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Cellular immunogenicity of a multi-epitope peptide vaccine candidate based on hepatitis C virus NS5A, NS4B and core proteins in HHD-2 mice

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To develop a vaccine against hepatitis C virus (HCV), a multi-epitope peptide was synthesized from nonstructural proteins containing HLA-A2 epitopes inducing mainly responses in natural infection. The engineered vaccine candidate, VAL-44, consists of multiple epitopes from the HCV NS5A, NS4B and core proteins. Immunization with the VAL-44 peptide induced higher CTL responses than those by the smaller VL-20 peptide. VAL-44 induced antigen-specific IFN-γ-producing CD4+ T cells and CD8+ T cells. VAL-44 elicited a Th1-biased immune response with secretion of high amounts of IFN-γ and IL-2, compared with VL-20. These results suggest that VAL-44 can elicit strong cellular immune responses. The VAL-44 peptide stimulated IFN-γ production from viral-specific peripheral blood mononuclear cells (PBMCs) of patients infected with HCV. These results suggest that VAL-44 could be developed as a potential HCV multi-epitope peptide vaccine.

1. Introduction

Hepatitis C virus (HCV), a positive stranded RNA virus belonging to the Flaviviridae family, is a major cause of chronic liver disease with 170 million infected people worldwide. The incidence of new cases each year is estimated at about 3 million (Cohen, 1999). HCV-associated chronic liver disease can result in cirrhosis and hepatocellular carcinoma, which seriously impact the quality of life of affected individuals (Ramos-Casals et al., 2004). The efficacies of interferon-α (IFN-α) and ribavirin-based therapies are limited and prohibitive for most patients in developing countries. Therefore, development of an effective HCV vaccine is the most feasible strategy to decrease the worldwide burden of HCV-induced liver diseases, including liver cirrhosis and hepatocellular carcinoma.

The HCV genome encodes three structural proteins, the core and envelope proteins E1 and E2, and at least seven nonstructural proteins, including NS1, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Bartenschlager et al., 2010). However, a major challenge to develop a vaccine to generate HCV neutralizing antibodies is the high mutation rate, especially in the antigenic sites of envelope glycoprotein E2 of HCV, due to the lack of proofreading ability of its RNA-dependent RNA polymerase.

Some studies have demonstrated that the spontaneous clearance of acute HCV infection occurs in association with a broadly specific and vigorous cellular immune response (Klenerman and Hill, 2005; Rehermann and Nascimbeni, 2005). T cell responses in chronic HCV patients are characteristically polyclonal and multispecific, but the virus cannot be cleared by low frequencies of specific CD8+ T cells in the peripheral blood (Cerny and Chisari, 1999). At the same time, the level of IFN-γ secretion of HCV-specific CD8+ T cells is suppressed in the liver (Neumann-Haefelin et al., 2008). Thus, chronic HCV infection is mainly attributed to the uncontrolled HCV replication due to CD8+ T cell dysfunction (Shin and Wherry, 2007).

In the past 10 years, significant progress has been made in understanding the pathogenesis of HCV infection, mechanisms of persistent infection and protective immune responses. As both strong CD4+ and CD8+ T cells and neutralizing antibodies have been

Abbreviations: ALT, alanine aminotransferase; DMSO, dimethyl sulfoxide; ELISPOT, enzyme-linked immunospot; HCV, hepatitis C virus; IFA, incomplete Freund's adjuvant; CFA, complete Freund's adjuvant; IFN-α, interferon-α; IFN-γ, interferon-γ; OD, optical density; LDH, lactate dehydrogenase; PBMCs, peripheral blood mononuclear cells; SFCs, spots forming cells; SI, stimulation index; Th, T helper.

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VL-20

VLSDFKVWLKKSMMAFSAAL

VAL-44

VLSDFKVWLKKKFPGGGQIVGGVYLLPRRGPRLKKSMMAFSAAL

Fig. 1. Amino acid sequences of the synthetic peptides.

associated with HCV clearance, inducing such immune responses is the goal of hepatitis C vaccine development.

Sustaining a strong level of HCV non-structural protein and multi-specific Th1 type cellular immune response is directly related to virus clearance and the course and outcome of disease (Lechner et al., 2000; Chang et al., 2001; Ward et al., 2002; Lang Kuhs et al., 2012). Because multiple virus subtypes exist among chronic HCV patients, a successful vaccine should induce strong and multi-specific immune responses, including CD4+ and CD8+ T cells. In this study, an HCV epitope vaccine was constructed from conserved regions selected by evaluating the score of nine peptides, as well as the proportion of virus genotypes and strains in the HCV database. The binding capacity of the nine peptides for different HLA molecules (super type) was predicted by bioinformatic analysis (http://peptibase.cs.biu.ac.il/PepCleave_II; http://www.syfpeithi.de; http://www.mpiib-berlin.mpg.de/MAPPP/). In addition, twelve CTL epitopes were considered based on HLA-A2 affinity testing and the responses in peripheral blood mononuclear cells (PBMCs) of chronic HCV-infected individuals, and two HCV CTL epitopes, NS5A (1991–1999) VLSDFKVWL (Yun et al., 2011) and NS4B (1793–1801) SMMAFSAAL, were finally selected (Duan et al., 2011). In order to stimulate both CD4+ and CD8+ T cells immune responses, a T helper (Th) epitope KFPGGGQIVGGVYLLPRRGPRL (Firbas et al., 2010) was added between two CTL epitopes. The KK sequence was added between each epitope of the HCV multi-epitope peptide vaccine in order to ensure proper processing and presentation. The goal of this study was to assess the safety and immunogenicity of the new HCV multi-epitope peptide vaccine.

2. Materials and methods

2.1. Synthetic polypeptides

Two polypeptides (Fig. 1) were synthesized according to the amino acid sequences of three CTL epitopes by ChinaPeptides (Shanghai, China). All multi-epitope peptides were analyzed for composition and purity using mass spectrometry, which showed purities of between 90% and 95%. The VL-20 peptide contains two of the CTL epitopes, while the VAL-44 peptide harbors a Th epitope along with two CTL epitopes and lysine linkers (-KK-) joining the different epitopes.

2.2. Cell lines

The cell line C1R.AAD (HMYC1R transfected with the HLA chimeric molecule containing α1 and α2 domains from human HLA-A2.1 and α3 from mouse H-2Dd) has been described previously (Newberg et al., 1996).

2.3. Mouse studies

2.3.1. Immunization of HHD-2 transgenic mice

HHD-2 transgenic mice at 6–8 weeks of age were purchased from the Fourth Military Medical University Animal Center. The HHD-2 mice, derived originally from C57BL mice with the mouse β_2-microglobulin and MHC I knock-out, contains a transgene of a chimeric HLA class-I molecule composed of the human β_2-microglobulin, HLA-A*0201 α-1 and α-2 domains and the mouse α-3 domain of H-2D^b (Huang et al., 2009). The mice were housed in isolated and ventilated cages in accordance with the American Physiological Society's Guiding Principles in the Care and Use of Animals. The mice were randomly divided into three groups of six each. At weeks 0, 2 and 4, the VL-20 peptide, VAL-44 peptide or PBS were injected subcutaneously at the tail base in a total volume of 200 μl at a 1:1 ratio with adjuvant. At week 0, peptide immunizations were carried out with a mixture of 100 μg multi-epitope peptide emulsified in complete Freund's adjuvant (CFA, Sigma, Saint Louis, USA). At weeks 2 and 4, experimental groups were boosted subcutaneously with 100 μg of multi-epitope peptide emulsified in incomplete Freund's adjuvant (IFA, Sigma, Saint Louis, USA). Control mice were immunized with 100 μl PBS combined with 100 μl CFA or IFA. Mice were sacrificed 10 days after the last immunization. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University.

2.3.2. T lymphocyte proliferation

T lymphocyte proliferation was determined by the MTT assay (Sigma, Saint Louis, USA). Briefly, spleen cells (1×10^5/well) isolated from the vaccinated mice were stimulated with the multi-epitope peptides (10 μg/ml) for 54 h and exposed to MTT (5 mg/ml) for 4 h (Livingston et al., 2001). After the medium was removed, 150 μl dimethyl sulfoxide (DMSO, Sigma, Saint Louis, USA) was added to each well of the plate, which was then incubated at 37 °C for 10 min, followed by measurement of the optical density (OD) value using a microplate reader. The stimulation index (SI) was calculated as follows: SI = OD of stimulated culture/OD of unstimulated culture. SI > 2 was considered significant (Olugbile et al., 2011).

2.3.3. Enzyme-linked immunospot (ELISPOT) assay

IFN-γ- and IL-2-secreting cells were quantified using an ELISPOT kit (Mabtech, Nacka Strand, Sweden). MultiScreen 96-well filtration plates were coated with the anti-mouse IFN-γ or IL-2 capture antibody (2.5 μg/ml) overnight at 4 °C. After washing, the plates were blocked for 1 h with 200 μl complete RPMI-1640 before adding splenocytes (1×10^6/well) for 24 h of culture with 10 μg/ml of corresponding antigens or 4 μg/ml of Con A (as positive control) in triplicate wells. After removing cells and washing with wash buffer (PBS with 0.1% Tween 20, Sigma, Saint Louis, USA), 1:2500 diluted biotinylated anti-IFN-γ or anti-IL-2 antibodies were added and incubated for 2 h at room temperature. After washing five times with PBS (0.05% Tween 20), 1:1000 diluted streptavidin-HRP was added into the wells and incubated for 1 h. TMB substrate solution was added into the wells until spots were clearly visible, and then the wells were washed with distilled water to stop the development. The spots were counted using an automated ELISPOT reader. A response was considered positive if the number of spots forming cells (SFCs) per 10^6 splenocytes was greater than 30 (Fournillier et al., 2006).

2.3.4. Flow cytometry

Relative proportions of CD4+ T and CD8+ T cells in the spleen cells of mice were analyzed by flow cytometry. Briefly, splenocytes (1×10^6/well) were cultured for 5 h (Rojas et al., 2011) in complete RPMI-1640 alone (negative control) or co-cultured with 10 μg/ml of corresponding antigens. Ten microliters of FITC-conjugated rat anti-mouse CD8 antibody (Ebioscience, San Diego, USA) and PE-conjugated rat anti-mouse CD4 antibody (Ebioscience, San Diego, USA) were incubated with 1.0×10^6 spleen cells in a 100 μl volume for 20 min at room temperature. The spleen cells were washed