti-HCV or anti-HBs antibodies reacted with the expressed vector protein Wt were determined.

Further ELISA was performed using the chimeric proteins as coating antigen to detect antibodies against different HCV genotypes. The results showed that the chimeric proteins carrying the HCV epitopes could react with serum antibodies collected from the hepatitis C patient infected with different genotypes or subtypes (see Table 3).

Western blot test results showed that the chimeric proteins carrying HCV epitopes could be specifically recognized by anti-HCV antibody in serum derived from hepatitis C patient, but not by anti-HBV antibody in serum derived from hepatitis B patient (see Fig. 4). The chimeric proteins carrying the HBV epitope S could be specifically recognized by anti-HBV

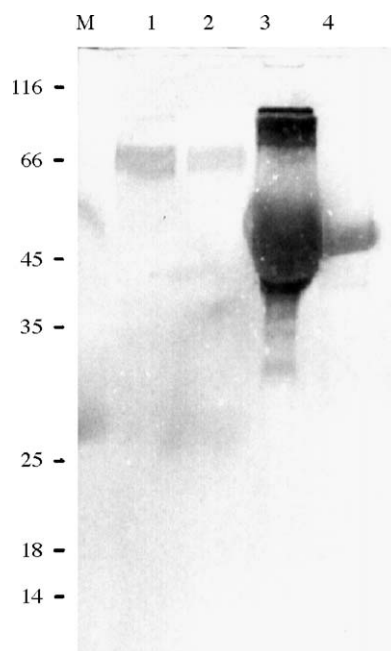


Fig. 4. Detection of chimeric proteins with antibodies in serum from the hepatitis C patient by Western blot. Protein molecular weight standard (M), recombinant vector (control) protein Wt (1), chimeric proteins carrying the HCV and HBV epitopes, I3S (2), L3C1-I2E-L1C2-L2E (3), L3C1-I2E-L1C2-L2E-I3S (4).

Table 3

Reactions of chimeric proteins as coating antigen with anti-HCV antibodies from different HCV genotypes infected patients by ELISA

Chimeric protein	Genotype 1		Genotype 2				Genotype 3			
	1b	1f	2a	2c	2d	2h	3a	3b	3h	3i
Wt	—	—	—	—	—	—	—	—	—	—
I3S	—	—	—	—	—	—	—	—	—	—
L3C1-I2E-L1C2-L2E	+	+	+	+	+	+	+	+	+	+
L3C1-I2E-L1C2-L2E-I3S	+	+	+	+	+	+	+	+	+	+

antibody in serum derived from hepatitis B patient, but not by anti-HCV antibody in serum derived from hepatitis C patient (see Fig. 5).

4.3. Production of anti-HCV and anti-HBV antibodies by immunization with the chimeric proteins

To test whether antibodies could be raised against the HCV epitopes in animal model, four groups of four guinea pigs were immunized with one of the chimeric proteins in the absence of adjuvant. After three injections, guinea pigs were bled. The serum samples were diluted in 1:10 to 1:50 and the anti-HCV and anti-HBs specific antibodies in the serum were detected by ELISA. The results showed that anti-HCV antibody (titer >1:50) was induced in animals inoculated with the chimeric proteins carrying the HCV epitopes, L3C1-I2E-L1C2-L2E and L3C1-I2E-L1C2-L2E-I3S. Anti-HBs antibody (titer >1:50) was induced in animals inoculated with the chimeric proteins carrying the HBV epitope S, I3S and L3C1-I2E-L1C2-L2E-I3S. No anti-HCV or anti-HBs an-

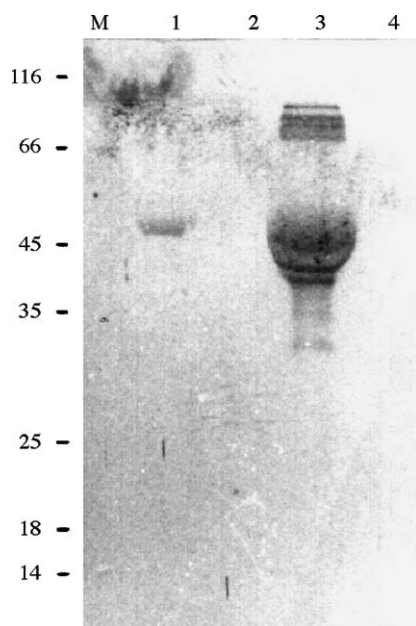


Fig. 5. Detection of chimeric proteins with antibodies in serum from the hepatitis B patient by Western blot. Protein molecular weight standard (M), recombinant vector (control) protein Wt (2), chimeric proteins carrying the HCV and HBV epitopes, I3S (1), L3C1-I2E-L1C2-L2E (3), L3C1-I2E-L1C2-L2E-I3S (4).

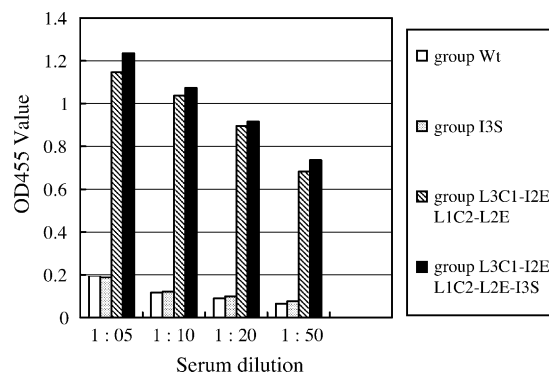


Fig. 6. Mean ELISA titers of antibodies against HCV in sera from each group of guinea pigs immunized with chimeric proteins by ELISA: group Wt (□), I3S (▨), L3C1-I2E-L1C2-L2E (▤), and L3C1-I2E-L1C2-L2E-I3S (■).

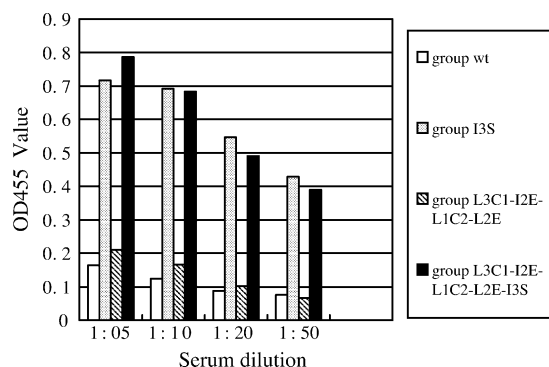


Fig. 7. Mean ELISA titers of antibodies against HBsAg in sera from each group of guinea pigs immunized with chimeric proteins by ELISA: group Wt (□), I3S (▨), L3C1-I2E-L1C2-L2E (▤), and L3C1-I2E-L1C2-L2E-I3S (■).

tibodies were detected in recombinant vector protein Wt inoculated animals (control group) (see Figs. 6 and 7).

The results also showed that the antibody titer induced by the HCV epitopes was comparatively higher than that by the HBV epitope S. This phenomenon might reflect difference in immunogenicity efficiency between the HCV epitopes and the HBV epitope S, or only reflected difference in serum antibody titer between the hepatitis C and hepatitis B patients.

5. Discussion

It is known that humoral immune response plays an important role in the host defense mechanism against virus.

However, it is difficult to demonstrate neutralizing antibodies to the virus and evaluate whether these antibodies could protect against hepatitis C, because there is no reproducible cell-culture systems could be used up to now. It was reported in some studies reinfections could take place in experimentally infected chimpanzees (Farci et al., 1992) and naturally infected humans (Duvoux et al., 1995; Lai et al., 1994; Laskus et al., 1996), suggesting that sufficient classical neutralizing immunity was not produced after resolution of hepatitis C. In the other studies the early development of antibodies to the HCV envelope proteins was observed, especially antibodies to the E2 glycoprotein hypervariable region (Choo et al., 1994; Zibert et al., 1997). These antibodies were thought to be correlative with recovery from the infection, suggesting such antibodies might play a role in clearance of the virus. The humoral immune response by neutralizing antibodies appeared to be restricted and isolate-specific, and HCV seemed to escape the antibody-mediated immune pressure by mutation. Despite its high variability, some conserved B cell epitopes in HCV structure proteins have been identified (Ferroni et al., 1993; Siemoneit et al., 1994, 1995; Lin et al., 1993). On the other hand, recombinant HBsAg particles have been used successfully worldwide for hepatitis B vaccination. The epitopes within the residues between aa120–160 of the HBsAg-S protein were demonstrated as cross neutralizing epitopes. The HBsAg-S epitope synthetic peptide was demonstrated to be group-specific, conformational determinant. This epitope possessed a high degree of cross-reactivity with polyclonal anti-HBsAg antibody, and could elicit an anti-HBsAg response (Manivel et al., 1992a,b) and a T-cell response in the early acute hepatitis, late stage and recovery patients (Mishra et al., 1992). The vaccines based on the conserved epitopes might induce strong and cross immune response against HCV and HBV.

The major deficiency for developing a peptide-based approach against infections was believed that a single peptide itself is not very immunogenic. This limitation could be overcome by the co-administration of a potent adjuvant, use of multiple epitopes, and use of a suitable carrier. It had been demonstrated that when a given peptide was inserted in an appropriate carrier protein, it could presume its spectrum of tertiary structures and could substantially improve its immunogenicity. This effect will be better if the epitopes are displayed on the surface of the virus particles. For recombinant epitopes, in order to obtain optimal immunogenicity, it will be better the epitopes were presented in several copies on a defined particulate structure (Tisminetzky et al., 1994; Ulrich et al., 1996). In a case using HBsAg envelope particles as a carrier, particles with foreign epitopes displayed on the external loop close to the transmembrane region showed to induce a B cell immune response against the foreign epitopes in the presence of Freund's adjuvant (Netter et al., 2001). The immunodominant hypervariable region (HVR1) of the HCV E2 protein displayed within the exposed "a"-determinant region of HBsAg (aa127–128) as virus-like particles could specifically react with the antiserum from a patient infected with the

same genotype HCV, and induce strain-specific antibody response in immunized mice (Netter et al., 2001, 2003). Cross-reactions of some HCV/HBV epitopes multiple-presented in a fused form with HCV/HBV antibodies were also found (Li et al., 2003).

In this study the FHV capsid protein was used as carrier matrix for the epitopes. The capsid protein after expression could self-assembly into particulate structure, and six sites on its exposed surface that can be used for insertion of foreign epitopes have been defined (Tisminetzky et al., 1994). Using this carrier protein, human immunodeficiency virus type 1 conformational epitopes (Tisminetzky et al., 1994), HCV core protein epitopes (Buratti et al., 1997) and rotavirus epitopes (Chen et al., 1998) displayed in a single form have been studied and their immunoreactions were demonstrated. A single HCV core epitope between residues aa1–20, aa21–40 or aa32–46 presented in this system could react with anti-HCV antibody, be able to induce immune responses against the corresponding synthetic peptides. In this study, the epitopes derived from the residues aa2–21 (C1), aa22–40 (C2) of the core protein and the residues aa315–328 (E) of the E1 protein of HCV were displayed in this system on four different positions on a single molecule of the vector protein. The results demonstrated that these epitopes presented in this form could cross-react with anti-HCV antibodies against different genotypes, induce high titers of HCV-specific antibodies in guinea pigs in the absence of adjuvant. When the HCV epitopes presented together with the HBV epitope S between residues aa124–147 of HBsAg-S, these epitopes could also cross-react with anti-HCV and anti-HBV antibodies. It should be mentioned that few serum samples from HC patients or HB patients were found react with the chimeric proteins carrying only HCV or HBV epitopes (Tables 1 and 2), implying these patients were coinfecting with both HCV and HBV mutants that could not be detected out by the commercial ELISA kit.

The epitopes displayed in this study are highly conserved in different genotypes of the HCV and HBV, could cross-react with different genotypes of HCV and HBV antibody, could induce serum antibodies against HCV and/or HBV. The results implied that the epitopes presented in this form may be used as epitope-based vaccines. However, since there is no sustainable cell-culture system can be used, it could not be determined whether the antibodies elicited by these epitopes displayed in this form could neutralize HCV and HBV infectivity or protect individuals from HCV and HBV infection. Whatever happens, more extensive studies should be done to solve these questions.

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