

Detection of IgE Antibody Specific to a Hepatitis C Virus–Derived Peptide Being Recognized by Cellular Immunity in Patients with HCV Infection

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Abstract

The determination of immunogenic peptides of hepatitis C virus (HCV) is pivotal for vaccine development. We previously reported that the majority of patients infected with HCV have significant levels of IgG specific to an HCV-derived peptide at positions 35–44 of core protein (C35–44), a major epitope recognized by cellular immunity. This study addresses whether or not the other subclasses of immunoglobulins to this peptide exist. As a result, IgE, but not IgM or IgA, specific to this peptide is consistently detectable in the majority of patients with HCV infection, regardless of the different HLA types and disease conditions. These results provide additional information on this immunogenic peptide with new insights that contribute to a better understanding of host responses to HCV.

Introduction

ONE HUNDRED AND SEVENTY MILLION PEOPLE AROUND THE WORLD are persistently infected by hepatitis C virus (HCV) with its associated high risk of liver cirrhosis (4) or cancer. The determination of immunogenic peptides of HCV is pivotal for vaccine development, a long-awaited treatment modality. Thus, extensive studies were carried out in the past decade to find immunogenic HCV peptides capable of inducing immune responses (2,9,10,14,15–19). As a result, an HCV-derived peptide at positions 35–44 of core protein (C35–44) was determined to be highly immunogenic because of its ability to induce cytotoxic T lymphocytes in HLA-A2-positive HCV-positive patients (2,15,16). We recently reported that IgG response to this C35–44 peptide was observed in the majority of HCV-positive patients without apparent HLA class I restriction (16). There is strong evidence that both cellular and humoral immune responses play major roles in the control of HCV infection. Furthermore, the region around C35–44 was very stable in the sequence of virus DNA, with only rare mutations. Therefore, C35–44 peptide could be an appropriate molecule for specific immunotherapy toward HCV. Immune responses to HCV, however, are suggested to be

partly responsible for damage to hepatocytes and oral epithelial cells (13). These results indicate that detailed studies of immune responses are needed in order to develop a clinically effective HCV vaccine with minimal adverse effects. This study extends our previous studies and investigates the other classes of immunoglobulins that are detectable in these patients.

Materials and Methods

Subjects

Sera from 50 HCV-positive patients, who were positive for anti-HCV antibodies (Abs) as determined by a second- or third-generation immunoassay test, were used in this study. The diagnoses of these patients as determined by biochemical and histological findings, ultrasound-sonography, and computed tomography were chronic hepatitis (CH, *n* = 25), liver cirrhosis (LC, *n* = 12), and hepatocellular carcinoma (HCC, *n* = 13) at the first serum samplings. Sera from two patients with human immunodeficiency virus-1 (HIV-1) infection and 34 healthy donors (HDS) with no history of either viral hepatitis or vaccination for HBV and normal liver function were also used as negative controls.

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Peptide and measurement of Abs against peptides

The peptide C35–44, a synthetic peptide that corresponds to the aa sequence (residues 35–44) of the HCV core region, and an HIV-derived peptide with an HLA-A2 binding motif as a negative control were purchased from Bio-Synthesis (Lewisville, TX). Desalted grade materials were used for the screening of peptides reactive to serum IgG, while >90% purified materials were used for further studies as assessed by reverse-phase high-pressure liquid chromatography. Each peptide was dissolved in dimethylsulfoxide and then stored at -20°C .

Preparation of xMAP beads

The xMAP carboxylate beads and Luminex[®] system platform were obtained from Luminex Corp. (Austin, TX) as reported previously (6). The 96-well filter plates (MABVN12) and vacuum manifold apparatus (MAVM 09601) were from Millipore Corp. (Bedford, MA). Goat anti-human IgE was purchased from Biosource International (Camarillo, CA). Streptavidin-PE (S-866) was purchased from Molecular Probes (Eugene, OR). 1-Ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride (EDC 22980) was obtained from Pierce (Rockford, IL). Peptides were coupled to xMAP beads according to the modified manufacturer's instructions as reported previously. In brief, 100 μL of xMAP beads were washed with 0.1 M MES buffer, pH 7.0, followed by mixing with 100 μL of peptide (1 mg/mL in 0.1 M MES buffer, pH 7.0). The peptide-loaded beads were incubated with EDC (1 mg/mL) at room temperature for 30 min in darkness, and then incubated twice more under the same conditions, and the beads were washed with 0.05% Tween 20-PBS. Finally, the beads were treated with 2-aminoethanol for 15 min at room temperature in darkness, then washed twice and resuspended with 1 mL of 0.05% NaN_3 in Block-Ace.

Anti-peptide antibody measurement by flowmetry assay

Peptide-specific IgE levels in plasma were measured by flowmetry assay using the Luminex system as reported previously (6). In brief, plasma or serum samples were diluted to 1:50 with 0.05% Tween 20-Block-Ace, and 100 μL /well of sample was added to each well. Plasma was incubated with

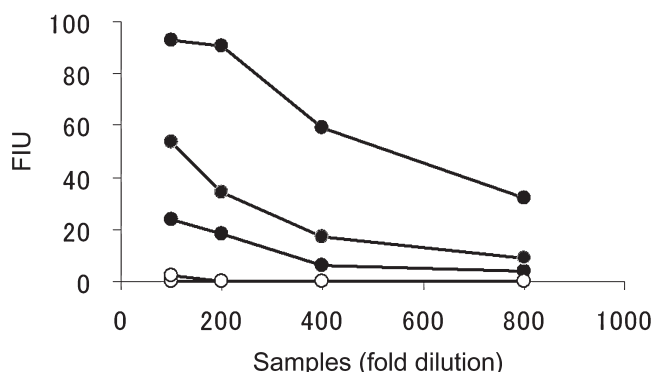


FIG. 1. Dose dependency. Anti-C35–44 peptide IgE antibody was measured by flowmetry analysis with Luminex at four different serum dilutions (100-, 200-, 400-, and 800-fold). Representative results of the dose-dependent curves are shown in this figure. The levels of IgE are shown in terms of FIU and the results are shown as closed circles (three patients) and as open circles (three HDs).

5 μL each of the peptide-coated beads for 2 h at 37°C in a 96-well filter plate on a plate shaker. After incubation, the plate was washed using a vacuum manifold apparatus and incubated with 100 μL of 1:100 diluted goat anti-human IgE for 1 h at 37°C on a plate shaker. The plate was then washed, 100 μL of 1:200 diluted streptavidin-PE was added to the wells, and the plate was incubated for 30 min at 37°C on a plate shaker. The bound beads were washed three times followed by the addition of 100 μL of Tween 20-PBS into each well, and the plate was placed for 3 min on a plate shaker.

The antibody absorption test

To prepare the peptide-immobilized ELISA plate for the antibody absorption test, peptides were diluted in 0.1 M carbonate buffer containing a chemical cross-linker, disuccinimidyl suberate (DSS) (Pierce), as reported previously (6). ELISA plates were coated overnight at 4°C with the target peptides (20 μg /well). The wells were rinsed three times with 0.05% Tween 20-PBS (PBST), and the plates were blocked overnight at 4°C with Block-Ace. To test the specificity of anti-peptide IgE in serum samples, 100 μL /well of serum samples (1:1000 dilution with 0.05% PBST) were absorbed with the immobilized peptide (20 μg /well) in wells kept for 2 h at room temperature. The absorption was repeated three times, and then the level of peptide-specific IgE in the resultant supernatant was measured by flowmetric analysis.

Statistical analysis

Data are presented as the median with 95% confidence intervals (CIs), and distributions were compared using non-parametric (Mann-Whitney U test) analysis. The results of the absorption test were analyzed by a two-tailed Student's *t*-test. All tests of significance were two-sided.

Results

First, we measured the levels of IgM, IgA, IgG, and IgE antibodies against C35–44 in sera from three patients infected

TABLE 1. THE LEVELS OF IgG, IgA, IgM, AND IgE ANTIBODIES AGAINST C35–44 PEPTIDE IN PATIENTS WITH HCV

Serum Ig classes	IgM	IgA	IgG	IgE
HD1	0	2	0	0
HD2	276	11	0	2
HD3	333	11	0	0
HCV1	1649	100	10280	613
HCV2	0	79	25525	466
HCV3	0	19	8481	60
HIV1	429	43	0	5
HIV2	291	26	0	6

The levels of IgG, IgA, IgM, and IgE antibodies against C35–44 peptide were assayed in sera (100-fold dilution) from HDs ($n = 3$) and patients with HCV ($n = 3$) and HIV ($n = 2$) by flowmetric analysis with Luminex. The representative results of net value of the levels of IgG, IgA, IgM, and IgE antibodies as indicated in units of mean fluorescent intensity units (FIU) are shown in the table.

with HCV by means of flowmetric analysis with Luminex. Sera from patients with HIV ($n = 2$) and HDs ($n = 3$) served as negative controls. As expected based on the previous results (16), the levels of IgG antibodies as indicated in mean fluorescent intensity units (FIU) were very high (19,280, 25,525, and 8481 FIU) in all three HCV-infected patients. The levels of IgE antibodies were also high (613, 466, and 60 FIU) in all three HCV-infected patients (Table 1). The higher titer of IgG compared to that of IgE was expected since the magnitude of serum IgG antibodies is genetically 10^6 times higher than that of IgE. In contrast to the sera of HCV patients, none of the sera from the negative controls showed such IgG or IgE responses to this C35–44 peptide (Table 1), suggesting that the response is specific. In contrast to IgE, low levels of IgM responses to C35–44 peptide were observed in certain samples from HDs, and those infected with HCV and HIV, suggesting that the IgM response is non-specific. A similar non-specific trend was observed in the IgA responses. Dose-dependent reactions of the IgE responses in three different patients with different levels of antibodies (Table 1) are shown in Fig. 1. The specificity of anti-C35–44 IgE antibodies was confirmed with an absorption experiment. Peptide-specific IgE was absorbed with the addition of C35–44, but not with an irrelevant control peptide (NS5A2132); representative results are shown in Fig. 2. These results suggest that the IgE class of anti-C35–44 antibody, but neither the IgM nor IgA class, is detectable in sera from patients with active HCV infection.

Next, we extended the study and measured the levels of IgE reactive to C35–44 peptide in sera from 50 HCV-positive patients and 34 HDs with no history of either viral hepatitis or HBV vaccination and normal liver function. The level of anti-C35–44 IgE antibody in HCV patients was significantly ($p < 0.001$; Mann-Whitney analysis) higher than that of HDs (Fig. 3). The results indicated that anti-C35–44 IgE antibody was specifically detected in HCV-infected patients. When the cut-off value for the FIU of sera from HDs taken as a nega-

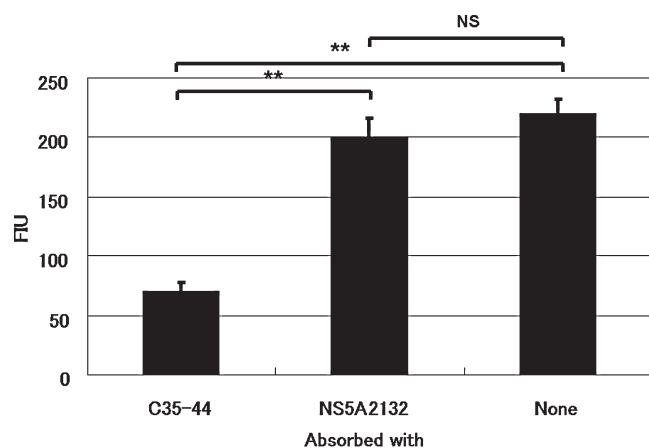


FIG. 2. Absorption test. The IgE antibody activity to C35–44 peptide was absorbed three times with an immobilized corresponding peptide (C35–44) and an irrelevant control peptide (NS5A2132). Serum at 100-fold dilution was used for this experiment. Values are given as the means $\pm$ SD of FIU. A two-tailed Student's *t*-test was employed for statistical analyses. ** $p < 0.001$. NS, not significant.

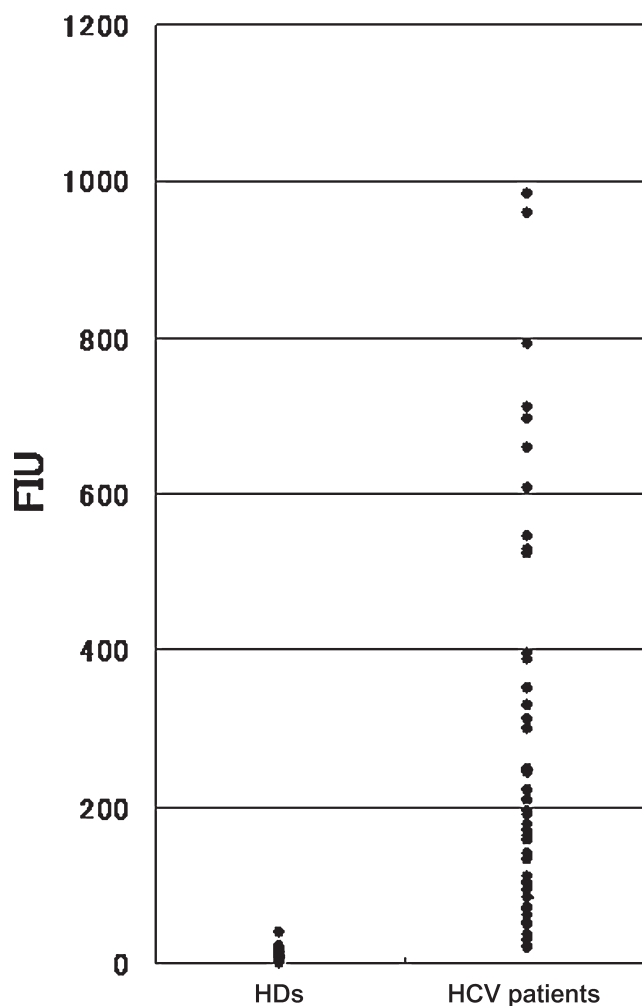


FIG. 3. Screening of patient sera. The levels of anti-C35–44 IgE antibody of the 50 HCV-infected patients were compared to those of the 34 HDs by means of flowmetry analysis with Luminex. The patient sera showed significantly higher levels of IgE ($p < 0.001$; Mann-Whitney U-test) than the means of the FIU scores from the sera of the HDs (100-fold dilution).

tive control was calculated as 22 (the mean $\pm$ 2 SD), there was one false-positive case among 34 HDs (specificity 98%), while there were two false-negative cases among 50 HCV-infected patients (sensitivity 96%).

We then studied sera from subjects ($n = 4$) with a history of HCV infection with no detectable levels of HCV-RNA (subjects with HCV serological conversion). IgG anti-C35–44 antibodies could be detected in all four (100, 142,000, 6200, and 10,000 FIU), and IgE anti-C35–44 antibodies could be detected in three (400, 190, and 200 FIU) of four HCV-RNA-negative patients. These results suggest that both the IgG and IgE classes of anti-C35–44 peptide are detectable in sera not only from patients with active HCV infection, but also in subjects with a history of HCV infection. Neither IgM nor IgA was detectable in those subjects ($n = 4$) with a history of HCV infection.

Finally, we addressed whether the levels of anti-C35–44 antibody were different among the different stages of HCV

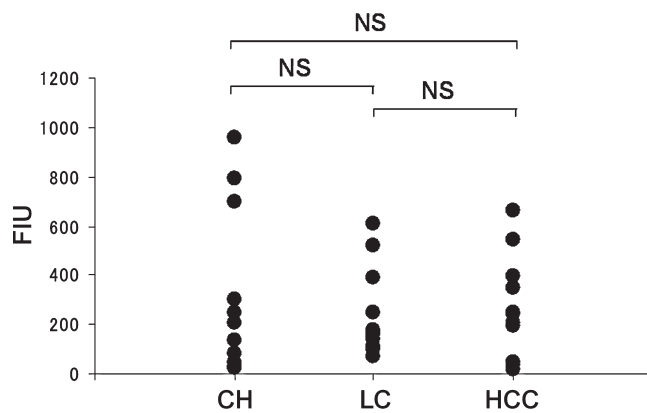


FIG. 4. The level of IgE among those with different stages of disease. Sera at 100-fold dilution from 11 patients of each group (chronic hepatitis, CH; liver cirrhosis, LC; hepatocellular carcinoma, HCC) were assayed, and estimation was done using the Mann-Whitney U-test. There were no significant differences (NS) in the levels of IgE antibody against C35–44 among those patients with different stages of liver disease with HCV infection.

infection. Namely, IgE levels were measured in sera from 11 patients of each group (CH, LC, and HCC). However, there was no statistical difference among these patients with different stages of liver disease with HCV infection (Fig. 4).

Discussion

This study extended the previous studies and reported that significant levels of IgE specific to the C35–44 peptide, but not IgM or IgA, were detectable in the majority of HCV-infected individuals without HLA-A2 restriction. It has been reported that HIV infection seemed to be associated with a marked decrease in the Th-1 cytokines IL-12 and IFN- γ , along with a marked increase of the Th-2 cytokines IL-4 and IL-5 (1,5,7,8,11,18). Th-2 cytokines promote IgE production (5), suggesting that an increase in IgE level in sera of HCV-infected patients is a risk factor for progression of the disease, such as to LC or HCC. Thus, in order to study the possible involvement of IgE antibody against C35–44 peptide in disease progression, the correlation of the level of IgE to C35–44 peptide and the stages of liver disease was analyzed. However, there was no difference in anti-C35–44 IgE antibody among the CH, LC, and HCC groups, suggesting that the level of anti-C35–44 IgE antibody was not a risk factor for disease progression.

HCV infection is a serious health problem, with 170 million people worldwide persistently infected with HCV and at high risk of LC and HCC at later stages. The presently available diagnostic and treatment modalities, such as third-generation enzyme immunoassay/immunoradiometric assays and interferon, are too expensive for patients in underdeveloped countries. Therefore the development of new, less expensive diagnostic or treatment modalities is needed. One way to achieve these goals would be to determine highly immunodominant HCV peptides recognized by both cellular and humoral immunity, and utilize them for such development. We reported that IgG and IgE specific to C35–44 pep-

tide were detectable in sera of at least 90% of patients with HCV infection, regardless of differing HLA types or differing stages of disease (16). Therefore, this C35–44 peptide, as well as proteins containing this sequence, could be a novel target for development of new diagnostic and therapeutic tools.

Of particular advantage to laboratories in developing countries, the IgE test can be rapidly performed without complex laboratory equipment, and can be run at room temperature, and the same technology may be used for both children and adults (12). Furthermore, IgE is one of the antibodies that does not cross the placenta, making it potentially valuable for viral detection in fetuses or neonates. In addition, the IgE response is rapid and reaches a peak earlier than the IgG antibody response (3), suggesting that an IgE-based assay may detect seroconversion earlier than conventional methods (3).

This C35–44 peptide, as well as proteins containing this sequence, could also be a novel target for development of specific immunotherapy for HCV infection to prevent disease progression, primarily because of higher immunogenicity. The translational clinical study of C35–44 peptide vaccination for HCV-positive patients who failed standard therapies is currently in progress at Kurume University Hospital.

The biological roles of anti-C35–44 IgE are unclear at the present time. We previously reported that the administration of CTL-epitope peptides to which IgE was detectable might not elicit a systemic reaction in animal models (19). Indeed, its subcutaneous administration did not elicit it in the translational clinical study (data not shown). Further studies are needed to elucidate the biological roles of anti-C35–44 IgE.

Conclusion

In conclusion, we can report the presence of anti-C35–44 peptide IgE antibody in HCV-infected patients. This information may provide better understanding of the host response against HCV.

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