Hepatitis C virus-specific cellular and humoral immune responses following immunization with a multi-epitope fusion protein

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Abstract. Hepatitis C virus (HCV) is an important causative agent of acute and chronic hepatitis worldwide. We prepared a fusion protein in the vector of pET-11d that included three conserved broadly neutralizing B-cell epitopes and a series of T-cell epitopes located in the HCV NS3 region. *In vivo* administration of this fusion construct resulted in specific $CD8^+$ cytotoxic lymphocytes in both PBMCs and splenocytes that could recognize specific antigen sites that could be detected by FACS. An HCVcc system was established and applied to detect HCV-specific neutralizing antibodies. These results suggest that the multi-epitope fusion protein is immunogenic and can elicit both humoral and cellular immune responses. In particular, this fusion protein is able to elicit HCV-specific neutralizing antibodies, which are critical for viral clearance. This construct may be significant for vaccine development and could be a potential candidate to be included in the design of a prophylactic and therapeutic vaccine against HCV.

Introduction

Hepatitis C is an infectious disease that seriously threatens public health. The clinical symptoms of an acute infection with hepatitis C virus (HCV) include fatigue, myalgia, nausea and vomiting. Other features of this infection are jaundice and a yellowish tone to the skin (1). Hepatitis C virus is the pathogen that causes this disease. This virus belongs to the *Hepacivirus* genus of the Flaviviridae virus family (2), and its genome consists of a single-stranded positive-sense RNA of ~9600 nucleotides containing a single open reading frame. This open reading frame encodes a precursor poly-protein of 3011 amino acids that is cleaved by viral proteases or host cell peptidases in subsequent co- and post-translational processes to yield at least 10 mature structural and non-structural proteins, including core protein C, envelope proteins E1 and E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (3).

The hepatitis C virus is transmitted mainly via the blood (4). According to the WHO's statistics, there are more than 170 million chronic HCV carriers and 3-4 million HCV new cases are reported every year (5). It is estimated that there are ~40 million HCV carriers and an HCV prevalence up to 3.2% in China (6). Moreover, a large portion of the acutely HCV-infected individuals will become chronically infected, and ~20% of the chronically infected will develop cirrhosis within a 10-30 year period (7). These patients have an elevated risk of developing hepatocellular carcinoma (8). Histological analysis of liver biopsies is an important tool to diagnose and follow chronic liver disease and the progression of fibrosis and cirrhosis (9). There are many reasons for the viral immune escape and persistent infection (10). One of the main factors is that the viral genome shows considerable genetic heterogeneity as the virus has a high mutation rate of $1.9x10^{-3}$ nucleotides per gene site every year (11). Due to host immune pressure and the drug selection pressure, viral mutations occur and new HCV quasispecies appear. As a result, the immune systems of some infected individuals cannot clear the virus efficiently. Antiviral treatment of HCV is now successful in about half of cases, but it is expensive, requires long-term treatment, and is associated with serious side effects (12). There is no vaccine currently available for the prevention of HCV infection (13). Therefore, developing an effective HCV vaccine to prevent HCV infection is a very urgent problem all over the world. However, until recently, HCV has not been able to be propagated efficiently in cell culture (14). This obstacle means that the use of inactivated or live, attenuated viral vaccines has not been feasible. Vaccine approaches against this virus have therefore included the use of recombinant polypeptide subunits of the virus along with adjuvant in attempts to prime viral neutralizing antibodies to the envelope glycoproteins 1 and 2 (gpE1 and gpE2), as well as priming MHC class-II-restricted $CD4^+$ T helper (Th) and MHC class-I-restricted $CD8^+$ cytotoxic lymphocyte (CTL) responses to these and other viral proteins (15).

An effective HCV vaccine would play a key role in the prevention of HCV infection (16). Recent studies have made great progress in developing many HCV vaccine candidates. These studies involved the use of recombinant HCV envelope

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glycoproteins gpE1 and gpE2 as vaccine antigens (17). The high yield of these vaccine antigens is very important for the evaluation of immune responses *in vitro*. However, the hypervariability of the HCV genome and the weakness of the humoral immune responses elicited by HCV subunit peptides have hindered the generation of HCV vaccines (18). To overcome these obstacles, we chose to generate a novel fusion protein vaccine including the conserved 780 bp HCV NS3 fragment, which contains a series of T-cell epitopes. Our fusion protein also included three broadly neutralizing B-cell epitopes that have been shown to improve the activities of HCV-specific B cells and to elicit HCV neutralization antibodies in many studies. We hoped that this novel vaccine construct would have strong immunogenicity and be able to neutralize many HCV strains of different genotypes. Moreover, our use of micro-neutralization tests and CTL cytotoxicity assays provided effective measures of the humoral and cellular immune responses generated by this HCV multi-epitope fusion protein.

Materials and methods

Plasmids and bacterial strains. The pET-11d-HCV-NS3 plasmid encoding a portion of the NS3 coding sequences of HCV (subtype 1b) was preserved in our laboratory. The vector pBluescript II SK encoding the HCV multi-epitope coding sequences (aa 192-202 YEVRNVSGVYH; 313-327 ITGHRMAWDMMMNWS; 702-710 PALSTGLIH) was provided by the Biomed Gene Company, Beijing. The *E. coli* strain BL21 (DE3) was used as a cloning and expression strain.

Expression and purification of HCV multi-epitope fusion proteins. After induction of protein expression in BL21 (DE3) host cells, 1.8 liters of cell culture were centrifuged (8,000 x g for 10 min) and the pellets were resuspended in 50 ml of lysis buffer (10 mM Tris-HCl, 0.5% Triton X-100, pH 8.0). The cell lysates were then clarified by centrifugation (8,000 x g for 10 min). The supernatants were discarded and the pellets were dissolved in 8 M urea. The supernatant was purified by DEAE negative ion exchange chromatography and His-tag affinity chromatography. The fusion protein was renatured after removal of urea by dialysis and then stored at -20˚C.

SDS-PAGE and Western blot analysis. For SDS gel electrophoresis, 10 μl of sample were mixed with an equal volume of sample buffer (63 mM Tris-HCl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.002% bromophenol blue, pH 6.8) and boiled for 3 min. The proteins (5-50 μg protein/lane) were submitted to 15% SDS-PAGE and stained with Coomassie Brilliant Blue R-250.

For immunoblotting, the proteins were electrophoretically transferred from the gel to a polyvinylidene difluoride (PVDF) membrane at 400 mA for 40 min. The membrane was blocked overnight with 5% non-fat milk in PBS containing 0.05% Tween-20 (PBST) at 4˚C. After washing with PBST, the blot was incubated at room temperature for 1 h with primary antibodies at a 1:1,500 dilution. After extensive washing with PBST, the blot was incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5,000). The blot was developed with 0.2 mM DAB (3,3'-diaminobenzidine tetrahydrochloride).

Culturing, transfection and infection of Huh7.5 cells. The culture of Huh7.5 cells was performed as previously described. One day before transfection or infection, Huh7.5 cells were plated at $3x10^5$ per well in 6-well plates. *In vitro* transcription of RNA for transfection was carried out on 5 μg of plasmid linearized with the *Xba*I restriction enzyme using T7 RNA polymerase (Promega) for 2 h. For transfection, 2.5 μg of unpurified RNA transcripts were incubated with 5 μl of Lipofectamine 2000 (Invitrogen) in 500 μl of Opti-MEM (Invitrogen) for 20 min at room temperature. RNA-Lipofectamine 2000 transfection complexes were left on cells for 12-24 h before washing. For infection, virus-containing supernatant was left on cells for 4 h followed by continuous cell culture in DMEM for 3 days. Supernatants collected during experiments were sterilized, filtered and stored at -80˚C.

Animal immunization and preparation of antisera, PBMC and splenocytes. Five rabbits were immunized intramuscularly with the fusion protein emulsified in complete Freud's adjuvant (Sigma) at doses of 10, 20, 50, 100 or 200 μg. A sixth rabbit received PBS as a negative control. Each animal received a second and third immunization at one-week intervals with the same quantity of antigen emulsified in incomplete Freud's adjuvant (Sigma). Blood was collected from the carotid artery at 7, 14 and 28 days after primary immunization and serum was prepared using standard procedures.

The immunization procedure of Balb/c mice was the same as described above for the rabbits, except that the injection doses of the fusion protein were 20 and 100 μg. All mice were sacrificed by cervical dislocation. Blood was collected into sterile tubes, and PBMCs were prepared by Ficoll-paque differential sedimentation and four washes in Dulbecco's phosphate buffer. Spleens from the mice were ground on a metal mesh to prepare single-cell suspensions in grinding media (RPMI-1640 supplemented with 100 ml/l FCS, 1 mmol/l sodium pyruvate and 50 μmol/l β-ME). Red blood cells were lysed by incubating splenocyte preparations with lysis buffer (0.15 mol/l NH_4Cl, 10 mmol/l $KHCO_3$, 0.1 mmol/l EDTA, pH 7.2-7.4) briefly and then washing with grinding media. The cells were resuspended in a small volume of grinding media and counted in the presence of trypan blue. Both PBMCs and splenocytes were immediately used for further experiments.

The animal protocols used in this study were approved by the Institutional Review Board and the Animal Care and Use Committee of the 309 Hospital of PLA (Beijing, China).

CTL assay. HCV tetramers were purchased from Xinlilai Biotechnology Company (Beijing, China). The resuspended PBMCs and splenocytes ($4x10^6$ cells/ml) were washed once with 0.1% BSA, 0.1% sodium azide in PBS and incubated with either of two HLA-A2 restricted HCV-specific tetramers (NS3-1287-1296 TGAPVTYSTY; NS3-1406-1415 KLVALGINAV) for 20 min at 37˚C. One microliter of antibody (anti-CD8-FITC) was added for 10 min at RT. After two washing steps, cells were analyzed by flow cytometry (FACSCalibur). One hundred thousand cells were collected for each analysis.

Anti-HCV antibody analysis. An enzyme-linked immunosorbent assay (ELISA) was used to determine the presence of antibodies against the HCV multi-epitope antigens in serum

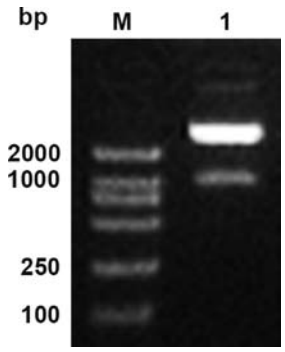

Figure 1. Digestion pattern of the recombinant plasmid. M, DNA molecular weight marker DL2000; 1, recombinant plasmid double digested by *Nco*I and *Xho*I.

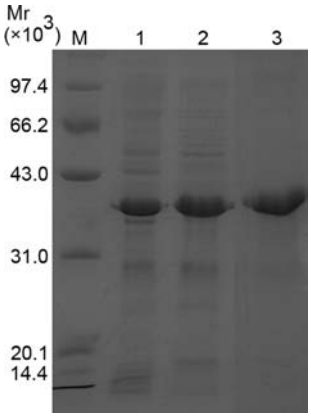

Figure 2. SDS-PAGE analysis of the HCV fusion protein. M, Standard markers for protein molecular weight; 1, whole cell lysate of cells induced by IPTG; 2, fusion protein after purification by DEAE-negative ion exchange chromatography; 3, fusion protein after purification by affinity chromatography.

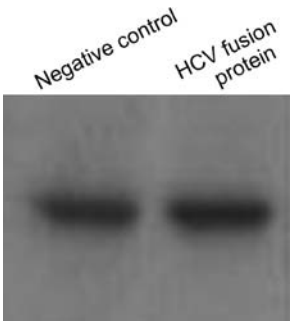

Figure 3. Western blot analysis of HCV fusion protein. 1, Negative control; 2, HCV fusion protein.

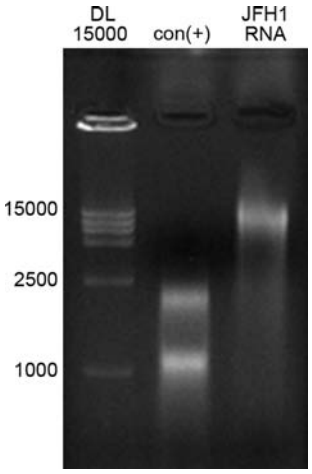

Figure 4. Full-length HCV RNA (JFH-1 strain). Con(+) stands for RNA band transcribed from the template provided by the Promega kit. The expected sizes are 1.1 and 2.4 kb.

samples. The fusion protein was used to coat 96-well plates at a concentration of 1 μg/ml and was incubated overnight at 4˚C. After the plates were washed with PBS plus 0.5 g/l Tween-20 (PBS-T) and blocked in blocking buffer (50 g/l fat-free milk powder in PBS-T), 2-fold serial dilutions of serum samples in blocking buffer were added and incubated for 2 h at 37˚C. After three wash steps with PBS-T, horseradish peroxidase (HRP)-conjugated goat-anti-rabbit IgG (1,000-fold dilution in blocking buffer) was added and incubated for 1 h at 37˚C. The plate was then developed with substrate buffer (50 mmol/l Na_2HPO_4, 25 mmol/l citric acid, 75 μg/ml 3,3',5,5'-tetramethylbenzidine, 0.15 ml/l H_2O_2). After 30 min of incubation at room temperature, the reaction was stopped by adding 0.5 mol/l H_2SO_4, and the absorbance was measured at 490 nm on a microplate reader. Antibody titers were calculated as the highest dilution that gave a positive reading. The cutoff value was 2.1-fold above the negative control. All tests were performed in duplicate.

Neutralization assay. To test neutralization capacity, serial dilutions of sera samples from the rabbits, in parallel with an NC, was mixed with the HCVcc before addition to the cells. After incubation at 37˚C for 2 h, the supernatants containing the HCVcc/antibody mixture were removed by washing with PBS, and then, complete DMEM was added to each well. The cells were continuously cultured in DMEM for 3 days, followed by cold methanol fixation and primary antibody staining. A FITC-conjugated secondary antibody was added after washing with PBS. Positive cells were visualized under a fluorescence microscope. The neutralization titer was expressed as the reciprocal of highest sera dilution, giving positive neutralization results.

Results

Recombinant plasmid construction. As shown in Fig.1 the recombinant plasmid was successfully constructed and two bands were visualized upon digestion with *Nco*I and *Xho*I (Fig. 1).

SDS-PAGE and Western blot analysis. SDS-PAGE (Fig. 2) and Western blot analysis (Fig. 3) of the HCV fusion protein demonstrate that the HCV multi-epitope fusion protein was expressed and purified successfully.

HCV full-length RNA preparation. A denaturing gel showing the HCV full-length is shown in Fig. 4.

Culture, transfection and infection of Huh7.5 cells. Immunofluorescence of Huh7.5 cells 12 and 24 h after full-length HCV RNA transfection is shown in Fig. 5. Positive staining

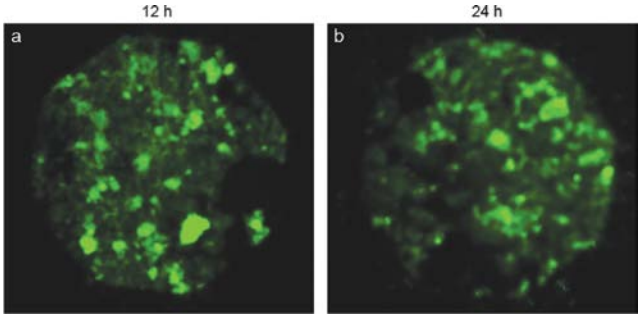

Figure 5. Immunofluorescence of Huh7.5 cells (a) 12 h and (b) 24 h after full-length HCV RNA transfection. Immunofluorescence could be detected in Huh7.5 cells 12 and 24 h after full-length HCV RNA transfection.

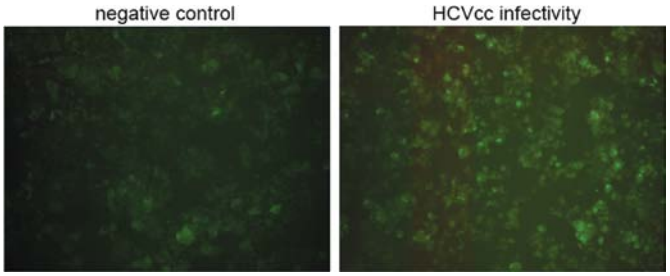

Figure 6. HCVcc infectivity. Immunofluorescence could be detected in Huh7.5 cells 3 days after HCVcc infection.

confirms the efficiency of transfection. Immunofluorescence was detected in Huh7.5 cells 3 days after HCVcc infection (Fig. 6).

CTL assay. The percentages of specific $CD8^+$ cytotoxic lymphocytes in PBMCs and the spleen are shown in Figs. 7 and 8, respectively. Cytotoxic T cell responses were much stronger in the mice immunized with 100 μg compared with the mice immunized with 20 μg of fusion protein. The percentages of $CD8^+$ cytotoxic lymphocytes were higher in PBMCs immunized with 20 than with 100 μg of the antigen, when incubated with the peptide NS3-1287-1296 (TGAPVTYSTY) (Fig. 7A vs. C) or with the peptide NS3-1406-1415 (KLVALGINAV) (Fig. 7B vs. D). Similarly, the percentages of $CD8^+$ cytotoxic lymphocytes were higher in splenocytes immunized with 20 than with 100 μg of the antigen, when incubated with either peptide (compare Fig. 8 panel A with C and B with D). There was little difference when PBMCs or splenocytes were incubated with the two different HCV T cell epitope peptides (TGAPVTYSTY and KLVALGINAV).

Anti-HCV antibody analysis. Increasing antibody titers against the multi-epitope fusion protein are shown in Fig. 9. Antibody titers increased 14 days after the priming immunization and seroconversion was observed in all animals except negative controls. Antibody titers continued to increase rapidly and one animal reached a titer of 1:8192 two weeks after the final boost.

Neutralization assay. Neutralizing antibody titers are shown in Fig. 10. The fusion protein was able to elicit neutralizing antibodies that were critical in viral clearance.

Discussion

Currently, there is no prophylactic or therapeutic vaccine for HCV, and HCV vaccine development has been difficult for multiple reasons (19). There is no small animal model of this viral infection, nor is there an *in vitro* replication system available to produce large quantities of virus to study viral replication and assay immune responses. Additionally, HCV is a highly heterogeneous virus consisting of six genotypes and multiple subtypes. The virus exists in chronically infected

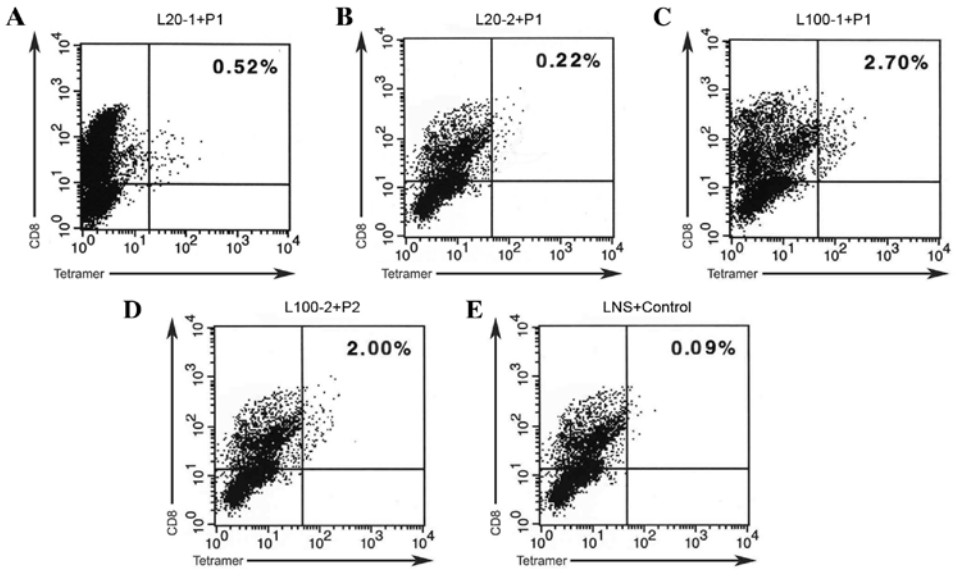

Figure 7. (A) PBMCs from mice immunized with 20 μg antigen were incubated with peptide NS3-1287-1296 (TGAPVTYSTY). (B) PBMCs from mice immunized with 20 μg antigen were incubated with peptide NS3-1406-1415 (KLVALGINAV). (C) PBMCs from mice immunized with 100 μg antigen were incubated with peptide NS3-1287-1296 (TGAPVTYSTY). (D) PBMCs from mice immunized with 100 μg antigen were incubated with peptide NS3-1406-1415 (KLVALGINAV). (E) Negative control.

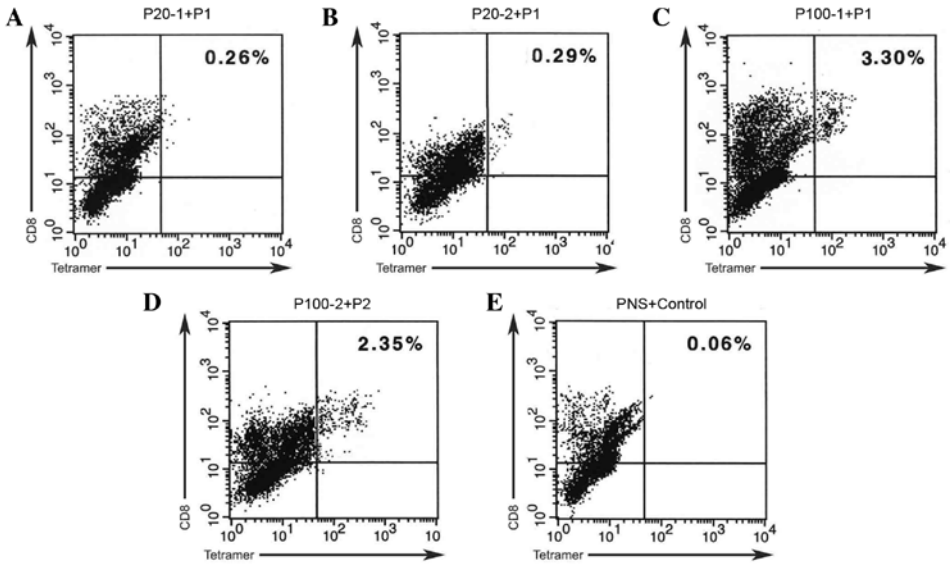

Figure 8. (A) Splenocytes from mice immunized with 20 μg antigen were incubated with peptide NS3-1287-1296 (TGAPVTYSTY). (B) Splenocytes from mice immunized with 20 μg antigen were incubated with peptide NS3-1406-1415 (KLVALGINAV). (C) Splenocytes from mice immunized with 100 μg antigen were incubated with peptide NS3-1287-1296 (TGAPVTYSTY). (D) Splenocytes from mice immunized with 100 μg antigen were incubated with peptide NS3-1406-1415 (KLVALGINAV). (E) Negative control.

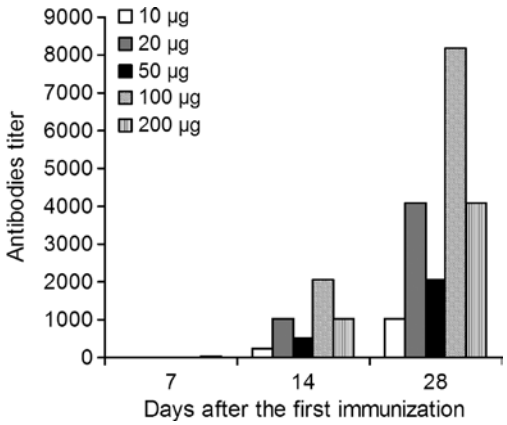

Figure 9. Antibody titers in rabbits immunized with different doses of antigen.

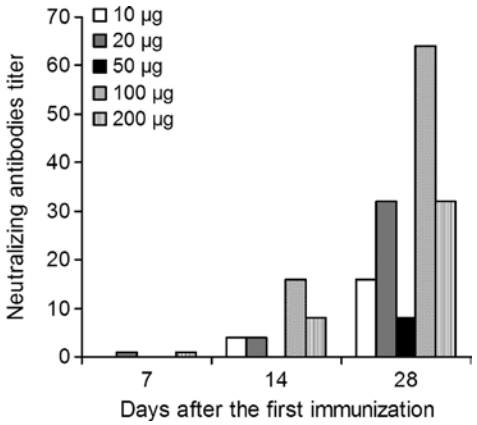

Figure 10. Neutralizing antibody titers of rabbits immunized with different doses of antigen.

individuals as a population of quasispecies with diverse but related sequences (20). However, there is evidence supporting the feasibility of an HCV vaccine (21). Fifteen to twenty-five percent of HCV-infected individuals are able to spontaneously clear the virus, which implies a role for the adaptive arm of the immune response in clearance of the virus (22). The majority of HCV-infected humans are protected from progression to the chronic state. These observations encouraged us to develop a prophylactic HCV vaccine that would be able to induce HCV-specific immune responses similar to those elicited in infected individuals and would be able to protect naive individuals post-infection.

Increasing evidence demonstrates that epitopes located within the HCV E1 and E2 proteins are important for HCV neutralization (23). In the present study, we designed a fusion protein containing multiple epitopes from E1 and E2 to generate potential neutralizing antibodies to the virus. This protein was expressed in *E. coli* and purified under denaturing conditions using DEAE negative ion exchange chromatography and affinity chromatography. SDS-PAGE and Western blotting demonstrated that the HCV multi-epitope fusion protein was expressed and purified successfully.

To study the effect of this protein, Balb/c mice and rabbits were immunized with the fusion protein at different doses. Antibody titers increased 14 days after the priming immunization and seroconversion was observed in all animals except negative controls. Antibody titers continued to increase rapidly and one animal reached a titer of 1:8192 two weeks after the final boost. The high antibody titers in these sera suggested that a prokaryotically expressed HCV multi-epitope fusion protein was able to elicit a significant humoral immune response in rabbits. The immunogenicity of this fusion protein was shown in animal experiments. Furthermore, we investigated HCV-specific neutralizing antibodies using a micro-neutralization assay and found that the fusion protein was able to elicit neutralizing antibodies that

were critical in viral clearance, although their titers were not high. This evidence allows us to hypothesize that the three broadly neutralized B-cell epitopes that we selected played an important role during the elicitation of neutralizing antibodies. These antibodies are worth further study.

The CTL assay indicated that specific $CD8^+$ cytotoxic lymphocytes could be detected in both PBMCs and splenocytes of the mice. Cytotoxic T cell responses in the mice immunized with 100 μg of fusion protein were much stronger than in the mice immunized with 20 μg of fusion protein. There was little difference when PBMCs or splenocytes were incubated with two different HCV T cell epitope peptides (TGAPVTYSTY and KLVALGINAV). We concluded that the fusion protein was capable of eliciting strong cellular immune responses, particularly in the mice immunized with 100 μg of fusion protein. Taken together, the humoral immune responses, viral neutralization ability and specific cellular responses suggest that this HCV multi-epitope fusion protein is a potential candidate for designing a prophylactic and therapeutic vaccine against HCV.

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