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Abstract

We detected an antibody to HCV envelope protein (E1) in sera of patients with HCV-related chronic liver diseases (20 patients with chronic hepatitis and 5 patients with liver cirrhosis) by Western blotting using the fusion protein of E1 envelope protein and beta-galactosidase as an antigen. The antibody to HCV E1 (anti-HCV E1) was detected in 8 (42%) of 19 patients positive for HCV-RNA (16 were positive and 3 were negative for antibody to C100-3) and in 1 (17%) of 6 patients negative for HCV-RNA but positive for antibody to C100-3. HCV-RNA was detected in 8 (89%) of 9 anti-HCV E1 positive sera. The value of alanine aminotransferase was significantly higher in patients positive for anti-HCV E1 than in patients negative for the antibody. Although an antibody to the envelope protein of HCV is suspected to be one of the candidates of virus-neutralizing antibodies, our results suggest this hypothesis appears to be unlikely.

KEYWORDS: hepatitis C virus, envelope, antibody, Western blotting

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Detection by Western Blotting of an Antibody to the Hepatitis C Virus E1 Envelope Protein in Sera of Patients with Chronic Liver Disease

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We detected an antibody to HCV envelope protein (E1) in sera of patients with HCV-related chronic liver diseases (20 patients with chronic hepatitis and 5 patients with liver cirrhosis) by Western blotting using the fusion protein of E1 envelope protein and beta-galactosidase as an antigen. The antibody to HCV E1 (anti-HCV E1) was detected in 8 (42%) of 19 patients positive for HCV-RNA (16 were positive and 3 were negative for antibody to C100-3) and in 1 (17%) of 6 patients negative for HCV-RNA but positive for antibody to C100-3. HCV-RNA was detected in 8 (89%) of 9 anti-HCV E1 positive sera. The value of alanine aminotransferase was significantly higher in patients positive for anti-HCV E1 than in patients negative for the antibody. Although an antibody to the envelope protein of HCV is suspected to be one of the candidates of virus-neutralizing antibodies, our results suggest this hypothesis appears to be unlikely.

Key words : hepatitis C virus, envelope, antibody, Western blotting

Hepatitis C virus (HCV) is reported to relate to most cases of post-transfusion non-A, non-B (NANB) hepatitis throughout the world (1-3). The entire nucleotide sequence of the HCV genome, which is a positive single-stranded RNA of 9.4kb, has been revealed, and its genome structure, which contains a single open reading frame encoding a large polypeptide of 3011 amino acids, is similar to that of flaviviruses or pestiviruses (3, 4). The large polypeptide is suggested to be cleaved to core, E1, E2/NS1, NS2, NS3, NS4, and NS5 proteins by its own or host cellular protease (2).

HCV infection can be diagnosed by detecting either antibodies to viral proteins, such as core, NS3 and NS4, or HCV-RNA through amplification by polymerase chain reaction (PCR)(2). However, the antibodies so far reported can not be a virus-neutralizing antibody, because those antibodies mostly coexist with viremia. By analogy to flaviviruses or pestiviruses, it is thought that the antibody to an envelope protein of HCV may have an ability to neutralize the virus (2). Recently, Matsuura *et al.* has reported the detection of antibody against HCV envelope protein in 2-17% of NANB hepatitis patients (5). They speculate in their paper that the reason for the low prevalence of the antibody is that the

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immunogenicity of the HCV envelope protein is low because of its hydrophobicity (5).

In this paper, we report the detection by Western blotting of an antibody to the envelope protein region (E1) of HCV in HCV-related chronic liver diseases, and investigate the relationship between the antibody and HCV-RNA or alanine aminotransferase (ALT) level which reflects the extent of liver damage.

Materials and Methods

Serum samples. Sera were collected from 29

Table 1 Serum hepatitis C virus (HCV) markers, antibody to HCV E1, and alanine aminotransferase (ALT) value in patients with chronic liver diseases

No.	Age, Sex	Clinical diagnosis	Anti-C100-3	HCV-RNA	Anti-HCV E1	ALT (IU/L)
Group I						
1	60, F	CH*	+	+	+	114
2	56, M	CH*	+	+	+	85
3	51, F	CH*	+	+	+	117
4	49, M	CH*	+	+	+	119
5	56, M	CH	+	+	+	65
6	62, M	CH	+	+	+	59
7	60, F	LC*	+	+	+	122
8	69, M	CH*	+	+	−	18
9	55, M	CH*	+	+	−	37
10	47, F	CH	+	+	−	24
11	71, F	CH	+	+	−	35
12	57, F	CH	+	+	−	26
13	54, M	CH	+	+	−	60
14	54, F	CH	+	+	−	20
15	51, F	LC	+	+	−	71
16	53, M	LC	+	+	−	64
Group II						
17	70, F	LC	−	+	+	94
18	79, M	CH	−	+	−	16
19	62, M	LC	−	+	−	92
Group III						
20	48, M	CH	+	−	+	18
21	63, F	CH*	+	−	−	17
22	64, M	CH*	+	−	−	26
23	59, M	CH*	+	−	−	118
24	63, F	CH*	+	−	−	69
25	38, M	CH*	+	−	−	23
Control						
26	21, F	Other	−	−	−	16
27	33, F	Other	−	−	−	216
28	18, M	Other	−	−	−	154
29	52, F	Other	−	−	−	11

*: histologically confirmed. HC: chronic hepafitis; LC: liver cirrhosis.

patients consisting of 16 (13 with chronic hepatitis (CH) and 3 with liver cirrhosis (LC)) positive for both antibody to C100-3 (anti-C100-3) (1) and HCV-RNA (Group I), 3 (1 with CH and 2 with LC) negative for anti-C100-3 but positive for HCV-RNA (Group II), 6 with CH positive for anti-C100-3 but negative for HCV-RNA (Group III), and 4 with other liver diseases (post-transfusion hepatitis, drug-induced hepatitis, fatty liver, and autoimmune hepatitis) negative for both anti-C100-3 and HCV-RNA (Control group) (Table 1). Serum ALT was determined using the same serum samples as those used for detection of anti-C100-3, anti-HCV E1 and HCV-RNA.

Preparation of antigen. Antigen for Western blotting was prepared as follows. A subgenomic fragment (E612) (Fig. 1), which spans nucleotide positions 787–1398 (6), was obtained by PCR (7). E612 was then cloned in frame into the EcoRI site of pEX2 expression plasmid in which the inserted fragment was induced at 42°C as a fusion protein with beta-galactosidase (8). According to the procedures by Stanley and Luzio (8), fifty microliters of competent TB1 cells were transformed with pEX2 containing E612 cDNA fragment, and a clone expressing a fusion protein with the expected size was selected. A hundred milliliters of LB media were inoculated with 1 ml of overnight bacterial culture, and were incubated at 30°C for 3h followed by incubation at 42°C for 2h in order to induce the fusion protein. The fusion protein containing an HCV protein (aa155-aa358, where aa indicates amino acid position) was partially purified by extraction with 8M urea, and showed predicted size (136kd) on 5% SDS-PAGE.

Western blotting. Beta-galactosidase without HCV protein (114kD) prepared in the same way was electrophoresed on the same gel served as a negative control (Fig. 2). After electrotransfer to a nitrocellulose membrane at 20V overnight, the membrane was incubated with 10 folds diluted patients'sera with PBS as the first antibody in the Screener blotter™ (Samplatec, Ltd., Tokyo, Japan) at 4°C overnight and then with horseradish peroxidase (HRPO)-labeled antibody to human IgG as the second antibody at room temperature for 1h. Each serum sample was tested at least twice.

Detection of anti-C100-3 and HCV-RNA Anti-C100-3 was detected using a commercially-available kit (Ortho Diagnostics, Co., NJ, USA), and HCV-RNA was detected by the two-stage PCR employing primers in the 5' non-coding region according to the procedures by Okamoto *et al.* (9).

Statistical analysis. Statistical analysis was done using the Student's *t*-test. These experiments were performed under permission from The Committee for Recombinant DNA Technology and Safety, Okayama University (Nos. 9041 and 9052).

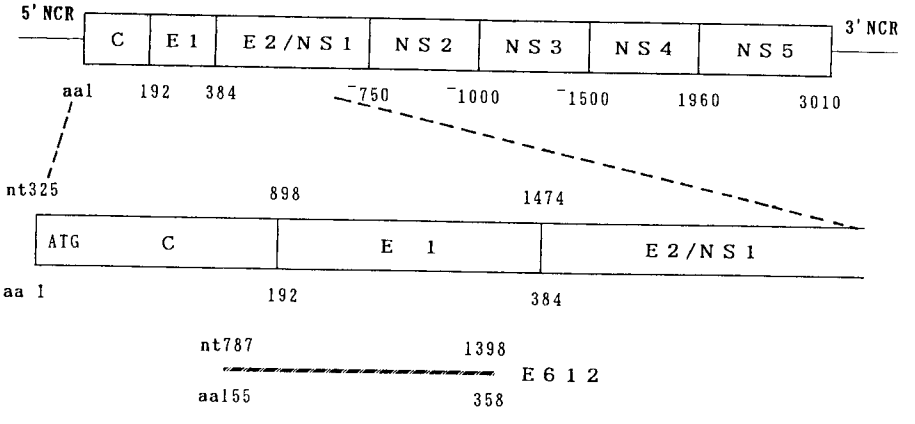

Fig. 1 Schematic illustration of the genome structure of HCV and location of E612. cDNA fragment corresponding to E1 region, E612, was expressed as a fusion protein with beta-galactosidase. Upper and lower halves indicate the whole genome structure and the structural protein region of HCV, respectively. Boxes indicate open reading frames. NCR: non-coding regin; C: core protein; E1 and E2: amino and carboxyl terminal portions of envelope protein, respectively. NS1 5: non-structural proteins 1–5; aa: amino acid position starting from the common ATG codon; nt: nucleotide position (7).

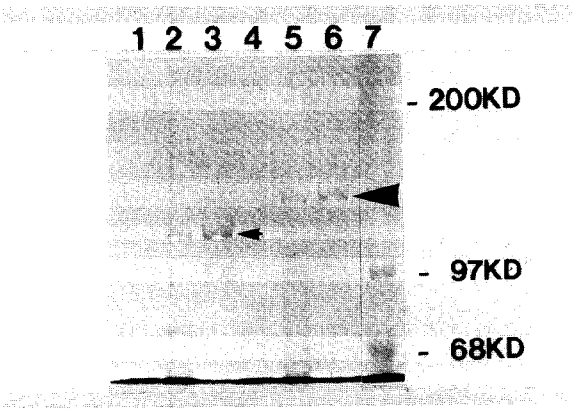

Fig. 2 SDS-PAGE of total bacterial proteins and partially purified fusion proteins. Total bacterial proteins and partially purified fusion proteins were electrophoresed on 5% SDS polyacrylamide gel and stained with Coomasie-Brilliant Blue. Lanes 1 and 4: total bacterial proteins without induction. Lanes 2 and 5: total bacterial proteins induced at 42°C for 2h. Lanes 3 and 6: fusion proteins partially purified by extraction with 8M urea. Lanes 1-3: from bacterial cells transformed with pEX2 plasmid without insert. Lanes 4-6: from bacterial cells transformed with pEX2 plasmid containing the insert, E612, in frame. Lane 7: size marker. Small arrowhead indicates beta-galactosidase. Large arrowhead indicates the fusion protein.

Results

Serum samples which showed specific binding only to the 136kD band were considered positive for the antibody to HCV E1 (anti-HCV E1) (Fig. 3).

Serum samples from 7 (44%) patients in Group I, one (33%) patient in Group II, one (17%) patient in Group III, and none in the Control group were positive for anti-HCV E1. HCV-RNA was also detected in 8 (89%) of these 9 positive samples. Seven (35%) of 20 CH patients and 2 (40%) of 5 LC patients were positive for anti-HCV E1. These results are summarized in Table 1.

In patients with HCV-related chronic liver diseases (Groups I, II and III), ALT value was significantly higher in patients positive for anti-HCV E1 ($n = 9$, 88 ± 35 IU/L) than in patients negative for the antibody ($n = 16$, 45 ± 31 IU/L) ($p < 0.01$) (Fig. 4).

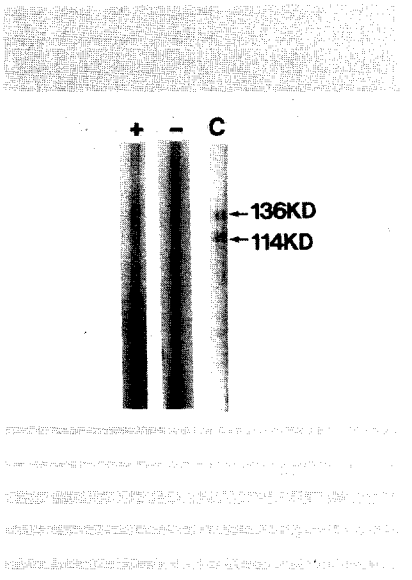

Fig. 3 Detection of antibody to HCV E1 by Western blotting. "+" and "−" on top of the figure indicate reprsentative positive and negative samples, respectively. Both bands on Lane C, the 136kD fusion protein and the 114kD beta-galactosidase, were visualized using a polyclonal rabbit antibody to beta-galactosidase and an HRPO-labeled antibody to rabbit IgG as the first and the second antibodies, respectively.

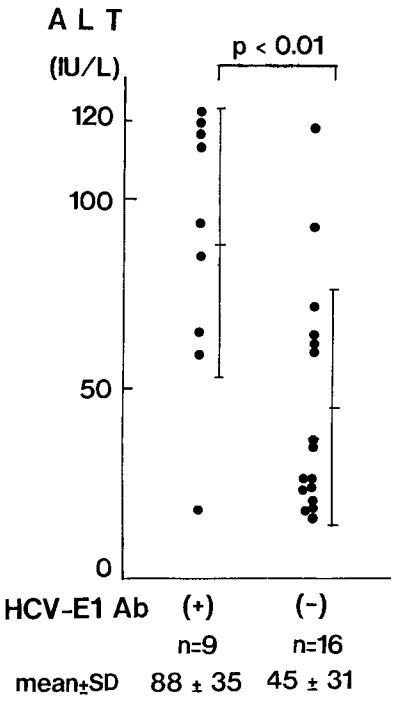

Fig. 4 Relationship between alanine aminotransferase (ALT) level and anti-HCV E1. ALT levels in patients positive for anti-HCV E1 were compared with those in patients negative for the antibody. The former was significantly higher than the latter ($p < 0.01$ by Student's t-test). Vertical bars indicate mean ± SD (standard deviation). Patients in Control were excluded from this analysis.

Discussion

The HCV E1 envelope protein spans aa192–aa383 (2), while E612 obtained by us is at aa 155–aa358 (amino acid numbers start from the same ATG codon) and contains a carboxyl terminus of core protein. However, it is reported that the carboxyl terminus of the core protein region was mainly hydrophobic, and that a synthetic peptide (C4) which corresponded to the only hydrophilic region (aa146–aa158) in the region of aa121–aa191 elicited an antibody response at a very low rate (2.4 %) in patients with NANB-CH (10). Therefore, the antibody we detected was presumed to be principally elicited by the HCV E1 envelope protein.

Very low positive rate (3/40 or 2–17 %) for the antibody to HCV envelope protein in patients with NANB chronic liver diseases was reported using a method similar to ours (11) or using a protein expressed in insect cells (5), respectively. In contrast, an antibody to HCV envelope was detected in 41 % of chronic NANB hepatitis patients using ELISA to synthetic peptides (12). It is interesting to note that in two of the studies a recombinant protein was used as an antigen (5, 11), while in the last report a synthetic peptide was used as an antigen. The reason why our results showed high positive rate in spite of using a recombinant protein is unknown.

In our study, there seemed to be no clear difference in the positive rate for anti-HCV E1 between in CH patients and in LC patients. This result might suggest that the existence of the antibody is not related to the progression of HCV infection.

Matsuura *et al.* reported three patients cured after acquiring the antienvelope antibody, but they also observed that the antibody sometimes coexisted with HCV-RNA (5). Therefore, they suggested that antienvelope antibody is neutralizing only in limited number of patients (5). Our findings that HCV-RNA was detected in most cases positive for anti-HCV E1 and that ALT level was significantly higher in patients positive for anti-HCV E1 suggested that the anti-HCV E1 lacks virus-neutralizing activity.

However, the possibility that an antibody to the remaining part of E1 (aa359–aa383) or to E2/NS1 (aa384–aa750) (2) might possess an activity to neutralize the virus still remains. The possibility also can not be denied that the antigen we used for the detection of the antibody differed from the original HCV E1 protein, because it was obtained from bacteria as a fusion protein. The meaning of the existence of anti-HCV E1 in HCV infection must be clarified.

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