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

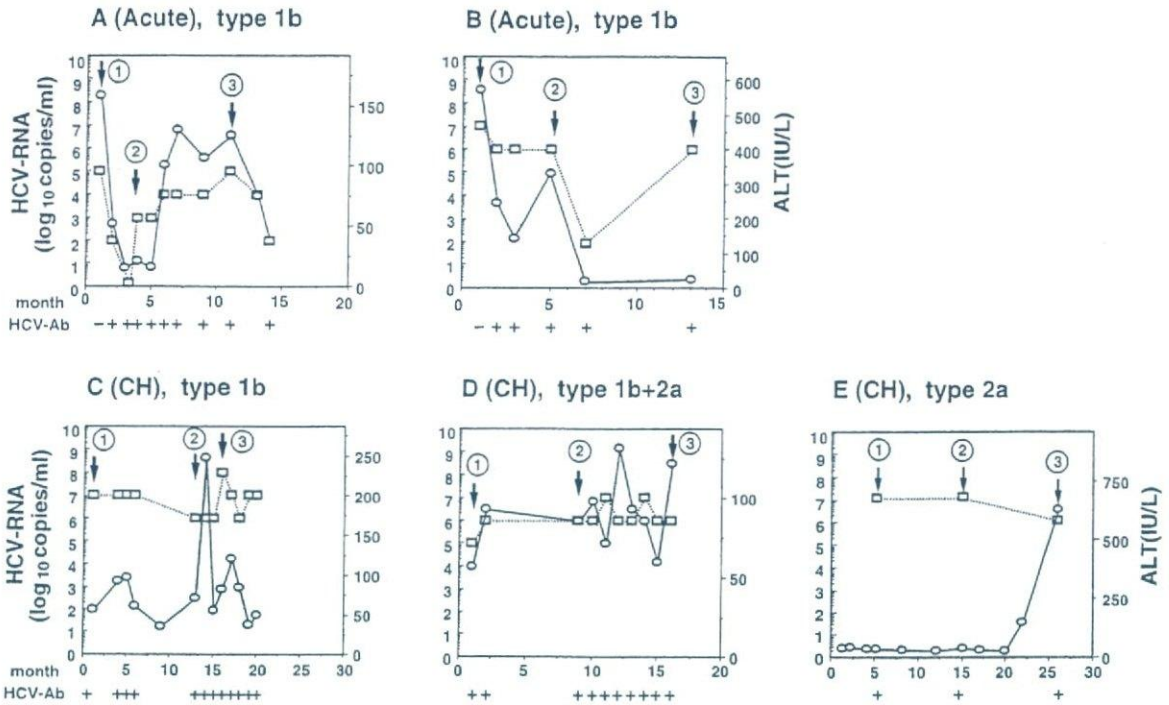

Fig. 1. Clinical course of hepatitis C patients. Changes in HCV RNA titer (broken line with open square), ALT level (black line with open circle) and HCV antibodies (− or +) for each patient. Numbers *1–3* in open circles indicate DNA sequencing points. In acute patients **A** and **B**, time point 1 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).

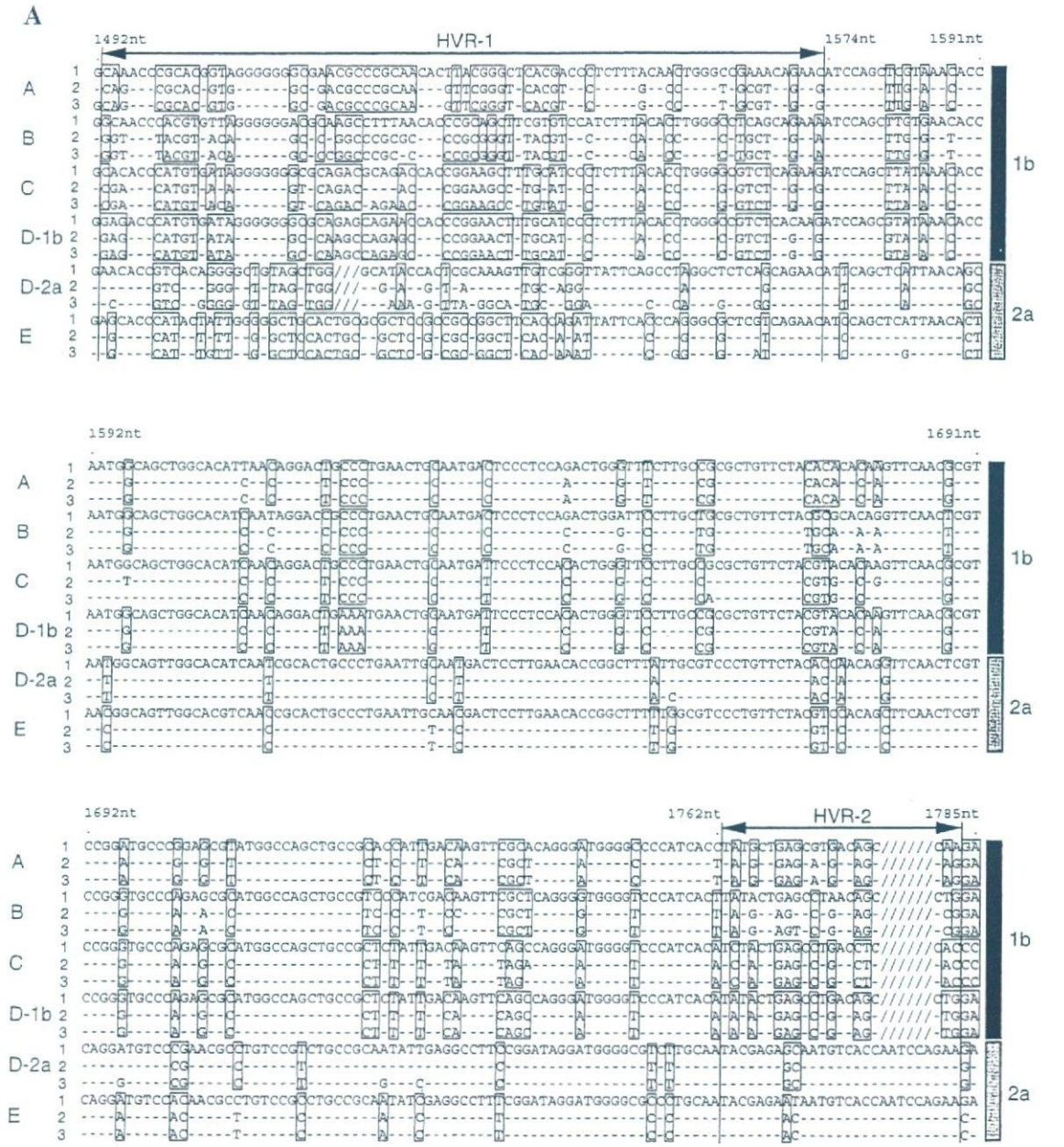

Fig. 2 *(continued)*

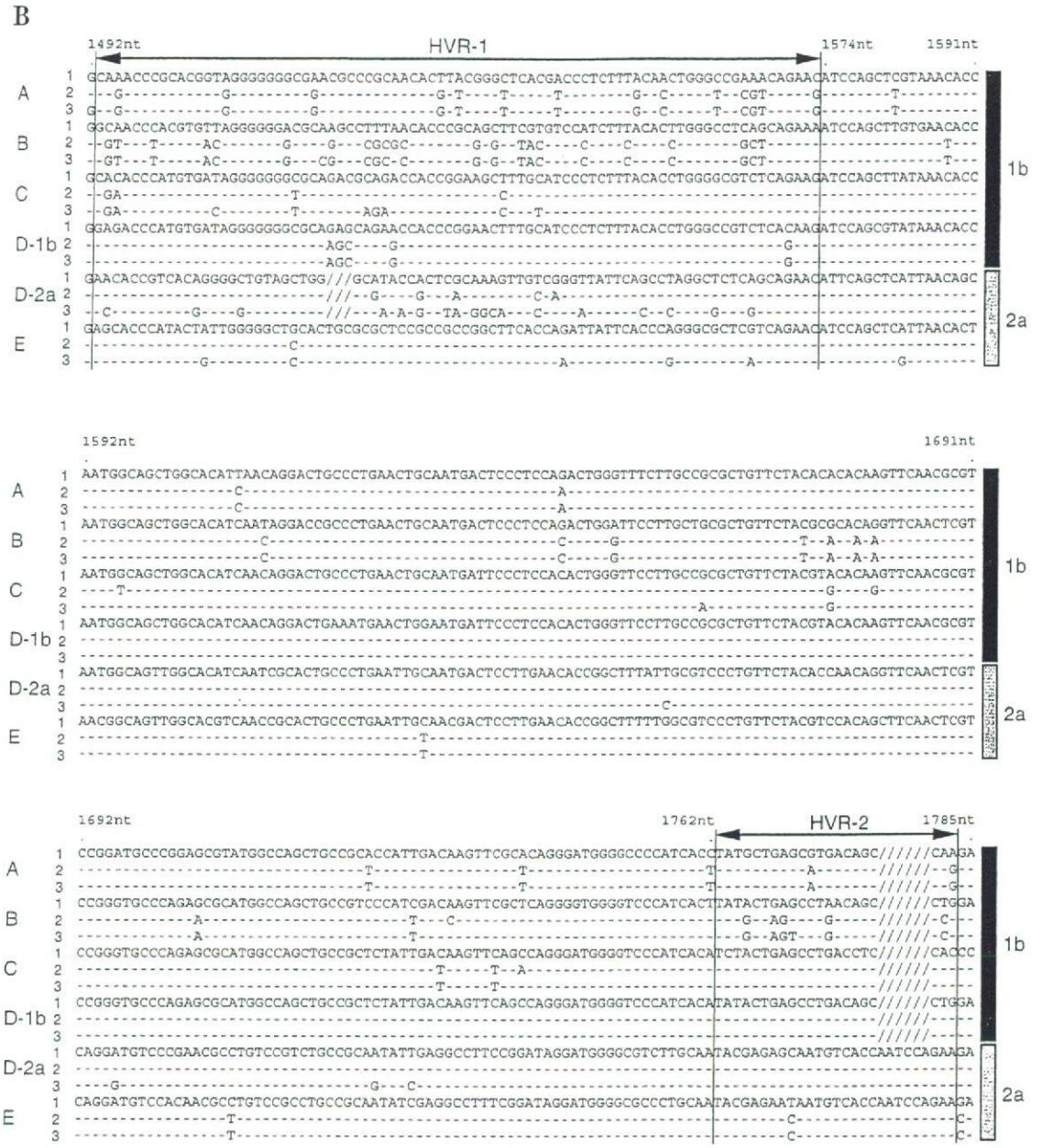

Fig. 2. Predominant nucleotide sequence comparison between each sequence from HCV patients at points 1–3. Region including nucleotides 1492–1785, including HVRs, was compared. **A** Patient-specific conserved sequences are enclosed in boxes. Each sequence column number indicates DNA sequencing point 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

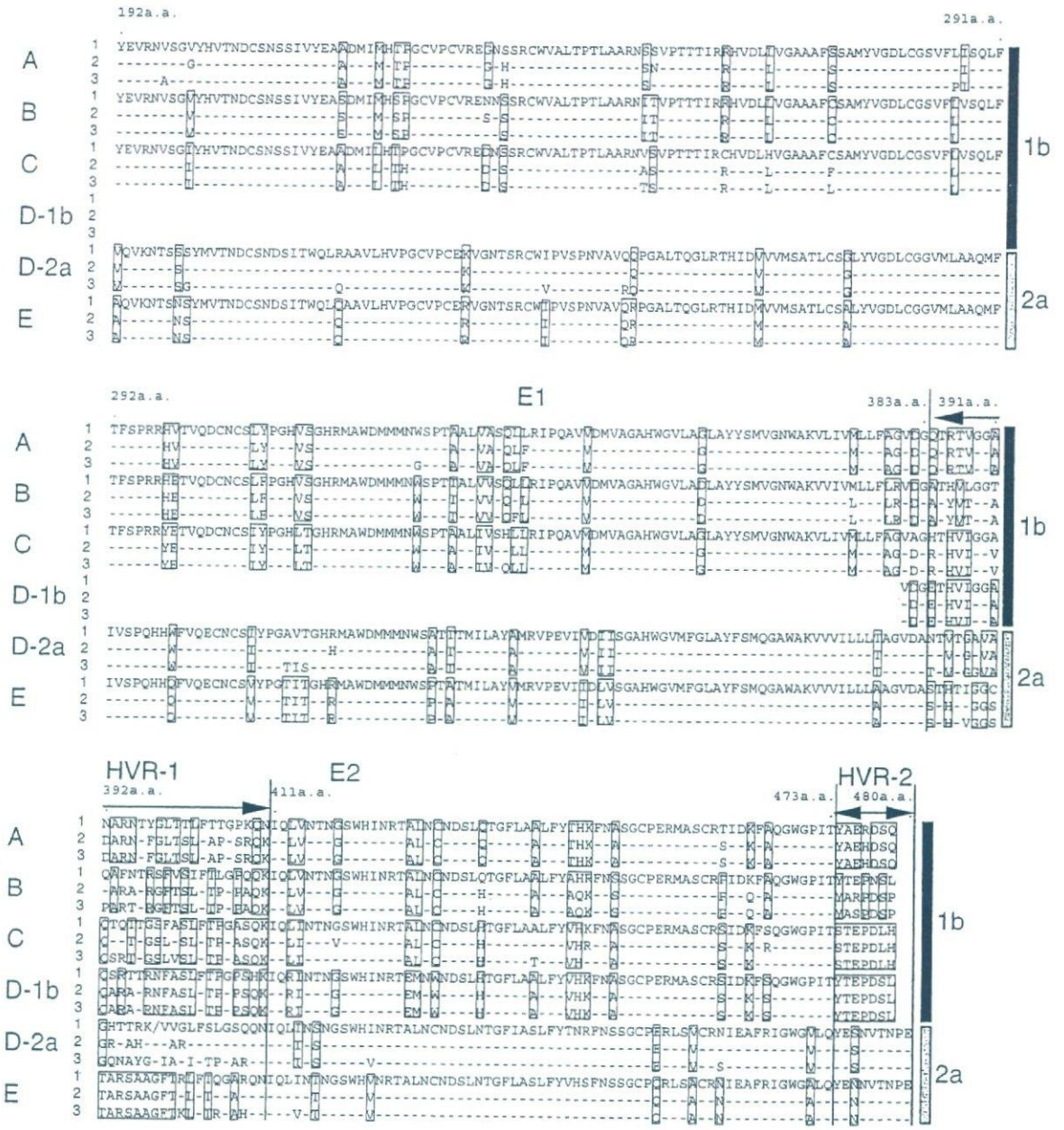

Fig. 3. Comparison of predicted amino acid sequences between each dominant HCV isolate from patients at points 1–3. E1/E2 protein sequences of HCV (a.a. 192–480) were compared. Sequence column number indicates DNA sequencing point for each patient. Patient-specific conserved sequences are enclosed in 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

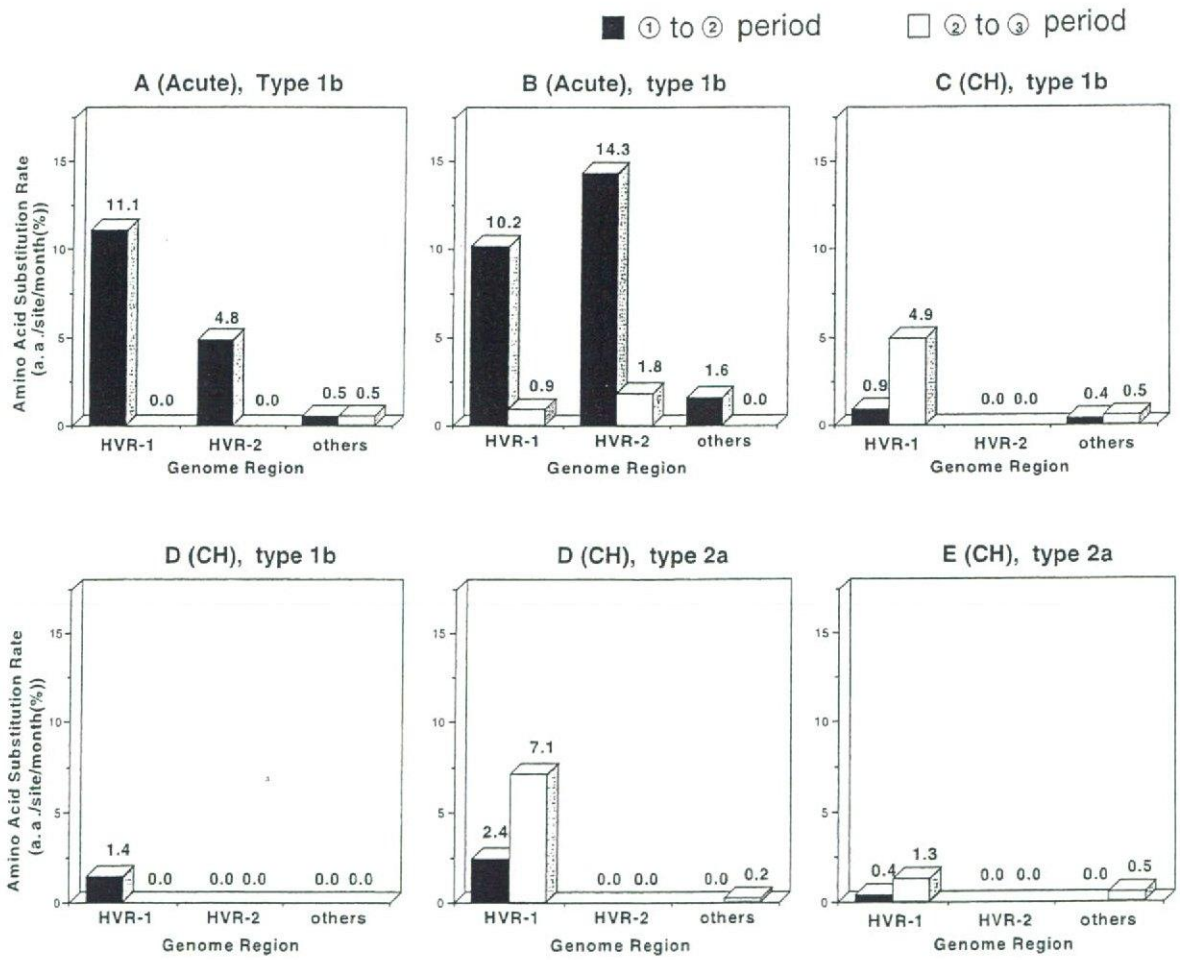

Fig. 4. Amino acid substitution speed in E1/E2 protein within HVR-1, HVR-2 and other regions. Amino acid substitution speed from DNA sequencing points 1 to 2 is indicated by black bars, and speed from points 2 to 3 is indicated by white bars. Regions HVR-1, HVR-2 and others represent a.a. 384–410, 474–480, 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).

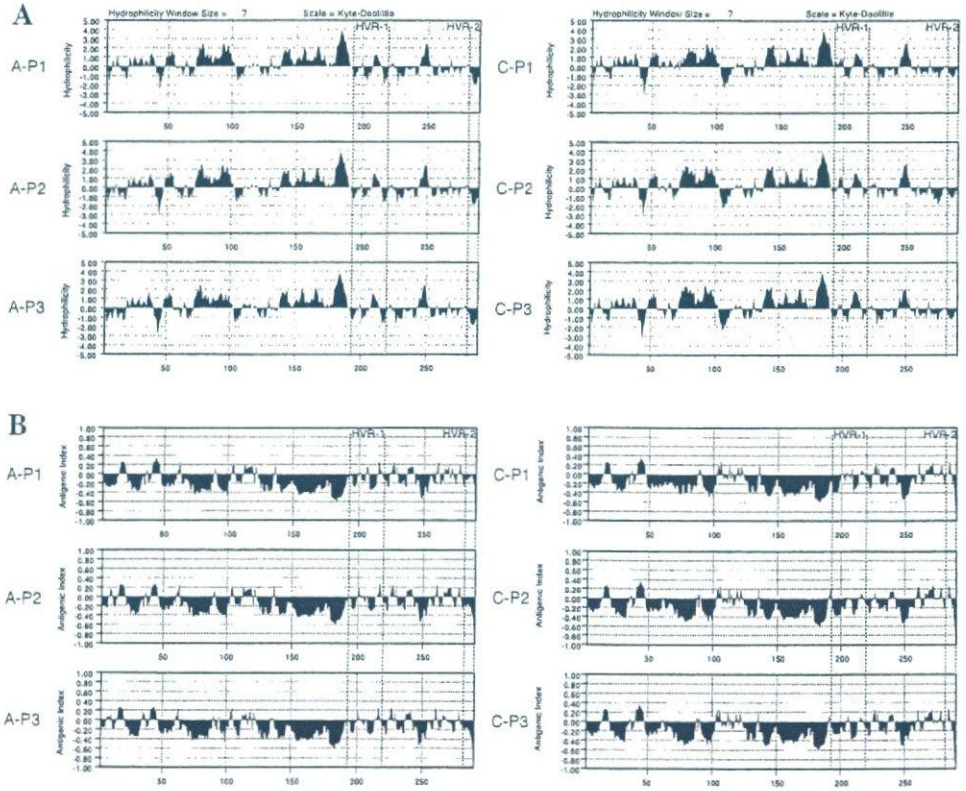

Fig. 5. Structural analysis of E1/E2 protein during HCV infection. Hydrophilicity profiles and antigenic indices of E1/E2 protein as predicted from direct DNA sequencing data were compared between points 1–3 for patients A and C. **A** Hydrophilicity profile. **B** Antigenic index. The presented region 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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Quantitative Analysis of Anti–Hepatitis C Virus Antibody–Secreting B Cells in Patients With Chronic Hepatitis C

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To investigate the quantitative characteristics of humoral immunity in patients with hepatitis C, we established an enzyme-linked immunosorbent spot (ELISpot) assay for detection of anti–hepatitis C virus (HCV)-secreting B cells. Receiver operating characteristic curve analysis demonstrated 100% specificity and 58% to 92% sensitivity for detecting B-cell responses to NS5b, NS3, E2, and core antigens. The median sum of anti-HCV–secreting B cells to all HCV antigens tested was significantly higher in 39 patients with chronic hepatitis C (47.3 spot forming cells [SFCs]/10^6 peripheral blood mononuclear cells [PBMCs]) than in 9 recovered subjects (15.3 SFCs/10^6 PBMCs; $P = .05$) or 11 uninfected controls (5.3 SFCs/10^6 PBMCs; $P < .001$); the significant difference ($P = .018$) in chronic versus recovered patients was in reactivity to nonstructural antigens NS3 and NS5b. Anti-HCV immunoglubulin M (IgM)–secreting B cells were also readily detected and persisted decades into HCV infection; there was no difference in IgM-positive cells between chronic and recovered patients. ELISpot reactivity to genotype 1–derived antigens was equivalent in patients of genotypes 1, 2, and 3. There was significant correlation between the numbers of anti-HCV IgG-secreting B cells and serum aminotransferase and to the level of circulating antibody. In conclusion, ELISpot assays can be adapted to study B-cell as well as T-cell responses to HCV. Measurement at the single-cell level suggests that humoral immunity plays a minor role in recovery from HCV infection and that B-cell immunity is strongest in those with persistent infection. (HEPATOLOGY 2006;43:91-99.)

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide. More than half of patients with acute HCV infection develop chronic hepatitis, leading to cirrhosis and/or hepatocellular carcinoma in at least 20% of these patients.[1-3] Chronic HCV infection results in the induction of a strong humoral immune response, and measurement of anti-HCV antibodies in serum is widely used to screen for HCV infection. Although several studies have examined the features of the humoral immune response to HCV,[4-7] the quantitative characteristics of HCV-specific antibody production during infection remain undefined. In patients with acute hepatitis C, an early HCV-specific T-cell response is associated with viral clearance,[8-11] but the role of humoral immune responses in HCV clearance is unclear and appears to be subsidiary, because strong antibody responses are detected in all immunocompetent chronic HCV carriers. It is also unknown whether anti-HCV antibodies serve to control the level of viremia during chronic infection and whether they ameliorate horizontal or vertical transmission.

An enzyme-linked immunosorbent spot (ELISpot) assay for detecting individual B cells secreting specific antibodies has enabled investigators to study B-cell immunity at a cellular level in a variety of clinical applications.[12,13] The advantages of the ELISpot assay are that it can detect even a single cell out of 10^6 peripheral blood mononuclear cells (PBMCs), whose secretion level may not be sufficient for detection of circulating antibody, and distinguishes and quantifies only active immunoglobulin-secreting

Abbreviations: ELISpot, enzyme-linked immunosorbent spot; HCV, hepatitis C virus; SFC, spot-forming cell; PBMC, peripheral blood mononuclear cell; Ig, immunoglobulin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PBS, phosphate-buffered saline; ROC, receiver-operating characteristics; AUC, area under the curve; IQR, interquartile range.

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cells. This assay thus provides a useful tool for better understanding immunity to infectious diseases and improved analysis of the immune response to vaccination.[14] Although studies of antigen-specific antibody-secreting cells in various viral infections have been conducted,[15-19] there are no published data on detection and quantification of anti-HCV antibody–secreting B cells.

The objective of this study was to adapt the ELISpot assay for the detection of anti-HCV antibody–secreting B cells to (1) clarify the HCV-specific humoral immune responses in patients with chronic hepatitis C, (2) examine the correlation between the numbers of anti-HCV antibody–secreting B cells and clinical outcomes, and (3) examine humoral immune responses in patients with chronic hepatitis C compared with those who spontaneously clear HCV.

Patients and Methods

Subjects. Individuals who were identified by the Greater Chesapeake and Potomac Region of the American Red Cross as being positive for anti-HCV via enzyme immunoassay at the time of blood donation were referred to the Department of Transfusion Medicine at the National Institutes of Health for participation in a long-term study of the natural history of HCV infection[20,21]; 750 participants were enrolled from 1990 through September 2003. Of these, 48 subjects were selected randomly to assess humoral immune responses at the B-cell level. The chronic hepatitis C group included 39 subjects who were positive for anti-HCV antibodies (EIA-2 and RIBA-3) and positive for HCV RNA. The recovered group included 9 anti-HCV–positive subjects who were HCV RNA–negative via qualitative polymerase chain reaction on at least two consecutive visits. The patients' characteristics are summarized in Table 1. Eleven volunteer blood donors without a history of HCV infection served as controls. All subjects were negative for hepatitis B surface antigen and antibodies to the human immunodeficiency virus. The study protocols were reviewed and approved by the appropriate institutional review boards, and all subjects gave written informed consent to participate in the study.

Laboratory Testing. Antibodies to HCV were measured in serum samples via second-generation enzyme immunoassay (EIA-2; Abbott Laboratories, North Chicago, IL). EIA-2 reactive samples were subsequently tested via third-generation recombinant immunoblot assay (RIBA-3; Chiron Corp., Emeryville, CA). Reactivity to at least two of four HCV antigens (5-1-1/C100-3, C33, C22, and NS5) was considered a positive RIBA-3 result, no reactivity was considered a negative result, and reactivity to only one antigen was considered an indeterminate result. The serum levels of HCV RNA were determined using the qualitative and quantitative COBAS AMPLICOR assays (Roche Diagnostic Systems, Branchburg, NJ), which amplify HCV RNA via reverse-transcription polymerase chain reaction. HCV genotypes were determined using INNO-LiPA HCV II (Innogenetics, Gent, Belgium). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and other relevant biochemical tests were performed using standard methods.

Table 1. Demographic and Clinical Characteristics of Patients With HCV Infection

Characteristics	All (N = 48)	Chronic (n = 39)	Recovered (n = 9)	*P* Value
Mean age, yrs (range)	51 (33-83)	52 (37-83)	49 (33-78)	.46
Male, n (%)	23 (48)	17 (44)	6 (67)	.28
Race, n (%)				
White	43 (90)	35 (90)	8 (89)	1.00
Black	5 (10)	4 (10)	1 (11)	
Source of infection, n (%)				
Transfusion	16 (33)	14 (36)	2 (22)	.30
Injection drug use	19 (40)	15 (38)	4 (44)	
Nasal cocaine use	4 (8)	2 (5)	2 (22)	
Occupational	6 (13)	6 (15)	0 (0)	
Unknown	3 (6)	2 (5)	1 (11)	
Genotype, n (%)				
1	25 (52)	24 (62)	1 (11)	.074
2	7 (15)	6 (15)	1 (11)	
3	2 (4)	1 (3)	1 (11)	
Unknown	14 (29)	8 (21)	6 (67)	
Mean values (range)				
ALT (IU/L)	52 (15-251)	58 (28-251)	25 (15-52)	.001
AST (IU/L)	43 (12-145)	48 (12-145)	24 (13-37)	.001
ALP (IU/L)	69 (32-171)	71 (35-171)	59 (32-74)	.20
Total bilirubin (mg/dL)	0.7 (0.3-1.5)	0.7 (0.3-1.5)	0.7 (0.4-1.4)	.72
Albumin (g/dL)	3.9 (3.3-4.5)	3.9 (3.3-4.5)	4.0 (3.6-4.3)	.53
GGTP (g/dL)	44 (8-286)	48 (8-286)	27 (8-102)	.025
HCV RNA level (10^6 IU/mL)	11.2 (<0.5-73)	14.1 (<0.5-73)	ND	<.001
Recombinant strip immunoblot assay				
C100	3.0 (0-4)	3.2 (0-4)	2.1 (0-4)	.042
C33	3.5 (1-4)	3.7 (1-4)	2.6 (1-4)	.011
C22	3.8 (0-4)	3.9 (1-4)	3.1 (0-4)	.068
NS5	2.1 (0-4)	2.3 (0-4)	1.3 (0-4)	.18

Abbreviations: ALP, alkaline phosphatase; GGTP, γ-glutamyltransferase; ND, below the limits of detection.

PBMCs. PBMCs were isolated from whole blood using cellular preparation tubes (Becton Dickinson, Franklin Lakes, NJ), washed one time in phosphate-buffered saline (PBS) and three times in medium (RPMI 1640 medium supplemented with 2 mmol/L L-glutamine, 5×10^{-5} mol/L 2 mercaptoethanol, 50 U/mL penicillin, 50 μg/mL streptomycin, and 10% fetal bovine serum), and were either studied immediately or cryopreserved in media containing 50% fetal bovine serum, 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO), and 10% RPMI 1640.

HCV Proteins. The recombinant full-length HCV core protein (amino acid residues 1-191), E2 protein

(amino acid residues 384-746), NS3 protein (amino acid residues 1027-1657), and NS5B protein (amino acid residues 2421-3011) were expressed and purified from *Escherichia coli* using the expression vector as previously described.[22,23] Control proteins were expressed as carboxy-terminal fusion proteins with human superoxide dismutase in *E. coli*.

ELISpot Assay. Ninety-six–well plates containing high-protein binding membranes (MAIP S4510; Millipore Co., Bedford, MA) were coated with a 10-μg/mL purified recombinant HCV core, E2, NS3, NS5b, or control antigens in carbonate coating buffer (0.1 mol/L Na_2CO_3, 0.1 mol/L $NaHCO_3$; pH 9.6). After incubation at 4°C overnight, the plates were washed twice with PBS and blocked with 3% bovine serum albumin for more than 30 minutes at 37°C. Cryopreserved PBMCs were thawed and incubated for 44 hours at 37°C in a humidified atmosphere of 5% CO_2 at 1.25×10^5 or 2.5×10^5 cells/well in AIM V Media (Invitrogen, Carlsbad, CA). All determinations were run in triplicate. After incubation, the cells were removed by washing 6 times with PBS containing 0.05% NP-40, and the plates incubated with horseradish peroxidase-linked anti-human IgG or IgM antibodies (1:1,000; KPL, Gaithersburg, MD) at 37°C for 2 hours. After the plates were washed twice with PBS and 6 times with PBS containing 0.05% NP-40, an optimal 4CN peroxidase substrate (Bio-Rad, Hercules, CA) was added and incubated for 20 to 30 minutes at room temperature to develop the spots. The reaction was stopped by washing with distilled water. The plates were dried overnight, and the spots were counted automatically by an ELISPOT reader (Carl Zeiss Vision, Hallbergmoos, Germany). The frequencies of anti-HCV antibody–secreting B cells were calculated by subtracting the mean number of spots in the control wells from the HCV antigen-coated wells, and expressed as the mean of triplicates of spot-forming cells (SFCs) per 10^6 PBMCs. Assays with a high background (>5 spots/well in the negative control) were excluded.

Assay of Anti-HCV/NS3 Antibodies. Anti-HCV/NS3 IgG was assayed via ELISA as described previously.[23] Briefly, MaxiSorp Nunc-Immuno plates were coated with recombinant HCV NS3 protein at 6 μg/mL in coating buffer (20 mmol/L sodium bicarbonate buffer [pH 9.6], 0.15 mol/L NaCl) and overcoated with 0.1% bovine serum albumin in PBS buffer (pH 7.4). The sera were tested via two-fold serial dilution in 0.3% IGEPAL CA-630 (Sigma), 5% milk diluent (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and PBS [pH 7.4], with initial dilution at 1:250. Biotinylated anti-human IgGγ (Kirkegaard & Perry Laboratories) and strepavidin–horseradish peroxidase (Kirkegaard & Perry Laboratories) were added sequentially. One hundred microliters per well ABTS microwell peroxidase substrate was used to develop the color and 100 μL per well peroxidase stop solution (Kirkegaard & Perry Laboratories) was added to stop the reaction. Absorbance was read at 405 nm. The IgG titer was determined via end point dilution.

Statistical Analysis. The Mann-Whitney *U* test or Student *t* test was used to analyze continuous variables as appropriate. Spearman's rank order correlations were used to evaluate the frequencies of anti-HCV antibody–secreting B cells to each antigen and to the clinical features. A *P* value of .05 or less was considered significant. Although SFCs/10^6 PBMCs were expressed in this study, the statistics were significant whether this was used or the raw counts were used. Statistical analyses were performed using SigmaStat (version 2.03; SPSS, Chicago, IL). Receiver-operating characteristic (ROC) curve analysis was performed using MedCalc 7.0 software (http://www.medcalc.be). The best cutoff values of the ELISpot assays were chosen automatically by MedCalc 7.0 as the SFCs with the highest diagnostic accuracy (*i.e.*, the sum of the false-negative and false-positive rates was minimized). The respective overall diagnostic values were expressed using the area under the curve (AUC).

Results

Optimal Cutoff Values for ELISPOT Assay. To determine the optimal cutoff values for the B-cell ELISpot assay in differentiating patients with HCV infection from HCV seronegative blood donors, ROC curve analysis was performed. The ROC curves for the ELISpot assay detecting anti-HCV IgG-specific B cells were obtained via calculations made using the values obtained from 48 patients with HCV infection and the 11 HCV-negative volunteer blood donors. The selection of the optimal cutoff point value was based on the level at which the accuracy was maximum (see Patients and Methods). The optimal cutoff values, sensitivity, specificity, positive predictive values, negative predictive values, and calculated AUCs to all HCV antigens are listed in Table 2. In our ELISpot assay, the values of sensitivity ranged from 58% to 92%, and the values of specificity were 100%. The AUC results were constantly high in the ELISpot assays for all antigens, and AUC values were between 0.71 (NS5B antigen) and 0.94 (core and E2 antigens).

After we defined the optimal cutoff value for each antigen, we determined the frequencies of anti-HCV IgG-secreting B cells in 48 patients with HCV infection. The prevalence of anti-HCV IgG-secreting B cells during HCV infection specific for the various antigens were:

Table 2. Optimal Cutoff Values, Sensitivity, Specificity, AUC, and Predictive Values of Anti-HCV IgG-Secreting B Cells in ELISpot Assay in 48 Patients With Chronic Hepatitis C and 11 Volunteer Blood Donors

Antigen	Cutoff Value	Sensitivity, % (95% CI)	Specificity, % (95% CI)	AUC (95% CI)	PPV, %	NPV, %
Core	13.4	92 (80-98)	100 (71-100)	0.94 (0.84-0.98)	100	73
E2	10.7	92 (80-98)	100 (71-100)	0.94 (0.85-0.99)	100	73
NS3	5.4	77 (63-88)	100 (71-100)	0.83 (0.71-0.92)	100	50
NS5B	5.4	58 (43-72)	100 (71-100)	0.71 (0.58-0.82)	100	36

NOTE. All AUC values were significantly higher than a 0.50 nonpredictive value ($P < .001$ for all comparisons).
Cutoff values were determined by making ROC curves and are expressed as SFCs/10^6 PBMCs.
Abbreviations: PPV, positive predictive value; NPV, negative predictive value.

core, 92%; E2, 92%; NS3, 77%; and NS5B, 58% (Table 2).

We further assessed the optimal cutoff values for the ELISPOT assay detecting anti-HCV IgM-secreting B cells using ROC curve analysis in 43 patients with HCV infection and in 6 HCV-negative blood donors (Table 3). The AUC values ranged from 0.73 (NS5B antigen) to 0.94 (core antigen). The prevalence of anti-HCV IgM-secreting B cells ranged from 54% (NS5B antigen) to 84% (core antigen) (Table 3).

Detection and Quantitation of Anti-HCV Antibody–Secreting B Cells. Forty-eight PBMC samples obtained from patients with HCV infection and 11 samples from healthy volunteer blood donors were examined for detection of anti-HCV IgG-secreting B cells. The median numbers of the sum of anti-HCV IgG-secreting B cells to all HCV antigens were significantly higher in patients with HCV infection (38.3 SFCs/10^6 PBMCs; interquartile range [IQR], 10.7-149.3) compared with control anti-HCV negative donors (5.3 SFCs/10^6 PBMCs; IQR, 2.7-8.0; $P < .001$). Figure 1A shows box plots for the numbers of anti-HCV IgG-secreting B cells to all 4 HCV antigens in patients with HCV infection and in the controls. Among 48 patients with HCV infection, the median numbers of anti-HCV IgG-secreting B cells ranged from 10.7 SFCs/10^6 PBMCs (NS5B antigen) to 119.0 SFCs/10^6 PBMCs (E2 antigen). The median numbers of anti-HCV IgG-secreting B cells in patients with HCV infection were significantly higher than those in controls for each HCV antigen (Fig. 1A).

Subsequently, we developed an ELISpot assay for detecting anti-HCV IgM-secreting B cells. Detection of the anti-HCV IgM-secreting B cells was performed in 43 patients with HCV infection and in 6 anti-HCV negative blood donors (Fig. 1B). The median numbers of the sum of anti-HCV IgM-secreting B cells to all HCV antigens were significantly higher in patients with HCV infection (21.3 SFCs/10^6 PBMCs; IQR, 9.2-48.0) compared with the controls (8.0 SFCs/10^6 PBMCs; IQR, 0.0-10.7; $P < .001$). The median numbers of anti-HCV IgM-secreting B SFC to the core (31.1 vs. 4.0 SFCs/10^6 PBMCs; $P < .001$) and E2 (32.0 vs. 8.0 SFCs/10^6 PBMCs; $P = .005$) antigens in patients with HCV infection were significantly higher than those in controls. (Fig. 1B).

Relationship Between Anti-HCV Antibody–Specific B Cells and HCV Genotypes. Because the antigens used were derived from HCV genotype 1a, the numbers of anti-HCV IgG-secreting B cells were compared between 25 patients with HCV genotype 1 infection (10 with 1a, 11 with 1b, and 4 not subtyped) and 9 infected with another single genotype (1 with 2a, 4 with 2b, 2 with 2 unsubtyped, and 2 with 3a). The median value of the anti-HCV IgG-secreting B cells to each antigen was not statistically different between the genotype 1 group and the other genotype groups (Fig. 2). In addition, there were no statistically significant differences in detecting anti-HCV IgM-secreting B cells to all HCV antigens in those with genotype 1 versus non-1 infections (data not shown).

Table 3. Optimal Cutoff Values, Sensitivity, Specificity, AUC, and Predictive Values of Anti-HCV IgM-Secreting B Cells in ELISpot Assay in 43 Patients With Chronic Hepatitis C and 6 Volunteer Blood Donors

Antigen	Cutoff Value	Sensitivity, % (95% CI)	Specificity, % (95% CI)	AUC (95% CI)	PPV, %	NPV, %
Core	12.1	84 (69-93)	100 (54-100)	0.94 (0.84-0.99)	100	46
E2	17.4	72 (56-85)	100 (54-100)	0.86 (0.73-0.94)	100	33
NS3	10.7	70 (54-83)	100 (54-100)	0.74 (0.60-0.86)	100	32
NS5B	8.1	54 (38-69)	100 (54-100)	0.73 (0.58-0.85)	100	23

NOTE. All AUC values were significantly higher than a 0.500 nonpredictive value ($P < .001$ for all comparisons). Cutoff values were determined by making ROC curves and are expressed as SFCs/10^6 PBMCs.
Abbreviations: PPV, positive predictive value; NPV, negative predictive value.

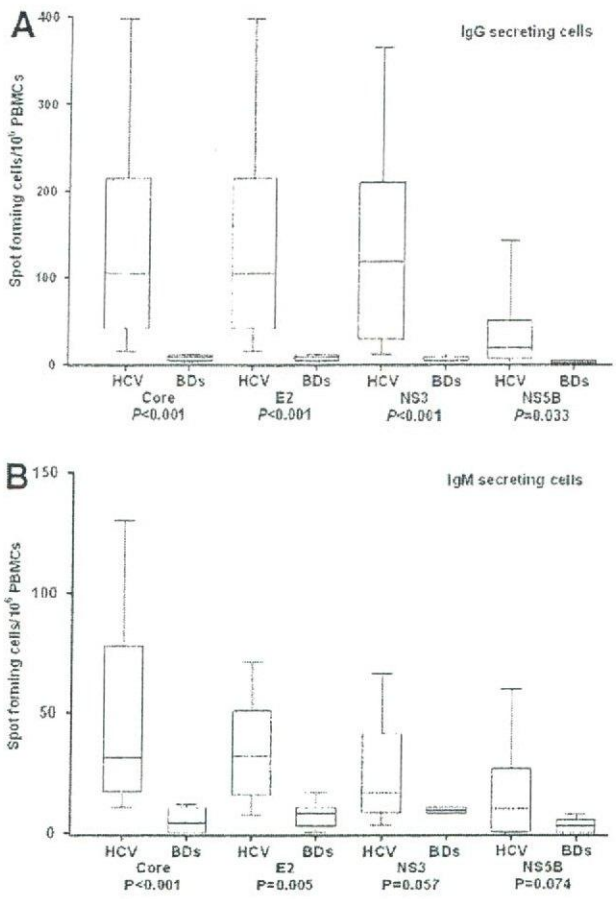

Fig. 1. Detection of anti-HCV antibody-secreting B cells in patients with HCV infection and volunteer blood donors. Boxes represent the IQR of the data. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively. (A) The frequencies of anti-HCV IgG-secreting B cells to 4 HCV antigens were detected in 48 patients with HCV infection and in 11 volunteer blood donors. (B) The frequencies of anti-HCV IgM-secreting B cells were detected in 43 patients with HCV infection and in 6 volunteer blood donors. PBMCs, peripheral blood mononuclear cells; IgG, immunoglobulin G; HCV, hepatitis C virus; BDs, blood donors; IgM, immunoblobulin M.

Correlation Between Anti-HCV IgG-Secreting B Cells and Clinical Features in Patients With HCV Infection. Several demographic (age and sex) and clinical (viral load, genotype, ALT, AST, alkaline phosphatase, total bilirubin, albumin, γ-glutamyltransferase, intensity of RIBA assay, and anti-HCV antibodies) findings were examined for their correlation with anti-HCV IgG-secreting B-cell frequency in patients with HCV infection. The circulating anti-HCV IgG-secreting B-cell frequency to the core antigen (Fig. 3A) was significantly correlated with the value of ALT ($P = .048$, $r = 0.29$) and inversely correlated with serum albumin ($P = .048$, $r = -0.33$). Similarly, the number of anti-HCV IgG-secreting B cells to the E2 antigen was significantly correlated with the value of ALT ($P = .037$, $r = 0.30$) (Fig. 3B) and AST ($P = .033$, $r = 0.31$) (Fig. 3C) and was inversely correlated with serum albumin ($P = .029$, $r = -0.36$). Furthermore, the number of SFCs to the NS3 antigen was significantly correlated with the circulating antibody level to the NS3 antigen in 38 patients with available serum samples ($P = .008$, $r = 0.43$) (Fig. 3D). There was no significant correlation between the numbers of anti-HCV IgG-secreting B cells to NS3 or NS5b antigens and any of the biochemical, demographic, or clinical parameters specified above.

Comparison of the Number of Anti-HCV Antibody-Secreting B Cells Between Patients With Chronic Hepatitis C and Patients Who Recovered. As shown in Table 1, patients with chronic hepatitis C had significantly higher mean serum levels of ALT (58 vs. 25 IU/L; $P = .001$), AST (48 vs. 24 IU/L; $P = .001$), and γglutamyltransferase (48 vs. 27 IU/L; $P = .025$) compared with the recovered patients. The mean HCV RNA level in the chronic group was 14.1×10^5 IU/mL. There were significant differences in the mean intensity of the RIBA assay against the C33 and C100 proteins in chronic vs. recovered subjects (C33, 3.2 vs. 2.1, $P = .042$; C100, 3.7 vs. 2.6, $P = .011$). We found no significant difference between patients with chronic hepatitis C and patients who had recovered when their age, sex, race, source of infection, HCV genotypes, total bilirubin, or albumin were compared.

The median numbers of the sum of anti-HCV IgG-secreting B cells to all HCV antigens were significantly higher in patients with chronic hepatitis C (47.3 SFCs/

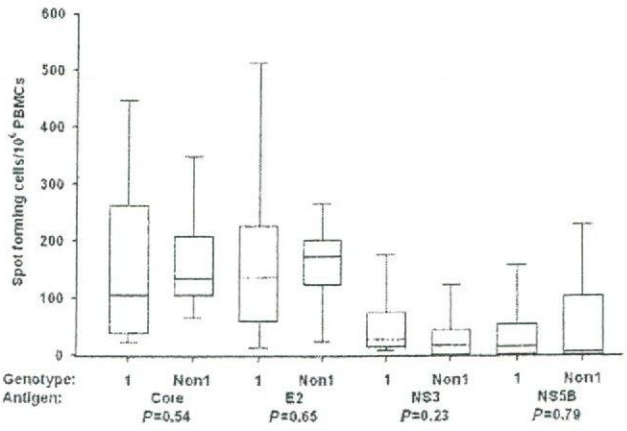

Fig. 2. Detection of anti-HCV IgG-secreting B cells in patients infected with HCV of genotype 1 and nongenotype 1. Boxes represent the IQR of the data. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively. The frequencies of anti-HCV-secreting B cells were detected in patients infected with genotype 1 (n = 25) and in those with other genotypes (n = 9). PBMCs, peripheral blood mononuclear cells; Non1, nongenotype 1.