neutralizing epitopes of HCV infection. The sequence variation in HVR-1 may instead indicate the existence of various clones in acute phase infection and the adaption of these clones is thought to have caused persistent and chronic infection in each patient.

Introduction

Hepatitis C virus (HCV) possesses a genome of single-strand RNA with positive polarity (about 9.6 kb), and is classified in the family *Flaviviridae*, genus *Hepacivirus* [24]. HCV is the major causative agent of post-transfusion-associated non-A, non-B hepatitis, and it an estimated 170 million people are infected worldwide. Persistent HCV infections often progress to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [3, 25]. Previous reports have suggested that variability of the HCV genome is likely to play crucial roles in facilitating escape from host immune surveillance [9, 12, 29]. In particular, high degrees of sequence variability have been observed in regions coding the E2 envelope protein, designated as hypervariable regions (HVR)-1 [13, 28] and 2 [22]. HVR-1 has been suggested as a dominant neutralizing epitope for HCV infection in chimpanzees [6]. Despite the confirmed presence of HCV-specific antibodies and cytotoxic T lymphocytes [1, 7], HCV causes frequently persistent infection. These results suggest that variation occurring in neutralizing epitopes within HVR-1 could produce escape variants able to elude the host immune system. Recent reports have indicated that the evolution of viral quasispecies may predict clinical course in viral hepatitis [8].

Although HCV preferably infects hepatocytes, as confirmed by the existence of negative-strand RNA [15], the mechanisms of adsorption into hepatocytes and transcription and replication of viral RNA in the cell remain unclear. The possibility of low-density lipoprotein (LDL)-receptor has been suggested as a virus receptor for HCV infection [2, 18]. CD81 belongs to a family of molecules called tetraspanins, characterized by four transmenbrane domains forming two extracellular loops [17], and interacts with E2 protein as a putative viral receptor [23]. So far, six hepatocyte-binding regions have been defined in the E1/E2 region using synthetic peptides [11]. Inhibition of natural killer cells through engagement of CD81 by E2 protein has been reported [5]. Moreover, no polymorphisms in CD81 amino acid (a.a.) sequences on peripheral blood mononuclear cells (PBMCs) have been observed between healthy volunteers and patients during HCV infection [10].

The mechanisms of adaptation and selection allowing HCV to establish chronic infection during the first phase of acute infection remain unclear. The present study characterized patient-specific conserved original nucleotide sequences of the E1 and E2 regions, and deduced amino acid (a.a.) substitutions during the course of HCV infection for acute and chronic phase using direct DNA sequencing methods and humoral immunity of patients to HVR-1 peptides during the course of chronic HCV infection.

Materials and methods

Patients and sera

Two patients displaying acute infection with hepatitis C virus by transfusion (patients A and B; Table 1, Fig. 1) were selected retrospectively, along with three randomly selected patients with chronic hepatitis C in which high levels of serum alanine aminotransferase (ALT) were maintained for more than six months after first medical examination (patients C–E; Table 1, Fig. 1). All serum samples were utilized to determine nucleotide sequences of the HCV E1 and E2 regions during disease progression, and deduced a.a. sequences were predicted. These selected sera were aliquoted and stored below −80 °C until characterization. Two patients were infected with HCV by transfusion: patient A (a 58-year-old woman) when she donated a kidney; and patient B (a 55-year-old man) during hip joint surgery. Patients A and B were followed up for 11 and 13 years, respectively. In patients A and B, serum ALT levels remained abnormal during the entire follow-up period. Patients C (54-year-old man), D (26-year-old man), and E (67-year-old man) displayed histological evidence of chronic active hepatitis C.

Informed consent was obtained from all patients in accordance with the Helsinki Declaration.

Detection of anti HCV antibody and HCV RNA

Second-generation enzyme-linked immunosorbent assay (Ortho Diagnostic Systems, Raritan, NJ) was used to detect HCV antibody in sera from the five patients during disease progression. Serum HCV RNA was extracted using the acid guanidium thiocyanate-phenol-chloroform (AGPC) method [4], and detected by reverse transcription and nested polymerase chain reaction (PCR) using primers for the 5-noncoding region of the HCV genome [19]. Results

Table 1. Clinical evaluation of patients and time points of characterization. Randomly selected patients with hepatitis C were analyzed

Patients	HCV genotype	Age (years)	Sex	Points[a]	Duration[b] (months)
(Acute)					
A	1b	58	F	1 to 2	3
				2 to 3	7
B	1b	55	M	1 to 2	4
				2 to 3	8
(CH)					
C	1b	54	M	1 to 2	12
				2 to 3	3
D	1b + 2a	26	M	1 to 2	8
				2 to 3	7
E	2a	67	M	1 to 2	10
				2 to 3	11

[a]Points, points of analysis

[b]Duration, duration between points of analysis

Acute: acute infection with hepatitis C virus by transfusion, with ALT and viral RNA levels rapidly decreased immediately after infection, then subsequently increased

CH: chronic hepatitis patient, with high levels of ALT maintained for more than six months after first medical examination

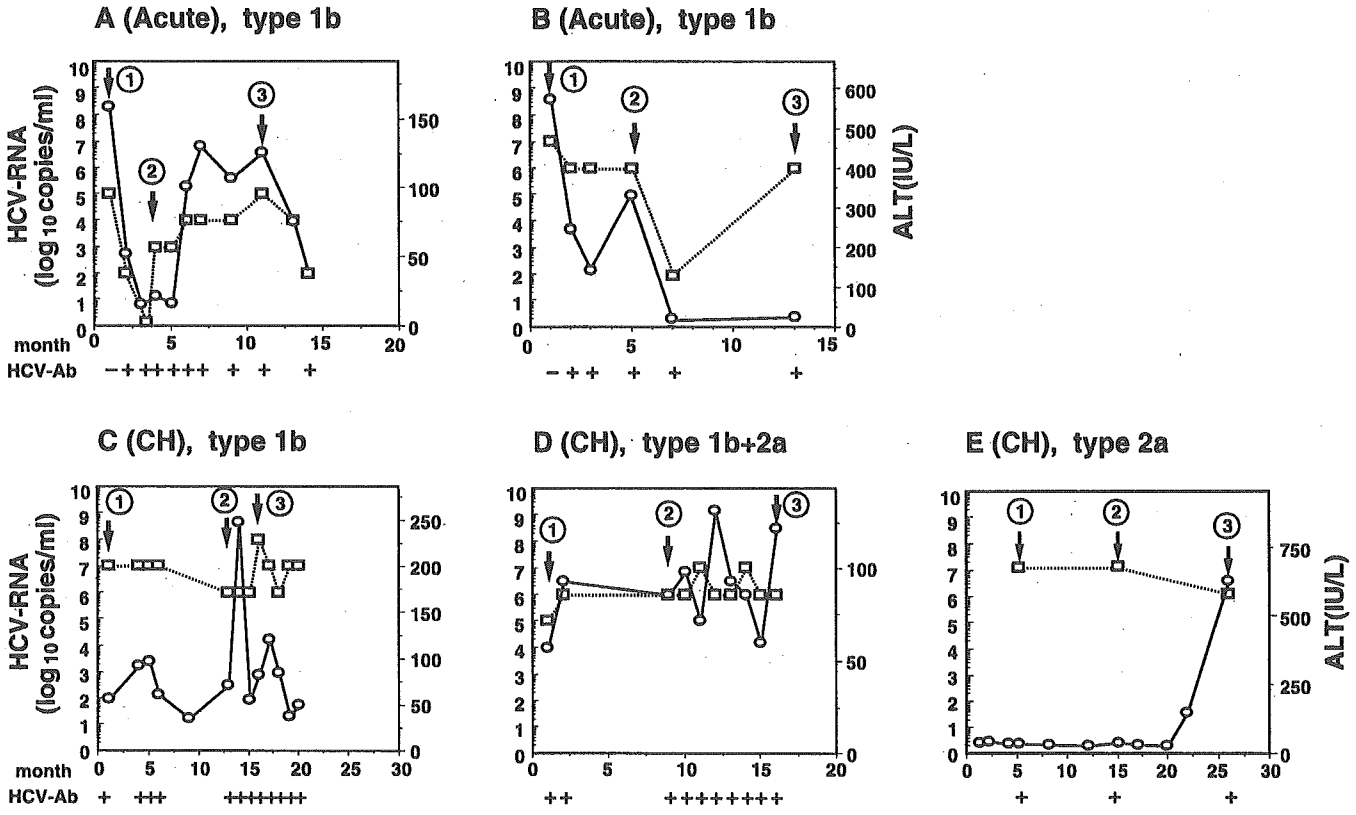

Fig. 1. Clinical course of hepatitis C patients. Changes in HCV RNA titer (broken line with open square), ALT level (black line with open circle) and HCV antibodies (− or +) for each patient. Numbers *1–3* in open circles indicate DNA sequencing points. In acute patients **A** and **B**, time point 1 represents onset by transfusion. In chronic patients **C** and **D**, time point 1 represents first medical examination. In patient **E**, time point 1 represents five months after first medical examination. *Acute*: acute HCV infection; *CH*: chronic HCV infection

were quantified using competitive PCR assay [30] in which cloned DNA (with a 15-bp deletion in the middle portion) used as a competitor [29].

Nucleotide sequence analysis in E1/E2 region

Nucleotide sequences for the E1/E2 region were analyzed according to direct DNA sequencing methods for PCR products using λ exonuclease (Gibco-BRL, Rockville, MD). HCV genome was extracted using AGPC methods [4], then amplified by reverse transcriptase and nested-PCR from serum for overlapping regions using two sets of primer pairs. PCR primers and amplified regions utilized for each patient are listed in Table 2. The second PCR product which was amplified by 5′-phosphorylated primer. PCR products were purified by 3% Nusieve 3:1 agarose gel electrophoresis (FMC BioProducts, Rockland, ME). One to four units of λ exonuclease was added to purified PCR products, including 67 mM glycine-KOH (pH 9.4) and 2.5 mM $MgCl_2$, and incubated at 37 °C for 1 h to form single-stranded DNA. Reaction mixtures were precipitated with ethanol and dried for DNA sequencing. Aliquoted DNA fragments were used for identification of nucleotide sequences in the E1/E2 region [27]. Nucleotide sequences of the E1/E2 region were determined for all five patients during disease progression. Characterization of nucleotide sequences and phylogenetic analyses of HVR-1 were performed using GENETYX version 10 software (Software Development, Tokyo, Japan). The phylogenetic tree for HVR-1 was constructed using the neighbor-joining (NJ) method [26].

Table 2. List of primer sequences for PCR of the HCV genome

Patients (Point*) [nt no.**]	Primer name	sequence
A (1, 3)	1st sense; (a)	5′-TGGGCAGGATGGCTCCTGTCN-3′
[618–1265]	1st anti-sense; (b)	5′-TAGATTGAGCAATTGCAATCTTGN-3′
	2nd sense; (c)	5′-CCGGTTGCTCTTTCTCTATCTTN-3′
[848–1265]	2nd anti-sense; (b)	5′-TAGATTGAGCAATTGCAATCTTGN-3′
A (2), B	1st sense; (a)	5′-TGGGCAGGATGGCTCCTGTCN-3′
[618–1385]	1st anti-sense; (d)	5′-GCCACCATGTCCACGACAGCTTGGTGG-3′
	2nd sense; (e)	5′-TGGTAAGGTCATCGATACCCTCACN-3′
[697–1365]	2nd anti-sense; (f)	5′-TTGTGGGATCCGGAGTAACTGCGACAC-3′
A, C	1st sense; (a)	5′-TGGGCAGGATGGCTCCTGTCN-3′
[618–1385]	1st anti-sense; (d)	5′-GCCACCATGTCCACGACAGCTTGGTGG-3′
	2nd sense; (c)	5′-CCGGTTGCTCTTTCTCTATCTTN-3′
[848–1365]	2nd anti-sense; (f)	5′-TTGTGGGATCCGGAGTAACTGCGACAC-3′
D-2a, E	1st sense; (a)	5′-TGGGCAGGATGGCTCCTGTCN-3′
[618–1387]	1st anti-sense; (p)	5′-CTAATGATGTCTATGATGACCTCGGGAACG-3′
	2nd sense; (c)	5′-CCGGTTGCTCTTTCTCTATCTTN-3′
[848–1357]	2nd anti-sense; (q)	5′-CGCATCACGTACGCCAGAATCATGG-3′
D-1b	1st sense; (h)	5′-ATGGCTTGGGATATGATGATGAACTGGTC-3′
[1290–1867]	1st anti-sense; (i)	5′-TGAAACAATACACTGGACCACACAC-3′
	2nd sense; (j)	5′-ATTCCATGGTGGGGAACTGGGCTAA-3′
[1424–1813]	2nd anti-sense; (k)	5′-TAGGTGCGTAGTGCCAGCAATAAGG-3′
B	1st sense; (l)	5′-CAAGATTGCAATTGCTCAATCTAN-3′
[1243–1887]	1st anti-sense; (m)	5′-ACTACAACAGGGCTCGGAGTGAAN-3′
	2nd sense; (n)	5′-ATGGCTTGGGATATGATGATGAACTGGTCN-3′
[1291–1867]	2nd anti-sense; (o)	5′-TGAAGCAATACACTGGACCACACACN-3′
D-2a	1st sense; (l)	5′-CAAGATTGCAATTGCTCAATCTAN-3′
[1243–1887]	1st anti-sense; (m)	5′-ACTACAACAGGGCTCGGAGTGAAN-3′
	2nd sense; (l)	5′-CAAGATTGCAATTGCTCAATCTAN-3′
[1243–1867]	2nd anti-sense; (o)	5′-TGAAGCAATACACTGGACCACACACN-3′
A, C, E	1st sense; (l)	5′-CAAGATTGCAATTGCTCAATCTAN-3′
[1243–1867]	1st anti-sense; (i)	5′-TGAAACAATACACTGGACCACACAC-3′
	2nd sense; (l)	5′-CAAGATTGCAATTGCTCAATCTAN-3′
[1243–1813]	2nd anti-sense; (k)	5′-TAGGTGCGTAGTGCCAGCAATAAGG-3′

*Point, point of analysis; **nt no., nucleotide number on HC-R6, accession no. AY045702

Protein structure and amino acid substitution speed analyses in E1/E2 region

The a.a. sequence of the E1/E2 region was deduced from corresponding nucleotide sequences for all five patients. Protein structural analyses (hydrophobic profile, antigenic index and surface probability) were performed using MacVector sequence analysis software (International Biotechnologies, New Haven, CT). Protein secondary structure (Chou-Fas) was determined using GENETYX version 10 software (Software Development). Amino acid substitution speed was analyzed for HVR-1 (27 a.a.), HVR-2 (7 or 9 a.a.), another region of

Table 3. Reactivities of patient sera to HVR-1 peptides

	HVR peptide		
	Point: 1	2	3
Patient C Serum:			
1	−	−	−
Point: 2	−	−	−
3	−	−	−
Patient E Serum:			
1	+	+	+
Point: 2	+	+	+
3	+	+	+

Patient point	HVR-1 peptide sequences
C-1	HTHVIGGAQTQTTGSFASLFTPGASQK
C-2	**R**THVIGG**V**QTQTTGS**L**ASLFTPGASQK
C-3	**R**THV**T**GG**V**Q**SR**TTGS**LV**SLFTPGASQK
E-1	STHTIGGCTARSAAGFTRLFTQGARQN
E-2	STHTIGG**S**TARSAAGFTRLFTQGARQN
E-3	STHT**V**GG**S**TARSAAGFT**K**LFT**R**GA**H**QN

E2 (between HVR-1 and HVR-2; 63 a.a.) and E1 as the monthly rate of a.a. substitutions per site (%) between each point during disease progression (points 1–3; Fig. 1).

Test of host immune response to HVR-1 peptide

Synthetic peptides of HVR-1 for patients C and E were synthesized for each point in the clinical course (points 1–3; Fig. 1, Table 3). Peptides were tested using ELISA to characterize host immune responses to HVR-1 during chronic infection.

Results

Characterization of HCV-RNA, anti-HCV antibody and ALT levels in acute and chronic infection of hepatitis C virus

To clarify the mechanisms of genetic variation during persistent HCV infection, 5 patients were retrospectively analyzed (Table 1, Fig. 1). Patients A and B displayed acute infection with HCV genotype 1b, with progression from first phase of acute infection to chronic infection, and persistent viremia (Fig. 1). In the first phase of acute infection, antibody to HCV became positive (after point 1; Fig. 1). In patient A, HCV-RNA and ALT levels in serum decreased immediately after infection (point 1 to 2; Fig. 1), then elevated in the second phase of acute infection (point 2 to 3; Fig. 1). In patient B, HCV-RNA and ALT levels in serum decreased immediately

after infection, with an elevation of HCV-RNA levels occurring only in the second phase of acute infection (point 2 to 3; Fig. 1). Patients C, D and E displayed chronic hepatitis and persistent infection of HCV. Patient C was infected with genotype 1b, Patient E was infected with genotype 2a, and Patient D displayed co-infection with genotypes 1b and 2a (Table 1). In Figure 1, quantity of HCV-RNA in patient D indicates combined total RNA for both genotypes. These three patients displayed continuously high levels of ALT for more than six months after first medical examination and did not display marked changes in HCV-RNA levels (points 1–3; Fig. 1). A peak in ALT value was detected between points 2 and 3 for patients C and D, while elevation of ALT values was detected between points 2 and 3 for patient E.

Nucleotide sequence variation and patient-specific nucleotide sequence in E1/E2 region during clinical course of hepatitis C

To clarify the predominant sequence of E1/E2 region during progression of hepatitis C, 5 patients (2 patients with acute hepatitis, 3 patients with chronic hepatitis) were retrospectively selected and sequences (nucleotides 620 ~ 1867; Table 2) from the sera of these patients were analyzed at three points (points 1–3; Fig. 1) using direct DNA sequencing methods as described. Analyzed HCV DNA sequences of the E1/E2 region for each patient were registered to Genbank (accession numbers AB107929–AB107949). Alignment of nucleotide sequences on one-third of the E2 region (nucleotide 1492 ~ 1785) is indicated in Fig. 2. Sequences categorized as patient-specific conserved nucleotide sequences displayed the following characteristics: 1) identical nucleotide sequences at each of the three points; 2) sequences that are not conserved within the same genotypes (Fig. 2A). Consistent with previous results [29], numerous nucleotide sequence variations in HVR-1 and 2 were identified in these HCV isolates from acute and chronic infection patients. However, patient-specific conserved nucleotide sequences were observed in this E2 region even within HVR-1 and -2 for each patient (boxed region; Fig. 2A). In the E1 region, patient-specific conserved nucleotide sequences were also observed in the five patients (data not shown).

Sequences categorized as substituted nucleotide sequences displayed (Fig. 2B). Substituted nucleotide sequences were present in this E1/E2 region for all 5 patients during the clinical course of infection.

Amino acid sequence variations in the E1/E2 protein region during the clinical course of hepatitis C

Deduced amino acid sequences of the E1/E2 region (a.a. 192 ~ 480) were compared in 5 patients (2 acute patients, 3 chronic patients) at points 1–3 (Fig. 3). Sequences categorized as patient-specific amino acid sequences displayed the same characteristics as those of patient-specific nucleotide sequences. Variations in a.a. sequence were particularly concentrated in HVR-1 and -2 for HCV genotype 1b isolates (patients A–C and 1b isolate from patient D; Fig. 3) and in HVR-1 alone

for HCV genotype 2a isolates (patient E and 2a isolate from patient D; Fig. 3) during the clinical course of infection. Although patient-specific conserved amino acid sequences were present in this E1/E2 region for all 5 patients during the clinical course of infection, the impact was not as strong as that of patient-specific conserved nucleotide sequences (Fig. 2).

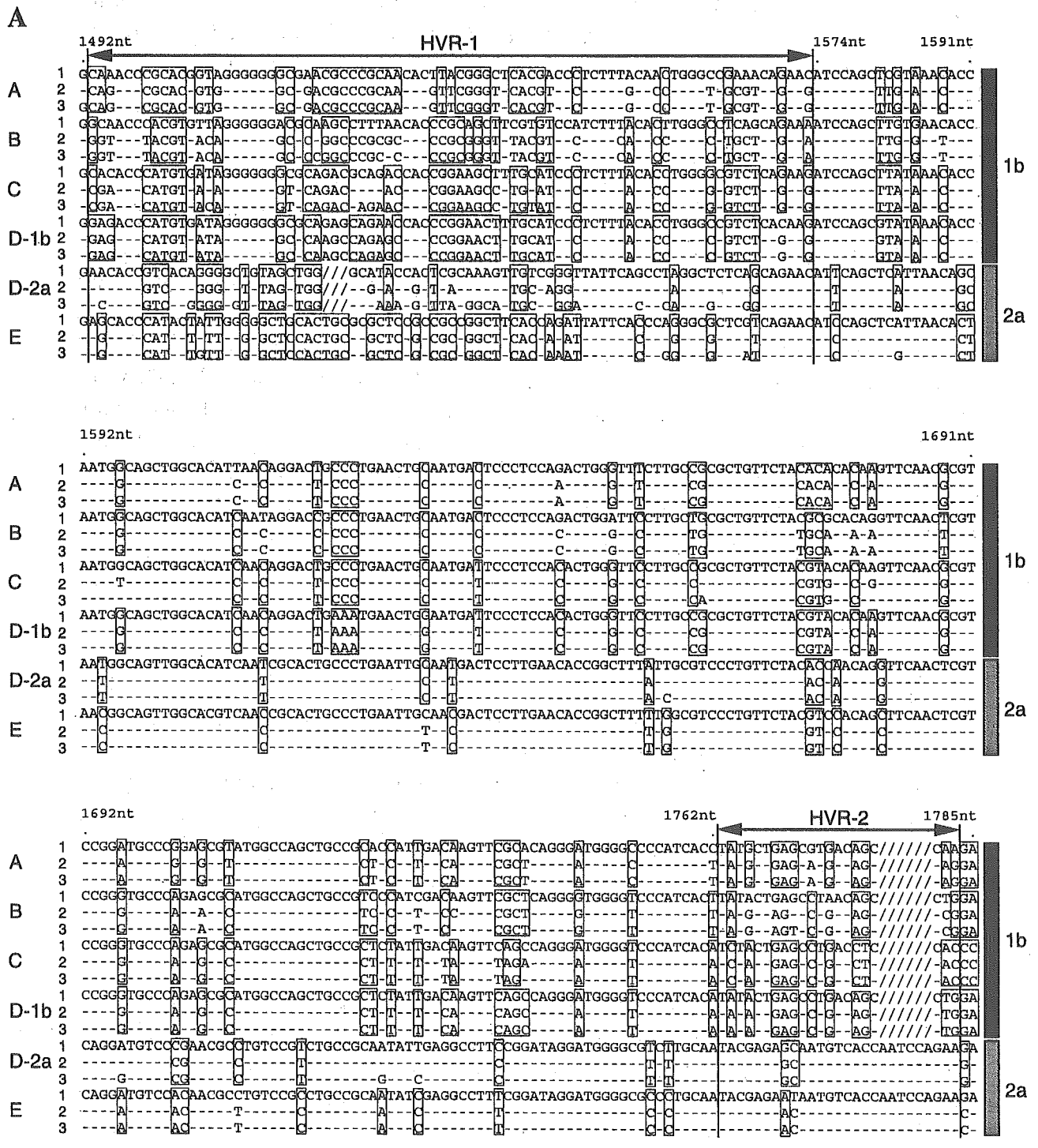

Fig. 2 (*continued*)

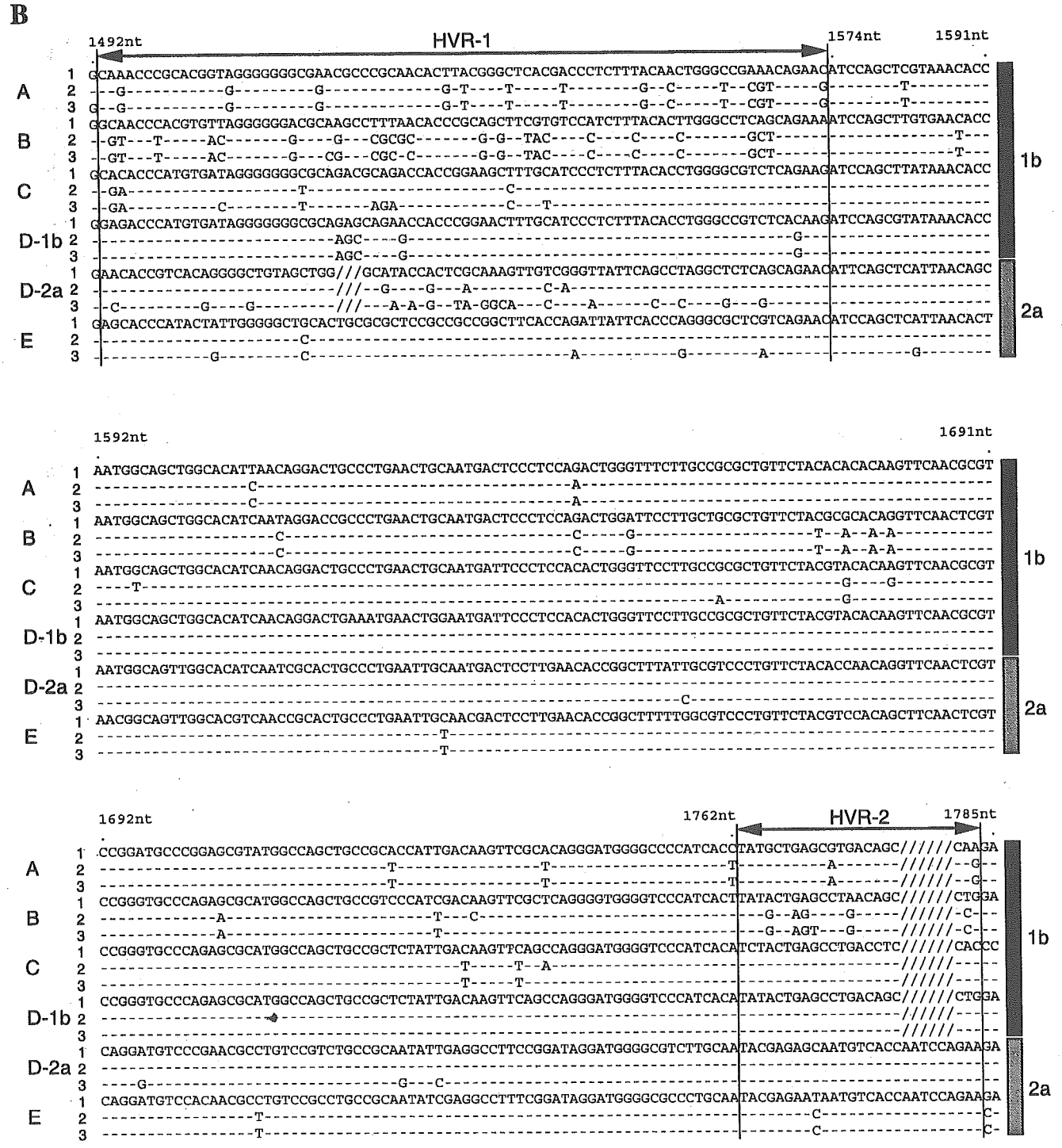

Fig. 2. Predominant nucleotide sequence comparison between each sequence from HCV patients at points 1–3. Region including nucleotides 1492–1785, including HVRs, was compared. **A** Patient-specific conserved sequences are enclosed in boxes. Each sequence column number indicates DNA sequencing point for each patient. Dash (–) indicates the same nucleotide as the first column sequence for each patient. Slash (/) indicates nucleotide deletion point. Column marked with a black box on the right side indicates HCV genotype 1b isolate. Column marked with a hatched box indicates HCV genotype 2a isolate. **B** Sequences categorized as substituted nucleotide sequences displayed

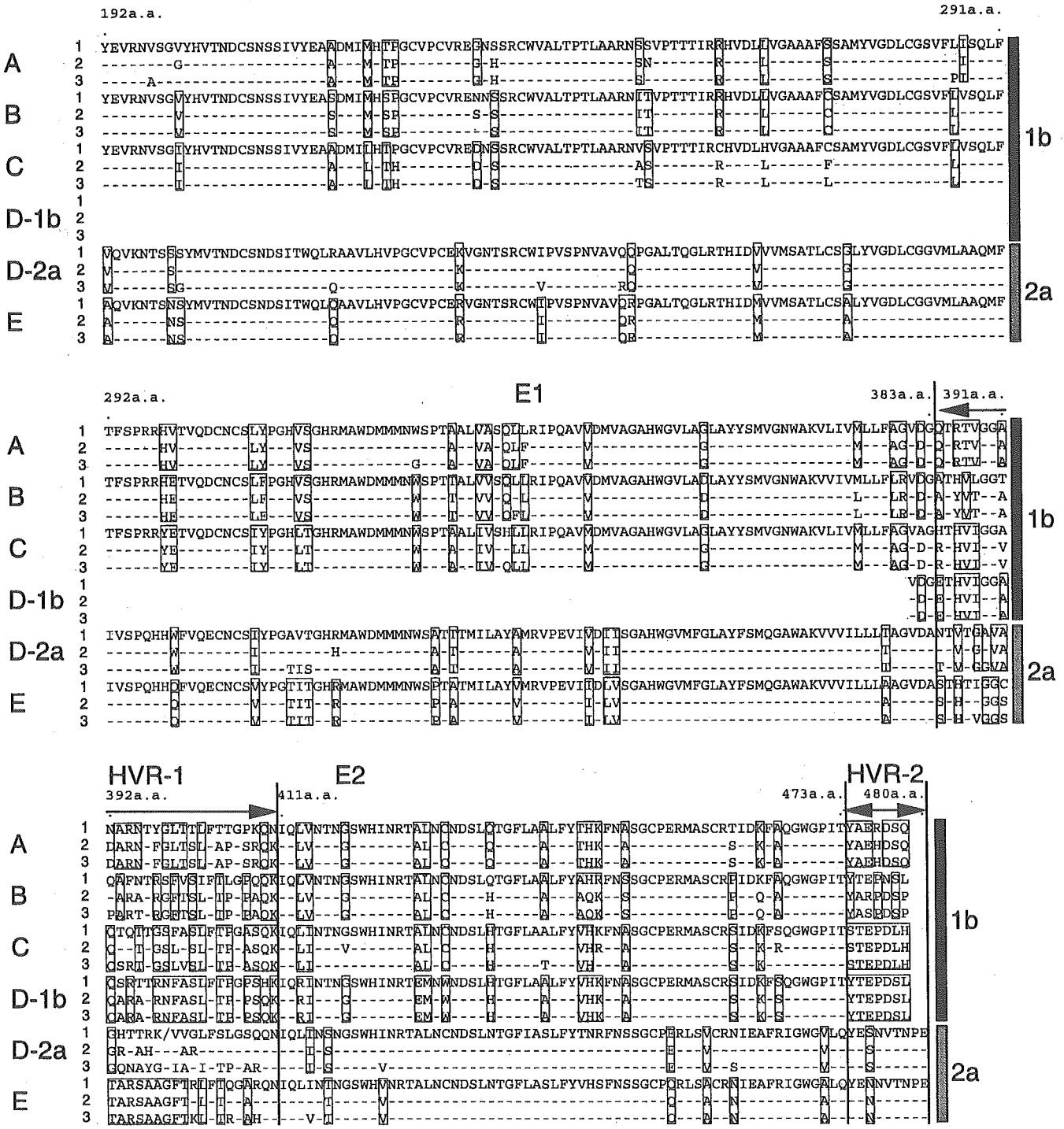

Fig. 3. Comparison of predicted amino acid sequences between each dominant HCV isolate from patients at points 1–3. E1/E2 protein sequences of HCV (a.a. 192–480) were compared. Sequence column number indicates DNA sequencing point for each patient. Patient-specific conserved sequences are enclosed in boxes. Dash (–) indicates the same a.a. residue as the first column sequence for each patient. Slash (/) indicates a.a. deletion point. Column marked with a black box on the right side indicates HCV genotype 1b isolate. Column marked with a hatched box indicates HCV genotype 2a isolate

In genotype 2a isolates from patient D (D-2a; Fig. 3), one a.a. deletion was identified in HVR-1 (residue 398, presented as a slash in Fig. 3). In genotype 2a isolates (patients D and E), two additional a.a.s in HVR-2 were noted, as reported elsewhere [21]. The deduced amino acid sequence of the E1 region (corresponding to a.a. 192 ~ 380) in genotype 1b isolates from patient D (D-1b; Fig. 3) could not be amplified by PCR at any time point (points 1–3; Figs. 1, 3).

Amino acid substitution speed in E1/E2 protein region and phylogenetic analysis of HVR-1 during progression of hepatitis C

To elucidate status of the HCV genome during infection, a.a. substitution speed between each point (point 1 to 2 and point 2 to 3; Fig. 1) was calculated as

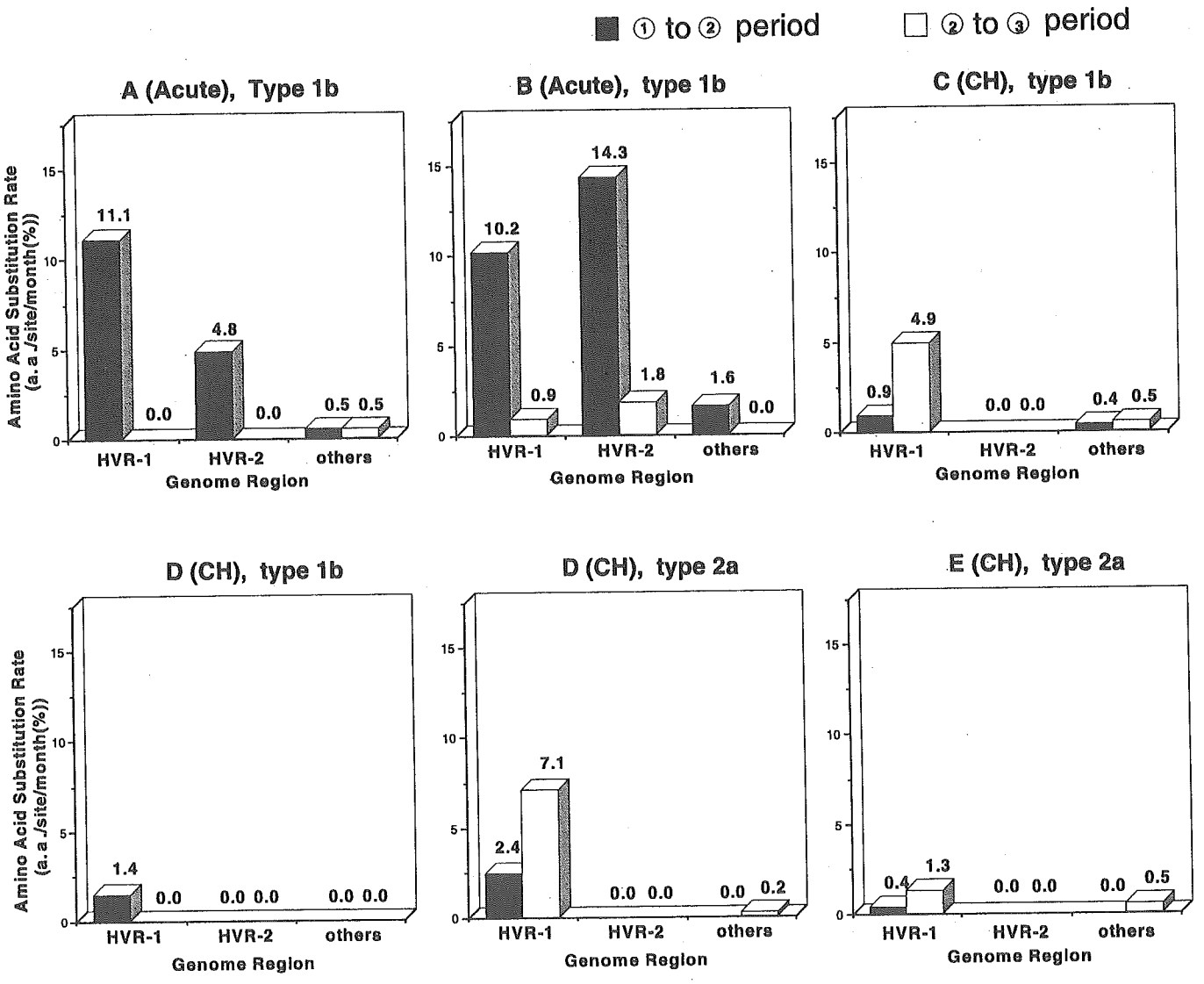

Fig. 4. Amino acid substitution speed in E1/E2 protein within HVR-1, HVR-2 and other regions. Amino acid substitution speed from DNA sequencing points 1 to 2 is indicated by black bars, and speed from points 2 to 3 is indicated by white bars. Regions HVR-1, HVR-2 and others represent a.a. 384–410, 474–480, and 411–473, respectively. *Acute*: acute HCV infection; *CH*: chronic HCV infection

the monthly rate of a.a. substitutions within each region (%; Fig. 4). In the first phase of acute infection (point 1 to 2), a.a. substitution speed in HVR-1 and HVR-2 was significantly faster than in the any other region of E1 and E2 in patients A and B (11.1% and 10.2% for HVR-1; 4.8% and 14.3% for HVR-2, respectively). In the second phase (point 2 to 3) of acute infection, a.a. substitution speed in HVR-1 and HVR-2 was slower than the first phase of acute infection in patients A and B (0% and 0.9% for HVR-1; 0% and 1.8% for HVR-2; 0.5% and 0% for other regions, respectively). In contrast, a.a. substitution speed in chronic patients was 0% in HVR-2 and below 0.5% in other regions (patients C–E; Fig. 4). Amino acid substitution speed in HVR-1 was fast during chronic HCV infection of ALT or when virus RNA levels underwent substantial transitions (patients C–E; Figs. 1, 4). In phylogenic tree analysis of HVR-1, sequence diversity of HVR-1 in the first phase of acute infection was phylogenetically distant from the original sequence (patients A and B), and the phylogenic tree of HVR-1 displayed clusters for each of the five patients (data not shown).

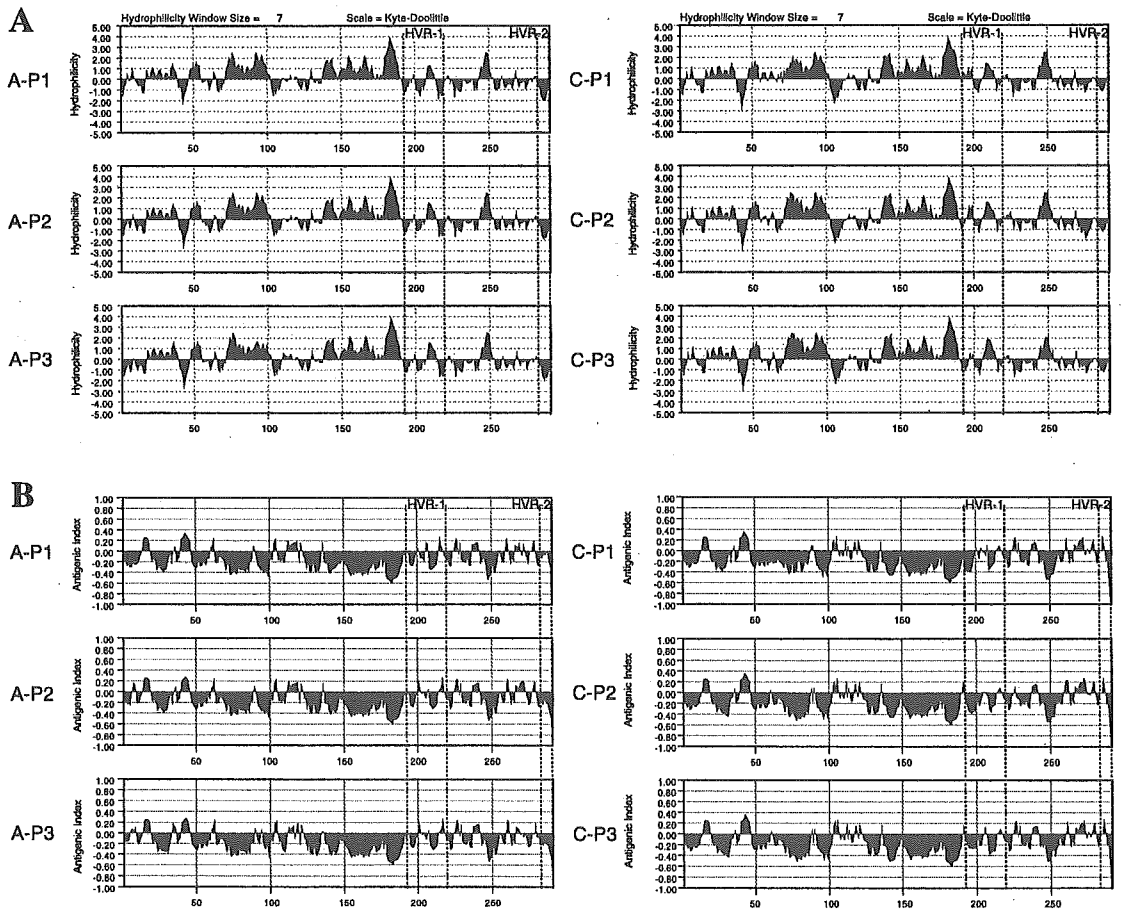

Fig. 5. Structural analysis of E1/E2 protein during HCV infection. Hydrophilicity profiles and antigenic indices of E1/E2 protein as predicted from direct DNA sequencing data were compared between points 1–3 for patients A and C. **A** Hydrophilicity profile. **B** Antigenic index. The presented region for HCV E1/E2 corresponds to a.a. 192–480

Comparison of protein characteristics for HVR-1 and -2 in the E1/E2 region

To examine the possibility that structural variation was generated in the E1/E2 region (a.a. 192～480) during disease progression in all five patients (Table 1), hydrophilicity, surface probability and antigenic indices were calculated from deduced a.a. sequences at each point. Figure 5 indicates the results of hydrophilicity (Fig. 5A) and antigenic index analyses (Fig. 5B) in patients A (acute infection) and C (chronic infection). These structural profiles displayed no significant changes during disease progression in patients A and C. The results of surface probability analysis in patients A and C likewise remained basically unchanged during disease progression. These three structural profiles demonstrate no significant changes in E1/E2 protein during the progression of HCV infection. Likewise, the remaining 3 patients (1 acute infection, 2 chronic infections; Table 1) displayed no significant changes in E1/E2 protein during disease progression. Moreover, the predicted secondary structure (chou-Fas) did not show any drastic changes between time points in any of the five patients (data not shown). Although some a.a. substitutions were observed in the E1/E2 region during disease progression in each patient, the major a.a. structure seems likely to have remained conserved in each case.

Humoral immune responses to each synthetic peptide from HVR-1

Synthetic HVR-1 peptides from chronic patients C and E were tested to characterize host immune responses during progression points using ELISA (points 1, 2, and 3; Fig. 1, Table 3). Patient C did not display antibody-positives against their own 3 HVR-1 peptides (C-1, C-2, C-3; Table 3) at any time point. In contrast, patient E displayed antibody-positives against their own 3 HVR-1 peptides (E-1, E-2, E-3; Table 3) at every time point (Table 3).

Discussion

The present study characterized nucleotide sequences of the E1/E2 protein region during clinical course from sera of 2 patients with acute HCV infection and 3 patients with chronic HCV infection using direct DNA sequencing methods. Furthermore, amino acid sequences and protein structures of the E1/E2 protein region (a.a. 192～480) were deduced during disease progression.

Nucleotide sequence variation in the E1/E2 region was mainly observed in HVR-1 and -2 for the 2 acute phase patients, and in only HVR-1 for the 3 chronic phase patients during clinical course. In the E1 protein region, a.a. substitution speed was below 0.69% in all five patients (2 acute patients, 3 chronic patients). This result indicates the possibility that E1 and HVR-2 may not be involved in escape mutation for chronic infection.

Previous reports have suggested that HVR-1 could serve as a target for neutralization of antibody and generation of escape mutants from humoral immune

responses, potentially contributing to the establishment of persistent HCV infection [14, 16, 20]. In our experiment, host immune responses to HVR-1 peptide during the course of chronic infection differed substantially between 2 patients (Table 3). For patient C, no antibody responses against 3 HVR-1 peptides (C-1, C-2 and C-3; Table 3) were observed at any time point. These data suggest two possible explanations. One is that antibodies were not produced in these stages, while the other is that the positions of C-1, C-2 and C-3 peptides might not be included in linear epitopes, instead being included in conformational epitopes. Patient E displayed a consensus sequence in each HVR-1 peptide (peptide sequence, TARSAAGFT; Table 3). For this reason, sera from patient E might react positively for E-1, E-2 and E-3 peptides at each time point. These results indicate the possibility that HVR-1 might not represent a significant epitope region for neutralization of HCV escape mutants in some cases.

Our results indicate the existence of patient-specific conserved nucleotide sequences in the E1/E2 region during clinical course of all HCV patients (Fig. 2). This finding may be useful for identifying HCV vertical transmission and other infection pathways. Furthermore, the existence of these patient-specific nucleotide sequences indicate the possible adaptation of the virus in patients and escape from the host immune surveillance systems in the early phase of HCV infection.

Rate of amino acid substitution speed between each point (point 1 to 2 and point 2 to 3; Fig. 1) during clinical course was calculated as the monthly rate of a.a. substitutions per site (%; Fig. 4). The data indicate that high a.a. substitution speed in HVR-1 and -2 was present in the first phase of acute infection, and that a.a. substitution speed in HVR-1 was elevated in chronic patients during major transitions in viral RNA or ALT levels. This phenomenon should support the understanding of HCV adaptation to host immune pressures and the establishment of persistent HCV infection.

The acute and secondary structures of the E1/E2 protein region (a.a. 192 ~ 380) from patients with hepatitis C displayed no significant change during clinical course. This observation suggests that HCV clones in hepatitis C patients may conserve a E1/E2 protein structure during persistent infection.

In conclusion, our observations suggest that the rapid substitution of amino acid sequences in the first phase of acute phase of infection may be involved the HCV adaptation to host immune pressures and the development of persistent HCV infection.

Acknowledgements

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Usefulness of a new immuno-radiometric assay to detect hepatitis C core antigen in a community-based population

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SUMMARY. A new immuno-radiometric assay (IRMA) to detect hepatitis C virus (HCV) core antigen (HCVcAg) has been developed. The aim of the present study was to investigate the sensitivity and specificity of this IRMA to measure HCV antigenemia, based on the detection of HCV RNA as the gold standard, and to assess the utility of the IRMA in a community-based population. Anti-HCV positive residents in a hyperendemic area of HCV infection in Japan were studied. Serum levels of HCVcAg were measured using IRMA, and the presence of HCV RNA was determined by a qualitative reverse transcription-polymerase chain reaction (RT-PCR) assay. The sensitivity and the specificity of the IRMA were 96.4 and 100%, respectively. The sensitivity of the IRMA was similar between serological HCV group I (HCV genotypes 1a and 1b) (97.6%) and group II (HCV genotypes 2a and 2b) (94.0%). There was a strong correlation between serum HCVcAg level and HCV-RNA measured by a quantitative RT-PCR ($r = 0.832$, $P < 0.0001$). There also was a very strong correlation of HCVcAg level between IRMA measurements performed on serum and those performed on plasma ($r = 0.984$, $P < 0.0001$). In conclusion, this new IRMA is useful for the detection of HCV core antigen in a community-based population.

Keywords: community-based population, hepatitis C virus core antigen, immuno-radiometric assay, serological hepatitis C virus group.

INTRODUCTION

Persistent infection with hepatitis C virus (HCV), as well as hepatitis B virus (HBV), is a primary cause of chronic liver disease, such as chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC). In Town C of Miyazaki Prefecture in Japan, the average annual mortality from liver disease was estimated to be 102.5/100 000 between 1995 and 1997, which was substantially higher than the estimate of 37.6/100 000 for all of the prefecture, as reported by the prefectural public health service. In 1993, over 4000 residents of Town C were tested for antibody to HCV (anti-HCV) and HBV surface antigen (HBsAg) in conjunction with the local, government-sponsored general health examination conducted in the town. The prevalence of anti-HCV positivity was found to be 22.5% and that of HBsAg positivity, 1.1%. Thus, the elevated mortality from liver disease in Town C is considered to be due to the high prevalence of HCV infection in this population.

Measurement of anti-HCV is generally used to screen for HCV infection. However, not all anti-HCV positive people are persistently infected with the virus. Therefore, a simple, quick, and inexpensive method for measuring HCV viremia is required for population-based testing. Although the detection of HCV RNA by reverse transcription-polymerase chain reaction (RT-PCR) represents the most sensitive method for determining persistent HCV infection, the assay is time-consuming, costly, and technically demanding. In contrast, enzyme immunoassays (EIAs) to detect HCV core antigen (HCVcAg) are simple and relatively inexpensive [1]. A number of reports have demonstrated the utility of measuring HCVcAg using EIAs [2–5]. Recently, a new immuno-radiometric assay (IRMA) to detect HCVcAg was developed.

The aim of the present study was to investigate the sensitivity and specificity of this new IRMA to measure HCV antigenemia, based on the detection of HCV RNA as the gold

Abbreviation: EIA, enzyme immunoassay; HCC, hepatocellular carcinoma; IRMA, immuno-radiometric assay; RT-PCR, reverse transcription-polymerase chain reaction; US, ultrasonography.

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standard, and to assess the utility of the IRMA in a community-based population such as Town C.

Methods and subjects

Since 1994, 1083 Town C residents have been identified as being positive for anti-HCV and have been screened annually for the early detection of HCC by ultrasonography (US) examination. To elucidate the natural history of HCV infection and the risk factors for HCC, additional virologic, epidemiologic, and clinical data also have been collected at these US examinations, beginning in 2001. Blood samples for CBC, liver function test, tumour markers (alpha-fetoprotein and PIVKA-II), hepatic fibrosis markers (hyaluronic acid and type IV collagen) and HCV-associated markers (anti-HCV antibody titre, serological genotyping of HCV, virus load, presence of HCV RNA) are collected after taking an informed consent. In 2002, 931 subjects were invited to the annual US examination, of whom 674 (72%) attended and provided a blood sample.

Anti-HCV antibody

Anti-HCV antibody was measured by chemiluminescent enzyme immunoassay using a third generation HCV antibody kit (Lumipulse Ortho II; Ortho-Clinical Diagonostics K. K., Tokyo, Japan).

HCV antigen load

At the 2002 US screening, the new IRMA (Ortho HCV Ag IRMA Test; Ortho-Clinical Diagnostics K. K.) was used to detect HCVcAg. Testing using the IRMA was carried out as indicated by the manufacturer. Briefly, serum or standard sample was added to pretreatment solution in a plastic tube and incubated at 56–60 °C. After adding the reaction solution and one bead coated with two different monoclonal antibodies against HCVcAg to each tube, the mixture was incubated at room temperature. Two different horse-radish peroxidase-conjugated monoclonal antibodies against HCVc Ag was added to each tube. ^{125}I-conjugated polyclonal antibodies against peroxidase then were added, and the radioactivity of the sample and the standard was measured with a γ-scintillation counter. The concentration of HCVcAg was expressed as femto-mol/L (fmol/L). The range of the IRMA was from 20 to 20 000 fmol/L. When the titre of HCVcAg was less than 20 fmol/L, the sample was judged as negative. The plasma levels of HCVcAg also were measured, by the same IRMA method, for a subset of 40 subjects.

HCV RNA detection and quantification

The presence of HCV RNA in serum was determined by a qualitative RT-PCR assay kit (Amplicore® HCV; Nippon Roche, Tokyo, Japan). The detectable HCV-RNA by this assay kit was 10 copy/mL. The serum HCV RNA level was measured using a quantitative RT-PCR assay kit (Amplicore® GT HCV Monitor v2.0; Nippon Roche) on a random sample of 100 HCV RNA positive subjects. However, four of the subjects had an insufficient sample for measuring HCV RNA level. The concentration of HCV RNA was expressed as KIU/mL, and the range was from 0.5 to 850 KIU/mL. When the titres of HCV-RNA were less than 0.5 KIU/mL, the sample was judged as negative.

Serological HCV group

Serological HCV groups were determined by a serological genotyping assay (Immunocheck F-HCV Grouping; International Reagents Co., Kobe, Japan) [6]. When the serological group could not be clearly classified by this assay, HCV genotypes were determined by the RT-PCR method [7]. Genotypes 1a and 1b were defined as serological HCV group I, and genotypes 2a and 2b as group II.

RESULTS

A total of 613 (90.9%) of the 674 residents who attended the 2002 US screening and provided a blood sample were anti-HCV antibody positive and 61 were negative. Thirty-eight per cent of the subjects studied were men ($n = 233$), and the overall mean age was 69.3 years.

The results of the IRMA testing are summarized in Table 1. HCVcAg was detected by IRMA in 424 (69.2%) of the 613 anti-HCV positive subjects; HCV RNA was detected by RT-PCR in 440 (71.8%). Based on the HCV RNA status as the gold standard, the sensitivity of the IRMA was 96.4% (424/440), with a specificity of 100%. All 61 subjects who were anti-HCV negative were negative for both HCVcAg and HCV-RNA. Ninety-six randomly selected serum samples were tested for HCV-RNA level using a quantitative RT-PCR assay (Table 2). Neither HCVcAg by IRMA nor HCV-RNA by RT-PCR was detected in four samples. In three samples, HCV-RNA was detected only by RT-PCR. The HCV-RNA level of these samples was 1.3, 7.1 and 19 KIU/mL. There were no samples that were HCVcAg positive but negative for HCV-RNA (Tables 1 and 2). Figure 1 shows a strong correlation

Table 1 Comparison of the detection of HCV core antigen by immuno-radiometric assay with the detection of HCV-RNA by RT-PCR

	HCV-RNA by qualitative RT-PCR	
HCVcAg by IRMA	+	−
+	424	0
−	16	173

HCV, hepatitis C virus; RT-PCR, reverse transcription-polymerase chain reaction.

Table 2 Comparison of the detection of HCV core antigen by IRMA and HCV-RNA by quantitative RT-PCR in HCV-RNA positive subjects

HCVcAg by IRMA	HCV-RNA by quantitative RT-PCR	
	+	−
+	89	0
−	3	4

HCV, hepatitis C virus; IRMA, immuno-radiometric assay; RT-PCR, reverse transcription-polymerase chain reaction.

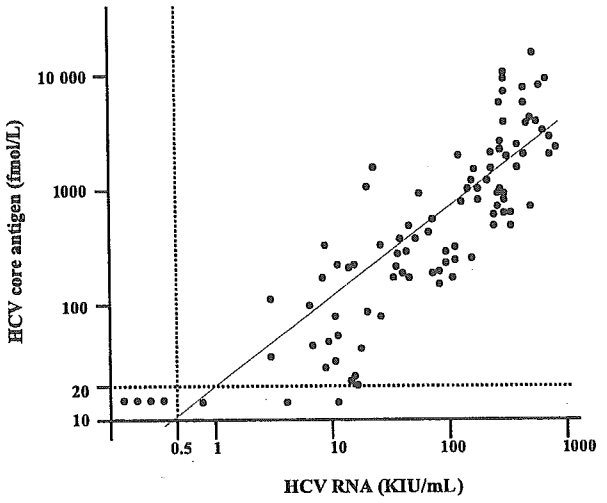

Fig. 1 Correlation between concentration of HCV RNA measured by AMPLICOR Monitor test and HCV core antigen measured by immuno-radiometric assay. The serum HCV RNA level was measured using a quantitative RT-PCR assay kit (Amplicore® GT HCV Monitor v2.0) on a random sample of ninety-six HCV RNA positive subjects. The levels of serum HCVcAg measured by IRMA were strongly correlated with HCV-RNA levels by RT-PCR (Pearson correlation coefficient, $r = 0.832$, $P < 0.0001$).

between serum HCVcAg level measured by IRMA and HCV-RNA level by RT-PCR ($r = 0.832$, $P < 0.0001$).

The HCV genotype group could be determined by serological genotyping or RT-PCR assay for 524 subjects who attended the 2002 screening (Table 3). The distribution of subjects by HCV genotype group was 356 (67.9%) of the 524 subjects in group I and 168 (32.1%) in group II. The sensitivity of the IRMA was similar in the two serological HCV genotype groups (97.6% in group I and 94.0% in group II) (Table 3). It is noteworthy that the average amount of HCVcAg detected by the IRMA was similar in the two-genotype groups (median of 3060 fmol/L in group I and median of 3350 fmol/L in group II).

The correlation between serum and plasma levels of HCVcAg detected by IRMA was analysed in a subset of the

Table 3 Comparison of the detection of HCV core antigen by IRMA with the detection of HCV RNA by RT-PCR, by serological HCV group

HCVcAg by IRMA	HCV-RNA by RT-PCR	
	+	−
Serological HCV group I		
+	282	0
−	7	67
Serological HCV group II		
+	141	0
−	9	18

HCV, hepatitis C virus; IRMA, immuno-radiometric assay; RT-PCR, reverse transcription-polymerase chain reaction.

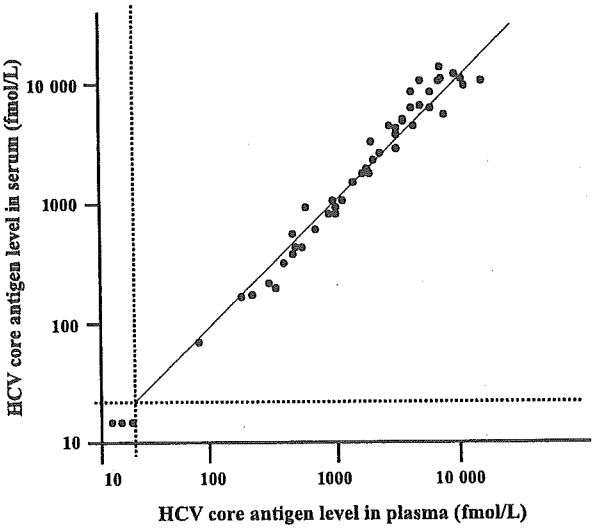

Fig. 2 Correlation between HCV core antigen levels in serum and in plasma measured by immuno-radiometric assay. The subjects were classified into six groups according to their serum HCVcAg levels (<20, 20–100, 100–1,000, 1000–10 000, 10 000–20 000, >20 000 fmol/L). Plasma samples from five or six subjects were randomly selected from each group for a total of 40 subjects and were measured by IRMA. The levels of HCVcAg in plasma were strongly correlated with those in serum (Pearson correlation coefficient, $r = 0.984$, $P < 0.0001$).

Town C residents who attended the 2002 US screening (Fig. 2). The levels of HCVcAg in plasma were strongly correlated with those in serum (Pearson correlation coefficient, $r = 0.984$, $P < 0.0001$).

DISCUSSION

HCV core antigen was first detected in the circulation of HCV-infected hosts using EIA-based methods [1,8,9]. Many

reports have demonstrated the utility of EIA to detect HCVcAg for monitoring the effect of interferon therapy [1], for following liver transplantation patients with HCV recurrence [2], for quantitative evaluation of HCV viremia in anti-HCV positive patients [3], and as a marker of HCV viremia in the serological window-phase period [4]. However, the first versions of the EIA for HCVcAg had some limitations. They could not detect HCVcAg below a level of 20 KIU/mL of HCV RNA, so that their use was limited to the monitoring of late events during and after antiviral treatment [10]. In addition, the pretreatment process of the sample was somewhat complicated, with three steps required to expose the epitopes of HCVcAg bound by low-density lipoprotein or anti-HCV core antibody. The sensitivity of the EIA also was affected by mutations in the HCV core region [11]. Moreover, differences in the detectable levels of HCVcAg titres between the serological HCV genotype groups resulted in a lower sensitivity of the EIA among people infected with HCV genotype 2 [12].

In 1999, Aoyagi and colleagues developed a modified version of the EIA for HCVcAg [5]. In this version, the epitope of HCVcAg could be easily exposed, and binding by anti-HCV core antibody in the serum could be reduced by incubation with three types of detergents. Since the modified EIA required only one pretreatment step, it was simpler than the first generation versions of the assay. In addition, it had a 100-fold increase in sensitivity over the earlier versions. Tanaka *et al.* demonstrated that the second generation of the EIA for HCVcAg was useful for the diagnosis of acute and chronic hepatitis C and for predicting and monitoring the effect of interferon treatment [13]. More recently, a new IRMA-based test for detecting HCVcAg, which is a further modification of the Aoyagi *et al.* EIA method, was developed.

In the present study, we investigated the sensitivity and specificity of this IRMA, to detect HCV persistent infection based on the presence of HCV RNA as the gold standard. The sensitivity of the IRMA was 96.4%. In contrast, we tested for HCVcAg by a first generation assay at the 2001 US screening and found its sensitivity was 85.4% (data not shown). Of the randomly selected 96 samples, there was a strong correlation between serum HCVcAg level measured by the IRMA and HCV-RNA level by a commercial quantitative RT-PCR assay. This result is the same as that previously reported by Tanaka *et al.* [13]. Moreover, the IRMA for HCVcAg overcomes the problem of the effect of serological HCV genotype group on the level of HCVcAg detectable by EIA in serum [12]. In our population, the sensitivity of the first generation EIA was significantly lower in serological group II (HCV genotypes 2a and 2b) (74.7%) than in group I (HCV genotypes 1a and 1b) (90.5%) (data not shown). In striking contrast, the sensitivity of the IRMA was 94% or higher in both serological HCV genotype groups. It is not clear why the sensitivity of the IRMA is not affected by HCV genotype group. Both the EIA [1] and the IRMA [5] use high-affinity monoclonal antibodies that recognize amino acid sequences known to be relatively well conserved across the six HCV genotypes. However, small differences in these amino acid sequences between genotype groups I and II may exist, which render the monoclonal antibodies in the first generation EIA less sensitive to detect genotype group II core antigen. Moreover, the four different monoclonal antibodies used in the second generation IRMA may enhance the ability of this assay to detect HCVcAg in persons infected with HCV group II genotypes.

The presence of HCV RNA is considered the gold standard for determining HCV persistence, and RT-PCR is a very sensitive method to detect HCV RNA. However, the RT-PCR assay requires additional amplification procedures and specially-trained personnel and carries the risk of contamination. In addition, it has been reported that the RT-PCR method produces false negative results for heparinized samples [14] and may not detect the lower HCV RNA levels found for persons infected with HCV genotypes 2a or 2b [15]. There also is a progressive and significant loss of HCV RNA activity when the time from the formation of the clot until centrifugation is longer than 2 h from the collection of the blood specimen [16]. In contrast, EIA-based methods to detect HCVcAg offer several advantages over RT-PCR for measuring HCV persistence. First, EIAs are not influenced by the anticoagulants EDTA, heparin, or sodium citrate [5]. In the current study, there was a very strong correlation between HCVcAg levels in serum and those in plasma as measured by the new IRMA. Second since the HCVcAg detected by EIA is stable [5], extra precautions in processing and storing specimens also should not be necessary. Moreover, the IRMA appears to be sufficiently sensitive to identify persistent group II HCV infection. Finally, the cost of the IRMA kit is less than one-third that of the RT-PCR assay.

The majority of HCV carriers are asymptomatic, and some may display advanced liver disease, including liver cirrhosis and HCC, without the awareness that they are infected with HCV. Thus it is important that persons with persistent HCV infection can be identified. In the present study, we demonstrated the usefulness of a new IRMA for the detection of HCV antigenemia in the community-based Town C population in Japan. The assay is relatively simple and inexpensive and has a high sensitivity to detect HCVcAg in people infected with HCV genotypes 1 or 2. Thus, this new IRMA is an economically viable option for identifying individuals with chronic HCV infection when screening large numbers of people on a population level.

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