Helper T cell determinant peptide contributes to induction of cellular immune responses by peptide vaccines against hepatitis C virus

Kiyohiko Hiranuma,1,2 Shigenori Tamaki,2 Yuki Nishimura,1 Shigenori Kusuki,2 Masanori Isogawa,1,2 Gisen Kim,1 Masahiko Kaito,2 Kagemasa Kuribayashi,1 Yukihiko Adachi2 and Yasuhiro Yasutomi1

Department of Bioregulation1 and Third Department of Internal Medicine2, Mie University School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan

The capacity of novel subunit vaccines to generate cytotoxic T lymphocytes (CTLs) against hepatitis C virus (HCV) was assessed. BALB/c mice were immunized with peptides based on the CTL and helper T cell (Th) epitopes of the HCV core, with a mixture of CTL and Th peptides (CTL Th) or with a conjugated Th–CTL peptide. Mice immunized with CTL, CTL Th and Th–CTL peptides, but not those immunized with Th peptide, developed HCV core CTL epitope-specific effector cells. Cytotoxic activity induced by immunization with Th–CTL was much higher than that induced by immunization with CTL Th or CTL alone. However, rapid and high cytotoxic activities against HCV core were not only detected after immunization with peptides containing the CTL epitope but also as a result of infection with recombinant vaccinia virus carrying the HCV core gene after immunization with the Th epitope alone. Immunization with peptides containing the Th epitope also elicited spleen cell proliferation. This study demonstrates the capacity of both Th and CTL activated peptide vaccines to elicit CD8+, MHC class I-restricted CTLs. The capacity of such CTLs to contribute towards a protective and/or pathogenic immune response against HCV can now be assessed in mouse models.

Introduction

Hepatitis C virus (HCV) is the major cause of non-A, non-B viral hepatitis (Choo et al., 1989; Houghton et al., 1991; Kuo et al., 1989). More than 50% of people infected develop chronic hepatitis and some progress to cirrhosis and perhaps to hepatocellular carcinoma (Saito et al., 1990). While the pathogenic mechanism of progression of hepatitis caused by HCV has not yet been clearly elucidated, much information has been accumulated showing that virus-specific cytotoxic T lymphocytes (CTLs) play a critical role in the host cellular immune response against HCV (Imawari et al., 1989; Rehermann et al., 1996). CTLs might contribute to hepatocellular damage in HCV infection as a consequence of killing virus-infected cells. On the other hand, CTLs play an important role in preventing the spread of virus and in clearing virus during infection (Nelson et al., 1997; Mochizuki et al., 1997). It may be important to identify immunodominant epitopes that elicit antiviral CTLs, which could contribute to the development of an antiviral vaccine and to an understanding of the pathogenesis of HCV infection.

Although exogenous lymphokines such as interleukin 2 (IL-2) can substitute for helper T (Th) cells in vitro, the role of Th cells in the maturation of CTL precursors remains poorly understood compared with their role in Th cell/B cell collaboration. CD4+ Th cells are crucial to the control of many virus infections, including HCV infection, and associated pathogenesis (Fujisawa et al., 1984; Greenberg et al., 1981; Ferrari et al., 1994). Some evidence for a Th requirement in CTL induction against viral antigens has been reported (Wagner et al., 1976; Zinkernagel et al., 1978; von Boehmer & Haas, 1979; Kast et al., 1986; Husmann & Bevan, 1988). Moreover, we and others have reported that linking of a Th epitope to a CTL determinant is effective in the generation of antiviral CTLs in murine and simian systems (Shirai et al., 1994a; Yasutomi et al., 1993).
An animal model of HCV infection is required for vaccine development and an understanding of the pathogenesis of HCV infection. Although chimpanzees can be infected with HCV, they are not readily available laboratory animals. Recently, CTL and Th epitope-associated MHC molecules have been reported not only in humans but also in mice (Battegay et al., 1995; Cerny et al., 1995; Shirai et al., 1992, 1994b, 1995). Moreover, HCV- and HLA-transgenic mice have been used in experiments aimed at vaccine development and examination of HCV pathogenesis (Koike et al., 1994; Shirai et al., 1995, 1997; Shirai et al., 1995; Moriya et al., 1997; Pasquinelli et al., 1997; Matsuda et al., 1998). These reports suggest that the mouse system might be useful as an animal model for exploring novel approaches to the preparation of vaccines and for understanding the pathogenesis of HCV infection. In the present study, we used the HCV/mouse model to explore the potential of the Th epitope peptide for vaccine elicitation of HCV-specific CTLs and assessed the effect of these CTLs against HCV infection by using recombinant vaccinia virus (rVV) expressing the HCV core gene rather than by HCV challenge.

Methods

**Peptide synthesis.** The peptides used in this study were the HCV core CTL epitope (C7A10; amino acids 133–142, LMGYIPVLGA) (Shirai et al., 1994b) and Th epitope (CP4; amino acids 72–91, EGRAWA-QPGYPWPPLYGNEGLLMGYIPLVGAG). The sequence of the control peptide (P18; HIV-1 EnvCTL epitope, residues 308–322) was RIQRGPGRAFVTIGK.

**Immunization.** Six- to eight-week-old BALB/c mice were immunized twice with peptides C7A10, CP4 or CP4–C7A10 or a mixture of CP4 and C7A10 peptides. For each immunization, 50 µg peptide in 50 µl PBS was emulsified with incomplete Freund’s adjuvant (IFA) and 100 µl of this emulsion was injected into the footpad.

**Generation of CTL effector cells.** Effector cells were derived from spleen cells as precursor CTLs. Aliquots containing 5 × 10⁶ spleen cells were co-cultured with 2.5 × 10⁶ mitomycin C-treated autologous spleen cells that had been labelled with peptide at 37 °C in a 5% CO₂ incubator. The effector cells generated were harvested after 5 days in culture.

**Generation of HCV core-specific T cell lines.** CTL effector cells derived from CP4–C7A10-immunized mice were re-stimulated in vitro for 7–10 days with mitomycin C-treated, C7A10-labelled spleen cells in RPMI medium containing 20 U/ml IL-2. T cell lines were assessed for lytic activity against HCV core antigen-labelled target cells.

**Cytotoxicity assay.** Target cells were MHC-matched (P815) or -mismatched (MBL-2 and FBL-3) tumour cells, incubated for 16 h at 37 °C in a 5% CO₂ atmosphere with 10 µg/ml peptide (either C7A10 or the control peptide) or with 2 × 10⁶ p.f.u. rVV expressing either HCV core (HCV–rVV) or an unrelated virus gene (control rVV), the env gene from human immunodeficiency virus (HIV). Target cells were then washed and labelled with ⁵¹Cr. The ⁵¹Cr-labelled target cells were incubated for 5 h with effector cells. Percent lysis was calculated as 100 × (experimental release — spontaneous release)/(total release — spontaneous release). C7A10-specific lysis was calculated as (% lysis of target cells labelled with C7A10)—(% lysis of target cells labelled with control peptide). All experiments were carried out at least three times with five mice in each group.

**Blocking of cytolysis.** Blocking of cytolysis was performed according to the method reported previously (Van Snick et al., 1982). ⁵¹Cr-labelled target cells were pre-incubated at 4 °C for 1 h with anti-H-2Kb or anti-H-2Dd monoclonal antibodies (MAb) (Meiji Institute of Health Science, Tokyo, Japan) and effector cells were then added. In a separate experiment, effector cells were pre-incubated with anti-CD4 MAb (GK1.5) or anti-CD8 MAb (Lyt2.2) at a 1:50 dilution under the same conditions, and then the labelled target cells were added. Blocking of cytolysis was confirmed by control MAb assays at a 5 h ⁵¹Cr-release assay.

**rVV infection.** Mice immunized with peptide were inoculated intravenously with 2 × 10⁷ p.f.u. HCV–rVV or control rVV and their HCV-specific CTLs and assessed for their ability to lyse HCV core peptide-pulsed target cells from spleen cells as precursor CTLs. All experiments were carried out at least three times with five mice in each group.

**Spleen cell proliferation assay.** The method of determination of proliferative responses against antigenic epitope peptides presented on MHC was described previously (Yasutomi et al., 1996; Lu et al., 1996). Spleen cells were maintained in triplicate cultures in 96-well microtitre plates for 4 days at 4 × 10⁶ cells per well with 1 × 10⁴ or 5 × 10⁴ autologous spleen cells that had been infected with HCV–rVV or control rVV and then fixed with paraformaldehyde. [³H]Thymidine (0.5 µCi) was added to each well 8 h before harvesting. Cells were harvested onto filters and the incorporation of [³H]thymidine was measured by liquid scintillation counting. The stimulation index was calculated as (mean c.p.m. of spleen cells cultured with HCV–rVV-infected spleen cells)/ (mean c.p.m. of spleen cells cultured with control rVV-infected spleen cells). All experiments were carried out at least three times with five mice in each group.

Results

**Induction of C7A10-specific CTL activity by immunization with conjugated peptide**

Spleen cells obtained from peptide-immunized mice were assessed for their ability to lyse HCV core peptide-pulsed MHC-matched target cells. Mice immunized with C7A10 alone, the CTL and Th mixture (CP4+C7A10) and CP4–C7A10 generated C7A10-specific effector responses (Fig. 1). The lytic activity of CP4–C7A10-immunized mice against C7A10-labelled target cells was much higher than those resulting from CP4+C7A10 or C7A10 immunization. Mice immunized with CP4 peptide alone did not show cytolytic activity (data not shown).

**CP4–C7A10 immunization generated CD8 effector cells**

C7A10-specific effector cells from mice immunized with CP4+C7A10 were cultured in medium containing anti-CD4 or -CD8 MAb during a ⁵¹Cr-release assay. Anti-CD8 MAb
Th peptide elicits HCV-specific immune response

Fig. 1. Spleen cells from mice immunized with peptides that include the CTL epitope develop HCV core CTL epitope-specific lytic activity. Mice were immunized with 50 µg peptide in IFA as follows: ○, conjugated Th–CTL epitope peptide (CP4–C10A7); □, Th and CTL epitope mixture (CP4 + C7A10); and △, CTL epitope peptide (C7A10) alone. Lytic activity was determined at various effector:target ratios.

Fig. 2. C7A10-specific lysis by effector cells elicited by CP4–C7A10 immunization is mediated by CD8+ cells. Spleen cells from mice immunized with CP4–C7A10 peptide were stimulated with C7A10 peptide and lytic activity against C7A10-labelled target cells was assessed after the addition of anti-CD4 MAb, anti-CD8 MAb or medium alone. Effector:target ratio was 80:1.

Fig. 3. (a) C7A10-stimulated spleen cells from mice immunized with CP4–C7A10 or C7A10 lysed C7A10-pulsed H-2d but not H-2b target cells. Cells were isolated from mice immunized with C7A10 (unfilled bars) or CP4–C7A10 (grey bars). Effector:target ratio was 80:1. (b) C7A10-specific lytic activity was inhibited by anti-H-2Dd MAb. Mice were immunized with C7A10 (unfilled bars), a mixture of CP4 and C7A10 (black bars) or CP4–C7A10 (grey bars). Effector cells were assessed for C7A10-specific lytic activity in the presence of anti-H-2Kd MAb or anti-H-2Dd MAb. Effector:target ratio was 80:1.

Inhibited cytolysis of target cells pulsed with C7A10 peptide, whereas anti-CD4 MAb did not affect this effector cell function (Fig. 2). This result therefore indicated that effector cells expressed CD8 and used this molecule to lyse the target cells.

Effector cells from mice immunized with CP4–C7A10 showed MHC class I restriction

The lytic activity of C7A10-specific effector cells against MHC-matched or -mismatched target cells labelled with C7A10 was assessed. C7A10-specific effector cells lysed MHC-matched (H-2d), but not MHC-mismatched (H-2b), target cells, whereas anti-CD4 MAb did not affect this effector cell function (Fig. 2). This result therefore indicated that effector cells expressed CD8 and used this molecule to lyse the target cells.

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Immunization with CP4–C7A10 elicited effector cells that recognized processed HCV core protein

The capacity of immunization with CP4–C7A10 to elicit effector cells that recognized virus-infected cells was assessed by using target cells infected with HCV–rVV or control rVV. As shown in Fig. 4, mice immunized with CP4–C7A10, CP4 + C7A10 or C7A10 alone demonstrated more lysis of HCV–rVV-infected target cells than of control rVV-infected target cells. Thus, peptide immunization of mice generated an
effect T cell response capable of recognizing endogenously processed viral protein.

**HCV core-specific CTLs could be derived from spleen cells of CP4–CP7A10-immunized mice**

HCV core-specific effector cells were re-stimulated in vitro with C7A10 in IL-2-containing medium. The HCV core specificity and MHC class I restriction of T cells are shown in Fig. 5. These T cells specifically lysed HCV-rVV-infected, MHC class I-matched target cells (H-2d) but not HCV-rVV-infected, MHC class I-mismatched cells (H-2b). Cytolytic activity of these T cells was inhibited by anti-CD8 MAb. These results demonstrate that CD8+, MHC class I-restricted, HCV core-specific CTLs could be derived from spleen cells of CP4–CP7A10-immunized mice.

**HCV core-specific responses were apparent in mice immunized with peptides containing the Th epitope soon after inoculation with rVV**

Immunized mice were inoculated with HCV-rVV and examined for cytolytic activity against C7A10-labelled target cells (Fig. 6). Cytolytic activity increased at 3 days post-inoculation in mice immunized with peptides containing CP4, i.e. CP4–C7A10, CP4 + C7A10 and CP4 alone, but not in mice immunized with C7A10 alone. Mice immunized with C7A10...
alone did not show increased cytolytic activity until 7 days post-inoculation. Cytolytic activity was not detected in control mice until 7 days post-inoculation. These results suggest that rapid and high CTL activity might be developed by Th-cell activation after infection with virus.

**Spleen cells obtained from mice immunized with Th-containing peptides showed an HCV core-specific proliferative response**

The generation of virus-specific CTLs through peptide vaccination is important for the delivery of a helper signal, as rapid and high CTL responses were detected after infection with HCV–rVV in mice immunized with peptides containing CP4. Spleen cells from immunized mice were assessed for proliferative responses after stimulation in vitro with autologous spleen cells infected with HCV–rVV (Fig. 7). Spleen cells from mice immunized with peptides containing the CP4 epitope exhibited high proliferative responses after stimulation in vitro with rVV-infected autologous spleen cells. However, spleen cells from mice immunized with C7A10 alone did not show proliferative responses after incubation with spleen cells infected with HCV–rVV.

**Discussion**

CTLs are a critical component of protective immunity against virus infections. However, cell-mediated immunity is not elicited through immunization with virus subunits or killed virus under usual conditions (Ada, 1990). Generally, MHC presentation of peptide antigens only occurs for proteins that are actively synthesized and processed intracellularly (Morrisson et al., 1986), so the usual subunit-based approaches to vaccination do not elicit cell-mediated immune responses. Strategies for vaccine-mediated induction of virus-specific CTLs have therefore relied on the use of live, attenuated viruses or of recombinant live organisms as vectors for the expression of viral proteins (Ada, 1990). A successful antiviral peptide vaccine should be capable of eliciting Th and CTL responses, as well as neutralizing antibody responses, in multiple MHC types. Immunization with a single CTL target peptide does not usually elicit effective CTL responses. In the present study, we demonstrated antiviral effects elicited by a Th epitope-containing vaccine.

Although exogenous lymphokines can substitute for Th cells in the maturation of CTL precursors in vitro, the role of Th cells in priming CTLs in vivo remains poorly understood compared with their role in Th–B cell collaboration (Singer & Hodes, 1983). Much evidence for a helper requirement in CTL induction during virus infection has been reported (Wagner et al., 1976; Zinkernagel et al., 1978; von Boehmer & Haas, 1979; Kast et al., 1986; Husmann & Bevan, 1988). Moreover, the requirement for the linking of Th and CTL determinants to generate antiviral CTLs was reported in mice (Shirai et al., 1994a, 1994b). In the present study, CTL responses induced by a conjugated CTL–Th peptide, CP4–C7A10, were much higher than those induced either by a CTL epitope peptide, C7A10, alone or by a mixture of Th and CTL epitope peptides (Fig. 1). The greater induction of CTLs in vivo by conjugated peptides is likely to be because longer peptides are taken up by specialized class II-expressing antigen-presenting cells (APCs), and the same cell may present both the CTL and Th epitopes through class I and class II MHC, respectively (Shirai et al., 1994a). This mode of presentation may be more efficient than the situation in which the two epitopes are presented independently by different APCs. In fact, recent studies have reported that induction of effective CTLs occurs in a cognate manner, such that both Th cells and CTLs recognize antigen on the same APC through the CD40 ligand and CD40 molecule (Ridg et al., 1998; Bennett et al., 1998; Schoenberger et al., 1998).

In a previous report on the mouse/HCV model, a CTL response was observed following immunization with a peptide composed of the HCV core CTL epitope conjugated to the Th epitope of an unrelated virus, HIV (Shirai et al., 1996). This observation, that a CTL response is induced by an unrelated conjugated Th epitope, might suggest that peptide vaccines may be active against a wider range of viruses. Mice immunized with Th epitope peptide showed a rapid and strong CTL response after injection of HCV–rVV (Fig. 6). The ability of immunization with Th epitope alone to activate antiviral effector cells has been reported, and immunization with the Th epitope has been reported to prevent development of disease following activation of CD8+ cells (Miyazawa et al., 1995; Ossendorp et al., 1998). However, although immunization with conjugates of unrelated viral Th and CTL epitope peptides induced high levels of CTL activity, the antiviral effects induced by Th epitope immunization were not obtained using the Th epitope of an unrelated virus. Our results suggest that for effective Th epitope immunization, it is important to use the CTL epitope from the target virus.

Various epitopes recognized by HCV-specific, MHC class II-restricted, CD4+ T cells have been reported, and immune responses by these cells are critical determinants of disease resolution and control of infection (Diepolder et al., 1995; Missale et al., 1996). The connection between CD4+ T-cell responses and a self-limited course of acute HCV infection is mediated by NS3 (Diepolder et al., 1997). One immunodominant CD4+ T-cell epitope was presented by five major HLA-DR molecules. From these reports, it appears that these epitopes could be promising candidates for the development of a peptide vaccine against HCV infection.

The use of Th epitopes in vaccines has several potential advantages. Firstly, Th cells activated by vaccination or infection may secrete several CTL-inducing or antiviral cytokines. Proliferative responses of spleen cells against rVV carrying the HCV core gene were indeed observed in immunized mice (Fig. 7). Secondly, Th cells might play an important role not only in optimal induction of CTL responses but also in maintenance of CTL memory, as reported for other
virus infections (Matloubian et al., 1994; Cardin et al., 1996). Thirdly, Th peptides are usually more promiscuous in binding to MHC class II molecules than CTL epitopes are for MHC class I. It is therefore possible that Th-based vaccines may be less allele-dependent than vaccines based on CTL and thereby more widely applicable.

Recently, a novel way to elicit CTLs was described through activation of immature CD8+ T cells. The present study suggests that activation of CD4+ Th cells by vaccines might be a useful way to elicit antiviral effector cells, including CTLs. Such vaccines containing CD4+ Th epitopes may contribute towards protective immune responses against HCV.

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