DNA immunization with fusion genes encoding different regions of hepatitis C virus E2 fused to the gene for hepatitis B surface antigen elicits immune responses to both HCV and HBV

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INTRODUCTION
Both Hepatitis B virus (HBV) and Hepatitis C virus (HCV) are major causative agents of transfusion-associated and community-acquired hepatitis worldwide. Development of a HCV vaccine as well as associated and community-acquired hepatitis (HCV) are major causative agents of transfusion-associated and community-acquired hepatitis worldwide. It is estimated that there are 250 million HCV carriers in the world and more than 10% of chronically infected HBV patients eventually develop cirrhosis and hepatocellular carcinoma. About 2-3% of the world population are HCV carriers. More than 70% of HCV infections become chronic, among which 5-20% progress to liver cirrhosis and hepatocellular carcinoma. Available HCV vaccines have proven to be safe and effective in preventing HBV infection. However, high costs, exclusion of some escape mutants and neonatal intolerance are eliminating their wide use. So far, no vaccine is available against HCV infection. IFN-γ treatment is the only useful therapy available. However, only 20-30% of treated patients develop long-term responses. Therefore, HBV and HCV infections pose a worldwide health threat and the development of uniformly effective vaccines of affordable prices is an urgent task.

DNA immunization, which allows the de novo synthesis of antigens in host’s cells, is able to elicit protective humoral and cellular immune responses in several animal models of viral infection. The cellular context for de novo synthesized proteins to achieve proper maturation is a particularly important advantage for proteins such as those constituting viral envelopes whose maturation requires the help of additional cellular factors. Increasing data showed that DNA immunization against HBsAg elicited strong humoral and cellular immune responses that protect chimpanzees against the challenge with HBV. Moreover, DNA immunization in transgenic mice expressing HBsAg in the liver resulted in the clearance of HBsAg and long-term control of transgene expression, suggesting that DNA immunization is a potential tool in the treatment of HBV chronic carriers. DNA immunization with HCV E2 protein that was believed to carry the major neutralization epitopes of HCV also was studied in several animal models including primates. These studies demonstrated that DNA immunization with HCV E2 elicited strong humoral and cellular immune responses in various animals, though it did not elicit sterilizing immunity in chimpanzees against the challenge with a monoclonal homologous virus. The DNA immunization did appear to modify the infection and might have prevented the progression to chronicity, suggesting that DNA vaccine could be a promising approach for HCV treatment.

The objective of this research was to simultaneously stimulate immune responses against both HBV and HCV by DNA immunization with fusion constructs comprising of various HCV E2 gene fragments fused to the HBsAg gene of HBV. HBsAg carries all the information required for membrane translocation, particle assembly and secretion from mammalian cells. We have previously shown that HBsAg carrying HBV preS1 (21-47) at its truncated carboxyl terminal end could present the preS1 epitope on the surface of the chimeric particle and induce preS1 specific antibodies in mice. Moreover, humoral and cellular immune responses were successfully induced via...
direct injection of the plasmid containing the HBsAg-preS1 fusion gene\(^{[20]}\). These data indicated that gene fragments of proper size could be fused to the C-terminal of HBsAg without affecting particle assembly and secretion, and were capable of inducing immune responses against both HBsAg and the fused epitope. Although the epitopes on envelope protein E2 are not very clear yet, there have been some successful experiments to determine the immune determinants\(^{[25-27]}\). Based on these previous findings, five fragments of HCV-E2 were selected in the hydrophilic region of E2 protein and fused to the truncated 3' end of HBsAg gene. The humoral and cellular immune responses of the plasmids expressing fusion proteins were evaluated. Furthermore, virus-capture ability of antibodies against those HCV-E2 fragments was also examined. The results are opening a new approach to the development of a bivalent vaccine against HBV and HCV, and lead to a better understanding of the immunological property of HCV-E2.

**MATERIALS AND METHODS**

**Expression plasmids**

Five HCV E2 gene fragments (A to E) were amplified from pUC18/E (E1-E2 gene of HCV genome type 1b inserted in pUC18)\(^{[28]}\) with the following primers:

| Fragment | Sense Primer | Anti-sense Primer |
|----------|--------------|------------------|
| A(384-413) | P1 CGGCGTTGTATACACACCTACG | P2 TGGGAATTCAAAGCTGGATTTTCT |
| B(414-443) | P3 GCGCAACCTATCAGGAACACGG | P4 CCGAATCCCATGAAACACGCCG |
| C(460-489) | P5 CATGGCCGTATACCGCTCCATTG | P6 GTCGAATTCAGTAGTGCCAGCA |
| D(490-519) | P7 TTTGTGTGTTATACCCACCTCGA | P8 GAGAATTCAGGGTCGTCCCCACCAC |
| E(529-549) | P9 GGTGGGGGTATACGATCGCCTC | P10 TACGAATTCACCAGTTGCCTTGCGG |

AccI and EcoRI sites were introduced in sense and anti-sense primers, respectively. After digestion with AccI and EcoRI, PCR products were inserted downstream from the HBV S gene in pcDNA3S\(^{[29]}\) to obtain pcDNA3SE2-A, pcDNA3SE2-B, pcDNA3SE2-C, pcDNA3SE2-D and pcDNA3SE2-E (Figure 1). A fragment coding for amino acids 384 to 649 of HCV-E2 was also amplified from pUC18/E with primers P1 (5’-CCCCGGGGATATCATGACCACC-3’) and P12 (5’-GCGAATCCATCTCGAGTCCAG-3’). pcDNA3E2 was constructed by inserting this PCR product digested with BamHI and EcoRI into the MCS of pcDNA3 (Figure 1).

**Western Blotting of transiently expressed products**

HeLa cells were maintained in DMEM supplemented with 5% fetal calf serum. To enhance the expression level of fusion genes, cells were first infected with vaccinia virus vTF7-8 that expresses T7 RNA polymerase at a M.O.I. of 10 pfu per cell\(^{[11]}\). Two hours post-infection, 1-2×10\(^5\) cells were transfected with 10\(\mu\)g of plasmid DNA by calcium phosphate method. Eighteen hours after transfection, cells were collected and cell lysate was electrophoresed on a 12.5% SDS-PAGE and subsequently electro-blotted onto nitrocellulose membrane. After blocking for 1h with 5% powdered lipid-free milk in PBST (PBS containing 0.1% Tween 20), the membrane was incubated with anti-HBs McAb H116 (kindly provided by Dr. E. Hilt, Munich, Germany) or anti-HCV E2 (450-565) polyclonal antiserum RE2-116\(^{[15]}\), followed by incubation with HRP conjugated secondary antibody (Dako Co., Denmark). Signals were detected by enhanced chemi-luminescence (ECL) blotting analysis system (Amersham-Pharmacia Co., UK).

**DNA immunization of mice**

C57BL/6 (H-2\(^b\)) mice were purchased from Shanghai Laboratory Animal Center. Groups of five female mice, 6-8 weeks old, were injected with 100\(\mu\)g of expression plasmids into regenerated tibialis anterior (TA) muscles 5 days after treatment of 100\(\mu\)l 10\(^{5}\)mol/l cardiotoxin (Sigma, USA)\(^{[15,29]}\). Four weeks later, the mice were boosted in the same way.

**Serologic tests**

Serum was collected by tail bleeding at different time points pre-and post-injection and assayed for anti-HBs and anti-HCV-E2 by ELISA. Microwell plates were coated with serum derived HBsAg particles\(^{[20]}\) (Kehua Co., Shanghai, China), or 0.1\(\mu\)g/well HCV-E2 (385-565) expressed in E. Coli\(^{[20]}\). After blocking with 5% powdered lipid-free milk in PBST (PBS containing 0.1% Tween 20), serial two-fold dilutions of sera were added. The bound antibodies were detected with HRP-conjugated rabbit anti-mouse IgG (1:1000) (Dako Co., Denmark) after extensive washing with PBST. Sera from mice immunized with pcDNA3 vector at 1 in 20 dilution were used as negative controls. A positive result was defined as an absorbance value of great than twice the absorbance of negative control with a cutoff of 0.050. Seroconversion was defined as giving a positive reading at 1:20 dilution. Antibody titres of pooled seroconverted animal sera were set as the highest dilution giving a positive reading (end-point dilution method)\(^{[22,31]}\).

To type the subclasses of IgG, mouse sera of each group were collected 6 weeks after the first immunization, and the titers of IgG1 and IgG2a antibodies assayed individually by a similar end-point dilution method, using the HRP-conjugated rabbit anti-mouse IgG1 and IgG2a (Serotec Co., Oxford, UK) for detection.
Splenic cytokine expression test

Expression of IFN-γ and IL-10 were assayed semi-quantitatively as described33,34. Mice were killed 10 weeks after the first injection and spleens were removed aseptically. The single cell suspensions of the splenocytes were prepared and maintained in DMEM supplemented with 5% fetal calf serum and 5×10−5 M β-ME at a density of 106 cells per well. Cells were stimulated with 0.5μg/ml RNA yeast derived HBsAg (National Vaccine and Serum Institute, Beijing, China), followed by culture at 37°C. At 0, 24, 48 hr post-stimulation, splenic cells were sampled. Total RNA was extracted with Trizol (Gibcol, BRL, USA) and reverse transcribed with random hexamer primers (New England Biolabs Inc, Beverly, USA.). Messenger RNAs for IFN-γ and IL-10 were semi-quantitated by competitive RT-PCR33,34. Splenic cells from mice immunized with pcDNA3 and mitogen ConA (5μg/ml) served as a negative and a positive control respectively.

Analysis of the virus capture capability of immune sera

The virus capture capability of antibodies in immune sera was assessed according to the method of affinity capture RT-PCR (AC-RT-PCR) detection of HEV in patient stool specimens35 with a few modifications. Briefly, 0.5ml microcentrifuge tubes were coated with 50μl goat anti-mouse IgG(F(ab')²-2 (0.02μg/μl, Jackson ImmunoResearch Laboratories, Inc., West Grove, USA), capped and incubated at room temperature for 1hr. After blocking with 5% powdered lipid-free milk in PBST for 15 min at 37°C and overnight at 4°C, tubes were aspirated and washed with 150μl PBST. The mouse sera were adjusted to same titre of 1:80 by dilution with PBS, to avoid the variations of the results caused by different antibody titer in different samples. Tubes were subsequently loaded with 25μl properly diluted mouse immune sera and 25μl HCV-RNA-PCR-positive patient’s serum (1.6×107 HCV genome Equivalent [Geq] per ml, provided by Dr. X. Zhang, Ruijin Hospital, Shanghai, China) diluted 1:50 in PBS. The mixtures were mixed gently and incubated for 1 hr at room temperature and overnight at 4°C. Tubes were then washed three times with 150μl wash buffer (25 mM Tris-HCl pH8.0, 75mM KCl, 2.5mM MgCl2).

Captured virus was detected with a commercial HCV PCR testing kit (Sino-American Co., Shanghai, China) diluted 1:50 in PBS. The mixtures were mixed gently and incubated for 1 hr at room temperature and overnight at 4°C. Tubes were then washed three times with 150μl wash buffer (25 mM Tris-HCl pH8.0, 75mM KCl, 2.5mM MgCl2).

Figure 3 Characterization of the fusion antigens transiently expressed in HeLa cells by Western blotting. After separation by a 12.5% SDS-PAGE, the proteins were blotted onto a nitrocellulose membrane and incubated with (A) anti-S McAb (H166, 1:300 diluted), (B) anti-HCV E2 (450:565) polyclonal antiserum RE2-116 (1:500 diluted) respectively. (A) lane 1-7, fusion proteins expressed by pcDNA3, pcDNA3SE2-A, pcDNA3SE2-B, pcDNA3SE2-C, pcDNA3SE2-D, pcDNA3SE2-E and pcDNA3SE2-E respectively. (B) lane 1-5, fusion proteins expressed by pcDNA3E2- pcDNA3, pcDNA3SE2-C, pcDNA3SE2-D, pcDNA3SE2-E respectively.
Humoral responses to fusion antigens in DNA-immunized mice

C57BL/6 mice were injected with HCV-E2 and HBV-S fusion constructs. As shown in Table 1, the immunized mice produced antibodies against both HBsAg and HCV-E2 after the first injection, and the seroconversion rate increased quickly after the boost, reaching 100% at week 6 or 8. Antibodies were still detectable after 24 weeks. Impressively, the anti-HCV E2 antibody titers elicited in mice immunized with fusion constructs were much higher than that induced in mice injected with pcDNA3E2 (up to 40 fold difference), which contained non-fused HCV E2(384-649). Compared with the humoral response induced by pcDNA3S, all the fusion constructs were able to contain non-fused HCV E2(384-649). Compared with the humoral response induced by pcDNA3S, all the fusion constructs were able to induce equivalent or slightly smaller anti-HBs antibody responses.

Subsets of IgG against both HBsAg and HCV-E2 were also determined. The IgG profiles specific to HBsAg indicated the obvious predominance of IgG2a (Figure 4A), while IgG profiles specific to HCV-E2 varied among different constructs (Figure 4B). Only pcDNA3E2-A and pcDNA3E2-D induced predominant IgG2a against HCV-E2, suggesting that the T helper responses elicited against these two fragments were Th1 dominant.

Expression of cytokines in the cultured splenic cells from DNA-immunized mice

After the in vitro stimulation of splenic cells derived from immunized mice, expressions of cytokines were analyzed by competitive RT-PCR. 24 hrs post-stimulation with rDNA yeast derived HBsAg (National Vaccine and Serum Institute, Beijing, China), high levels of IFN-γ were detected in the splenic cells from the mice immunized with pcDNA3E2-A, pcDNA3E2-B, pcDNA3E2-C and pcDNA3E2-E (Figure 5). The IL-10 expression was also detectable, but weaker than IFN-γ expression.

Table 1A Sero-conversion Ratios and Titres of Anti-HBsAg in DNA Immunized Mice

| DNA Immunogen | pcDNA3S | pcDNA3SE2-A | pcDNA3SE2-B | pcDNA3SE2-C | pcDNA3SE2-D | pcDNA3SE2-E |
|---------------|--------|-------------|-------------|-------------|-------------|-------------|
| 0 (n=5)       | 0/5    | 0/5         | 0/5         | 0/5         | 0/5         | 0/5         |
| 2             | 0/5    | 0/5         | 0/5         | 0/5         | 0/5         | 0/5         |
| 4             | 0/5    | 0/5         | 0/5         | 0/5         | 0/5         | 0/5         |
| 6             | 0/5    | 0/5         | 0/5         | 0/5         | 0/5         | 0/5         |
| 8             | 0/5    | 0/5         | 0/5         | 0/5         | 0/5         | 0/5         |
| 10            | 0/5    | 0/5         | 0/5         | 0/5         | 0/5         | 0/5         |
| 12            | 0/5    | 0/5         | 0/5         | 0/5         | 0/5         | 0/5         |

Note: Groups of mice (n=5) were injected i.m. with the respective plasmids, and anti-HBsAg antibody titers were measured at different time points by ELISA. Sera from mice immunized with pcDNA3 vector were used as negative controls and showed no reactivity with any coated antigens (data not shown). For sero-conversion, 1:20 starting dilution were assayed and the sera giving positive reading were taken as converted one. To determine antibody titers, serial two-fold dilutions of pooled sera were incubated in antigen-coated wells. A positive result was defined as an absorbance value of greater than twice the absorbance of the negative control with a cutoff of 0.050 (also see Materials and Methods).

Virus-capture ability of antisera from DNA-immunized mice

To test the virus-capture capability of antibodies elicited in response to DNA immunization, affinity-capture-RT-PCR (AC-RT-PCR) was performed as described in “Materials and Methods”. The HCV virions bound to anti-HCV-E2 were detected by nested RT-PCR to amplify a specific fragment in the 5'-UTR of HCV genome. The results indicated that immune sera of mice immunized with pcDNA3SE2-A, pcDNA3SE2-B and pcDNA3SE2-D were able to capture HCV virions in the serum of a hepatitis C patient in vitro (Figure 6).

Figure 5 IFN-γ and IL-10 mRNA expression. mRNA was isolated from splenic cells of immunized mice after stimulation with HBsAg for 0, 24, 48 hours. IFN-γ and IL-10 mRNA expression was...
determined by semi-quantitative RT-PCR after standardization of the cDNA concentration by amplification of HPRT. Level of expression is indicated as ratio of target to competitor, calculated from densitometric analysis of their PCR products. (High ratio indicates high expression of target mRNA).

**DISCUSSION**

In this report, five fusion constructs comprising various fragments within the hydrophilic region of HCV-E2 fused to the 3’ end of the S gene of HBV were constructed. The immune responses of mice to immunization with these fusion constructs indicated that all five constructs were able to elicit strong antibody responses against both HBsAg and HCV-E2. Importantly, antibody typing showed the HBsAg specific Th1 like responses, which were consistent with the high levels of IFN-γ expression in cultured spleen cells from the immunized mice after stimulation with HBsAg. The IgG subtype responses to HCV-E2 also showed E2 specific Th1 like responses to E2-A and E2-D. Since Th1 like response is essential for triggering a wide range of cellular responses against infectious agents, including NK cell responses, CTL responses and inflammatory reactions, the results of the DNA immunization with our fusion plasmids suggest a promising strategy to capture both HBV and HCV in the host.

The excellent vaccine potential of our constructs may be ascribed to the use of HBsAg as a carrier. HBsAg has the advantage of assembling into large secretable particles, which form a virus-like polymeric structure that enhances antigenic stability and provides a high-density presentation to antigen-presenting cells (APC). HBsAg has been used successfully as vaccine carrier to present various antigens[15,23,29,36,37]. The successful immune responses to both HBV S and HCV E2 in these reports support our preliminary observation that antigens of a proper size fused to C-terminal of HBsAg could be successfully presented without affecting the immunogenicity of HBsAg. Moreover, our fusion constructs were able to elicit higher levels of antibody responses to HCV-E2, compared to pcDNA3SE2 that encodes only a truncated HCV-E2 (384-649). This of course suggests that fusion to HBsAg may help these fragments to be presented more effectively to APCs, and hence induce stronger humoral responses against HCV-E2. These fusion constructs may turn out to be promising candidates for the vaccines against HCV and HBV.

It is well established that the intact E2 glycoproteins expressed alone or together with E1 (E1-E2 heterodimer) are retained in the endoplasmic reticulum[20,21] and that E2 proteins expressed in mammalian cells without the signal sequence at the C-terminus of E1 are not glycosylated[9,50]. Consistently, we also observed that the truncated E2 (384-649) was expressed in unglycosylated form in mammalian cells. However, the use of HBsAg as carrier seems to give rise to expression of fusion proteins carrying HCV-E2 epitopes in glycosylated and secretable form, which emphasizes the advantage of HBsAg as a carrier. Notably, two products, SE2-B and SE2-D expressed by pcDNA2SE2-B and pcDNA3SE2-D, produced an additional 31000 glycosylated form, suggesting that in addition to the glycosylation site in HBsAg, some sites in fragment B and D also may be glycosylated. Since glycosylation is a decisive factor for biological molecules to carry out correct biological functions, the additional glycosylation of the fusion constructs may elicit immune responses similar to that of natural infections by the HCV. This may be an additional reason that our constructs are able to elicit strong immune responses.

The results presented here also suggest that the DNA immunization with different HCV E2 fragments fused to HBsAg may be a useful approach to screen for epitopes or immune determinants on HCV-E2. Naturally, in_tDNA inserted different regions of HCV-E2 into the preS2 region of HBV surface protein, and evaluated the humoral immune responses of these fusion constructs via DNA immunization. Their study also suggest that fusion constructs of E2 with HBsAg might be an efficient way to identify restricted immunogenic domains within E2. In our study, each of the chosen E2 fragments contained only 30 amino acids, it provides a possibility to study the antigenic domains of HCV E2 more precisely. Antibody responses and IgG subtype typing against these E2 fragments suggest that B- and T- epitopes were present in fragment D (490-519). Furthermore, the results of virus-capture assay showed that HCV virions in the serum of a patient with hepatitis C patient could be captured by immune sera against SE2-A, SE2-B and SE2-D in vitro. In particular, antibodies against fragment D exhibited the strongest ability to capture HCV virions. Because the ability of antibodies to bind viruses is a prerequisite for neutralizing viruses in vivo and preventing bodies from being infected, our data suggest that besides HVR1-containing fragment A, fragments B and D probably also harbour neutralizing epitopes. So far, only HVR1 has been confirmed to be an important epitope[40-43] whose corresponding antibody can neutralize the binding of HCV-E2 to susceptible cells in vitro, and prevent HCV infection in vitro neutralization[42,43]. Recently, correlation between circulating antibodies against HVR1 and resolution of chronic hepatitis C has been confirmed[49]. However, the high variability of HVR1 interferes with the development of HVR1-based vaccines. Some researchers suggested that there may be B-cell epitopes located downstream from HVR1[17,18,50]. Rosa et al[40] speculated that there may be at least one additional neutralizing epitope located somewhere other than HVR1 of HCV E2, this was based on the finding that HVR-1 peptide failed to completely block the binding of HCV to susceptible cells. Our results suggest that potential neutralizing epitopes may be present within fragments B (414-443) and D (490-519) that are downstream from HVR1. Since fragments B and D are relatively more conservative than HVR1, it would seem that vaccines containing fragments B and/or D may be more promising than that containing only HVR1. Further studies on these neutralizing epitopes are under way, which hopefully will lead to a better understanding of the immunological property of HCV E2, and facilitate the design of efficient HCV vaccines.

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